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Influence of dietary factors on the macro and micro- composition of bovine milk for use in protein ingredient powder manufacture

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

by

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Submitted: December 2021

Viva voce: April 5th 2022

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Dedicated to my brother James, who propelled me into science.

Table of Contents

Declaration.....	i
Acknowledgements.....	ii
Abstract.....	iv
List of abbreviations used.....	vii
List of publications and awards.....	ix

Chapter 1 (Part 1): Composition of bovine milk 1

1.1.1. Gross composition of bovine milk	2
1.1.2. Milk components	2
1.1.2.1. Milk protein composition.....	2
1.1.2.2. Milk fat composition.....	3
1.1.2.3. Milk carbohydrate, mineral and vitamin composition.....	4
1.1.2.4. Minor compounds in milk.....	8
1.1.3. Factors influencing milk composition	9
1.1.3.1. Individual variation and genetics.....	9
1.1.3.2. Cow breed, age and health status.....	9
1.1.3.3. Seasonality and stage of lactation.....	10
1.1.3.4. Cow diet.....	12
1.1.4. Conclusion	12

Chapter 1 (Part 2): Compositional and functional properties of milk and dairy products derived from cows fed pasture or concentrate-based diets13

1.2.1. Abstract	14
1.2.2. Introduction	14
1.2.3. Milk, nutrition and recent trends in dairy consumption	18
1.2.3.1. Nutritional significance of milk.....	18
1.2.3.2. Trends in milk and dairy product consumption.....	19
1.2.3.2.1. Lactose intolerance and milk allergy.....	20
1.2.3.2.2. A1 & A2 milk.....	21
1.2.3.2.3. “Pasture-fed” dairy.....	22
1.2.4. Impact of feeding system on milk composition	23
1.2.4.1. Protein and amino acids.....	23
1.2.4.1.1. Milk protein yield, crude protein and true protein.....	23
1.2.4.1.2. Milk urea and non-protein nitrogen.....	31
1.2.4.1.3. Amino acid composition.....	33
1.2.4.2. Fat and fatty acids.....	34
1.2.4.2.1. Milk fat yield/gross milk fat.....	34
1.2.4.2.2. Fatty acid composition.....	38
1.2.4.2.3. Milk fat globule size.....	43

1.2.4.3. Water-soluble components and ash	45
1.2.4.3.1. Carbohydrate content	45
1.2.4.3.2. Mineral composition	46
1.2.4.3.3. Vitamin composition	49
1.2.5. Impact of milk composition on dairy product functionality	51
1.2.5.1. Liquid milk properties	51
1.2.5.2. Yoghurt, butter and cheese	55
1.2.5.3. Sensory quality	63
1.2.5.4. Dairy product verification	68
1.2.6. Conclusion	70
1.2.7. References	70
 Statement of research objectives	 94

Chapter 2: Impact of bovine diet on metabolomic profile of skim milk and whey protein ingredients 95

2.1. Abstract	96
2.2. Introduction	97
2.3. Materials and Methods	99
2.3.1. Materials	99
2.3.2. Experimental design	99
2.3.3. Ingredient manufacture	100
2.3.3.1. Skim milk preparation	100
2.3.3.2. Sweet whey preparation	101
2.3.3.3. Acid whey preparation	102
2.3.3.4. Micellar casein whey preparation	102
2.3.4. Determination of nitrogen content	102
2.3.5. Total amino acid analysis	103
2.3.6. Liquid chromatography – Mass Spectrometry	103
2.3.7. Statistical analysis	104
2.4. Results and discussion	104
2.4.1. Nitrogen composition	104
2.4.2. Total amino acid composition	107
2.4.3. Metabolomic profiles of protein ingredients	111
2.5. Conclusion	119
2.6. References	119

Chapter 3: Effect of diet on the vitamin B profile of bovine milk-based protein ingredients124

3.1. Abstract	125
3.2. Introduction	125
3.3. Materials and Methods	128
3.3.1. Materials	128
3.3.2. Experimental design	128
3.3.3. Protein ingredient manufacture	128

3.3.4. Liquid chromatography-mass spectrometry	129
3.3.4.1. Sample preparation	129
3.3.4.2. Operating conditions	129
3.3.5. Nephelometry	130
3.3.6. Statistical analysis	130
3.4. Results and discussion	131
3.4.1. Overall distribution	131
3.4.2. Vitamin composition	137
3.4.2.1. Vitamin B1 (Thiamine)	137
3.4.2.2. Vitamin B2 (Riboflavin)	138
3.4.2.3. Vitamin B3 (Nicotinic acid)	139
3.4.2.4. Vitamin B5 (Pantothenic acid) and B6 (Pyridoxine)	140
3.4.2.5. Vitamin B7 (Biotin)	141
3.4.2.6. Vitamin B12 (Cobalamin)	142
3.4.3. Relationship between skim milk and recommended daily allowances	143
3.4.4. Vitamin content of skim milk for use in infant milk formula manufacture	145
3.5. Conclusion	147
3.6. References	148

Chapter 4: Compositional and physical properties of skim milk and whey protein concentrate powders prepared from milk of cows fed perennial ryegrass or total mixed ration154

4.1. Abstract	155
4.2. Introduction	156
4.3. Materials and methods	158
4.3.1. Materials	158
4.3.2. Experimental design	158
4.3.3. Protein ingredient manufacture	158
4.3.4. Compositional analysis	159
4.3.4.1. Total nitrogen and non-protein nitrogen content	159
4.3.4.2. Size exclusion chromatography	159
4.3.4.3. Inductively coupled plasma mass spectrometry	160
4.3.4.4. Ionic calcium concentration	160
4.3.5. Heat coagulation time	161
4.3.6. Rheological measurements	161
4.3.6.1. Viscosity of skim milk concentrate	161
4.3.6.2. Oscillation of whey protein concentrate	161
4.3.6.3. Viscosity of skim milk and whey protein concentrate dispersions..	162
4.3.7. Statistical analysis	162
4.4. Results and discussion	163
4.4.1. Composition	163
4.4.1.1. Nitrogen composition	163
4.4.1.2. Whey protein profile	166
4.4.1.3. Mineral composition	168
4.4.1.4. Ionic calcium concentration	173
4.4.2. Heat coagulation time of reconstituted skim milk powder	174
4.4.3. Rheology of skim milk and whey protein concentrates	175
4.4.3.1. Low amplitude oscillation	175
4.4.3.2. High shear viscosity measurement	178

4.5. Conclusion	185
4.6. References	186

Chapter 5: Physicochemical properties of whole milk powder derived from milk of cows fed pasture or total mixed ration diets191

5.1. Abstract	192
5.2. Introduction	193
5.3. Materials and Methods	195
5.3.1. Materials	195
5.3.2. Experimental design	195
5.3.3. Whole milk powder production	196
5.3.4. Compositional analysis of powder	196
5.3.4.1. Determination of nitrogen and fat content	196
5.3.4.2. High-performance liquid chromatography	197
5.3.5. Colour measurements	197
5.3.6. Whey protein nitrogen index	198
5.3.7. Heat coagulation time	198
5.3.8. Milk fat globule size of rehydrated whole milk powders.....	198
5.3.9. Rheological properties of yoghurt	199
5.3.9.1. Yoghurt production	199
5.3.9.2. Texture profile analysis	200
5.3.10. Statistical analysis	200
5.4. Results and Discussion	201
5.4.1. Whole milk powder composition	201
5.4.2. Colour analysis of whole milk powders	205
5.4.3. Heat coagulation time of reconstituted whole milk powders	208
5.4.4. Rheological properties of yoghurt	212
5.4.5. Texture profile analysis	217
5.5. Conclusion	220
5.6. References	221

Chapter 6: Effect of pasture and total mixed ration-based diets on the composition and digestion of whole milk powder224

6.1. Abstract	225
6.2. Introduction	226
6.3. Materials and Methods	228
6.3.1. Materials	228
6.3.2. Experimental design	228
6.3.3. Whole milk powder production	229
6.3.4. In-vitro digestion	230
6.3.5. Whole milk powder and digestate analysis	230
6.3.5.1. Determination of nitrogen and fat content	230
6.3.5.2. Mineral composition	230
6.3.5.3. Total amino acid composition	231

6.3.5.4.	Free amino acid composition	231
6.3.5.5.	Fatty acid methyl ester composition	231
6.3.5.6.	Free fatty acid composition	232
6.3.5.6.1.	Lipid extraction.....	233
6.3.5.6.2.	Solid phase extraction.....	233
6.3.5.7.	Raman spectroscopy	234
6.3.5.8.	Buffering capacity of reconstituted WMP	234
6.3.5.9.	Gel electrophoresis	235
6.3.5.10.	Confocal microscopy.....	235
6.3.5.11.	Proteomic and peptidomic analysis	236
6.3.5.11.1.	Proteomic analysis of whole milk powders	236
6.3.5.11.2.	Peptidomic analysis of Infogest samples	237
6.3.6.	Statistical analysis	238
6.4.	Results and Discussion	238
6.4.1.	Properties of whole milk powders and reconstituted whole milk powders	238
6.4.1.1.	Gross composition of whole milk powders	238
6.4.1.2.	Mineral composition of whole milk powders	239
6.4.1.3.	Total amino acid composition of whole milk powders	241
6.4.1.4.	Fatty acid methyl ester profile of whole milk powders	242
6.4.1.5.	Raman spectroscopy of reconstituted whole milk powders	245
6.4.1.6.	Buffering capacity of reconstituted whole milk powders	248
6.4.1.7.	Proteomic profile of reconstituted whole milk powders	250
6.4.2.	Effect of in-vitro digestion on the protein and fat components of reconstituted whole milk powders	255
6.4.2.1.	Gel electrophoresis and confocal microscope images	255
6.4.2.2.	Free amino acid composition	256
6.4.2.3.	Free fatty acid profile	258
6.4.2.4.	Peptidomic profile of digested whole milk	259
6.5.	Conclusion	262
6.6.	References	262

Chapter 7: General discussion	267
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7.1. Summary of key findings	268
7.2. Scientific and industrial impact of this research	270
7.3. Future considerations	273
7.4. References	276

Appendix i: Supplementary materials	277
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Appendix ii: Published papers	301
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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 and intellectual property. All the work described herein is the original work of the author, with the following exceptions:

Chapter 2 - Jiamin Zheng and Lun Zhang contributed to the metabolomics analysis;

Chapter 3 - Jiamin Zheng and Lun Zhang contributed to the vitamin analysis;

Chapter 6 – Rebecca Owens contributed to the proteomics and peptidomics analysis. Laura Gómez-Mascaraque contributed to the confocal microscopy and Raman spectroscopy analysis.

Signed: 

Date: 25th August 2022

Jonathan Magan

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Fortitudine vincimus

Abstract

The increasing frequency of “grass-fed” labelling claims in dairy product marketing, predicated in part on improved product quality and nutritional benefits associated therewith, has necessitated both rigorous experimental data to determine the relative differences in the composition of milk from grass-fed and non-grass-fed cows and robust methods for verifying these labelling claims. The “Grass-fed Standard” established by Bord Bia in 2020 is the first scientifically-backed standard in use internationally, which is founded on the somewhat unique pasture-based dairy production system practiced in Ireland. The compositional and functional benefits associated with products derived from pasture-fed cows have thus far been focused on business-to-consumer products. The effect of pasture-based production relative to the conventional indoor production system utilised most widely throughout the world on the composition and functional properties of “business-to-business” milk powder products, which represent a major aspect of Irish dairy production, remained to be established. Variation in the composition and functional properties of dairy products arises due to numerous factors, including feed composition, which can differ depending on the type of production system practiced. Therefore, the objective of this research was to investigate the effect of perennial ryegrass only (GRS), perennial ryegrass/white clover pasture (CLV) and indoor total mixed ration (TMR) feeding systems on the composition and functional properties of bovine milk-based protein ingredients.

There was a significant effect of feeding system on the concentration of metabolite compounds and B vitamins in skim milk powder (SMP) and whey ingredients, which were distinguishable by liquid chromatography-mass spectrometry. Concentrations of vitamins B1, B2 and B7 were significantly higher in GRS and CLV samples than TMR samples, for which concentrations of vitamin B3 and B3-amide were higher than in CLV and GRS samples, respectively. Gross compositional parameters were similar between the feeding systems;

however, the CLV and TMR systems were associated with high concentrations of non-protein nitrogen and high heat stability in whole milk powder and SMP samples. Some differences in total amino acid (AA) composition were also exhibited between WMP and SMP samples from each diet, which suggests that AA profile may be responsive to dietary variation. Yoghurts produced from GRS and CLV-derived WMP were typified by significantly higher gel strength and textural firmness than those derived from TMR, despite significantly higher concentrations of palmitic acid in TMR samples. Fatty acid (FA) profiles of WMP samples from each diet were similar to those in the literature produced from pasture or concentrate-based systems. The level of FA unsaturation and carotenoid content could also distinguish between pasture and TMR samples using Raman spectroscopy.

The mineral composition of WMP, SMP and whey protein concentrate (WPC) samples from each diet did not vary significantly, with the exception of selenium and iodine, which were consistently, and significantly, higher in TMR samples than GRS and CLV samples. The viscosity of GRS-derived skim milk concentrate was significantly higher than that for TMR, but the effect of diet on the heat-dependent viscosity of dispersions of skim milk with WPC was not as substantial as the differences between WPC types, with micellar casein whey being most stable on heating, and acid WPC the least stable. Proteomic and peptidomic analysis of WMP and digested WMP samples determined qualitative and quantitative differences in proteins and peptides arising from each diet.

In conclusion, this research demonstrated that the influence of bovine diet on milk components is not limited to gross compositional factors, but the micro-composition and functional properties, such as heat stability and acid-induced gelation, of value-added dairy products can also be influenced by feeding practices. This research will be of most interest to the manufacturers of dairy powder ingredients, such as SMP and WPC and premium WMP products in establishing the commercial point-of-difference for pasture or concentrate-derived

products along with the unique compositional elements and functional behaviour associated with products from the three feeding systems investigated herein. Furthermore, this research will aid in ingredient selection for manufacturers of value-added dairy commodities, such as infant milk formula. Finally, this research contributes to the list of compositional variables which demonstrate potential for differentiation between feeding systems, providing preliminary information, relating to the establishment of robust analytical methods for verification of “Grass-fed” labelling claims for milk powder products to international entities responsible for policy implementation, such as Bord Bia.

List of abbreviations used

°C	Degrees Celcius
μL	MicroLitre
μm	Micrometre
AA	Amino acid
ANOVA	Analysis of variance
AWP	Acid whey protein
AWPC	Acid whey protein concentrate
CCP	Colloidal calcium phosphate
CLA	Conjugated linoleic acid
CLV	Grass/white clover feeding system
CP	Crude protein
DM	Dry matter
FA	Fatty acid
FAA	Free amino acids
FAME	Fatty acid methyl esters
FFA	Free fatty acids
g	Gram
G'	Elastic modulus
G''	Viscous modulus
GC-FID	Gas chromatography flame ionisation detection
GRS	Grass feeding system
HCl	Hydrochloric acid
HCT	Heat coagulation time
Hz	Hertz
IMF	Infant milk formula
kcal	KiloCalories
kDa	KiloDalton
kg	Kilogram
L	Litre
LC-MS/MS	Liquid Chromatography –Mass Spectrometry/Mass Spectrometry
M	Molar
MCWP	Micellar casein whey protein
MCWPC	Micellar casein whey protein concentrate
MFGM	Milk fat globule membrane
mg	Milligram
min	Minutes
mL	MilliLitre
mm	Millimetre
mM	MilliMolar
MUFA	Monounsaturated fatty acid
M _w	Molecular weight
N	Nitrogen
NaOH	Sodium Hydroxide
NCN	Non-casein nitrogen
NMR	Nuclear magnetic resonance
NPN	Non-protein nitrogen

Pa.s	Pascal seconds
PCA	Principal component analysis
PLS-DA	Partial least square-discriminant analysis
PUFA	Polyunsaturated fatty acid
RDA	Recommended daily allowance
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
s	Seconds
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SEC	Size Exclusion Chromatography
SFA	Saturated fatty acid
SMP	Skim milk powder
SWP	Sweet whey protein
SWPC	Sweet whey protein concentrate
TAA	Total amino acids
TCA	Trichloroacetic acid
TMR	Total mixed ration feeding system
TN	Total nitrogen
TP	Total protein
TS	Total solids
w/v	Weight/volume
w/w	Weight/weight
WMP	Whole milk powder
WPC	Whey protein concentrate
WPNI	Whey Protein Nitrogen Index
η^*	Complex viscosity

List of publications and awards

Peer-reviewed publications:

Magan, J.B., Tobin, J.T., O’Callaghan, T.F., Kelly, A.L., Fenelon, M.A., Hennessy, D., McCarthy, N.A. (2019). Physicochemical properties of whole milk powder derived from cows fed pasture or total mixed ration diets. *Journal of Dairy Science*. 102, 9611–9621. DOI: <https://doi.org/10.3168/jds.2019-16415>

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Chapter 1 (Part 1)
Composition of bovine milk

1.1.1. Gross composition of bovine milk

Milk is a highly complex dispersion of macro and micro-nutrients in a continuous phase of water produced to meet the specific nutritional demands of mammalian neonates. Interspecies variation in overall milk composition can be considerable and is primarily determined by nursing frequency and neonatal growth rates (Skibieli *et al.*, 2013). The high moisture content of milk is necessary to both fulfil the calf's requirement for water and maintain the relative osmotic balance of the blood plasma and interstitial fluid of the secretory cells from which the milk is produced (Sinha, 2007). The high fat (3.8%) and protein (3.4%) contents (Fox *et al.*, 2015) of milk are attributable to the calf's need for rapid growth and muscle development from a young age (Kertz *et al.*, 2017). The relatively high ratio of casein protein in comparison to whey (80:20) allows for the formation of a harder, enzyme-induced curd in the calf's abomasum, maximising entrapment of fat within the casein matrix, residence time and nutrient digestibility (Longenbach & Heinrichs, 1998). In addition to fat, the other principal energy source in milk is the disaccharide lactose, representing approximately 5% of the total mass, while the remaining solids present constitute the ash fraction (mineral and organic salts) (Fox *et al.*, 2015).

1.1.2. Milk components

1.1.2.1. Milk protein composition

Among the bovine milk proteins, the phosphate-containing casein protein fraction comprises four principal genetic products; α_{S1} -casein, α_{S2} -casein, β -casein and κ -casein (Gallinat *et al.*, 2013, along with γ -caseins, plasmin-induced breakdown products of β -casein (Eigel *et al.*, 1979). The further two sub-forms of α_{S1} -casein and four sub-forms of α_{S2} -casein vary by degree of post-translational phosphorylation (Holland, 2008). The whey protein fraction consists of a heterogeneous mixture of globular proteins which are structurally

dissimilar (de Wit & Klarenbeek, 1984) and are rather associated by their collective solubility at pH 4.6 and separation from casein during cheese-making (Smithers, 2008). The primary whey protein is β -lactoglobulin, accounting for approximately 50% of total whey protein nitrogen, followed by α -lactalbumin (~20%), immunoglobulins (~12%) and bovine serum albumin (~6%) (deWit, 1998). The remaining ~12% is comprised of proteose-peptones, produced from the plasmin-induced cleavage of β -casein, similar to γ -casein (Andrews & Alichanidis, 1983). Bovine milk also contains low levels of the iron-binding glycoprotein lactoferrin, a natural protective factor, the concentration of which is correlated with somatic cell count and, thus, is elevated at times of mastitic infection (Cheng *et al.*, 2008; Newman *et al.*, 2009).

1.1.2.2. Milk fat composition

Bovine milk contains an average of 3.8% total fat (Fox *et al.*, 2015), though considerable variation may be present between breeds and indeed between individual cows (US National Research Council, 1988). As the most energy-dense macronutrient (37 kJ g⁻¹), fat plays a significant role in the growth and development of the calf, while also providing essential fatty acids (FA) and fat-soluble vitamins. The fat fraction of milk is dominated by triglyceride esters (~98% of total milk fat), composed of FA of varying chain length and degree of saturation and, as such, their constituent FA distribution determines the melting and rheological behaviour of milk fat (Gresti *et al.*, 1993) and, hence, the texture and mouthfeel of milk fat-based products. The remaining proportion of total milk fat comprises of phospholipids (~1%), cholesterol (~0.5%), free FA (~0.1%) and diacylglycerol (~2%) (Lindmark Månsson, 2008). Over 400 individual FA are present in bovine milk (Lindmark Månsson, 2008), the most significant of which are shown in Table 1.1.1, adapted from those reported by Markiewicz-Kęszycka *et al.* (2013). Palmitic acid (C16:0) and oleic acid (C18:1 *cis*-9) are by far the most

abundant FA present in bovine milk; however, ruminant milks also contain high concentrations of short chain FA such as caproic acid (C6:0) and butyric acid (C4:0), of which milk is one of few food sources (Talpur *et al.*, 2008).

Table 1.1.1: Major fatty acid composition (%) of bovine milk.

Carbon number	Fatty acid name	% weight of total fatty acids
C4:0	Butyric acid	2.87
C6:0	Caproic acid	2.01
C8:0	Octanoic acid	1.39
C10:0	Decanoic acid	3.03
C12:0	Lauric acid	3.64
C14:0	Myristic acid	10.92
C16:0	Palmitic acid	28.7
C18:0	Stearic acid	11.23
C18:1 n9c	Oleic acid	22.36
C18:2 c9t11	<i>cis</i> -9, <i>trans</i> -11 conjugated linoleic acid	0.57
C18:2 c9c12	<i>cis</i> -9, <i>cis</i> -12 linoleic acid	2.57
C18:3 n3	α -linolenic acid	0.5

Adapted from Markiewicz-Kęszycka *et al.* (2013).

1.1.2.3. Milk carbohydrate, mineral and vitamin composition

The principal carbohydrate in milk is lactose, which is present at an average content of 4.8% (Fox *et al.*, 2015) and is a disaccharide which is unique to milk, as it is produced exclusively in the mammary gland through condensation of blood glucose and galactose (Silanikove *et al.*, 2015). There are two anomeric forms of lactose, α -lactose and β -lactose, which are present in an approximately 1:2 ratio in standard conditions and are nutritionally equivalent but differ in behavioural characteristics (e.g., solubility, crystallisation) (Costa *et al.*, 2019). Oligosaccharides present in milk (approx. 100 mg L⁻¹) (Robinson, 2019) are derived from hydrolysis of lactose by glycosyltransferase enzymes (Urashima *et al.*, 2001), though they represent a functionally insignificant proportion of the total carbohydrate content of bovine milk (Boehm & Stahl, 2007).

The ash content of milk exists both as a soluble dispersion among the aqueous phase and in association with milk proteins (Fox *et al.*, 2015) and is present in a comparatively narrow range of occurrence (0.7 - 0.8%), maintaining a relatively constant osmotic balance with lactose (Fox *et al.*, 2015). Table 1.1.2 shows the composition of major and trace minerals present in milk, adapted from Fox *et al.* (2015), Hunt & Nielsen (2009), Park *et al.* (2007) and Murthy *et al.* (1972). The major anions present in highest concentrations are calcium, phosphorous, potassium, chloride and citrate, while sodium, magnesium and zinc are also present at appreciable levels. Approximately 66% of total calcium in milk is associated within the casein micelle, with the remaining 33% present as soluble calcium (including free ionic calcium) within the serum phase (Lewis, 2011). Concentrations of calcium and phosphorous tend to be too high to be maintained in the soluble phase at native milk pH and consequently, associate with the casein micelle to form insoluble colloidal calcium phosphate (CCP) (Lenton *et al.*, 2014).

Table 1.1.2 :Average mineral composition of bovine whole milk.

Mineral	Chemical symbol	Average concentration (mg L ⁻¹ or µg L ⁻¹)
Major		
Calcium	Ca	1178 mg L ⁻¹
Phosphate	PO ₄ (3-)	1984 mg L ⁻¹
Magnesium	Mg	124 mg L ⁻¹
Sodium	Na	556 mg L ⁻¹
Chloride	Cl ⁻	1070 mg L ⁻¹
Potassium	K	1356 mg L ⁻¹
Citrate	C ₆ H ₅ O ₇ (3-)	1882 mg L ⁻¹
Trace		
Zinc	Zn	3500 µg L ⁻¹
Copper	Cu	200 µg L ⁻¹
Iron	Fe	500 µg L ⁻¹
Nickel	Ni	25 µg L ⁻¹
Manganese	Mn	30 µg L ⁻¹
Sulphur	S	3200 µg L ⁻¹
Iodine	I	260 µg L ⁻¹
Fluoride	F ⁻	51 µg L ⁻¹
Selenium	Se	20 µg L ⁻¹
Molybdenum	Mo	73 µg L ⁻¹
Cobalt	Co	1 µg L ⁻¹
Strontium	Sr	174 µg L ⁻¹
Chromium	Cr	10 µg L ⁻¹

Adapted from Fox *et al.* (2015), Hunt & Nielsen (2009), Park *et al.* (2007) and Murthy *et al.* (1972).

Although numerous models have been proposed to describe the structure of the casein micelle, including those of Schmidt (1982), Holt (1992), Walstra (1990), de Kruif & Holt (2003), Horne (2006), Dalgleish (2011) and Huppertz *et al.* (2017), the nanocluster model (de Kruif & Holt, 2003) is recently the generally preferred understanding (McMahon & Oommen, 2008). This model proposes the distribution of α - and β -caseins in an homogenous matrix in which CCP nanoclusters are enclosed, where the “hairy” κ -casein layer is less distinct, as is proposed in submicelle models, though each model recognises the presence of CCP either distributed throughout or within the interior of the micelle. Zinc, in turn, is extensively

associated with CCP in bovine milk, which can limit its bioavailability (Singh *et al.*, 1988). Among the trace elements present in milk, concentrations of heavy metal ions, earth elements and iodine vary considerably depending on feed composition (Flachowsky *et al.*, 2013) or levels of soil contamination (Rajaganapathy *et al.*, 2011).

Water-soluble and fat-soluble vitamins in milk are present in the aqueous phase and fat globules, respectively (Luisa, 1995) and, therefore, their relative concentrations in subsequently produced dairy products will be reflective of their fat content. Table 1.1.3 shows the water-soluble and fat-soluble vitamin composition of milk, adapted from Cheung & Mehta (2015) and Haroon *et al.* (1982). The most abundant water-soluble vitamins in milk are riboflavin (B2), pantothenate (B5), niacin (B3) and thiamine (B1); with the exception of Vitamin C, water-soluble vitamins are generally stable to standard processing temperatures (excepting sterilisation treatment) (Goldsmith *et al.*, 1983), allowing for transfer into downstream products without significant losses. As mentioned above, concentrations of fat-soluble vitamins are more variable in different dairy products as increasing fat concentration will consequently increase the fat-soluble vitamin content of products such as cream or butter, although this would therefore necessitate supplementation of these vitamins in low-fat products. Vitamin A (particularly in the form of β -carotene) and Vitamin D are most abundant in milk, and concentrations of Vitamin E and Vitamin K are substantially lower.

Table 1.1.3: Average vitamin composition of bovine whole milk.

Vitamin	Average concentration (mg L ⁻¹ or µg L ⁻¹)
Water-soluble	
Thiamine (B1)	400 µg L ⁻¹
Riboflavin (B2)	1700 µg L ⁻¹
Niacin (B3)	1000 µg L ⁻¹
Pantothenate (B5)	3500 µg L ⁻¹
Pyridoxine (B6)	600 µg L ⁻¹
Biotin (B7)	30 µg L ⁻¹
Folate (B9)	50 µg L ⁻¹
Cobalamin (B12)	5 µg L ⁻¹
Vitamin C	20 mg L ⁻¹
Fat-soluble	
Retinol (A)	400 µg L ⁻¹
Calciferol (D)	1.0 µg L ⁻¹
Tocopherol (E)	1.0 mg L ⁻¹
Phylloquinone (K)	5 µg L ⁻¹

Adapted from Cheung & Mehta (2015) and Haroon *et al.* (1982).

1.1.2.4. Minor compounds in milk

Milk also contains over 200 indigenous metabolites (Boudonck *et al.*, 2009) primarily transferred from blood through the interstitial junctions of secretory cells (Sundekilde *et al.*, 2011) or produced through the action of rumen microflora (Saleem *et al.* 2013), in addition to numerous indigenous enzymes. Free amino acids, FA and their respective derivatives, particularly volatile short chain FA, vitamin cofactors and peptides constitute the majority of minor compounds present in milk (Boudonck *et al.*, 2009; O’Callaghan *et al.*, 2018), with concentrations varying significantly in response to the health, nutrition and lactation status of the individual cow (Sundekilde *et al.*, 2011). Choline, a water-soluble metabolite which displays similar biological activity to the B vitamin group (US Institute of Medicine, 1998), is present in particularly high concentrations in milk and milk-derived products such as whey products and is of major significance to both cow (Sharma & Erdman, 1988) and human (Zeisel

& da Costa, 2009) health. Choline functions as a precursor to the synthesis of other metabolites in milk (O'Callaghan *et al.* 2018) and fulfils several key physiological functions in the body, such as neurotransmitter and phospholipid biosynthesis (Zeisel & da Costa, 2009).

1.1.3. Factors influencing milk composition

1.1.3.1. Individual variation and genetics

The variability of milk composition is influenced by several factors, though variation between individual cows within a herd and between separate milkings from an individual cow is perhaps the most significant. This is largely addressed by the bulking process at milk collection, which acts to effectively “dilute” individual compositional variables into an homogenous mixture (Fox, 2012). Percentages of fat, protein and lactose in milk are also genetically influenced, with respective heritability scores of 0.22, 0.35 and 0.29 estimated by Berry *et al.* (2013) for Holstein/Friesian cows. Genetic selection on this basis is practiced to meet the requirements of milk processors who offer milk-quality-based payment schemes (Dillon *et al.*, 2006).

1.1.3.2. Cow breed, age and health status

Just as major differences in milk composition are observed between different mammalian species, considerable variation exists in milk composition (particularly fat and protein content) between different cow breeds (Malossini *et al.*, 1996). Although Holstein and Holstein/Friesian crossed cows (~3.8% fat & 3.4% protein; Palladino *et al.*, 2010) typically produce the highest milk yields, milk with greater average total solids content is produced by breeds such as Jersey (~5% fat & 4% protein; Palladino *et al.*, 2010), Fleckvieh (~4.1% fat & 3.5% protein; Goni *et al.*, 2015) and Ayrshire (~4% fat & 3.4% protein; McEwan & Knight, 2018), which are often included within Holstein/Friesian herds in an effort to increase milk

solids yields. However, overall Holstein milk solids yield is greater than most other breeds, due to increased volumes being produced over the course of an entire lactation. The age and health status of the cow will also affect milk yield and the efficiency of milk synthesis within the mammary gland (US National Research Council, 1988). Total solids content generally decreases as cows age, while mammary infections (most commonly sub-clinical mastitis) also lead to reduced milk yield and milk solids yield (Gonçalves *et al.*, 2016) and incur high costs to both processors and farmers (More *et al.*, 2010). High somatic cell count, along with leakage of blood constituents and ionic species through the secretory cells of the mammary gland, renders mastitic milk compositionally unsuitable for processing (Auldist & Hubble, 1998; Atroshi *et al.*, 1996).

1.1.3.3. Seasonality and stage of lactation

Table 1.1.4 shows the variation in the gross composition of milk which occurs throughout lactation in a seasonal grass-based milk production system, typical of the Irish manufacturing milk pool. The compositional variation which occurs throughout the lactation period and between seasons is a major challenge to milk processors. The pattern of milk production for the Irish manufacturing milk pool largely reflects the trend in grass growth rates, leading to greater volumes of milk in mid-lactation and lower volumes in early and late-lactation. This can therefore affect milk quality at these times, through possible inclusion of colostrum in spring bulk milk or high volumes of late-lactation milk in autumn and winter. Colostrum produced immediately *post-partum* is characterised by a considerably high total solids content (about twice that of standard bulk milk), containing elevated levels of protein (particularly whey proteins due to significantly greater immunoglobulin content), fat, casein and ash, but low levels of lactose (McGrath *et al.*, 2016). This compositional difference is sufficient to lead to markedly reduced heat stability and increased viscosity, making colostrum

inappropriate for processing (McGrath *et al.*, 2016; Tsioulpas *et al.*, 2007), particularly under standard conditions.

Table 1.1.4: Change in major compositional variables (%) of Holstein-Friesian milk throughout lactation from a seasonal grass-based production system.

%	Early-lactation	Mid-lactation	Late-lactation	Yearly average
Total solids	13.60	13.56	14.58	13.95
Protein	3.33	3.51	3.89	3.65
Fat	4.56	4.46	4.90	4.65
Lactose	4.98	4.92	4.75	4.87
Casein	2.66	2.78	3.31	2.95
Whey	0.48	0.54	0.65	0.56

Adapted from O’Callaghan *et al.* (2016a).

Milk composition gradually tends towards normal over the course the first week *post-partum*, with overall yield increasing and consequently, fat and protein content decreasing over the following 5-7 weeks (Silvestre *et al.*, 2009). Throughout the remaining lactation period, the inverse is observed, where milk yield gradually declines as fat and protein content increases, with lactose content remaining consistent, before declining in late lactation (Hettinga, 2019), likely in response to increasing flux of ionic species from blood into the milk (Fox *et al.*, 2015). Farmers paid on milk quality payment schemes can be required to meet minimum lactose content requirements in order to limit the volume of late-lactation milk in the supply.

The declining heat stability of late lactation milk is also influenced by increasing milk pH (up to pH 7.0 or greater) (Fox *et al.*, 2016), increasing total protein content and decreasing casein:whey protein ratio, along with increasing levels of ionic species such as sodium (Na⁺), chloride (Cl⁻) and particularly ionic calcium (Ca⁺⁺) (Fox & McSweeney, 2013). These factors also lead to reduced cheese yields and, hence, limit cheese production in late lactation. Similarly, production of butter is affected by the use of late lactation milk, whereby the increasingly saturated FA profile (Samková *et al.*, 2012) results in a harder, less spreadable butter (O’Callaghan *et al.*, 2016b). Lactational and seasonal effects are limited by the extended

calving pattern utilised in the Irish liquid milk pool, where variations in milk composition are reduced through the bulking effect of continually supplied mid-lactation milk.

1.1.3.4. Cow diet

In the context of Irish milk composition, a notable factorial confluence exists between lactation stage, seasonality and cow diet, given the seasonal production pattern utilised thereby. As previously mentioned, the lactation curve reflects that of grass growth, allowing production of high volumes of mid-lactation milk at the period of maximal grass growth. For the manufacturing milk pool, dietary supplementation of conserved forage such as silage or hay, along with cereal concentrates, is practiced throughout late lactation, when seasonal changes necessitate indoor housing of cows, and up to the beginning of the following lactation after the drying-off period. This combined effect may lead to a notable difference in milk composition from countries where pasture-based milk production is widespread, in comparison to those which typically utilise conventional TMR systems. The importance of country-specific information on milk composition to milk processors and consumers has previously been highlighted by Schönfeldt *et al.* (2012).

1.1.4. Conclusion

Bovine milk is a highly complex dispersion of nutritionally significant components, which is subject to compositional variation due to a variety of factors, the most notable from an Irish perspective being stage of lactation and feed composition, which can vary substantially between regions and throughout the year. Various feed types have been investigated in previous studies, which primarily focused on the gross composition of milk. The effect of dietary factors on the composition and functional properties of milk and milk-derived products will be discussed through review of existing literature in Chapter 1 (Part 2).

Chapter 1 (Part 2)

Compositional and functional properties of milk and dairy products derived from cows fed pasture or concentrate-based diets

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Published paper shown in Appendix ii.

1.2.1. Abstract

Worldwide milk production is predominantly founded on indoor, high-concentrate feeding systems, whereas pasture-based feeding systems are most common in New Zealand and Ireland, but have received greater attention recently in countries utilising conventional systems. Consumer interest in “pasture-fed” dairy products has also increased, arising from environmental, ethical and nutritional concerns. A substantial body of research exists describing the effect of different feeding strategies on the composition of milk, with several recent studies focusing on the comparison of pasture and concentrate-based feeding regimes. Significant variation is typically observed in the gross composition of milk produced from different supplemental feeds, but various changes in the discrete composition of macromolecular components in milk have also been associated with dietary influence, particularly in relation to the fatty acid profile. Changes in milk composition have also been shown to have implications for milk and dairy product processability, functionality and sensory properties. Methods to determine the traceability of dairy products or verify marketing claims such as “pasture-fed” have also been established, based on compositional variation due to diet. This review explores the effects of feed types on milk composition and quality, along with the ultimate effect of diet-induced changes on milk and dairy product functionality, with particular emphasis placed on pasture and concentrate-based feeding systems.

1.2.2. Introduction

Milk provides a comprehensive source of nutrition for mammalian neonates. Although milk composition varies substantially between species, the milk of other species can be consumed by mature humans and children of sufficient renal development (Ziegler, 2007), providing a greater distribution of essential macro and micro nutrients than most other food sources (Drewnowski, 2005). Thus, milk has been produced from livestock for human

consumption since antiquity (Silanikove, Leitner, & Merin, 2015), with bovine milk being the most commercially viable for large-scale production.

As ruminants, cows produce milk primarily from the consumption of forageable grasses, making pasture-based milk production the most historically prevalent system. Modern dairy farming practices founded on increasing mechanisation and the availability of alternative feed sources allow for greater scale and intensification and have led to the widespread utilisation of indoor total mixed ration (TMR)-based milk production systems, accounting for approximately 90% of global milk production (Coleman *et al.*, 2009). These systems are widely implemented in the USA and large-scale European producers such as France and Germany (van Arendonk & Liinamo, 2003). By virtue of the indoor housing used in TMR systems, producers can also prevent exposure of their livestock to weather extremes and maintain year-round production in harsher climates (Arnott *et al.*, 2017).

Conversely, pasture-based milk production systems remain dominant in Ireland and New Zealand, where mild, temperate climates and abundant rainfall allow for consistent grass growth throughout the majority of the year and potentially up to 300 days of grazing per year (O'Donovan *et al.*, 2011). This offers an economical source of feedstuff, while also allowing the cow to perform their natural foraging behaviour outdoors. Approximately 85% of milk supply in Ireland is produced on a seasonal basis, forming the “manufacturing milk” pool (Hennessy & Roosen, 2003), and allowing for a correspondence between the availability of grass throughout the year and the lactation cycle of the cow. This results in most dairy herds calving within a narrow window in the spring months and a period of maximal milk production in the summer months, gradually decreasing in the same pattern as the yield of the individual cow into the winter (O'Connell *et al.*, 2015).

The challenge posed by the disparity in milk supply volume in Ireland between the summer and winter months is addressed through the focus of the industry on the production of

long shelf-life commodities such as butter and cheese, which maximises the economic value of a seasonal milk supply. An emphasis on the production of harder cheese varieties using mid-lactation milk (particularly Cheddar) is also necessitated by the unsuitability of late lactation milk for processing into cheese (Lucey & Fox, 1992). A continuous year-round supply of milk which is appropriate for further processing is produced by suppliers who contribute to the “liquid milk” pool, forming the remaining 15% of the total Irish milk supply. This necessitates a broader calving distribution throughout the year in order to consistently meet the market demand for short shelf-life products such as liquid milk, cream and infant milk formula throughout the winter months. Farmers are incentivised to continue production in this way by a “Winter milk” payment scheme, which offers a premium price on off-peak milk to offset the higher cost of milk production based on conserved forage in winter months (Hopps & Maher, 2007).

Although pasture-based milk production is generally established on exclusively grass-based swards, the inclusion of white clover within the sward is widely practiced and has been shown to improve cow performance in terms of milk yield and milk solids yield (Egan *et al.*, 2015), along with fixing N₂ in the soil (Ledgard *et al.*, 2001). Typically, Irish milk processing co-operatives pay farmers on the basis of milk solids mass (kg of protein + kg of fat), which incentivises farmers to produce milk with higher protein and fat contents (Sneddon *et al.*, 2013). Milk solids composition is influenced by a variety of factors, leading to substantial variation in the nutritive values and functionalities of the diverse range of end-products derived from milk.

Dietary-based interventions have been widely investigated and have highlighted significant implications for milk product processability (Gulati *et al.*, 2019a; Barłowska *et al.*, 2012), traceability (O’Callaghan *et al.*, 2018; Coppa *et al.*, 2012) and sensory (Bendall, 2001; Kilcawley *et al.*, 2018; Clarke *et al.*, 2019) and nutritional quality (O’Callaghan *et al.*, 2016a;

Benbrook *et al.*, 2013; Kelly *et al.*, 1998). Considering this, the objective of this review is to outline the effect of bovine feeding systems on the composition and functional properties of a variety of milk products, with a particular focus on perennial ryegrass, perennial ryegrass/white clover and indoor total mixed ration-based systems. A graphical summary of notable effects of pasture and indoor, concentrate-based feeding systems on milk product composition and functionality is shown in Fig. 1.2.1.

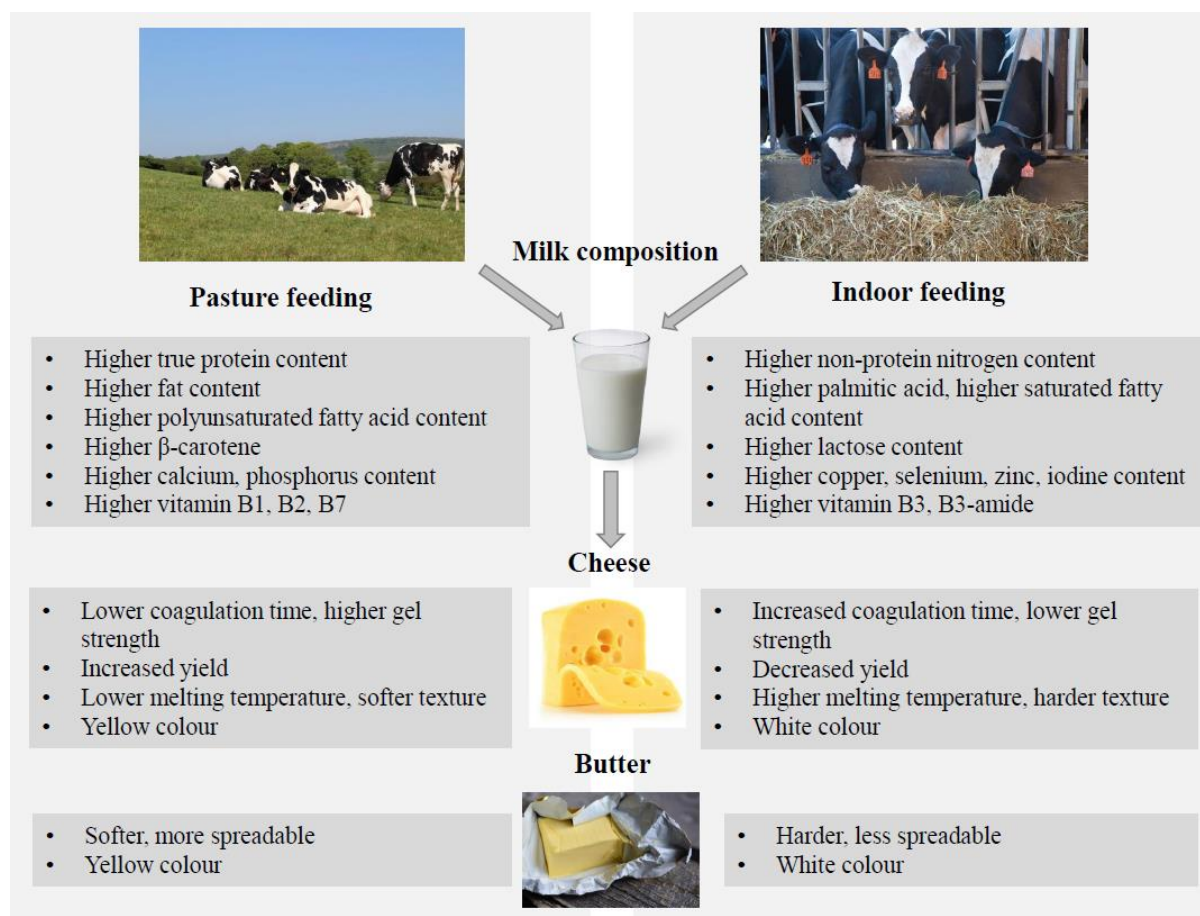


Figure 1.2.1: Summary of notable effects of pasture and indoor concentrate feeding on milk composition and product functionalities.

1.2.3. Milk, nutrition and recent trends in dairy consumption

1.2.3.1. *Nutritional significance of milk*

Milk is regarded to function as a “complete food”, necessitated by the requirement of the neonate to receive complete nutrition from milk alone. Despite this optimisation for the neonate, the extensive balance of nutrients in milk makes it an ideal source of continued nutrition into adulthood (Pereira, 2014). As a complex colloidal dispersion of fat, protein, lactose and soluble salts, milk can contribute to a range of daily human dietary requirements. A 500-mL portion of whole milk can provide significant proportions of daily fat, protein, vitamin and mineral requirements (Haug, Høstmark, & Harstad, 2007), with concentrated end-products such as butter or cream (fat), casein or whey powders (protein) and cheese (fat and casein protein) offering even greater quantities of these respective nutrients (Finglas *et al.*, 2014).

Milk is a particularly good source of calcium, magnesium and potassium, minerals which are considered to be widely under-consumed in adult diets (Decker & Park, 2010). Milk also contains water-soluble vitamins in high concentrations and is an important source of thiamine (B1), riboflavin (B2) and cobalamin (B12). Depending on fat content, vitamins A and D can also be present in high concentrations in milk or milk-derived products (Fox *et al.*, 2015). Milk is a particularly important source of cobalamin, as it is almost exclusively an animal-derived vitamin and cannot be acquired from most plant sources (Herbert, 1988). A 500-mL serving of whole milk provides up to 90% of the daily adult cobalamin requirement (Haug, Høstmark, & Harstad, 2007).

The relationship between calcium consumption and bone development from childhood to adulthood is well documented (Peterlik, Kállay, & Cross, 2013; Ondrak & Morgan, 2007). Matkovic *et al.* (2004) observed an association between increased dairy product consumption

and increased volumetric bone mineral density in adolescent females, particularly in relation to spinal development, whereas calcium supplementation did not have a comparable effect. Milk and dairy products have also been suggested to have an important role in geriatric nutrition as a readily available source of protein for maintenance of muscle tissue (Philips, Tang & Moore, 2009) and a concentrated source of calcium to prevent the development of osteoporosis (Elbon, Johnson, & Fischer, 1998). Massler (1979) reported that diminishing secretion of hydrochloric acid in the stomachs of elderly individuals may limit mineral absorption in the small intestine, also suggesting that calcium absorption may be promoted through the consumption of acidified dairy products such as yoghurt and cottage cheese. However, it is generally accepted that sufficient intake of calcium in childhood and adolescence is a greater contributing factor to bone mineral density in the elderly than milk consumption in later life (Wadolowska, 2013; Renner, 1994).

1.2.3.2. Trends in milk and dairy product consumption

Despite the increasingly positive perception of the potential health effects of regular intake of milk and dairy products (Ebringer, Ferenčík, & Krajčovič, 2008; Pereira, 2014), negative consumer attitudes towards milk remain widespread (McCarthy *et al.*, 2017), reflected in trends of decreasing milk consumption in many developed countries (Zingone *et al.*, 2017). Some consumers have recently opted for a diet completely free of dairy products, though this decision may often be influenced by ethical concerns, such as in the practice of vegan diets. In these cases, food fortification or supplementation of omega-3 polyunsaturated fatty acids (Russell & Meital, 2018) and essential trace elements such as vitamin D (Dunn-Emke *et al.*, 2005), calcium and particularly cobalamin (US Department of Agriculture; US Department of Health and Human Services, 2010) is often needed to meet advised daily nutritional requirements. Despite the recent overall decrease in liquid milk consumption in developed

countries, per-capita consumption of dairy products is increasing (OECD, Food, & Nations, 2018). This is primarily attributable to the widespread popularity of added-value products such as whey protein powders, strained yoghurt and convenient cheese products, and may be influenced by the continually changing understanding of the potential health benefits of dairy consumption (OECD, Food, & Nations, 2018).

1.2.3.2.1. *Lactose intolerance and milk allergy*

Despite the increasing consumption of dairy products, approximately 75% of the world population remains intolerant to lactose (Suarez, Savaiano, & Levitt, 1995), with the highest incidences of intolerance found among East Asian, African and Native American populations (Silanikove, Leitner, & Merin, 2015). The activity of the β -galactosidase (lactase) enzyme necessary to hydrolyse lactose to glucose and galactose does not typically persist into adulthood (Suarez, Savaiano, & Levitt, 1995), though the historical prevalence of milk products in northern and continental Europe has led to a wide distribution of lactase persistence within populations in these regions (Sahi, 1994), where as little as 2% of the population now experiences lactase deficiency (Heyman, 2006). Milk products do, however, remain widely consumed in many areas in which lactose intolerance is widespread, primarily as products in which the lactose has been hydrolysed (e.g. fermented milk/yoghurt) or removed (e.g. hard cheese varieties) (Silanikove, Leitner, & Merin, 2015). In more severe cases, an individual may be allergic to milk proteins, most commonly the whey proteins β -lactoglobulin and α -lactalbumin, or α_{S1} -casein (Lifschitz & Szajewska, 2015).

The recent popularity of raw milk consumption may partly be predicated on the suggestion of reduced allergenicity associated with raw milk when compared to pasteurised milk (Sozańska, 2019). This may in part be due to misinterpretation of the subject of the PARSIFAL study by Waser *et al.* (2007) which observed an inverse association between

consumption of farm milk and asthma and allergy. The farm milk referenced in this study may have been widely confused for raw milk, though the authors indicate that the status of the farm milk as raw could not be determined. It is widely accepted that any potential health benefits of raw milk consumption are outweighed by its pathogenic risk factors (Ombarak *et al.*, 2016; Lucey, 2015; Claeys *et al.*, 2013).

1.2.3.2.2. A1 & A2 milk

Recently, the qualitative difference in the casein composition of milk has received attention regarding the presence of the A1 or A2-type genotypes of β -casein, the sequences of which differ by a single amino acid (European Food Safety Authority, 2009). It has been suggested that the presence of histidine in the A1 sequence and subsequent release of β -casomorphin-7 (BCM7) may lead to increased risk of Type-1 diabetes, cardiovascular disease or even schizophrenia (Sodhi *et al.*, 2012). Pal *et al.* (2015) also stated that BCM7 may be responsible for many instances of milk intolerance through immunomodulatory or proinflammatory effects, opposing the singular association between lactose and milk intolerance. Substitution of histidine with proline in the A2 sequence is suggested to prevent the enzymatic hydrolysis required to release BCM7 (European Food Safety Authority, 2009).

Reviews carried out by Parashar & Saini (2015) and Truswell (2005) propose that little evidence exists to support the suggested risks of A1 milk consumption. However, a randomized controlled study carried out by Sheng *et al.* (2019) comparing gastrointestinal symptoms in Chinese preschool children consuming conventional milk (containing A1 and A2 β -casein variants) or milk containing the A2 β -casein variant only reported improved gastrointestinal symptoms and cognitive performance in children consuming the A2-only variant. This study provides some evidence to support the difference in pathological effects between A1 and A2 milk, but is limited to preschool children in a population group in which dairy consumption is

particularly low. There is certainly considerable scope for investigation of the potential effects of A1 and A2 milk consumption in older groups and among populations where dairy consumption is more widespread. The A1 and A2 variants of β -casein are genetic products and, therefore, their relative abundance in milk will vary between species and breeds of cow, but are unlikely to vary substantially depending on the use of different feeding systems, which are discussed throughout this review.

1.2.3.2.3. “Pasture-fed” dairy

As many consumers continue to make more conscious dietary considerations, interest in pasture-derived milk and dairy products is increasing (Getter *et al.*, 2015). Animal welfare, environmental impact and health concerns are reported by Conner & Oppenheim (2008) as contributory motivators to the choice of pasture-derived milk products over conventionally-derived milk products among American consumers, who are also willing to pay a premium price for products of known pasture-based origin. Health-based marketing of pasture-derived products is often focused on the fatty acid profile of milk, with particular attention directed to conjugated linoleic acid and omega-3 fatty acids such as α -linolenic acid (Clancy, 2006). Labelling such as “pasture-fed” or “pasture-raised” is used widely for milk products in the USA and may be conflated with organic milk by consumers, which was also reported as experiencing substantial yearly growth by Conner & Oppenheim (2008), who identify it as a subset of pasture-raised milk.

However, organic dairy farming standards only require cows to spend a minimum of 120 days per year at pasture (Liu *et al.*, 2018; US Department of Agriculture, 2000) and larger, higher producing organic dairy farms in the USA tend to utilise proportionally lower average levels of pasture-derived forage (McBride & Greene, 2009). In contrast, cows spend an average of 240 days per year at pasture on standard grazing operations in Ireland, where grass

constitutes at least 90% of the diet (Bord Bia, 2020). Seasonal changes which arise in pasture-based milk production systems are largely dependent on dietary factors, the effects of which on the composition and functional properties of milk and milk-derived products will be the focus of the remainder of this review.

1.2.4. Impact of feeding system on milk composition

1.2.4.1. Protein and amino acids

1.2.4.1.1. Milk protein yield, crude protein and true protein

The differences in gross milk composition observed between cow breeds are of considerably greater magnitude than those which can be achieved through dietary interventions and, although genetic effects are the primary determinants of the amino acid (AA) composition of proteins, changes to cow diets nonetheless have a notable influence on concentrations of gross milk proteins and can be more readily altered by the farmer. A summary of the literature discussed in this section is shown in Table 1.2.1. In previous decades, attempts at altering crude protein (CP) levels in milk were dependent on manipulation of both the protein content and energy supply of supplemented feedstuffs (Emery, 1978; Thomas, 1984; DePeters *et al.*, 1985; Coulon & Rémond, 1991; Stockdale, 1994; Khalili *et al.*, 2002; Broderick, 2003; Patton *et al.*, 2006).

Other important considerations for planning dietary regimes which were identified include the level of energy supplied through dietary fibre (Emery, 1978) and the source of energy used in the supplied feed, such as the addition of rapeseed meal or field pea (Khalili *et al.*, 2002) or soybean meal (Broderick, 2003). Despite the high energy-density of lipids, supplementation of dietary fats and oils has been observed to decrease milk protein and casein content (Carroll *et al.*, 2006). DePeters *et al.* (1985) recorded a decrease in milk protein and

casein content and increase in non-protein nitrogen (NPN) content in response to increased dietary supplementation with whole cottonseed (high fat content). Even as far back as four decades ago, Emery (1978) suggested that increased milk protein concentration is not determined by caloric load alone, but may rather be dependent on feed quality, particularly carbohydrate content. Due to the high starch content and metabolic availability of maize, Stockdale (1994) suggested that a ration high in supplemented maize silage would be optimal for provision of high energy levels for increased milk protein production. Indeed, a study by Dalley *et al.* (2019) observed a relatively high crude protein content of 3.99% for Holstein-Friesian cows fed on pasture supplemented with maize silage, though this was lower than that of milk of cows fed on pasture supplemented with fodder beet (4.3%). Patton *et al.* (2006) observed increased protein yield while feeding a high-energy diet consisting of grass silage and corn silage with a high concentrate inclusion; however, this effect was less substantial than the effect of reducing daily milking frequency from three to one. Considering these studies, it seems reasonably well defined that milk protein content can be influenced by providing high levels of dietary energy through feeding high quality carbohydrate.

Table 1.2.1: Summary of significant dietary effects on milk composition.

Component	Dietary factor	Effect relative to comparison diets	References
Protein	Grass and clover feeding	Increase in protein / true protein %.	O'Callaghan <i>et al.</i> (2016a), Gulati <i>et al.</i> (2018a)
	Clover and concentrate feeding	Increase in non-protein nitrogen	O'Callaghan <i>et al.</i> (2016a), Magan <i>et al.</i> (2019b)
	Fat supplementation	Decrease in protein content	Carroll <i>et al.</i> (2006)
	Maize feeding	Increase in protein content	Patton <i>et al.</i> (2006)
	Excess crude protein intake	Decrease in protein content	Colmenero & Broderick (2006)
Fat	Maize silage and fodder beet feeding	Increase in fat %, but decrease with excess fodder beet feeding	Dalley <i>et al.</i> (2019)
	Grass feeding	Increase in fat %	O'Callaghan <i>et al.</i> (2016a)
	Concentrate feeding	Increase in fat yield	O'Callaghan <i>et al.</i> (2016a), Gulati <i>et al.</i> (2018a)
	Overfeeding of fat or concentrates	Decrease in fat %	Flachowsky <i>et al.</i> (2006), Broderick (2003)
	Grass and clover feeding	Increase in polyunsaturated fatty acid and conjugated linoleic acid content	Chilliard <i>et al.</i> (2007), Couvreur <i>et al.</i> (2006), O'Callaghan <i>et al.</i> (2016a)
	Concentrate feeding	Increase in palmitic acid content	O'Callaghan <i>et al.</i> (2016a)
	Lipid supplementation	Increase in long-chain fatty acid content and decrease in <i>de novo</i> fatty acid content	Shingfield <i>et al.</i> (2003), Chilliard <i>et al.</i> (2009), Hoffman <i>et al.</i> (2016)
Lactose	Clover and concentrate feeding	Increase in lactose yield	Harris <i>et al.</i> (1998), Panthi <i>et al.</i> (2019), O'Callaghan <i>et al.</i> (2016a).
	Concentrate feeding	Increase in lactose yield and %	Gulati <i>et al.</i> (2018a)
Minerals	Concentrate feeding	Increase in zinc, copper and selenium concentrations	Gulati <i>et al.</i> (2018a), Rey-Crespo <i>et al.</i> (2013)
	Grass feeding	Increase in calcium and phosphorus concentrations	Gulati <i>et al.</i> (2018a)
	Fat supplementation	Increase in concentration of magnesium and decrease in concentration of phosphorus	Carroll <i>et al.</i> (2006)
Vitamins	Organic production	Increase in riboflavin content	Poulsen <i>et al.</i> (2015)
	Grass and clover feeding	Higher thiamine, riboflavin and biotin content	Magan <i>et al.</i> (2020)
	Concentrate feeding	Higher nicotinic acid and nicotinamide content	Magan <i>et al.</i> (2020)
	Grass and grass silage feeding	Increase in retinol content	Agabriel <i>et al.</i> , (2007)
	Maize feeding	Increase in folate and cobalamin content	Chassaing <i>et al.</i> , (2011)

A positive correlation between supplementation of corn grain with canola meal and milk protein concentration was also observed by Auld *et al.* (2016b) across a series of four feeding strategies applied to grazing cows supplemented with formulated grain mix or partial mixed rations. Notably, this occurred across all four feeding strategies, in which levels of metabolizable energy were kept the same. Mackle *et al.* (2000) proposed that an increase in dietary energy content promoted increased N synthesis by rumen microbiota, thus increasing milk protein content, although recent research has suggested that more specifically, this may be dependent on the supply of particular AA, which will be discussed in section 1.2.4.1.3. A study by Zimmerman *et al.* (1991) also investigated the effect of dietary CP levels on milk production and compositional parameters, noting that total milk yield (kg/day) and milk CP yield increased with increasing dietary protein; however, it is important to note that the percentage protein in the milk remained the same.

Overfeeding of dietary CP can, however, result in a decline in milk protein content. Leonardi *et al.* (2003) found that milk protein content decreased from 3.24 to 3.18% when dietary CP content was increased from 16.1 to 18.8 % of DM. Excess dietary protein was demonstrated by Colmenero & Broderick (2006) to limit the nitrogen conversion efficiency of rumen microflora, resulting in increased urinary excretion of nitrogen and, consequently, depressed nitrogen secretion in milk. The authors proposed a dietary CP content of 16.5% for optimal milk production and minimal urinary nitrogen losses. Notably, this study involved a high proportion of concentrate inclusion prior to the substitution of corn silage with soybean meal to increase protein content in the ration, which would suggest that the observed decrease in milk protein occurred in spite of the high level of energy intake attributable to the level of concentrates in the ration. An additional consideration for overfeeding of CP is the effect of excessive N excretion by the cow on subsequent water pollution (Kalscheur *et al.*, 1999). This

is an effect of considerable relevance in the efforts to move towards more sustainable farming and dairy production practices, particularly as global herd numbers increase.

The influence of varying dietary forage and forage:concentrate ratios on percentage milk protein has been more widely investigated in recent years (Harris *et al.*, 1998; Mackle *et al.*, 1999; Couvreur *et al.*, 2006; McAuliffe *et al.*, 2016; O’Callaghan *et al.*, 2016a; Gulati *et al.*, 2018a; Panthi *et al.*, 2019). Harris *et al.* (1998) investigated the effect of varying the level of white clover in perennial ryegrass fed to cows housed indoors, and found milk protein concentrations did not change at 200, 500 and 800 g white clover per kg dry matter (DM), both at restricted and *ad-libitum* feeding levels, despite increased milk yields, which were attributed to the high nutritional value of white clover. Similarly, Mackle *et al.* (1999) found no significant differences in the CP content of milk derived from three groups of cows assigned to diets comprising pasture only, pasture supplemented with maize silage, and pasture supplemented with maize and grass silage. Total casein content was also unaffected, but the β -casein level and casein:crude protein ratio increased significantly with maize and grass silage supplementation. This effect would certainly seem to warrant investigation in further studies, as it is unusual that individual protein components would be directly affected by dietary changes. A curvilinear increase in milk yield and milk protein content was observed by Couvreur *et al.* (2006) as a function of increasing fresh grass (0, 30, 60 & 100% DM) in place of corn silage, though each group also received varying ratios of soybean:cereal concentrate in a 3 kg supplemental mixture. The increase in percentage milk protein was significant from 0 – 30% DM but again, what seems of note is that this increase in milk protein content plateaued at the higher supplementation rates.

Several recent studies have been undertaken using milk derived from three specific feeding systems at the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Ireland. Cows were randomly assigned to the following feeding systems:

Group 1 was housed indoors and fed a TMR diet (8.3 kg of concentrates, 7.15 kg of grass silage and 7.15 kg of maize silage on a DM basis), Group 2 was maintained outdoors at pasture consuming ~18 kg DM/day of perennial ryegrass (GRS) and Group 3 was also maintained outdoors consuming ~18 kg DM/day of perennial ryegrass/white clover pasture (CLV) with an average annual white clover content of 24%. The TMR formulation and chemical composition of the concentrate used by O’Callaghan *et al.* (2016a) is shown in Table 1.2.2. Table 1.2.3 shows the chemical composition of the grass silage and maize silage used in the TMR ration and Table 1.2.4 shows the chemical composition of the GRS and CLV swards. The values presented in Tables 1.2.2, 1.2.3 and 1.2.4 are typical of the diets used in each of the studies based on the TMR, GRS and CLV feeding systems implemented at Teagasc Animal and Grassland Research and Innovation Centre. The TMR diet provided higher concentrations of crude protein and a higher dry matter intake, relative to the GRS and CLV systems, while the CLV system also provided higher dry matter intake and protein digestibility than the GRS only diet. The research herds used in these studies represent best practice commercial milk supplies from different production systems; the GRS and CLV herds represent typical pasture-based systems, whereas the TMR herd represents a conventional indoor and concentrate-based system.

Table 1.2.2. Typical ingredient formulation (% as fed) and chemical composition (%) of TMR diet (Megalac concentrate sourced from Volac Ireland, Co. Cavan, Ireland), adapted from O'Callaghan *et al.* (2016a).

TMR ingredient	(% of ration)
Soybean meal (48% crude protein)	30.00
Molassed beet pulp	15.50
Rolled barley	15.00
Maize	13.00
Maize distillers	12.00
Rapeseed meal	7.50
Megalac	3.30
Maize/beet mineral balancer	2.50
Acid buffer	0.70
Salt	0.50
Chemical composition (%)	(%)
Organic matter	93.50 ± 0.94
Dry matter	86.76 ± 0.75
Moisture	13.24 ± 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
Neutral cellulose plus gamanase digestibility	83.35 ± 1.15

Table 1.2.3. Chemical composition (g/kg of DM; mean \pm SD) and nutritional content of silages from TMR diet (grass silage and maize silage) collected weekly throughout analyzed by near-infrared spectroscopy, adapted from O'Callaghan *et al.* (2016a).

Component	Grass silage	Maize silage
Dry matter	389.37 \pm 61.35	343.03 \pm 43.45
Organic matter	917.94 \pm 7.45	972.53 \pm 3.22
Acid detergent fibre	296.82 \pm 23.40	NA
Neutral detergent fibre	452.02 \pm 39.31	434.80 \pm 49.57
Crude protein	114.55 \pm 12.55	68.97 \pm 9.91
Starch	NA	285.37 \pm 28.81
Ash	82.06 \pm 6.75	27.47 \pm 3.22

NA = not available.

Table 1.2.4. Chemical composition (g/kg of DM; mean \pm SD) and nutritional content of pasture systems (grass and clover) collected weekly throughout lactation, analyzed by near-infrared spectroscopy, adapted from O'Callaghan *et al.* (2016a).

Component	Grass	Clover
Organic matter	928.00 \pm 9.31	931.49 \pm 7.18
Organic matter digestibility	764.43 \pm 19.34	769.22 \pm 18.97
Acid detergent fibre	218.89 \pm 16.91	220.67 \pm 14.05
Neutral detergent fibre	427.62 \pm 23.83	423.46 \pm 18.94
Crude protein	210.90 \pm 23.71	220.67 \pm 14.05
Ash	72.00 \pm 9.31	68.51 \pm 7.18

From the Teagasc research group, McAuliffe *et al.* (2016), O'Callaghan *et al.* (2016a), Gulati *et al.* (2018a) and Panthi *et al.* (2019) consistently observed significantly higher concentrations of total protein in milk produced from both the GRS and CLV systems when compared to the TMR system. O'Callaghan *et al.* (2016a) also observed the highest true protein content of the three diets in GRS-derived milk. Contrasting results were earlier found by Schroeder *et al.* (2003), where the total protein content of milk from TMR-fed cows was significantly higher than two groups of pasture-fed cows supplemented with corn-based concentrate and a combination of corn-based concentrate with calcium salts of fatty acids,

although the true protein content of each type of milk was not determined. In addition, O'Callaghan *et al.* (2016a) and Gulati *et al.* (2018a) found that milk yield and milk protein yield were higher for cows assigned to the TMR system than both the GRS and CLV systems. This may imply that milk yield and milk solids yield may be primarily influenced by the overall energy density of the feed, whereas percentage milk protein and quality is more dependent on levels of dietary roughage, higher proportions of which have been suggested by Couvreur *et al.* (2006) to increase propionic acid content in the rumen, thus modifying energy supply to the udder. The effect of lactation stage was also considered by O'Callaghan *et al.* (2016a) and Gulati *et al.* (2018a), who both found higher total protein concentrations in late-lactation milk than in early or mid-lactation, though, again, this may be augmented by variability in the quality of forage consumed throughout the lactation cycle.

The availability and quality of forage are quite variable depending on climatic and seasonal factors. Consumption of grasses which mature later in the growing season has been shown to significantly increase milk protein yield and milk protein concentration (Gowen *et al.*, 2003). Likewise, white clover, which matures more slowly than perennial ryegrass and is more sensitive to low soil temperatures, can be included within a grass sward to increase milk yield and milk solids yield, although percentage milk protein is similar to that of grass-derived milk (Egan *et al.*, 2015; Hennessy *et al.*, 2018).

Overall, there has been a multitude of well defined studies performed relating diet to milk CP; however, the exact biochemical mechanisms for how dietary factors translate to changes in milk CP still remains to be fully elucidated.

1.2.4.1.2. *Milk urea and non-protein nitrogen*

Among the components of NPN in milk, urea is the most abundant, accounting for approximately 50% of NPN, which itself represents 3 – 5% of the total nitrogen content of milk. Creatine, free amino acids, uric acid, phospholipids, peptides and organic acids constitute

the remaining 50 % of NPN. These nitrogenous compounds are transferred from the cow's blood to milk following protein metabolism (Ruska & Jonkus, 2014) and, despite representing a small proportion of the overall nitrogen content of milk, the concentration of milk NPN is an important indicator of milk protein quality.

O'Callaghan *et al.* (2016a), O'Callaghan *et al.* (2018) and Magan *et al.* (2019b) found higher concentrations of NPN and urea, in CLV-derived milk, when compared to GRS and TMR. Harris *et al.* (1998) also observed increased blood and milk urea in response to increasing white clover content in feed. This is a direct dietary effect arising from the uptake of soil N by N-fixing bacteria present in the root nodules of white clover. O'Callaghan *et al.* (2016a) and O'Callaghan *et al.* (2018) also observed lower concentrations of NPN and urea, respectively, in GRS-derived milk in comparison to TMR-derived milk, but contrasting results were reported by Mackle *et al.* (1999), who observed both higher NPN and urea contents in milk from cows fed pasture only, when compared to those receiving pasture supplemented with maize grain and pasture with maize grain and pasture silage. The group consuming pasture only received the highest CP and lowest metabolizable energy intakes in this case, which may indicate that increasing the energy intake rather than CP intake may have a more favourable effect in reducing milk NPN content. However, it is unusual that the true protein content (Total nitrogen - NPN x 6.38) of milk did not vary between feeding systems in this study, considering the differences observed for NPN.

The milk urea nitrogen (MUN) content of milk from pasture fed cows was also found to decrease with concentrate supplementation by Bargo *et al.* (2002), whereas conflicting results were again reported by Utama *et al.* (2018) and Magan *et al.* (2019a). In the former study, MUN content increased significantly with increasing concentrate:forage ratio and, in the latter, TMR-derived skim milk powder had significantly higher NPN content than that produced from GRS-derived milk. This variation in MUN between studies may be influenced

by differences in the forage quality or pasture composition and specific composition of concentrate rations used in each study. Similar to CP content, the relative levels of MUN and NPN appear to be influenced by the quality of dietary carbohydrate content and the metabolizable energy provided thereby. However, this also seems to be directly increased by increased N supply in the feed.

1.2.4.1.3. *Amino acid (AA) composition*

An effect of dietary factors on the AA profile of milk has not been conclusively determined. Rather, dietary alterations are understood to affect ruminal AA composition and flow of AA to the duodenum (Overton *et al.*, 1995; Pacheco-Rios *et al.*, 1999; Li *et al.*, 2012) and, consequently, availability of precursory AA to the mammary gland, influencing total milk protein yield and concentration (Li *et al.*, 2012; O’Callaghan *et al.*, 2018), but not the relative proportions of milk AA.

Methionine and lysine have been shown to be the primary limiting AA in milk protein production, and the effect of their dietary supplementation (particularly in rumen-protected form) on milk production has been widely investigated (Socha *et al.*, 2005; Wang *et al.*, 2010; Lee *et al.*, 2015). The efficiency at which these limiting AA are utilised for milk protein production has been shown to increase with decreasing dietary availability of metabolizable protein (Socha *et al.*, 2005; Lee *et al.*, 2015), indicating a stabilising effect in response to low CP in the diet. O’Callaghan *et al.* (2018) found increased concentrations of methionine and L-lysine in the rumen fluid of cows fed GRS and CLV diets in comparison to those fed a TMR diet, a relationship which reflects that of the milk protein content of these diets and is supported by the findings of Leonardi *et al.* (2003), who observed an increase in percentage milk protein with methionine supplementation at two levels of dietary CP. Schwab *et al.* (1976) and Hanigan *et al.* (2002) suggested that milk protein synthesis is dependent on the supply and interaction

of multiple essential AA (cysteine, threonine, methionine, lysine, histidine, phenylalanine) to the udder, rather than individual limiting AA.

A limited effect of feeding system on the total AA composition of three types of whey powders was suggested by the results of a study by Magan *et al.* (2019a). Concentrations of cysteine, glycine, phenylalanine and valine were found to vary between GRS, CLV and TMR feeding systems, though no clear explanation for why or how the diet may affect these AA was proposed. A compositional study carried out by Lindmark Månsson *et al.* (2003) on milk collected from 9 dairies across Sweden throughout the year also suggested a possible effect of feeding changes on milk AA profile. Although significant geographic variation was only observed for total concentrations of arginine and tyrosine, total concentrations of 16 AA were found to vary significantly by season. The authors suggested that feeding strategy and feed quality may vary by region, but are also likely to vary considerably between seasons, as cows are transitioned from outdoor grazing in summer to silage and concentrate feeding in winter housing. This would seem to imply that if geographical variation in AA profile due to differences in feeding practices occurs within one country, AA profiles would also vary considerably internationally. However, the geographic variation observed in this study may also be influenced by genotypic differences, as relative numbers of particular breeds on farms contributing to dairies throughout the country may be variable.

1.2.4.2. *Fat and fatty acids (FA)*

1.2.4.2.1. *Milk fat yield/gross milk fat*

Much like milk protein, the lipid fraction of milk displays significant variation between breeds (Woodford *et al.*, 1986), seasons (Heck *et al.*, 2009) and throughout lactation (Chilliard *et al.*, 1991), and has also been demonstrated to be the milk component most susceptible to variation due to dietary interventions (Santos, 2002; Dewhurst *et al.*, 2006; Chilliard *et al.*,

2007, O'Callaghan *et al.*, 2016a); for these reasons, it has been the most thoroughly investigated component. Variation in the forage:concentrate ratio has been identified as a major dietary consideration for influencing milk FA profile (Soita *et al.*, 2005; Chilliard *et al.*, 2007; Sterk *et al.*, 2011), though various supplemental feedstuffs which can be easily introduced to the cow's diet have been shown to determine an effect on the gross content of milk fat.

Whole cottonseed feeding was shown by DePeters *et al.* (1985) to result in decreased milk protein content, but resulted in increasing fat yield and milk fat content with increasing proportions of supplemental whole cottonseed. Dalley *et al.* (2019) also observed a high milk fat content of 5.3% for Holstein-Friesian cows when receiving supplemental maize silage. This was compared to fodder beet, which increased milk fat content to 5.54% when fed as a supplement to perennial ryegrass (25% of DM intake). Increasing the fodder beet supplementation rate to 40% resulted in a decrease in milk fat content to 5.07%, which is suggested to be due to the occurrence of sub-clinical acidosis in some of the cows assigned to this diet, whereby rumen pH was reduced due to the high starch content of the supplementary fodder beet.

Incidences of sub-clinical acidosis on high-producing conventional dairy farms are commonly attributed to overfeeding of high-starch, grain-based concentrates (Nocek, 1997; Plaizier *et al.*, 2014; Fiorentin *et al.*, 2018), which provide excessive levels of ruminally fermentable carbohydrate, leading to lactic acid and volatile FA accumulation and, consequently, reduced rumen pH (Nocek, 1997; Broderick, 2003). This effect is often associated with the occurrence of milk fat depression (MFD) (Stone, 2004; Krause & Oetzel, 2006) caused by inhibition of rumen microflora and, thus, reduced biohydrogenation of dietary FA, though this may be compounded by the behavioural effects of acidosis in cows, such as reduced feed intake and the resultant insufficient nutritional status (Plaizier *et al.*, 2008). Cows in early lactation may be particularly at risk to sub-clinical acidosis for similar reasons, due to

condition loss and the high energy requirement to maintain milk production relative to feed intake *peripartum* (Doepel, *et al.*, 2002).

The occurrence of MFD is multi-faceted, as a variety of dietary factors can lead to its development, from spring grazing (Rivero & Anrique, 2015) to low dietary roughage content (Gama *et al.*, 2008), overfeeding of fat (Flachowsky *et al.*, 2006) and high concentrate inclusion (Broderick, 2003; Auldist, 2016b). For pasture-based milk production systems, MFD in spring milk arises due to the lower proportion of fibrous material in comparison to leaves and the low concentrations of triglycerides in grass at an early developmental stage (Rivero & Anrique, 2015). Attempts to mitigate MFD typically involve the use of hay or concentrate supplementation to introduce additional dietary fibre, though balancing the ration is particularly important in avoiding concentrate overfeeding, which can exacerbate the issue by depressing ruminal pH, leading to sub-clinical acidosis and inhibiting ruminal fibre digestion (Broderick, 2003), ultimately further depressing milk fat content. Rumen function and MFD can also be influenced by levels of “effective fibre”, whereby the reduced particle size of ground or pelleted fibre present in concentrate rations reduces the effectiveness of ruminal fibre digestion (Grant *et al.*, 1990).

In addition, direct supplementation of dietary fat or oils with high polyunsaturated fatty acid (PUFA) content can lead to MFD (Bauman & Grinari, 2001). The *trans-10 cis-12* isomer of conjugated linoleic acid can inhibit milk fat synthesis when present in high concentrations (Gama *et al.*, 2008). Consideration of the level of FA saturation in supplemental dietary fat was also previously highlighted by Coppock & Wilks (1991). Reduced milk fat content may nonetheless be desirable to producers who receive payment on the basis of milk yield rather than milk fat or protein content, such as those contributing to the “beverage product” class in the USA (Sneddon *et al.*, 2013) or where preference is given to milk protein content over fat content.

The importance of dietary fibre levels in preventing MFD was also stated by Zimmerman *et al.* (1991), who observed a significant increase in both milk fat yield and % with increasing dietary CP in a high fibre diet, which was attributed to increased synthesis of short-chain fatty acids (SCFA), with a less substantial effect observed for a low fibre diet. It was also noted that the extent of MFD when transitioning from high fibre to low fibre diets was reduced in cows fed grass hay when compared to alfalfa hay, which may be influenced by the lower particle size of alfalfa hay relative to grass. Other efforts to influence milk composition by increasing CP intake have produced inconsistent results for milk fat. Broderick (2003) recorded a linear increase in milk fat yield (kg/day) but not % milk fat with increasing CP content, although the inverse was seen by Colmenero & Broderick (2006) and Wildman *et al.* (2007), whereas Leonardi *et al.* (2003) observed a significant increase in both fat yield and fat content in response to increased dietary CP.

Despite the consistent results seen for protein yield and concentration in milk derived from GRS, CLV and TMR feeding systems investigated by McAuliffe *et al.* (2016), O'Callaghan *et al.* (2016a), Gulati *et al.* (2018a) and Panthi *et al.* (2019), measurements for milk fat were more variable. Both McAuliffe *et al.* (2016) and O'Callaghan *et al.* (2016a) observed that milk fat concentrations were significantly higher for GRS than both CLV and TMR, whereas Gulati *et al.* (2018a) observed significantly higher fat content in GRS than in CLV only and Panthi *et al.* (2019) recorded no difference in fat content between the diets. The similarities in the former two studies and variation in the latter may indicate that milk fat content is more responsive to variation in feed composition or forage quality between years than milk protein content.

Harris *et al.* (1998) found that, on increasing white clover content from 500 to 800 g per kg dry matter, milk fat yield increased due to increased overall milk yield, which is typically observed with increasing white clover addition, but % milk fat was unaffected. Both

O'Callaghan *et al.* (2016a) and Gulati *et al.* (2018a) also found that milk fat yield was consistently higher for TMR than both GRS and CLV and that minima and maxima % milk fat occurred in April and September, respectively, in all diets. This effect is more attributable to variation by lactation stage than to seasonal changes, as these diets were maintained throughout complete lactation cycles. However, the importance of changes in feeding regimes in the seasonal variations observed in other production systems has been shown by Heck *et al.* (2009), who determined that seasonal variation in Dutch milk composition was most likely due to dietary changes, as calving is evenly distributed throughout the year in the Netherlands. These dietary changes throughout the year would most likely constitute changes in forage availability and quality, along with decreasing forage:concentrate ratio, which would have a pronounced effect on the FA composition of milk.

1.2.4.2.2. *Fatty acid composition*

Milk FA arise from two sources, depending on chain length; short to medium-chain FA (C4:0 – C14:0) are derived from *de novo* synthesis in the epithelial cells of the mammary gland, whereas long-chain fatty acids (LCFA) are sourced from the diet of the cow or a combination of both diet and *de novo* synthesis in the case of palmitic acid (C16:0) (Knutsen *et al.*, 2018). In addition to the overt effect of diet on LCFA content, the extent of *de novo* synthesis and, thus, SCFA content, may also be influenced by dietary factors, through modulation of rumen microflora based on levels of substrate available for biohydrogenation (Palmquist *et al.*, 1993; Bauman & Griinari, 2003; Sterk *et al.*, 2011). Relative levels of saturated (SFA) and unsaturated (UFA) fatty acids are significantly influenced by dietary factors, particularly depending on forage content (Chilliard *et al.*, 2001). Indeed, apart from the supplementation of dietary fat, varying the level of pasture feeding is perhaps the most frequently practiced means of FA profile manipulation (Castillo *et al.*, 2006).

Despite recent evidence to the contrary (Drouin-Chartier *et al.*, 2016; Pereira, 2014), the widely-held negative health associations between the SFA content of milk fat and increased risk of cardiovascular disease (CVD) are likely an influence on the broad interest in the potential for alteration or improvement of milk FA composition through relatively simple means at primary production level. Although the consensus is changing surrounding the health impact of milk fat on CVD, it remains of considerable interest to reduce the levels of lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids which have been shown to have adverse effects on low-density lipoprotein cholesterol (LDL) levels, which is considered an important risk factor for CVD (Lordan, *et al.*, 2018). Thus, the effect of varying levels of pasture inclusion and pasture:concentrate ratios on milk FA composition have been extensively studied (Bär *et al.*, 2020; O’Callaghan *et al.* 2016a; Benbrook *et al.*, 2013; Chilliard *et al.*, 2007; Couvreur *et al.*, 2006; Kelly *et al.*, 1998), with generally consistent results and significant effects conclusively determined.

In comparing raw milk produced from GRS, CLV and TMR diets, O’Callaghan *et al.* (2016a) found that concentrations of a range of minor SFA (C11:0, C13:0, C15:0, C17:0, C23:0) and UFA (C18:2 n-6*trans*, C18:2 *cis*-9*trans*-11, C18:3 n-3, C20:1, C20:4 n-6) were significantly higher in both pasture-derived milks than in TMR, whereas several UFA (C18:2 n-6*c*, C18:3 n-6*c*, C22:1 n-9, C18:2 *cis*-10*trans*-12), along with the SFA C16:0 and C22:0 were found to be significantly higher in TMR milk than in both GRS and CLV. In addition, both pasture-based systems produced milk with significantly higher levels of omega-3 (n-3) FA and significantly lower levels of omega-6 (n-6) FA than the TMR system. Further studies on butter (O’Callaghan *et al.*, 2016b) and full-fat Cheddar cheese (O’Callaghan *et al.*, 2017) yielded similar results, with the largest variation between diets being amongst the medium- to long-chain FA, particularly PUFA content, n-3:n-6 ratio and concentrations of conjugated linoleic acid (CLA) isomers, α -linolenic acid (GRS & CLV > TMR), vaccenic acid (GRS & CLV >

TMR) and behenic acid (TMR > GRS & CLV). Increased concentrations of palmitic acid (C16:0), which has a high melting point (63°C), in TMR-derived milk has a significant impact on the properties of butter and cheese, which will be discussed in section 1.2.5.2.

The C18:2 *cis-9trans-11* (rumenic acid) isomer of CLA has been the subject of widespread interest in recent years for its potential anti-carcinogenic, anti-hypertensive and adiposity-mediating physiological effects (Lehnen *et al.*, 2015). Produced by ruminal biohydrogenation of UFA (principally vaccenic acid and α -linolenic acid), CLA isomers are present predominantly in ruminant-derived fats (Palmquist *et al.*, 2005), making milk a major dietary source which has received extensive study. In agreement with the previously referenced studies by O'Callaghan *et al.* (2016a, 2016b & 2017), various authors have seen a linear increase in milk CLA content with increasing pasture consumption (Kelly *et al.*, 1998; White *et al.*, 2001; Lock & Garnsworthy, 2003; Schroeder *et al.* 2005; Couvreur *et al.*, 2006; Talpur *et al.*, 2008), which has been attributed to the higher levels of rumen biohydrogenation intermediates such as vaccenic acid and α -linolenic acid in grass and other fresh forages than in concentrates, ensiled or lower quality, late season, forages.

The importance of consideration of forage type in devising feeding systems for FA manipulation was discussed by Sterk *et al.* (2011), who found that transitioning from 80% grass silage to 80% corn silage (on a DM basis) resulted in increased concentrations of linoleic acid (C18:2 n-6) and of *trans-10* C18:1, which is typically present in low concentrations in ruminant fats (Kuhnt *et al.*, 2011), along with a decrease in α -linolenic acid and *trans-11, cis-15* C18:2 (a stearic acid intermediate). The high starch content of corn silage was also suggested to influence the relative abundance and function of rumen microflora, leading to the observed changes, particularly in *trans-10* C18:1 content.

Lourenço *et al.* (2008) compared the effects of feeding red clover, white clover and botanically diverse forages against ryegrass, finding a reduction in short- to medium-chain FA

content (C6:0 – C18:0) and an increase in medium- to long-chain FA content (C18:1 – C18:3 n-3) when feeding a diverse forage diet. This diet also resulted in increased CLA *cis-9trans-11* in milk, associated with increased supply of vaccenic acid. Concentrations of α -linolenic acid were increased by red clover feeding, though neither red or white clover feeding had a substantial effect on the overall FA profile of milk when compared to grass. Lee *et al.* (2009) observed a greater effect of red clover and particularly chopped red clover feeding on milk FA profile; however, this was at a comparably high rate of red clover feeding.

Increased concentrations of CLA *cis-9trans-11* in summer milk, when forage quality is highest, also appear to be consistent between ryegrass (Stanton *et al.*, 1997) and alfalfa pastures (Castillo *et al.*, 2006). Comparison of fresh and ensiled grass has shown a nutritionally unfavourable trend in milk FA composition upon transition from grazing to silage feeding, which resulted in a linear increase in concentrations of myristic, palmitic, stearic and oleic acids and a linear decrease in vaccenic acid, total CLA and the proportion of rumenic acid to total CLA (Kelly *et al.*, 1998; Elgersma *et al.*, 2004). Consistent results for CLA, α -linolenic acid and oleic acid were seen by Larsen *et al.* (2014) when comparing milk from a grass-based organic dairy to two conventional dairies. A seasonal effect was also observed within the organic system, although the palmitic acid content and, consequently, melting point of the low-temperature melting fraction, was increased in milk from this system. This result contrasted with those of other studies which determined higher palmitic acid content in concentrate-derived milk (Chilliard *et al.*, 2001; Couvreur *et al.*, 2006; O’Callaghan *et al.* 2016a).

Despite seeing similar overall results to other studies which compared the FA profiles of milks produced from diets of varying forage:concentrate ratios, Jaakamo *et al.* (2019) also recorded increased PUFA content in milk from cows fed a low (30:70) compared to high (70:30) forage:concentrate ratio, although the forage source used in these diets was grass silage administered in a TMR, rather than fresh grass forage. A 50:50 red clover:concentrate diet

increased milk monounsaturated FA content and further increased milk PUFA content over the low forage:concentrate diet, which has also been seen in previous studies (Vanhatalo *et al.*, 2007; Moorby *et al.*, 2009), though not at the high concentrate inclusion rate administered in the foregoing red clover:concentrate diet.

Efforts to alter milk FA composition through dietary lipid supplementation have utilised diverse sources, including fish oil (Shingfield *et al.*, 2003), linseed and linseed extracted oil (Chilliard *et al.*, 2009), soya oil (Agenäs *et al.*, 2002), soy bean meal (Murphy *et al.*, 1995) rapeseed (Hoffman *et al.*, 2016), tallow (Dunkley *et al.*, 1977) and calcium salts of fatty acids (Castañeda-Gutiérrez *et al.*, 2007). The general trend among these feeding trials was similar to that seen in pasture-feeding trials for concentrations of LCFA such as vaccenic acid, oleic acid, linoleic acid and CLA, which tended to increase significantly. However, these diets appear to have a more pronounced depressive effect on concentrations of *de novo* synthesised FA than interventions based on varying forage:concentrate ratios. Supplementation with crushed rapeseed or rapeseed oil (Chilliard *et al.*, 2009) produced similar results to high forage diets, as the FA profile tended towards increased LCFA content and increased UFA content (Chilliard *et al.*, 2001, O’Callaghan *et al.*, 2016a). High concentrations of omega-3 FA, such as eicosapentaenoic acid and docosahexaenoic acid, resulting from fish oil supplementation (Donovan *et al.*, 2000) are of interest from a nutritional perspective, particularly for their beneficial effects on cardiovascular health, foetal development and cognitive performance in Alzheimer’s patients (Stark *et al.*, 2016).

In a study which examined the effect of various lipid sources, feed processing treatments, and heat treatments on the CLA content of milk, Chouinard *et al.* (2001) found significant increases in CLA content with supplementation of Ca salts of canola oil, linseed oil and soybean oil when compared to a basal diet consisting of a grass silage, corn silage and soybean meal ration. Increased CLA content was also observed with increasing soybean meal

extrusion temperature, and CLA content increased significantly in response to fish oil supplementation. As an animal fat source, tallow is high in CLA; however, the effect of tallow supplementation in this study was minor when compared to the other oils used, particularly fish oil. The results of this comparison between tallow and fish oil are in agreement with the report of Jones *et al.* (2000). Dunkley *et al.* (1977) found that feeding tallow resulted in a reduction in *de novo*-synthesised FA, while also decreasing milk protein content, an effect which should be considered for all fat supplementation diets, depending on inclusion rates. However, feeding beef tallow to cows has seemingly fallen largely out of favour in recent years, possibly due to ethical concerns.

Levels of *de novo*-synthesised FA in milk may also be influenced by supplementation of individual FA. In addition to greater milk and fat yields (Mosley *et al.*, 2007), increased *de novo* FA synthesis has also resulted from palmitic acid supplementation, modulated by increased synthesis of the *de novo* FA precursor butyrate in the mammary gland (Hansen & Knudsen, 1987). Other FA supplements were found to have neutral (lauric acid, oleic acid) or inhibitory (stearic acid, linoleic acid) effects on *de novo* FA concentrations. This may indicate a potential means of influencing short- to medium-chain length FA composition, which is otherwise largely unresponsive to dietary variation. However, feeding supplements with high palmitic acid content has also been shown to have a limited effect on *de novo* FA synthesis and, rather, lead directly to considerably high palmitic acid deposition in milk (Lock *et al.*, 2013), which would be a noteworthy concern for butter or cheese manufacturers with regard to product texture and hardness.

1.2.4.2.3. *Milk fat globule size*

The differential between *de novo*-synthesised and diet-derived FA can also influence milk fat globule (MFG) size and secretion. The diameter of the MFG ranges from 0.1 to 15 μm , with an average of 2.5 to 4.6 μm (Fleming *et al.*, 2017). Wiking *et al.* (2004) determined

that variation in MFG size is influenced by the relative proportions of LCFA derived from the diet and not by SCFA (C4:0 – C14:0) arising from *de novo* synthesis. The authors noted that increasing MFG size was correlated with diurnal fat yield in this study, attributing this observation to a limitation in the availability of MFG membrane (MFGM) material with increased fat production. This may be due to the nature of the structure of the MFG as a core of bulk triglycerides surrounded by a membrane rich in polar lipids which, in turn, contain high LCFA content (Lopez *et al.*, 2014). Increased dietary intake of LCFA may increase the polar lipid content of the MFGM, increasing the ratio of MFGM to bulk triglycerides, thus reducing MFG size. Feeding diets either naturally high or enriched in LCFA was also suggested by Logan *et al.* (2014) to have an influence on MFG size. Indeed, Carroll *et al.* (2006) showed that MFG size (D_{90} and D_{50}) increased linearly with increasing fat supplementation using yellow grease (vegetable oil), a fat source with particularly high LCFA content (Plascencia *et al.*, 2003).

Couvreur *et al.* (2007) found several differences in MFG characteristics between the milk of cows fed pasture supplemented with cereal concentrates and those fed corn silage supplemented with soybean meal. The pasture treatment resulted in reduced sauter diameter ($d_{3,2}$) (3.38 to 3.15 μm), volume mean diameter ($d_{4,3}$) (3.94 to 3.65 μm) and size distribution range, with increased specific surface area (1.95 to 2.09 m^2/g of fat). Conflicting results were reported by Argov-Argaman *et al.* (2014), who found that larger milk globules were secreted by cows assigned to a low concentrate, high forage diet (3.51 μm) compared to those on a high concentrate, low forage diet (3.3 μm), which was attributed to increased concentrations of phosphatidylserine, which reduces the interfacial surface tension between droplets, leading to fewer droplets of larger size. However, despite the association between feeding practice and MFG size made by the authors in the cited study, the observed values for average MFG

diameter did not vary significantly ($P > 0.05$), rather the difference was reported as a tendency ($P = 0.1$) towards change in MFG size.

Jaakamo *et al.* (2019) also recorded increased volume mean diameter and decreased specific surface area in milk derived from a low concentrate, high forage diet (30:70), compared to high concentrate, low forage (70:30), but found that the application of a diet based on a 50:50 red clover:concentrate ratio resulted in increased PUFA content and a lower MFG size than a grass-based diet. The authors did, however, acknowledge that this variation may have been influenced by cow-to-cow variation in MFG secretion as much as any dietary effect. Overall, MFG size appears to be influenced by LCFA content, variation of which can influence both the nutritional profile and functional properties of milk (Logan *et al.*, 2014), such as butter churning time (Avramis *et al.*, 2003) and cheese yield and texture (O'Mahony *et al.*, 2005).

1.2.4.3. *Water-soluble components and ash*

1.2.4.3.1. *Carbohydrate content*

In contrast to milk protein and fat content, concentrations of lactose are relatively constant and are not subject to significant variation outside of lactational changes (Walker *et al.*, 2004, O'Callaghan *et al.*, 2016a). However, feeding studies have previously indicated that lactose yield may increase with increasing dietary energy supply, as is often observed for protein content. As seen for protein and fat yield, the increased lactose yield observed by Harris *et al.* (1998) with increasing dietary white clover content was attributable only to increased milk yield, with % lactose remaining unaffected. Mackle *et al.* (1999) reported minimal variation in lactose yield in milk from cows fed pasture, pasture supplemented with maize grain, or pasture supplemented with maize grain and pasture silage, despite a significant increase in milk yield and the significantly higher levels of metabolizable energy in both

supplemented diets. Notably, however, milk protein yield was also not significantly affected in this study.

Consistent reports of a greater lactose yield in CLV- and particularly TMR-derived milk over that of GRS were made by Panthi *et al.* (2019), Gulati *et al.* (2018a) and O'Callaghan *et al.* (2016a). Both O'Callaghan *et al.* (2016a) and Panthi *et al.* (2019) observed significantly higher lactose yield, but not % lactose in CLV and TMR milk than in GRS, whereas, for Gulati *et al.* (2018a), TMR lactose yield was significantly greater than both CLV and GRS, with a significant effect also observed for % lactose between each diet, where TMR>CLV>GRS. These studies seem to substantiate the understanding that increased milk lactose yield is correlated with increased overall milk yield (Shahbazkia *et al.*, 2010), attributable in this case to the increased dietary energy content of TMR and CLV diets over a GRS diet (Tables 2, 3 and 4).

1.2.4.3.2. *Mineral composition*

The previously referenced investigations of varying pasture and concentrate feeding primarily focused on changes in gross composition and FA profile, with the micronutrient composition of milk receiving relatively limited focus. One study which did investigate this topic in detail is that of Gulati *et al.* (2018a), who, in comparing GRS, CLV and TMR feeding systems, found notable differences in mineral composition between milks produced from each system. The GRS system produced milk with significantly higher Ca and P levels than both the CLV and TMR systems, which was attributed to the greater casein content of the GRS milk, as the majority of Ca and P in milk is incorporated into the casein micelle as colloidal calcium phosphate. Concentrations of Mn were also significantly higher in GRS milk than in CLV milk.

Despite observed variation by lactational stage in the other macroelements measured (Mg and Na), feeding system did not have a significant effect on their levels in milk. Along with Cl, Na concentration is a primary influence on the osmotic balance of milk, increasing

markedly in late lactation milk, thus leading to reduced lactose content (Fox *et al.*, 2015). Gulati *et al.* (2018a) observed differences in lactose yield and % between GRS, CLV and TMR samples in both mid- and late-lactation milk, which is therefore likely to be due in part to the relative energy contents of the diets. Among the trace elements measured, greater concentrations of Zn, Cu and Se were present in TMR-derived milk than those of both pasture-based systems. This partition in the distribution of macro- and micro-elements between pasture-derived and concentrate-derived milk could be ascribed to uptake of Ca and P from the soil ingested during grazing and the provision of supplemental trace elements in concentrate rations (Rey-Crespo *et al.*, 2013).

Conflicting results were found by Gabryszuk *et al.* (2008) in a study comparing conventional indoor-feeding farms with organic farms. Concentrations of Ca, Mg and P were greater in milk produced on an intensive conventional farm feeding TMR than in milk from organic farms where cattle grazed from March to October. For this study, milk sampling took place in September, when declining forage quality may be responsible for reduced micronutrient content in grass (Nantapo & Muchenje, 2013). In addition, milk mineral profiles from organic farming systems may not be reflective of more widely practiced pasture-based systems, as restrictions on organic production may exclude the use of most mineral supplements (Blanco-Penedo *et al.*, 2009), which can otherwise be used as required. In a meta-analysis which compared the mineral composition of milk produced from conventional and organic feeding systems in various countries, Zwierzchowski & Ametaj (2018) determined that milk from conventional farms was characterised by significantly higher concentrations of both macro elements (Ca, Mg, P, K, S) and trace elements (Cu, Mn, Se, Zn, I) than that produced on organic farms, citing the use of mineral supplementation in conventional systems as the most significant factor, while also noting that mineral composition varied considerably between different countries, where production factors also varied.

In agreement with Gulati *et al.* (2018a), Rey-Crespo *et al.* (2013) determined significantly higher concentrations of Zn, Cu and Se in conventionally- than in organically-produced milk, although the Fe content of organic milk was higher, attributed to soil consumption during grazing. Therefore, a noteworthy seasonal effect which this study also highlights is that of increasing adhesion of soil to forages and thus, soil ingestion, in autumn/winter, as the Fe content of organic milk increased significantly during this period.

Additional effects of increasing dietary fat supplementation reported by Carroll *et al.* (2006) were a linear decrease in P content and a linear increase in Mg content. However, the authors did not provide an explanation or suggest a mechanism for these findings. Supplemental dietary fats have previously been suggested to reduce Mg absorption in the bovine digestive tract by the formation of insoluble Mg soaps (Ramirez & Zinn, 2000). Overall, the variability of the mineral profile of milk from different feeding systems may be reflective of variation in several factors influencing the composition of the feed used, such as forage quality, soil type, levels of mineral fertilisers applied or concentrate formulation. Consideration of the mineral content of the feed provided in a given system therefore seems to offer a relatively responsive means of influencing the mineral composition of milk derived from that system.

Of particular note is that changes in the trace-element composition of milk in relation to bovine diet is of significance to the infant milk formula (IMF) industry. The prevalent use of skim milk in IMF manufacture means that where significant quantities of concentrates are fed in the bovine diet, mineral specifications in IMF may be in excess of CODEX regulations (European Commission Directive 2006/141/EC, 2006). This is often most significant for iodine, where supplementation in the feed can lead to substantial increases in milk (O'Brien *et al.*, 2013).

1.2.4.3.3. *Vitamin composition*

Despite the importance of milk as a source of fat- and water-soluble vitamins, there is a significant lack of information regarding the effects of varying feeding strategies on the overall vitamin profile of milk. The effect of dietary interventions on levels of vitamins and vitamin precursors in rumen fluid (Hayes *et al.*, 1966; Miller *et al.*, 1986; Santschi *et al.*, 2005; Schwab *et al.*, 2006) and the effects of vitamin supplementation on cow performance and health (Zimmerly & Weiss, 2001; Bergsten *et al.*, 2003; Rosendo *et al.*, 2004) have been well characterised, but relatively few studies have compared the final vitamin composition of milk produced from different feeding systems. In what is seemingly the first study to compare the vitamin B2 (riboflavin) content of milk from conventional and organic farms, Poulsen *et al.* (2015) found higher concentrations of this vitamin in organic milk and, interestingly, seasonal variation, which occurred only for the organic milk, resulted in higher riboflavin content in winter compared to summer milk. This seems quite unusual given the high proportion of riboflavin in fresh, leafy forage (Pinto & Zempeni, 2016). However, the authors suggested that this may have been due to increased ruminal synthesis of riboflavin in response to decreased intake of forage; however, the riboflavin content of the organic milk in this study was consistently higher than that produced from conventional farms, which utilised lower forage content all year-round.

Magan *et al.* (2020) compared the water-soluble B vitamin composition of skim milk, sweet whey, micellar casein whey and acid whey powders produced from mid-lactation milk derived from cows assigned to the GRS, CLV and TMR diets previously described. Concentrations of vitamin B1 (thiamine), riboflavin and vitamin B7 (biotin) were significantly higher in both GRS and CLV-derived ingredients compared to those from TMR, which contained higher levels of vitamin B3 (nicotinic acid) and B3-amide (nicotinamide), with significant variation also present between ingredient types. The differences observed in

riboflavin content, in particular, were likely to have been due to direct transfer from the proportion of fresh forage available in the diet, but differences in biotin content were attributed to potential diet-induced modulation of rumen microbiota activity.

Within grazing systems, riboflavin content tends to be higher in milk produced from grass forage than from maize (Laverroux *et al.*, 2014) or hay (Havemose *et al.*, 2006), whereas comparison of grass and maize feeding has shown increased vitamin B9 (folate) in milk-derived from grass and greater B12 (cobalamin) in milk produced from maize feeding (Chassaing *et al.*, 2011). Concentrations of the fat-soluble vitamin A (retinol) precursor β -carotene and vitamin E have also been observed to increase with increasing grass or grass silage feeding (Agabriel *et al.*, 2007; Adler *et al.*, 2013; O'Callaghan *et al.*, 2016b). Indeed, the characteristic yellow colour of grass-derived milk and dairy products is attributable to their high β -carotene and riboflavin contents. Diet-based effects on the cobalamin content of milk have been observed to be marginal, with a slight positive correlation between percentage of chopped mixed silage in the diet and milk cobalamin concentrations being determined by Duplessis *et al.* (2019).

In addition to dietary intake, B vitamin synthesis occurring in the rumen provides sufficient levels to prevent deficiency (Schwab *et al.*, 2006). However, by depression of rumen cellulolytic microflora, sub-clinical acidosis arising from concentrate over-feeding can lead to insufficient synthesis of thiamine (Pan *et al.*, 2018) and biotin (Rosendo *et al.*, 2003). The ability to influence the relative abundance of vitamins in milk through dietary means is an important nutritional consideration, as standard portions of milk and dairy products offer a significant proportion of the RDA for some vitamins (Magan *et al.*, 2020).

1.2.5. Impact of milk composition on dairy product functionality

1.2.5.1. Liquid milk properties

Milk is subjected to various processing conditions during the manufacture of products such as powders, yoghurt, cheese or milk-based beverages and must, therefore, be stable to the environmental stresses applied. As milk is most often pasteurised before sale, thermal stability is a particularly important property in dairy processing. Ultra-high temperature (UHT) processing involves heating milk to over 135°C for 1 – 8 s (Penfield & Campbell, 1990) and can result in whey protein denaturation, Maillard browning reactions, lactulose production and release of volatile compounds (Dursun *et al.*, 2017), the extents of each depending on the specific thermal loads applied. Indeed, surface fouling or “burn-on” can occur at various processing conditions, even at lower temperatures. The total protein and whey protein contents of milk are, therefore, important factors determining its susceptibility to heat-induced aggregation and coagulation (Rosmaninho *et al.*, 2007). A summary of the literature discussed in this section is shown in Table 1.2.5.

Table 1.2.5: Summary of significant dietary effects on milk and dairy product functionality.

Functionality	Dietary factor	Effect relative to comparison diets	References
Heat coagulation time (HCT)	Clover feeding	Increased HCT	Magan <i>et al.</i> (2019b)
	Organic production	Increased calcium ion activity	Akkerman <i>et al.</i> (2019)
	Crude protein supplementation	Decreased HCT	Reid <i>et al.</i> (2015)
Ethanol stability	Concentrate supplementation	Increased ethanol stability	O'Brien <i>et al.</i> (1999a)
	Grass and clover feeding	Increased gel strength	Magan <i>et al.</i> (2019b)
Acid gelation	Concentrate feeding	Increased gel strength	Jasińska <i>et al.</i> (2010)
	Soy bean meal, palm kernel, beet pulp and standard concentrate feeding	Gel strength of soya-derived milk > palm kernel > beet pulp > standard concentrate.	O'Callaghan <i>et al.</i> (2019)
Butter properties	Linseed oil supplementation	Increased moisture content and decreased firmness	Hurtaud <i>et al.</i> (2010)
	Fish oil supplementation	Improved spreadability	Avramis <i>et al.</i> (2003)
	Pasture feeding	Lower melting temperature and hardness	Couvreur <i>et al.</i> (2006), O'Callaghan <i>et al.</i> (2016b), Hurtaud <i>et al.</i> (2007)
	Concentrate feeding	Higher melting temperature and hardness	Hurtaud <i>et al.</i> (2007)
	Pasture feeding	Improved spreadability	Couvreur <i>et al.</i> (2006)
Rennet coagulation	Grass feeding	Lower rennet coagulation time and higher gel strength	Gulati <i>et al.</i> (2019b)
	Grass feeding	Higher flowability of Mozzarella cheese	Gulati <i>et al.</i> (2018b)
	Grass and clover feeding	Softer texture of Cheddar cheese	O'Callaghan <i>et al.</i> (2017)
	Concentrate feeding	Higher firmness in Maasdam cheese	Panthi <i>et al.</i> (2019)
	Partial mixed ration or formulated grain mix feeding	Increased cheddar cheese yield	Auldish <i>et al.</i> (2016a)
Sensory quality	Grass and clover feeding	Thicker texture in the mouth	Faulkner <i>et al.</i> (2018)
	Clover feeding	Higher creaminess	Clarke <i>et al.</i> (2019)
	Pasture feeding	Yellower colour	O'Callaghan <i>et al.</i> (2016b), Magan <i>et al.</i> (2019b), Martin <i>et al.</i> (2005)
	Concentrate feeding	Whiter colour	Martin <i>et al.</i> (2005)
	Grass and clover feeding	“Cooked milk” flavour and “barnyard” aroma	Clarke <i>et al.</i> (2019), Faulkner <i>et al.</i> (2018)
Traceability	Concentrate feeding	“Hay-like” flavour	Faulkner <i>et al.</i> (2018)
	Pasture feeding	Lutein, β -carotene, toluene, p-cresol and dimethyl sulfone biomarkers	Nozière <i>et al.</i> (2006), O'Callaghan <i>et al.</i> (2017), Faulkner <i>et al.</i> (2018), Clarke <i>et al.</i> (2019), Panthi <i>et al.</i> (2019), Glover <i>et al.</i> (2012)
	Concentrate feeding	2,3-butanediol, citrate and 2-pentanone biomarkers	Clarke <i>et al.</i> (2019), Panthi <i>et al.</i> (2019), Glover <i>et al.</i> (2012)

Pasture and concentrate feeding	Differentiation by fatty acid profiling.	Pagano & Marcella (2010), O'Callaghan <i>et al.</i> (2017)
Pasture and concentrate feeding	Differentiation by nuclear magnetic resonance.	O'Callaghan <i>et al.</i> (2018), Panthi <i>et al.</i> (2019)
Pasture and concentrate feeding	Differentiation by Fourier-transform infrared spectroscopy.	Capuano <i>et al.</i> (2014)
Pasture and concentrate feeding	Differentiation by liquid-chromatography-mass spectrometry.	Magan <i>et al.</i> (2019a)
Pasture and concentrate feeding	Differentiation by Raman spectroscopy.	Gómez-Mascaraque <i>et al.</i> (2020)

Increases in the NPN content of milk arising due to increased urea concentrations, such as those seen in clover-derived milk by Harris *et al.* (1998), O'Callaghan *et al.* (2018) and Magan *et al.* (2019b) will markedly increase its heat coagulation time (HCT) and stability in thermal processing applications. Indeed, Magan *et al.* (2019b) observed significantly higher HCT and NPN content in CLV-derived whole milk powder than that produced from TMR. Urea undergoes heat-induced decomposition to ammonia, resulting in a buffering effect against heat-induced acidification, which is also significantly influenced by the lactose concentration; HCT decreases with increasing lactose content (Huppertz, 2016). Increased acidity due to formic acid produced through heat-induced decomposition of lactose is primarily responsible for this trend (Singh, 2004). Huppertz (2016) suggested that lactose can, however, have a stabilisation effect on the HCT of milk through complex formation with homocitrulline, formed from the reaction of lysine residues with isothiocyanate, another heat-induced decomposition product of urea. Murphy *et al.* (2014) have previously shown a stabilising effect of lactose in heat-treated infant milk formula emulsions, suggesting that the presence of carbohydrates causes proteins to be preferentially hydrated, as interactions between carbohydrates and the hydrophobic regions of whey proteins are reduced at higher temperatures. As discussed in Section 1.2.4.1.2., the urea content of milk can vary substantially by diet, but lactose concentrations are generally less susceptible to dietary influence.

In addition to the improved thermal stability of milk with high NPN content, the contribution of NPN to overall crude protein content may appear to be beneficial to producers operating within a crude protein payment scheme; however, this will result in milk of reduced protein quality or true protein content, which would be of particular concern to cheese manufacturers, as cheese yield is positively correlated with the true protein and casein contents of milk and negatively impacted by increased levels of NPN (Amenu *et al.*, 2006).

The HCT of milk is also negatively influenced by increasing levels of ionic calcium (Ca^{++}) (Sievanen *et al.*, 2008), which represents approximately 10% of the total calcium content of milk (Akkerman *et al.*, 2019). Akkerman *et al.* (2019) compared the calcium and citrate content of milk from two organic and one conventional dairy farm, finding significantly higher levels of total Ca^{++} and proportions of Ca^{++} relative to total Ca in milk from both organic farms when compared to that from the conventional farm, but noted that the differences observed were unlikely to be sufficient to lead to significant differences in milk processability. Levels of Ca^{++} were also observed to increase significantly as the season progressed, which had also been previously observed by Chen *et al.* (2014) in milk from a year-round-calving herd, indicating that the seasonal variation recorded by Akkerman *et al.* (2019) may have occurred independent of lactational changes, which were likely to have occurred in both organic systems.

Gulati *et al.* (2019a) found higher levels of Ca^{++} in reconstituted low-heat skim milk powder produced from GRS, than those produced from CLV and TMR, but did not observe any significant variation in HCT between the samples, suggesting that the increased protein and Ca^{++} content of the GRS sample may be offset by its reduced lactose content. Reid *et al.* (2015) observed decreasing HCT of milk with increasing dietary crude protein supplied through concentrate supplementation, which was attributed to increased total protein content, but also correlated increased HCT with increasing NPN content. Magan *et al.* (2019b) observed

the highest and lowest Ca⁺⁺ in TMR- and CLV-derived whole milk powder, respectively, which was inversely associated with the trend observed in HCT between the samples.

Ethanol stability is an important quality parameter and rapid indicator of the suitability of milk for thermal processing. Ethanol-induced aggregation and precipitation in milk occurs due to inhibition of the κ -casein “hairy layer” on the exterior of the casein micelle, reducing the steric barrier and thus promoting micelle flocculation (Horne, 2016). The ethanol stability of GRS, CLV and TMR-derived skim milk was compared by Gulati *et al.* (2019a), who did not observe any significant differences in stability between the samples. Similarly, Machado *et al.* (2014) and Grimley *et al.* (2009) did not observe significant variation in ethanol stability in milk from cows fed with varying forage:concentrate ratios or when turning out to pasture from indoor housing, although O’Brien *et al.* (1999a) observed increased ethanol stability of milk with concentrate supplementation in a grazing system.

Potential effects of diet-induced compositional variation on the functional properties (e.g. solubility, wettability, bulk density, flowability) of milk powder products have not yet been investigated, but are unlikely to be significant, as powder properties are primarily dependent on processing parameters such as dryer temperatures and the type of atomiser used (Sharma *et al.*, 2012).

1.2.5.2. *Yoghurt, butter and cheese*

The functional characteristics of concentrated end-products of milk processing such as butter or cheese tend to experience greater variation due to compositional variation than those of liquid milk and acidified milk products such as yoghurt, in which the major components (fat and protein) are not concentrated with respect to the milk used for their production. The production of yoghurt, fermented dairy beverages or acid cheese involves acid coagulation of heat-treated liquid milk, typically through the addition of lactic acid-producing bacterial

cultures such as *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Hill & Kethireddipalli, 2013). The application of a high thermal load to milk for use in yoghurt production has two critical functions: (1.) the removal of spoilage microorganisms which are potentially competitive to the starter cultures used and (2.) extensive denaturation of whey proteins, thus exposing sulphhydryl groups to allow complex formation of covalent bonds with κ -caseins. As the pH of the acidified milk declines to the isoelectric point of casein (pH 4.6), the surface charge of the casein micelle is reduced, causing the κ -casein “hairy layer” to flatten, reducing the steric and electrostatic repulsion between the micelles and facilitating micelle aggregation and the subsequent formation of a 3-dimensional gel matrix (Lee & Lucey, 2010). In research studies, acidification is often achieved through the addition of glucono- δ -lactone to milk (Gastaldi *et al.*, 2003).

Yoghurt gel formation is influenced by a variety of factors such as the extent of heat treatment applied, homogenisation pressure and the fat, protein and mineral composition and casein:whey ratio of milk (Lee & Lucey, 2010). Kamal *et al.* (2017) showed that increasing the Ca content of cows’ milk through the addition of calcium chloride (CaCl_2) at two concentrations (10 and 20 mM) resulted in a significant decrease in gelation time and significant increase in gel firmness with increasing Ca concentration. However, increasing P content through the addition of hydrogen phosphate dehydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) resulted in a significant increase in gelation time, a significant delay in the onset of gelation and a significant decrease in gel firmness, only at a concentration of 20 mM. The differences in compositional factors due to diet which have been previously discussed would, therefore, be expected to influence yoghurt gel formation and gel strength. The higher protein, casein, Ca and P content of GRS-, compared to CLV and TMR-derived milk observed by Gulati *et al.* (2018a) would imply that it is the most suitable for this purpose. However, in spite of these compositional differences, Gulati *et al.* (2019a) did not observe significant differences in the gelation time or

gel strength (storage modulus) of yoghurts produced from reconstituted skim milk powder made from milk from each feeding system, suggesting that the differences in protein content were insubstantial relative to the natural variation in starter-culture activity and fermentation rates between replicates. However, Magan *et al.* (2019b), observed significantly higher gel strength and textural firmness in yoghurts produced from both GRS and CLV-derived reconstituted whole milk powder than those from TMR, though gelation time was unaffected. The FA distribution of each whole milk powder would be expected to have an inverse effect on gel strength to that observed, as concentrations of the high-melting point FA palmitic acid are typically significantly higher in TMR-derived milk fat than that derived from pasture (Couvreur *et al.*, 2006; O'Callaghan *et al.*, 2016b).

Jasińska *et al.* (2010) compared the textural properties of yoghurts produced from the milk of cows grazing pasture supplemented with concentrates and with those fed a conventional indoor TMR, observing significantly higher gel firmness values for TMR-fed cows, although this was not correlated to differences in the protein content of milk from each diet, and a consistent significant effect on gel firmness was not observed throughout the entire lactation period. O'Callaghan *et al.* (2019) observed more substantial differences in acid gels produced from the milks of four groups of cows fed different supplementary feed types (16% crude protein parlour concentrate, palm kernel plus parlour concentrate, soya hulls plus parlour concentrate and molassed beet pulp plus parlour concentrate) in a pasture-based system. The storage moduli and loss moduli of feed type differed significantly, in the order: soya feed > palm kernel > beet pulp > standard parlour concentrate. Increasing gel strength was also inversely correlated with decreasing casein micelle size in these samples, indicating that gel strength may have increased with increasing casein micelle surface area availability for aggregation.

The production of butter involves concentration of the fat content of cream which has been allowed to partially coalesce, following centrifugal separation from heat-treated milk (Buldo *et al.*, 2013). The shearing forces applied during the churning process destabilise the cream emulsion and disrupt the milk fat globule membrane enclosing the fat globule, inverting the oil-in-water emulsion to a water-in-oil emulsion as the aqueous phase (buttermilk) is released (Vanderghem *et al.*, 2010). The efficiency of this process is determined by the % recovery of fat in the solid phase, which has been shown to be reduced when using cream with lower MGF size (Hurtaud *et al.*, 2010). Hurtaud *et al.* (2010) reported that increasing levels of supplementation of extruded linseed oil (2.1% and 4.3% of a TMR) compared to a control ration caused a linear decrease in MFG size (from 4.18 μm to 4.07 μm at 2.1% and 3.49 μm at 4.3%) and increase in UFA content in milk. The decrease in MFG size also caused an increase in the moisture content of butter as the proportion of fat lost in buttermilk increased. These changes resulted in reduced firmness values for butter and, consequently, increased spreadability. A similar effect was recorded by Avramis *et al.* (2003), who observed decreasing MFG size (2.31 μm to 1.84 μm) and improved spreadability of butter produced from the milk of cows supplemented with fish oil in comparison to those fed a corn-based TMR control diet. The authors also stated that the improved spreadability was influenced by the presence of lower-melting point FA, but that this was unlikely to be the primary determinant, as the iodine values of the butter samples did not vary significantly. The iodine value of a fat measures the degree of unsaturation of its constituent FA (Sanders, 2003) and is an important indicator of the susceptibility of butter to oxidation. The potential for reduced oxidative stability due to increasing UFA content arising from pasture feeding, as observed by Couvreur *et al.* (2006) and O'Callaghan *et al.* (2016a & 2016b), may be counter-balanced by the increased levels of the antioxidant species β -carotene and α -tocopherol present in milk derived from these systems (Nozière *et al.*, 2006; Butler *et al.*, 2008).

In contrast to the results of Avramis *et al.* (2003), differences in the FA composition of milk have been shown to significantly influence the hardness and spreadability of butter (Couvreur *et al.*, 2006; Hurtaud & Peyraud, 2007; Hurtaud *et al.*, 2007; O'Callaghan *et al.*, 2016b). Both Couvreur *et al.* (2006) and O'Callaghan *et al.* (2016b) observed lower butter hardness and melting temperatures and improved texture in pasture-derived butter, when compared to concentrate-derived butter, attributing these differences to the increased UFA content of pasture-derived milk and, in particular, the increased concentrations of the high melting point FA palmitic acid (C16:0) in concentrate-derived milk. Significant variation in spreadability scores were observed by Couvreur *et al.* (2006), but not by O'Callaghan *et al.* (2016b), despite the differences in textural and melting properties. On balance, it should be accepted that the texture and spreadability of butter is influenced by variation in FA melting points. This is most likely the primary cause for the improved spreadability observed by Avramis *et al.* (2003), despite the recorded iodine values.

The final major aspect of milk processability which will be discussed is rennet-induced coagulation and cheese formation. The cheese-making properties of milk are highly susceptible to variation due to compositional changes, as influenced by seasonal and lactational factors (Sapru *et al.*, 1997; Coulon *et al.*, 1998). Rennet coagulation of milk requires destabilisation of the casein micelle and is achieved through the addition of rennet, a blend of mucosal enzymes present in the abomasum of ruminants which contains the protease chymosin, which selectively hydrolyses κ -casein at the surface of the casein micelle (Anema *et al.*, 2007). This facilitates coagulation in two ways; proteolysis of the κ -casein layer removes the steric barrier between casein micelles and, in addition, the loss of the negatively charged glycomacropeptide region on the κ -caseins reduces electrostatic repulsion between the micelles, allowing calcium-induced micelle aggregation and the formation of an ordered three-dimensional matrix to occur.

Heat treatment of milk prior to rennet addition has been used as a means of increasing cheese yield (Anema *et al.*, 2007) by incorporating denatured whey proteins, which would otherwise be removed when cutting the curd, into the gel matrix by binding to κ -casein *via* disulphide linkages. However, the level of whey protein denaturation is dependent of the heat load applied during pasteurisation, which is negligible at standard high temperature-short time conditions of 72°C x 15 s (van Lieshout *et al.*, 2020). More severe heat treatments (80 – 140°C x 4 s) have been shown to result in increased rennet coagulation times (RCT) when more than 60% of β -lactoglobulin has been denatured and reduced gel strength at more than 10% denatured β -lactoglobulin (Waungana *et al.*, 1996). Excessive complex formation between denatured whey proteins and κ -casein inhibits the action of chymosin, inhibiting micelle flocculation and subsequent curd formation (Salih & Abdalla, 2020). Furthermore, the decrease in syneresis which would result from whey protein incorporation would be undesirable in most cheese-production applications. As NPN is present in milk serum and not involved in gel formation, increased proportions of NPN relative to total protein and, thus, a lower true protein content in milk, will be linked to decreased cheese yield (Panthi *et al.*, 2019).

The coagulation time and gel strength of rennet gels are highly variable due to the influence of a variety of factors, including the level of preheat-treatment of milk, concentration of rennet used, temperature during coagulation, total protein and casein content, casein micelle size, distribution of casein fractions, milk pH and levels of ionic and colloidal calcium (Avramis *et al.*, 2003; Martin *et al.*, 2008; Corredig & Salvatore, 2016). The effect of dietary factors on the rennet coagulation properties of milk were determined by Gulati *et al.* (2019a; 2019b), who investigated the effect of varying daily herbage allowance (DHA) on the processability of milk throughout lactation (Gulati *et al.*, 2019b) and the effect of pasture and concentrate feeding on the processability of reconstituted low-heat skim milk powder (Gulati *et al.*, 2019a). In agreement with a similar study by O'Brien *et al.* (1997b), rennet coagulation

properties did not vary significantly according to DHA in early-, mid- or late-lactation milk, which corresponded to the lack of significant compositional variation (total and soluble casein content and ratios of Ca and P to casein) in milk produced at each level of DHA. Interestingly, when compared to mid- and early-lactation milk, significantly reduced RCT and increased gel strength were observed in late-lactation milk (Gulati *et al.*, 2019b), when cheese-making properties are typically negatively affected by changes in the protein and mineral profiles of milk (Lucey & Fox, 1992). The authors suggested that the significantly increased casein content was sufficient to counteract the lower Ca- and P- to casein ratios and increased level of soluble casein which occurred in late lactation.

Further evidence for the effect of increased protein content in milk on RCT and gel strength was provided by Gulati *et al.* (2019a) using reconstituted skim milk powder. Milk derived from GRS displayed significantly lower RCT and significantly higher gel strength than that of TMR, with intermediate values observed for CLV for both variables. This was correlated with the significantly higher protein content of the GRS sample. In addition, the same lactational effect as was previously observed by Gulati *et al.* (2019b) also occurred in this study. Further to these studies, Gulati *et al.* (2018b) measured the functional properties of mozzarella cheese produced from GRS, CLV and TMR diets. Compositional variation between the raw milk samples from each feeding system was standardised in the resultant cheese samples, with the exception of the levels of I, Se and Cu, which were significantly higher in TMR-derived milk than in both GRS and CLV. Yield, extent of proteolysis, water-holding capacity and textural properties were unaffected by feeding system. Despite this, the flowability and loss tangent (ratio of storage modulus to loss modulus) of GRS-derived cheese was significantly higher at room temperature than that from TMR, indicating that grass feeding results in a more fluid mozzarella product that melts at a lower temperature, which is a notable consideration for mozzarella functionality. Although FA profiling was not carried out on the

samples used in this study, the difference in fat liquefaction was attributed to the significantly higher proportion of palmitic acid measured previously in TMR samples (O’Callaghan *et al.*, 2016a) derived from the same feeding systems used in this study. This may also have been due to increased proportions of UFA relative to SFA in the GRS sample, as has previously been observed in pasture-derived milk (Couvreur *et al.*, 2006; O’Callaghan *et al.*, 2016b).

A more pronounced effect of feeding system on the textural properties of full-fat Cheddar cheese was reported by O’Callaghan *et al.* (2017), despite a lack of significant variation in proteolysis between samples. Both GRS and CLV-derived cheese samples were significantly softer at room temperature than TMR samples, although no significant differences in firmness were observed at refrigeration temperature. This variation was attributed to the considerable variation in FA composition between the cheese samples, as has been observed for milk samples from these feeding systems. As reported for butter, the greater palmitic acid content of TMR-derived cheese is likely the most substantial contributor to its higher firmness values at room temperature. Panthi *et al.* (2019) also reported no significant variation in the yield or gross composition of standardised Maasdam cheeses produced from GRS, CLV and TMR feeding systems. Significant differences in sensory attributes were observed, however, including textural qualities which correspond to those observed by O’Callaghan *et al.* (2017), as TMR-derived cheese scored highest for hardness and rubbery texture in the mouth. Provision of corn grain and canola meal in a partial mixed ration or formulated grain mix to grazing cows receiving standard parlour meal and alfalfa hay was observed to have a beneficial effect on Cheddar cheese yield by Auld *et al.* (2016a), with no changes to coagulation properties, cheese composition or sensory acceptability.

1.2.5.3. *Sensory quality*

Sensory evaluation of dairy products encompasses all aspects of organoleptic perception, although olfactory stimuli are perhaps the most significant, as aromatic compounds are generally considered to account for a considerable majority of flavour perception, relative to gustatory stimuli. Flavour chemistry techniques can quantify volatile compounds present to elucidate differences in perception identified through sensory evaluation. Compositional variation due to feeding system has notable effects on the appearance and texture of dairy products and the relative abundance of numerous volatile aromatic compounds has also been seen to be associated with feeding practices (Faulkner *et al.*, 2018; Clarke *et al.*, 2019, Panthi *et al.*, 2019). Volatile compounds are transferred into milk by direct and indirect means, the former being direct inhalation of airborne volatiles which are diffused into the bloodstream through the lungs and ultimately to the mammary gland and the latter being absorption during digestion and subsequent diffusion through the blood supply to the mammary gland (Faulkner *et al.*, 2018).

As discussed in Section 1.2.5.2., the substantial variation due to diet in milk FA composition results in variation in the textural properties of milk and products such as butter and cheese. This is primarily due to variation in the ratio of high- (palmitic acid) to low-melting point (oleic acid) FA and fat globule size, although perceptible textural differences are not limited to products of high fat content. In a comprehensive study combining volatile analysis with hedonic sensory acceptance and ranked descriptive analysis, Faulkner *et al.* (2018) compared pasteurised samples of the whole milk collected from the GRS, CLV and TMR feeding systems by O'Callaghan *et al.* (2016a). Milk derived from the GRS system was scored significantly higher for the "viscosity" attribute than both CLV and TMR-derived milks by an Irish consumer panel, despite the higher palmitic acid content of TMR milk. The observed effect may be due to the higher gross fat content of GRS-derived milk relative to the other

samples (O’Callaghan *et al.*, 2016a) or possibly due to variation in free fatty acid content between samples, which can influence the surface tension of milk (Kamath *et al.*, 2008). The GRS sample also scored significantly higher for “liking of texture” and “overall liking” than both CLV and TMR samples in hedonic ranking, indicating that the panel consisting of Irish consumers seemed to assign the highest ranking to the sample with which they were most familiar. The perceived difference in viscosity may, therefore, be linked to the overall preference for the texture of the familiar sample.

The abundance of free SCFA, which are present in greater concentrations in TMR-derived milk can also influence the flavour of cheese (Kilcawley, 2017). Likewise, increased LCFA content, as observed for pasture-derived milk, provides greater levels of substrate for oxidation or reduction to odour-active compounds (Villeneuve *et al.*, 2013). As stated in Section 1.2.5.2., the higher PUFA content resulting from pasture feeding also increases the susceptibility of milk to oxidation (Hedegaard *et al.*, 2006), though this may be counteracted by the presence of antioxidants such as β -carotene, as perceptible sensory differences due to lipid oxidation have not been observed between milks produced from pasture and concentrate (Kilcawley *et al.*, 2018).

Visual differences between pasture and concentrate-derived products are generally dependent on lightness (L^*) and yellowness (b^*) values (as determined by CIE LAB colour space), whereby pasture-derived products have a characteristic “golden hue” of increasing intensity in higher-fat products, with concentrate-derived products displaying a whiter appearance (Martin *et al.*, 2005; Nozière *et al.*, 2006; O’Callaghan *et al.*, 2016b; Faulkner *et al.*, 2018; Magan *et al.*, 2019b). This can be attributed to the higher carotenoid (chiefly β -carotene) and riboflavin contents of grass and other leafy forages, transferring a yellow pigmentation to milk and other products manufactured therefrom (O’Callaghan *et al.*, 2016b; Magan *et al.*, 2019b).

Milk flavour is influenced by a range of classes of volatile compounds, including ketones, sulphur compounds, acids, alcohols, esters, phenols, aldehydes and lactones (Kilcawley *et al.*, 2018), primarily arising from rumen AA metabolism (Faulkner *et al.*, 2018). Dimethyl-sulfone is frequently determined to be present in significantly higher concentrations in pasture-derived milk (Coppa *et al.*, 2011; Villeneuve *et al.*, 2013; Faulkner *et al.*, 2018; Clarke *et al.*, 2019) and cheese (Carpino *et al.*, 2004; Panthi *et al.*, 2019) and is an important compound in sensory evaluation due to its low odour-threshold (Kilcawley *et al.*, 2018) and “cooked milk” flavour (Clarke *et al.*, 2019). Notably, release of volatile sulphur-compounds is strongly associated with heating of milk (Al Attabi *et al.*, 2009), though Villeneuve *et al.* (2013), Faulkner *et al.* (2018) and Clarke *et al.* (2019) all applied low-heat pasteurisation treatments (approx. 72°C x 15 s) to their milk samples, whereas Coppa *et al.* (2011) utilised unpasteurised milk. Increased concentrations of dimethyl-sulfone have been associated with increased methionine metabolism due to the higher protein content of pasture-derived milk, which provides greater substrate for deamination, resulting in higher levels of volatile sulphur compounds (Faulkner *et al.*, 2018).

Volatile analysis carried out by Clarke *et al.* (2019) identified sulphur compounds and hydrocarbons as being associated with GRS and CLV feeding, whereas TMR feeding was correlated with increased concentrations of aldehydes and esters of short-chain FA, which corresponds to the increased SCFA content previously identified in TMR-derived milk. Faulkner *et al.* (2018) also observed increased concentrations of carbohydrate esters in TMR milk samples, and attributed this to alcohols produced through fermentation of the high carbohydrate content of the ration.

Levels of the hydrocarbon toluene in milk vary considerably by level of pasture feeding. Toluene is primarily a breakdown product of β -carotene metabolism and is present in correspondingly high concentrations in pasture-derived products (Villeneuve *et al.*, 2013;

Faulkner *et al.*, 2018; Panthi *et al.*, 2019), though its odour-threshold is comparatively high. The phenolic compound *p*-cresol is an important odour-active compound in comparing milk and cheese derived from pasture and concentrate-based feeding systems. The sensory evaluation carried out by Faulkner *et al.* (2018) identified a “barnyard” aroma, alongside colour and viscosity, as the most significant discriminating factors between GRS, CLV and TMR-derived milk samples. This trait scored higher for both the GRS and CLV samples, in comparison to TMR, and was associated with the concomitant high levels of *p*-cresol identified in these samples. Like toluene, *p*-cresol is derived from rumen degradation of β -carotene, in addition to degradation of tryptophan, tyrosine and isoflavones present in white clover pasture (Faulkner *et al.*, 2018). A similar association between *p*-cresol and pasture-feeding was observed by Lopez & Lindsay (1993) and O’Callaghan *et al.* (2018). Moio *et al.* (1996) also showed evidence for direct transfer of *p*-cresol arising from lignin degradation in forage to milk.

Terpenoids are another major class of odour-active compounds in milk which undergo variation due to feed type. They are secondary plant metabolites with concentrations in milk which are directly correlated to their concentrations in the feed consumed by the cow (Bugaud *et al.*, 2001), varying considerably between different forage types. The terpenoid content of dicotyledonous forages is particularly high relative to monocotyledons (Tornambé *et al.*, 2006; Agabriel *et al.*, 2007) and, similarly, diverse forages contain substantially greater levels than monocultures (O’Callaghan *et al.*, 2016a; Faulkner *et al.*, 2018). Despite the diversity of feeds supplied in indoor TMR systems, pasture-derived milk has demonstrated greater diversity in terpenoid content, though this is also dependent on forage diversity (Fernandez *et al.*, 2003). Sensory discrimination between milk and dairy products from different feeding systems based on terpenoid content may be limited, however, by their high odour threshold and the development of terpenoid compounds during cheese ripening (Kilcawley *et al.*, 2018).

Using a trained descriptive sensory panel, Clarke *et al.* (2019) compared pasteurised milk samples from GRS, CLV and TMR feeding systems, which could be clearly discriminated. The CLV samples scored significantly higher than GRS for creaminess, and TMR samples scored highest for white colour, with both GRS and CLV samples scoring higher for creamy colour and TMR scoring significantly higher than both GRS and CLV for hay-like flavour. Hedonic sensory analysis was carried out on pasteurised milk samples from these feeding systems by Faulkner *et al.* (2018) using an Irish consumer panel. The GRS sample scored highest for all preferential attributes and scored statistically higher than both CLV and TMR for liking of texture, liking of flavour and overall acceptability. The CLV sample scored lowest for all attributes other than liking of texture, which is surprising, given the unfamiliarity that Irish consumers are likely to have with TMR-derived milk.

Similar results were found by O'Callaghan *et al.* (2016b) for hedonic and ranked descriptive analysis of sweet-cream butter produced from GRS, CLV and TMR milk, where an untrained consumer panel preferred GRS samples over both CLV and TMR samples, but did not determine significant descriptive differences between GRS and CLV samples. In this study, GRS butter scored highest for liking of appearance and flavour and scored significantly higher than TMR for intensity of colour, diacetyl aroma, diacetyl flavour and cream flavour. The CLV-derived butter scored significantly higher for texture than TMR, as seen by Faulkner *et al.* (2018) for pasteurised milk. However, a study by Croissant *et al.* (2007) found that a trained descriptive panel was capable of discriminating between pasture and concentrate-derived pasteurised milks, where an untrained consumer panel was not. Where consumers can differentiate between products derived from different feeding systems, preference and overall acceptance are likely to reflect their familiarity with products from a given system. American consumers are likely to prefer products manufactured from TMR milk, whereas Irish

consumers will favour those from GRS or CLV (Cheng *et al.*, 2020). Analysis of cross-cultural differences is therefore an area which is particularly appropriate for further research.

1.2.5.4. Dairy product verification

The differences in milk composition arising from types of feeding practices also offer potential means for verification of milk and dairy product origin, which will be increasingly important as “grass-fed” product labelling becomes more widespread. Certain carotenoids and volatile compounds which are particularly associated with pasture- and concentrate-based feeding have been identified as potential biomarkers for products derived from these systems. The significantly higher concentrations of the carotenoids “lutein” and “ β -carotene” in pasture-derived milk are frequently cited as being applicable for this purpose (Nozière *et al.*, 2006; O’Callaghan *et al.* 2016a; O’Callaghan *et al.* 2017; Faulkner *et al.* 2018; Mirzad *et al.*, 2018), along with the volatile degradation products of β -carotene metabolism, toluene and p-cresol (O’Callaghan *et al.* 2017; Faulkner *et al.* 2018; Clarke *et al.*, 2019). Other volatiles correlated with particular feeding systems include 2,3-butanediol in TMR-derived cheese (O’Callaghan *et al.* 2017), dimethyl-sulfone and pentanal in pasture-derived milks (Coppa *et al.*, 2011; Faulkner *et al.* 2018; Clarke *et al.*, 2019) and 2-pentanone in concentrate-derived milk (Glover *et al.*, 2012). Volatile analysis offers an effective method of determination of product origin, although the evaluation made by the consumer, while subjective, can be determined with reasonable certainty based on the perceptible colour difference in fat-containing products arising from the relative levels of β -carotene.

Multivariate statistical analysis has also been used in conjunction with FA (Pagano & Marcella, 2010; O’Callaghan *et al.*, 2017) and metabolomics profiling (O’Callaghan *et al.*, 2018; Panthi *et al.*, 2019; Magan *et al.* 2019a) and spectroscopic methods (Pagano & Marcella, 2010; Coppa *et al.*, 2012; Capuano *et al.*, 2014; Gómez-Mascaraque *et al.*, 2020) to clearly

discriminate between pasture and concentrate-derived dairy products. Using Fourier-transform infrared (FTIR) spectroscopy, Capuano *et al.* (2014) distinguished between grass-fed and conventional milk, though this method could not as effectively discern between grazed grass and grass silage fed indoors or between organic and conventional milk. The substantial effect of feeding system on the FA profile of milk also presents an effective means of differentiation, as demonstrated by Pagano & Marcella (2010) in milk and by O'Callaghan *et al.* (2017) for Cheddar cheese. Using quantitative nuclear magnetic resonance (¹H-NMR), consistent effects of pasture and concentrate feeding on the metabolome of milk and maasdam cheese were reported by O'Callaghan *et al.* (2018) and Panthi *et al.* (2019), respectively, showing clear separation between both the GRS and CLV diets and the TMR diet. O'Callaghan *et al.* (2018) identified particular correlations with urea, dimethyl sulfone and p-cresol and the CLV system, whereas hippuric acid was indicative of pasture feeding in general and particularly of GRS feeding. Panthi *et al.* (2019), also utilising gas chromatography-mass spectrometry (GC-MS), similarly identified toluene, dimethyl sulphide and citrate as clear biomarkers for the GRS, CLV and TMR feeding systems, respectively. The separation between GRS, CLV and TMR feeding systems was also supported by the results of Magan *et al.* (2019a), using liquid chromatography-mass spectrometry (LC-MS) to distinguish skim milk powder and whey protein ingredients derived from each system.

The application of ¹H-NMR, GC-MS or LC-MS is useful for resolution of a large number of metabolite compounds and ¹H-NMR is non-destructive by nature. However, these methods are expensive and require relatively long sample run-times. A recent application of Raman spectroscopy in verification of butter samples may offer a rapid and cost effective alternative to the foregoing methods. Using Raman spectroscopy, Gómez-Mascaraque *et al.* (2020) determined a clear distinction between pasture and TMR-derived butter on the basis of FA and micronutrient quantification, providing further insight into the nutritional quality of

butter originating from different feeding systems, which would be of particular interest to the consumer making a selection on the basis of “grass-fed” labelling.

1.2.6. Conclusion

As new trends in the consumption of dairy products have evolved in recent years, a substantial body of work has been developed to characterise the effect of commonly-practiced pasture and concentrate-based feeding systems on milk and dairy product quality and processability. Considerable differences in the compositional, functional and organoleptic properties of milk derived from these systems have already been determined, but the opportunity remains to establish further knowledge on the effect of dietary factors and milk powder functionality, protein and micro-composition and, ultimately, possible nutritional benefits of milk produced from a given system. The practice of seasonal, pasture-based feeding systems is largely determined by economic and environmental factors, but evidence for potential nutritional benefits associated with both consumer-focused and value-added dairy commodities produced therefrom could also provide valuable considerations in this regard.

1.2.7. References

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Statement of research objectives

The overall objective of the research reported in this thesis was focused on determining the potential for variation in the composition and functional properties of milk protein ingredients due to perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) or total mixed ration (TMR) feeding. The objectives of the studies carried out as part of this thesis are outlined below:

- Previous studies on pasture and concentrate-based feeding of cows have largely focused on the macro-compositional factors of milk and dairy products, with little information available on their effects on minor compounds and metabolites. The effect of GRS, CLV and TMR feeding on the metabolome, vitamin and mineral composition of SMP and whey protein ingredients was thus determined;
- The increasing prevalence of “grass-fed” and “pasture-fed” product labelling claims has necessitated the establishment of methods for composition-based verification of dairy product origin. The use of LC-MS/MS-based metabolomics and proteomics, and Raman spectroscopy for identification of potential biomarkers and differentiation between products derived from GRS, CLV and TMR feeding systems was investigated;
- The functional properties of milk powders in end-use applications and behaviour throughout processing are critical quality attributes governed by milk composition. The effect of GRS, CLV and TMR feeding on the heat stability and gelation properties of powder products was examined;
- Infant milk formula represents one of the primary uses for SMP and whey ingredients; as such, the composition and functional properties of these ingredients were reported with reference to their suitability or implications for infant milk formula manufacture.

Chapter 2

Impact of Bovine Diet on Metabolomic Profile of Skim Milk and Whey Protein Ingredients

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Jiamin Zheng, Lun Zhang, Rupasri Mandal and David S. Wishart facilitated metabolomics analysis at The Metabolomics Innovation Centre, University of Alberta, Edmonton, Alberta, Canada.

Deirdre Hennessy (Teagasc Animal and Grassland Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland) and Mark A. Fenelon (Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland) conceptualized the project experimental for the feeding systems used in this study and contributed to project administration.

2.1. Abstract

The influence of bovine diet on the metabolome of reconstituted skim milk powder (SMP) and protein ingredients produced from the milk of cows fed on pasture or concentrate-based diets was investigated. Cows were randomly assigned to diets consisting of perennial ryegrass only (GRS), perennial ryegrass/white clover sward (CLV), or indoor total mixed ration (TMR) for an entire lactation. Raw milk obtained from each group was processed at pilot scale, to produce SMP and sweet whey, and SMP was further processed at laboratory scale, to yield micellar casein whey and acid whey. The total amino acid composition and metabolome of each sample were analyzed, using high-performance cation exchange and a targeted combination of direct-injection mass spectrometry and reverse-phase liquid chromatography–tandem mass spectrometry (LC–MS/MS), respectively. The nitrogen composition of the products from each of the diets was similar, with one exception being the significantly higher nonprotein nitrogen content in TMR-derived skim milk powder than that from the GRS system. Total amino acid analysis showed significantly higher concentrations of glycine in GRS- and CLV-derived sweet whey and acid whey than in those from TMR. The cysteine contents of CLV-derived micellar casein whey and acid whey were significantly higher than for TMR, whereas the valine content of GRS-derived acid whey was significantly higher than TMR. The phenylalanine content of GRS-derived micellar casein whey was significantly higher than that from CLV. Metabolomic analysis showed significantly higher concentrations of the metabolites glutamine, valine, and phosphocreatine in each ingredient type derived from TMR than those from GRS or CLV, whereas the serine content of each GRS-derived ingredient type was significantly higher than that in TMR-derived ingredients. These results demonstrate that the type of bovine feeding system used can have a significant effect on the amino acid composition and metabolome of skim milk and whey powders and may aid in the selection of raw materials for product manufacture, while the clear separation between the samples gives further evidence

for distinguishing milk products produced from different feeding systems based on LC-MS/MS.

2.2. Introduction

Skim milk powder (SMP) and whey typically form the basis of various dairy product formulations, particularly in infant milk formula (IMF) manufacture. Whey is obtained from the side-streams of other milk processing applications, such as cheese manufacture (Ulber *et al.*, 2001) or directly from skim milk by membrane microfiltration (Pouliot, 2008). All essential amino acids (EAA) are present in whey (Smithers, 2008), including branched-chain amino acids (BCAA), with high bioavailability relative to other dietary protein sources (Kalman, 2014). It is widely accepted that the concentration of amino acids (AA) in bovine milk is primarily influenced by genetic factors (Michaelidou, 2008). While previous studies have investigated the effect of bovine dietary supplementation of individual AAs on milk gross composition (Canale *et al.*, 1990; Swanepoel *et al.*, 2010) and protein synthesis (Lee *et al.*, 2015), little information exists on the potential effect of standard bovine feeding systems on the overall AA composition of milk. Recently, consumer interest has increasingly been focused on dairy products derived from what is perceived as a healthier (Sajdakowska *et al.*, 2018), natural, or more sustainable, origin (Park, 2018). While previous research has demonstrated the effect of diet on the fat fraction of milk, the micronutrient composition of various protein ingredients is an important consideration in the manufacture of dairy products which aim to meet this demand. The relative abundance of low-molecular-weight metabolites may also provide an insight into verification methods for milk from different systems. The increasing prevalence of “grass-fed” product labeling will necessitate such verification methods.

The production of milk from cows fed outdoors on pasture is generally regarded by consumers as a more environmentally sustainable method, with distinct welfare advantages for

cows free to forage naturally (Krohn, 1994; Redbo *et al.*, 2001). Despite this developing consumer interest, pasture-based production systems are estimated to represent only 10% of the global milk supply (Coleman *et al.*, 2009). The dominance of this production system in Ireland and New Zealand is primarily attributable to their mild, temperate climates, with plentiful rainfall enabling long, consistent grass-growing seasons. Larger, more-intensive milk-producing industries in the Americas or Central Asia are almost exclusively based on indoor, concentrate-based feeding systems, allowing for more independence from climatic variances (Redbo *et al.*, 2001). The extent of pasture grazing for milk production in the USA has been observed to decrease substantially with increasing herd size (United States Department of Agriculture, 2016).

The application of quantitative nuclear magnetic resonance ($^1\text{H-NMR}$)-based metabolomics to distinguish between rumen fluid and raw milk from pasture-based and total-mixed-ration-based feeding systems has previously been reported (O’Callaghan *et al.*, 2018). This nontargeted method has proven to be suitable for the purpose of verification of milk product origin, particularly in reference to claims of “pasture-fed” provenance. Reverse-phase liquid chromatography–tandem mass spectrometry (LC–MS/MS) is a highly sensitive analysis technique which utilizes liquid chromatography for the separation of compounds within a sample and subsequent compound analysis, using mass spectrometry. In targeted format, this method can be used for the detection and quantification of known compounds or metabolites, which can be identified from the established metabolome database for that sample type (e.g., milk metabolome). The potential to now extensively analyze the metabolome of dairy ingredients allows for greater understanding of the potential health effects from their consumption. Metabolomics has been demonstrated to offer potential as a mechanism for the verification of milk product origin claims (O’Callaghan *et al.*, 2018).

The objectives of this study were to determine (1) the influence of perennial ryegrass (*Lolium perenne* L.), perennial ryegrass/white clover (*Trifolium repens* L.), and indoor total-mixed-ration-based feeding systems on the metabolome of SMP and whey protein ingredients and (2) the potential of LC–MS-based metabolomics for differentiating between milk and dairy product samples derived from different feeding systems.

2.3. Materials and Methods

2.3.1. Materials

Raw milk was obtained from Teagasc Animal and Grassland Research and Innovation Centre (Moorepark, Fermoy, Co. Cork, Ireland). Hydrochloric acid and sodium hydroxide used for acid whey production were sourced from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

2.3.2. Experimental Design

The experimental design for this study was the same as that previously described in studies which investigated the quality of butter (O’Callaghan *et al.*, 2016b), cheddar cheese (O’Callaghan *et al.* 2017), and raw milks (O’Callaghan *et al.* 2018; O’Callaghan *et al.* 2016a) from these feeding systems. For a detailed description of the feeding system experimental design used in this study, see Egan *et al.* (2016). For the chemical composition of the feeds used during the period of milk collection for this study, see Appendix i (Supplementary Materials, Tables S1, S2 and S3). Briefly, fifty-one spring-calving Holstein-Friesian, with some Holstein-Friesian×Jersey cross-bred cows from the Teagasc Moorepark dairy herd were randomly assigned to three groups ($n=17$) with separate feeding systems. Cows were randomised by breed, calving date (mean 17 February; ± 16 days), parity (2.45) and milk yield (23.7 kg) and milk solids (fat+protein) yield (2.04 kg) for the first 2 weeks post-calving. Mean body weight at the beginning of the experimental period was 519 kg.

Group 1 was fed a total mixed ration (TMR) diet and housed indoors, Group 2 was maintained outdoors on perennial ryegrass only pasture (GRS), and Group 3 was also maintained outdoors on a perennial ryegrass/white clover pasture (CLV) with an average annual sward white clover content of 24%. On a dry matter (DM) basis, the TMR diet consisted of 8.3 kg of concentrates, 4.5 kg of grass silage, and 9 kg of maize silage. Individual electronically controlled Griffith Elder Mealmaster feed bins (Griffith Elder and Company Ltd., Suffolk, England) were used to administer *ad libitum* feed to the cows within the TMR system at 08:30 h daily. Both groups of pasture based cows consumed ~18 kg DM/day. This was allocated using estimates of pre-grazing herbage mass and daily post grazing sward heights as described by Egan et al. (2016). Each group of cows was milked twice daily at 07:30 and 15:30 h. Cows from each of the three feeding systems were milked separately and their milk was segregated into designated 5000 L refrigerated tanks. Tanks were maintained at 4 °C with morning and evening milk being added together and agitated prior to sample collection.

The raw milk of each of the three groups of 17 cows was bulked into three designated bulk tanks over 5 consecutive milkings (3 morning milkings, 2 evening milkings) and collected on two separate occasions over a two-week period in July 2017 (mean days in milk 143) and processed into two batches of skim milk powder (SMP) from each feeding system at Moorepark Technology Ltd. (Moorepark, Fermoy, Co. Cork, Ireland). All of the milk powders within each batch were manufactured on the same day.

2.3.3. Ingredient Manufacture

2.3.3.1. Skim Milk Preparation

Raw whole milk (approximately 1000 kg) was obtained from bulk milk tanks designated to each dietary treatment. This milk was preheated to 50 °C in an APV plate heat exchanger (SPX Flow Technology, Crawley, West Sussex, UK), followed by separation in a

Westfalia centrifugal disk separator (GEA Westfalia, Oelde, Germany), and then it was pasteurized at 72 °C, for 15 seconds. The pasteurized skim milk was preheated to approximately 78 °C and concentrated to ~43% total solids (TS) in a Niro three-effect falling film evaporator (GEA Niro A/S, Soeborg, Denmark), at sequential effect temperatures of 73, 64, and 55 °C, respectively. The concentrate feed was then spray-dried, using a Niro Tall-Form Anhydro three-stage spray dryer (air inlet and outlet temperatures were set at 180 and 85 °C, respectively), using a high-pressure atomization system. External first and second fluid bed temperatures were set at 74 and 24 °C, respectively. All fines were returned from the cyclone to the second fluid bed, yielding a low-heat non-agglomerated skim milk powder (SMP) (~3% moisture).

2.3.3.2. Sweet Whey Preparation

Raw whole milk from each dietary treatment was set aside from the bulk collection described in Section 3.3.1 and pasteurized at 72 °C, for 15 s, using a Microthermics™ tubular heat-exchanger (Microthermics Inc., Raleigh, North Carolina, USA), and then stored in sterilized containers. Milk samples (~10 kg) were then added to a laboratory scale jacketed cheese-production vessel and preheated to 33 °C. Chymosin (Chy-Max Plus, 200 IMCU mL⁻¹; Chr Hansen Ireland Ltd., Cork, Ireland) was diluted in 30 mL of deionized water and added to the milk (0.272 mL L⁻¹), followed by controlled stirring for 3 min. The stirring paddles were then removed from the vessel and replaced with cutters. An aliquot (17 g) of the inoculated milk was weighed into a concentric cylinder in a Discovery HR-1 Hybrid Rheometer (TA Instruments, New Castle, Delaware, USA). After ~35 min, at an elastic modulus (G') reading of 30 Pa, curd cutting was carried out for 1 hour, at 45 °C. The whey was then separated from the curd, using cheesecloth, and stored at -80 °C. The whey was later filtered, using Whatman No. 1 filter paper, and clarified, using a 0.1 µm Sartoclon Slice polyethersulfone cassette membrane (Sartorius AG, Göttingen, Germany), before being freeze-dried in a Labconco

stopping tray dryer equipped with a Freezone 12 plus vacuum collector/refrigerator unit (Labconco, Kansas City, Missouri, USA).

2.3.3.3. Acid Whey Preparation

Skim milk powder from each dietary treatment was reconstituted to 9% TS and maintained at 20 °C in a water bath. Hydrochloric acid (HCl) (2 M) was added to the milk in order to decrease the pH to the isoelectric point of pH 4.6, and the precipitated casein curd was then removed from the whey, using cheesecloth. The pH of the whey was then readjusted to pH 6.7, using sodium hydroxide (NaOH), and it was later filtered, clarified, and freeze-dried, as described in Section 3.3.2.

2.3.3.4. Micellar Casein Whey Preparation

Skim milk powder from each dietary treatment was reconstituted to 9% TS and filtered through a 0.1 µm Sartocon Slice polyethersulfone cassette membrane, at approximately 2.0 bar under recirculation mode (30 °C) in order to separate micellar casein and whey protein. Both micellar casein from the retentate and the whey permeate stream were then freeze-dried, as described in Section 3.3.2.

2.3.4. Determination of Nitrogen Content

The total nitrogen content of each sample was determined through the Kjeldahl method, as described in ISO 8968-1 (2001), using a nitrogen-to-milk protein conversion factor of 6.38. Nonprotein nitrogen content was determined by precipitation of the protein component of each sample, using trichloroacetic acid (15% w/v). The precipitate was removed from the mixture by using Whatman No. 1 filter paper, and a Kjeldahl determination was then carried out on the filtrate, as described above.

2.3.5. Total Amino Acid Analysis

Acid hydrolysis was carried out to break complete proteins and peptides down to individual AAs. Proteins were hydrolyzed with 6 N HCl at 110 °C, for 23 h, using a Gals-Col combo mantle (Gals-Col, Terre Haute, USA) for the determination of all AAs except sulfur AAs and tryptophan. Methionine and cysteine were oxidized with performic acid to methionine sulfone and cysteic acid, respectively, and then hydrolyzed with HCl. The resulting hydrolysates were then diluted 1 in 2 with the internal standard, norleucine, to give a final concentration of 125 nm/ml. Amino acids were quantified by using a Jeol JLC-500/V amino acid analyzer (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na⁺ high-performance cation-exchange column. The analyzer uses an ion-exchange column with post-column online reactor derivatization with ninhydrin.

2.3.6. Liquid Chromatography–Mass Spectrometry (LC–MS)

Metabolite analysis was carried out at The Metabolomics Innovation Centre (University of Alberta, Edmonton, Alberta, Canada). A targeted quantitative metabolomics approach was used to analyze the milk samples, using a combination of direct injection mass spectrometry (DI-MS) with a reverse-phase LC–MS/MS assay. The method used combines the derivatization and extraction of analytes and the selective mass-spectrometric detection, using multiple reaction monitoring (MRM) pairs. Isotope-labeled internal standards were used for metabolite quantification. All the milk samples were thawed on ice and were vortexed and centrifuged at 13,000x g. Ten µL of each milk sample was loaded and dried in a stream of nitrogen, and 50 µL of a 5% solution of phenyl-isothiocyanate was then added for derivatization. After incubation, samples were dried again, using a nitrogen evaporator. Extraction of the metabolites was then achieved by adding 300 µL of methanol containing 5 mM of ammonium acetate. Extracts (150 µL) were diluted in a 1:1 ratio with water for LC–MS/MS analysis of

AAs and biogenic amines. The remaining 150 μL of extracts was mixed with 400 μL of running solvent for DI-MS analysis of lipids and carnitines.

Mass spectrometric analysis was performed on a 4000 QTrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, California, USA) equipped with an Agilent 1100 LC system. The samples were delivered to the mass spectrometer by an LC method, followed by a direct injection (DI) method. Data analysis was performed, and concentrations were calculated by using Analyst software 1.6.2.

2.3.7. Statistical Analysis

Statistical analysis was performed by using Genstat v18.1 (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK). Datasets were analyzed for normality, using the Shapiro–Wilk’s test. Data were deemed normally distributed, and the analysis was carried out by using a 4 x 3 factorial ANOVA with post hoc Tukey test. The p -values < 0.05 were considered significant. Multivariate statistical analysis was carried out by using Metaboanalyst 3.0 (Xia & Wishart, 2016) software, from which the figures were also generated. All ingredients were produced in duplicate to give 24 separate powders. LC-MS/MS analysis was then carried out in duplicate for each sample.

2.4. Results and Discussion

2.4.1. Nitrogen Composition

Average concentrations of total nitrogen and nonprotein nitrogen (NPN) for SMP and whey powder samples are shown in Table 2.1. The total protein content of SMP and acid whey samples derived from both pasture-based systems were higher than those from the TMR system, whereas TMR-derived sweet whey and micellar casein whey samples contained higher total protein levels than samples derived from either pasture-based system. Nonprotein nitrogen content was highest in all TMR-derived ingredients and lowest in all samples derived from

GRS. Indeed, the NPN content of TMR-derived SMP was significantly higher than that produced from the GRS-derived milk (Table 2.1). Increased NPN content has previously been observed in raw milk (O’Callaghan *et al.*, 2016a) and whole milk powder (Magan *et al.* 2019b) derived from a CLV feeding system. Urea is the primary component of NPN (Bryant *et al.*, 1999) and products derived from the CLV system exhibit increased urea levels, resulting from the inclusion of nitrogen-fixing white clover in this system (O’Callaghan *et al.*, 2018). The increased concentrations of NPN observed in the TMR sample may arise from greater levels of dietary crude protein in the concentrate ration, which does not necessarily correlate with increased milk true protein content, but rather increased milk urea (Broderick, 2003).

A study which analyzed the rumen fluid of cattle fed varying proportions of roughage in a total mixed ration diet found decreasing NPN content with increasing roughage content relative to concentrate content, though these figures did not differ significantly (Sinha *et al.*, 2017). In the present study, increased average total protein content was observed in sweet whey samples derived from each feeding system, relative to micellar casein whey and acid whey, both of which were equivalent. The higher level of protein in sweet whey is likely attributable to the presence of soluble glycomacropeptide (GMP) resulting from the renneting process. Sweet whey derived from cheese manufacture contains 20–25% glycomacropeptide (Sharma *et al.*, 2013).

Table 2.1. Average total nitrogen and nonprotein nitrogen content of skim milk powder, sweet whey, micellar casein whey, and acid whey, as determined by Kjeldahl analysis.

Sample Type	Total Protein (% w/w)			Nonprotein Nitrogen (% w/w)		
	GRS	CLV	TMR	GRS	CLV	TMR
Skim milk powder	37.2 (\pm 0.57) ^b	37.5 (\pm 0.06) ^d	36.1 (\pm 0.61) ^c	0.27 (\pm 0.01) ^{b,A}	0.32 (\pm 0.06) ^{b,A,B}	0.37 (\pm 0.00) ^{c,B}
Sweet whey powder	9.27 (\pm 0.29) ^a	9.41 (\pm 0.68) ^{b,c}	9.64 (\pm 0.17) ^b	0.25 (\pm 0.01) ^{a,b}	0.27 (\pm 0.04) ^{a,b}	0.32 (\pm 0.01) ^{a,b,c}
Micellar casein whey powder	8.07 (\pm 0.45) ^a	6.98 (\pm 0.61) ^a	8.23 (\pm 0.86) ^{a,b}	0.21 (\pm 0.01) ^{a,b}	0.23 (\pm 0.04) ^a	0.28 (\pm 0.03) ^{a,b}
Acid whey powder	7.64 (\pm 0.17) ^a	7.98 (\pm 0.17) ^{a,b}	7.50 (\pm 0.47) ^a	0.19 (\pm 0.01) ^a	0.21 (\pm 0.04) ^a	0.25 (\pm 0.01) ^a

GRS—cows fed perennial ryegrass only.

CLV—cows fed perennial ryegrass/20% white clover sward.

TMR—cows fed indoor total mixed ration *ad-libitum*.

^{a,b,c,d} indicates values within a column not sharing a common lower-case superscript letter differed significantly ($p < 0.05$).

^{A,B} indicates values within a row not sharing a common upper-case superscript letter differed significantly ($p < 0.05$).

2.4.2. Total Amino Acid Composition

Average concentrations (g per kg total protein) for nineteen AAs in each ingredient type from each feeding system are shown in Table 2.2. The feeding system had a significant effect on concentrations of glycine, cysteine, valine, and phenylalanine in sweet whey, micellar casein whey, and acid whey samples. Concentrations of glycine in GRS-derived acid whey were significantly higher than those from TMR, though concentrations were significantly higher in sweet whey derived from both pasture-based systems when compared to TMR. Glycine is a nonessential proteinogenic AA, primarily utilized in the synthesis of collagen, with limited function in the synthesis of other proteins and metabolic pathways (de Paz-Lugo *et al.*, 2018). Previous work by Meléndez-Hevia and de Paz-Lugo (2008) suggests that a restriction in the stoichiometry of the glycine synthesis reaction may lead to insufficient glycine production relative to metabolic demand, making glycine an essential or conditionally essential AA.

The CLV feeding system produced both micellar casein whey and acid whey with significantly higher concentrations of cysteine than the TMR system. Cysteine is a nonessential proteinogenic AA which, uniquely amongst the AAs, contains a thiol group. Cysteine also has a function in energy metabolism, along with some antioxidant capacity, owing to the affinity for redox reactions due to the presence of the thiol group (Poole, 2015). Acid whey derived from GRS feeding had a significantly higher valine content than that from the TMR system. Valine, alongside leucine and isoleucine, is one of the three essential BCAAs (Brosnan & Brosnan, 2006). It plays a structural role in the synthesis of globular proteins, where it forms the nonpolar center surrounded by polar residues (Manavalan & Ponnuswamy, 1977), along with other metabolic functions, such as insulin secretion (Zhang *et al.*, 2017). The only significant difference between both pasture-based feeding systems was that of the phenylalanine content of micellar casein whey, with GRS > CLV. Phenylalanine is an EAA

which acts as a precursor for the synthesis of the nonessential AA tyrosine in the body. Both phenylalanine and tyrosine are utilized in the synthesis of amine-based hormones, such as dopamine, tyramine, and adrenaline, released within the body in stress responses (Fernstrom & Fernstrom, 2007).

The overall average values for each diet (combining each protein ingredient type) indicate a significant effect of feeding system over a wider range of AAs than in each ingredient type alone. These differences can be briefly summarized as follows: GRS > TMR for glutamine, alanine, and isoleucine; GRS and CLV > TMR for glycine and valine; CLV > GRS and TMR for cysteine; GRS > CLV and TMR for phenylalanine ($p < 0.05$). These effects are noteworthy, given the dominant influence of genetic factors in the total AA distribution of milk and the randomized distribution of cows into each feeding system in this study. Vanhatalo et al. (2009) observed a significant effect of forage-feed type on plasma AA concentrations, with generally increased concentrations recorded for cows fed red clover silage in comparison to those fed grass.

Variations in the AA composition of each whey ingredient type are apparent when expressed as a combined average of the three feeding systems (Table 2.3). When compared to micellar casein whey and acid whey, sweet whey samples contained a significantly higher average concentration of the AAs most associated with the GMP component of cheese: isoleucine, proline, serine, valine, and threonine. Threonine, in particular, is an EAA present in high concentrations within GMP. Significantly higher concentrations of glycine, cysteine, and tyrosine were present in micellar casein whey, when compared to sweet whey and acid whey, whereas the histidine content of acid whey was significantly higher than both other whey types. While the total protein content of the SMP samples is markedly higher than that of each whey type, increased values for cysteic acid, taurine, aspartic acid, glycine, alanine, and cysteine were observed for each whey type when compared to SMP, owing to concentration of

these AAs in the whey protein fraction of milk protein. Gorissen and Witard (2017) reported similarly increased relative concentrations of glycine, cysteine, alanine, and aspartic acid in whey protein, when compared to skimmed milk and particularly casein. The protein used in the manufacture of IMF is selected on the basis of the amino acid profile that best mimics that of human milk. Variations due to feeding in the amino acid profile of skim milk powder and whey-protein powders, which typically form the primary ingredients for conventional IMF production, would be a noteworthy consideration for IMF manufacturers.

Table 2.2. Total amino acid composition of skim milk powder, sweet whey, micellar casein whey, and acid whey, determined by high-performance cation exchange.

TAA g/kg Total Protein	Skim milk powder			Sweet whey powder			Micellar casein whey powder			Acid whey powder		
	GRS	CLV	TMR	GRS	CLV	TMR	GRS	CLV	TMR	GRS	CLV	TMR
Cysteic acid	11.3	11.9	10.7	30.4	30.2	30.1	35.5	33.4	34.3	36.0	34.4	35.8
Methionine Sulfone	33.6	34.7	35.0	21.8	22.1	20.9	24.8	22.3	22.2	20.8	21.4	21.4
Asparagine	74.0	74.5	74.9	101	100	98.1	104	99.6	101	105	103	104
Threonine	41.5	41.3	41.6	64.3	62.9	60.0	43.3	40.0	41.9	43.0	42.1	41.3
Serine	50.6	50.3	50.4	42.7	41.3	39.5	36.9	32.9	35.2	36.0	34.7	33.8
Glutamine	192	195	192	165	161	149	162	148	150	166	161	147
Glycine	16.4	16.8	15.7	21.0 ^b	19.7 ^b	16.5 ^a	21.5	21.2	19.0	20.4 ^b	19.5 ^{a,b}	17.2 ^a
Alanine	28.6	28.2	28.1	41.0	42.8	38.5	40.3	34.0	32.8	39.5	37.3	36.7
Cysteine	7.21	8.21	7.97	21.4	25.4	22.4	26.8 ^{a,b}	36.7 ^b	22.6 ^a	23.1 ^{a,b}	31.9 ^b	16.2 ^a
Valine	55.9	58.4	56.8	56.6	55.0	54.2	54.1	49.5	45.2	53.4 ^b	51.3 ^{a,b}	38.6 ^a
Isoleucine	44.1	46.0	44.9	55.7	53.6	50.5	46.0	40.5	42.7	48.5	46.2	44.9
Leucine	94.4	96.4	95.6	91.3	92.0	85.6	103	97.3	96.6	108	106	98.5
Tyrosine	30.5	34.5	33.0	9.25	10.0	12.5	14.3	13.3	13.8	9.23	11.0	9.20
Phenylalanine	42.8	42.6	41.2	25.2	23.7	24.2	32.5 ^b	26.0 ^a	29.6 ^{a,b}	31.2	31.4	28.3
Histidine	36.0	35.8	36.1	26.3	29.3	26.7	35.1	37.9	33.1	41.8	43.2	37.5
Lysine	73.4	73.8	74.5	78.4	77.5	73.9	81.2	72.7	74.6	85.2	81.6	78.6
Arginine	31.7	32.3	31.8	20.5	20.3	20.9	25.2	24.7	26.0	26.3	25.3	24.2
Proline	85.0	86.9	89.6	45.6	50.0	36.5	28.3	25.7	34.3	31.5	26.3	23.0

GRS—cows fed perennial ryegrass only.

CLV—cows fed perennial ryegrass/20% white clover sward.

TMR—cows fed indoor total mixed ration *ad-libitum*.^{a,b} indicates values within a row for each ingredient not sharing a common superscript letter differed significantly ($p < 0.05$).

Table 2.3. Average values for total amino acid composition of sweet whey, micellar casein whey, and acid whey, determined by high-performance cation exchange.

TAA g/kg Total Protein	Sweet whey powder	Micellar casein whey powder	Acid whey powder
Cysteic acid	30.2 (\pm 0.18) ^a	34.4 (\pm 1.09) ^b	35.4 (\pm 0.84) ^b
Methionine Sulfone	21.6 (\pm 0.63)	23.1 (\pm 1.50)	21.2 (\pm 0.36)
Asparagine	99.7 (\pm 1.36)	102 (\pm 2.31)	104 (\pm 1.25)
Threonine	62.4 (\pm 2.22) ^b	41.7 (\pm 1.64) ^a	42.1 (\pm 0.89) ^a
Serine	41.1 (\pm 1.60) ^b	35.0 (\pm 2.00) ^a	34.8 (\pm 1.09) ^a
Glutamine	158 (\pm 8.39)	153 (\pm 7.95)	158 (\pm 9.86)
Glycine	19.0 (\pm 2.33) ^a	20.6 (\pm 1.37) ^b	19.0 (\pm 1.63) ^a
Alanine	40.8 (\pm 2.16) ^b	35.7 (\pm 4.01) ^a	37.8 (\pm 1.48) ^{a,b}
Cysteine	23.1 (\pm 2.09) ^a	28.7 (\pm 7.27) ^b	23.7 (\pm 7.86) ^a
Valine	55.3 (\pm 1.23) ^b	49.6 (\pm 4.46) ^a	47.8 (\pm 7.98) ^a
Isoleucine	53.3 (\pm 2.66) ^b	43.1 (\pm 2.80) ^a	46.5 (\pm 1.80) ^a
Leucine	89.6 (\pm 3.51) ^a	98.9 (\pm 3.41) ^b	104 (\pm 4.93) ^b
Tyrosine	10.6 (\pm 1.72) ^a	13.8 (\pm 0.54) ^b	9.80 (\pm 1.02) ^a
Phenylalanine	24.4 (\pm 0.74) ^a	29.3 (\pm 3.24) ^b	30.3 (\pm 1.76) ^b
Histidine	27.4 (\pm 1.66) ^a	35.4 (\pm 2.43) ^b	40.8 (\pm 2.97) ^c
Lysine	76.6 (\pm 2.39)	76.2 (\pm 4.47)	81.8 (\pm 3.32)
Arginine	20.5 (\pm 0.30) ^a	25.3 (\pm 0.63) ^b	25.3 (\pm 1.04) ^b
Proline	44.0 (\pm 6.90) ^b	29.5 (\pm 4.40) ^a	27.0 (\pm 4.28) ^a

GRS—cows fed perennial ryegrass only.

CLV—cows fed perennial ryegrass/20% white clover sward.

TMR—cows fed indoor total mixed ration *ad-libitum*.

^{a,b,c} indicates values within a row not sharing a common superscript letter differed significantly ($p < 0.05$).

2.4.3. Metabolomic Profiles of Protein Ingredients

Liquid Chromatography–Mass Spectrometry/Mass Spectrometry analysis identified 46 individual metabolite compounds (47 in total) in SMP and whey protein samples, 25 of which were free AAs, including 19 of the 20 standard proteinogenic AAs. The average concentration of each metabolite is shown for SMP, sweet whey, micellar casein whey, and acid whey in Appendix i Tables S4–S7, respectively, and as an overall average by feeding system in Table S5. Glutamic acid was the most abundant free AA in all samples from each feeding system. Glutamic acid has previously been shown to be the free AA present in the highest concentration in milk (McDermott *et al.*, 2016; Ferchaud Roucher *et al.*, 2013).

Four compounds were found to be significantly different between diets across the four ingredient types and are shown in abbreviated form in Table 2.4. In each of the four ingredient types, concentrations of glutamine in the TMR sample were significantly higher than both the GRS and CLV samples. Glutamine is generally regarded as a nonessential AA, although it has recently been suggested to be considered conditionally essential, following investigation of stress-response requirements (Smith, 1990). It is primarily utilized in the biosynthesis of proteins, with additional functions in glycogen synthesis and the maintenance of the intestinal mucous membrane (Smith, 1990). Concentrations of serine in GRS-derived SMP, sweet whey, and micellar casein whey samples were significantly higher than in those derived from TMR. Serine is regarded as a conditionally essential AA, which, like most other AAs, plays a role in protein synthesis, with additional functions in cell proliferation, hepatic gluconeogenesis (de Koning *et al.*, 2003), and immune response (Ma *et al.*, 2017). Similarly, significantly higher concentrations of phosphocreatine were observed in the TMR sample in each of these three ingredient types. Phosphorylation of the endogenous AA creatine occurs within muscle tissue by the action of creatine kinase (Wyss & Kaddurah-Daouk, 2000). Phosphocreatine is integral to adenosine triphosphate generation within the muscle and subsequent control of muscle contraction (Guimarães-Ferreira, 2014). Concentrations of valine were significantly higher in TMR-derived SMP, micellar casein whey, and acid whey than both pasture-based systems. This is notable, given the lower concentrations of valine in TMR samples in the aforementioned total AA analysis, which may imply a greater proportion of total valine in the GRS and CLV samples is present in the bound form.

Although the concentrations of most total AAs were lower in the TMR-derived samples than both the GRS and CLV samples (Table 2.2), the inverse can be seen for the concentrations of most free AAs in the metabolome analysis (Appendix i, Tables S4–S8). As free AAs are constituents of the nonprotein nitrogen component of milk (Huber & Kung,

1981), the overall increased concentrations of free AAs in the TMR samples in each product may be correlated to their increased nonprotein nitrogen contents (Table 2.1), which represent a greater proportion of the total nitrogen of these samples than those from GRS or CLV. This trend may also suggest that, while the quaternary structure of milk proteins assembled from amino acids transferred to the bovine mammary gland may be genetically determined, the concentrations of free amino acids in the serum phase of milk (i.e., aqueous phase containing whey proteins) may be influenced by dietary interventions. In comparison to the other protein ingredients, acid whey samples exhibited greater variation in the average concentrations of a number of metabolites. Average concentrations of acetylmethionine, alpha-aminoadipic acid, and leucine in acid whey were 3 to 4 times higher than the other ingredient types, with concentrations of tyrosine extremely low or absent, in comparison to the other products.

Metabolomic analysis of raw milk produced from cows on the three feeding systems used in this study, using ¹H-NMR (O'Callaghan et al., 2018), identified 11 metabolites in common with this study: aspartic acid, betaine, choline, creatine, creatinine, isoleucine, glutamic acid, leucine, proline, tyrosine, and valine. Among these compounds, a significant effect of feeding system was found for concentrations of betaine, choline, creatinine, proline, tyrosine, and valine (Appendix i, Table S8) in both studies. Of all metabolites identified in the present study, choline was present in the highest concentrations in all samples, though average concentrations for GRS samples were significantly higher than those from TMR (Appendix i, Table S8). A similar result was observed for raw milk by O'Callaghan et al. (2018), although the inverse was found in rumen fluid samples with significantly higher choline concentrations observed in TMR samples than in both GRS and CLV samples. Choline is considered an essential nutrient both in bovine (Sharma & Erdman, 1988a) and human (Zeisel & da Costa, 2009) nutrition, wherein it has a number of important functions, such as phospholipid biosynthesis and neurotransmitter synthesis via conversion to

acetylcholine (Zeisel & da Costa, 2009). In cattle, dietary choline is susceptible to extensive ruminal degradation and must be supplemented in a rumen-protected form (Sharma & Erdman, 1988a), whereas the majority of choline secreted into milk is the product of de novo synthesis of phosphatidylcholine (José *et al.*, 2009). It is therefore unlikely that the differences in choline content observed between milk and whey produced from cows from each feeding system can be attributed to direct absorption and transfer of choline from each feed type. Rather, it may infer that the differences observed are attributable to modulation due to diet of the rumen microflora (in this case, protozoa), as was previously suggested by O'Callaghan *et al.* (2018), leading to variation in rates of de novo choline synthesis. José *et al.* (2009) suggest that a high-concentrate diet may reduce rumen protozoa numbers, leading to a decrease in the availability of choline to the cow. An additional consideration regarding the choline content of milk and whey protein ingredients is the higher level present in human milk compared to bovine milk (Holmes-McNary *et al.*, 1996). As choline is essential to the organ growth and synthesis of cell membranes for the neonate (Artegoitia *et al.*, 2014), the production of IMF from SMP or whey with low innate choline concentrations would necessitate a higher level of supplementation within the formulation.

In the present study, the overall metabolome of the protein ingredients was shown to be significantly influenced by the type of feeding system when analyzed by principal component analysis (Appendix i, Figure S1) of the metabolomics analysis data. While both pasture-based systems show similar distributions, the distribution of the TMR system is narrow and distinct from both GRS and CLV, displaying low axial variance. Partial-least-square discriminant analysis (Figure 2.1) shows the significant overlap between the samples from the GRS and CLV systems and the pronounced separation between both of these systems and that of TMR. The overlap between the GRS and CLV samples is to be expected, given the similarity of these diets. This trend can also be shown clearly by using hierarchical

clustering analysis (Figure 2.2), which represents the degree of positive or negative correlation of each metabolite to each feeding system. A clear separation between the TMR and pasture-based samples was also demonstrated by this analysis, offering evidence to support the applicability of LC–MS-based metabolomics for differentiation between dairy products derived from different feeding systems. This method may be appropriate for implementation into a milk-quality-determination laboratory setting, where the preparatory equipment used is typically readily available, and operators may already be trained in chromatographic and mass spectroscopy methods. However, in comparison to NMR, this method requires more extensive sample preparation (e.g., derivatization) and longer sample run times, though LC–MS offers greater sensitivity and greater potential for resolution of a larger number of compounds.

Table 2.4. Average concentrations (μM) of metabolites which showed significant differences between feeding systems for skim milk powder, sweet whey, micellar casein whey, and acid whey, determined by LC–MS/MS.

Ingredient	Metabolite (μM)	GRS	CLV	TMR
Skim milk powder	Glutamine	4.38 (± 0.54) ^a	4.95 (± 1.24) ^a	12.4 (± 0.28) ^b
	Phosphocreatine	8.05 (± 1.70) ^{a,b}	6.32 (± 0.56) ^a	16.3 (± 4.45) ^b
	Serine	22.1 (± 0.78) ^b	18.3 (± 1.20) ^{a,b}	10.0 (± 0.55) ^a
	Valine	7.49 (± 0.12) ^a	7.27 (± 1.09) ^a	11.3 (± 1.34) ^b
Sweet whey powder	Glutamine	1.36 ^{*a}	0.187 (± 0.02) ^a	7.02 (± 3.20) ^b
	Phosphocreatine	9.65 (± 0.93) ^a	6.49 (± 0.70) ^a	22.6 (± 6.01) ^b
	Serine	25.3 (± 3.11) ^b	18.7 (± 2.33) ^{a,b}	9.31 (± 0.64) ^a
Micellar casein whey powder	Glutamine	4.04 (± 0.23) ^a	3.19 (± 1.77) ^a	11.5 (± 2.64) ^b
	Phosphocreatine	7.21 (± 1.64) ^a	5.37 (± 0.61) ^a	17.1 (± 1.41) ^b
	Serine	21.9 (± 1.13) ^b	18.8 (± 1.27) ^{a,b}	9.62 (± 1.10) ^a
Acid whey powder	Valine	7.00 (± 0.74) ^a	7.92 (± 1.43) ^{a,b}	10.6 (± 0.07) ^b
	Glutamine	2.39 (± 1.74) ^a	2.97 (± 1.07) ^a	12.3 (± 0.42) ^b
	Valine	6.80 (± 1.97) ^a	7.29 (± 0.69) ^a	12.0 (± 1.63) ^b

GRS—cows fed perennial ryegrass only.

CLV—cows fed perennial ryegrass /20% white clover sward.

TMR—cows fed indoor total mixed ration *ad-libitum*.^{a,b} indicates values within a row not sharing a common superscript letter differed significantly ($p < 0.05$).

*Denotes where a replicate was below the limit of detection or limit of quantification.

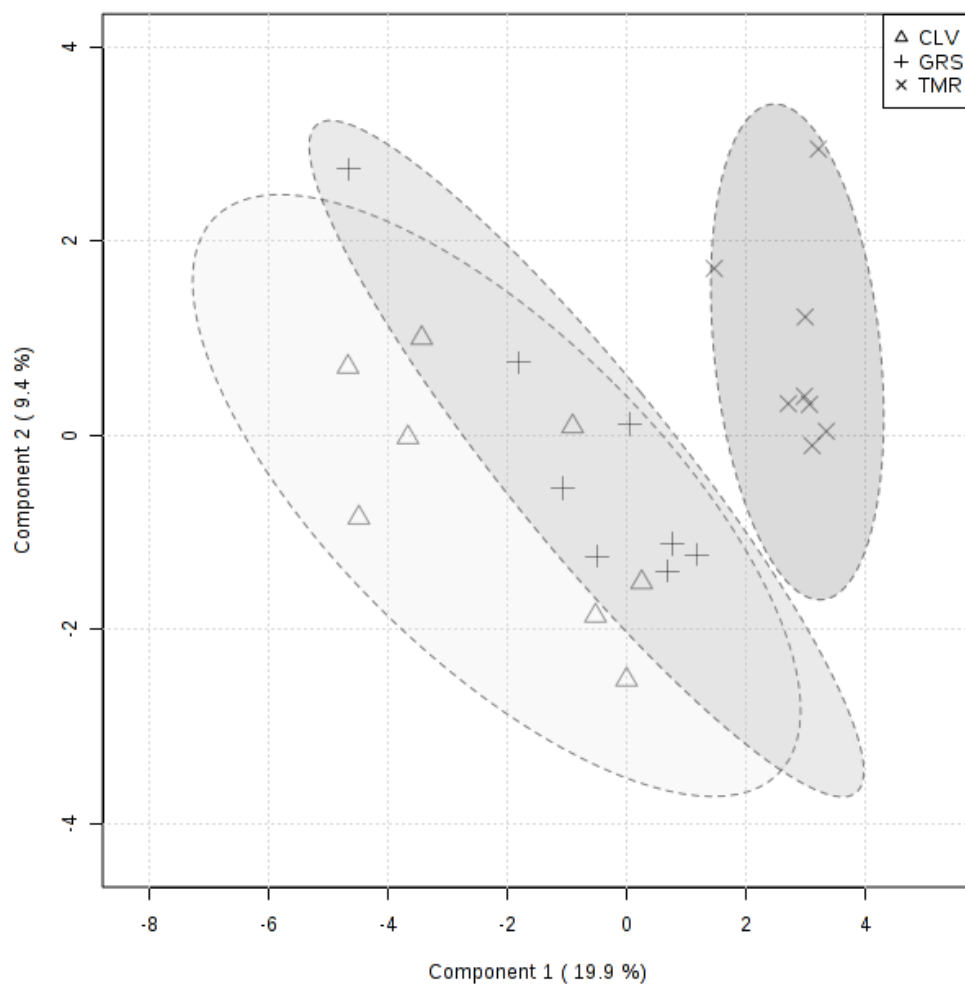


Figure 2.1. Partial-least-square discriminant analysis (PLS-DA) score plot for protein ingredient metabolome from milk of cows fed perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

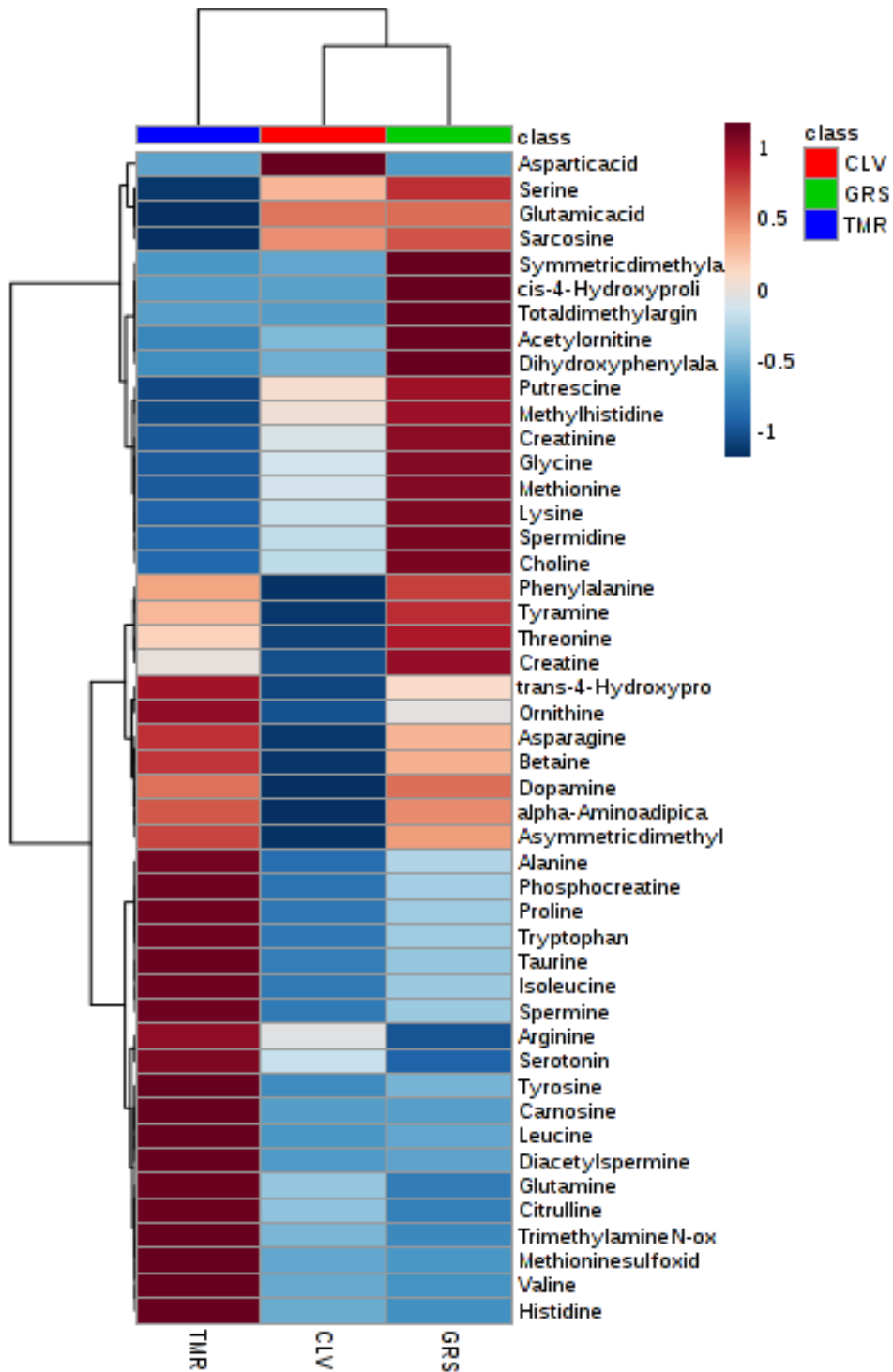


Figure 2.2. Heat map showing average SMP and whey ingredient metabolites from cows fed on perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), or total mixed ration (TMR) feeding systems, determined by LC–MS/MS. Degree of positive and negative correlation between metabolite and diet is indicated by +1 (red) to –1 (blue).

2.5. Conclusion

The data presented in this study contribute to the overall characterization of the composition of milk products derived from the milk of cows fed via three widely practiced feeding systems. This lends an insight into the effect of these diets on the AA composition of milk, which has received limited attention to date. The significant differences observed in the total AA analysis suggest that the AA composition of milk may be more responsive to variation due to diet than previously assumed. Significant variation was observed in the average AA composition between each whey type (acid, sweet, and micellar casein), which may be an important consideration for nutritional formulations. The greater overall concentrations of free AAs in TMR-derived samples may be linked to the increased nonprotein nitrogen content of these milks. The significant effect of the type of feeding system on the metabolome of each ingredient type supports previous work examining the metabolome of pasture and concentrate-derived milk. As such, this implies that LC–MS-based metabolomics may be a suitable method for the differentiation of milks and whey products from different feeding systems.

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Chapter 3

Effect of Diet on the Vitamin B Profile of Bovine Milk-Based Protein Ingredients

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Deirdre Hennessy (Teagasc Animal and Grassland Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland) and Mark A. Fenelon (Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland) conceptualized the project experimental for the feeding systems used in this study and contributed to project administration.

3.1. Abstract

The influence of diet on the water-soluble vitamin composition of skim milk powder and whey protein ingredients produced from the milk of cows fed pasture or concentrate-based diets was examined. Fifty-one Holstein-Friesian cows were randomly assigned into three diets ($n=17$) consisting of outdoor grazing of perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), or indoor feeding of total mixed ration (TMR) for an entire lactation. Raw mid-lactation milk from each group was processed into skim milk powder and further processed to yield micellar casein whey and acid whey. Sweet whey was also produced by renneting of pasteurised whole milk from each system. The water-soluble vitamin profile of each sample was analysed using a combination of direct injection mass spectrometry and reverse-phase liquid chromatography–mass spectrometry. Vitamin B3 and B3-amide concentrations were significantly higher ($p < 0.05$) in TMR-derived samples than in those from CLV and GRS, respectively. Vitamin B1, B2, and B7 concentrations were significantly higher in GRS and CLV-derived samples than those from TMR. Significant differences in vitamins B1, B2, and B3-amide were also observed between protein ingredient types. This study indicates that bovine feeding systems have a significant effect on B vitamin composition across a range of protein ingredient types.

3.2. Introduction

Protein-based “value-added” commodities such as skim milk powder (SMP) and derivatives thereof (e.g., whey produced from milk acidification or membrane filtration) are widely used as the primary materials in dairy-based product formulations, particularly in the manufacture of infant milk formula, providing much of the protein, lactose and water-soluble micronutrients required for such formulations. Due to the high value of milk fat in comparison to skim milk, fat-filled milk powders can be produced at low cost by blending

vegetable oils with SMP. However, with studies on the potential health benefits of milk fatty acids becoming increasingly positive (Gómez-Córtés *et al.*, 2018; German *et al.*, 2009), applications for milk fat in added-value nutritional beverages may become more widespread. This may contribute to reducing dependency on palm oil, the production of which is widely recognised as a major environmental issue (Vijay *et al.*, 2016).

Consumer awareness of sustainable food production is increasing (Vermeir & Verbeke, 2006; Park, 2018), along with professional interest in communicating the concept of high nutrient-density foods to the consumer (Mobley *et al.*, 2009). Milk and dairy products provide a good dietary source of water-soluble B vitamins, particularly vitamins B2 (riboflavin), B5 (pantothenic acid), and B12 (cobalamin), each with comparatively high bioavailability (Hewson *et al.*, 2007; Stokstad *et al.*, 1981; Matte *et al.*, 2011). Milk is also a particularly good source of cobalamin as it is exclusively produced by soil bacteria and archaea (Matte *et al.*, 2011), which can be consumed by grazing ruminants or by ruminal synthesis through the uptake of precursory cobalt from the soil (Walker & Elliot, 1972). Cobalamin cannot be acquired by humans from most dietary plant sources (Herbert, 1988). Previously, Jensen (1995a; 1995b) reported the standard quantities of water-soluble and fat-soluble vitamins in milk.

Consumer perceptions of healthier milk and dairy products increasingly tend towards pasture-based production systems (Croissant *et al.*, 2007). Rising interest in pasture-derived milk is reflected by the increasing popularity of dairy products marketed on the basis of claims of “pasture-fed” provenance. However, pasture-based milk production systems are climate-dependent and represent only a minor proportion of overall global milk production (Coleman *et al.*, 2009). These systems are the most widely practiced in New Zealand and Ireland, where recent marketing efforts have been increasingly focused on the potential environmental (Arsenault *et al.*, 2009) and health (Benbrook *et al.*, 2013) benefits of their use. “Grass-fed” marketing claims have also become widespread among the protein

supplement industry in the USA, where milk production is almost exclusively based on indoor, concentrate feeding-based systems (United States Department of Agriculture, 2016). Indeed, the vast majority of international milk suppliers use a concentrate-based system. For the consumer, concerns often emerge regarding animal welfare and the environmental impact of these systems (Weinrich *et al.*, 2014). These changing considerations have resulted in renewed interest in pasture-based milk production in the USA, where research into their economic merit is on-going.

A significant effect of feeding system on the fatty acid profile of milk and dairy products has previously been shown (O'Callaghan *et al.*, 2016a; Mitani *et al.*, 2016). While the effect of pasture or concentrate feeding on levels of fat-soluble vitamins such as retinol (vitamin A) and alpha-tocopherol (vitamin E) has been investigated in meat (Daley *et al.*, 2010), neither the fat-soluble nor water-soluble vitamin profiles have been established for the milk of cows fed on these systems. Numerous studies (Dufva *et al.*, 1983; Zimmerly & Weiss, 2001; Milda *et al.*, 1998) have investigated the effect of dietary supplementation of water-soluble vitamins (particularly biotin) on cow performance and health, though not on the subsequent vitamin composition in milk. Similarly, studies (Santschi *et al.*, 2005; Nuernberg *et al.*, 2005) have determined the effect of grass and concentrate feeding on water-soluble vitamin levels in rumen fluid and muscle tissue, but not in milk or milk-derived ingredients such as whey. Nonetheless, the importance of milk and dairy products as major sources of water soluble vitamins in human nutrition merits investigation of the variation in vitamin composition between milks derived from cows fed on different commonly practiced feeding systems.

Bovine milk is a source of water-soluble vitamins which are present in quantities that can contribute substantially to the minimum recommended daily allowance for children or adults. Skim milk powder also typically forms the nutritional base for infant milk formula (IMF) manufacture, where it is combined with lactose, demineralised whey, or whey protein

concentrate to achieve an overall composition similar to that of human breast milk. Therefore, potential effects of ruminant feeding systems on the vitamin B content of skim milk and whey ingredients may be important considerations for formulation design and nutritional quality, particularly in relation to IMF production.

With this considered, the objective of this study was to determine the influence of perennial ryegrass (*Lolium perenne* L.), perennial ryegrass/white clover (*Trifolium repens* L.), and indoor total mixed ration-based feeding systems on the water-soluble vitamin composition of SMP and whey protein ingredients.

3.3. Materials and Methods

3.3.1. Materials

All materials were as described in Chapter 2.

3.3.2. Experimental Design

This study utilised the same SMP and whey powders used in Chapter 2 and, as such, all experimental design conditions and powder production methods are as previously described. Details of diet composition and administration, milking times, milk segregation and storage and milk collection were as described in Chapter 2. Chemical composition tables for each feed type are shown in Appendix i, Supplementary Materials, Tables S1, S2 and S3.

3.3.3. Protein Ingredient Manufacture

Each SMP, sweet whey, micellar casein whey, and acid whey powder was produced as described in detail in Chapter 2.

3.3.4. Liquid Chromatography–Mass Spectrometry (LC-MS/MS)

3.3.4.1. Sample Preparation

Water-soluble vitamin (B1, B2, B3, B3-amide, B5, B6-Pyridoxine, and B7) analysis was carried out at The Metabolomics Innovation Centre (University of Alberta, Edmonton, Alberta, Canada) with a targeted mass spectrometry (multiple reaction monitoring) method using a reverse-phase LC-MS/MS assay. Standard solutions, internal standard solution, and quality control solutions were all diluted using 0.1% formic acid in deionised water. Internal standard solution (10 μL) was first added to 0.6 mL Eppendorf tubes. Calibration standard solutions (50 μL), quality control standard solutions (50 μL), and reconstituted skim milk/whey samples (50 μL) were then added to their corresponding Eppendorf tubes. Protein was precipitated by the addition of 60 μL of trichloroacetic acid (50 mg mL^{-1}) to each tube, after which each sample was vortexed for 30 s. All tubes were stored on ice for 4 h, followed by centrifugation at 13,000 rpm for 15 min. The supernatant from each tube was then transferred into a 96 deep-well collection plate and sealed with a pre-slit mat.

3.3.4.2. Operating Conditions

The aqueous phase (solvent A) consisted of 5 mM ammonium formate and 0.1% formic acid in water and the organic phase (solvent B) consisted of 5 mM ammonium formate and 0.1% formic acid in methanol. Samples were separated using an Agilent reversed-phase Zorbax Eclipse XDB C18 (3.0 mm \times 100 mm, 3.5 μm particle size, 80 \AA pore size) column (Agilent Technologies, Santa Clara, California, USA) at a column temperature of 40 $^{\circ}\text{C}$. Samples were injected at a volume of 10 μL at an auto-sampler temperature of 4 $^{\circ}\text{C}$. Flow rate was 400 $\mu\text{L min}^{-1}$ and run time was 9.5 min. Mass spectrometric analysis was performed on an AB SciexQTrap[®] 4000 tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, California, USA). Multiple reaction

monitoring was carried out in positive ionisation mode at a temperature of 450 °C and an ion spray voltage of 5500 V. Curtain gas, gas stream 1, and gas stream 2 pressures were 20, 40, and 60 psi, respectively. All 48 samples were analysed together in the same run (24 samples in duplicate). Validation parameters (calibration range, calibration regression coefficient, concentrations (μM) of quality control solutions, % accuracy, precision and recovery, limit of detection, and limit of quantitation) for each vitamin in the assay are shown in Appendix i Table S9. Quality control samples were run in triplicate to calculate average accuracy (80%–120%) and precision (within 20%) percentages. Unspiked samples, together with low, medium, and high-spiked samples were measured in triplicate to calculate recovery percentages (80%–120%). Data analysis and calculations of vitamin concentrations were carried out using Analyst Software 1.6.2.

3.3.5. Nephelometry

The vitamin B12 content of each SMP sample was determined by nephelometry by an external laboratory (Eurofins Food Testing Ireland—EFTI Cork, Glanmire Industrial Estate, Glanmire, Co. Cork) using the AOAC 952.20 microbiological assay method (Association of Official analytical Chemists, 1990). Briefly, vitamin B12 was extracted from the sample in an autoclave using a buffered solution. After dilution with basal medium (containing all required growth nutrients except cobalamin), the growth response of *Lactobacillus leichmanii* (ATCC 7830) to extracted cobalamin was measured turbidimetrically and compared to calibration solutions of known concentrations. Vitamin B12 concentrations were measured only in the SMP samples.

3.3.6. Statistical Analysis

Statistical analysis was performed using Genstat v18.1 (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK). The mean of two replicates was used for each sample

value. Datasets were analysed for normality using the Shapiro–Wilk’s test. Data was deemed normally distributed and analysis was carried out using a 4×3 factorial ANOVA with post hoc Tukey test. The effect of ingredient type (4 levels: SMP, sweet whey, MCW, and acid whey) and the effect of cow diet (3 levels: GRS, CLV, and TMR) were considered in the ANOVA. p -values < 0.05 were considered significant. Multivariate analysis of the vitamin profiles was also performed. A supervised multivariate model was built using partial-least-square discriminant analysis (PLS-DA). To validate the model, a permutation test with 2000 repetitions was performed to check that the model differed from a random model ($p < 0.05$). In addition, the R^2 and Q^2 parameters were obtained to assess the performance of the model using 10-fold cross validation approach. The variables which have the greater influence on the latent variables of the built model were determined using a variable importance plot (VIP). Unsupervised hierarchical clustering analysis (HCA) was performed to observe patterns in the data, and is shown as a heat map. Each of these tests and generation of subsequent figures were carried out using Metaboanalyst 3.0 software (www.metaboanalyst.ca) (Xia & Wishart, 2016). All ingredients were produced in duplicate to give 24 separate powders. LC-MS/MS analysis was then carried out in duplicate for each sample.

3.4. Results and Discussion

3.4.1. Overall Distribution

Table 3.1 shows the average concentration of each water-soluble vitamin expressed as μg per g of protein in each ingredient type. Average concentrations (μM) for each vitamin in SMP at 9.5% total solids and each whey type at 6.5% total solids from each feeding system are shown in Appendix i Table S10. Table 3.2 compares the average concentration of each water-soluble vitamin in the sweet whey, micellar casein whey (MCW), and acid whey samples, expressed as μg per g of protein. Average concentrations (μM) for each vitamin in

each whey type at 6.5% total solids are shown in Appendix i Table S11. Table S12 in Appendix i shows average concentrations ($\mu\text{g/g}$ protein) for each vitamin in sweet whey, micellar casein whey, and acid whey powders derived from each feeding system. The overall significant effect of feeding system on vitamin profile can be shown using partial-least-square discriminant analysis (PLS-DA) (Figure 3.1, panel A) and hierarchical clustering analysis (Figure 3.2). Figure 3.1A shows a substantial overlap in the distribution of GRS and CLV samples and a more pronounced separation between both and the TMR samples. In contrast to the clustered variables of the GRS and CLV systems, each TMR variable was distinct from those of the other two systems. In this PLS-DA, $R^2 = 0.80$ and $Q^2 = 0.71$, indicating a close fit to predicted variation, with 83% of the observed variance also being explained by the model. Figure 3.1B shows the variable importance plot (VIP) generated from this PLS-DA, which determines the variables which contribute most to the observed variance in the model and indicates that vitamins B7, B3, and B2 contribute most to the discrimination between classes.

The degree of positive or negative correlation of each vitamin to a particular feeding system is shown in Figure 3.2, which shows the notably positive correlation between the level of vitamin B3 complex and the TMR feeding system and the respective negative correlations of nicotinamide with the GRS system and nicotinic acid to the CLV system. The positive correlation of riboflavin and biotin to both the GRS and CLV systems is also apparent from Figure 3.2, with these vitamins exhibiting a strongly negative correlation with the TMR system. The relative abundance of particular vitamins in milk from different feeding systems may thus be used as an effective means of differentiation between products derived from these different systems. The potential to distinguish between pasture-derived and concentrate-derived milks based on the metabolomics profile has also previously been determined in raw milk (O'Callaghan *et al.*, 2018), SMP, and whey ingredients (Magan *et*

al., 2019a) using quantitative nuclear magnetic resonance ($^1\text{H-NMR}$) and reverse-phase liquid chromatography–mass spectrometry (LC-MS/MS), respectively.

Historically, common chromatographic methods based on HPLC or Ultra-HPLC have been used for vitamin analysis in food matrices, gradually replacing individual microbiological assays, with use of LC-MS widely adopted in recent years due to the higher sample throughput, specificity and sensitivity it affords, and the capacity to simultaneously quantify multiple analytes (Hampel *et al.*, 2021; Shetty *et al.*, 2020; Márquez-Sillero *et al.*, 2013). However, LC-MS methods are subject to limitations on the effective quantification of all vitamers or bound forms of individual vitamins due to matrix interference, which can occur due to protein binding or co-elution of lactose in dairy samples (Hampel *et al.*, 2021; Shetty *et al.*, 2020). Moreover, specific time-consuming sample preparation is required for liberation of cobalamin (vitamin B12) prior to LC-MS analysis (Campos-Giménez *et al.*, 2008). For this reason, a microbiological assay was carried out to quantify vitamin B12 in the present study. The most recent and generally more favourable alternatives to LC-MS in vitamin analysis are ultra-high performance liquid chromatography-mass spectrometry/selected reaction monitoring and supercritical fluid chromatography, which allow for the use of more environmentally friendly mobile phase materials, faster sample run times, greater sample resolution and high selectivity for structurally-similar compounds (Tomai *et al.*, 2022; Oberson *et al.*, 2020; Shetty *et al.*, 2020).

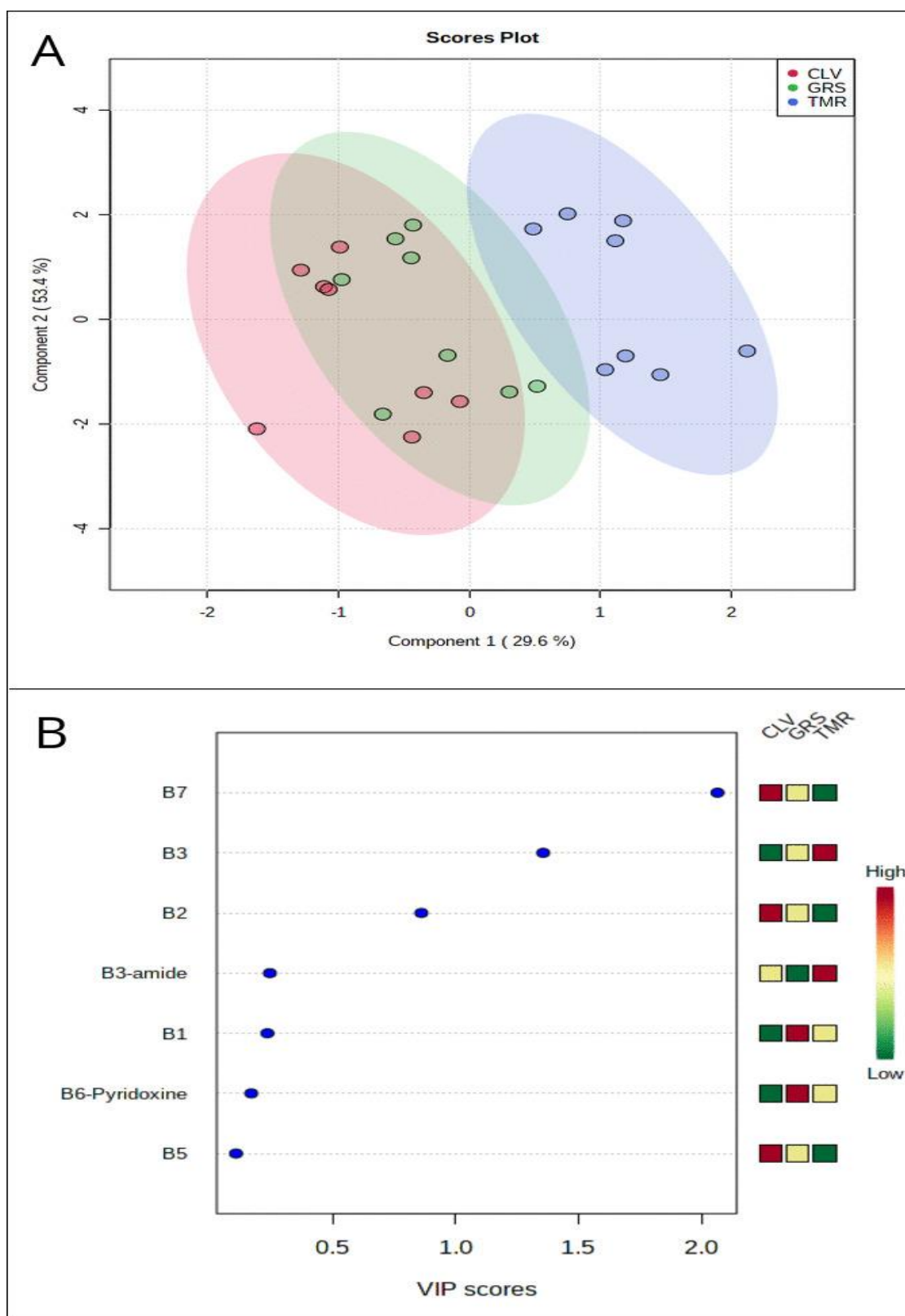


Figure 3.1. Panel (A): partial-least-square discriminant analysis (PLS-DA) score plot for water-soluble vitamins in reconstituted skim milk powder and whey ingredients from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), and total mixed ration (TMR) feeding systems, determined by LC-MS/MS ($R^2=0.80$, $Q^2=0.71$). Panel (B): variable importance plot of vitamins most responsible for separation observed in PLS-DA.

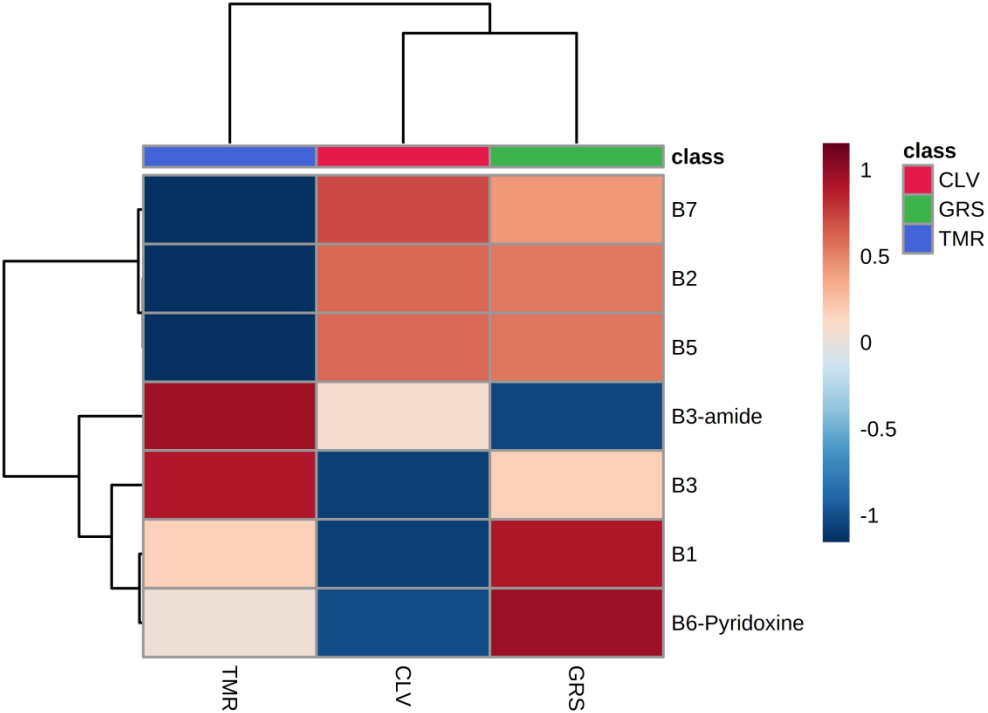


Figure 3.2. Hierarchical clustering analysis of average reconstituted skim milk powder and whey ingredient vitamins from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), or total mixed ration (TMR) feeding systems, determined by LC-MS/MS. Degree of positive and negative correlation between vitamin and diet is indicated by +1 (red) to -1 (blue).

Table 3.1. Average concentrations ($\mu\text{g/g}$ protein) of water-soluble vitamins for reconstituted skim milk, sweet whey, micellar casein whey, and acid whey powders derived from the milk of Holstein-Friesian cows assigned to perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

Sample Type	Water-Soluble Vitamin ($\mu\text{g/g}$ protein)	GRS	CLV	TMR
Skim milk powder	B1	5.47 ^b	5.44 ^b	4.31 ^a
	B2	422 ^b	432 ^b	250 ^a
	B3	0.86	0.63	0.94
	B3-amide	15.4	15.4	18.0
	B5	112	117	125
	B6-Pyridoxine	0.13	0.11	0.13
	B7	0.46	0.53	0.25
Sweet whey powder	B1	29.5	27.9	36.3
	B2	1489 ^b	1400 ^b	636 ^a
	B3	4.60 ^a	3.37 ^a	5.41 ^b
	B3-amide	74.9	82.0	81.9
	B5	738	695	675
	B6-Pyridoxine	0.71	0.77	0.77
	B7	3.04 ^{a,b}	3.12 ^b	1.23 ^a
Micellar casein whey powder	B1	23.2	22.1	20.9
	B2	191 ^b	232 ^b	85.2 ^a
	B3	4.48	4.51	4.91
	B3-amide	117	150	137
	B5	869	1067	811
	B6-Pyridoxine	0.77	0.86	0.74
	B7	3.97 ^b	5.65 ^b	1.77 ^a
Acid whey powder	B1	30.9	22.4	29.4
	B2	81.7	83.8	49.6
	B3	5.89	4.03	8.03
	B3-amide	110 ^a	115 ^{a,b}	129 ^b
	B5	929	843	933
	B6-Pyridoxine	1.03	0.77	0.80
	B7	3.55 ^b	3.75 ^b	1.95 ^a

Values are presented as the average of duplicate samples. GRS—Cows fed perennial ryegrass only. CLV—Cows fed perennial ryegrass/white clover. TMR—Cows fed total mixed ration ad libitum. Vitamins: B1—Thiamine, B2—Riboflavin, B3—Nicotinic acid, B3-amide—Nicotinamide, B5—Pantothenic acid, B7—Biotin. Note: Only the pyridoxine form of vitamin B6 is represented in the data. ^{a,b} Different superscripts within a row indicate significant differences ($p < 0.05$).

Table 3.2. Average concentrations ($\mu\text{g}/\text{g}$ protein) of total water-soluble vitamins for reconstituted sweet whey, micellar casein whey, and acid whey powders derived from the milk of Holstein-Friesian cows assigned to each feeding system, determined by LC-MS/MS.

Water-Soluble Vitamin ($\mu\text{g}/\text{g}$ protein)	Sweet Whey Powder	Micellar Casein Whey Powder	Acid Whey Powder
B1	31.2 ^b	22.1 ^a	27.6 ^a
B2	1175 ^b	170 ^a	71.7 ^a
B3	4.46	4.64	5.98
B3-amide	79.6 ^a	135 ^b	118 ^b
B5	703	916	902
B6-Pyridoxine	0.75	0.79	0.87
B7	2.46	3.80	3.08

Values are presented as the average of data from duplicate samples. Vitamins: B1—Thiamine, B2—Riboflavin, B3—Nicotinic acid, B3-amide—Nicotinamide, B5—Pantothenic acid, B7—Biotin. Note: Only the pyridoxine form of vitamin B6 is represented in the data. ^{a,b} Different superscripts within a row indicate significant differences ($p < 0.05$).

3.4.2. Vitamin Composition

3.4.2.1. Vitamin B1 (Thiamine)

Concentrations of thiamine in GRS and CLV-derived SMP samples were significantly higher ($p < 0.05$) than those derived from TMR (Table 3.1), despite higher concentrations of thiamine typically being present in the germ and seed of cereal grains than other plant sources (Marks, 1975). Differences between diet were not observed in any of the whey samples, however, Pan et al. (2018) suggested that ruminal thiamine production may be reduced in low rumen pH caused by subacute ruminal acidosis arising from feeding of high levels of concentrates. A previous study by Duckett et al. (2009) also found thiamine concentrations to be three times greater in the muscle tissue of grass-fed bulls compared to those fed on a high-concentrate diet, whereas Shingfield et al. (2005) found no significant variation in the thiamine content of milk derived from cows fed varying levels of concentrates. Thiamine is, however, stored in particularly high concentrations in animal muscle tissue (McDowell, 2000).

Thiamine content was also shown to be significantly different between whey ingredient types in the present study (Table 3.2). Average concentrations of thiamine in sweet whey (31.2 $\mu\text{g/g}$ protein) were significantly higher ($p < 0.05$) than for both MCW (22.3 $\mu\text{g/g}$ protein) and acid whey (27.3 $\mu\text{g/g}$ protein). Thiamine content is associated with protein content, as serum thiamine is primarily bound to albumin (Frank *et al.*, 1970). The SMP and whey powders used in this study are the same as those previously used in Chapter 2, where the total protein content of the powders was determined, with sweet whey exhibiting higher average total protein content (9.44%) than MCW (7.76%) and acid whey (7.71%).

3.4.2.2. Vitamin B2 (Riboflavin)

Average concentrations of riboflavin were significantly ($p < 0.05$) higher in both GRS and CLV samples when compared to the TMR sample (Table 3.1). Bovine dietary riboflavin is primarily sourced from green, leafy forage, though riboflavin synthesis also occurs in the rumen (Hunt *et al.*, 1941). Riboflavin provides pigmentation in leaves (Pinto, 2016), conferring a yellow colour similar to β -carotene, the relative abundance of which is primarily responsible for the intensity of yellow colour in fat-containing dairy systems (O'Callaghan *et al.*, 2016b; O'Callaghan *et al.*, 2017). Although each of the ingredient types in the present study are derived from non-fat systems and β -carotene is fat-soluble, visible differences in yellowness were observed between samples derived from each feeding system. Riboflavin is present in considerably lower concentrations in cereal grains, compared to fresh leafy forage (i.e., grass) (Edelman & Colt, 2016).

Previous studies by Hayes *et al.* (1966) and Duckett *et al.* (2009) reported increased riboflavin concentrations in the rumen fluid of bulls receiving increased dietary roughage (i.e., hay) content and in the muscle tissue of bulls assigned to a pasture-based, rather than high concentrate-based finishing system, respectively. Conflicting results were found in a study by Santschi *et al.* (2005), where increased riboflavin content was recorded in the rumen

fluid of cows fed at a high concentrate to forage ratio. Poulsen et al. (2014) compared the riboflavin content of bulk milk from three dairies in Denmark, recording higher riboflavin concentrations in milk from an organic dairy derived from high dietary proportions of grass and legume-based forage, when compared to the milk from two conventional dairies. The average riboflavin concentration of sweet whey (1169 $\mu\text{g/g}$ protein) was significantly higher ($p < 0.05$) than both MCW (166 $\mu\text{g/g}$ protein) and acid whey (71.9 $\mu\text{g/g}$ protein) (Table 3.2). Previously, Mavropoulou and Kosikowski (1973) examined commercially produced spray dried whey samples and found higher concentrations of riboflavin in sweet whey than in acid whey powders (data presented on a g per kg powder basis). Glass and Hedrick (1977) reported similarly increased riboflavin content in commercial dried sweet whey compared to acid whey (data presented on a mg per 100 g powder basis). However, the differences observed in both of the previous studies are significantly lower than those for the present study. The exact reason for the present finding is still not fully understood.

3.4.2.3. Vitamin B3 (Nicotinic Acid)

The vitamin B3 complex comprises two common forms; nicotinic acid, nicotinamide, and a third recently discovered form; nicotinamide riboside (Bogan & Brenner, 2008). Both nicotinic acid and nicotinamide are identified in the LC-MS/MS analysis used in the present study, though nicotinamide is present in substantially higher concentrations in bovine milk than nicotinic acid as the latter is converted to the amide form in the rumen (Erickson *et al.*, 1991). The average concentration of nicotinic acid in ingredients derived from TMR was significantly higher ($p < 0.05$) than those from CLV, whereas the nicotinamide concentration of TMR-derived ingredients was significantly higher than those derived from GRS (Table 3.1). The bioavailability of both forms of vitamin B3 is equivalent (Erickson *et al.*, 1991).

Primary bovine dietary sources of nicotinic acid are cereal grains, although, similar to riboflavin, synthesis of nicotinic acid also occurs in the rumen (Niehoff *et al.*, 2008). The

high inclusion rate of cereal-derived concentrates in the TMR feeding system is likely the most significant contributor to the increased nicotinic acid content in these samples. Hayes et al. (1966) showed increased levels of nicotinic acid in the rumen fluid of bulls fed a concentrate-based diet. Nicotinic acid is synthesised from the essential amino acid tryptophan (Goldsmith, 1958), though previous analysis of the tryptophan content of the samples used in the present study did not reveal significant differences between the diets (Magan *et al.*, 2019a). The lack of significant variation in tryptophan content between the diets relative to nicotinic acid content may be explained by the primary utilisation of tryptophan for protein synthesis (Yao *et al.*, 2011) and the relative inefficiency of the synthesis of nicotinic acid from tryptophan (Fukuwatari & Shibata, 2013). Contrary to riboflavin, average nicotinamide concentrations of MCW (134 $\mu\text{g/g}$ protein) and acid whey (138 $\mu\text{g/g}$ protein) were significantly higher than those of sweet whey (79.6 $\mu\text{g/g}$ protein) (Table 3.2).

3.4.2.4. Vitamin B5 (Pantothenic Acid) and B6 (Pyridoxine)

While concentrations of pantothenic acid were high in all samples, particularly when compared to the low concentrations of pyridoxine (Table 3.1), both vitamin B5 and pyridoxine were not found to be significantly different between feeding systems or ingredient types. However, the forage:grain ratio of foodstuffs consumed by the cow is generally regarded as an influence on pantothenic acid synthesis (Ragaller, *et al.*, 2011). The effect of varying this ratio on the levels of pantothenic acid transferred into the milk of the cow has not previously been investigated. Sources of vitamins B5 and B6 are consistent in the bovine diet, with relatively similar concentrations present in leafy forages and cereal grains (Ragaller, *et al.*, 2011; McDowell, 1989). A study comparing the vitamin B6 content of commercial milk with that of milk produced from cows fed a diet restricted in B6 found consistent levels of the vitamin between both milk sources, indicating that the concentrations

of vitamin B6 present in milk may be independent of diet and it may instead be entirely supplied through ruminal synthesis (McElroy & Goss, 1940).

The vitamin B6 complex consists of six vitamers: pyridoxine, pyridoxal, pyridoxamine, and the phosphate ester form of each; pyridoxine phosphate, pyridoxal phosphate, and pyridoxamine phosphate (Yagi *et al.*, 2013). The multiple reaction monitoring assay carried out in the present study exclusively measured the pyridoxine vitamer, whereas the predominant vitamer present in bovine milk is the active form pyridoxal phosphate (Schmidt *et al.*, 2017). Consequently, concentrations for vitamin B6 observed in this study were negligible in comparison to average values for total vitamin B6 observed for milk in the literature (Jensen, 1995a; Graulet, 2014; Alm, 1982).

3.4.2.5. Vitamin B7 (Biotin)

The average biotin concentrations of CLV and GRS were significantly higher ($p < 0.05$) than that of TMR (Table 3.1). Biotin is present in low concentrations in milk (Woollard & Indyk, 2013), though milk remains a good dietary source as requirements for the vitamin are comparatively low (US Institute of Medicine, 1998). As with the other B-complex vitamins, biotin is a product of rumen metabolism (Zimmerly & Weiss, 2001), though conflicting information exists on the relationship between dietary intake, net ruminal synthesis, and duodenal flow of biotin (Miller *et al.*, 1986; Frigg *et al.*, 1994; Schwab & Shaver, 2006). However, the analysis carried out herein suggests that biotin content was significantly affected by feeding system.

The role of nutrition in biotin synthesis has been widely investigated. Briggs *et al.* (1964) suggested that feeding of dietary urea may increase rumen biotin synthesis, which corresponds to the increased concentration of the vitamin in the CLV sample in the present study. Increased urea content has previously been observed in samples derived from this system (O'Callaghan *et al.*, 2018), with increasing dietary biotin supplementation previously

found to significantly increase milk biotin concentrations (Zimmerly & Weiss, 2001). Other studies have outlined a decrease in rumen biotin content with increasing grain content (Abel *et al.*, 2001) or decreasing dietary forage content (Santschi *et al.*, 2005) and an increase in rumen acidity with increasing grain supplementation, leading to inhibition of cellulolytic rumen microflora (Rosendo *et al.*, 2003), as previously suggested for thiamine content in Section 3.2.1. This is supported by O'Callaghan *et al.* (2018), who showed no significant effect of bovine feeding system on the overall composition of rumen microflora, but rather rumen microflora functionality. The results of the present study support this indirect effect of the feeding system, whereby the functionality of rumen microflora and, hence, efficiency of rumen biotin synthesis may be increased or decreased depending on the substrate derived from the type of feed consumed by the cow. However, this is a notable contrast to pantothenic acid and pyridoxine (Section 3.1.4), which are primarily products of rumen metabolism, but do not display a significant effect of the feeding system on their concentrations in the SMP and whey samples.

3.4.2.6. Vitamin B12 (Cobalamin)

Cobalamin concentrations did not differ significantly ($p > 0.05$) between SMP samples. The highest concentrations were present in CLV-derived SMP ($38.6 \mu\text{g kg}^{-1}$), followed by TMR ($37.2 \mu\text{g kg}^{-1}$) and GRS ($34.3 \mu\text{g kg}^{-1}$). This is likely due to increased levels of cobalt present in the concentrate used in the TMR ration and in the root nodules of the CLV sward. Leguminous plants such as *Trifolium repens* L. exhibit a high affinity for the concentration of cobalt (Andrews, 1966), which is required by nitrogen-fixing microflora present in the root nodule (Lowe & Evans, 1962) and forms the central constituent in cobalamin synthesis (Huwait *et al.*, 2015). The lower concentrations observed in GRS-derived SMP may therefore be due to the absence of cobalt from the leaves of the perennial ryegrass exclusively consumed by the cows assigned to this feeding system.

3.4.3. Relationship between Skim Milk and Recommended Daily Allowances

Table 3.3 shows the recommended daily allowance (RDA) and adequate intake (AI) values for each water-soluble vitamin for mature females and males (aged 14+) (US Institute of Medicine, 1998), along with the mass of each vitamin present in a 200-mL serving of skim milk derived from each feeding system and the percentage which they contribute to the RDA for each vitamin. Skim milk from each diet offers a low proportion of the RDAs for vitamins B1 and B3, though the stated allowance for vitamin B3 represents “niacin equivalent”, a figure which incorporates the typical daily intake of tryptophan, the mass of which is not included in the skim milk sample values in the table. As discussed in Section 3.1.4, the proportion of vitamin B6 in each SMP refers to the pyridoxine form only and is, therefore, omitted from Table 3.3, as an approximate comparison to the full RDA for vitamin B6 cannot be made for the individual vitamer. The vitamin B5 content of skim milk from each feeding system would account for 16% to 17% of its RDA, whereas, notably, almost one third of the average RDA (RDA based on recommendations of US Institute of Medicine, 1998) for vitamin B12 would be provided from a 200-mL serving of skim milk from each feeding system (Table 3.3). As previously described, concentrations of vitamins B2 and B7 were approximately twice as high in GRS and CLV-derived samples with respect to TMR-derived samples. Thus, approximately 10% of the RDA of vitamin B7 may be provided by a skim milk serving from either pasture-derived sample, with approximately 5% available from TMR-derived milk. While this ratio is similar for vitamin B2, the riboflavin content of a 200-mL serving of skim milk from each feeding system would provide substantially higher than the average RDA for the vitamin.

Table 3.3. Recommended daily allowances (US Institute of Medicine, 1998) for females and males (aged 14+) and average mass (mg) of water-soluble vitamins present in skim milk powder reconstituted at 9.5% total solids derived from Holstein-Friesian cows assigned to each feeding system.

Water-Soluble Vitamin	Recommended Daily Allowance (mg)		Mass (mg) in 200 mL Skim Milk		% of Adult Human RDA		Mass (mg) in 200 mL Skim Milk		% of Adult Human RDA		
	Female	Male	GRS	Female	Male	CLV	Female	Male	TMR	Female	Male
B1	1.1	1.2	0.038	3.5	3.2	0.038	3.5	3.2	0.029	2.6	2.4
B2	1.1	1.3	2.964	269	228	3.049	277	235	1.697	154	131
B3 complex	14	16	0.114	0.8	0.7	0.113	0.8	0.7	0.128	0.9	0.8
B5	5.0*	5.0*	0.789	16	16	0.829	17	17	0.846	17	17
B7	0.03*	0.03*	0.0033	11	11	0.0037	12	12	0.0017	5.7	5.7
B12	0.0024	0.0024	0.0006	27	27	0.0007	30	30	0.0007	29	29

Skim milk values are presented as the average of duplicate samples. GRS—Cows fed perennial ryegrass only. CLV—Cows fed perennial ryegrass/white clover. TMR—Cows fed total mixed ration ad libitum. Vitamins: B1—Thiamine, B2—Riboflavin, B3 complex—Nicotinic acid and Nicotinamide, B5—Pantothenic acid, B7—Biotin, B12—Cobalamin. * Indicates adequate intake values where recommended daily allowance values have not been derived. Note: Recommended daily allowance values for the vitamin B3 complex are expressed as “niacin equivalent”, comprising vitamin B3, B3-amide, and tryptophan. Values for the vitamin B3 complex are expressed as the sum of vitamin B3 and B3-amide.

3.4.4. Vitamin Content of Skim Milk for Use in Infant Milk Formula Manufacture

Table 3.4 shows the B vitamin contribution of SMP in an IMF (1.4%, w/w, protein), if 50% (w/w) of the total protein is obtained from SMP. In addition, shown are the minimum amount of each vitamin required (mg/100 mL), based on the recommendations of the Codex Alimentarius standard 72 (1981) for IMF with an energy density of 65 kcal/100 mL. Although further quantities of water-soluble vitamins would be introduced to an IMF mixture through the addition of whey protein ingredients or by supplementation, the SMP base from any of the three feeding systems would provide substantially more riboflavin than the minimum required level. Similarly, the required cobalamin content would be achieved using CLV (0.000068 mg/100 mL) or TMR-derived SMP (0.000065 mg/100 mL) alone, with GRS-derived SMP only slightly lower at 0.000060 mg/100 mL. The use of SMP derived from GRS, CLV, and TMR would provide 30%, 35%, and 16% of the required biotin content, respectively, while each SMP type would provide approximately 30% of the pantothenic acid requirement.

Quantitative differences in thiamine, nicotinic acid, and pyridoxine content between the three feeding systems are unsubstantial when compared to the high requirements for these vitamins. This necessitates the use of vitamin concentrate pre-mixes to supplement the required levels for IMF. As discussed in Section 3.1.4, the predominant form of vitamin B6 in bovine milk is pyridoxal phosphate, whereas in human milk, pyridoxal is the dominant form, followed by pyridoxal phosphate, with the other vitamers present in very low concentrations (Yagi *et al.*, 2013). Fortification of most foods, including IMF, is most commonly achieved through the addition of pyridoxine alone in the form of the salt pyridoxine hydrochloride (Clayton, 2006).

Table 3.4. B-vitamin contribution (mg) of skim milk powder derived from each feeding system in infant milk formula (65 kcal per 100 mL).

Water-Soluble Vitamin	Mass (mg/100 mL)			
	GRS	CLV	TMR	Minimum Requirement (CODEX STAN 72, 1981).
B1	0.004	0.004	0.003	0.039
B2	0.279	0.287	0.160	0.052
B3 complex	0.011	0.011	0.012	0.195
B5	0.074	0.078	0.080	0.260
B6-Pyridoxine	0.000088	0.000075	0.000086	0.023
B7	0.0003	0.0003	0.0002	0.001
B12	0.000060	0.000068	0.000065	0.000065

SMP values are presented as the average of duplicate samples. GRS—Cows fed perennial ryegrass only. CLV—Cows fed perennial ryegrass / white clover. TMR—Cows fed total mixed ration ad libitum. Vitamins: B1—Thiamine, B2—Riboflavin, B3 complex—Nicotinic acid and Nicotinamide, B5—Pantothenic acid, B7—Biotin, B12—Cobalamin. Note: Only the pyridoxine form of vitamin B6 is represented in the data. Values for the vitamin B3 complex are expressed as the sum of vitamin B3 and B3-amide.

3.5. Conclusion

The utilisation of pasture or concentrate-based bovine feeding systems significantly affected the relative concentrations of a limited number of water-soluble vitamins in the skim milk and whey protein powder ingredients used in this study and varied across ingredient types. Significant differences in thiamine (B1) content were observed only in SMP, whereas significantly higher riboflavin (B2) content was apparent in GRS and CLV-derived SMP, sweet whey, and MCW, when compared to TMR. This may be primarily attributable to the forage content of the GRS and CLV diets which contain high concentrations of riboflavin. Despite the higher proportions of the nicotinic acid/amide complex in cereal grains, significantly higher concentrations of vitamin B3 and B3-amide were observed only in TMR-derived sweet whey and acid whey, respectively. Variations in the concentration of biotin (B7) may, however, be indirectly affected by bovine diet, through substrate-based modulation of rumen microflora and subsequent variation in the relative efficiency of rumen biotin synthesis.

The PLS-DA, VIP, and HCA plots provide a visual representation of the distinguishable difference in milk protein ingredients derived from each feeding system based on their vitamin profile, which offers potential as a means of milk product verification. Riboflavin and biotin exhibited the most biologically significant differences in SMP when compared to average recommended daily allowances and requirements for infant milk formulation. The data presented also suggest that significant differences in vitamin content may arise due to the type of whey production method used, independent of dietary effects.

3.6. References

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Chapter 4

Compositional and physical properties of skim milk and whey protein concentrate powders prepared from milk of cows fed perennial ryegrass or total mixed ration

4.1. Abstract

This study investigated the effect of bovine dietary variations on the composition and physical behaviour of skim milk powder (SMP) and whey protein concentrates (WPC) derived from the milk of cows fed on specific dietary treatments. Raw milk was obtained from three groups of 17 cows randomly assigned to perennial ryegrass only (GRS) or total mixed ration (TMR) based diets for a full lactation. This milk was processed into SMP under typical industrial conditions at pilot scale and further processed to produce acid whey, micellar casein whey and sweet whey protein concentrate powders. All samples were analysed for protein and mineral composition. Reconstituted SMP was analysed for heat coagulation time and ionic calcium concentration, and the heat-dependent rheological behaviour of reconstituted WPC and blends of SMP and WPC was also measured. Skim milk powder samples derived from the TMR system were characterised by significantly higher non-protein nitrogen and significantly lower ionic calcium contents than those from GRS and displayed higher heat stability and lower viscosity on heating. Concentrations of selenium and iodine in TMR samples were significantly higher than in GRS samples. Irrespective of diet, the mineral profiles of the WPC sample types varied substantially by calcium, phosphorus, zinc, magnesium, sulphur and sodium contents. The gel strength of GRS-derived heated WPC powders standardised to 5% true protein and the dynamic viscosity of heated mixtures of SMP with WPC were higher overall than those from TMR samples, although this difference was less substantial than the differences between WPC types, among which acid WPC displayed significantly lower thermal stability than both sweet and micellar casein WPC. The study determined a limited effect of feeding system on the composition and rheological properties of protein ingredients relative to the differences observed between ingredient types from different whey processing streams, with the results suggesting that micellar casein WPC may be more suitable for infant milk formula manufacture on the basis of thermal stability and mineral composition.

4.2. Introduction

In recent years, increasing consumer interest has been focused on dairy products with a more natural image or sustainable production process (Park, 2018; Conner & Oppenheim, 2008). This interest is reflected in increased research in to the distinction between milk and its derivatives produced from cows fed on different dietary regimes. Widely practiced in Ireland and New Zealand, pasture-based production systems are generally more favourably perceived by consumers in comparison to indoor housing-based systems (Croissant *et al.*, 2007), which represent the worldwide convention for large-scale milk production. Concerns typically arise over animal welfare and the use of concentrate-based feeds. Recent studies have identified significant differences in the fatty-acid (O’Callaghan *et al.*, 2016a; O’Callaghan *et al.*, 2016b) and sensory (Clarke *et al.*, 2019; Kilcawley *et al.*, 2018) profiles of raw milk and products such as butter and cheese derived from pasture and concentrate-based diets. Limited information exists, however, concerning differences in the protein component of products in which the fat content has been removed, such as skim milk powder (SMP) and whey protein powders.

Although whey is produced as a by-product of other milk processing streams such as cheese manufacture (renneting of whole milk) (González Siso, 1996), pressed yoghurt production (acidification of whole milk) (Jørgensen *et al.*, 2019), casein precipitation and caseinate production (acidification of skim milk) (Sarode *et al.*, 2016) or fractionation of skim milk by membrane filtration (Zydney, 1998), it has become increasingly valued in recent years for its use both as an ingredient in other product formulations such as infant milk formula (IMF) (Królczyk *et al.*, 2016) and as a concentrated form of protein for supplementation (Andrade *et al.*, 2019). Concentration of whey protein is achieved by membrane ultrafiltration, using ceramic or polyethersulfone membranes typically with a molecular weight cut-off of 10 to 20 kDa to separate the whey protein (retentate) from lactose, minerals and water (permeate). To achieve high protein concentrations (85 – 90%), a diafiltration process is required to dilute the

retentate and effectively “flush” other permeable components out (Regan, Ennis & Mulvihill, 2009).

The formulation of IMF primarily utilises SMP as a base material, to which SMP-derived lactose, whey protein or whey protein concentrate (WPC) are added (McCarthy *et al.*, 2017). The ultrafiltration process used in whey concentration can result in reduced concentrations of soluble salts, as they are removed in the permeate (Vyas & Tong, 2003), meaning that selection of raw whey ingredients over whey protein concentrates may be preferable when formulating a product on the basis of mineral or vitamin content. However, demineralisation of whey by ion exchange or electrodialysis is required for use in IMF production, as the mineral content of bovine milk is considerably higher than that of human milk (Flynn & Cashman, 1997).

As milk-based protein ingredients are widely used for further processing, their behaviour during the application of high thermal loads is particularly important. The resistance of process materials to heating without experiencing destabilisation, coagulation, development of fouling on equipment surfaces and blocking equipment entirely is an essential aspect of dairy product quality, which is governed largely by their mineral and protein compositions. While the protein and amino acid composition of milk is generally accepted to be primarily influenced by cow breed and genetics (Schopen *et al.*, 2009; Linn, 1988), some studies (Magan *et al.*, 2019; Schwendel *et al.*, 2017; Vanhatalo *et al.*, 2009) have suggested that dietary factors may also have an effect. Therefore, the objective of this study was to determine the effect of practicing feeding systems based on perennial ryegrass (*Lolium perenne* L.) or indoor total mixed ration on the composition and physical properties of skim milk powder and three types of whey protein concentrate powders derived from the milk produced from each system.

4.3. Materials and methods

4.3.1. Materials

All skim milk powders were produced at Moorepark Technology Ltd. (Moorepark, Fermoy, Co. Cork, Ireland) using raw milk produced at Teagasc Animal and Grassland Research and Innovation Centre (Moorepark, Fermoy, Co. Cork, Ireland). All sweet whey and acid whey samples were produced at laboratory scale. All micellar casein whey was produced by membrane filtration at laboratory scale.

4.3.2. Experimental design

The experimental design for this study was as described in Chapter 2, with the exclusion of the CLV system. Details of diet composition and administration, milking times, milk segregation and storage and milk collection were as described in Chapter 2. Chemical composition tables for each feed type are shown in Appendix i, Supplementary Materials, Tables S1, S2 and S3.

4.3.3. Protein ingredient manufacture

Production of SMP, sweet whey, micellar casein whey and acid whey from each feeding system was as described in Chapter 2. Each whey type was concentrated to approximately 40% protein using a volumetric concentration factor of 15X by recirculation through a 10 kDa Vivaflow 200 polyethersulfone cassette membrane (Sartorius AG, Göttingen, Germany). The recirculating whey was maintained at 40°C throughout the concentration process. Permeate was collected and frozen for analysis and use in dilution of retentate as required. Retentate was freeze-dried in a Labconco stoppering tray dryer equipped with a Freezone 12 plus vacuum collector/refrigerator unit (Labconco, Kansas City, Missouri, USA).

4.3.4. Compositional analysis

4.3.4.1. Total nitrogen and non-protein nitrogen content

The total nitrogen content of each powder was determined using the Kjeldahl method, as described in ISO 8968-1 (2001) using a nitrogen-to-milk protein conversion factor of 6.38. Non-protein nitrogen content was measured as described in Chapter 2.

Based on the nitrogen content of each WPC powder, the true protein content was standardized to 34% (w/w) by adding permeate produced during the ultrafiltration step to the corresponding WPC powder. WPC ingredients with a standardised protein content were used for all further analysis.

4.3.4.2. Size exclusion chromatography (SEC)

Whey protein determination was carried out using TSK G2000SW (300 x 7.5 mm) and TSK G2000swxl (300 x 7.8 mm) columns (Tosoh Hass, Minato-Ku, Tokyo, Japan) linked in series, fitted to a Waters Alliance 2695 separation module (Waters Corporation, Milford, Mass, USA). The eluent used was 30% (v/v) acetonitrile (HPLC grade) containing 0.1% trifluoroacetic acid and was run at a flowrate of 1 mL min⁻¹ and continually monitored at 214 nm using a Waters 2487 dual wavelength detector. The samples were standardised at a protein content of 0.25% (w/w) and filtered through 0.22 µm Sarstedt Filtropur filters (Thermo Fisher Scientific, Waltham, Massachusetts, USA) prior to injection on to the column (injection volume 20 µL). Chromatographic data was collected and analysed using Empower data handling software package (Waters Corporation, Milford, MA, USA.). Calibration curves were generated using 1 mg mL⁻¹ solutions of the whey proteins bovine serum albumin, β-lactoglobulin, α-lactalbumin and GMP (Sigma Aldrich, Missouri, USA) which were mixed to produce a 100 µg mL⁻¹ solution of each standard. The retention of each standard had previously

been determined. The mixed standards were then run at 4 different concentrations (10, 20, 50 and 100 $\mu\text{g mL}^{-1}$) and calibration curves were generated using Empower software package.

4.3.4.3. Inductively coupled plasma mass spectrometry (ICP-MS)

Mineral analysis was carried out using an external laboratory (FBA Laboratories, Cappoquin, Co. Waterford, Ireland). Briefly, an aliquot (0.25 g) of each sample was digested in nitric acid at 100°C for 160 min. The samples were then filtered and made to a final volume of 50 mL using deionised water, HCl and butanol. A portion of each sample was transferred to a tube on an auto-sampler tray for analysis. Samples were analysed against a matrix-matched calibration curve on an Agilent ICP-MS 7700 series. Included in the run were AQC and CRM samples for quality control.

4.3.4.4. Ionic calcium concentration

Concentrations of ionic calcium in skim milk powders reconstituted to 3.5% protein (w/w) in deionised water were determined using a Hach Sension+ MM340 Multi Meter fitted with a Sension+ calcium ion selective electrode (Hach Co., Loveland, Colorado, USA). Calcium chloride (CaCl_2) calibration standards (1 L) were prepared at concentrations of 0.5, 1.0, 2.5 and 5.0 mM by addition of anhydrous CaCl_2 to deionised water in 1 L volumetric flasks. The electrode was placed in a 0.09 M solution of CaCl_2 for 30 min prior to calibration. Aliquots (30 mL) of each skim milk sample were adjusted to pH 6.2, 6.4, 6.6, 6.8, 7.0, and 7.2, using 0.1 N HCl or NaOH in order to correspond to the pH values of the HCT-pH curve. An aliquot (100 μL) of ionic strength adjustor (3 M KCl) was added to 10 mL aliquots of each calibration standard or sample and mixed for 30 s using a magnetic stirrer, prior to measurements.

4.3.5. *Heat coagulation time*

The heat coagulation time (HCT) of reconstituted skim milk powders was determined using an Elbanton Oil Bath (Hettich Benelux Laboratory Equipment, Geldermalsen, Netherlands). Each SMP sample was reconstituted in deionised water to 3.5% protein (w/w) and divided into 6 aliquots (approx. 30 mL), the pH values of which were adjusted to 6.2, 6.4, 6.6, 6.8, 7.0, and 7.2, using 0.1N HCl or NaOH. Samples were allowed to equilibrate and further pH adjustment was made where necessary. Aliquots (~3.4 g) of each sample were pipetted into 4 mL glass tubes, stoppered and secured in the oil bath rack. The rack was then inserted into the temperature controlled oil bath at 140°C and rocked at 8 oscillations min⁻¹. The time taken in minutes for visual coagulation of each sample was then recorded.

4.3.6. *Rheological measurements*

4.3.6.1. *Viscosity of skim milk concentrate*

The viscosity of skim milk powder samples reconstituted to 10% protein (w/w) was determined using a starch pasting cell geometry unit in an AR-2000ex Rheometer (TA Instruments, New Castle, Delaware, USA). An aliquot (28 g) of each sample was added to the cylinder and maintained at an angular velocity of 16.8 rad s⁻¹ throughout the experiment. An initial peak hold was carried out at 25°C for 5 min, followed by a temperature ramp from 25 - 90°C at 10° min⁻¹, a 10 min peak hold at 90°C, a temperature ramp from 90 - 25°C at 10° min⁻¹ and a final 5 min peak hold at 25°C.

4.3.6.2. *Oscillatory measurements of whey protein concentrate dispersions*

The temperature-dependent flow behaviour of each WPC was measured using an AR-2000ex Rheometer (TA Instruments, New Castle, Delaware, USA) equipped with 60 mm aluminium parallel plate geometry. Samples were reconstituted to a protein content of 5% w/w

and oscillated at a constant frequency of 1 Hz and 0.5% strain during a temperature ramp from 20 - 85°C at 10° min⁻¹. A time sweep was carried out at 85 °C for 3 minutes at a frequency of 1 Hz and 0.5% strain, followed by a temperature ramp from 85-20°C at 10° min⁻¹. Samples were maintained at 20°C for the duration of a frequency sweep from 1 – 100 Hz at 0.5% strain.

4.3.6.3. *Viscosity of skim milk and whey protein concentrate dispersions*

Dispersions of skim milk and WPC were prepared in order to simulate the protein component of an infant milk formula dispersion for each GRS and TMR replicate. Each SMP was reconstituted using deionised water and combined with the corresponding WPC mixture (e.g. TMR-derived SMP combined with TMR-derived AWPC), yielding a final dispersion standardised at an overall true protein content of 5%. An aliquot (28 g) of each mixture was added to the sample holding cylinder of an AR-2000ex Rheometer (TA Instruments, New Castle, Delaware, USA) equipped with a starch pasting cell geometry. Samples were measured at a constant angular velocity of 16.8 rad s⁻¹ at 20°C for 3 min and maintained at 16.8 rad s⁻¹ during a temperature ramp from 20 - 90°C at 10° min⁻¹. Samples were maintained at this angular velocity and temperature for 3 min, before a temperature ramp from 90-20°C at 10° min⁻¹ and a further 3 min peak hold at a constant angular velocity of 16.8 rad s⁻¹ at 20°C.

4.3.7. *Statistical analysis*

Statistical analysis was performed using Genstat v18.1 (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK). Datasets were analysed for normality using the Shapiro-Wilk's test. Data was deemed normally distributed and analysis was carried out using a 4 × 2 factorial ANOVA with *post hoc* Tukey test. The effect of ingredient type (4 levels: SMP, SWPC, MCWC and AWPC) and the effect of cow diet (2 levels: GRS and TMR) were considered in the ANOVA. *P*-values < 0.05 were considered significant. All ingredients were

produced in duplicate to give 16 separate powders. All analyses, with the exception of ICP-MS, were then carried out in duplicate for each sample.

4.4. Results and discussion

4.4.1. Composition

4.4.1.1. Nitrogen composition

The average total protein and non-protein nitrogen (NPN) contents (% w/w) of GRS and TMR-derived SMP, sweet whey protein concentrate (SWPC), micellar casein whey protein concentrate (MCWPC) and acid whey protein concentrate (AWPC) are shown in Table 4.1. The total protein and NPN content of both SMPs was previously reported in Chapter 2 and is shown here for comparison to each WPC, as the MCWPCs and AWPCs were produced from each corresponding SMP. For each product type, the total protein contents were similar between the GRS and TMR samples, whereas concentrations of NPN were significantly higher in the TMR-derived SMP and MCWPC, but not SWPC or AWPC. The greater overall concentration of NPN is most likely due to increased urea content, which may arise from the high proportion and relatively lower digestibility of crude protein in the TMR concentrate (Bargo *et al.*, 2002; Schroeder *et al.*, 2003). As the WPC samples were concentrated volumetrically, a greater extent of membrane fouling due to the casein component of the precursory skim milk, which is otherwise largely removed prior to membrane filtration of sweet whey and acid whey, resulted in lower total protein content in the MCWPC samples, relative to SWPC and AWPC.

The NPN content of the SWPC samples was higher than would be expected, relative to the other sample types, given the concentration of the TCA (15%) used for protein extraction prior to measurement of NPN. While GMP remains soluble in lower concentrations of TCA, at concentrations of 14% and greater (Rao *et al.*, 2012), it would be expected to precipitate with

the protein component and thus, not be quantified within the NPN fraction. Therefore, the higher concentrations of NPN in the SWPC samples may indicate that the TCA solubility of the GMP component in these samples is variable, potentially due to variable levels of glycosylation and, thus, hydrophobicity (Li & Mine, 2004; Kawakami *et al.*, 1992).

The concentration of NPN in AWPC samples was notably lower than those of SWPC and MCWPC; however, the total nitrogen content of the corresponding permeate was not concomitantly higher than those of the permeates produced from the other WPC types, indicating that some NPN constituents may have been trapped within the acid gel matrix and removed prior to the microfiltration step during processing.

Table 4.1: Average total nitrogen and non-protein nitrogen content of skim milk powder, sweet whey protein concentrate, micellar casein whey protein concentrate, and acid whey protein concentrate powders, determined by Kjeldahl analysis.

Sample Type	Total protein (% w/w)		Non-protein nitrogen (% w/w)		True protein (% w/w)	
	GRS	TMR	GRS	TMR	GRS	TMR
Skim milk powder	37.2 (\pm 0.57)	36.1 (\pm 0.61)	0.27 (\pm 0.01) ^a	0.37 (\pm 0.00) ^b	35.4 (\pm 0.66)	33.8 (\pm 0.60)
Sweet whey protein concentrate powder	47.0 (\pm 0.29)	47.8 (\pm 1.33)	0.37 (\pm 0.00)	0.37 (\pm 0.03)	44.7 (\pm 0.30)	45.4 (\pm 1.15)
Micellar casein whey protein concentrate powder	38.1 (\pm 0.37)	36.6 (\pm 3.11)	0.28 (\pm 0.02) ^a	0.36 (\pm 0.02) ^b	36.3 (\pm 0.26)	34.4 (\pm 3.21)
Acid whey protein concentrate powder	43.2 (\pm 4.25)	43.8 (\pm 5.19)	0.14 (\pm 0.01)	0.18 (\pm 0.01)	42.3 (\pm 4.19)	42.7 (\pm 5.15)

GRS—cows fed perennial ryegrass only

TMR—cows fed indoor total mixed ration *ad-libitum*^{a,b,c,d} indicates values within a row not sharing a common lower-case superscript letter differed significantly ($p < 0.05$).

4.4.1.2. *Whey protein profile*

Average concentrations (mg g^{-1}) of constituent proteins within each WPC sample are shown in Table 4.2. The presence of GMP exclusively within sweet whey is responsible for its qualitative difference to other whey types and contributes to the elevated NPN content observed within the SWPC samples relative to MCWPC and AWPC. The GMP contents of the GRS and TMR samples represent 26% and 24% of the total nitrogen content of each SWPC, respectively, levels which are similar to those reported in the literature (Sharma *et al.*, 2013). No significant variation was observed in bovine serum albumin, β -lactoglobulin, α -lactalbumin or GMP content between the GRS and TMR samples of each product type. The only significant difference observed was the higher concentration of β -lactoglobulin in both AWPC samples relative to the TMR-derived MCWPC sample; however, both of these product types were derived from the same reconstituted SMP samples. This difference may, therefore, be due to the greater extent of membrane fouling which occurred during microfiltration of the skim milk samples to yield micellar casein whey samples prior to ultrafiltration, limiting the permeability of β -lactoglobulin, which both represents the largest proportion of bovine whey protein (58%) and, as present in the dimeric form at native milk pH, has the second highest molecular weight (36 kDa) after bovine serum albumin (66 kDa) (Sharma, 2019), which was also present in lower concentrations in the final MCWPC powder and protein-standardised aliquot.

Table 4.2: Average concentration (mg g^{-1}) of whey proteins in sweet whey protein concentrate (WPC), micellar casein WPC, and acid WPC powders, as determined by size exclusion chromatography.

Whey protein (mg/g)	Sweet WPC powder		Micellar casein WPC powder		Acid WPC powder	
	GRS	TMR	GRS	TMR	GRS	TMR
Bovine serum albumin	37.0 (\pm 3.05)	41.6 (\pm 1.30)	25.9 (\pm 8.60)	26.6 (\pm 2.99)	47.7 (\pm 12.5)	46.4 (\pm 7.44)
β-lactoglobulin	257 (\pm 0.17)	258 (\pm 5.12)	263 (\pm 9.23)	220 (\pm 20.6) ^a	323 (\pm 12.4) ^b	299 (\pm 23.7) ^b
α-lactalbumin	117 (\pm 1.01)	156 (\pm 34.9)	135 (\pm 3.49)	132 (\pm 5.39)	133 (\pm 7.88)	144 (\pm 9.89)
Glycomacropeptide	147 (\pm 0.04)	146 (\pm 5.62)	-	-	-	-

GRS—cows fed perennial ryegrass only

TMR—cows fed indoor total mixed ration *ad-libitum*^{a,b,c,d} indicates values within a row not sharing a common lower-case superscript letter differed significantly ($p < 0.05$).

4.4.1.3. *Mineral composition*

The average mineral profiles ($\text{mg } 100\text{g}^{-1}$) of each product type derived from GRS and TMR feeding systems standardised to 34% protein are shown in Table 4.3. In agreement with Gulati *et al.* (2018), who previously compared skim milk samples derived from the same feeding systems utilised in the present study, the manganese content of GRS-derived samples were higher overall, possibly due to direct transfer from the high manganese content of pasture (Teagasc, 2017), although this difference was not statistically significant. Concentrations of some trace elements were also substantially higher in TMR-derived products. The overall higher concentrations of copper and zinc and significantly higher selenium and iodine concentrations observed in all TMR samples can be attributed to the provision of these minerals in high concentrations within the components of the ration consumed by the TMR group. Moreover, Gulati *et al.* (2018; 2019) also recorded significantly higher concentrations of selenium and iodine in TMR samples than in those from GRS.

Iodine is an essential nutrient for maintenance of thyroid health (Zimmermann & Boelaert, 2015) and is particularly important to pregnant women and infants in promoting the brain development of the neonate (Monahan *et al.*, 2015). Iodine deficiency is common among many populations (Zimmermann & Boelaert, 2015), although milk represents a good dietary source of the nutrient and is the primary source in the UK and Ireland, where adolescent girls have been identified as a group which is both deficient and of particular concern (Mullan *et al.*, 2020).

The magnitude of the difference in iodine content between the TMR-derived milk and the GRS -derived milk may be of concern in the manufacture of infant milk formula (IMF), in which skim milk powder and whey or whey protein powders are the primary ingredients (O'Brien *et al.*, 2013). The upper tolerable limit for iodine content in IMF defined by the Codex Alimentarius (European Commission Directive 2006/141/EC, 2006) is $130 \mu\text{g}$ per kg of IMF

powder, equating to 250 µg per kg of fresh milk (O'Brien *et al.*, 2013). On a fresh milk basis, the concentrations of iodine observed in the skim milk samples used in the present study would correspond to 209 µg per kg for GRS and 912 µg per kg for TMR. These quantities would be further augmented in IMF manufacture by the addition of whey protein powder or permeate used for standardisation, as iodine was found to be present in similar proportions in each ingredient fraction within the present study (mineral concentration of permeates not shown).

Gulati *et al.* (2018) previously determined significant differences in certain macroelements (calcium, phosphorus, sodium) between GRS and TMR milks, which were not observed in the present study. However, significantly different concentrations of some macroelements are apparent between the product types analysed in the present study, despite the similar total protein contents between each. Various quantitative differences arose as artefacts of the each WPC production method employed which warrant some discussion. The significantly higher calcium and phosphorus contents of SMP relative to each WPC arise due to the presence of the majority of calcium and phosphorus in casein-bound form as colloidal calcium phosphate (CCP). By extension, a much greater proportion of zinc was also determined in SMP, relative to each WPC, as zinc is predominantly associated with CCP in bovine milk, limiting its bioavailability (Singh *et al.*, 1988). Likewise, the high magnesium content of the SMP samples may be attributed to its association with the casein micelle (Oh & Deeth, 2017). In comparison to SMP, the higher concentration of sulphur in WPC ingredients is due to the greater proportion of sulphur-containing amino acids in whey than in casein on a dry matter basis (Baldwin *et al.*, 2020).

The low concentrations of calcium in the SWPC samples, relative to the other whey types, is likely due to the formation of complexes of calcium with the rennet casein which are subsequently entrapped within the gel matrix formed by the action of chymosin (Durham & Hourigan, 2007; Wong *et al.*, 1978). The lower concentrations of phosphorus observed in

AWPC relative to SWPC and MCWPC may be due to solubilisation of CCP during the acidification process, which subsequently formed complexes with residual suspended casein from the removal of the curd, as the pH of the whey was adjusted to pH 6.7. These complexes would then have been too large to permeate the membrane through which the whey was subsequently filtered. The calcium content of the AWPC samples would also be expected to be substantially higher than the other samples due to increased partitioning of colloidal to ionic calcium in the serum phase (whey) during the acidification process (Lee & Lucey, 2010; Wong *et al.*, 1978). However, prior to micro-/ultrafiltration of the acid whey, the pH was re-neutralized causing the re-formation of insoluble calcium phosphate and its precipitation prior to AWPC production. The aforementioned complex formation, precipitation and removal during acid whey production may, therefore, be responsible for some losses in Ca content, resulting in final concentrations equivalent to the other WPC types.

It should be noted that re-adjustment of pH to 6.7 is not a standardised procedure during industrial-scale re-neutralisation of AWPC and is not universally applied at the same point during the production process as that of the current study. Sequential neutralisation steps may be applied throughout the process or the pH of the acid whey product may be maintained in acidic conditions for use in fortification of yoghurt products, for example. In the present study, the AWPC samples were intended to simulate ingredients for use in IMF manufacture, which would require neutralisation to limit the potential acidification effect of the addition of AWPC to the other materials during formulation. On addition of AWPC at pH 4.6 to a skim milk base in IMF manufacture, a decline in the overall pH of the system would lead to increased dissociation of calcium from the casein micelle in the skim milk component, leading to a decline in the thermal stability of the formulation during subsequent heating cycles.

The ratios of calcium to phosphorus in the AWPC samples were also higher than the SWPC and MCWPC samples (Table 4.3). The ratio for both SMP samples (55:45) aligns with

that reported for milk from Holstein Friesian cows in the literature (Ceballos *et al.*, 2009), whereas the ratio was variable between the WPC samples, whereby the SWPC and MCWPC samples displayed lower proportions of calcium and higher proportions of phosphorus than the AWPC samples, implying that the specific methods used for removal of casein have variable effects on the Ca:P ratio of WPC. The use of NaOH to adjust the pH of the whey back to pH 6.7 prior to membrane filtration is also the reason for the significantly higher sodium content of AWPC relative to all other samples. Similar increases in calcium or potassium content may occur depending on the alkaline compound used during caseinate production (Wiley-VCH, 2017).

Table 4.3: Average mineral concentrations (mg 100 g⁻¹) of skim milk powder and whey protein concentrate powders derived from grass or total mixed ration-based diets, determined by ICP-MS.

Mineral mg 100g ⁻¹	SMP		SWPC		MCWPC		AWPC	
	GRS	TMR	GRS	TMR	GRS	TMR	GRS	TMR
Calcium	1259 ^b	1237 ^b	350 ^a	307 ^a	452 ^a	461 ^a	411 ^a	417 ^a
Phosphorus	1034 ^d	1001 ^d	390 ^{abc}	433 ^{abc}	444 ^{bc}	540 ^c	268 ^a	329 ^{ab}
Ca:P Ratio	55:45	55:45	47:53	41:59	50:50	46:54	61:39	56:44
Potassium	1662 ^a	1661 ^a	1929 ^{ab}	1753 ^{ab}	2035 ^b	1904 ^{ab}	1777 ^{ab}	1757 ^{ab}
Sodium	338 ^a	326 ^a	357 ^a	331 ^a	376 ^a	362 ^a	1071 ^b	1084 ^b
Magnesium	111 ^d	118 ^{cd}	88.0 ^{ab}	86.1 ^a	100 ^{abc}	105 ^{bcd}	92.4 ^{ab}	93.5 ^{ab}
Sulphur	338 ^a	307 ^a	461 ^b	455 ^b	491 ^{bc}	466 ^b	535 ^c	519 ^{bc}
Manganese	0.049	0.015	0.018	0.010	0.012	0.012	0.017	0.011
Iron	0.313	0.186	0.362	0.449	0.378	0.300	0.401	0.477
Cobalt	< LOQ	< LOQ	0.0004	< LOQ	0.004	< LOQ	0.0004	0.001
Copper	0.095	0.135	0.077	0.095	0.169	0.101	0.099	0.139
Zinc	6.295 ^b	5.425 ^b	0.308 ^a	0.597 ^a	0.437 ^a	0.673 ^a	0.355 ^a	0.399 ^a
Selenium	0.017 ^a	0.034 ^c	0.018 ^{ab}	0.033 ^c	0.020 ^{ab}	0.035 ^c	0.015 ^a	0.028 ^{bc}
Molybdenum	0.036	0.032	0.065	0.076	0.113	0.072	0.043	0.041
Iodine	0.222 ^a	0.980 ^b	0.133 ^a	1.091 ^b	0.231 ^a	0.905 ^b	0.221 ^a	1.006 ^b

GRS—cows fed perennial ryegrass only

TMR—cows fed indoor total mixed ration *ad-libitum*

SMP – skim milk powder

SWPC – sweet whey protein concentrate (34%, w/w, protein)

MCWPC – micellar casein whey protein concentrate (34%, w/w, protein)

AWPC – acid whey protein concentrate (34%, w/w, protein)

^{a,b,c,d} indicates values within a row not sharing a common lower-case superscript letter differed significantly ($p < 0.05$).

LOQ – limit of quantification

4.4.1.4. *Ionic calcium concentration*

The average concentrations of ionic calcium (Ca^{2+}) in GRS and TMR-derived SMP reconstituted to 3.5% protein are shown in Figure 4.1. Concentrations of Ca^{2+} were similar between the GRS and TMR samples, the GRS sample being consistently higher at each pH point, with significant differences exhibited at pH 6.4, 6.8, 7.2 and 7.4. These differences are surprising, given the similarity in ionic composition between mid-lactation GRS-derived milk and that from a TMR system, as seasonal variance in Ca^{2+} is typically most extreme in early and particularly late lactation (Li *et al.*, 2019). However, the overall values observed for both systems were relatively low. Standard values for Ca^{2+} at native milk pH, for example, would be expected to be between approx. 2.0 and 2.5 mmol L⁻¹, whereas the concentrations recorded at the point nearest native pH (pH 6.6) were comparatively low at 1.37 and 1.12 mmol L⁻¹ for GRS and TMR samples, respectively. Within the serum phase of milk, approx. 20% of soluble calcium (10% of total calcium including colloidal calcium) is present in ionic form (Lewis, 2010), however the partitioning of ionic calcium between these phases may be more variable in reconstituted, heat treated SMP, relative to raw skim milk, as levels of soluble calcium have been shown to decrease in response to heat treatment (On-Nom *et al.*, 2010; Pouliot *et al.*, 2009). While the values recorded at each pH point are indicative of the levels of Ca^{2+} in each sample analysed for HCT, the true concentrations corresponding to each HCT value are likely considerably altered by increased conversion of ionic calcium into the colloidal phase on exposure to high temperatures in the oil bath.

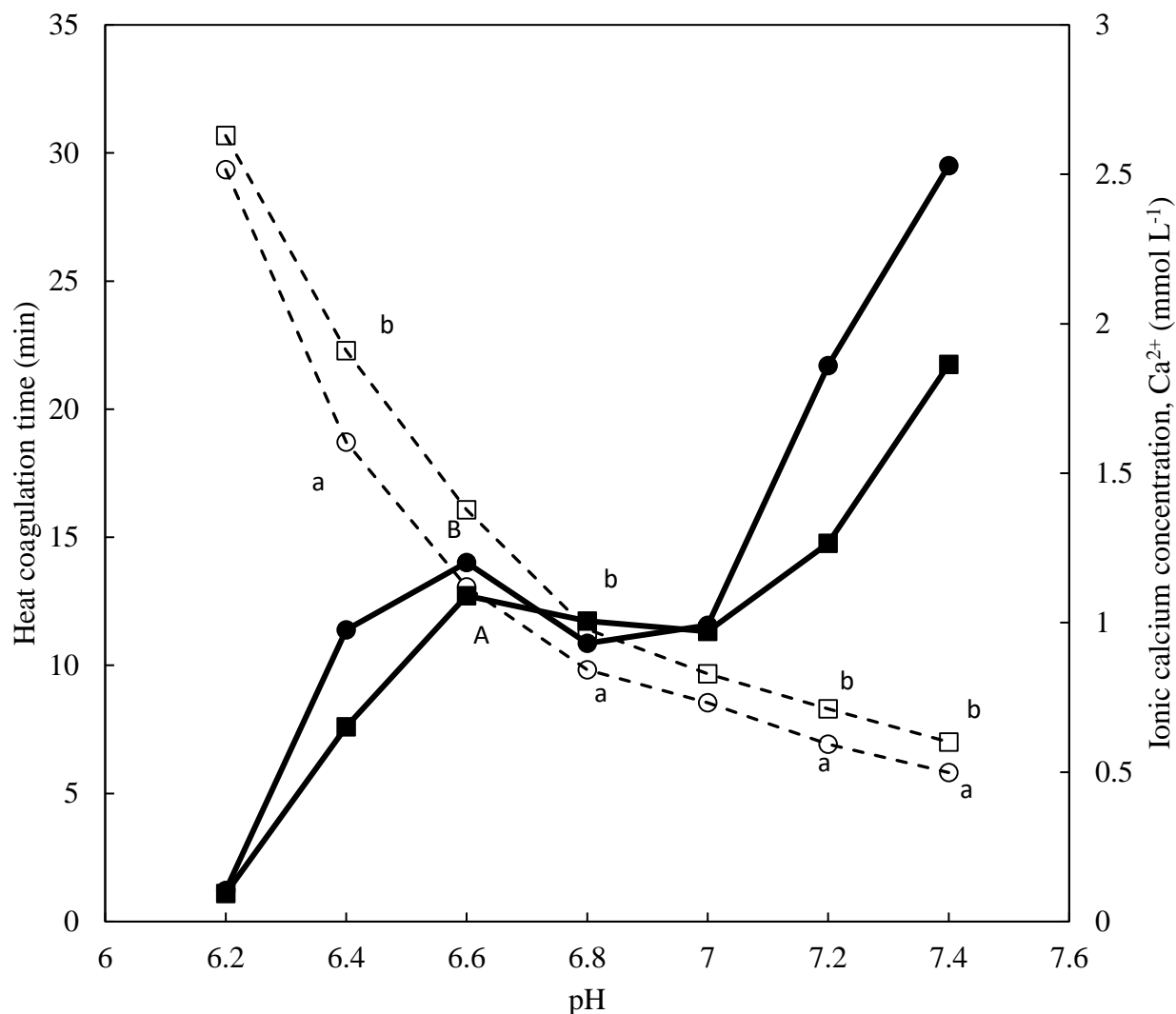


Fig. 4.1. Average heat coagulation time (filled symbols, bold series; min) and ionic calcium concentration (open symbols, dashed series; mmol L⁻¹) of skim milk powder dispersions (3.5% protein, w/w) heated at 140°C at 8 oscillations min⁻¹ obtained from cows fed perennial ryegrass (■, □) or total mixed ration (●, ○). Data points not sharing a common lower case (ionic calcium concentration) or upper case (heat coagulation time) letter differed significantly ($P < 0.05$).

4.4.2 Heat coagulation time of reconstituted skim milk powder

The average heat coagulation time (HCT)-pH curves for GRS and TMR-derived SMP reconstituted to 3.5% protein are shown in Figure 4.1. Both sample types displayed type A HCT-pH profiles, characterised by an increase in HCT with increasing pH to a local maximum at pH 6.6, followed by a decline to pH 7.0 and a further increase to an overall maximum at pH

7.4, with significantly higher heat stability exhibited by the TMR sample at pH 6.6, close to native pH. Substantially higher HCT values were also recorded in the TMR samples at pH 6.4, 7.2 and 7.4, although considerable variation was also present between replicates from each feeding system at these pH points. These results are contrary to those recorded by Gulati *et al.* (2019), who previously measured the HCT of reconstituted skim milk powder samples produced from the feeding systems utilised in the present study, between which little variation in HCT was observed. The overall increased heat stability in the TMR sample in the current study may be influenced by a combination of lower Ca^{2+} and higher NPN content, of which urea is the primary component. Heat-induced decomposition of urea to ammonia is considered to provide a buffering effect against heat-induced acidification and consequent micelle destabilisation (Huppertz, 2016).

4.4.3. Rheology of skim milk and whey protein concentrates

4.4.3.1 Low amplitude oscillation

The average elastic moduli (G') of each WPC reconstituted to 5% protein (w/w) is shown in Figure 4.2. The onset of gelation occurred during the peak hold at 85°C in all samples, with the GRS samples displaying significantly higher overall gel strength than the TMR samples. The average G' curves for MCWPC and SWPC were broadly similar, whereas both AWPC types produced significantly higher G' values than the other product types, in addition to lower gelation onset temperatures (between 77 and 80°C). Relative to AWPC and SWPC, heating of MCWPC also resulted in more consistent gel formation, indicated by the lack of fluctuation in the viscosity of these samples throughout the measurement (Fig. 4.2).

Profiles for the frequency sweep step applied after heating and gel formation are shown in Figure 4.3. The overall G' values for GRS-derived samples were again higher than TMR and the average G' values of both AWPC samples were significantly higher than each other product

type. Both SWPC types and GRS-derived MCWPC experienced gel fracture at 100 HZ. The rheological behaviours of SMP/WPC dispersions subsequently analysed by high shear measurements were largely similar to those of the WPC components alone and, as such, compositional differences potentially resulting in the behaviours observed will be discussed in more detail in the following section. It should also be noted that, due to the use of freeze-drying during sample production necessitated by the low volumes used within this study, the WPCs did not receive a level of heat treatment representative of typical industrial practice, which would incorporate pre-heating and evaporation steps, prior to spray-drying. These heating cycles and greater total thermal load would be expected to significantly increase thermal stability in further applications.

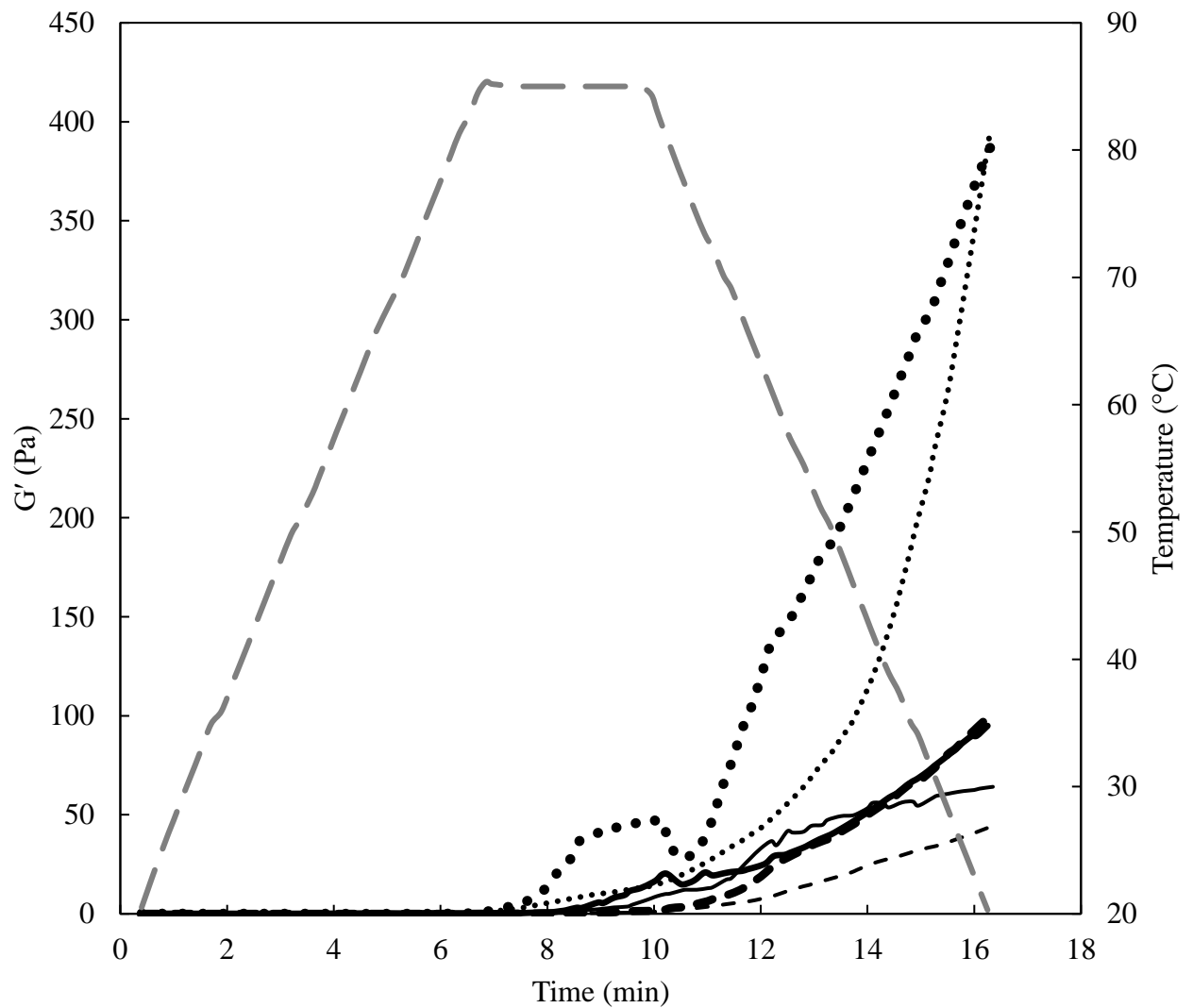


Fig. 4.2. Average elastic moduli, G' (Pa) of whey protein concentrate dispersions (5% protein w/w) for perennial ryegrass (bold series) and total mixed ration (light series)-derived acid whey protein concentrate (●●●, ...), micellar casein whey protein concentrate (— — —, - - -) and sweet whey protein concentrate (—, —).

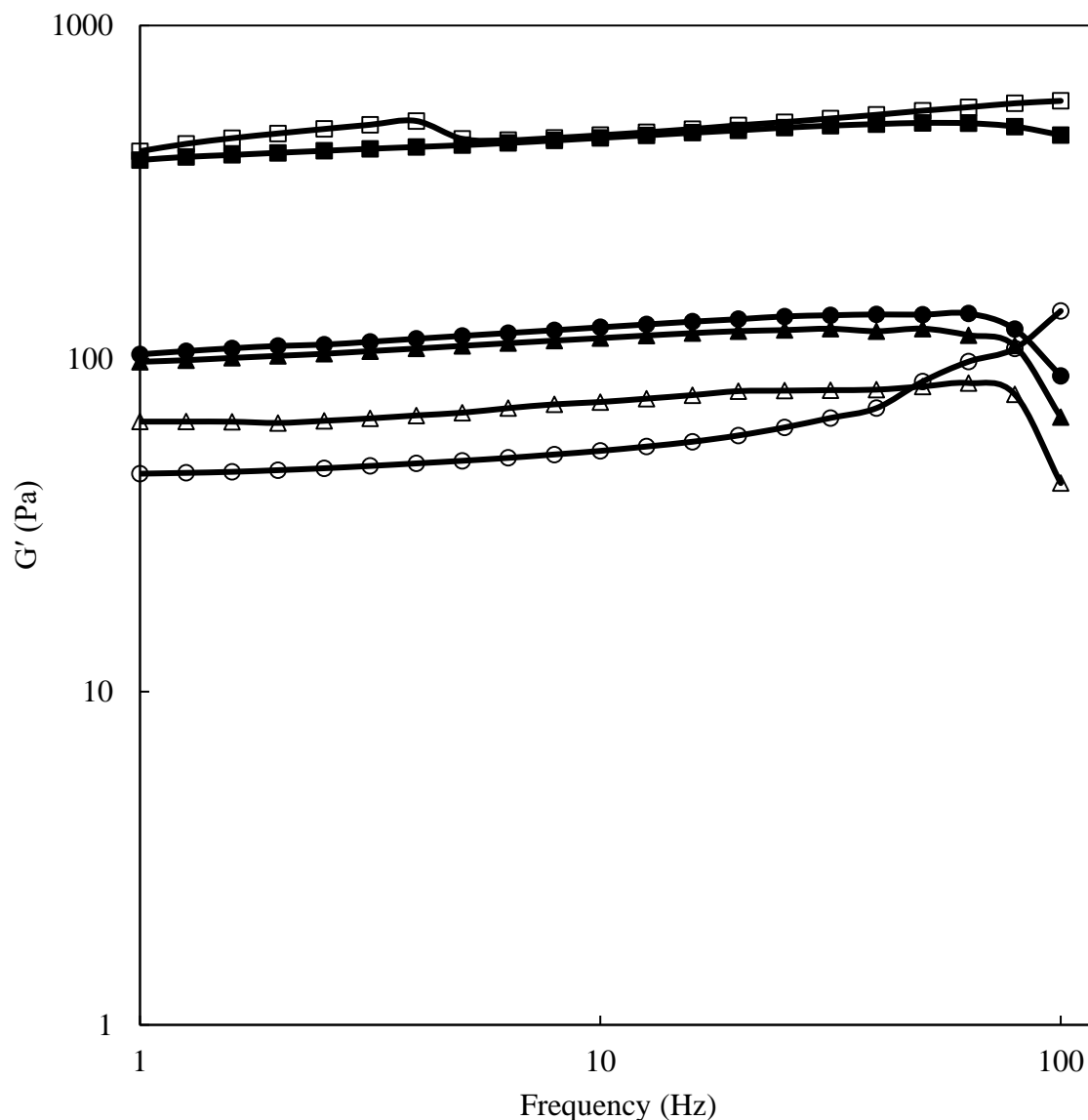


Fig. 4.3. Average elastic moduli, G' (Pa) of whey protein concentrate dispersions (5% protein w/w) for perennial ryegrass (filled symbols) and total mixed ration (open symbols)-derived acid whey protein concentrate (\blacksquare , \square), micellar casein whey protein concentrate (\bullet , \circ) and sweet whey protein concentrate (\blacktriangle , \triangle), as a function of oscillation frequency (1 - 100 Hz).

4.4.3.2. High shear viscosity measurements

Average values for the dynamic viscosity (Pa.s) of heated dispersions (5%, protein) of SMP and WPC are shown in Figure 4.4. The TMR-derived AWPC dispersions displayed significantly higher viscosity than those from GRS (Fig. 4.4C), despite 50% of the protein content of the dispersion being derived from SMP. Figure 4.5 compares GRS and TMR-derived

SMP reconstituted to 10% w/w protein using a similar heating profile in the starch pasting cell geometry, with significantly higher average viscosity observed in the GRS sample. Both GRS-derived SWPC (Fig. 4.4B) and MCWPC (Fig. 4.4A) dispersions displayed higher overall viscosities than the respective TMR samples. The average viscosity of both AWPC dispersions were again significantly higher than the other product types, between which MCWPC dispersions displayed the lower overall viscosity and the most consistent increase in viscosity (lack of fluctuation in viscosity values throughout the heating cycle) of the three product types, even following extensive heating at temperatures exceeding 65°C.

Both SWPC dispersions displayed erratic profiles with a distinct spike in viscosity at approx. 40°C observed in all SWPC replicates. The reason for this occurrence is not yet known, but could be influenced by the apparent losses in Ca content during SWPC production, as Ca removal has been suggested to cause a decline in the denaturation temperature of α -lactalbumin (Shimada & Matsushita, 1981), the tertiary structure of which is stabilised by Ca binding residues (Anderson *et al.*, 1997). However, this alone is unlikely to reduce the denaturation temperature to the extent observed in the present study.

This spike appears to indicate a potential reversible heat-dependent self-aggregation of GMP or supramolecular aggregation between GMP and other proteins. This complexation could potentially occur with α -lactalbumin, but would be unusual at low temperature and without a change in pH (Sharma *et al.*, 2013). The observed activity is therefore more likely to be related to interactions between β -lactoglobulin and GMP. The high heat stability and “chaperone” activity of GMP in whey protein dispersions would be expected to maintain lower viscosity values in SWPC samples on heating (Gaspard *et al.*, 2021), however, the kinetics of denaturation of β -lactoglobulin have previously been shown to be accelerated due to the presence of GMP in solutions at pH 6.7 (Croguennec *et al.*, 2014), whereby a combination of

hydrophobic interactions and the strong electrostatic interactions arising from the negative surface charge of GMP increase the rate of β -lactoglobulin denaturation.

However, this activity is dependent on a number of compositional factors, including levels of glycosylation and phosphorylation of GMP, ratio of GMP: whey protein and ratio of calcium:protein, and the rate of heating applied to the dispersions (Gaspard *et al.*, 2020). A relatively fast heating rate of $10^{\circ}\text{C min}^{-1}$ was applied in the measurements of the present study, which may have influenced the apparent reversibility of the aggregation shown in Figure 4.4, by providing insufficient time for the completion of disulphide bond formation between GMP and β -lactoglobulin (Relkin *et al.*, 1992), although this phenomenon has previously been observed only at low pH values (pH 3.5). Martinez *et al.* (2009) also observed a decrease in the temperature of the onset of gelation of β -lactoglobulin in the presence of GMP, which was highlighted by Gaspard *et al.* (2020) as demonstrating the dependence of this behaviour on GMP and whey protein composition. However, this does not account for the apparent reversal of aggregation in the sample after the initial spike in viscosity at approx. 40°C and prior to full gelation at approx. 90°C (Figure 4.4). This activity may, therefore, be due to electrostatic interactions between GMP and β -lactoglobulin and consequent aggregate formation prior to the application of heat (Martinez *et al.*, 2010), followed by reversal of this aggregation as the temperature was increased from 40°C to 65°C , at which point full denaturation of the whey protein component occurred.

The sharp increase in dynamic viscosity at approx. 40°C was followed by full coagulation of the samples at 90°C , with the samples displaying particularly uneven profiles thereafter. In contrast, the viscosity of both AWPC dispersions increased only upon reaching 90°C , but displayed significantly higher viscosities both prior to the point of coagulation and thereafter. Fig. 4.4B and 4.4C do not display full profiles corresponding to the complete

experiment run time as shown in Fig. 4.4A as, beyond the points displayed, each sample was considered fully coagulated following the occurrence of extensive protein precipitation.

Both MCWPC dispersions (Fig. 4.4A) displayed profiles dissimilar to the AWPC and SWPC dispersions, characterised by a consistent increase in viscosity throughout heating to 90°C and thereafter. The average time to onset of gelation also varied significantly between the dispersions of each product type. A sharp increase in dynamic viscosity was observed at 5.75 min (40.4°C) for the SWPC dispersions. This was significantly lower than the AWPC dispersions at 9.38 min (77.1°C), which were also significantly lower than the MCWPC dispersions at 10.3 min (85.7°C).

Following removal from the geometry, all SWPC and AWPC dispersions were also visibly coagulated and separated into a distinct serum phase in which a large precipitated coagulum was suspended. In contrast, each MCWPC dispersion remained in solution, within which small white flecks of protein were suspended. Representative images displaying these visual differences on removal of the samples from the geometry *post* heating are shown in Appendix i Figure S2. Despite the presence of GMP in SWPC, MCMPC dispersions were clearly more stable during heat treatment. The higher NPN content and associated resistance to thermal denaturation of the protein component of the TMR samples may in part be responsible for the lower viscosities observed in the TMR dispersions relative to those from GRS. Similarly, the NPN content of the AWPC samples was notably lower than the other WPC types (Table 4.1); however, the NPN contents of the highly heat stable MCWPC samples were comparable to those of the SWPC samples.

The thermal instability of the AWPC dispersions will have been further promoted by the higher absolute concentrations of β -lactoglobulin relative to MCWPC and proportionally higher concentrations of the same relative to SWPC, as β -lactoglobulin undergoes extensive conformational change during heating at temperatures exceeding 80°C (Chen *et al.*, 2005).

Moreover, the high sodium content unique to the AWPC samples will have caused further destabilisation of the system. The capacity for high concentrations of ionic calcium to promote protein aggregation is well accepted; however, at pH values above the isoelectric point of whey proteins (pH 5.5), high concentrations of sodium have also been shown to facilitate whey protein denaturation and aggregation (Xiong *et al.*, 1993; Varunsatian *et al.*, 1983). As the specific mineral content of WPC powders is influenced by the type of alkaline solution used to adjust the pH of the MF permeate, further comparative study could be carried out to determine if a similar destabilising effect occurs due to the use of potassium hydroxide during AWPC production.

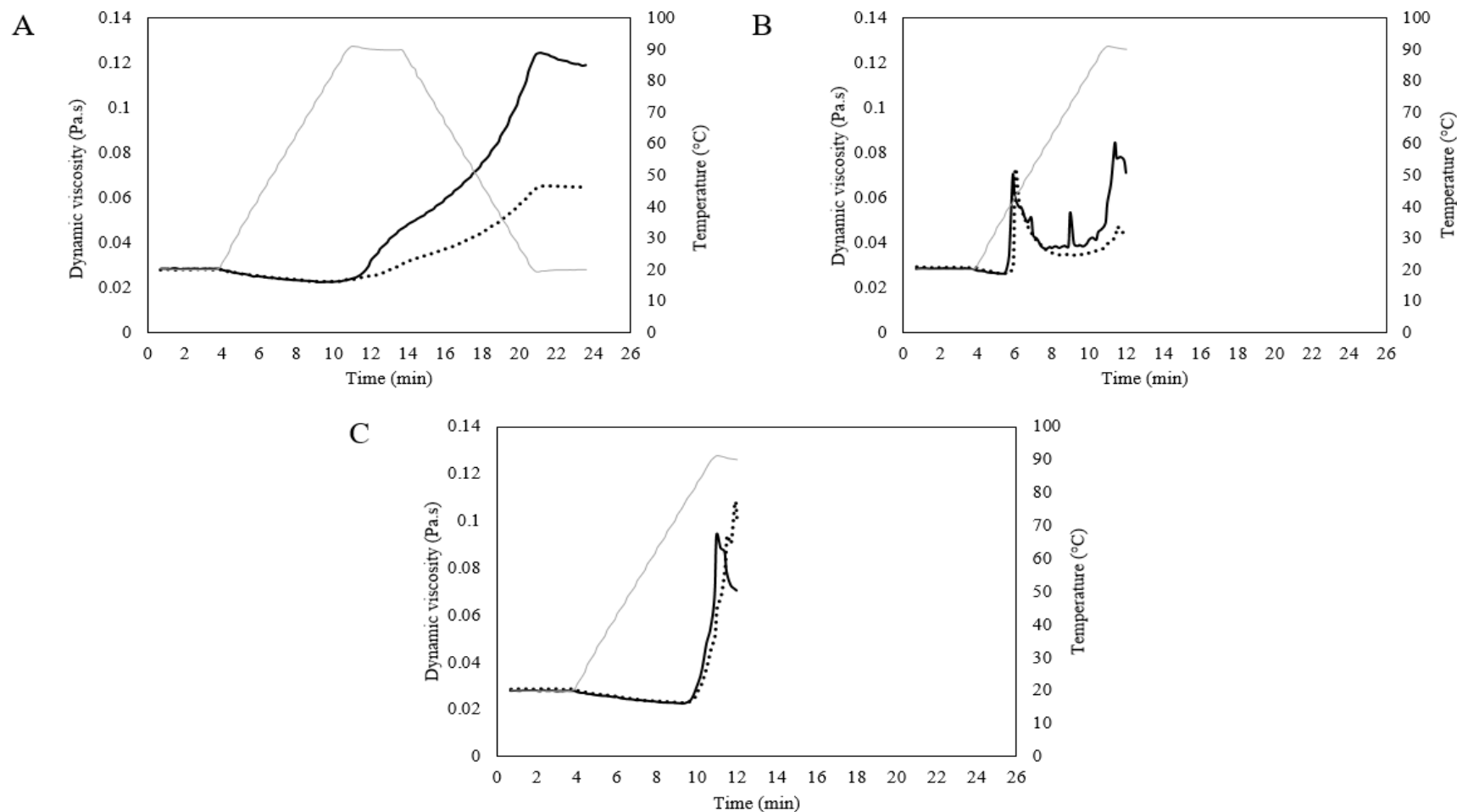


Fig. 4.4. Average dynamic viscosity (Pa.s) of (A) dispersions of 5% protein (w/w) skim milk powder/micellar casein whey protein concentrate, (B) dispersions of 5% protein (w/w) skim milk powder/sweet whey protein concentrate and (C) dispersions of 5% protein (w/w) skim milk powder/acid whey protein concentrate from perennial ryegrass (—) and total mixed ration (···).

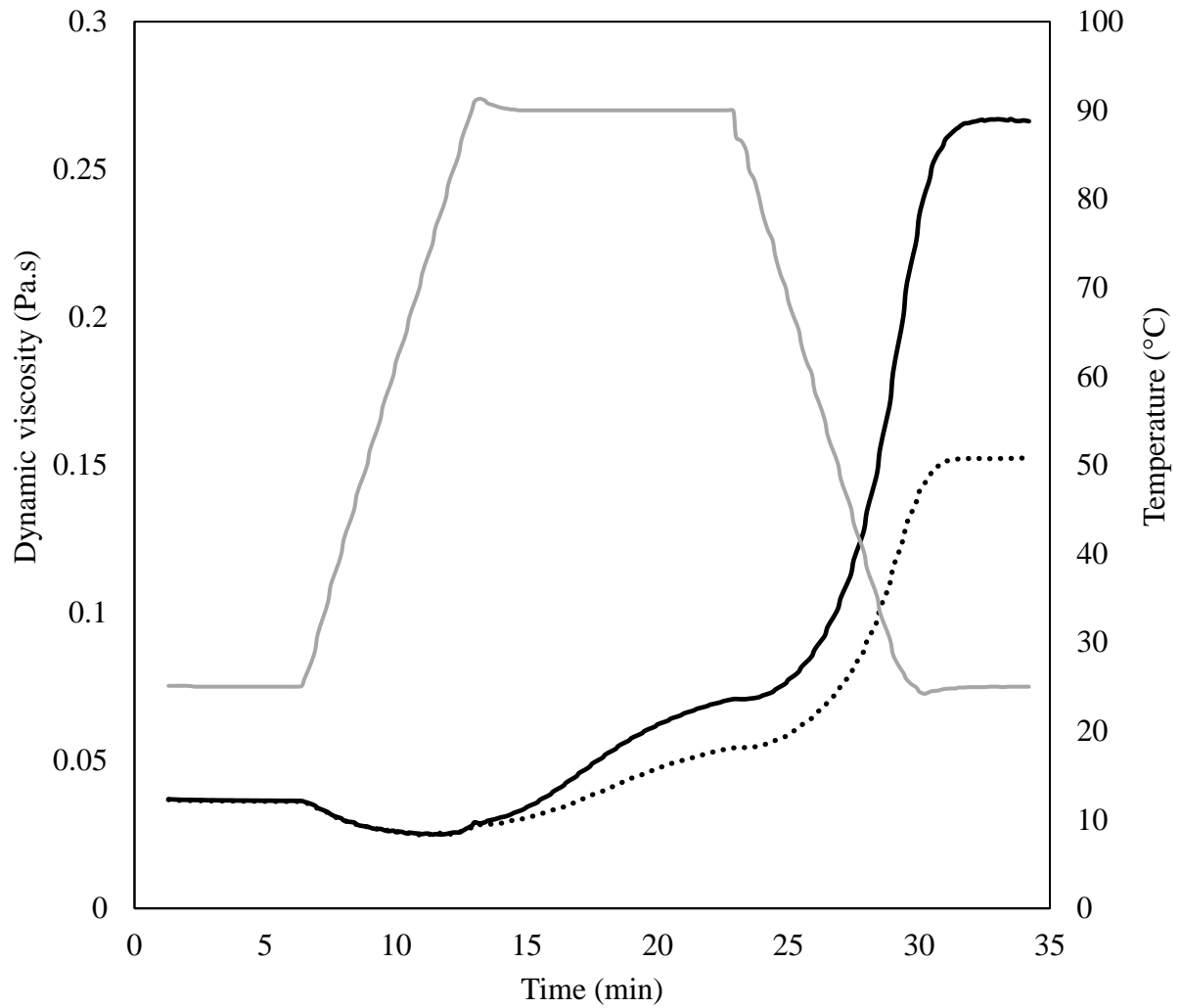


Fig. 4.5. Average dynamic viscosity (Pa.s) of skim milk powder dispersions (10% protein w/w) from perennial ryegrass (—) and total mixed ration (•••).

4.5. Conclusion

This work demonstrated a limited effect of bovine feeding system on the composition and functional properties of skim milk powder and whey protein concentrates, with more substantial differences observed between the protein ingredient types analysed. The comparative nitrogen and mineral compositions of the GRS and TMR skim milk samples were similar to those previously reported in milk derived from these systems, however, the compositional variances observed relative to previous work in milk derived from the same feeding systems may be indicative of the variance in feed and pasture quality between seasons. The high concentrations of selenium and iodine in TMR-derived milk and WPC samples indicates a high level of direct mineral transfer from feed into milk. The high concentrations of iodine are also a noteworthy consideration for milk selection for IMF production. The TMR-derived reconstituted skim milk powder exhibited greater stability, as determined by HCT and rheological analysis. The considerable variation in gelation characteristics between the WPC types suggests that selection of sweet whey and particularly micellar casein whey may be more appropriate than acid whey for use in IMF manufacture on the basis of thermal stability and particularly mineral composition.

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Chapter 5

Physicochemical properties of whole milk powder derived from milk of cows fed pasture or total mixed ration diets

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John T. Tobin (Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland) facilitated whole milk powder production trials.

5.1. Abstract

This study determined the effect of dietary factors on compositional and functional properties of whole milk powder (WMP) produced from bovine milk. Raw milk samples were obtained from three groups of 18 Holstein Friesian Spring calving cows randomly assigned to diets based on perennial ryegrass (GRS), perennial ryegrass/white clover sward (CLV) and total mixed ration (TMR). Raw milks obtained in late lactation were subsequently standardised for fat, heat-treated (90 °C for 30 s), evaporated and homogenised prior to spray-drying. WMP produced from each diet was analysed to determine differences in colour, fat globule size distribution, heat coagulation time, yoghurt gelation, texture profile and protein profile due to each diet. Significant differences in heat coagulation time were observed between the CLV and TMR samples and colour values were significantly different between the GRS and TMR samples. No significant differences in gross composition, protein profile or whey protein nitrogen index were found between the three WMP samples. Average D_{90} values for fat globule size were significantly lower in the TMR sample, relative to both the GRS and CLV samples. Yoghurts produced from GRS and CLV-derived WMP samples exhibited significantly higher elastic moduli (G') than those derived from TMR. Similarly, texture profile analysis showed significantly higher firmness values in yoghurt samples derived from CLV, in comparison to TMR samples. The data presented characterises the effect of these diets on the composition and functional properties of fat standardised WMP, suggesting better yoghurt functionality and thermal stability in WMP derived from pasture-based bovine diets.

5.2. Introduction

Whole milk powder (**WMP**) is a high value dairy commodity produced through processing and spray-drying of standardised whole milk. While WMP retains the inherent fat composition of the whole milk from which it is produced, it typically undergoes the same centrifugal separation process as skim milk in order to yield skim and cream, which are subsequently recombined to standardise the fat content of the whole milk (Kelly *et al.*, 2002). WMP is used extensively in the production of chocolate and ice-cream, but is also frequently rehydrated as a source of whole milk or for use as the base ingredient in yoghurt production.

The temperate climate of countries such as Ireland and New Zealand are most suited to pasture-based milk production systems, the utilisation of which has been shown to result in a range of compositional differences to milk, compared to that from concentrate-based production systems. The latter systems are widely implemented in the Americas and parts of Europe (Chilliard *et al.*, 2001; Couvreur *et al.*, 2006). Previous studies have demonstrated greater levels of carotenoids (particularly β -carotene) and, hence, a more yellow colour, greater unsaturated fatty acid content, most notably conjugated linoleic acid (Kelly *et al.*, 1998) and greater true protein content in pasture-derived milk, compared to concentrate-derived milk (O'Callaghan *et al.*, 2016b).

Due to the increased energy-density of high-protein TMR diets, their application has been observed to result in an increase in milk yield, but not milk protein content (Kolver *et al.*, 2000). Indeed, over-feeding of dietary crude protein may not necessarily lead to an increase in milk protein content, as excess nitrogen is excreted as urinary nitrogen (Broderick, 2003). Similarly, increasing dietary oil supplements and reducing dietary fibre content can affect rumen biohydrogenation pathways, producing inhibitory fatty acid intermediates which limit milk fat synthesis (Bauman and Griinari, 2003). Conversely, pasture-based diets which are higher in roughage content tend to promote the action of cellulolytic rumen microflora, leading

to increased milk fat biosynthesis and ultimately higher milk fat content (Bauman and Grinari, 2003).

Differences in milk composition due to dietary variation may have a significant effect on the thermal stability and processability of products derived from WMP. Gel strength, viscosity and textural qualities of acidified reconstituted WMP are properties of significant importance in determining the behaviour of set-yoghurts. Gelation can be influenced by various factors, such as milk fat and protein (particularly casein) content, heat treatment and homogenisation pressure (Lee and Lucey, 2010). Given the various heating processes to which whole milk is subjected in the production of WMP, thermal stability is an important functional parameter (Singh and Creamer, 1991). Heat-induced changes are primarily caused by the denaturation/aggregation of whey protein fractions (particularly β -lactoglobulin) or the interaction of κ -casein and whey proteins (Anema & Li, 2003). The whey protein nitrogen index (**WPNI**) quantifies the level of un-denatured whey protein in dairy powders, indicating the level of heat treatment a powder product has received during processing. These WPNI values are of particular importance for dictating the gel-strength of yoghurts.

While the effect of pasture-based and concentrate-based bovine feeding systems on the composition of raw milk has been reported, limited information exists to characterise their effect on the composition of standardised milk powder products. Moreover, O'Callaghan *et al.* (2016b) have previously determined significant variation in the fatty acid profile of milks derived from the feeding systems investigated in the current study, although the potential effect of diet on the protein profile of milk from each system has not yet been investigated. As the gel-strength of yoghurt is influenced by the relative proportions of casein and whey in milk (Lee and Lucey, 2010), the concentrations of the individual casein and whey proteins present in WMP derived from each feeding system are factors of interest relative to gel formation. The objective of this study was to characterise the influence of perennial ryegrass (*Lolium perenne*

L.), perennial ryegrass/white clover (*Trifolium repens* L.) sward and total mixed ration (TMR)-based feeding systems on the colour, thermal stability, fat globule size distribution and protein profile of standardised WMP produced from whole milk derived from each system and the subsequent gelation and textural properties of yoghurt.

5.3. Materials and Methods

5.3.1. Materials

Raw milk was obtained from the Teagasc Animal and Grassland Research and Innovation Centre dairy unit (Moorepark, Fermoy, Co. Cork, Ireland). The mesophilic starter culture, MO1, used for yoghurt production (mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*) was provided by Chr. Hansen, Cork, Ireland.

5.3.2. Experimental Design

The experimental design for the bovine feeding systems was similar to that described in Chapter 2 and in previous work by O'Callaghan *et al.* (2016b). For this lactation cycle, 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. The GRS and CLV groups were handled as described in Chapter 2. The chemical and nutritional values of each of the feeding systems are shown in Chapter 1, Part 2, Section 1.2.4.1.1. (Tables 1.2.2, 1.2.3 and 1.2.4). The TMR system consisted of, on a dry matter basis, 7.15 kg of grass silage, 7.15 kg of maize silage and 8.3 kg concentrates. All other details relating to feed administration, milk collection and handling were as described in Chapter 2.

5.3.3. Whole Milk Powder Production

All milk powder production was carried out at Moorepark Technology Limited (MTL) pilot plant facility (Fermoy, Co. Cork, Ireland). Raw whole milk (~1000 kg) was pre-heated to 50°C in an APV plate heat-exchanger (SPX Flow Technology, Crawley, West Sussex, UK) before separation into skim and cream in a GEA Westfalia centrifugal disk separator (GEA Westfalia, Oelde, Germany). The separate skim and cream fractions were then recombined to produce a whole milk of standardised fat content (3.5%, w/w). This standardised whole milk was then pasteurised at 90°C for 30 s using an APV plate heat-exchanger, before being evaporated to ~52% total solids (TS) in a Niro three-effect falling film evaporator (GEA Niro A/S, Soeborg, Denmark). The pasteurised concentrate was pre-heated to 65°C in an APV plate heat-exchanger and homogenised using an APV Gaulin two-stage homogeniser (Lake Mills, Wisconsin, USA) at a first and second-stage pressure of 150 and 50 bar, respectively. The homogenised whole milk concentrate was then dried using a Niro Tall-Form Anhydro three-stage spray-dryer (air inlet temperature 180°C; air outlet temperature 80°C). First and second fluid bed temperatures were set at 65°C and 25°C, respectively. Fines were returned from the second fluid bed and the cyclone to the top of the spray-dryer to yield an agglomerated WMP of approximately 97% TS. The WMP production was carried out in triplicate from three independent raw milk collections.

5.3.4. Compositional Analysis of Powder

5.3.4.1. Determination of Nitrogen and Fat Content.

The total nitrogen content of each powder was determined using the Kjeldahl method, as described in ISO 8968-1 (2001). A nitrogen-to-milk protein conversion factor of 6.38 was used. The fat content of each powder was determined using the Röse-Gottlieb gravimetric method, as described by the International Dairy Federation (1996). The ash content of each

powder was determined by ashing approximately 3 g of each sample in a Carbolite muffle furnace (Carbolite Gero Ltd, Hope, Sheffield, UK) overnight. The moisture content of each powder was determined using a HR83 Halogen rapid moisture analyser (Mettler Toledo, Columbus, Ohio, USA). The lactose content of each powder was calculated by difference.

Non-casein nitrogen content was determined by precipitation of the casein component of a whole milk sample. The sample was diluted with deionised water at 40°C and acidified to pH 4.6 by addition of acetic acid and sodium acetate. The mixture was cooled to 20°C and allowed to settle before filtration using Whatman No. 1 filter paper. A Kjeldahl determination was then carried out on the filtrate as described above. Non-protein nitrogen content was measured as described in Chapter 2.

5.3.4.2 High-performance Liquid Chromatography (HPLC).

The protein profile of each WMP was determined using Reversed-Phase HPLC as described by Mounsey and O'Kennedy (2009). The aqueous (phase A) and organic (phase B) phases consisted of Acetonitrile, HPLC grade water and Trifluoroacetic acid (TFA) in respective ratios of 100:900:0.1 (v/v/v) and 900:100:0.1 (v/v/v). Samples at a dilution factor of 1:20 sample:buffer were filtered through a 0.2- μm filter and separated using an Agilent 300SB Poroshell (2.1 x 75mm) column (Agilent Technologies, Santa Clara, California, USA) at 35°C. Detection wavelength was set at 214 nm, injection volume was 5 μL and flow rate was 0.5 mL min^{-1} .

5.3.5. Colour Measurements

The CIE $L^*a^*b^*$ method was used to measure the colour of each WMP. Lightness, red/green and yellow/blue values were determined using a Konica Minolta CR-400 Chroma

Meter (Chiyoda, Tokyo, Japan). Powdered and reconstituted (13% w/w) samples of each WMP were placed into plastic cuvettes and measured in triplicate.

5.3.6. Whey Protein Nitrogen Index (WPNI)

WPNI was determined using the GEA Niro Method No. A 21a, modified from the Harland-Ashworth method (Kuramoto *et al.*, 1959).

5.3.7. Heat Coagulation Time

The heat coagulation time (**HCT**) of raw whole milk and reconstituted powders was determined using an Elbanton Oil Bath (Hettich Benelux Laboratory Equipment, Geldermalsen, Netherlands). Each WMP sample was reconstituted to 1.5% (w/w) protein using deionised water. Milk samples were divided into a further 13 aliquots (approx. 30mL), the pH values of which were adjusted in pH increments of 0.1 between pH 6.2, and 7.4 using 0.1N HCl or NaOH. Following approximately 2 hours of equilibration and final pH adjustment, where necessary, ~3.4 g aliquots of each sample were pipetted into 4 mL glass tubes, stoppered and inserted into the oil bath rack. The rack was then inserted into the temperature-controlled oil bath at 140°C and rocked at 8 oscillations min⁻¹. The time taken in minutes for visual coagulation of each sample was recorded.

5.3.8. Milk Fat Globule Size of Rehydrated Whole Milk Powders

The size distribution of milk fat globules in WMP dispersions reconstituted at 15% (w/w) was measured in triplicate by static light scattering (SLS) using a Malvern Mastersizer laser-light diffraction unit (Hydro MV, Mastersizer 3000, Malvern Instruments Ltd, Worcestershire UK) equipped with a 300 RF lens. Refractive indices were set at 1.462 and 1.33 for the analyte and dispersant (water), respectively. Size measurements were determined as the

median diameter, D_{50} , and cumulative diameters, D_{90} and D_{10} , in which 50, 90 and 10% of the volume of globules were smaller than the specified size. Size distributions were determined by polydisperse analysis, and measurements were taken when laser obscuration reached ~3%.

5.3.9. Rheological Properties of Yoghurt

5.3.9.1 Yoghurt Production.

Whole milk powder from each group of cows was reconstituted to 15% TS in deionised water and refrigerated overnight at 4°C to ensure complete hydration. Dispersions (1 L) were then tempered at 30°C prior to inoculation in a laminar flow hood. Freeze-dried pellets of the starter culture (0.2 g) were then added to ~20 mL aliquots of the tempered milk before being added back into the dispersions and mixed thoroughly. Sub-samples (100 mL) not intended for rheological measurements were added to sealed cups and placed in an incubator at 30°C (temperature chosen based on supplier recommendation). The pH of the inoculated milk was constantly monitored until a pH of 4.6 was reached. At pH 4.6, the incubated samples were immediately steeped in an ice bath to cease starter culture activity. On reaching a temperature of 10°C, these samples were then refrigerated overnight.

Low-amplitude oscillation measurements were carried out using a Discovery HR-1 hybrid rheometer (TA Instruments, New Castle, Delaware, USA), equipped with a concentric cylinder, maintained at 30°C. An aliquot (17 g) of the freshly inoculated milk was immediately weighed into the concentric cylinder. A time sweep was initiated using the following pre-test conditions: 5 s temperature equilibration at 30°C, 15 s pre-shear at a shear rate of 50 s⁻¹ and 10 s equilibration. The sample was then oscillated at 1% strain, at a frequency of 1 Hz over a 10 s sampling interval, until pH 4.6 was reached. This was monitored by measuring the pH of parallel incubated samples of inoculated milk, which were maintained at the same temperature as the sample in the rheometer for the same duration. Once the pH reached pH 4.6, the time

sweep was stopped. This was followed immediately by a logarithmic frequency sweep from 1 to 63.1 Hz at a constant strain of 1%.

5.3.9.2. Texture Profile Analysis.

Following overnight refrigerated storage, set yoghurt samples (100 mL) were analysed at 4°C using a 35-mm flat disk backward extrusion rig on a Texture Expert Exceed system (Stable Microsystems, Godalming, Surrey, UK). Probe force was calibrated using a 2-kg weight, mounted on a 5-kg load cell. Trigger force was set at 2 g. The probe penetrated the sample to a depth of 25 mm and returned to the starting point. Pre-test, test and post-test probe speeds were set at 1 mm s⁻¹. The sample firmness, consistency, cohesiveness and index of viscosity were recorded. Yoghurt gels from each WMP sample were also tested at 30°C to determine the influence of variation in fatty acid melting points between the samples.

5.3.10. Statistical Analysis

All analyses were carried out on WMP from three independent trials from each dietary treatment. 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. Data was deemed normally distributed and analysis was carried out using one-way ANOVA with post hoc Tukey test. *P*-values < 0.05 were considered significant. All WMPs were produced in triplicate to give 9 separate powders. Gross compositional and heat coagulation analyses were carried out in duplicate for each sample. Colour and fat globule size analyses were carried out in triplicate for each sample. For the rheological and textural analyses, duplicate yoghurt samples were produced from each sample and measured.

5.4. Results and Discussion

5.4.1. Whole Milk Powder Composition

Total nitrogen, total fat, lactose, non-protein nitrogen and non-casein nitrogen content of WMP samples are shown in Table 5.1. The highest mean protein content was present in the GRS sample (31.5%, w/w), followed by CLV (30.7%, w/w) and TMR (30.3%, w/w), though these values were not significantly different ($P > 0.05$). Similarly, no significant difference in mean fat content was found between the powders, with mean fat contents of 25.8, 25.7 and 25.5% (w/w) for CLV, TMR and GRS milk samples, respectively. No significant differences in lactose, ash or free moisture content were found between the samples. However, non-protein nitrogen and non-casein nitrogen values for CLV were significantly higher ($P < 0.05$) than those from TMR. Increased non-protein nitrogen content may be attributed to increased urea content arising from the inclusion of white clover in the CLV feeding system. Significantly higher levels of urea have previously been reported in milk from this system, when compared to milk from the TMR system (O'Callaghan *et al.*, 2018). Significant differences in protein and fat content in pasture-derived raw unstandardized whole milk, relative to concentrate-derived raw whole milk, have been previously observed (O'Callaghan *et al.*, 2016b); however, no information exists on the effect of these feeding systems on the composition of standardised WMP. Another study which utilised these feeding systems recorded significant differences in total protein and casein content between pasture-derived and concentrate-derived raw whole milks, though a significant difference in total fat was only observed between the two pasture-derived diets (Gulati *et al.*, 2018a).

Table 5.1. Compositional analysis data for whole milk powders

Sample	Total protein	Total fat	Lactose	Non-protein nitrogen %, w/w	Non-casein nitrogen	Ash	Free moisture
GRS	31.5 ± 0.99 ^a	25.5 ± 0.78 ^a	35.6 ± 0.52 ^a	0.32 ± 0.05 ^{ab}	0.99 ± 0.02 ^{ab}	5.42 ± 0.54 ^a	1.94 ± 0.29 ^a
CLV	30.7 ± 0.53 ^a	25.8 ± 0.63 ^a	36.2 ± 0.79 ^a	0.37 ± 0.02 ^b	1.03 ± 0.02 ^b	5.57 ± 0.13 ^a	1.83 ± 0.02 ^a
TMR	30.3 ± 1.35 ^a	25.7 ± 0.48 ^a	36.4 ± 1.22 ^a	0.29 ± 0.01 ^a	0.97 ± 0.02 ^a	5.72 ± 0.03 ^a	1.84 ± 0.12 ^a

GRS – Cows fed perennial ryegrass only.

CLV – Cows fed perennial ryegrass / 20 % white clover sward.

TMR – Cows fed indoor total mixed ration *ad-libitum*.

Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

The mass of protein fractions present in each WMP dispersion (13% w/w) are shown in Table 5.2. No significant differences ($P > 0.05$) were observed in protein profile between the WMP samples. Slightly higher amounts of κ -CN, α_{s2} -CN, α_{s1} -CN, β -CN, β -LG a and β -LG b were observed in the GRS sample than in both the CLV and TMR samples. A previous study by Mackle *et al.* (1999) investigated the effect of feeding cows on ryegrass-white clover pasture, pasture supplemented with maize grain and pasture supplemented with a combination of maize grain and pasture silage. The above study, using sodium dodecyl sulphate polyacrylamide gel electrophoresis, identified a significantly lower proportion of β -CN in milk from cows supplemented with maize grain only, with higher β -CN content in milk from cows fed on pasture only. This trend for β -CN appears to be similar to the present study; however, the lack of significance ($P > 0.05$) could be related to reduced sample set size ($n = 3$) in the present study.

Table 5.2. Mass of protein fractions (mg/mL) present in whole milk powder dispersions (13% w/w), determined by reversed-phase high performance liquid chromatography

Sample	κ -CN	α 2-CN	α s1-CN	β -CN	α -LA	β -LGa	β -LGb
GRS	3.4 ± 0.32	2.9 ± 0.08	12.4 ± 0.20	12.1 ± 0.48	0.4 ± 0.10	1.5 ± 0.26	1.4 ± 0.51
CLV	3.1 ± 0.44	2.5 ± 0.26	11.4 ± 0.44	11.3 ± 0.49	0.5 ± 0.15	1.4 ± 0.09	1.3 ± 0.39
TMR	2.6 ± 0.69	2.5 ± 0.56	10.9 ± 1.47	10.8 ± 1.23	0.6 ± 0.15	1.4 ± 0.43	1.4 ± 0.30

GRS – Cows fed perennial ryegrass only.

CLV – Cows fed perennial ryegrass / 20% white clover sward.

TMR – Cows fed indoor total mixed ration *ad-libitum*.

Average WPNI values for the CLV, GRS and TMR powder samples were 4.2 ± 0.19 , 3.8 ± 0.11 and 4.0 ± 0.05 mg of undenatured whey protein per g of powder, respectively. Excessive heat treatment will lead to increased whey protein denaturation, represented by a low WPNI value of <1.5 (Harland and Ashworth, 1947), whereas a high WPNI value (> 6.0) indicates a high level of native whey protein in dairy-based powders (Sikand et al., 2008). All WMP samples exhibited WPNI values in the medium heat treatment range ($>1.5 - <6.0$), indicating a consistent heat treatment process and no significant difference in the level of denatured whey protein content between the samples.

5.4.2. Colour Analysis of Whole Milk Powders

Colour measurements of WMP samples are shown in Table 5.3. Lightness (L^*) indicates the degree of whiteness of a sample, ranging from 0 (black) to 100 (white). Positive values on the green/red (a^*) component indicate redness and negative values indicate greenness. Similarly, positive values on the yellow/blue (b^*) component indicate sample yellowness and negative values indicate blueness.

Table 5.3. Average Lightness (L*), red/green (a*) and yellow/blue (b*) values for whole milk powders and whole milk powder dispersions (15% w/w)

Sample	Powder				Dispersion			
	L*	a*	b*	ΔE	L*	a*	b*	ΔE
GRS	93.0 ± 0.71 ^a	-5.44 ± 0.56 ^a	18.5 ± 1.40 ^b	1.10 ^(GRS-CLV)	85.0 ± 1.64 ^a	-4.58 ± 0.86 ^a	11.0 ± 1.67 ^a	2.78 ^(GRS-CLV)
CLV	93.3 ± 0.75 ^a	-5.24 ± 0.63 ^a	17.5 ± 1.13 ^{ab}	2.59 ^(CLV-TMR)	86.2 ± 0.27 ^a	-3.69 ± 0.10 ^a	8.66 ± 0.49 ^a	0.86 ^(CLV-TMR)
TMR	93.5 ± 0.85 ^a	-4.65 ± 0.70 ^a	15.0 ± 1.15 ^a	3.68 ^(GRS-TMR)	85.7 ± 1.53 ^a	-3.84 ± 1.00 ^a	7.96 ± 1.72 ^a	3.21 ^(GRS-TMR)

GRS – Cows fed perennial ryegrass only.

CLV – Cows fed perennial ryegrass / 20 % white clover sward.

TMR – Cows fed indoor total mixed ration *ad-libitum*.

Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

ΔE denotes total colour difference between samples which can be visibly determined. Values < 1 are imperceptible. Values between 1 – 3 are visibly perceptible. Values > 3 are visually distinct.

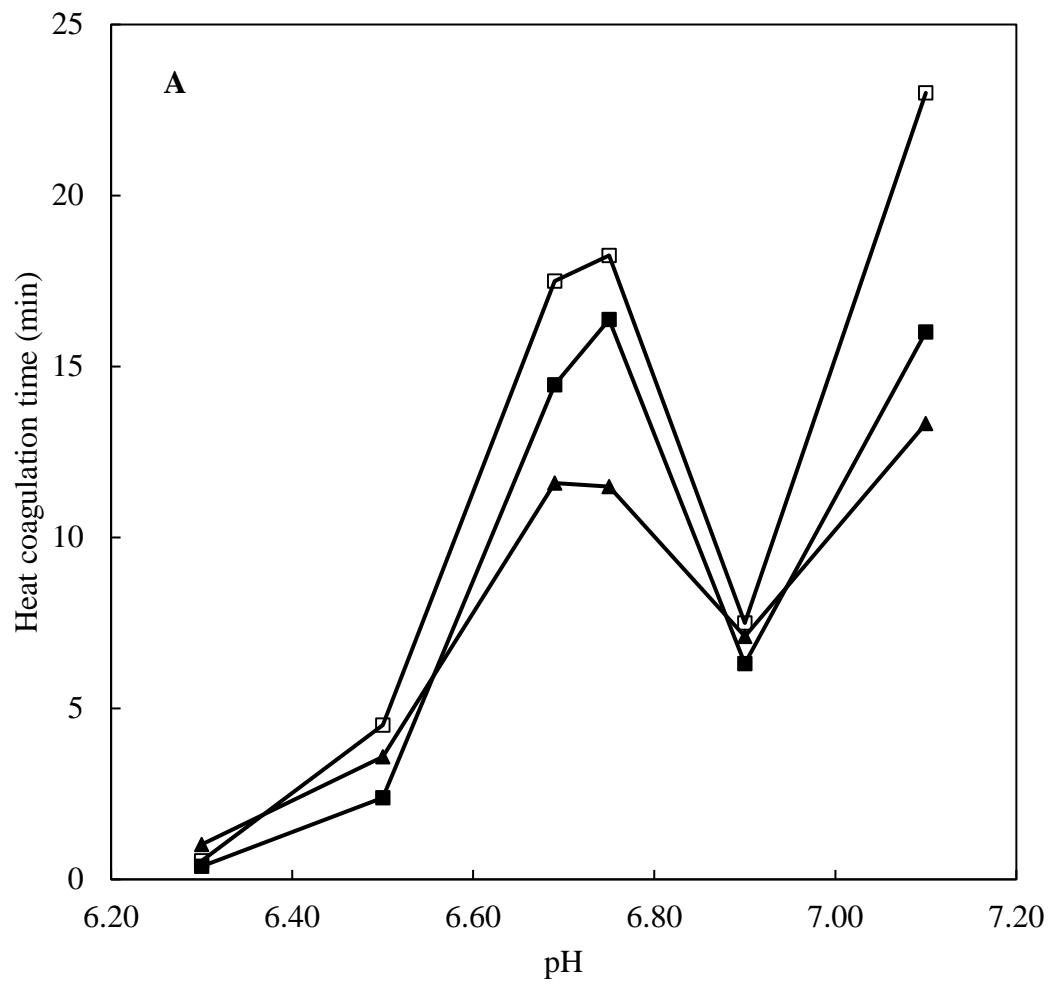
Differences in green/red (a^*) and lightness (L^*) values were not significant between milk powders from each of the diets, though significant differences ($P < 0.05$) were observed in yellow/blue (b^*) values. These differences were apparent between both the GRS and CLV samples and the TMR sample. The powder samples exhibited higher overall colour intensity and L^* values than the rehydrated powder samples. Maximum powder L^* values were observed in the TMR sample, followed by CLV and GRS. The highest L^* value of the rehydrated WMP samples was observed in the CLV sample, followed by TMR and GRS. The highest a^* values in milk powder and rehydrated WMP samples were observed in the GRS sample, followed by CLV and TMR, respectively. However, there were no significant differences in L^* or a^* values between the milk powders or in the rehydrated samples. GRS powder b^* values were significantly higher ($P < 0.05$) than those of the TMR sample. CLV b^* values were also higher than TMR b^* values, though not significantly ($P > 0.05$). Table 5.3 includes ΔE values for each sample, denoting the difference between each sample which can be visibly determined. A difference of 3.21 in ΔE value between the TMR and GRS samples indicates that these samples were visually distinct. Differences between GRS and CLV were visually perceptible, whereas differences between the TMR and CLV samples were imperceptible.

Similar differences in the colour of milk and milk products derived from TMR or pasture-based diets have been previously identified (Hurtaud *et al.*, 2002). The slightly lower L^* and more negative a^* values exhibited by the GRS sample, along with the more positive b^* values exhibited by the GRS and CLV samples, may be attributed to increased β -carotene content in both pasture-based samples (Nozière *et al.*, 2006; O'Callaghan *et al.*, 2016a). Present in high concentrations in grass, β -carotene functions as red/orange pigmentation and as a precursor compound to retinol (vitamin A) synthesis in the liver (Darwish *et al.*, 2016). As vitamin A is fat-soluble, the quantity of β -carotene in various dietary fat sources has been widely investigated. Studies which investigated the effect of pasture and concentrate-based

feeding systems on the composition of beef muscle (Duckett *et al.*, 2009), butter (O'Callaghan *et al.*, 2016a) and cheddar cheese (O'Callaghan *et al.*, 2017) have identified significantly higher concentrations of β -carotene in pasture-derived samples than in concentrate-derived samples.

5.4.3. Heat Coagulation Time of Reconstituted Whole Milk Powders

All raw whole milk samples exhibited typical “type A” HCT-pH profiles, characterised by clear HCT maxima and minima and a sharp decline in HCT to a local minimum at pH 6.9 (O'Sullivan *et al.*, 2001). Figure 5.1A shows typical HCT-pH profiles for raw milk samples from each feeding system. The trend of HCT increasing as a function of pH to a local maximum at pH 6.7 and then decreasing before further increasing is typically attributed to heat-induced dissociation of κ -casein from the casein micelle and subsequent complex formation with β -lactoglobulin at pH > 6.6 (Anema, 2008; Singh and Fox, 1985). The κ -casein-depleted casein micelle is then susceptible to calcium-induced aggregation, leading to protein precipitation (McSweeney *et al.*, 2004). This is followed by increased HCT above pH 6.9, as the charge of the casein micelle increases due to the loss of κ -casein (Panda, 2011).



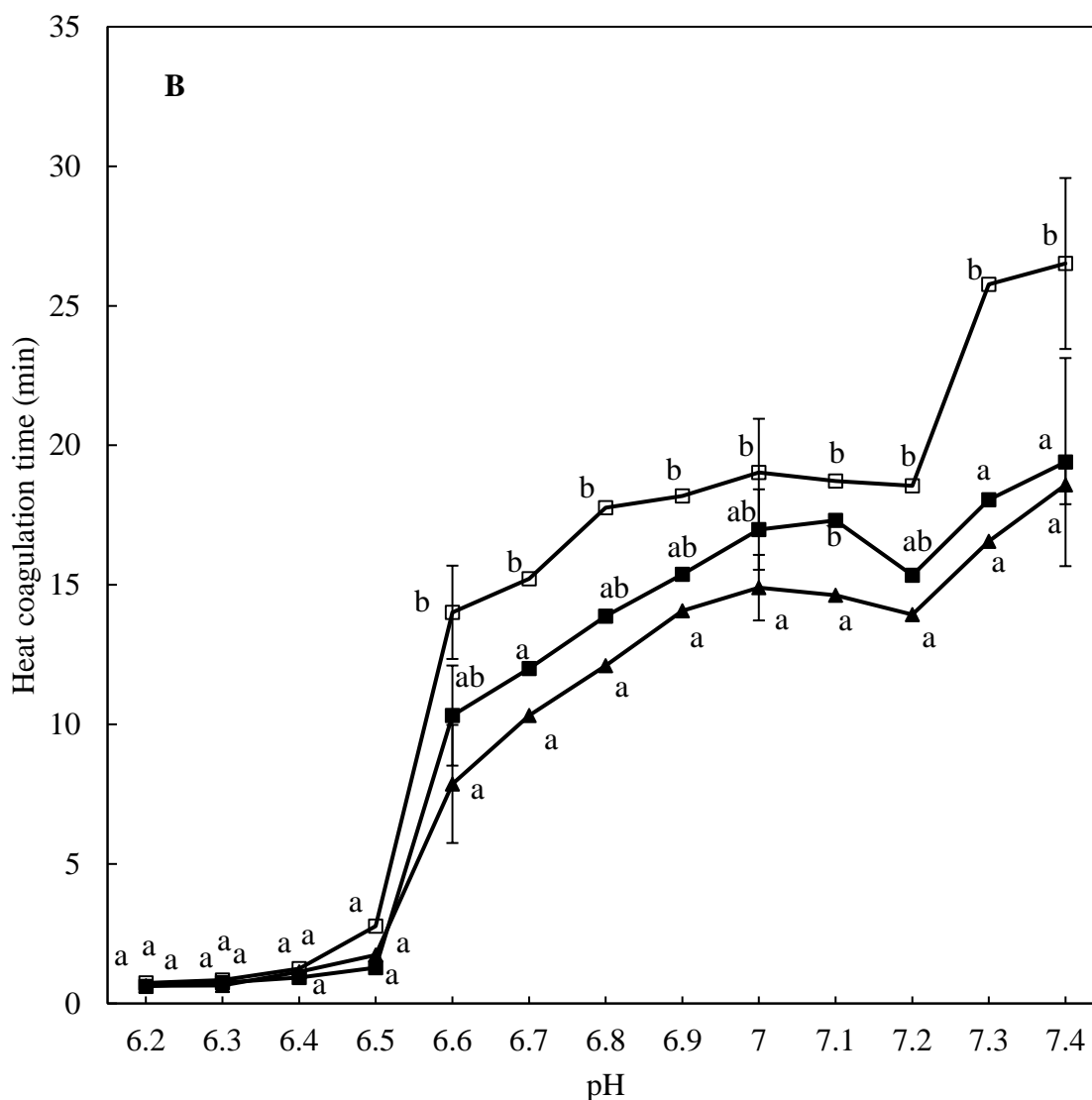


Fig. 5.1. Heat coagulation time of (A) raw whole milk and (B) whole milk powder dispersions (1.5%, w/w, protein) heated at 140°C at 8 oscillations min⁻¹ obtained from cows fed grass (■), grass/clover (□) or total mixed ration (▲). Values in Fig. 5.1A represent a single measurement. Values in Fig. 5.1B are the means of data from triplicate trials and duplicate analysis. Error bars represent standard deviation. Superscript letters at each pH point not sharing a common superscript differed significantly ($P < 0.05$).

In contrast, all WMP samples exhibited unusual HCT-pH profiles, whereby HCT increased as a function of pH (type B profile); however, a slight decrease in HCT was observed at pH 7.1 for both the TMR and CLV samples and 7.2 for the GRS sample, followed by HCT maxima at pH 7.4 in all samples (Fig. 5.1B). The highest overall HCT was observed in the CLV sample, followed by GRS and TMR samples, respectively. Visible coagulation occurred after approximately 26, 19 and 18 minutes at pH 7.4 and 19, 15 and 14 minutes at pH 7.2 for the CLV, GRS and TMR samples, respectively. There were significant differences ($P < 0.05$) between the CLV and TMR samples at each pH increment between pH 6.6 and 7.4. The HCT of the GRS sample was significantly higher than the TMR sample at pH 7.1 ($P < 0.05$), whereas the HCT of the CLV sample was significantly higher than the GRS sample at pH 6.7, 7.3 and 7.4 ($P < 0.05$). These samples, however, did not exhibit a decrease in HCT between pH 6.7 and pH 6.9.

Similar type-B HCT-pH profiles have previously been observed in low-heat ($72^{\circ}\text{C} \times 15 \text{ s}$) skim milk concentrates heated at 120°C (Lin *et al.*, 2018). Skim milk powder samples reconstituted at low concentration (9.4 % TS) showed trends of steadily increasing HCT with increasing pH, followed by a slight decline at \sim pH 7.0, although HCT decreased significantly at higher pH values in samples reconstituted at higher total solids concentrations (Lin *et al.*, 2018). In the present study, standardisation of reconstituted WMP samples at 1.5% total protein content resulted in a total solids content of approximately 5%. This reduced concentration may contribute to the type-B HCT-pH profile and lack of decline in HCT at pH 6.9 observed in all three samples. The significant differences in HCT between feeding systems is notable, as a previous study on mid-lactation skim milk produced from these same feeding systems did not observe a similar trend of significant variation of HCT (Gulati *et al.*, 2018b).

Results for calcium ion activity (Ca^{2+}) showed a higher average concentration of ionic calcium in TMR-based WMP (2.26 mM), compared to the GRS (2.21 mM) and CLV (2.12

mM) samples, though these quantities did not differ significantly ($P > 0.05$). While Ca^{2+} level decreases with increasing pH (Tsioulpas *et al.*, 2007), milk HCT decreases with increasing Ca^{2+} (Sievanen *et al.*, 2008). The significant differences in HCT observed between the CLV and TMR samples may be attributable to increased non-protein-nitrogen content in the CLV sample, arising from increased urea levels (Huppertz, 2016) due to the white clover content of the diet (Harris *et al.*, 1998). The addition of high concentrations of urea to unconcentrated milk has been shown to increase milk HCT (Muir and Sweetsur, 1976). Heat-induced decomposition of urea to ammonia reduces the susceptibility of milk to heat-induced acidification, leading to increased heat-induced dissociation of κ -casein and a decrease in Ca^{2+} level (Huppertz, 2016). As previously discussed, significantly higher concentrations of urea have been observed in milk derived from the CLV feeding system compared to that derived from the TMR system (O'Callaghan *et al.*, 2018).

The lower HCT values in the TMR sample may also be due to reduced fat droplet size. D_{90} values for TMR WMP dispersions were significantly lower than those for both GRS and CLV dispersions. Decreases in HCT due to homogenisation and consequently reduced fat globule size have been previously described (McCrae, 1999).

5.4.4. Rheological Properties of Yoghurt

Rheological values showing the onset of gelation of yoghurts produced from each WMP sample are shown in Table 5.4 and Figure 5.2 and 5.3. There were no significant differences in the time and pH values at which the elastic modulus (G') values of each sample exceeded 1 Pa or the time at which G' values exceeded viscous modulus (G'') values. At pH 4.6, the TMR sample yielded an average maximum G' of 51.5 Pa after 514 min. In contrast, the GRS and CLV samples yielded average G' maxima of 92.6 Pa and 94.3 Pa after 525 and 541 min, respectively. G' and G'' values were significantly ($P < 0.05$) higher in the CLV and

GRS samples, relative to the TMR sample. A previous study investigating characteristics of mid-lactation milk from these feeding systems reported significantly higher G' values and gel-firming rates in rennet gels from GRS-derived milk than those from TMR-derived milk, though no significant differences were found between CLV and TMR rennet gels (Gulati *et al.*, 2018b). No significant differences were found between the other gelation properties in the present study (Table 5.4).

Table 5.4. Gelation properties of yoghurt gels produced from whole milk powder dispersions (15%, w/w, total solids) inoculated with 0.2 g mesophilic starter culture

Sample	Time at which $G' =$ 1 Pa	pH at which $G' =$ 1 Pa	Time at which $G' >$ G''	Time at which pH = 4.6	Final G' value at pH 4.6
	min		min	min	Pa
GRS	469 ± 10 ^a	4.77 ± 0.09 ^a	462 ± 10 ^a	525 ± 13 ^a	92.6 ± 10 ^b
CLV	473 ± 20 ^a	4.76 ± 0.04 ^a	471 ± 20 ^a	541 ± 23 ^a	94.3 ± 5 ^b
TMR	467 ± 31 ^a	4.77 ± 0.02 ^a	463 ± 27 ^a	514 ± 29 ^a	51.5 ± 12 ^a

GRS – Cows fed perennial ryegrass only.

CLV – Cows fed perennial ryegrass / 20 % white clover sward.

TMR – Cows fed indoor total mixed ration *ad-libitum*.

Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

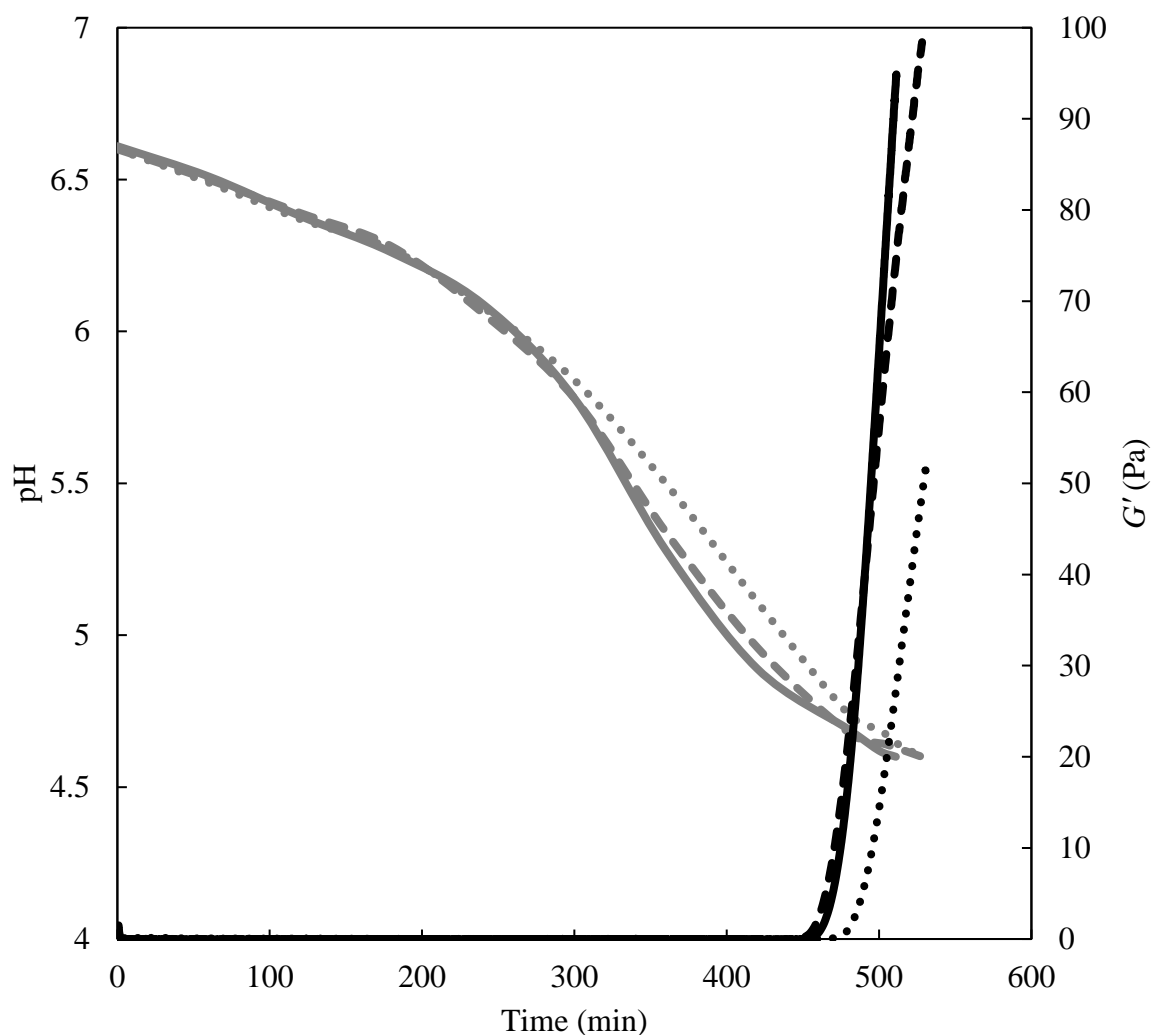


Fig. 5.2. Typical elastic moduli, G' (black series; Pa), and pH profiles (grey series) of yoghurt gels produced from whole milk powder dispersions (15% w/w) for Grass (—), Grass/Clover (- -) and TMR (●●●), as a function of incubation time at 30°C. Measurements were ceased at pH 4.6.

Logarithmic frequency sweeps recorded typical viscoelastic behaviour in each set yoghurt sample up to a frequency 63.1 Hz (Fig. 5.3). At this frequency, the highest average G' value was observed in the GRS sample (236 Pa), followed by CLV (152 Pa) and TMR (105 Pa). The highest average G' values for the CLV (165 Pa) and TMR (109 Pa) samples were observed at 25.1 Hz and 39.8 Hz, respectively. All samples exhibited overall thixotropic (time-dependent shear-thinning) behaviour, characterised by a substantial decrease in complex viscosity (η^*) as a function of increasing frequency, along with a decrease in G' and

concomitant increase in G'' at 63.1 Hz (Fig. 5.3). The GRS sample, however, did not exhibit a decrease in G' at this frequency, despite an increase in G'' . The highest average η^* value at 1 Hz was observed in the GRS sample (18.2 Pas^{-1}), followed by CLV (16.0 Pas^{-1}) and TMR (9.37 Pas^{-1}). The η^* value of the GRS sample was significantly higher ($P < 0.05$) than that of the TMR sample. The higher G' , G'' and η^* values observed in the GRS and CLV samples indicate the formation of stronger, more cohesive gel matrices, which are more resistant to deformation, than those from the TMR sample.

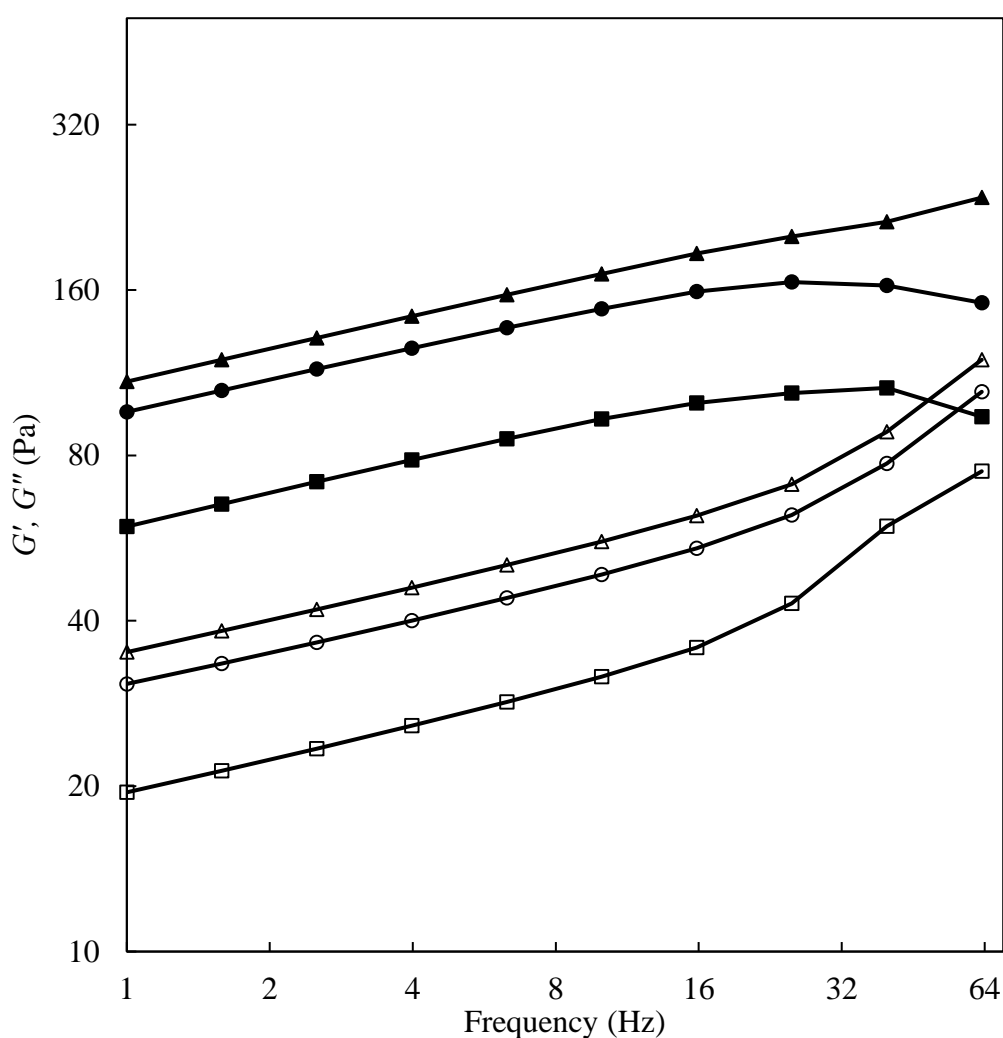


Fig. 5.3. Average elastic, G' (filled symbols; Pa) and viscous G'' (open symbols; Pa) moduli of yoghurt gels produced from whole milk powder dispersions (15% w/w) for Grass (\blacktriangle , \triangle), Grass/Clover (\bullet , \circ) and TMR (\blacksquare , \square), as a function of oscillation frequency (1 - 63.1 Hz).

Standardisation of yoghurt samples at 4% true protein content did not result in significant differences in gel strength to yoghurt samples from the same dietary treatments standardised at 15% TS. This implies that variations in the gel strength of yoghurts between dietary treatments were not attributable to variations in their true protein content, although the potential influence of varying casein:whey ratios and β -casein concentrations between feeding systems may have been slightly more pronounced at a standardised true protein content when compared to the concentrations shown in Table 5.2., which may have contributed to the observed differences in gel strength. The significantly higher G' values observed in both pasture-based samples relative to the TMR sample may therefore be attributable to variations in the composition and structure of their fat components.

The fat globule size distribution indicates the relative abundance of fat droplets of varying sizes in WMP dispersions. No significant difference in volume mean diameter ($D_{[4,3]}$) was observed between the samples (Fig. 5.4); however, the TMR sample had a significantly lower ($P < 0.05$) D_{90} value compared to both the GRS and CLV samples. This indicates a lower abundance of large fat globules in the TMR sample, which may be caused by increased fat globule flocculation/coalescence in the GRS and CLV samples, due to the presence of lower melting point fatty acids (O'Callaghan *et al.*, 2016a). The degree of electrostatic repulsion between protein-adsorbed fat globules in milk at the onset of gelation may be an influencing factor in the gel strength of the final gel matrix.

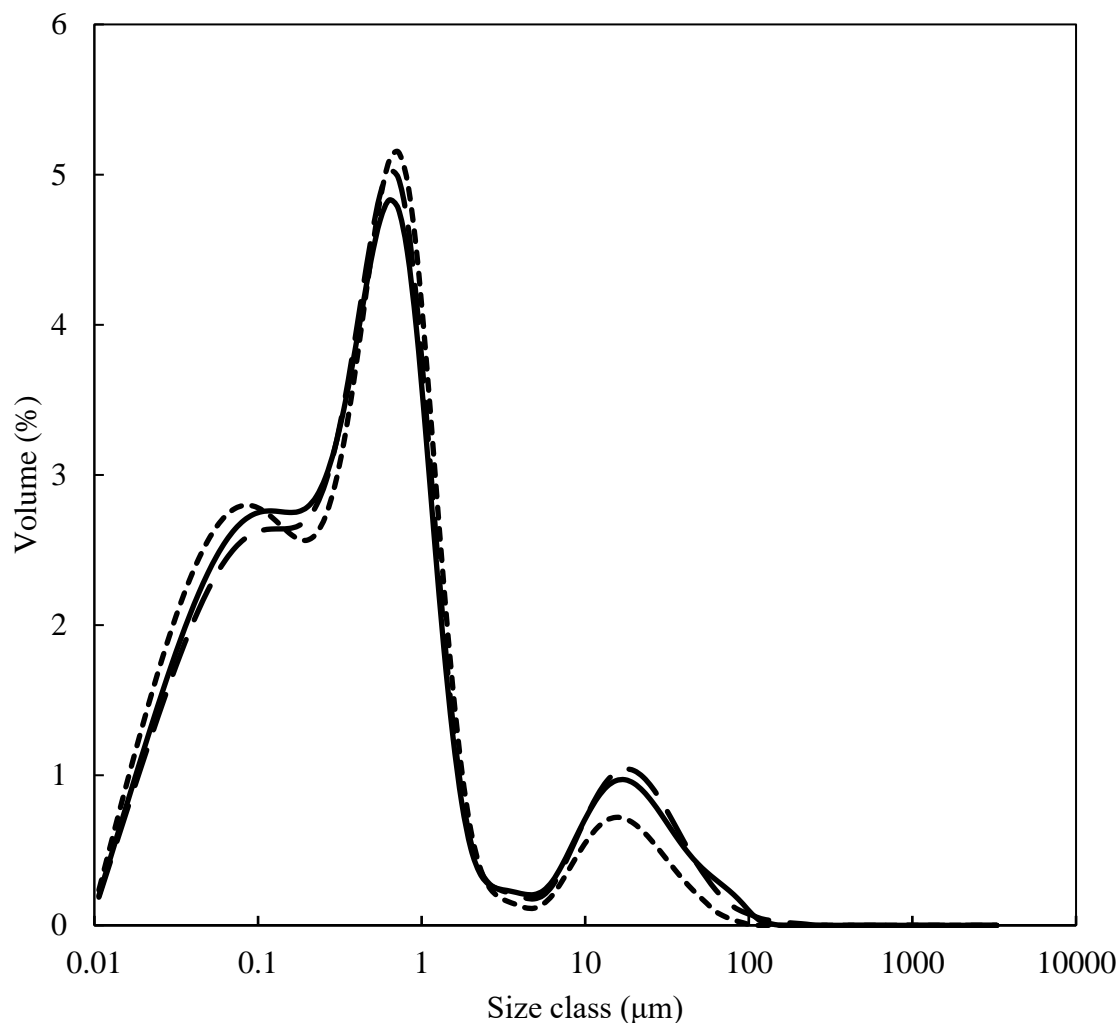


Fig. 5.4. Milk fat globule size distribution of whole milk powder dispersions (15%, w/w, total solids), for Grass (—), Grass/Clover (- - -) and TMR (●●●), represented as a function of triplicate trials.

5.4.5. Texture Profile Analysis

Average texture profile data for each yoghurt gel is shown in Table 5.5 and Figure 5.5. Firmness, cohesiveness and consistency are defined as the force required to penetrate the gel structure, the degree of deformation the gel matrix can withstand before it ruptures (Rawson and Marshall, 1997) and the resistance of the gel matrix to deformation (do Espírito Santo *et al.*, 2012), respectively. The highest gel firmness (192.8 g) and consistency values (4218 g s⁻¹) were exhibited by the CLV yoghurt sample. The CLV-derived yoghurt gel yielded significantly

higher ($P < 0.05$) firmness values than those of the TMR sample. Maximum gel cohesiveness (-60.9 g) and index of viscosity (-940 g s^{-1}) were exhibited by the GRS sample. Lower values were observed in the TMR sample for all texture profile components. The WMP from the CLV feeding system produced the thickest and firmest yoghurt samples overall, whereas WMP from the GRS system produced a more cohesive, elastic gel.

Table 5.5. Textural properties for yoghurts produced from whole milk powder dispersions (15%, w/w, total solids) inoculated with 0.2 g mesophilic starter culture

Sample	Firmness	Consistency	Cohesiveness	Index of viscosity
	g	g s^{-1}	g	g s^{-1}
GRS	175 ± 36.1 ^{ab}	4089 ± 805 ^a	-60.9 ± 12.3 ^a	-940 ± 172 ^a
CLV	192 ± 20.9 ^b	4218 ± 477 ^a	-57.3 ± 8.87 ^a	-878 ± 123 ^a
TMR	130 ± 6.96 ^a	3095 ± 194 ^a	-40.4 ± 2.95 ^a	-644 ± 32.7 ^a

GRS – Cows fed perennial ryegrass only.

CLV – Cows fed perennial ryegrass / 20 % white clover sward.

TMR – Cows fed indoor total mixed ration *ad-libitum*.

Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

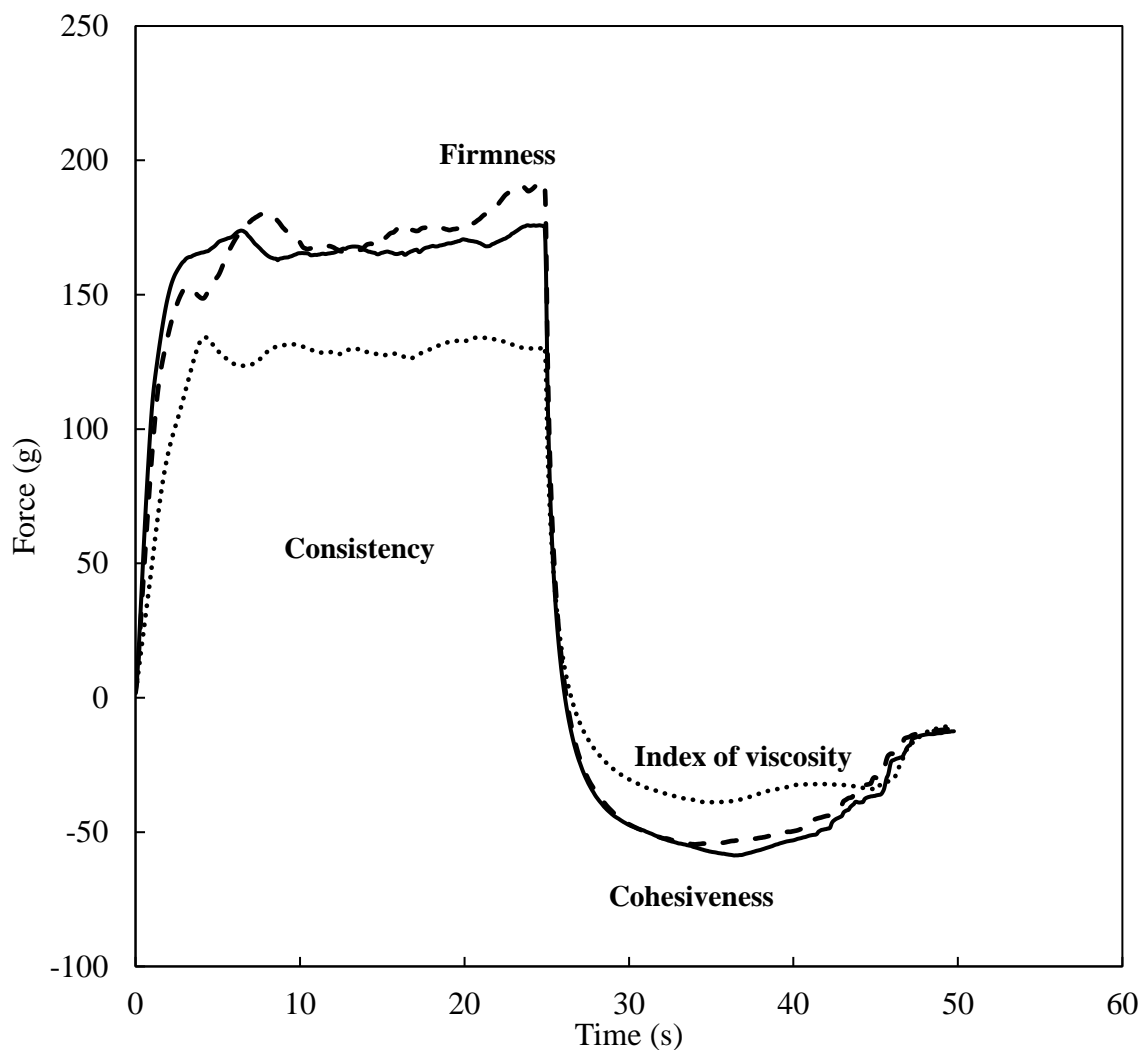


Fig. 5.5. Average texture profile of yoghurt gels produced from whole milk powder dispersions (15% w/w) Grass (—), Grass/Clover (- - -) and TMR (●●●). Data labels: Firmness indicates maximum force (g) recorded on initial probe stroke; Consistency (area) indicates the force per second (gs^{-1}) on initial probe stroke; Cohesiveness indicates maximum negative force (g) recorded on probe return stroke; Index of viscosity (area) indicates the force per second (gs^{-1}) on probe return stroke.

Warming yoghurt samples to 30°C prior to texture profile analysis resulted in an approximately 30% decrease in firmness and consistency and approximately 50% decrease in cohesiveness and index of viscosity across all samples. This implies that the textural variations identified between yoghurts produced from WMP from each diet were not attributable to variation in their fatty acid melting points. The effect of inter-seasonal variation in milk composition must also be noted as a potential source of variation in WMP composition and

functionality. The characteristic behaviour of the late-lactation milk processed and analysed in this study may differ substantially from that of milk produced at a different stage of lactation or in a separate lactation cycle. Indeed, previous studies carried out by O'Callaghan *et al.* (2016b) and Gulati *et al.* (2018a) on milks from the feeding systems analysed in the present study recorded significant compositional variation between early-, mid- and late-lactation, suggesting that the functional properties of yoghurts produced from milk from these feeding systems may, in practice, be variable depending on the time of year at which they are produced.

5.5. Conclusion

Pasture-based feeding of cows resulted in some significant differences in WMP functionality compared to a TMR diet, characterised by increased thermal stability of WMP (particularly CLV), in addition to significantly higher yoghurt gel strength and firmness. However, the WMP samples were not found to differ significantly over a range of characteristics, including WPNI and gross composition (it is noted that powders were standardised for fat content), although NPN values did differ between samples. The differences in minor compositional constituents and behaviour of the WMP samples used in this study may be attributed to the feeding system employed. The significantly higher b^* values observed in both WMP and reconstituted WMP produced from the GRS feeding system than both the CLV and TMR systems supports previous studies on the colour profile of pasture-derived milk products. Overall, this study suggests that the application of pasture-based dietary treatments confers increased thermal stability in WMP and increased gel strength in yoghurt derived from these systems, suggesting increased functionality when used as a base material for set-style yoghurt manufacture.

5.6. References

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Chapter 6

Effect of pasture and total mixed ration-based diets on the composition and digestion of whole milk powder

6.1 Abstract

The composition of whole milk powders (WMP) produced from the milk of three groups of cows fed diets consisting of perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) or indoor total mixed ration (TMR) was determined. Raw milk from each group was heat treated (90°C x 30 s), homogenised, evaporated and spray-dried to yield WMP. Reconstituted WMPs were also enzymatically digested using the INFOGEST 2.0 method to produce corresponding digestate samples. No significant differences in gross composition were observed between the WMP samples. Significantly higher ($p < 0.05$) concentrations of selenium and iodine were present in TMR-derived WMP than those derived from both GRS and CLV. Total fatty acid profiles of milk from each diet were similar to those typically reported for pasture or concentrate-based diets, i.e., higher palmitic acid content in the TMR samples and higher conjugated linoleic acid and alpha-linolenic acid in both the GRS and CLV samples. Minor differences in total amino acid, free amino acid and free fatty acid composition between the diets were also recorded, although the overall effect of digestion was similar between the feeding systems. Raman spectroscopy displayed distinct spectra for each diet based on levels of carotenoids and fatty acid unsaturation. Further significant differences in proteomic profiles were observed between WMP samples from each diet, although differences in the peptidomic profiles of the digested samples were less distinct. Bioactivities associated with the peptides identified within the digested samples include: angiotensin-converting enzyme (ACE)-inhibitory, dipeptidyl-peptidase 4 (DPP-IV)-inhibitory, immunomodulatory, anti-inflammatory, antioxidant and antimicrobial effects and stimulation of trabecular bone growth. This study determined a significant effect of feeding system on the composition of WMP, but significant variation in the composition of digestate between feeding systems could not be conclusively identified by static *in-vitro* digestion, which may receive further insights using dynamic methods in future work.

6.2. Introduction

Milk occupies a significant position in human nutrition, as it functions as a complete food for mammalian neonates, while also offering a comparatively high density of bioavailable macro and micronutrients throughout maturation and into adulthood relative to most other food types (Drewnowski, 2017). This nutritional significance, coupled with their long-established consumption in most Western cultures, make milk and dairy products highly important both as food sources and economic commodities. Whole milk powder (WMP) is one such premium commodity, its high value owing to the presence of milk fat, which is often removed from whole milk for production of added-value fat-based products such as butter and cream (Kaylegian & Lindsay, 1995), with the resulting skim milk being combined with cheaper vegetable oils for use in other emulsion-based formulations (Schmidmeier *et al.*, 2019). Whole milk powder is frequently used as the basis of full-fat yoghurt manufacture, both at industrial and cottage-scale (Tamime & Robertson, 1999), though another major application of WMP is its use as a comprehensive source of nutrition in food aid programmes (Steinfeld *et al.*, 2013), where it can be provided in large quantities as powder and later reconstituted.

Worldwide milk production is conventionally established on the use of indoor total mixed ration-based feeding systems, which allow greater control over feeding and climatic exposure (Legrand *et al.*, 2009). These systems have increased in popularity with the increasing intensification of the dairy industry over the past 50 years, particularly on farms with large herd sizes (Schingoethe, 2017). Pasture-based milk production systems are more prevalent in countries with temperate grassland climates, such as Ireland and New Zealand, where they are practiced on the majority of farms, whereas smaller herds and less intensive farms in France and The Netherlands still maintain a regular, albeit declining, annual grazing period (van den Pol-van Dasselaar *et al.*, 2015). Pasture-based milk production accounts for approximately 10 % of global milk supply (Coleman *et al.*, 2009). The use of TMR-based systems has been

shown to result in increased milk yield (O'Neill *et al.*, 2011); however, the potential beneficial effect of pasture-feeding on milk quality has received increased attention in recent years.

Variations in bovine feeding regimes have been demonstrated to significantly affect milk and dairy product composition (Panthi *et al.*, 2019; Gulati *et al.*, 2018; O'Callaghan *et al.*, 2017, 2016a, 2016b; Couvreur *et al.*, 2006) particularly on milk fatty acid profile. Previous studies by O'Callaghan *et al.* (2016a, 2017), Kelly *et al.* (1998) and White *et al.* (2001) have demonstrated a favourable effect of pasture feeding on milk fatty acid (FA) composition, particularly with respect to FA, which have received attention for their potential nutritional benefits. Pasture-derived milk and dairy products were shown to comprise higher proportions of polyunsaturated FA, Omega-3 FA, vaccenic (C18:1 *trans*-11) acid (O'Callaghan *et al.*, 2017; Kelly *et al.*, 1998) and *cis*-9,*trans*-11 conjugated linoleic acid (O'Callaghan *et al.*, 2016; Kelly *et al.*, 1998; White *et al.*, 2001), whereas significantly higher concentrations of unfavourable compounds such as Omega-6 FA and palmitic (C16:0) acid have been observed in TMR-derived products (O'Callaghan *et al.*, 2016; Kelly *et al.*, 1998). Minor differences in the amino acid profile of pasture and TMR-derived milk products have been previously reported (Magan *et al.*, 2019a); however, it is generally accepted that the protein profile and amino acid composition of milk is primarily influenced by genetic, rather than dietary, factors (Schopen *et al.*, 2009).

Moreover, the potential for unique behaviours or variation in the release of free amino acids, free fatty acids and bioactive peptides during digestion of dairy products derived from pasture or TMR-based feeding systems has not yet been examined, either *in-vivo* or using a simulated *in-vitro* digestion process, such as the INFOGEST method established by Brodkorb *et al.* (2019), which may provide further insights into the potential nutritional benefits arising from the unique compositional factors associated with particular feeding systems.

The proteomic analysis field combines genomic analysis with comprehensive protein profiling and identification of the physiological functions attributable to particular proteins or peptides. The milk proteome is highly complex, as substantial variation exists between bovine milk protein gene products due to levels of post-translational glycosylation, phosphorylation and inter-protein associations (Chevalier, 2011). The potential for variation in the proteome of milk derived from pasture or concentrate-based feeding systems has yet to be comprehensively investigated. Therefore, the primary objective of this study was to determine the effect of perennial ryegrass (*Lolium perenne* L.), perennial ryegrass/white clover (*Trifolium repens* L.) and indoor total-mixed-ration-based feeding systems on the mineral, fatty acid, amino acid, proteomic and peptidomic profiles of whole milk powders. An additional aim of this study was to compare the composition of digestate samples generated from static *in-vitro* digestion of each reconstituted WMP, using the INFOGEST 2.0 protocol (Brodkorb *et al.*, 2019).

6.3. Materials and methods

6.3.1. Materials

Raw milk was collected from the Teagasc Animal and Grassland Research and Innovation Centre dairy unit (Moorepark, Fermoy, Co. Cork, Ireland). All digestive enzymes and bile used for *in-vitro* digestion of whole milk samples were sourced from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

6.3.2. Experimental Design

The experimental design for this study was similar to that described in Chapters 2 and 5. In brief, fifty-four Spring calving Holstein/Friesian cows were assigned 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 parity (2.22), calving date (mean

calving date 07th February, 2019 \pm 14 days), milk yield and milk solids yield for the first two weeks *post-partum*. The GRS, CLV and TMR groups were maintained as described in Chapter 2. The average annual white clover content of the sward was 24%. Raw milk was collected from each group for WMP manufacture on three separate occasions over a three-week period in May 2019 (mean days in milk of 110).

6.3.3. *Whole Milk Powder Production*

Whole milk powder production was carried out at Moorepark Technology Limited (MTL) Biofunctional Food Engineering pilot plant facility (Fermoy, Co. Cork, Ireland). Raw whole milk (~1000 kg) was pre-heated to 50°C and pasteurised at 90°C for 30 s in an APV plate heat-exchanger (SPX Flow Technology, Crawley, West Sussex, UK), before homogenisation using an APV Gaulin two-stage homogeniser (Lake Mills, Wisconsin, USA) at first and second-stage pressures of 150 and 50 bar, respectively. The homogenised milk was then evaporated to ~40% total solids (TS) in a Scheffers single-effect recirculating evaporator (Scheffers, Schiedam, The Netherlands). The concentrate was pre-heated to 65°C in a plate heat-exchanger and transferred to an Anhydro three-stage spray dryer (SPX Flow Technology Denmark A/S, Søborg, Denmark) (air inlet and outlet temperatures were set at 170 and 65°C, respectively). First- and second-fluid bed temperatures were set at 65 and 25°C, respectively. Fines were returned to the top of the spray-dryer from the second fluid bed and the cyclone, yielding a final, agglomerated WMP. Production of WMP from each feeding system was carried out in triplicate from three independent raw milk collections.

6.3.4. *In-vitro digestion*

Static *in-vitro* digestion was carried out using the INFOGEST 2.0 method as described by Brodkorb *et al.* (2019). Briefly, simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were produced using the following reagents: KCl, KH₂PO₄, NaHCO₃, NaCl, MgCl₂(H₂O)₆, and (NH₄)₂CO₃. Porcine pancreatin was dissolved in SIF, porcine pepsin and rabbit gastric lipase were dissolved in SGF and bovine bile was dissolved in H₂O. Aliquots (4 mL) of SGF were added to 5 mL samples of reconstituted whole milk powder (14% w/v). To these mixtures, 2.5 µL of 0.3 M CaCl₂, 0.25 mL of rabbit gastric lipase and 0.25 mL of pepsin were added. The pH of the mixture was adjusted to pH 3.0 using 0.5 M HCl and incubated in a rotating incubator at 37°C for 1 hour. After this period, 4.25 mL of SIF was added to the mixture, along with 20 µL of 0.3 M CaCl₂, 2.5 mL of pancreatin and 1.25 mL of bile. The pH was then adjusted to pH 7.0 using 0.5 M NaOH and the complete mixture was incubated for a further hour at 37°C in a rotating incubator. Following incubation, 1 mL of aqueous protein inhibitor (Pefabloc SC Plus, Merck KGaA, Darmstadt, Germany) was added to stop enzyme activity and the samples were stored at -20°C.

6.3.5. *Whole milk powder and digestate analysis*

6.3.5.1. *Determination of nitrogen and fat content*

The total nitrogen and non-protein nitrogen contents of each WMP were measured as described in Chapter 2. The fat content of each WMP was measured as described in Chapter 5.

6.3.5.2. *Mineral composition*

Mineral analysis was carried out by an external laboratory (FBA Laboratories, Cappoquin, Co. Waterford, Ireland), according to the methodology for inductively coupled plasma-mass spectrometry described in brief in Chapter 4.

6.3.5.3. Total amino acid composition

The total amino acid profile of each WMP was measured as described in Chapter 2.

6.3.5.4. Free amino acid composition

Protein precipitation was carried out by mixing each sample with an equal volume of 24% (w/v) tri-chloroacetic acid (TCA). Samples were allowed stand for 10 min before centrifugation at 14400 x g (Sigma Laborzentrifugen GmbH, Germany) for 10 min. The supernatant from each sample was removed and diluted with 0.2 M sodium citrate buffer (pH 2.2), resulting in approximately 250 nmol of each amino acid residue. Samples were diluted 1 in 2 with the internal standard, norleucine, to a final concentration of 125 nm mL⁻¹. Quantifications of AAs was carried out using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na⁺ high performance cation-exchange column, with post-column on-line reactor derivatization with ninhydrin.

6.3.5.5. Fatty acid methyl ester composition

Lipid extraction was performed as described by O'Callaghan et al. (2016, 2019), using the method outlined by De Jong and Badings (1990). Briefly, 10 mL of each sample was mixed with 10 mL of ethanol (98% purity) and 1 mL of 2.5 M H₂SO₄. Each mixture was thrice extracted with 15 mL diethyl ether/heptane (1:1) and centrifuged at 1500 × g for 5 min. Extracts were pooled and dried at 55°C under N₂ gas, before 4.8 mL of C19:0 triacylglyceride (200 ppm) in heptane was added to 60 mg of each extracted lipid sample. To this mixture, 200 µL of 2 M sodium methoxide solution was added and mixed vigorously for 30 s, followed by 1 g of sodium hydrogen sulphate monohydrate (Sigma Aldrich) which was again mixed vigorously. After settling, the upper layer containing methyl esters was decanted into a clean

test tube and diluted with 8 mL of heptane. Fatty acid methyl esters were then stored at 20°C, before being transferred to 2 mL amber vials (Part no: 5182-0716, Agilent Technologies, Little Island, Cork) capped with PTFE/white silicone septa (part no: 5185-5864, Agilent Technologies, Little Island, Cork).

Fatty acid methyl esters were analysed using an Agilent 7890A gas chromatograph system, equipped with an Agilent 7693 autosampler (Agilent Technologies, Cork, Ireland) and flame ionisation detector (FID). A Select FAME capillary column (100 m \times 250 μ m I.D., 0.25 μ m phase thickness, part number: CP7420) (Agilent Technologies, Little Island, Cork, Ireland) was used. The injector was maintained at 250°C throughout the run, while the column oven was held at 80°C for 8 min, raised to 200°C at 8.5°C min⁻¹ and held for 55 min. Total run time was 77.1 min. The FID was operated at 300°C. Hydrogen carrier gas was held at a constant flow of 1.0 mL min⁻¹. Results were processed using OpenLab CDS Chemstation edition software version Rev.C.01.04 (35) (Agilent Technologies). All standard mixtures were prepared in heptane and stored at 18°C. During dilution prior to GC-FID analysis, C19 FAME was added as an internal standard (ISTD), to give a final concentration of 200 ppm. Quantitation of individual FAMES was based on correction factors relative to the ISTD. The FAME reference mix was also used as an in-run quality control sample and analysed once for every 10 samples.

6.3.5.6. Free fatty acid composition

The method undertaken was a modified version of that used by De Jong & Badings (1990), where fat is extracted in solvent and the free fatty acids separated by amino-propyl solid phase extraction. Identification and quantification were carried out using a gas chromatography flame ionization detector with on-column injection equipped with a CP FFAP CB column (25m \times 0.32mm \times 0.3 μ m).

6.3.5.6.1. Lipid Extraction

Lipid extraction was carried out as described by De Jong and Badings (1990). Sample volumes of 10 mL and 5 mL were used for extraction for whole milk and digestate samples, respectively. Aliquots (0.3 mL) of 2.5 M H₂SO₄ and 1 mL of internal standard (ISTD) (C_{5:0}, C_{11:0}, C_{17:0} each at 1000 ppm in heptane) were added to each sample. This mixture was extracted with 15 mL of diethyl ether/heptane (1:1) and 10 mL of ethanol. The solution was clarified by centrifugation at 3000 x g for 5 min. Following centrifugation, the top layer of supernatant was transferred to a sterile tube and the bottom layer of supernatant was further extracted with diethyl ether/heptane (1:1) and centrifuged. Extraction with diethyl ether/heptane was carried out 3 times in total, after which the collected extracts were pooled for solid phase extraction.

6.3.5.6.2. Solid Phase Extraction

Pre-conditioning was carried out on 500 mg aminopropyl columns using 10 mL of heptane. The lipid extract was applied to the column and the neutral lipids removed using 10 mL of 20% diethyl ether in hexane. Columns were not allowed to dry. The FFAs were collected using 5 mL of 2% formic acid/diethyl ether (2% FA/DE) in glass test tubes. The entire extract was immediately separated and stored in 2-mL amber vials to prevent ultraviolet light degradation of any PUFAs that may be present in the extract and capped with PTFE/white silicone septa. The FFA extract was derivatised to butyl esters and 1 µL was injected for GC-FID analysis.

6.3.5.7. Raman spectroscopy

Reconstituted whole milk powder samples were transferred to cavity slides (15-18 mm diameter and 0.6 - 0.8 mm depth) and covered with 0.13 mm coverslips to prevent evaporation. Raman spectra were generated following a method adapted from Gómez-Mascaraque *et al.* (2020), using an Alpha300 R confocal Raman microscope (WITec, Ulm, Germany) equipped with a 532 nm laser, a 50× microscope objective (0.55 numerical aperture), and an ultra-fast Raman imaging CCD camera. At least 10 different Raman spectra were collected from each sample at randomly selected positions and averaged. Laser power was set at 60 mW and the integration time was set at 2 s. Raw spectral data was processed using Project Five software v5.0 (WITec, Ulm, Germany). A shape function for background subtraction (shape size 250, noise factor 1) and a cosmic ray removal correction function (filter size 3, dynamic factor 8) were applied to each individual spectrum.

6.3.5.8. Buffering capacity of reconstituted WMP

The buffering capacity of reconstituted whole milk powders was measured according to the method described by Kim *et al.* (2018) using a Titrando 842 Autotitrator with TIAMO v.2.2 software (Metrohm Ireland Ltd., Carlow, Ireland). A three-point calibration of the pH probe was carried out at pH 4.0, 7.0 and 9.0 prior to sample measurements. A two-step titration method was carried out on 50 mL sample aliquots, maintained under constant stirring at 25°C. Samples were first acidified to pH 2.0 by controlled addition of 0.1M HCl in 20 µL increments with 20 s equilibration intervals. Samples were then alkalized to pH 8.0 by addition of 0.1 M NaOH, with the same increment and equilibration maintained. The buffering index (dB/dpH) of each sample was determined using the following equation, as defined by Van Slyke (1922):

$$\frac{dB}{dpH} = \frac{(\text{volume of base or acid added}) \times (\text{normality factor of base or acid})}{(\text{volume of the sample}) \times (\Delta pH)}$$

6.3.5.9. *Gel electrophoresis*

The protein profile of reconstituted whole milk powder (14%, w/v) and digestate samples was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using the method described by Buggy *et al.* (2017). Samples were prepared under reducing and non-reducing conditions on pre-cast 12% Bis-Tris gels (Novex Technologies, ThermoFischer Scientific, Dublin, Ireland) and run at 200 V for 50 min in an Invitrogen mini gel tank connected to an Invitrogen PowerEase 90 W power supply unit (ThermoFischer Scientific). Whole milk samples in buffer were centrifuged at 800 x g for 5 min to remove fat. Following electrophoresis, gels were stained overnight using 0.05% (w/v) R-250 Coomassie brilliant blue in 25% (v/v) isopropanol and 10% (v/v) acetic acid. Following staining, gels were de-stained using 10% (v/v) isopropanol / 10% (v/v) acetic acid until a clear background was achieved.

6.3.5.10. *Confocal microscopy*

Aliquots of 0.1% aqueous Fast green FCF (20 μ L) and 0.02% Nile red in 1, 2-propanediol (20 μ L) (Sigma Aldrich, Wicklow, Ireland) were added to 1.5 mL reconstituted WMP and digestate samples. Aliquots of the mixtures were placed on glass microscopy slides and covered with 0.13 mm coverslips. Stained samples were then observed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) using a 63 \times oil immersion objective (numerical aperture 1.4), maintaining the pinhole diameter at 1 Airy Unit. Fast green FCF was excited at 633 nm using a He/Ne laser, and the corresponding emission filter was set at 660 - 710 nm. Nile red was excited at 561 nm using a diode-pumped solid-state laser, and the corresponding emission filter was set at 571 - 606 nm. Leica LAS AV software (v 2.7.3.9723) was used to acquire digital images of 1024 x

1024 pixels in size. Images were taken at 50, 25 and 10 μm and 3 lines were averaged to reduce noise.

6.3.5.11. Proteomic and peptidomic analysis

Proteomic and peptidomic analysis was carried out externally at the Department of Biology at Maynooth University (Maynooth, Co. Kildare, Ireland), utilising an existing liquid chromatography-mass spectrometry assay intended for “bottom-up” quantification of peptides in bovine milk samples for comparison to the established Milk Bioactive Peptide Database (Nielsen et al., 2017). This method allowed for quantification of the complex range of peptides derived from bovine milk proteins and was more suitable for analysis of the products of non-specific cleavages generated from the INFOGEST method than traditional “top-down” proteomics methods utilising a combination of two-dimensional electrophoresis and mass spectrometry.

6.3.5.11.1. Proteomic analysis of whole milk powders

Whole milk powder samples (1 g) were reconstituted in 10 mL of deionised water and centrifuged at 2200 x g for 15 min at 4 °C to separate the skim milk from the fat and cellular portions. Skim milk was recovered from below the fat layer and protein was recovered by precipitation using TCA/acetone. Samples were added to 15% TCA, incubated on ice for 30 min, centrifuged at 12,000 x g and the resulting pellets were washed twice with ice-cold acetone. Protein pellets were re-suspended in urea buffer (8 M Urea, 0.1 M Tris-HCl pH 8.0) and vortexed. A Bradford assay was performed to determine protein concentration, compared to a standard curve of bovine serum albumin. Proteins (50 $\mu\text{g}/15 \mu\text{L}$) were diluted with 50 mM ammonium bicarbonate (78.5 μL), and reduced with dithiothreitol (1 μL ; 0.5 M DTT) at 56 °C

for 20 min, followed by alkylation with iodoacetamide (IAA; 2.7 μ L; 0.55 M) at room temperature in the dark.

Proteins were then digested using sequencing-grade trypsin and ProteaseMax overnight at 37 °C, and the digestion was stopped by the addition of trifluoroacetic acid (TFA). Millipore C18 Zip-tip clean-up of peptides was performed according to manufacturer's instructions. Peptides (0.75 μ g) were separated on a 2.25 h gradient on a Q-Exactive followed by MaxQuant analysis (v 1.6.17.0) against a Uniprot *Bos taurus* database (Downloaded 31/08/2020). Trypsin was selected as the enzyme for database comparison, allowing for 2 missed cleavages. Proteins were removed if they matched to a reverse or a contaminants database, if they were only identified in a single replicate from only one group, or if they were only identified by a single peptide.

6.3.5.11.2. Peptidomic analysis of Infogest samples

Frozen digestate samples were thawed on ice and centrifuged at 5,000 x g for 15 min at 4 °C to remove insoluble material. Supernatants were further clarified by centrifugation and an aliquot (300 μ L) was processed using Vivacon centrifugal filter units (500 μ L units; 30 kDa cut-off filter). Samples in Vivacon units were centrifuged (10,000 x g for 5 min at 4 °C) and this was repeated as necessary until the total volume was processed. Filtrate (peptides < 30 kDa) was recovered from each sample and processed further for mass spectrometry analysis. An aliquot of the filtrate was reduced using DTT and alkylated using IAA as described in Section 6.3.5.11.1 and then dried. Millipore C18 Zip-tip clean-up and peptide separations were carried out as described in Section 6.3.5.11.1. For comparison to the database, the digestion parameters were set as unspecific, so that no cleavage rule was applied.

6.3.6. Statistical analysis

Statistical analysis was performed using Minitab v17.1 (Minitab Inc., State College, Pennsylvania, USA). Datasets were analysed for normality using the Shapiro-Wilk's test. Data was deemed normally distributed and analysis was carried out using one-way ANOVA with *post hoc* Tukey test. *P*-values < 0.05 were considered significant. For proteomic analysis, quantitative differences between pairs of WMP samples were determined using a two-sample Student's T-test with a *p* value of 0.05 used as a threshold. All WMPs were produced in triplicate to give 9 separate powders, with all 9 powders digested *in-vitro* to produce 9 digestate samples. Gross compositional analysis was carried out in duplicate for each sample, whereas confocal microscopy, Raman spectroscopy, proteomic and peptidomic analyses were carried out in triplicate for each sample.

6.4. Results and discussion

6.4.1. Properties of whole milk powders and reconstituted whole milk powders

6.4.1.1. Gross composition of whole milk powders

Average total nitrogen, non-protein nitrogen, true protein and total fat values for each whole milk powder are shown in Table 6.1. No significant differences ($P > 0.05$) in gross compositional parameters were observed between the samples. The average NPN content of the TMR sample (0.28%) was substantially higher than the average concentration for the GRS sample (0.23%), but did not differ significantly. Significantly higher NPN values were previously observed in the TMR-derived skim milk powders analysed in Chapters 2 and 4 when compared to GRS; however, the NPN content of CLV-derived WMP was also observed to be significantly higher than that of TMR-derived WMP in Chapter 5, which was attributed to the increased urea levels of milk produced from white clover. As described in Chapter 4, the increased NPN content of the TMR samples produced for the present study may be due to the

high dietary crude protein content provided by the high concentrate inclusion rate in the TMR (Broderick, 2003).

Table 6.1: Average gross protein, fat and non-protein nitrogen content (\pm standard deviation) of whole milk powders produced from grass, grass/clover and total mixed ration diets.

Feeding system	Total protein %	Non-protein nitrogen %	True protein %	Total fat %
GRS	26.9 \pm 0.12	0.23 \pm 0.01	25.4 \pm 0.15	27.9 \pm 1.04
CLV	27.1 \pm 1.23	0.25 \pm 0.01	25.5 \pm 1.28	28.2 \pm 2.74
TMR	26.1 \pm 1.51	0.28 \pm 0.05	24.3 \pm 1.29	28.2 \pm 4.03

GRS – cows fed perennial ryegrass.

CLV – cows fed perennial ryegrass/white clover.

TMR – cows fed total mixed ration indoors.

6.4.1.2. Mineral composition of whole milk powders

Average mineral concentrations for each whole milk powder sample are shown in Table 6.2. Overall, mineral concentrations were comparable to those for skim milk in Chapter 4 and in the literature (Gulati *et al.*, 2018), on a fresh milk basis. Significantly higher concentrations of selenium and iodine were present in TMR-derived samples than in both GRS and CLV samples. No significant differences were observed between the samples for other minerals. In agreement with the present study, Gulati *et al.* (2018) and Gulati *et al.* (2019) observed significantly higher concentrations of selenium and iodine in TMR-derived skim milk samples than those from GRS and CLV. Gulati *et al.* (2018) also observed significant differences between the feeding systems for the macroelements calcium, phosphorus and sodium, which were not observed in a subsequent study using these feeding systems (Gulati *et al.*, 2019). The lack of differences between feeding systems for these elements in the latter study and the

present WMP samples may be indicative of variation between years in the mineral composition of the feeds used for the experimental herds in this project, and possibly changes in soil composition or supplementation within the TMR system.

Table 6.2. Average mineral concentrations (\pm standard deviation) of whole milk powders produced from grass, grass/clover and total mixed ration diets, determined by ICP-MS.

mg kg ⁻¹	GRS	CLV	TMR
Sodium	2357 \pm 109	2677 \pm 238	2560 \pm 233
Magnesium	747 \pm 32.3	771 \pm 59.5	759 \pm 76.0
Phosphorus	7680 \pm 656	7142 \pm 468	7817 \pm 1127
Sulphur	2394 \pm 124	2501 \pm 236	2351 \pm 233
Potassium	10830 \pm 686	11105 \pm 1233	11425 \pm 1152
Calcium	8878 \pm 558	8806 \pm 837	8535 \pm 953
Manganese	0.18 \pm 0.01	0.18 \pm 0.03	0.16 \pm 0.02
Iron	1.88 \pm 0.83	1.55 \pm 0.39	1.34 \pm 0.14
Cobalt	<0.01 \pm 0.00	<0.01 \pm 0.00	<0.01 \pm 0.00
Copper	0.85 \pm 0.19	0.66 \pm 0.13	1.08 \pm 0.24
Zinc	30.2 \pm 3.50	32.3 \pm 6.20	29.4 \pm 4.77
Selenium	0.10 \pm 0.01 ^a	0.09 \pm 0.01 ^a	0.20 \pm 0.07 ^b
Molybdenum	0.31 \pm 0.05	0.36 \pm 0.04	0.28 \pm 0.02
Iodine	0.90 \pm 0.22 ^a	0.61 \pm 0.17 ^a	3.13 \pm 1.13 ^b

GRS – cows fed perennial ryegrass.

CLV – cows fed perennial ryegrass/white clover.

TMR – cows fed total mixed ration indoors.

^{a,b}. Values within a row not sharing a common superscript differed significantly ($P < 0.05$).

As discussed in Chapter 4, the significantly higher concentrations of selenium and iodine present in the TMR-derived samples likely arose due to mineral supplementation within the feed provided in the TMR diet. Indeed, concentrate feeding and supplementation have been shown to be effective methods of direct permeation of feed minerals into milk (Dunshea *et al.*, 2019), although this would be dependent on the specific formulation in use. The present study further demonstrates the considerable effect of TMR-feeding on the iodine content of milk identified in Chapter 4. Considering the previously cited upper tolerable limit of 130 μ g iodine per kg of IMF powder defined by the Codex Alimentarius (European Commission Directive

2006/141/EC, 2006), the concentrations of iodine observed in the samples used in the present study would correspond to 126 µg per kg for GRS, 84 µg per kg for CLV and 407 µg per kg for TMR, on a fresh milk basis.

Large, but statistically insignificant differences between samples for some mineral concentrations (i.e., phosphorus, iron, copper) are likely due to considerable variation existing between replicates within those sample groups, as standard deviations were high between the replicates for these variables.

6.4.1.3. *Total amino acid composition of whole milk powders*

The average total amino acid (TAA) composition (including methionine sulphone) of each WMP is shown in Table 6.3. The concentration of methionine sulphone was significantly higher in the CLV sample than the TMR sample, the tyrosine content of both the GRS and CLV samples were significantly higher than the TMR sample and the histidine content of the TMR sample was significantly higher than the GRS sample. No significant differences were observed for any of the other AAs measured. The SMP samples derived from these diets which were analysed in Chapter 2 did not display significant variation in TAA composition, despite the presence of differences in whey protein types derived from the SMP samples.

Sulphur containing volatile compounds derived from the metabolism of methionine, such as dimethyl-sulfone, have previously been observed in higher concentrations in samples from both the GRS and CLV feeding systems than those from TMR (Faulkner *et al.*, 2018). A potential mechanism for the higher concentrations of tyrosine in both the pasture-derived samples is not currently known; however, tyrosine has previously been identified as a biomarker for a hay-based diet in cheese samples by Segato *et al.* (2019), although those authors did not identify a correlation between tyrosine and the fresh pasture-based diet which was also being investigated. The higher concentration of histidine associated with the TMR

sample in the present study may arise from histidine supplementation within the ration provided to this group to maintain lactational performance, as histidine has been identified as a limiting AAs in corn silage-based diets low in metabolizable protein (Giallongo *et al.*, 2017).

Table 6.3. Total amino acid composition of whole milk powders derived from grass, grass/clover and total mixed ration diets, determined by high-performance cation exchange.

g Kg protein ⁻¹	GRS	CLV	TMR
Cysteic acid	9.94	11.6	11.7
Methionine Sulphone	37.3 ^{a,b}	37.4 ^b	35.9 ^a
Asparagine	77.6	80.1	79.3
Threonine	42.6	43.6	43.0
Serine	53.2	53.3	53.6
Glutamine	216	218	219
Glycine	19.1	19.2	18.9
Alanine	31.1	31.3	31.6
Cysteine	3.91	4.72	3.26
Valine	64.9	66.4	63.9
Isoleucine	50.2	50.4	50.5
Leucine	94.4	95.6	96.5
Tyrosine	44.8 ^b	43.0 ^b	38.7 ^a
Phenylalanine	48.4	48.4	48.8
Histidine	33.9 ^a	34.9 ^{a,b}	36.4 ^b
Lysine	76.5	77.1	76.9
Arginine	34.7	34.8	34.4
Proline	93.7	93.8	95.1

GRS – cows fed perennial ryegrass.

CLV – cows fed perennial ryegrass/white clover.

TMR – cows fed total mixed ration indoors.

^{a,b}, Values within a row not sharing a common superscript differed significantly ($P < 0.05$).

6.4.1.4. Fatty acid methyl ester profile of whole milk powders

The total fatty acid (FA) profile of each WMP is shown in Table 6.4. A total of 25 fatty acid triglyceride esters were quantified, with 10 displaying significant variation between feeding systems. Average concentrations of palmitic acid (C16:0), linoleic acid (C18:2 n6c) and eicosatrienoic acid (C20:3 n6) in the TMR sample were significantly higher than those

from both GRS and CLV samples, whereas both pasture-derived samples exhibited significantly higher average concentrations of alpha-linolenic acid (C18:3 n3), *cis-9, trans-11* conjugated linoleic acid (CLA) and eicosapentaenoic acid (C20:5) than the TMR sample. Average concentrations of myristoleic acid (C14:1) and linolelaidic acid (C18:2 n6t) were significantly higher in the CLV sample than both GRS and TMR. The CLV sample also displayed a significantly higher concentration of pentadecanoic acid (C15:0) than the TMR sample, whereas the concentration of arachidic acid (C20:0) was significantly higher in the TMR sample compared to the CLV sample.

Overall FA concentrations were comparable to those reported by O'Callaghan *et al.* (2016) for raw milk produced from the feeding systems utilised in this study. Unique FAs which were quantified in the samples produced in the present study, but not in those analysed by O'Callaghan *et al.* (2016a) were as follows: ginkgolic acid (C17:1), arachidic acid (C20:0), eicosapentaenoic acid (C20:5) and lignoceric acid (C24:0), whereas the present study did not quantify γ -linoleic acid (C18:3n-6c), *cis-12, trans-10* CLA, eicosenoic acid (C20:1), erucic acid (C22:1n-9), tricosanoic acid (C23:0) and arachidonic acid (C20:4n-6, where the study by O'Callaghan *et al.* (2016a) did. The referenced FAs which were unique to either study are present in low concentrations in milk and, while all would have been qualitatively present in the milk samples analysed in each study, inter-seasonal variation in their relative concentrations may leave the respective FAs below the limit of quantification for the GC-FID method used.

Table 6.4. Fatty acid methyl ester results (\pm standard deviation) for total fatty acids in whole milk powder samples derived from grass, grass/clover and total mixed ration diets, determined by GC-FID.

Fatty acid (g per 100g fat)	GRS	CLV	TMR
C4:0	5.00 \pm 0.14	4.81 \pm 0.43	4.49 \pm 0.08
C6:0	2.93 \pm 0.08	2.93 \pm 0.31	2.67 \pm 0.07
C8:0	1.78 \pm 0.06	1.83 \pm 0.22	1.61 \pm 0.05
C10:0	4.30 \pm 0.20	4.49 \pm 0.62	3.99 \pm 0.13
C11:0	0.09 \pm 0.03	0.12 \pm 0.05	0.05 \pm 0.02
C12:0	4.92 \pm 0.21	5.22 \pm 0.70	4.76 \pm 0.19
C13:0	0.12 \pm 0.03	0.16 \pm 0.04	0.09 \pm 0.03
C14:0	13.2 \pm 0.13	14.00 \pm 0.54	13.4 \pm 0.33
C14:1	0.97 \pm 0.07 ^a	1.14 \pm 0.06 ^b	0.93 \pm 0.07 ^a
C15:0	1.47 \pm 0.11 ^{a,b}	1.70 \pm 0.11 ^b	1.28 \pm 0.05 ^a
C16:0	30.7 \pm 1.79 ^a	30.3 \pm 1.23 ^a	34.8 \pm 1.48 ^b
C16:1	1.51 \pm 0.10	1.44 \pm 0.15	1.40 \pm 0.05
C17:0	0.60 \pm 0.00	0.58 \pm 0.05	0.54 \pm 0.01
C17:1	0.26 \pm 0.01	0.25 \pm 0.03	0.21 \pm 0.02
C18:0	9.69 \pm 0.48	8.65 \pm 0.46	9.47 \pm 0.36
C18:1 n9c	17.7 \pm 0.46	16.4 \pm 1.42	15.9 \pm 1.28
C18:2 n6c	0.83 \pm 0.04 ^a	0.78 \pm 0.02 ^a	1.49 \pm 0.16 ^b
C18:2 n6t	1.35 \pm 0.05 ^a	1.89 \pm 0.15 ^b	1.40 \pm 0.10 ^a
C18:3 n3	0.61 \pm 0.04 ^b	0.61 \pm 0.08 ^b	0.30 \pm 0.04 ^a
CLA: C9t11	1.32 \pm 0.15 ^b	1.42 \pm 0.21 ^b	0.35 \pm 0.06 ^a
C20:0	0.08 \pm 0.01 ^{a,b}	0.06 \pm 0.02 ^a	0.12 \pm 0.02 ^b
C20:3n6	0.02 \pm 0.00 ^a	0.02 \pm 0.01 ^a	0.06 \pm 0.02 ^b
C20:5	0.05 \pm 0.00 ^b	0.06 \pm 0.01 ^b	0.02 \pm 0.01 ^a
C22:0	0.03 \pm 0.00	0.02 \pm 0.01	0.03 \pm 0.01
C24:0	0.10 \pm 0.04	0.08 \pm 0.04	0.09 \pm 0.04

GRS – cows fed perennial ryegrass.

CLV – cows fed perennial ryegrass/white clover.

TMR – cows fed total mixed ration indoors.

^{a,b}, Values within a row not sharing a common superscript differed significantly ($P < 0.05$).

An association between concentrate feeding and increased C16:0 content of milk has been widely reported (Couvreur *et al.*, 2006; Rego *et al.*, 2016; O’Callaghan *et al.*, 2016a), with increased concentrations of this high melting-point saturated FA resulting in altered dairy product textural qualities, such as increased butter hardness (Couvreur *et al.*, 2006; Hurtaud &

Peyraud, 2007; O’Callaghan *et al.*, 2016b). Similarly, the significantly higher CLA content of pasture-derived milk products has also been regularly reported in previous studies (Kelly *et al.*, 1998; Lock & Garnsworthy, 2003; Talpur *et al.*, 2008; O’Callaghan *et al.*, 2016a). Intake of the CLA isomer has been associated with beneficial physiological effects, including mediation of obesity, and anti-carcinogenic and anti-hypertensive capacity (Koba & Yanagita, 2014).

6.4.1.5. *Raman Spectroscopy of reconstituted whole milk powders*

Normalized average Raman spectra for each reconstituted WMP are shown in Figure 6.1. The most significant bands corresponding to these spectra are shown in Table 6.5, which were identified based on previous studies by Gallier *et al.* (2011), Czamara *et al.* (2015), Gómez-Mascaraque *et al.* (2020) and Gómez-Mascaraque *et al.* (2021). The bands identified were comparable to those of Gómez-Mascaraque *et al.* (2020), who compared the Raman spectra of butter produced from the GRS, CLV and TMR feeding systems utilised in the present study, although three additional bands were identified in the reconstituted WMP samples in the present study; an additional $\nu(\text{C}-\text{C})$ peak identified at 1087 or 1088 rel. cm^{-1} in all sample types; a (C-C backbone) peak identified at 1196 rel. cm^{-1} in both the GRS and CLV samples, but not in TMR; and a $\delta(=\text{CH})$ peak uniquely identified at 1280 rel. cm^{-1} in the CLV sample.

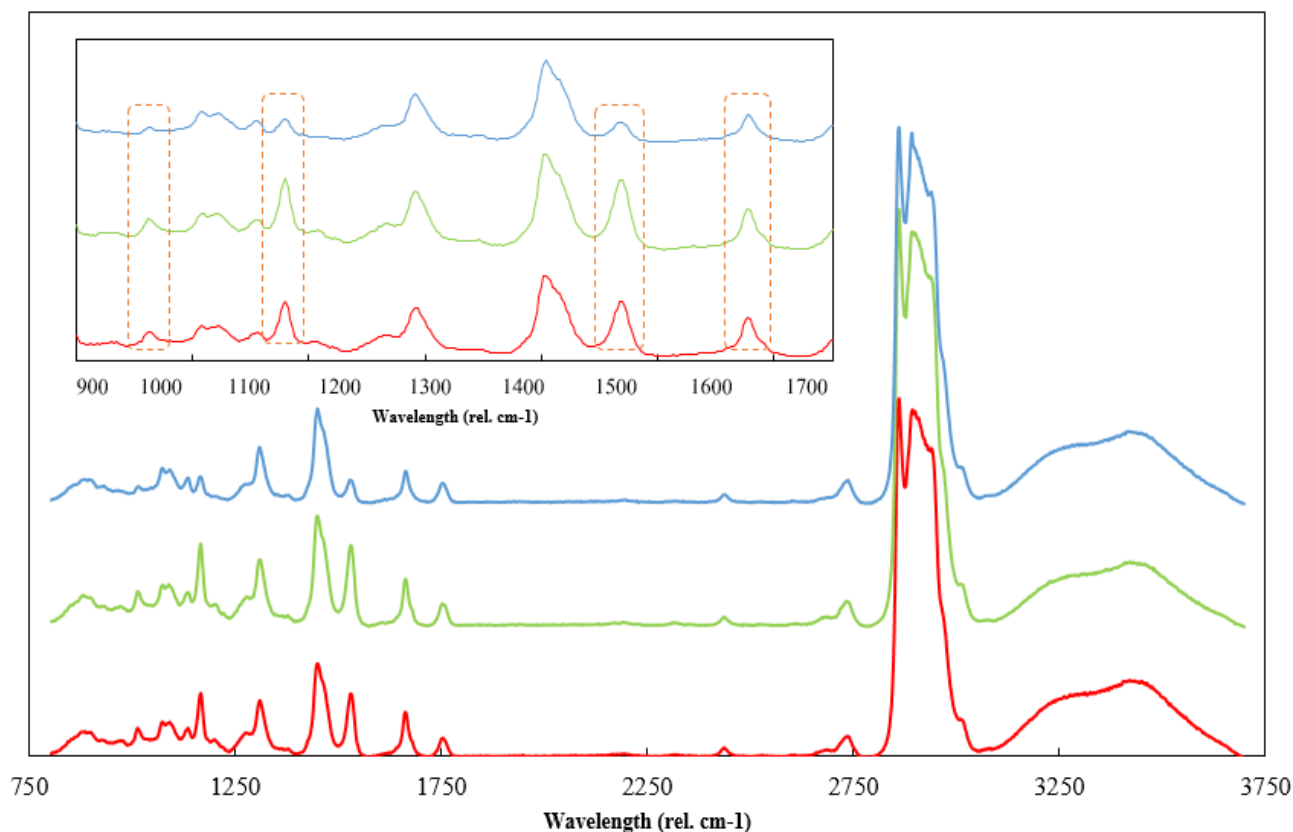


Fig. 6.1. Normalised average Raman spectra for reconstituted whole milk powders derived from grass/clover (CLV, red series), grass (GRS, green series) and total mixed ration (TMR, blue series) diets. Inset: Peaks highlighted within dotted lines indicate distinctions between diets.

As was observed by Gómez-Mascaraque *et al.* (2020), clear distinctions could be identified between both the pasture-derived samples and the TMR sample based on the relative intensity of peaks attributed to the presence of carotenoid compounds (1013 -1015, 1164 -1166 and 1529 rel. cm^{-1}) and the stretching vibration of C=C double bonds (1662-1663 rel. cm^{-1}), which were all lower in the TMR sample (Fig. 6.1). The distinction between the carotenoid levels of both pasture-derived samples and the TMR sample provide some substantiation for the significant differences in colour observed between the GRS and TMR WMP samples analysed in Chapter 5. In addition, the level of FA unsaturation in the reconstituted WMP samples was estimated, as described by Gallier *et al.* (2011), as the ratio of intensities at 1659 and 1445 rel. cm^{-1} . The levels of unsaturation for the CLV, GRS and TMR samples were 0.46,

0.40 and 0.29, respectively, which were comparable to those observed for the butter samples produced from these feeding systems by Gómez-Mascaraque *et al.* (2020) and reflect the significant differences in the relative levels of unsaturated FAs shown in Table 6.4.

Table 6.5. Assignments of major Raman bands identified in reconstituted WMP samples.

Assignment	Average peak maximum (rel. cm ⁻¹)		
	GRS	CLV	TMR
C–O–O	881	882	882
	-	-	926
β(CH)	-	972	-
Carotenoids	1013	1014	1015
ν(C–C)	1076	1076	1073
ν(C–C)	1087	1087	1088
ν(C–C)	1133	1133	1133
ν(C–C), carotenoids	1164	1165	1166
(C-C backbone)	1196	1196	-
δ(=CH)	-	1280	-
τ(-CH ₂)	1308	1308	1308
	-	-	1375
α(-CH ₂)	1448	1448	1448
Carotenoids	1529	1529	1529
ν(C=C)	1662	1662	1663
ν(C=O)	1753	1751	1752
	2434	2435	2436
	2732	2733	2733
ν _s (-CH ₂)	2859	2859	2859
ν _s (-CH ₃)/ν _{as} (-CH ₂)	2890	2892	2892
ν _{as} (=CH)	3007	3007	3008

GRS – cows fed perennial ryegrass.

CLV – cows fed perennial ryegrass/white clover.

TMR – cows fed total mixed ration indoors.

Assignments of bands were based on studies by Gallier *et al.* (2011), Czamara *et al.* (2015), Gómez-Mascaraque *et al.* (2020) and Gómez-Mascaraque *et al.* (2021).

α = scissoring; β = bending; δ = deformation; τ = twisting; ν = stretching (s = symmetric, as = asymmetric).

The rapid nature of Raman spectroscopy makes it a method of interest for differentiation between products derived from different feeding systems and verification of

labelling claims. Partial least square-discriminant analysis of the average Raman spectra from each reconstituted WMP sample displayed a clear distinction between the TMR sample and the two pasture-derived samples, which showed some overlap (Appendix i, Figure S3). This distinction is similar to that observed in the SMP and whey ingredients analysed by LC-MS/MS in Chapters 2 and 3.

6.4.1.6. *Buffering capacity of reconstituted whole milk powders*

Typical buffering indices for reconstituted WMP derived from GRS, CLV and TMR are shown in Fig. 6.2. The distinct increase in buffering index between pH 6.0 and 4.0 and local maximum at approx. pH 5.0 is typically observed during acidification of milk samples (Olsen et al., 2015). A slightly lower buffering index in the region of pH 4.0 to 3.0 was observed in the CLV sample. The buffering index of the GRS sample was also lower at low pH (near pH 2.0) in both the acidification and alkalisation steps. No substantial differences in buffering index at the pH points corresponding to the gastric (pH 3.0) and intestinal phases (pH 7.0) of the Infogest method and overall buffering capacity were observed between the GRS, CLV and TMR samples, which may be due to their similar protein, amino acid composition and mineral salt content. These results may also indicate that the differences in the gel strength of yoghurts derived from WMP in Chapter 5 were not substantially influenced by the relative buffering capacities of the GRS, CLV or TMR samples.

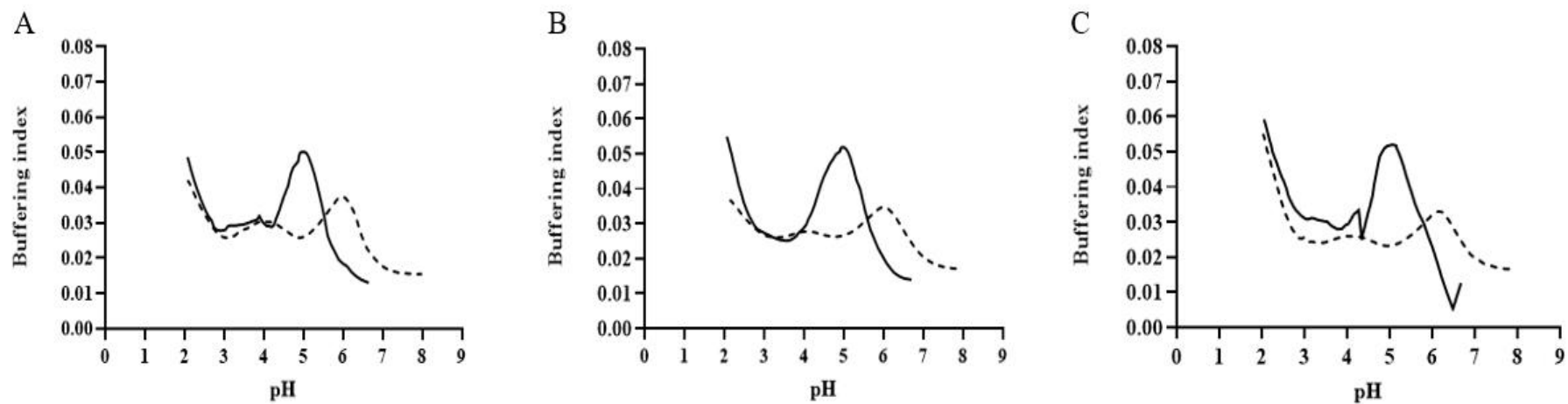


Fig. 6.2. Typical buffering index profiles for reconstituted whole milk powders derived from (A) perennial ryegrass (GRS), (B) perennial ryegrass/white clover (CLV) or (C) total mixed ration (TMR) feeding systems during acidification (—) with 0.1M HCl and alkalisation (- - -) with 0.1 M NaOH.

6.4.1.7. *Proteomic profile of reconstituted whole milk powders*

Between the nine WMP samples screened, 190 protein groups were identified in total. As shown in previous chemometric analysis applied to samples from the GRS, CLV and TMR feeding systems (Chapter 2, Chapter 3), the TMR replicates were clustered and distinct from both the GRS and CLV replicates, which were overlapping (Appendix i, Figure S4). Hierarchical clustering analysis (Appendix i, Figure S5) also displayed grouping within the TMR replicates and within the GRS replicates, although there was some distinction between the CLV replicates. The T1 CLV and T3 CLV samples were clustered within the same branch, but the T2 CLV sample was distinct to the other samples.

The comparison groups used for quantitative analysis of proteins were CLV v GRS, CLV v TMR and TMR v GRS. The results of these comparisons are shown in Table 6.6. Considering the number of proteins identified, there were relatively few statistically significant quantitative differences between the comparator groups, at a fold-change cut-off of 1.5 (a 1.5-fold increase or decrease in the concentration of a protein). As this is a low fold change cut-off for proteomics, this indicated a considerable degree of similarity between the quantifiable proteins detected.

The comparison with the lowest number of quantitative differences was CLV v GRS, between which the levels of abundance of only 2 proteins (1 higher and 1 lower in CLV compared to GRS) were significantly different between the samples ($p < 0.05$, fold change ≥ 1.5). The comparison of TMR v CLV displayed 5 proteins which were significantly up-regulated in the CLV samples compared to TMR and 4 proteins which were significantly down-regulated. For the GRS v TMR comparison, 4 proteins were significantly up-regulated and 4 were significantly down-regulated in the TMR samples. This grouping of protein profiles between the two pasture-based sample types and distinction from the TMR sample was also observed in the principal component analysis (Appendix i, Figure S3) and the hierarchical

clustering analysis (Appendix i, Figure S5). The variance in significant up-regulation and down-regulation of proteins between comparator groups are also shown using Volcano plots in Figure 6.3.

Table 6.6: Summary of quantitative differences in the abundance of proteins between pairs of reconstituted WMP samples ($p < 0.05$; fold change ≥ 1.5).

	Control groups in comparison		
	TMR	GRS	CLV
TMR		↑4 (*15) ↓4 (*13)	
GRS			
CLV	↑5 (*11) ↓4 (*12)	↑1 (*6) ↓1 (*11)	

Number of proteins with higher (↑) or lower (↓) abundance in each sample in comparison to the control group.

* Number of significantly differentially abundant proteins in a comparison when not restricted to proteins with at least 1.5 fold change in abundance.

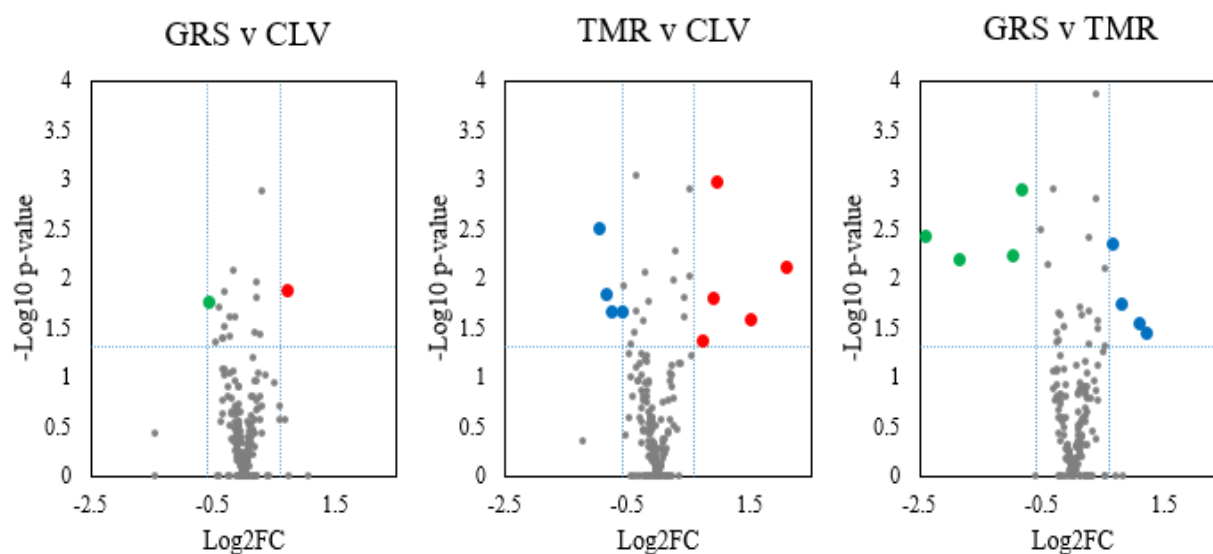


Figure 6.3: Volcano plots displaying quantitative proteomic comparisons between pairs of samples. Each point represents a protein mapped by the $-\log_{10}$ p-value and the \log_2 FC from a student's t-test comparison between each pair of samples. Coloured points represent proteins with a significantly higher abundance in GRS (green), CLV (red) and TMR (blue) in each comparison ($p < 0.05$, $FC \geq 1.5$). Grey points represent proteins that were outside the significance cut-off or the minimum fold change ($p > 0.05$, $FC < 1.5$). The cut-offs are indicated by dotted lines on each plot.

In addition to the quantitative comparison, a qualitative analysis was also carried out to identify proteins which were uniquely detected in a particular sample within a comparator group. These qualitative differences are summarised in Table 6.7. The criteria for definition of a qualitative difference between samples was a protein that: (a) was detected in at least 2/3 replicates of one group and was not detected in any of the control sample replicates and (b) was uniquely detected, with a protein intensity of at least 2 fold above the minimum intensity threshold (mean minimum protein intensity across all samples + 2 standard deviations). The second criteria eliminated proteins that were close to the minimum intensity threshold. The comparison of CLV v TMR identified 3 proteins unique to CLV and 3 unique to TMR, whereas the TMR v GRS comparison identified 2 proteins unique to TMR and 1 unique to GRS and the CLV v GRS comparison identified 2 proteins unique to CLV and 1 unique to GRS.

Table 6.7: Summary of qualitative differences in proteins detected between pairs of whole milk samples.

	Control groups in comparison		
	TMR	GRS	CLV
TMR		2 (*6), 1 (*3)	
GRS			
CLV	3 (*8), 3 (*7)	2 (*4), 1 (*2)	

Number of proteins which were detected uniquely in the comparator sample (in rows; red) or the control sample (in columns; green) for each comparison.

* Number of proteins uniquely detected when criteria (b) above is not applied.

When the quantitative ($p < 0.05$, fold change ≥ 1.5) and qualitative (proteins uniquely detected and above the minimum intensity threshold) differences were combined, a total of 11 proteins were identified with variable levels between TMR and GRS, 15 proteins which varied between CLV and TMR, and only 5 proteins that were present in variable levels between CLV and GRS. These proteins are summarised in Table 6.8. While the number of proteins with qualitative or quantitative differences between WMP samples from the separate feeding

systems were relatively low, these findings indicate that undigested whole milk samples, between which diet was the sole variable, were distinguishable on the basis of the presence or absence and relative abundance of genetic products. This may imply a modulation of rumen function due to diet, resulting in variation in the generation of protein components available to the mammary gland for subsequent protein synthesis.

Table 6.8: Proteins with quantitative or qualitative changes between comparator samples.

Majority protein IDs	Gene Name	Protein Name	GRS v CLV		TMR v CLV		GRS v TMR	
			p-value	Log2FC (*predicted min)	p-value	Log2FC (*predicted min)	p-value	Log2FC (*predicted min)
P22226	CATHL1	Cathelicidin-1	0.269	0.670	0.382	-0.525	0.035	1.196
P19660	CATHL2	Cathelicidin-2	-	0	1	-1.181	1	1.181
		G protein-coupled receptor class C group						
Q1JPD9	GPRC5B	5 member B	1	-1.452	0.014	-0.854	1	-0.598
A0A3Q1ML26		Ig-like domain-containing protein	1	-2.442	1	-2.319	0.644	-0.123
A0A3Q1LWV8		Ig-like domain-containing protein	0.017	-0.594	0.036	-0.400	0.170	-0.194
F1MLW8		Uncharacterized protein (Ig-like domain)	0.040	-0.340	0.003	-0.978	0.004	0.638
		Jacalin-type lectin domain-containing						
G3MZ19	ZG16B	protein	0.167	-0.336	0.007	2.079	0.004	-2.416
		Uncharacterized protein (Jacalin-like						
F1N1Z8		lectin domain)	0.282	-0.367	0.025	1.493	0.006	-1.859
P01045	KNG2	Kininogen-2	-	0	1	-1.169	1	1.169
P24627	LTF	Lactotransferrin	0.449	0.121	0.001	0.950	0.001	-0.829
E1B6Z6	LCN2	Lipocalin 2	0.432	-0.183	0.021	-0.603	0.075	0.420
A0A3Q1LR40	LIPG	Lipoprotein lipase	0.115	0.501	0.043	0.712	0.023	-0.212
Q8SPP7	PGLYRP1	Peptidoglycan recognition protein 1	0.265	0.592	0.260	-0.490	0.028	1.081
F1MR22	PIGR	Polymeric immunoglobulin receptor	1	2.086	1	2.086	-	0
O02853	PTGDS	Prostaglandin-H2 D-isomerase	0.508	-0.112	0.016	0.877	0.006	-0.989
Q2KIX7		Protein HP-25 homolog 1	0.082	-0.335	1	1.204	1	-2.916
Q2KIU3		Protein HP-25 homolog 2	1	2.108	1	2.108	-	0
P61823	RNASE1	Ribonuclease pancreatic	0.309	-0.244	0.022	-0.769	0.008	0.525
A0A3Q1M3H8	SAA3	Serum amyloid A protein	0.013	0.693	0.681	-0.088	0.018	0.780

Proteins with a higher level or uniquely detected in the comparator sample compared to the control are denoted in red, and those with lower abundance or uniquely detected in the control are in green. * A minimum predicted fold change (Log2FC) is included for the qualitative results, showing the levels relative to the minimum intensity threshold cut-off. Qualitative results have a p-value of 1.

6.4.2. *Effect of in-vitro digestion on the protein and fat components of reconstituted whole milk powders*

Static *in-vitro* digestion was carried out on each reconstituted WMP to determine potential variation in the the protein, AA, FA and peptidomic profiles of samples from each diet *post* digestion.

6.4.2.1. *Gel electrophoresis and confocal microscope images*

Protein profiles of reconstituted whole milk and corresponding digestates as determined by SDS-PAGE under reducing and non-reducing conditions are shown in Appendix i Figure S6. No qualitative differences in the protein profiles of the GRS, CLV and TMR samples were apparent. The absence of clear protein bands in the digestate samples indicate the extent of protein hydrolysis resulting from the *in-vitro* digestion process. Under reducing conditions, only one band remained visible after digestion, between the casein and minor whey protein (immunoglobulins, bovine serum albumin, lactoferrin) fractions. This may be indicative of the presence of residual gastric enzymes or autolysis products (Brodkorb *et al.*, 2019) added during the digestion process and visible only under disulphide reducing conditions.

Representative confocal micrographs of reconstituted whole milk and corresponding digestates from GRS, CLV and TMR-derived WMP are also shown in Appendix i Figure S7. Fat globules (red) and protein aggregates (green) can be clearly seen in non-digested samples. Relative levels of protein and adsorbed protein were variable between replicates for GRS, CLV and TMR, across triplicate images from each sample replicate. Micrograph images corresponding to the digested samples displayed complete hydrolysis, with a distinct loss of fluorescent protein and fat.

6.4.2.2. *Free amino acid composition*

The free amino acid composition of each reconstituted WMP and each corresponding digestate is shown in Table 6.9. The extent of AA liberation due to the digestion process did not differ between the feeding systems and, with the exception of tyrosine, there were no differences in fully liberated FAAs post digestion corresponding to the differences in TAA concentrations shown in Table 6.3. It should be noted that the quantities for liberated FAAs shown in Table 6.9 do not equate to the total AA concentrations shown in Table 6.3, as peptides were still present within the digestate samples examined in the peptidomic analysis in Section 6.4.2.4. The digestion process also liberated tryptophan for quantification, which could not otherwise be quantified by the method of TAA analysis used, as it is degraded during the acid hydrolysis step (Delgado-Andrade *et al.*, 2013).

Table 6.9. Free amino acid contents of reconstituted whole milk powders and digested reconstituted whole milk powders derived from grass, grass/clover and total mixed ration diets, determined by high-performance cation exchange.

$\mu\text{g mL}^{-1}$	Whole milk			Digestate		
	GRS	CLV	TMR	GRS	CLV	TMR
Cysteic acid	3.33	4.34	4.56	205	211	218
Asparagine	1.68	1.29	1.69	137	140	138
Threonine	1.25	1.94	2.04	421	438	414
Serine	2.62	2.21	2.09	252	258	252
Glutamine	50.5	44.2	46.8	524	531	528
Glycine	7.23	5.66	6.38	257	260	258
Alanine	3.10	2.83	3.40	234	240	233
Cysteine	9.37	4.02	3.64	170	170	169
Valine	2.42	1.74	1.85	227	233	226
Methionine	0.26	0.16	0.14	140	144	139
Isoleucine	0.49	0.61	0.67	131	136	130
Leucine	0.73	2.00	1.30	606	629	600
Tyrosine	0.62	0.68	0.73	498 ^{a,b}	524 ^b	487 ^a
Phenylalanine	0.70	0.65	0.77	539	559	527
Histidine	5.98	2.89	4.05	374	383	370
Lysine	1.68	1.91	1.67	945	978	941
Arginine	2.80	2.51	2.66	694	712	693
Proline	0.80	1.26	1.72	35.0	36.2	34.9
Tryptophan				334	346	329

GRS – cows fed perennial ryegrass.

CLV – cows fed perennial ryegrass/white clover.

TMR – cows fed total mixed ration indoors.

^{a,b}. Values within a row not sharing a common superscript differed significantly ($P < 0.05$).

6.4.2.3. *Free fatty acid profile*

The free fatty acid (FFA) profile of each WMP and each corresponding digestate is shown in Table 6.10. Milk FAs comprise esterified FAs bound to glycerides and their hydrolysis products, present as FFA (Kilcawley & Mannion, 2017). Levels of FFA in milk contribute to the development of volatile compounds which influence the sensory quality of dairy products, while also influencing the texture and foaming properties of milk (Kilcawley & Mannion, 2017). No significant variation in FFA content between feeding systems was observed in the WMP samples; however, the significantly higher concentration of C18:2 liberated in the digested TMR sample when compared to GRS and CLV and higher concentrations of C18:3 in both the pasture-derived samples compared to the TMR sample were somewhat consistent with the relative levels of total C18:2 n6c and C18:3 n3 between the WMP samples in Table 6.4. Previous analysis of butter samples produced from the GRS, CLV and TMR systems by O'Callaghan *et al.* (2016b) also observed no significant differences in levels of FFA between similar dietary treatments.

Table 6.10. Average free fatty acid content (\pm standard deviation) of whole milk powder and corresponding digestate samples derived from grass, grass/clover and total mixed ration diets, determined by GC-FID.

FFA (mg L ⁻¹)	WMP			Digestate		
	GRS	CLV	TMR	GRS	CLV	TMR
C4	21.3	22.7	23.7	264	257	267
C6	22.7	25.7	25.7	190	187	192
C8	19.3	22.3	22.0	132	127	134
C10	24.3	26.7	27.3	247	241	249
C12	28.0	30.3	31.3	228	227	233
C14	35.3	38.7	38.3	527	529	544
C16	62.0	66.3	63.3	1159	1126	1302
C18	40.0	42.3	40.3	458	446	468
C18:1	38.3	42.3	37.7	577	565	554
C18:2	18.3	23.3	21.3	111 ^a	107 ^a	124 ^b
C18:3	17.3	22.7	20.7	53.7 ^{a,b}	55.7 ^b	47.0 ^a
Total	326	362	351	3949	3868	4115

GRS – cows fed perennial ryegrass.

CLV – cows fed perennial ryegrass/white clover.

TMR – cows fed total mixed ration indoors.

^{a,b}. Values within a row not sharing a common superscript differed significantly ($P < 0.05$).

6.4.2.4. Peptidomic profile of digested whole milk

Database searching was performed using an unspecific digestion search with minimum peptide length set to 8 and maximum set to 25. Variable modifications included oxidation of methionines and acetylation of protein N-terminals, with carbamidomethylation of cysteines as a fixed modification (from reduction/alkylation). A database of non-bovine contaminants was also included to detect common contaminants of proteomic experiments (e.g., keratin) and decoy database searching was also included to reduce the number of false positive identifications reported. Throughout the nine replicates analysed (GRS, CLV and TMR, each in triplicate), 73 distinct peptides were detected, within 35 protein groups. The number of peptides detected from these protein groups ranged from a single identified peptide to the

identification of 26 peptides from one protein group (A0A452DHW7 β -casein). When including peptides only detected in at least 2 replicates from a group, 49 peptides were detected in the CLV samples, 47 were detected in the GRS samples, and 37 were detected in the TMR samples.

Qualitative comparison of the GRS, CLV and TMR digestates identified one peptide from a Histone-lysine N-methyltransferase, H3 lysine-79 specific protein (QQVYNHSVTDPE) which was detected in both the CLV and TMR samples, but not in the GRS samples. A second peptide from this protein (MKEGGRIVSSKP) was also detected in CLV and TMR samples, but not GRS samples. Interestingly, this protein was not detected in the proteomics analysis carried out on the WMP samples prior to *in-vitro* digestion. A peptide from a fatty acid synthase (IALSLGCRVFPL) was detected in GRS, but not TMR samples. A peptide from elongation factor 1-alpha (DCILPPTTRPT) was detected exclusively in TMR. Two peptides from β -casein were detected in GRS but not in CLV (YQEPVLGPV, SLVYFPFGPIH). Another β -casein peptide (TDVENLHLPLPL) was detected in both the GRS and CLV samples, but not TMR.

Effective quantitative analysis, as described for the WMP samples in Section 6.4.1.7 could not be carried out due to the range of proteases with non-specific cleavage points used in the Infogest method, and the limited number of proteins/peptides which were detected. Label-free quantitative (LFQ) analysis would typically be performed on protein groups, based on the generation of specific tryptic peptides and a combination of all contributing peptides associated with the original protein sequence. As this analysis required a comparison of individual peptides, the MaxLFQ algorithm of MaxQuant was not suitable for this application. The peptides identified in the analysis of the digestate samples were searched against the Milk Bioactive Peptide Database (<http://mbpdb.nws.oregonstate.edu/>; Nielsen et al., 2017), for established sequences and activities.

The search was performed 3 times, using the search types (i) Sequence, or (ii) Truncated, or (iii) Precursor. The similarity threshold was set to 90 % and the Scoring matrix used was BLOSUM62.

- (i) The “sequence” search screened for peptides in the database with sequences which matched those in the search input. Peptides from β -casein and β -lactoglobulin matched to sequences in the database using this search.
- (ii) The “truncated” search screened for peptides in the database that contain the search peptide sequence. Peptides from β -casein, β -lactoglobulin, κ -casein and haemoglobin subunit alpha matched to sequences in the database using this search.
- (iii) The “precursor” search sequence screened for peptides that are contained within the search input. This search obtained the most results; however, some of the peptides identified were as small as di- and tri-peptides, which could be found in a range of protein sequences. Peptides from α -lactalbumin, α_{S1} -casein, β -casein, β -lactoglobulin, κ -casein, lactotransferrin and serum albumin matched to sequences in the database using this search.

Among the peptides identified in the digestate samples using the above search types, associated bioactivities included: angiotensin-converting enzyme (ACE)-inhibitory, dipeptidyl-peptidase 4 (DPP-IV)-inhibitory, immunomodulatory, anti-inflammatory, antioxidant and antimicrobial effects and stimulation of trabecular bone growth. Further analysis of the specific peptides generated by the *in-vitro* digestion process could be carried out to investigate their potential to elicit these activities. Moreover, dynamic *in-vitro* digestion, involving multiple set sampling points may allow for more direct comparison between the proteomes of whole and digested samples in further analysis.

6.5. Conclusion

The data presented in this study provided further characterisation of the composition of WMP produced from GRS, CLV and TMR diets, expanding on the factors analysed in Chapter 5. The mineral composition of the WMP samples did not vary to the extent previously observed in milk samples produced from these systems; however, the data confirms the consistent effect of pasture or TMR feeding on the relative levels of FAs, particularly long-chain FAs. The use of Raman spectroscopy provided confirmation of this effect on the basis of relative levels of FA unsaturation and of the significant colour differences between powders derived from each diet, offering another effective means of differentiation between milk from separate feeding systems. Interesting variations were observed in the proteomic and peptidomic profiles of the samples analysed, in which unique proteins were identified which could act as potential biomarkers for each feeding system. The overall effect of static digestion on the FAA and FFA profiles of the WMP samples did not differ substantially between samples from each feeding system, but more mechanistic insights into the change in protein and fat composition throughout digestion could be provided by dynamic *in-vitro* digestion in future work.

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Chapter 7
General discussion

7.1. Summary of key findings

This research analysed WMP, SMP and three varieties of whey powders and WPCs produced from milk derived from GRS, CLV and TMR feeding systems and identified unique compositional and functional properties associated with each system throughout. While relative protein contents of the WMP and SMP samples did not vary significantly, the TMR and CLV feeding systems were associated with significantly increased concentrations of NPN in SMP (Chapters 2 and 4) and WMP (Chapter 5), respectively, which corresponded to increased heat stability in these samples. The greater heat stability of TMR-derived SMP, relative to the GRS sample also resulted in significantly lower viscosity values in heated dispersions of reconstituted SMP (10% protein, w/w). Interestingly, despite the lower relative NPN content and greater concentrations of high-melting-point FAs in the TMR-derived WMP analysed in Chapter 5, yoghurt gels produced from reconstituted, TMR-derived WMP were characterised by significantly lower gel strength and firmness than those from GRS and CLV.

The AA composition of the powder samples did not display clear, significant variation due to diet. While this is to be expected, significant differences in the average concentrations of cysteine, glycine, phenylalanine and valine were observed between the diets in sweet whey, micellar casein whey and acid whey, whereby concentrations were generally higher in either the GRS or CLV samples compared to TMR. Significant variation in the concentrations of fourteen amino acids was also observed between the whey product types, irrespective of diet (Chapter 2). These differences were not consistently observed in the WMP samples analysed in Chapter 6, in which concentrations of methionine sulphone, tyrosine and histidine varied significantly between diets.

Clear distinctions between samples produced from each feeding system were made using a number of methodologies throughout this research. Using LC-MS/MS, distinctions between pasture and TMR samples were identified in SMP and whey ingredients on the basis

of metabolomics (Chapter 2) and water-soluble B-vitamin (Chapter 3) profiles. Concentrations of vitamins B1 (thiamine), B2 (riboflavin) and B7 (biotin) were significantly higher in pasture-derived samples in comparison to TMR samples, whereas the concentrations of vitamin B3 (nicotinic acid) and B3-amide (nicotinamide) were significantly higher in the TMR samples than those from CLV and GRS. This study also calculated the proportion of the RDA for each vitamin which would be provided by a 200 mL serving of skim milk from each feeding system and the mass of each vitamin which would be provided by SMP from each feeding system in a typical first-stage IMF.

Further distinction between pasture- and TMR-based feeding systems was identified by the FAME profiles of the WMP samples analysed in Chapter 6, which were similar to those previously reported for milk from these feeding systems (O'Callaghan *et al.*, 2016), with 10 FAs differing significantly between diets, including high concentrations of palmitic acid in the TMR sample and high concentrations of CLA and α -linolenic acid in both pasture-derived samples. This study also demonstrated the applicability of Raman spectroscopy to differentiate between milk derived from pasture and TMR feeding systems based on the unsaturation level of the FAs identified and by relative levels of carotenoids, which were also likely responsible for the significantly higher b^* values (yellowness) observed in GRS-derived WMP, relative to TMR, in Chapter 5. The visual distinction between the samples was also quantified by a ΔE value of 3.21. These methods demonstrated potential for rapid verification of product labelling claims, although the CLV and GRS systems could not be effectively differentiated by these analyses.

Proteomic and peptidomic analysis carried out on whole milk and digested whole milk samples in Chapter 6 displayed applicability for this purpose, as unique peptides were identified in samples from each feeding system using this methodology, which may act as suitable biomarkers for dairy products derived from these systems. Differences in the

hydrolysis and release of FAA and FFA due to diet could not be determined using the static *in-vitro* digestion method. However, the differences observed between feeding systems in this proteomic analysis and in concentrations of choline (Chapter 2) and vitamin B7 (Chapter 3) may indicate a diet-induced modulation of rumen synthesis and subsequent epithelial synthesis of these components, rather than direct transfer from feed to milk. In contrast, the respective levels of vitamins B1, B2, B3 and B3-amide (Chapter 3) and the trace minerals selenium and iodine (Chapters 4 and 6) were likely influenced by the respective levels of these components in each feed type.

Significant differences have previously been observed in the macroelement composition of milk derived from these feeding systems (Gulati *et al.*, 2018), but were limited to significantly higher concentrations of selenium and iodine present in each of the WMP, SMP and WPC products derived from TMR (Chapters 4 and 6). The levels of iodine observed were of particular note for exceeding maximum levels for IMF outlined in the Codex Alimentarius. The heat-dependent viscosity of SMP/WPC dispersions formulated to represent a first stage IMF emulsion was also determined in Chapter 4, with no significant differences observed between the diets; however, the differences in viscosity observed between the WPC types (i.e., sweet, micellar and acid WPC) were more substantial than between diets. Concentrations of NPN and the mineral profiles of each WPC also differed significantly, depending on production method.

7.2. Scientific and industrial impact of the research

This thesis formed part of a wider multidisciplinary research programme entitled: “Profiling milk from grass”, which sought to extensively characterise the compositional and functional properties of milk and dairy products derived from GRS, CLV and TMR feeding systems, encompassing numerous methodologies and aspects of dairy product quality. Within

this research programme, this thesis work was carried out to investigate the effect of these feeding systems on the composition and functional properties of milk protein ingredient powders, specifically. Extensive bovine dietary intervention studies have been carried out in other countries in recent decades (Stockdale, 1994; Mackle *et al.*, 1999; Chilliard *et al.*, 2007; Lourenço *et al.*, 2008), but full characterization of milk derived from the unique pasture-based production system practiced in Ireland, and a comparison of this system to conventional indoor systems practiced worldwide, was lacking. Additionally, modification of the standard perennial ryegrass sward grazed in Ireland to include white clover was investigated and compared with the other feeding systems.

The increased demand among consumers for pasture-derived dairy products in the past decade and associated increase in “pasture-fed” or “grass-fed” labelling claims among these products has necessitated the establishment of a universal standard in this regard. The “Grass-Fed Standard” (Bord Bia, 2020) introduced by Bord Bia, the Irish national food board, requires products displaying this label to be derived from the milk of cows receiving a minimum of 95% of their feed from grazed grass on a fresh weight basis, although the commercial point-of-difference for these products required robust experimental substantiation. The Grass-Fed Standard is perhaps most relevant to “business-to-consumer” end products such as butter and cheese, for which organoleptic qualities are most readily considered. Despite differences in the colour of WMPs derived from the different feeding systems being visually discernible and significant textural differences between yoghurts derived from these WMPs, the composition and functionality of value-added products intended for use as ingredients in further manufacturing processes will be of great interest to milk processors.

Regarding the relative composition of the products derived from each feeding system, clear distinctions were made between both the GRS and CLV systems and the TMR system on the basis of NPN content, metabolite concentrations, water-soluble vitamins, trace minerals

and fatty acid profile. The CLV samples were, predictably, similar to GRS; however, while the CLV system is of particular interest for its effects at primary production level on milk yield, milk solids yield and nitrogen efficiency (Egan *et al.*, 2018), it also has an industrially-relevant effect on concentrations of vitamins B2 and B7 and NPN, increases in which have implications for true protein content and the yield of protein-based products (DePeters & Ferguson, 1992). Indeed, the high NPN content of CLV and TMR samples was identified throughout this research as an important factor influencing the heat stability, viscosity and gelation of reconstituted powder products.

This research also demonstrated a significant effect of bovine diet on the functional properties of SMP, but a lack of significant effect on WPC functionality. This was in contrast to distinct differences in the mineral, nitrogen and whey protein composition between whey powder and WPC powders produced by different methods of casein removal, resulting in distinct rheological behaviours, particularly the greater thermal instability of AWPC. This data lends insight into understanding the heat-dependent behaviour of WPC derived from different product streams and will also be of interest to IMF manufacturers utilising SMP and WPC ingredients in product formulation and processing. The dietary effects observed in mineral, vitamin and AA composition on SMP and whey powders will similarly be of interest to IMF manufacturers for the purposes of ingredient selection.

The use of LC-MS/MS and Raman spectroscopy within this thesis provides two robust, high-throughput and rapid methodologies to distinguish between milk powder ingredients derived from pasture- and TMR-fed cows. These methods can be implemented for verification of pasture or concentrate-based feeding in products marketed using the established Grass-Fed Standard. This research also substantiates previous data (O'Callaghan *et al.*, 2016; Schroeder *et al.*, 2003) that demonstrated the effect of pasture and TMR feeding systems on the FA profile of milk, which is directly influenced by dietary factors, while providing further experimental

data to suggest that serum components (trace minerals and water-soluble vitamins) are also readily transferred from feed into milk. Furthermore, this research confirms the understanding that the amino acid and protein profiles of milk are largely unaffected by diet, but may otherwise be influenced by animal genetics and breeding, although some limited effects of diet on the protein profile were observed which may warrant further investigation. In addition, the unique peptides identified in WMP derived from each feeding system may be of nutritional interest for their potential physiological effects *in vivo* and of commercial interest as biomarkers for particular feeding systems.

7.3. Future considerations

This research has presented data relevant to academic interest in dairy chemistry and the dairy industry, particularly milk producers, processors and companies manufacturing “business-to-business” dairy commodities. Future work which could expand on the research established herein may be based on the following areas:

1. Various methods for detecting potential markers associated with milk derived from particular feeding systems are available and have been investigated throughout this thesis work and previous studies. An in-depth review providing comparison between these methods and their applicability for verification of feed-based product labelling claims would prove a useful resource for research bodies and those responsible for the establishment of food policy;
2. The major water-soluble vitamins present in milk protein ingredients were quantified as part of this research. Considering the high value of milk fat-based products, quantification of the fat-soluble vitamins in dairy products derived from different feeding systems will provide further insight into the nutritional value of “business-to-

consumer” foods which represent considerable economic value to the Irish export market;

3. Unique compositional factors and rheological behaviours were identified within the SWPC samples analysed in this work. Further work to elucidate the causes and effects of these aspects of SWPC may include; characterization of the macropeptides present in SWPC by mass-spectrometry or immunochemical analysis, investigation of the potential variation in the TCA solubility of GMP using sequential concentrations during NPN extraction, quantification of serum calcium enclosed within the rennet curd and the potential for heat-induced aggregation of GMP in SWPC dispersions. This will provide useful information for prediction of SWPC behaviour during processing and aid in ingredient selection for processors utilising same;
4. Similarly, unique compositional factors were identified in AWPC samples relative to the other WPC types. Recording full mineral and NPN mass balances during WPC production to account for potential losses and partitioning between fractions would provide further information relating to the processability and functionality of WPC products, particularly in the case of AWPC;
5. In cases of neutralization during industrial production of AWPC, NaOH is most commonly used and was identified as a cause of AWPC destabilization during heating in the present work. Examining the potential destabilizing effect of other alkaline compounds (e.g. KOH) in the neutralization of acid WPC and their comparative effects on composition and rheological properties, compared to NaOH may offer alternative options to ensure a more stable WPC product;
6. The protein ingredients analysed within this thesis are principal ingredients in the manufacture of IMF, which represents a key product for the Irish dairy export market, for which nutritional quality is a paramount consideration for the end-user. Production

and full characterization of model IMF powders/emulsions utilizing ingredients produced from different feeding systems will provide insight into the potential nutritional or economic benefits of selecting ingredients derived from the milk supplied from areas in which these systems are prevalent;

7. The static *in-vitro* digestion method carried out in the experimental work in Chapter 6 resulted in the near-complete hydrolysis of the fat and protein components of the WMP samples analysed. Dynamic *in-vitro* digestion of milk products from each feeding system, utilizing multiple sampling points, would allow for examination of the kinetics of AA and FA hydrolysis and peptide formation throughout digestion and any potential variation thereof due to cow diet, which may be of interest in future research focusing on the nutritional quality of milk from pasture or concentrate-based feeding systems;
8. Considerable variation occurs in the relative concentrations of the compositional elements of milk throughout the year in seasonal milk production systems, such as the representative GRS system analysed within this thesis. The gross composition and fatty acid profile of seasonally-produced milk has been extensively characterized in previous studies, but there remains scope for further characterization of seasonal variation in select minor milk components analysed throughout this research, such as water and fat-soluble vitamins, protein profile and amino acid profile;
9. Extensive research is currently being carried out to determine practicable means of reducing agricultural greenhouse gas emissions, including examination of the potential for alternative forage sources to reduce methane generation in ruminants, increase biodiversity and facilitate nitrogen fixation within soil. However, the influence of consumption of alternative pasture types and supplemental feeds on milk composition and quality will also need to be established in order to characterize or, where possible,

avoid significant changes in the processing behaviour, functionality and consumer acceptance of dairy products produced therefrom.

7.4 References

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Appendix i

Supplementary material

Table S1. Ingredient formulation (% as fed) and chemical composition (%) of TMR diet (Megalac concentrate sourced from Volac Ireland, Co. Cavan, Ireland).

TMR ingredient	% of ration
Soyabean meal 48% CP	20.9
Beet pulp unmolassed	20.0
Rolled Barley	16.0
Maize	15.0
Maize distillers	12.3
Rapeseed meal	11.5
Fat (vegetable)	1.0
Limestone Flour	1.1
Salt	0.8
Di Cal Phosphate	0.6
AcidBuf	0.5
Calcined Magnesite (Calmag)	0.2
Trace Element/Vitamin/Additive Pack*	0.1
* To supply	mg kg⁻¹ as fed (approx)
Iodine	1.8
Zinc	75
Selenium	0.5
Cobalt	1.5
Copper	38
Manganese	45
Yeast (Vitacel)	2000
Biotin	2.2
Vitamin E	60 iu
Vitamin D	2000 iu
Vitamin A	7500 iu
Chemical composition	(%)
Dry matter	87.3
UFL	0.11
Protein	23.5
PDIN	16.3
PDIE	13.8
Starch	21.1
Fibre	8.54
Oil	3.85
Ash	5.44
Sugar	5.54
Ca	1.17
P	0.63

UFL = Unité fourragère lait

PDIN = true protein absorbable in the small intestine when degradable N is limiting microbial protein synthesis in the rumen

PDIE = true protein absorbable in the small intestine when rumen fermentable energy (organic matter) is limiting microbial protein synthesis in the rumen

Table S2. Chemical composition (mean \pm SD) and nutritional content of silages from TMR diet (grass silage and maize silage) collected weekly throughout analysed by near-infrared spectroscopy.

Component		Grass silage	Maize silage
pH		4.4 (\pm 0.17)	3.8 (\pm 0.00)
Dry matter	%	39.6 (\pm 0.7)	23.8 (\pm 4.53)
Ash	%	9 (\pm 0.5)	4.37 (\pm 0.68)
Neutral detergent fibre	%	47.6 (\pm 1.82)	50.1 (\pm 0.4)
Starch	%	N/A	16.0 (\pm 4.05)
Crude protein	%	14.3 (\pm 1.21)	8.8 (\pm 2.07)
Metabolisable energy	MJ kg ⁻¹	10.6 (\pm 0.26)	10.5 (\pm 0.44)

N/A = not available

Table S3. Chemical composition (g kg⁻¹ of DM; mean \pm SD) and nutritional content of pasture systems (grass and clover) analysed by near-infrared spectroscopy.

Component	Grass	Clover
Organic matter digestibility	842 (\pm 28)	837 (\pm 23.5)
Acid detergent fibre	201 (\pm 2.69)	198 (\pm 7.07)
Neutral detergent fibre	369 (\pm 12.7)	345 (\pm 11.2)
Crude protein	189 (\pm 40.9)	185 (\pm 23.8)
Ash	72.5 (\pm 3.32)	77.3 (\pm 1.98)

Table S4: Average concentrations of total metabolites (μM) for skim milk powder derived from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

Metabolite (μM)	GRS	CLV	TMR
Acetylmornitine	0.97 (\pm 0.07)	0.60 (\pm 0.08)	0.88 (\pm 0.54)
Alanine	34.1 (\pm 2.90)	30.2 (\pm 3.32)	41.9 (\pm 1.20)
alpha-Aminoadipic acid	6.86 (\pm 0.47)	5.45 (\pm 2.26)	7.42 (\pm 1.83)
Arginine	14.2 (\pm 0.42)	14.6 (\pm 1.70)	16.6 (\pm 0.14)
Asparagine	1.41 (\pm 0.84)	2.11*	1.29 (\pm 0.16)
Aspartic acid	10.2 (\pm 3.34)	17.3 (\pm 4.38)	11.5 (\pm 2.38)
Asymmetric dimethylarginine	0.09 (\pm 0.09)	0.05 (\pm 0.03)	0.09 (\pm 0.01)
Betaine	74.2 (\pm 20.01)	61.0 (\pm 19.30)	94.2 (\pm 12.52)
Carnosine	0.35 (\pm 0.02)	0.40 (\pm 0.04)	0.47 (\pm 0.08)
Choline	1245 (\pm 190.92)	1024 (\pm 319.61)	1035 (\pm 35.36)
cis-4-Hydroxyproline	0.31 (\pm 0.00)	0.34 (\pm 0.01)	0.32 (\pm 0.01)
Citrulline	1.89 (\pm 0.16)	1.71 (\pm 0.94)	2.29 (\pm 0.47)
Creatine	512 (\pm 7.07)	507 (\pm 4.24)	567 (\pm 65.76)
Creatinine	111 (\pm 2.83)	93.1 (\pm 14.00)	92.6 (\pm 3.82)
Diacetylspermine	0.03 (\pm 0.00)	0.03 (\pm 0.00)	0.03 (\pm 0.00)
Dihydroxyphenylalanine	0.18 (\pm 0.02)	0.16 (\pm 0.00)	0.15 (\pm 0.02)
Dopamine	0.08 (\pm 0.00)	0.08 (\pm 0.00)	0.08 (\pm 0.00)
Glutamic acid	256(\pm 7.07)	284 (\pm 12.73)	241 (\pm 7.07)
Glutamine	4.38 (\pm 0.54) ^a	4.95 (\pm 1.24) ^a	12.4 (\pm 0.28) ^b
Glycine	65.0 (\pm 28.35)	54.3 (\pm 13.86)	74.1 (\pm 16.55)
Histidine	2.35 (\pm 0.57)	2.37 (\pm 0.42)	3.41 (\pm 0.43)
Isoleucine	2.49 (\pm 0.62)	2.40 (\pm 0.03)	4.32 (\pm 0.23)
Leucine	2.67 (\pm 1.14)	3.10 (\pm 0.95)	5.59 (\pm 1.20)
Lysine	15.8 (\pm 2.05)	13.8 (\pm 1.48)	12.9 (\pm 0.28)
Methionine	0.66 (\pm 0.03)	0.71 (\pm 0.18)	0.42 (\pm 0.08)
Methionine sulfoxide	0.07*	0.00*	0.22 (\pm 0.13)
Methylhistidine	1.20 (\pm 0.15)	0.99 (\pm 0.07)	1.00 (\pm 0.07)
Ornithine	2.24 (\pm 0.68)	2.11 (\pm 0.87)	4.14 (\pm 0.57)
Phenylalanine	1.44 (\pm 0.01)	1.19 (\pm 0.06)	1.42 (\pm 0.00)
Phosphocreatine	8.05 (\pm 1.70) ^{ab}	6.32 (\pm 0.56) ^a	16.3 (\pm 4.45) ^b
Proline	15.3 (\pm 0.71)	14.0 (\pm 0.64)	19.1 (\pm 1.41)
Putrescine	0.07 (\pm 0.00)	0.08 (\pm 0.01)	0.04 (\pm 0.00)
Sarcosine	0.48 (\pm 0.05)	0.56 (\pm 0.03)	0.42 (\pm 0.09)
Serine	22.1 (\pm 0.78) ^b	18.3 (\pm 1.20) ^{ab}	10.0 (\pm 0.55) ^a
Serotonin	0.01 (\pm 0.00)	0.01 (\pm 0.00)	0.01 (\pm 0.00)
Spermidine	0.83 (\pm 0.13)	0.77 (\pm 0.06)	0.82 (\pm 0.03)
Spermine	0.48 (\pm 0.05)	0.50 (\pm 0.13)	0.59 (\pm 0.02)
Symmetric dimethylarginine	4.36 (\pm 0.28)	3.26 (\pm 0.24)	3.38 (\pm 0.87)
Taurine	26.5 (\pm 2.69)	25.0 (\pm 1.84)	30.3 (\pm 1.84)
Threonine	4.49 (\pm 0.61)	3.82 (\pm 0.35)	4.99 (\pm 0.67)
Total dimethylarginine	4.45 (\pm 0.19)	3.31 (\pm 0.21)	3.47 (\pm 0.86)
trans-4-Hydroxyproline	4.39 (\pm 0.04)	4.25 (\pm 0.15)	4.53 (\pm 0.22)

Appendix i

Trimethylamine N-oxide	3.35 (\pm 0.69)	2.83 (\pm 0.08)	3.22 (\pm 0.02)
Tryptophan	0.83 (\pm 0.02)	0.74 (\pm 0.06)	0.83 (\pm 0.11)
Tyramine	0.01*	0.01 (\pm 0.01)	0.00 (\pm 0.00)
Tyrosine	0.31 (\pm 0.01)	0.28 (\pm 0.08)	0.64 (\pm 0.02)
Valine	7.49 (\pm 0.12) ^a	7.27 (\pm 1.09) ^a	11.3 (\pm 1.34) ^b

^{a,b} indicates values within a row not sharing a common superscript letter differed significantly ($p < 0.05$).

* denotes where a replicate was below the limit of detection or limit of quantification.

Table S5: Average concentrations of total metabolites (μM) for sweet whey powder derived from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

Metabolite (μM)	GRS	CLV	TMR
Acetylmornitine	1.26 (\pm 0.25)	0.87 (\pm 0.15)	0.80 (\pm 0.04)
Alanine	44.6 (\pm 7.64)	35.2 (\pm 2.90)	39.2 (\pm 5.30)
alpha-Aminoadipic acid	10.6 (\pm 3.15)	9.32 (\pm 2.67)	7.69 (\pm 1.15)
Arginine	16.0 (\pm 2.90)	14.7 (\pm 0.00)	14.9 (\pm 0.64)
Asparagine	1.25*	1.70*	3.24 (\pm 1.13)
Aspartic acid	10.5 (\pm 0.28)	18.7 (\pm 11.31)	10.2 (\pm 0.66)
Asymmetric dimethylarginine	0.18 (\pm 0.06)	0.04 (\pm 0.02)	0.06 (\pm 0.04)
Betaine	80.7 (\pm 16.48)	63.9 (\pm 23.05)	73.0 (\pm 19.09)
Carnosine	0.40 (\pm 0.06)	0.39 (\pm 0.04)	0.48 (\pm 0.08)
Choline	1415 (\pm 219.20)	1224 (\pm 574.17)	892 (\pm 1.41)
cis-4-Hydroxyproline	0.34 (\pm 0.00)	0.33 (\pm 0.01)	0.32 (\pm 0.01)
Citrulline	1.50 (\pm 0.28)	1.38 (\pm 1.20)	2.46 (\pm 0.69)
Creatine	761 (\pm 108.19)	543 (\pm 26.16)	561 (\pm 101.82)
Creatinine	115 (\pm 14.85)	93.6 (\pm 6.93)	72.9 (\pm 5.59)
Diacetylspermine	0.03 (\pm 0.00)	0.03 (\pm 0.00)	0.03 (\pm 0.00)
Dihydroxyphenylalanine	0.21 (\pm 0.02)	0.17 (\pm 0.02)	0.17 (\pm 0.00)
Dopamine	0.08 (\pm 0.00)	0.08*	0.08 (\pm 0.00)
Glutamic acid	318 (\pm 46.67)	297 (\pm 33.23)	238 (\pm 9.19)
Glutamine	1.36* ^a	0.187 (\pm 0.02) ^a	7.02 (\pm 3.20) ^b
Glycine	93.9 (\pm 55.37)	61.7 (\pm 1.13)	47.8 (\pm 11.17)
Histidine	2.61 (\pm 0.74)	2.50 (\pm 0.16)	3.11 (\pm 0.53)
Isoleucine	3.10 (\pm 0.71)	2.49 (\pm 0.39)	3.81 (\pm 0.28)
Leucine	3.20 (\pm 0.48)	3.24 (\pm 0.24)	4.16 (\pm 2.14)
Lysine	18.6 (\pm 2.90)	14.0 (\pm 2.12)	12.5 (\pm 1.13)
Methionine	1.34 (\pm 0.08)	1.23 (\pm 0.04)	0.95 (\pm 0.14)
Methionine sulfoxide	0.42 (\pm 0.14)	0.47 (\pm 0.06)	0.25 (\pm 0.31)
Methylhistidine	1.26 (\pm 0.25)	1.13 (\pm 0.28)	0.92 (\pm 0.04)
Ornithine	2.89 (\pm 1.20)	1.92 (\pm 0.03)	2.76 (\pm 0.28)
Phenylalanine	1.72 (\pm 0.08)	1.51 (\pm 0.06)	1.46 (\pm 0.02)
Phosphocreatine	9.65 (\pm 0.93) ^a	6.49 (\pm 0.70) ^a	22.6 (\pm 6.01) ^b
Proline	16.8 (\pm 2.33)	15.5 (\pm 0.07)	18.3 (\pm 0.49)
Putrescine	0.06 (\pm 0.01)	0.06 (\pm 0.02)	0.04 (\pm 0.04)
Sarcosine	0.54 (\pm 0.21)	0.47 (\pm 0.17)	0.33 (\pm 0.03)
Serine	25.3 (\pm 3.11) ^b	18.7 (\pm 2.33) ^{a,b}	9.31 (\pm 0.64) ^a
Serotonin	0.01 (\pm 0.00)	0.01 (\pm 0.00)	0.01 (\pm 0.00)
Spermidine	0.34 (\pm 0.04)	0.35 (\pm 0.04)	0.30 (\pm 0.00)
Spermine	0.05 (\pm 0.00)	0.06 (\pm 0.03)	0.08 (\pm 0.01)
Symmetric dimethylarginine	4.60 (\pm 0.95)	3.30 (\pm 0.63)	3.06 (\pm 0.39)
Taurine	29.8 (\pm 6.36)	28.4 (\pm 0.49)	30.5 (\pm 1.06)
Threonine	5.19 (\pm 1.20)	3.41 (\pm 0.50)	4.41 (\pm 0.18)
Total dimethylarginine	4.79 (\pm 1.01)	3.35 (\pm 0.64)	3.12 (\pm 0.43)
trans-4-Hydroxyproline	4.84 (\pm 0.30)	4.45 (\pm 0.17)	4.41 (\pm 0.37)

Appendix i

Trimethylamine N-oxide	3.33 (\pm 0.41)	3.19 (\pm 0.01)	3.32 (\pm 0.35)
Tryptophan	0.89 (\pm 0.35)	0.79 (\pm 0.01)	0.70 (\pm 0.10)
Tyramine	0.01 (\pm 0.00)	0.01 (\pm 0.00)	0.01 (\pm 0.00)
Tyrosine	0.42 (\pm 0.26)	0.28 (\pm 0.02)	0.51 (\pm 0.22)
Valine	8.50 (\pm 2.83)	7.78 (\pm 0.72)	10.6 (\pm 0.00)

^{a,b} indicates values within a row not sharing a common superscript letter differed significantly ($p < 0.05$).

* denotes where a replicate was below the limit of detection or limit of quantification.

Table S6: Average concentrations of total metabolites (μM) for micellar casein whey powder derived from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

Metabolite (μM)	GRS	CLV	TMR
Acetylmornitine	0.96 (\pm 0.30)	0.98 (\pm 0.22)	0.78 (\pm 0.07)
Alanine	36.9 (\pm 4.03)	33.0 (\pm 1.84)	42.3 (\pm 4.03)
alpha-Aminoadipic acid	7.56 (\pm 1.16)	6.01 (\pm 2.51)	6.00 (\pm 0.57)
Arginine	13.4 (\pm 1.20)	13.8 (\pm 0.21)	15.6 (\pm 0.21)
Asparagine	1.45 (\pm 0.37)	0.34*	3.18 (\pm 0.95)
Aspartic acid	11.6 (\pm 0.99)	10.8 (\pm 2.93)	15.2 (\pm 2.19)
Asymmetric dimethylarginine	0.07 (\pm 0.03)	0.06 (\pm 0.02)	0.15 (\pm 0.01)
Betaine	85.5 (\pm 17.96)	66.8 (\pm 9.90)	83.8 (\pm 25.81)
Carnosine	0.38 (\pm 0.04)	0.37 (\pm 0.00)	0.43 (\pm 0.01)
Choline	1225 (\pm 120.21)	1020 (\pm 282.84)	906 (\pm 19.80)
cis-4-Hydroxyproline	0.33 (\pm 0.01)	0.31 (\pm 0.01)	0.32 (\pm 0.01)
Citrulline	1.40 (\pm 0.25)	1.67 (\pm 0.13)	2.66 (\pm 0.16)
Creatine	513 (\pm 29.70)	519 (\pm 12.02)	500 (\pm 55.86)
Creatinine	125 (\pm 12.02)	107 (\pm 17.47)	101 (\pm 8.84)
Diacetylspermine	0.03 (\pm 0.00)	0.03 (\pm 0.00)	0.03 (\pm 0.00)
Dihydroxyphenylalanine	0.18 (\pm 0.01)	0.18 (\pm 0.01)	0.15 (\pm 0.00)
Dopamine	0.08 (\pm 0.00)	0.08 (\pm 0.00)	0.08 (\pm 0.00)
Glutamic acid	266 (\pm 27.58)	269 (\pm 70.00)	282 (\pm 26.87)
Glutamine	4.04 (\pm 0.23) ^a	3.19 (\pm 1.77) ^a	11.5 (\pm 2.64) ^b
Glycine	66.8 (\pm 15.49)	61.2 (\pm 5.37)	56.4 (\pm 7.85)
Histidine	2.35 (\pm 0.50)	2.39 (\pm 0.45)	3.02 (\pm 0.17)
Isoleucine	2.76 (\pm 0.08)	2.78 (\pm 0.44)	3.86 (\pm 0.38)
Leucine	2.56 (\pm 1.63)	2.68 (\pm 1.61)	5.54 (\pm 0.10)
Lysine	15.8 (\pm 1.20)	12.8 (\pm 0.64)	13.2 (\pm 2.33)
Methionine	0.41 (\pm 0.29)	0.32 (\pm 0.37)	0.31 (\pm 0.07)
Methionine sulfoxide	0.20 (\pm 0.09)	0.29 (\pm 0.07)	0.35 (\pm 0.04)
Methylhistidine	1.26 (\pm 0.13)	0.92 (\pm 0.11)	0.83 (\pm 0.03)
Ornithine	2.54 (\pm 0.64)	2.37 (\pm 1.01)	2.79 (\pm 0.75)
Phenylalanine	1.46 (\pm 0.08)	1.38 (\pm 0.06)	1.38 (\pm 0.01)
Phosphocreatine	7.21 (\pm 1.64) ^a	5.37 (\pm 0.61) ^a	17.1 (\pm 1.41) ^b
Proline	15.8 (\pm 0.07)	14.7 (\pm 1.27)	19.0 (\pm 1.13)
Putrescine	0.05 (\pm 0.01)	0.05 (\pm 0.04)	0.02 (\pm 0.02)
Sarcosine	0.56 (\pm 0.12)	0.51 (\pm 0.04)	0.48 (\pm 0.05)
Serine	21.9 (\pm 1.13) ^b	18.8 (\pm 1.27) ^{a,b}	9.62 (\pm 1.10) ^a
Serotonin	0.01 (\pm 0.00)	0.01 (\pm 0.00)	0.01 (\pm 0.00)
Spermidine	0.29 (\pm 0.04)	0.26 (\pm 0.05)	0.24 (\pm 0.06)
Spermine	0.07 (\pm 0.01)	0.07 (\pm 0.01)	0.07 (\pm 0.00)
Symmetric dimethylarginine	3.82 (\pm 0.08)	2.80 (\pm 0.46)	2.73 (\pm 0.25)
Taurine	26.9 (\pm 2.33)	26.4 (\pm 1.56)	29.6 (\pm 1.70)
Threonine	4.88 (\pm 0.09)	3.90 (\pm 0.55)	4.08 (\pm 1.00)
Total dimethylarginine	3.90 (\pm 0.05)	2.86 (\pm 0.48)	2.88 (\pm 0.26)
trans-4-Hydroxyproline	4.21 (\pm 0.40)	4.03 (\pm 0.76)	4.53 (\pm 0.07)

Appendix i

Trimethylamine N-oxide	2.77 (\pm 0.30)	2.82 (\pm 0.04)	3.10 (\pm 0.29)
Tryptophan	0.70 (\pm 0.06)	0.64 (\pm 0.11)	0.75 (\pm 0.02)
Tyramine	0.01 (\pm 0.00)	0.00*	0.01 (\pm 0.01)
Tyrosine	0.27 (\pm 0.04)	0.28 (\pm 0.16)	0.48 (\pm 0.18)
Valine	7.00 (\pm 0.74) ^a	7.92 (\pm 1.43) ^{a,b}	10.6 (\pm 0.07) ^b

^{a,b} indicates values within a row not sharing a common superscript letter differed significantly ($p < 0.05$).

* denotes where a replicate was below the limit of detection or limit of quantification.

Table S7: Average concentrations of total metabolites (μM) for acid whey powder derived from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

Metabolite (μM)	GRS	CLV	TMR
Acetylmornitine	4.18 (\pm 0.16)	2.58 (\pm 0.11)	2.36 (\pm 1.53)
Alanine	27.7 (\pm 2.19)	34.1 (\pm 5.37)	40.6 (\pm 3.04)
alpha-Aminoadipic acid	23.9 (\pm 7.14)	28.0 (\pm 28.61)	40.8 (\pm 2.26)
Arginine	12.9 (\pm 1.63)	16.4 (\pm 3.11)	16.0 (\pm 1.13)
Asparagine	2.98 (\pm 0.16)	0.32*	1.32 (\pm 0.12)
Aspartic acid	6.98 (\pm 1.03)	13.3 (\pm 12.47)	4.95 (\pm 0.34)
Asymmetric dimethylarginine	0.06 (\pm 0.01)	0.07 (\pm 0.00)	0.11 (\pm 0.04)
Betaine	75.9 (\pm 26.87)	70.7 (\pm 37.19)	84.9 (\pm 6.43)
Carnosine	0.33 (\pm 0.09)	0.30 (\pm 0.06)	0.39 (\pm 0.01)
Choline	1160 (\pm 98.99)	1076 (\pm 501.34)	950 (\pm 84.85)
cis-4-Hydroxyproline	0.31 (\pm 0.00)	0.31*	0.33*
Citrulline	0.33 (\pm 0.13)	0.66 (\pm 0.11)	0.86 (\pm 0.32)
Creatine	483 (\pm 60.10)	661 (\pm 119.50)	612 (\pm 144.96)
Creatinine	99.4 (\pm 3.68)	104 (\pm 14.35)	94.0 (\pm 5.52)
Diacetylspermine	0.03 (\pm 0.00)	0.03 (\pm 0.00)	0.03 (\pm 0.00)
Dihydroxyphenylalanine	0.14 (\pm 0.01)	0.12 (\pm 0.01)	0.15 (\pm 0.05)
Dopamine	0.08 (\pm 0.00)	0.08 (\pm 0.00)	0.08 (\pm 0.00)
Glutamic acid	262 (\pm 37.48)	255 (\pm 69.30)	234 (\pm 25.46)
Glutamine	2.39 (\pm 1.74) ^a	2.97 (\pm 1.07) ^a	12.3 (\pm 0.42) ^b
Glycine	56.1 (\pm 9.62)	57.3 (\pm 0.28)	41.2 (\pm 0.49)
Histidine	2.34 (\pm 0.55)	2.49 (\pm 0.01)	3.21 (\pm 0.15)
Isoleucine	2.80 (\pm 0.02)	2.58 (\pm 1.53)	3.73 (\pm 1.13)
Leucine	12.0 (\pm 5.82)	8.28 (\pm 3.57)	14.6 (\pm 0.71)
Lysine	12.7 (\pm 4.04)	12.5 (\pm 1.13)	11.4 (\pm 7.95)
Methionine	0.47 (\pm 0.10)	0.49 (\pm 0.10)	0.43 (\pm 0.24)
Methionine sulfoxide	0.16 (\pm 0.21)	0.44 (\pm 0.25)	0.14 (\pm 0.08)
Methylhistidine	1.27 (\pm 0.14)	1.36 (\pm 0.13)	0.97 (\pm 0.16)
Ornithine	3.22 (\pm 0.83)	2.68 (\pm 0.04)	3.35 (\pm 0.82)
Phenylalanine	1.22 (\pm 0.09)	1.09 (\pm 0.21)	1.41 (\pm 0.29)
Phosphocreatine	3.56 (\pm 0.24)	3.66 (\pm 0.84)	4.35 (\pm 0.47)
Proline	14.8 (\pm 2.47)	14.7 (\pm 2.12)	19.1 (\pm 2.26)
Putrescine	0.08 (\pm 0.02)	0.05*	0.01*
Sarcosine	0.41 (\pm 0.06)	0.39 (\pm 0.01)	0.40 (\pm 0.01)
Serine	20.2 (\pm 0.64)	17.5 (\pm 8.27)	11.2 (\pm 2.63)
Serotonin	0.01 (\pm 0.00)	0.01 (\pm 0.00)	0.01 (\pm 0.00)
Spermidine	0.71 (\pm 0.04)	0.58 (\pm 0.15)	0.56 (\pm 0.12)
Spermine	0.26 (\pm 0.07)	0.22 (\pm 0.08)	0.30 (\pm 0.21)
Symmetric dimethylarginine	3.40 (\pm 0.38)	2.94 (\pm 1.58)	2.77 (\pm 0.03)
Taurine	26.0 (\pm 5.16)	26.0 (\pm 3.04)	30.0 (\pm 3.46)
Threonine	1.69 (\pm 0.76)	1.02 (\pm 0.40)	1.28 (\pm 0.41)
Total dimethylarginine	3.46 (\pm 0.37)	3.01 (\pm 1.58)	2.88 (\pm 0.07)
trans-4-Hydroxyproline	4.09 (\pm 1.12)	4.01 (\pm 0.11)	4.48 (\pm 0.38)

Appendix i

Trimethylamine N-oxide	2.57 (\pm 0.05)	3.21 (\pm 0.69)	3.13 (\pm 0.48)
Tryptophan	0.45 (\pm 0.11)	0.57 (\pm 0.10)	0.58 (\pm 0.12)
Tyramine	0.01 (\pm 0.00)	0.00 (\pm 0.00)	0.00*
Tyrosine	0.00*	0.00*	0.08*
Valine	6.80 (\pm 1.97) ^a	7.29 (\pm 0.69) ^a	12.0 (\pm 1.63) ^b

^{a,b} indicates values within a row not sharing a common superscript letter differed significantly ($p < 0.05$).

* denotes where a replicate was below the limit of detection or limit of quantification.

Table S8: Average concentrations of total metabolites (μM) from skim milk powder, sweet whey powder, micellar casein whey powder and acid whey powder derived from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

Metabolite (μM)	GRS	CLV	TMR
Acetylmornitine	1.84 (\pm 1.57)	1.26 (\pm 0.90)	1.20 (\pm 0.77)
Alanine	35.8 (\pm 7.02) ^{a,b}	33.1 (\pm 2.15) ^a	41.0 (\pm 1.40) ^b
alpha-Aminoadipic acid	12.2 (\pm 7.93)	12.2 (\pm 10.66)	15.5 (\pm 16.90)
Arginine	14.1 (\pm 1.36)	14.9 (\pm 1.11)	15.8 (\pm 0.74)
Asparagine	1.77 (\pm 0.81) ^{a,b}	1.12 (\pm 0.92) ^a	2.26 (\pm 1.10) ^b
Aspartic acid	9.83 (\pm 1.99)	15.0 (\pm 3.62)	10.5 (\pm 4.22)
Asymmetric dimethylarginine	0.10 (\pm 0.05)	0.06 (\pm 0.01)	0.10 (\pm 0.04)
Betaine	79.1 (\pm 5.10) ^{a,b}	65.6 (\pm 4.16) ^a	83.9 (\pm 8.66) ^b
Carnosine	0.36 (\pm 0.03) ^a	0.36 (\pm 0.05) ^a	0.44 (\pm 0.04) ^b
Choline	1261 (\pm 108.73) ^b	1085 (\pm 95.49) ^{a,b}	945 (\pm 64.43) ^a
cis-4-Hydroxyproline	0.32 (\pm 0.01)	0.32 (\pm 0.01)	0.32 (\pm 0.01)
Citrulline	1.28 (\pm 0.67) ^a	1.35 (\pm 0.49) ^a	2.07 (\pm 0.82) ^b
Creatine	567 (\pm 130.27)	557 (\pm 70.49)	559 (\pm 46.08)
Creatinine	112 (\pm 10.36) ^b	99.3 (\pm 6.97) ^{a,b}	90.1 (\pm 11.96) ^a
Diacetylspermine	0.03 (\pm 0.00)	0.03 (\pm 0.00)	0.03 (\pm 0.00)
Dihydroxyphenylalanine	0.18 (\pm 0.03)	0.16 (\pm 0.03)	0.16 (\pm 0.01)
Dopamine	0.08 (\pm 0.00)	0.08 (\pm 0.00)	0.08 (\pm 0.00)
Glutamic acid	275 (\pm 28.76)	276 (\pm 18.09)	248 (\pm 22.43)
Glutamine	3.04 (\pm 1.42) ^a	2.82 (\pm 1.97) ^a	10.8 (\pm 2.56) ^b
Glycine	70.4 (\pm 16.30)	58.6 (\pm 3.49)	54.9 (\pm 14.26)
Histidine	2.41 (\pm 0.13) ^a	2.44 (\pm 0.07) ^a	3.18 (\pm 0.17) ^b
Isoleucine	2.78 (\pm 0.25) ^a	2.56 (\pm 0.16) ^a	3.93 (\pm 0.27) ^b
Leucine	5.10 (\pm 4.60) ^{a,b}	4.32 (\pm 2.65) ^a	7.47 (\pm 4.80) ^b
Lysine	15.7 (\pm 2.37)	13.3 (\pm 0.74)	12.5 (\pm 0.78)
Methionine	0.72 (\pm 0.42)	0.69 (\pm 0.40)	0.53 (\pm 0.29)
Methionine sulfoxide	0.21 (\pm 0.15)	0.40 (\pm 0.10)	0.24 (\pm 0.09)
Methylhistidine	1.25 (\pm 0.03) ^b	1.10 (\pm 0.19) ^{a,b}	0.93 (\pm 0.07) ^a
Ornithine	2.72 (\pm 0.42) ^{a,b}	2.27 (\pm 0.33) ^a	3.26 (\pm 0.65) ^b
Phenylalanine	1.46 (\pm 0.20) ^b	1.29 (\pm 0.19) ^a	1.41 (\pm 0.03) ^{a,b}
Phosphocreatine	7.12 (\pm 2.58) ^a	5.46 (\pm 1.30) ^a	15.1 (\pm 7.74) ^b
Proline	15.6 (\pm 0.85) ^a	14.7 (\pm 0.61) ^a	18.9 (\pm 0.41) ^b
Putrescine	0.06 (\pm 0.01) ^b	0.06 (\pm 0.02) ^b	0.03 (\pm 0.02) ^a
Sarcosine	0.50 (\pm 0.07)	0.48 (\pm 0.07)	0.41 (\pm 0.06)
Serine	22.4 (\pm 2.15) ^c	18.3 (\pm 0.60) ^b	10.0 (\pm 0.85) ^a
Serotonin	0.01 (\pm 0.00)	0.01 (\pm 0.00)	0.01 (\pm 0.00)
Spermidine	0.54 (\pm 0.27)	0.49 (\pm 0.23)	0.48 (\pm 0.27)
Spermine	0.21 (\pm 0.20)	0.21 (\pm 0.21)	0.26 (\pm 0.24)
Symmetric dimethylarginine	4.04 (\pm 0.54) ^b	3.08 (\pm 0.25) ^a	2.98 (\pm 0.30) ^a
Taurine	27.3 (\pm 1.72) ^{a,b}	26.4 (\pm 1.41) ^a	30.1 (\pm 0.38) ^b
Threonine	4.06 (\pm 1.61) ^b	3.03 (\pm 1.36) ^a	3.69 (\pm 1.65) ^{a,b}
Total dimethylarginine	4.15 (\pm 0.59) ^b	3.13 (\pm 0.24) ^a	3.09 (\pm 0.28) ^a

Appendix i

trans-4-Hydroxyproline	4.38 (\pm 0.33)	4.18 (\pm 0.21)	4.49 (\pm 0.06)
Trimethylamine N-oxide	3.00 (\pm 0.40)	3.01 (\pm 0.22)	3.19 (\pm 0.10)
Tryptophan	0.72 (\pm 0.19)	0.68 (\pm 0.10)	0.71 (\pm 0.10)
Tyramine	0.01 (\pm 0.00)	0.01 (\pm 0.00)	0.01 (\pm 0.00)
Tyrosine	0.33 (\pm 0.08) ^{a,b}	0.28 (\pm 0.00) ^a	0.43 (\pm 0.24) ^b
Valine	7.45 (\pm 0.76) ^a	7.56 (\pm 0.34) ^a	11.09 (\pm 0.66) ^b

^{a,b} indicates values within a row not sharing a common superscript letter differed significantly ($p < 0.05$).

Table S9: Validation parameters for water-soluble vitamin determination by LC-MS/MS.

		Vitamin B1	Vitamin B2	Vitamin B3	Vitamin B3- amide	Vitamin B5	Vitamin B6- Pyridoxine	Vitamin B7
Calibration Ranges (μM)		0.01 - 1	0.01 - 1	0.1 - 10	0.05 - 5	0.05 - 5	0.01 - 1	0.01 - 1
Calibration Regression R^2		0.9974	0.9985	0.9999	0.9994	0.9995	0.9965	0.9997
Quality control standard concentrations (μM)	QC1	0.08	0.08	0.8	0.4	0.4	0.08	0.08
	QC2	0.25	0.25	2.5	1.25	1.25	0.25	0.25
	QC3	0.75	0.75	7.5	3.75	3.75	0.75	0.75
Accuracy (%)	Low	104	93.1	96	99.6	110	105	106
	Mid	112	105	108	114	108	115	107
	High	104	107.5	107	102	107	98.5	100
Precision (%)	Low	0.51	13.58	2.67	0.93	8.23	8.75	3.88
	Mid	2.27	5.39	2.30	4.51	6.39	5.47	4.55
	High	1.38	7.66	6.51	7.03	2.38	3.50	1.12
Recovery (%)	Low	109	92.4	92.3	101	105	111	103
	Mid	107	96.7	102	111	91.6	104	105
	High	96.2	90.2	91.7	100	97.6	104	102
Limit of detection (μM)		0.0006	0.0008	0.0330	0.0060	0.0010	0.0003	0.0007
Limit of quantitation (μM)		0.0020	0.0027	0.1100	0.0200	0.0033	0.0010	0.0023

Table S10: Average concentrations (μM) of water-soluble vitamins for reconstituted skim milk (9.5% total solids), sweet whey (6.5% total solids), micellar casein whey (6.5% total solids) and acid whey (6.5% total solids) powders derived from the milk of Holstein-Friesian cows assigned to perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

Sample type	Water-soluble vitamin (μM)	GRS	CLV	TMR
Skim milk powder	B1	0.72 ^b	0.72 ^b	0.55 ^a
	B2	39.4 ^b	40.5 ^b	22.6 ^a
	B3	0.24	0.18	0.26
	B3-amide	4.42	4.44	5.00
	B5	18.0	18.9	19.3
	B6-Pyridoxine	0.03	0.02	0.03
	B7	0.07	0.08	0.03
Sweet whey powder	B1	0.65	0.63	0.83
	B2	23.2 ^b	22.1 ^b	10.2 ^a
	B3	0.22 ^a	0.16 ^a	0.27 ^b
	B3-amide	3.60	3.99	4.06
	B5	19.8	18.8	18.7
	B6-Pyridoxine	0.02	0.03	0.03
	B7	0.07 ^{a,b}	0.08 ^b	0.03 ^a
Micellar casein whey powder	B1	0.45	0.36	0.41
	B2	2.60 ^b	2.71 ^b	1.17 ^a
	B3	0.19	0.16	0.21
	B3-amide	4.88	5.40	5.80
	B5	20.3	21.4	19.1
	B6-Pyridoxine	0.02	0.02	0.02
	B7	0.08 ^b	0.10 ^b	0.04 ^a
Acid whey powder	B1	0.56	0.43	0.52
	B2	1.05	1.13	0.62
	B3	0.23	0.17	0.31
	B3-amide	4.37 ^a	4.78 ^{a,b}	4.98 ^b
	B5	20.5	19.4	20.1
	B6-Pyridoxine	0.03	0.02	0.02
	B7	0.07 ^b	0.08 ^b	0.04 ^a

Values are presented as the mean of duplicate samples. GRS – Cows fed perennial ryegrass only. CLV – Cows fed perennial ryegrass / white clover. TMR – Cows fed total mixed ration ad libitum. Vitamins: B1 – Thiamine, B2 – Riboflavin, B3 – Nicotinic acid, B3-amide – Nicotinamide, B5 – Pantothenic acid, B7 – Biotin.

Note: Only the pyridoxine form of vitamin B6 is represented in the data.

^{a,b} different superscripts within a row indicate significant differences ($P < 0.05$).

Table S11: Average concentrations (μM) of total water-soluble vitamins for reconstituted sweet whey (6.5% total solids), micellar casein whey (6.5% total solids) and acid whey (6.5% total solids) powders derived from the milk of Holstein-Friesian cows assigned to each feeding system, determined by LC-MS/MS.

Water-soluble vitamin (μM)	Sweet whey powder	Micellar casein whey powder	Acid whey powder
B1	0.70 ^b	0.41 ^a	0.50 ^a
B2	18.5 ^b	2.16 ^a	0.93 ^a
B3	0.22	0.18	0.23
B3-amide	3.88 ^a	5.36 ^b	4.71 ^b
B5	19.1	20.2	20.0
B6-Pyridoxine	0.03	0.02	0.03
B7	0.06	0.07	0.06

Values are presented as the mean of duplicate samples. Vitamins: B1 – Thiamine, B2 – Riboflavin, B3 – Nicotinic acid, B3-amide – Nicotinamide, B5 – Pantothenic acid, B7 – Biotin. Note: Only the pyridoxine form of vitamin B6 is represented in the data.

^{a,b} different superscripts within a row indicate significant differences ($P < 0.05$).

Table S12. Average concentrations ($\mu\text{g/g}$ protein) of water-soluble vitamins for sweet whey, micellar casein whey and acid whey powders derived from the milk of Holstein-Friesian cows assigned to from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

Diet	Ingredient type	Water-soluble vitamin ($\mu\text{g/g}$ protein)						
		B1	B2	B3	B3-amide	B5	B6-Pyridoxine	B7
GRS	Sweet whey powder	29.5 ^{a,b,c,d}	1489 ^c	4.60	74.9 ^a	738	0.71	3.04 ^{a,b,c}
	Micellar casein whey powder	23.2 ^{a,b,c}	191 ^{a,b}	4.48	117 ^{a,b,c}	869	0.77	3.97 ^c
	Acid whey powder	30.9 ^{a,b,c,d}	81.7 ^a	5.89	110 ^{a,b,c}	929	1.03	3.55 ^{a,b,c}
CLV	Sweet whey powder	27.9 ^{a,b,c,d}	1400 ^c	3.37	82.0 ^{a,b}	695	0.77	3.12 ^{b,c}
	Micellar casein whey powder	22.1 ^a	232 ^{a,b}	4.51	150 ^{b,c}	1067	0.86	5.65 ^c
	Acid whey powder	22.4 ^{a,b}	83.8 ^a	4.03	115 ^{a,b,c}	843	0.77	3.75 ^{b,c}
TMR	Sweet whey powder	36.3 ^d	636 ^b	5.41	81.9 ^{a,b}	675	0.77	1.23 ^a
	Micellar casein whey powder	20.9 ^a	85.2 ^a	4.91	137 ^c	811	0.74	1.77 ^{a,b}
	Acid whey powder	29.4 ^{a,b,c}	49.6 ^a	8.03	129 ^{a,b,c}	933	0.80	1.95 ^{a,b}

Values are presented as the average of duplicate samples.

GRS – Cows fed perennial ryegrass only. CLV – Cows fed perennial ryegrass / white clover. TMR – Cows fed total mixed ration ad libitum.

Vitamins: B1 – Thiamine, B2 – Riboflavin, B3 – Nicotinic acid, B3-amide – Nicotinamide, B5 – Pantothenic acid, B7 – Biotin.

Note: Only the pyridoxine form of vitamin B6 is represented in the data.

^{a,b,c,d} different superscripts within a column indicate significant differences ($P < 0.05$).

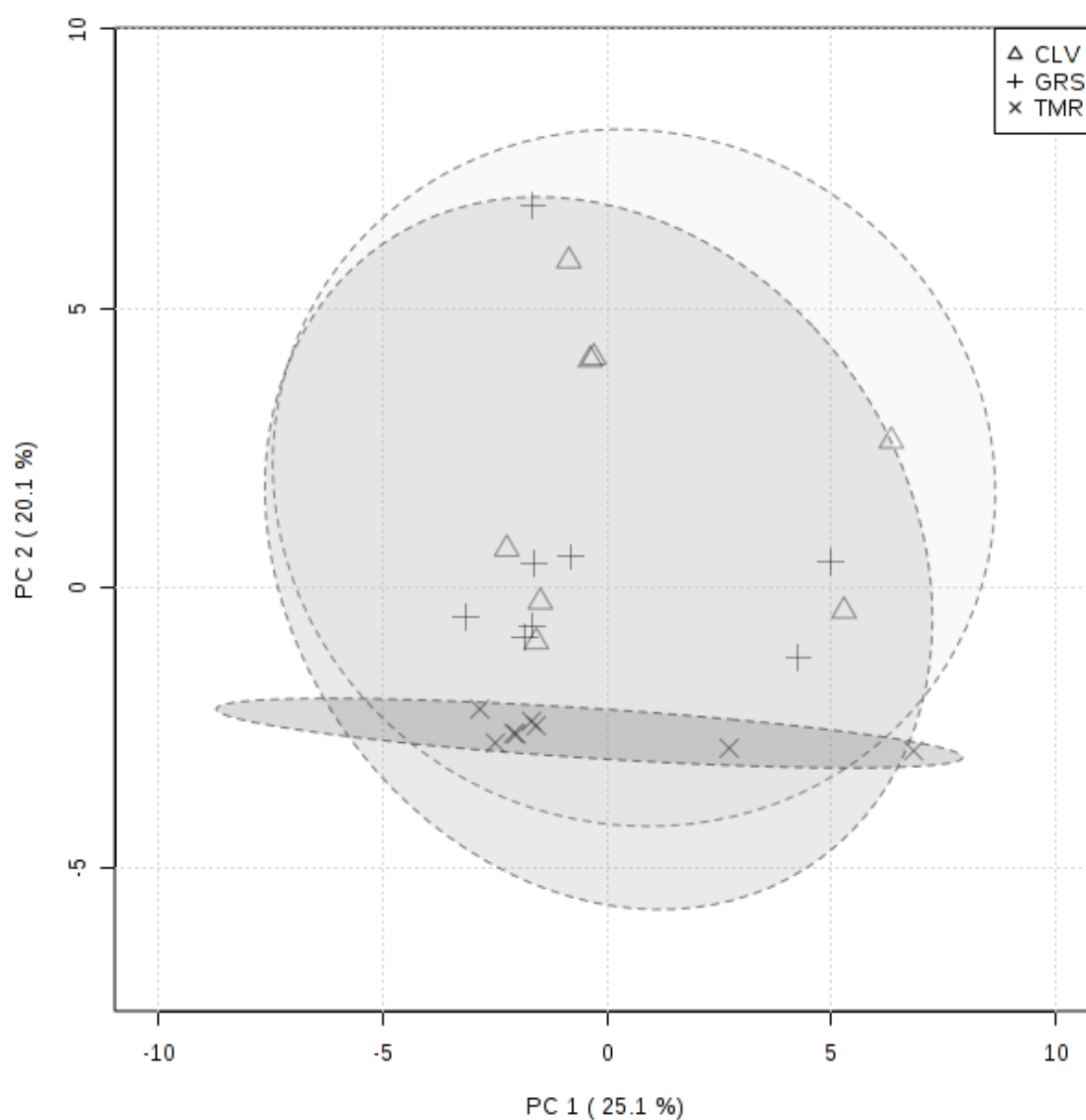


Figure S1. Principal component analysis (PCA) score plot for metabolomics analysis of protein ingredients from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

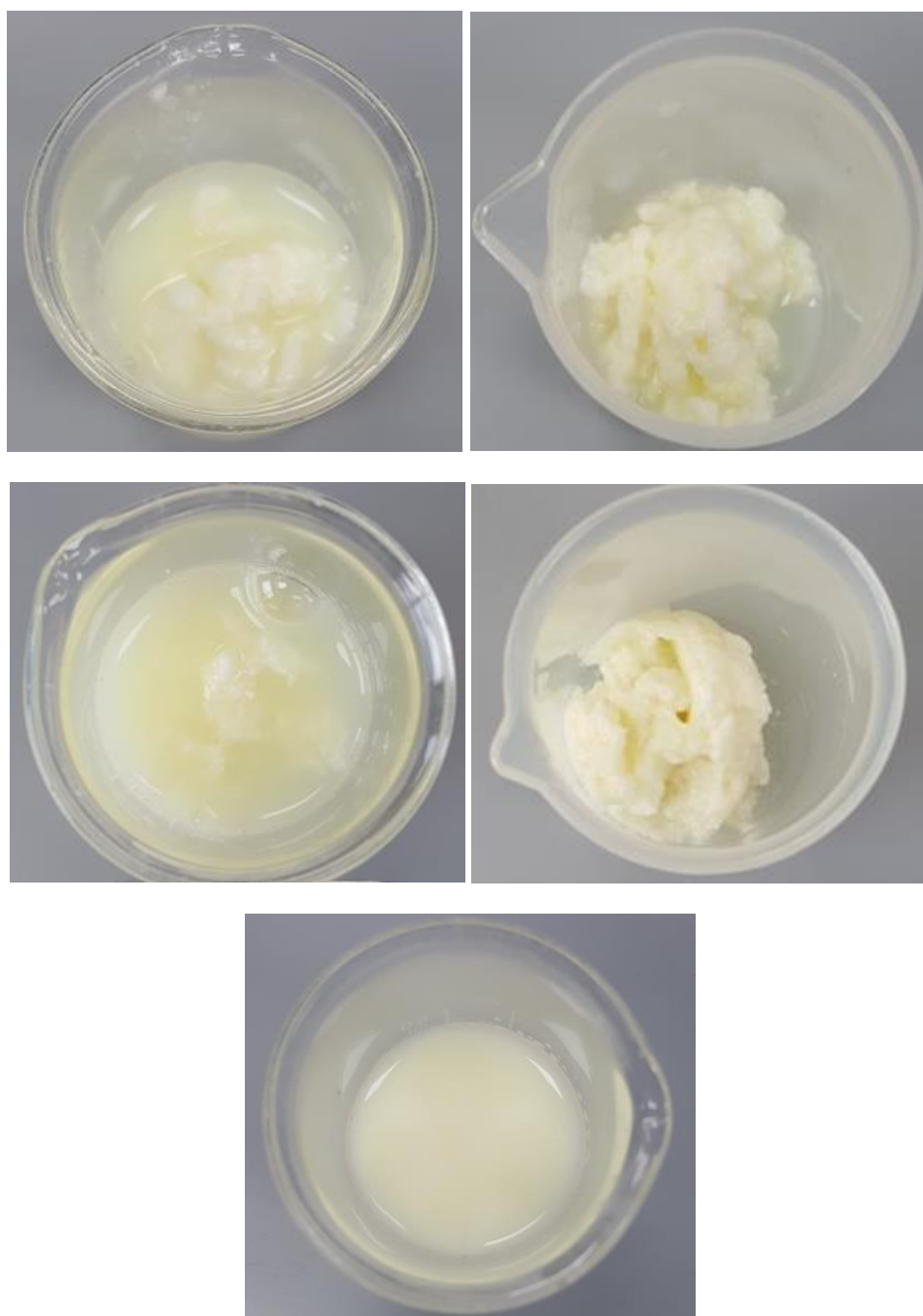


Figure S2. Representative images of reconstituted SMP and WPC dispersions following viscosity measurements throughout heat treatment in a starch pasting cell geometry.

Top row: Skim milk powder/acid whey protein concentrate dispersion (left) and drained coagulum (right) from perennial ryegrass replicate 2.

Middle row: Skim milk powder/sweet whey protein concentrate dispersion (left) and drained coagulum (right) from perennial ryegrass replicate 2.

Bottom: Skim milk powder/micellar casein whey protein concentrate dispersion from perennial ryegrass replicate 2. No precipitated coagulum was present in samples containing micellar casein whey protein concentrate.

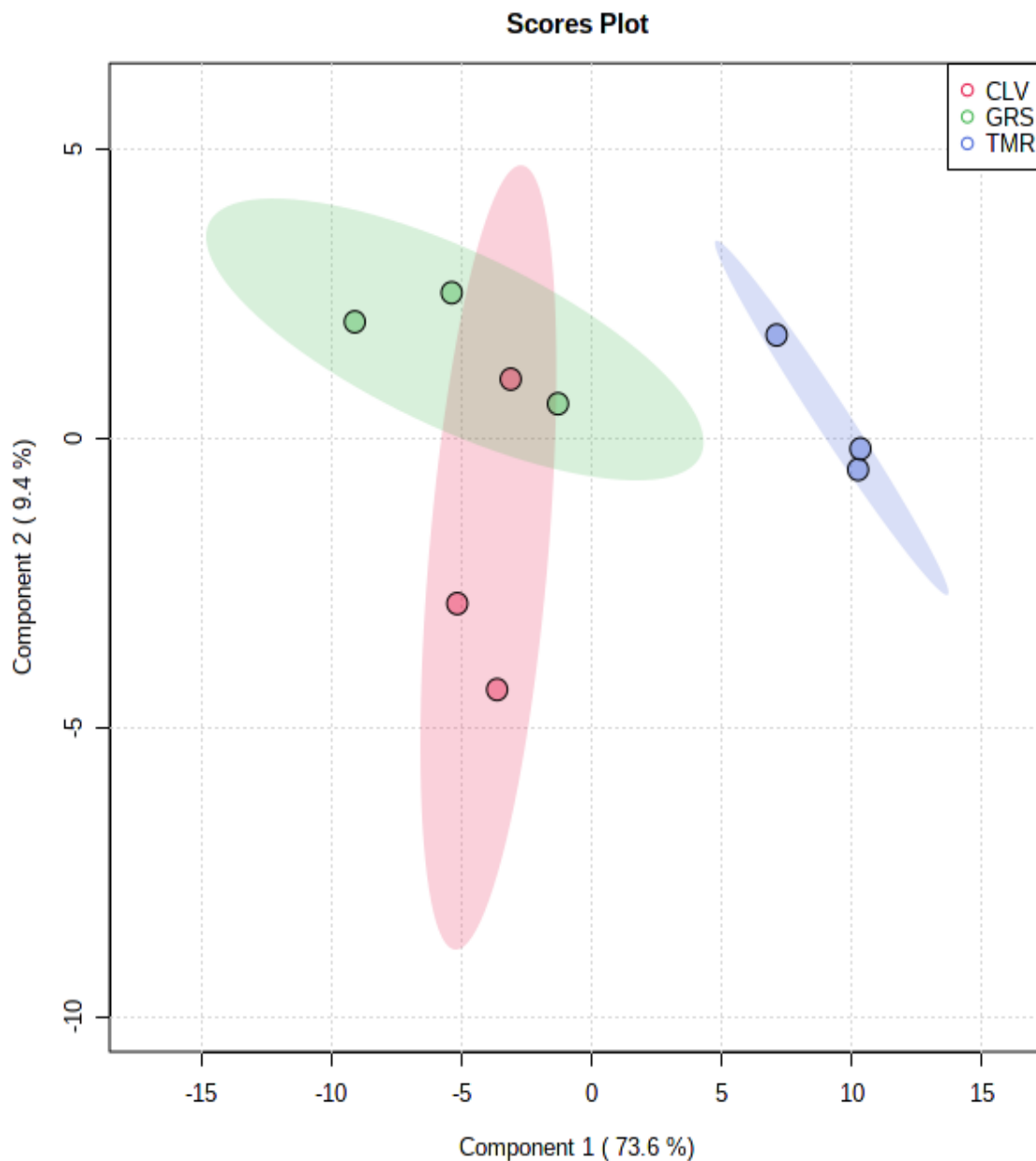


Figure S3: Partial least square-discriminant analysis plot of Raman spectra for reconstituted WMP samples from GRS, CLV and TMR feeding systems.

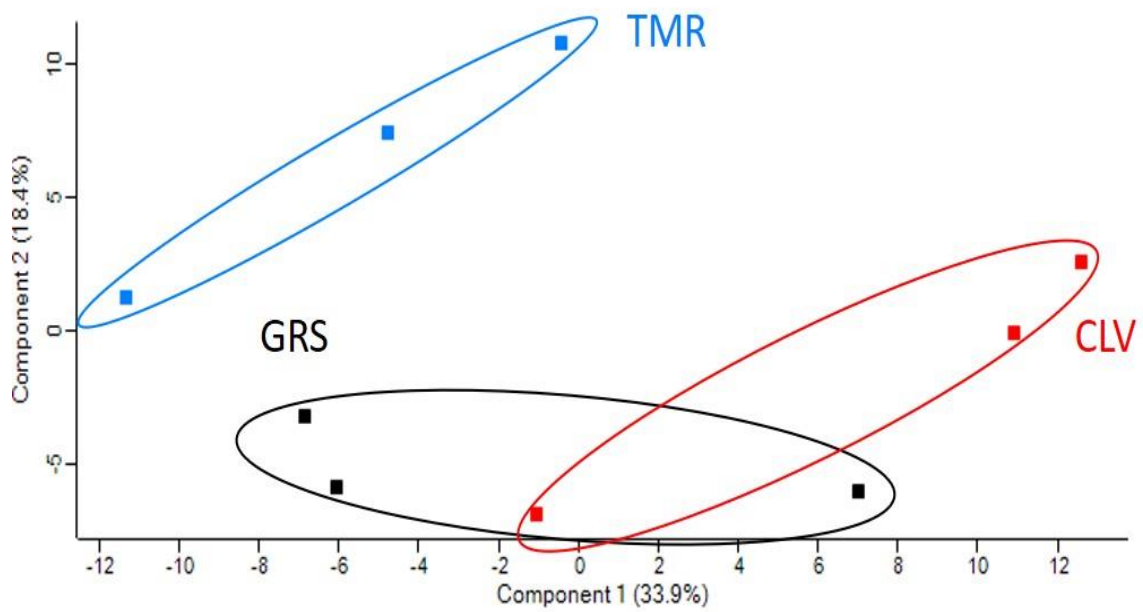


Figure S4: Principal components analysis (PCA) plot of bovine skim milk samples from TMR (blue), CLV (red) and GRS (black).

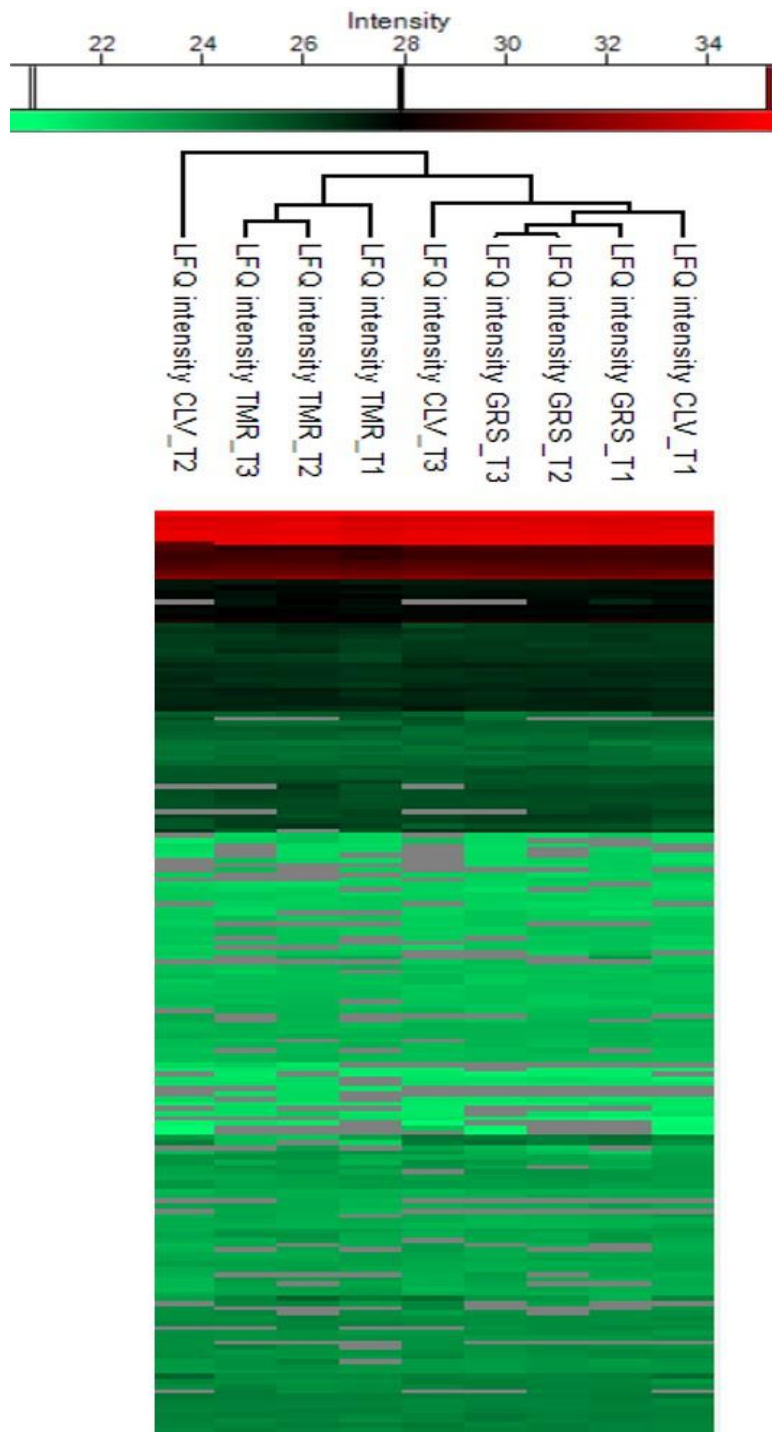


Figure S5: Hierarchical clustering of skim milk proteins from TMR, GRS and CLV samples. Each row represents a separate protein and each column is a sample. Colours indicate the relative abundance of the protein from low (green) to high (red) abundance. Grey indicates the protein was not detectable in the relevant sample.

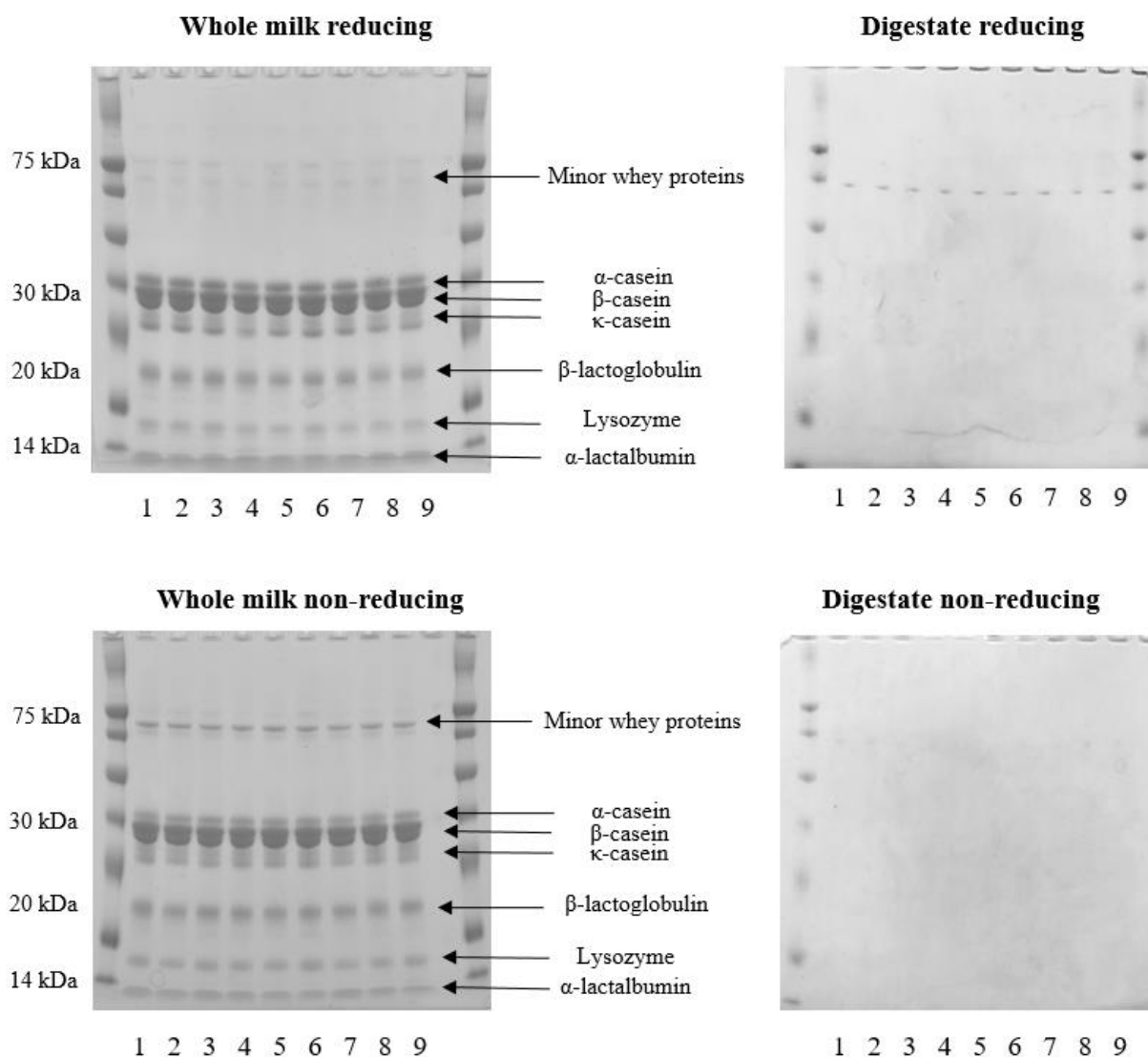


Figure S6: Reducing and non-reducing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein analysis of reconstituted whole milk powders and corresponding digestates.

Numbers 1 – 9 under each image correspond to lanes within each gel. From left to right, lanes 1, 2 and 3 show protein bands for triplicate GRS samples; lanes 4, 5 and 6 for triplicate CLV samples; lanes 7, 8 and 9 for triplicate TMR samples.

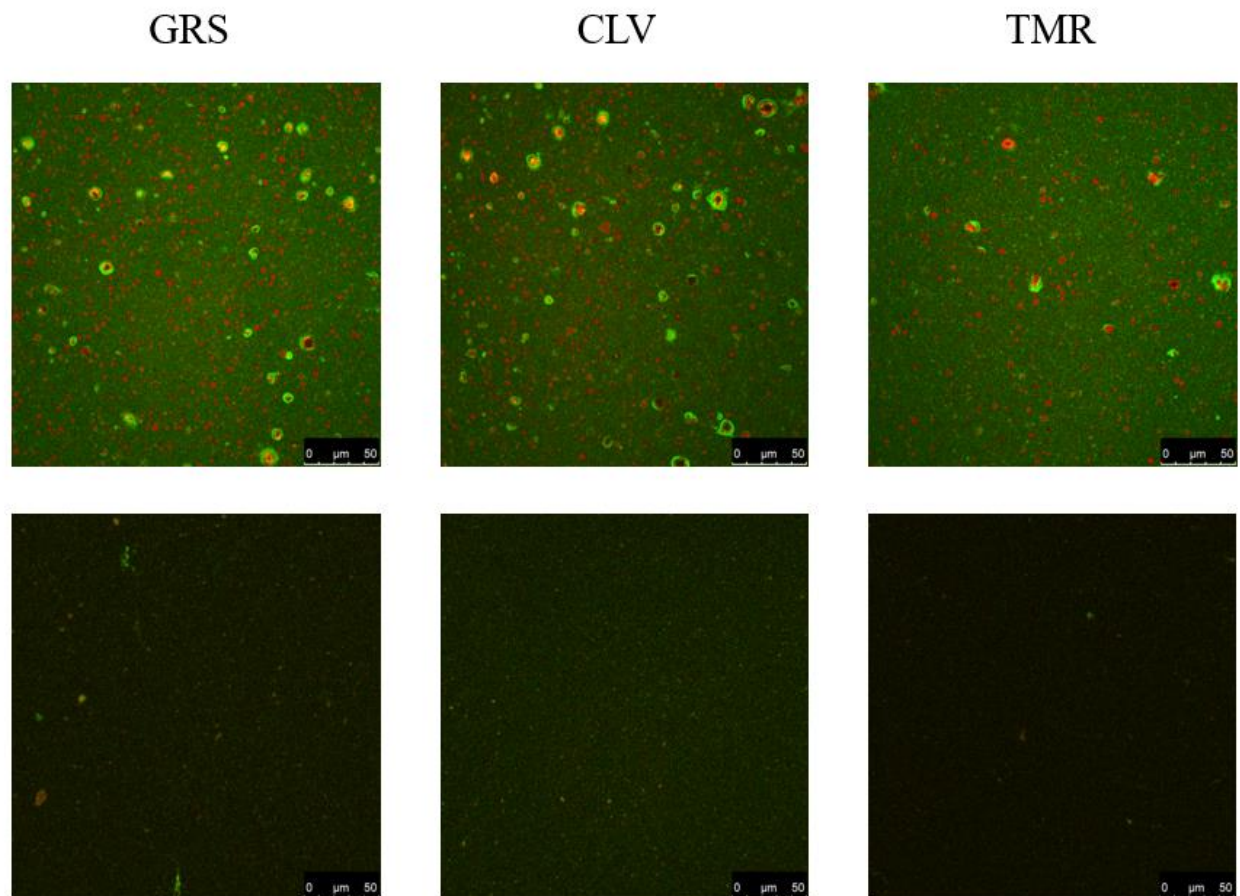


Figure S7: Confocal laser scanning microscopy 2D images of reconstituted whole milk (top row) and corresponding digestates (bottom row) derived from grass (GRS), grass/clover (CLV) and total mixed ration (TMR) diets. Red components represent fat droplets; bright green components represent protein aggregates.

Appendix ii
Published papers

Compositional and functional properties of milk and dairy products derived from cows fed pasture or concentrate-based diets

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Abstract

Worldwide milk production is predominantly founded on indoor, high-concentrate feeding systems, whereas pasture-based feeding systems are most common in New Zealand and Ireland but have received greater attention recently in countries utilizing conventional systems. Consumer interest in 'pasture-fed' dairy products has also increased, arising from environmental, ethical, and nutritional concerns. A substantial body of research exists describing the effect of different feeding strategies on the composition of milk, with several recent studies focusing on the comparison of pasture- and concentrate-based feeding regimes. Significant variation is typically observed in the gross composition of milk produced from different supplemental feeds, but various changes in the discrete composition of macromolecular components in milk have also been associated with dietary influence, particularly in relation to the fatty acid profile. Changes in milk composition have also been shown to have implications for milk and dairy product processability, functionality and sensory properties. Methods to determine the traceability of dairy products or verify marketing claims such as 'pasture-fed' have also been established, based on compositional variation due to diet. This review explores the effects of feed types on milk composition and quality, along with the ultimate effect of diet-induced changes on milk and dairy product functionality, with particular emphasis placed on pasture- and concentrate-based feeding systems.

KEYWORDS

Bovine diet, pasture, concentrate, milk composition, processing

1 | INTRODUCTION

Milk provides a comprehensive source of nutrition for mammalian neonates. Although milk composition varies substantially between species, the milk of other species can be consumed by mature humans and children of sufficient

renal development (Ziegler, 2007), providing a greater distribution of essential macro- and micro-nutrients than most other food sources (Drewnowski, 2005). Thus, milk has been produced from livestock for human consumption since antiquity (Silanikove, Leitner, & Merin, 2015), with bovine milk being the most commercially viable for large-scale production.

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As ruminants, cows produce milk primarily from the consumption of forgeable grasses, making pasture-based milk production the most historically prevalent system. Modern dairy farming practices founded on increasing mechanisation and the availability of alternative feed sources allow for greater scale and intensification and have led to the widespread utilisation of indoor total mixed ration (TMR)-based milk production systems, accounting for approximately 90% of global milk production (Coleman et al., 2009). These systems are widely implemented in the United States and large-scale European producers such as France and Germany (van Arendonk & Liinamo, 2003). By virtue of the indoor housing used in TMR systems, producers can also prevent exposure of their livestock to weather extremes and maintain year-round production in harsher climates (Arnott et al., 2017).

Conversely, pasture-based milk production systems remain dominant in Ireland and New Zealand, where mild, temperate climates and abundant rainfall allow for consistent grass growth throughout the majority of the year and potentially up to 300 days of grazing per year (O'Donovan et al., 2011). This offers an economical source of feedstuff, while also allowing the cow to perform their natural foraging behavior outdoors. Approximately 85% of milk supply in Ireland is produced on a seasonal basis, forming the 'manufacturing milk' pool (Hennessy & Roosen, 2003), and allowing for a correspondence between the availability of grass throughout the year and the lactation cycle of the cow. This results in most dairy herds calving within a narrow window in the spring months and a period of maximal milk production in the summer months, gradually decreasing in the same pattern as the yield of the individual cow into the winter (O'Connell et al., 2015).

The challenge posed by the disparity in milk supply volume in Ireland between the summer and winter months is addressed through the focus of the industry on the production of long-shelf life commodities such as butter and cheese, which maximizes the economic value of a seasonal milk supply. An emphasis on the production of harder cheese varieties using mid-lactation milk (particularly Cheddar) is also necessitated by the unsuitability of late-lactation milk for processing into cheese (Lucey & Fox, 1992). A continuous year-round supply of milk which is appropriate for further processing is produced by suppliers who contribute to the 'liquid milk' pool, forming the remaining 15% of the total Irish milk supply. This necessitates a broader calving distribution throughout the year in order to consistently meet the market demand for short-shelf life products such as liquid milk, cream, and infant milk formula (IMF) throughout the winter months. Farmers are incentivized to continue production in this way by a 'Winter milk' payment scheme, which offers a premium price on off-peak milk to offset the higher cost of milk

production based on conserved forage in winter months (Hopps & Maher, 2007).

Although pasture-based milk production is generally established on exclusively grass-based swards, the inclusion of white clover within the sward is widely practiced and has been shown to improve cow performance in terms of milk yield and milk solids yield (Egan et al., 2015), along with fixing N₂ in the soil (Ledgard et al., 2001). Typically, Irish milk processing co-operatives pay farmers on the basis of milk solids mass (kg of protein + kg of fat), which incentivizes farmers to produce milk with higher protein and fat contents (Sneddon et al., 2013). Milk solids composition is influenced by a variety of factors, leading to substantial variation in the nutritive values and functionalities of the diverse range of end products derived from milk.

Dietary-based interventions have been widely investigated and have highlighted significant implications for milk product processability (Barłowska et al., 2012; Gulati, Hennessy, et al., 2019), traceability (Coppa et al., 2012; O'Callaghan et al., 2018) and sensory (Bendall, 2001; Clarke et al., 2019; Kilcawley et al., 2018) and nutritional quality (Benbrook et al., 2013; Kelly et al., 1998; O'Callaghan, Hennessy, et al., 2016). Considering this, the objective of this review is to outline the effect of bovine feeding systems on the composition and functional properties of a variety of milk products, with a particular focus on perennial ryegrass, perennial ryegrass/white clover, and indoor TMR-based systems. A graphical summary of notable effects of pasture and indoor, concentrate-based feeding systems on milk product composition and functionality is shown in Figure 1.

2 | MILK, NUTRITION AND RECENT TRENDS IN DAIRY CONSUMPTION

2.1 | Nutritional significance of milk

Milk is regarded to function as a 'complete food', necessitated by the requirement of the neonate to receive complete nutrition from milk alone. Despite this optimisation for the neonate, the extensive balance of nutrients in milk makes it an ideal source of continued nutrition into adulthood (Pereira, 2014). As a complex colloidal dispersion of fat, protein, lactose, and soluble salts, milk can contribute to a range of daily human dietary requirements. A 500-mL portion of whole milk can provide significant proportions of daily fat, protein, vitamin, and mineral requirements (Haug et al., 2007), with concentrated end products such as butter or cream (fat), casein or whey powders (protein), and cheese (fat and casein protein) offering even greater quantities of these respective nutrients (Finglas et al., 2014).

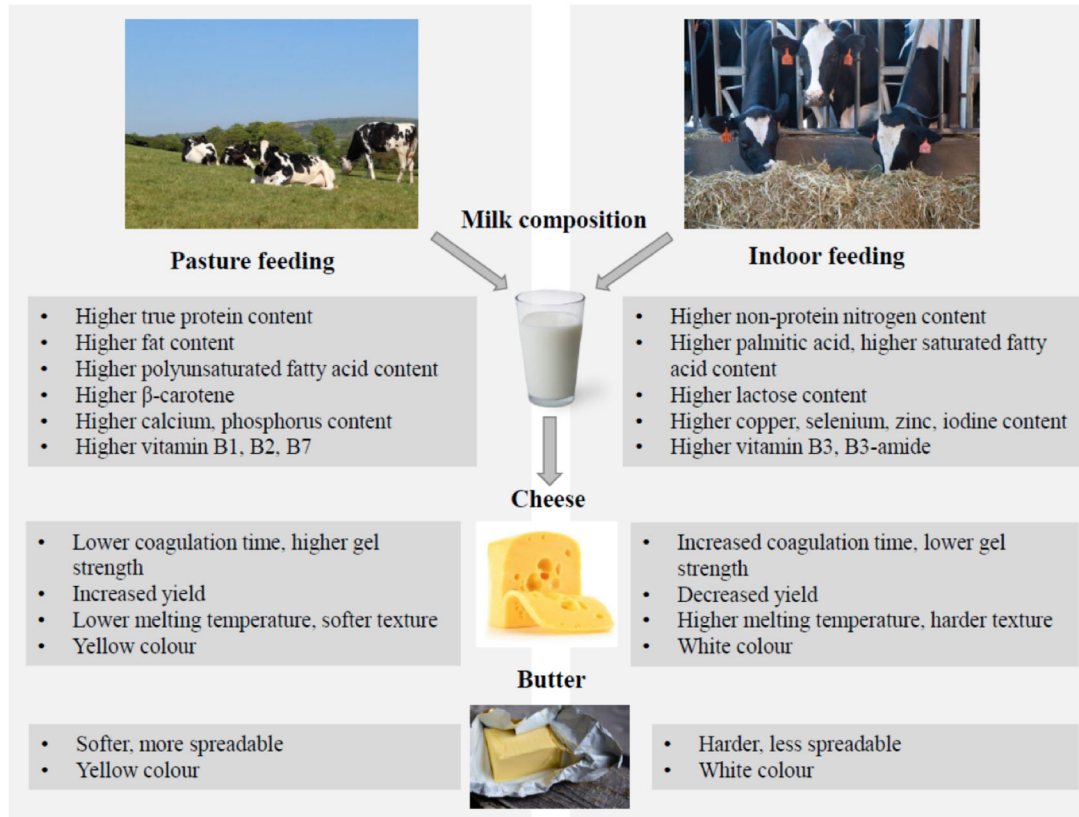


FIGURE 1 Summary of notable effects of pasture and indoor concentrate feeding on milk composition and product functionalities

Milk is a particularly good source of calcium, magnesium, and potassium, minerals that are considered to be widely under-consumed in adult diets (Decker & Park, 2010). Milk also contains water-soluble vitamins in high concentrations and is an important source of thiamine (B1), riboflavin (B2), and cobalamin (B12). Depending on fat content, vitamins A and D can also be present in high concentrations in milk or milk-derived products (Fox et al., 2015). Milk is a particularly important source of cobalamin, as it is almost exclusively an animal-derived vitamin and cannot be acquired from most plant sources (Herbert, 1988). A 500-mL serving of whole milk provides up to 90% of the daily adult cobalamin requirement (Haug et al., 2007).

The relationship between calcium consumption and bone development from childhood to adulthood is well documented (Ondrak & Morgan, 2007; Peterlik et al., 2013). Matkovic et al. (2004) observed an association between increased dairy product consumption and increased volumetric bone mineral density in adolescent females, particularly in relation to spinal development, whereas calcium supplementation did not have a comparable effect. Milk and dairy products have also been suggested to have an important role in geriatric nutrition as a readily available source of protein for maintenance of muscle tissue (Phillips et al., 2009) and a concentrated source of calcium to prevent the development of

osteoporosis (Elbon et al., 1998). Massler (1979) reported that diminishing secretion of hydrochloric acid in the stomachs of elderly individuals may limit mineral absorption in the small intestine, also suggesting that calcium absorption may be promoted through the consumption of acidified dairy products such as yogurt and cottage cheese. However, it is generally accepted that sufficient intake of calcium in childhood and adolescence is a greater contributing factor to bone mineral density in the elderly than milk consumption in later life (Renner, 1994; Wadolowska et al., 2013).

2.2 | Trends in milk and dairy product consumption

Despite the increasingly positive perception of the potential health effects of regular intake of milk and dairy products (Ebringer et al., 2008; Pereira, 2014), negative consumer attitudes towards milk remain widespread (McCarthy et al., 2017), reflected in trends of decreasing milk consumption in many developed countries (Zingone et al., 2017). Some consumers have recently opted for a diet completely free of dairy products, though this decision may often be influenced by ethical concerns, such as in the practice of vegan diets. In these cases, food fortification or supplementation of omega-3 polyunsaturated fatty

acids (PUFAs) (Russell & Meital, 2018) and essential trace elements such as vitamin D (Dunn-Emke et al., 2005), calcium, and particularly cobalamin (U.S. Department of Agriculture & U.S. Department of Health and Human Services, 2010) is often needed to meet advised daily nutritional requirements. Despite the recent overall decrease in liquid milk consumption in developed countries, per-capita consumption of dairy products is increasing (OECD & FAO, 2018). This is primarily attributable to the widespread popularity of added-value products such as whey protein powders, strained yogurt, and convenient cheese products, and may be influenced by the continually changing understanding of the potential health benefits of dairy consumption (OECD & FAO, 2018).

2.2.1 | Lactose intolerance and milk allergy

Despite the increasing consumption of dairy products, approximately 75% of the world population remains intolerant to lactose (Suarez et al., 1995), with the highest incidences of intolerance found among East Asian, African and Native American populations (Silanikove, Leitner, & Merin, 2015). The activity of the β -galactosidase (lactase) enzyme necessary to hydrolyse lactose to glucose and galactose does not typically persist into adulthood (Suarez et al., 1995), though the historical prevalence of milk products in northern and continental Europe has led to a wide distribution of lactase persistence within populations in these regions (Sahi, 1994), where as little as 2% of the population now experiences lactase deficiency (Heyman, 2006). Milk products do, however, remain widely consumed in many areas in which lactose intolerance is widespread, primarily as products in which the lactose has been hydrolysed (e.g. fermented milk/yogurt) or removed (e.g. hard cheese varieties) (Silanikove, Leitner, & Merin, 2015). In more severe cases, an individual may be allergic to milk proteins, most commonly the whey proteins β -lactoglobulin and α -lactalbumin, or α_{S1} -casein (Lifschitz & Szajewska, 2015).

The recent popularity of raw milk consumption may partly be predicated on the suggestion of reduced allergenicity associated with raw milk when compared to pasteurized milk (Sozańska, 2019). This may in part be due to misinterpretation of the subject of the PARSIFAL study by Waser et al. (2007) which observed an inverse association between consumption of farm milk and asthma and allergy. The farm milk referenced in this study may have been widely confused for raw milk, though the authors indicate that the status of the farm milk as raw could not be determined. It is widely accepted that any potential health benefits of raw milk consumption are outweighed by its pathogenic risk factors (Claeys et al., 2013; Lucey, 2015; Ombark et al., 2016).

2.2.2 | A1 and A2 milk

Recently, the qualitative difference in the casein composition of milk has received attention regarding the presence of the A1- or A2-type genotypes of β -casein, the sequences of which differ by a single amino acid (AA) (European Food Safety Authority, 2009). It has been suggested that the presence of histidine in the A1 sequence and subsequent release of β -casomorphin-7 (BCM7) may lead to increased risk of type 1 diabetes, cardiovascular disease (CVD) or even schizophrenia (Sodhi et al., 2012). Pal et al. (2015) also stated that BCM7 may be responsible for many instances of milk intolerance through immunomodulatory or proinflammatory effects, opposing the singular association between lactose and milk intolerance. Substitution of histidine with proline in the A2 sequence is suggested to prevent the enzymatic hydrolysis required to release BCM7 (European Food Safety Authority, 2009).

Reviews carried out by Parashar and Saini (2015) and Truswell (2005) propose that little evidence exists to support the suggested risks of A1 milk consumption. However, a randomized controlled study carried out by Sheng et al. (2019) comparing gastrointestinal symptoms in Chinese preschool children consuming conventional milk (containing A1 and A2 β -casein variants) or milk containing the A2 β -casein variant only reported improved gastrointestinal symptoms and cognitive performance in children consuming the A2-only variant. This study provides some evidence to support the difference in pathological effects between A1 and A2 milk, but is limited to preschool children in a population group in which dairy consumption is particularly low. There is certainly considerable scope for investigation of the potential effects of A1 and A2 milk consumption in older groups and among populations where dairy consumption is more widespread. The A1 and A2 variants of β -casein are genetic products and, therefore, their relative abundance in milk will vary between species and breeds of cow, but will not vary depending on the use of different feeding systems, which are discussed throughout this review.

2.2.3 | 'Pasture-fed' dairy

As many consumers continue to make more conscious dietary considerations, interest in pasture-derived milk and dairy products is increasing (Getter et al., 2015). Animal welfare, environmental impact and health concerns are reported by Conner and Oppenheim (2008) as contributory motivators to the choice of pasture-derived milk products over conventionally derived milk products among American consumers, who are also willing to pay a premium price for products of known pasture-based origin. Health-based marketing of pasture-derived products is

often focused on the fatty acid (FA) profile of milk, with particular attention directed to conjugated linoleic acid (CLA) and omega-3 FAs such as α -linolenic acid (Clancy, 2006). Labelling such as 'pasture-fed' or 'pasture-raised' is used widely for milk products in the United States and may be conflated with organic milk by consumers, which was also reported as experiencing substantial yearly growth by Conner and Oppenheim (2008), who identify it as a subset of pasture-raised milk. However, organic dairy farming standards only require cows to spend a minimum of 120 days per year at pasture (Liu et al., 2018; U.S. Department of Agriculture, 2000) and larger, higher-producing organic dairy farms in the United States tend to utilize proportionally lower average levels of pasture-derived forage (McBride & Greene, 2009). In contrast, cows spend an average of 240 days per year at pasture on standard grazing operations in Ireland, where grass constitutes at least 90% of the diet (Bord Bia, 2020).

In the context of Irish milk composition, a notable factorial confluence exists between lactation stage, seasonality and cow diet, given the seasonal production pattern utilized thereby. As previously mentioned, the lactation curve reflects that of grass growth, allowing production of high volumes of mid-lactation milk at the period of maximal grass growth. For the manufacturing milk pool, dietary supplementation of conserved forage such as silage or hay, along with cereal concentrates, is practiced throughout late lactation, when seasonal changes necessitate indoor housing of cows, and up to the beginning of the following lactation after the drying-off period. This combined effect may lead to a notable difference in milk composition from countries where pasture-based milk production is widespread, in comparison to those which typically utilize conventional TMR systems. The importance of country-specific information on milk composition to milk processors and consumers has previously been highlighted by Schönfeldt et al. (2012). The effect of dietary factors on the composition and functional properties of milk and milk-derived products will be the focus of the remainder of this review.

3 | IMPACT OF FEEDING SYSTEM ON MILK COMPOSITION

3.1 | Nitrogenous compounds

3.1.1 | Milk protein yield, crude protein and true protein

The differences in gross milk composition observed among cow breeds are of considerably greater magnitude than those which can be achieved through dietary interven-

tions and, although genetic effects are the primary determinants of the AA composition of proteins, changes to cow diets nonetheless have a notable influence on concentrations of gross milk proteins and can be more readily altered by the farmer. A summary of the literature discussed in this section is shown in Table 1. In previous decades, attempts at altering crude protein (CP) levels in milk were dependent on manipulation of both the protein content and energy supply of supplemented feedstuffs (Broderick, 2003; Coulon & Rémond, 1991; DePeters et al., 1985; Emery, 1978; Khalili et al., 2002; Patton et al., 2006; Stockdale, 1994; Thomas, 1984).

Other important considerations for planning dietary regimes which were identified include the level of energy supplied through dietary fiber (Emery, 1978) and the source of energy used in the supplied feed, such as the addition of rapeseed meal or field pea (Khalili et al., 2002) or soybean meal (Broderick, 2003). Despite the high energy density of lipids, supplementation of dietary fats and oils has been observed to decrease milk protein and casein content (Carroll et al., 2006). DePeters et al. (1985) recorded a decrease in milk protein and casein content and increase in non-protein nitrogen (NPN) content in response to increased dietary supplementation with whole cottonseed (high fat content). As far back as four decades ago, Emery (1978) suggested that increased milk protein concentration is not determined by caloric load alone, but may rather be dependent on feed quality, particularly carbohydrate content. Due to the high starch content and metabolic availability of maize, Stockdale (1994) suggested that a ration high in supplemented maize silage would be optimal for provision of high energy levels for increased milk protein production. Indeed, a study by Dalley et al. (2020) observed a relatively high CP content of 3.99% for Holstein-Friesian cows fed on pasture supplemented with maize silage, though this was lower than that of milk of cows fed on pasture supplemented with fodder beet (4.3%). Patton et al. (2006) observed increased protein yield while feeding a high-energy diet consisting of grass silage and corn silage with a high concentrate inclusion; however, this effect was less substantial than the effect of reducing daily milking frequency from three to one. Considering these studies, it seems reasonably well defined that milk protein content can be influenced by providing high levels of dietary energy through feeding high-quality carbohydrate.

A positive correlation between supplementation of corn grain with canola meal and milk protein concentration was also observed by Auldlist, Marett, et al. (2016) across a series of four feeding strategies applied to grazing cows supplemented with formulated grain mix or partial mixed rations. Notably, this occurred across all four feeding strategies, in which levels of metabolizable energy were kept the same. Mackle et al. (2000) proposed that an increase in dietary

TABLE 1 Summary of significant dietary effects on milk composition

Component	Dietary factor	Effect relative to comparison diets	References
Protein	Grass and clover feeding	Increase in protein/true protein %	Gulati, Galvin, Lewis, et al., 2018; O'Callaghan, Hennessy, et al., 2016
	Clover and concentrate feeding	Increase in non-protein nitrogen	Magan, Tobin, et al., 2019; O'Callaghan, Hennessy, et al., 2016
	Fat supplementation	Decrease in protein content	Carroll et al., 2006
	Maize feeding	Increase in protein content	Patton et al., 2006
	Excess crude protein intake	Decrease in protein content	Colmenero & Broderick, 2006
Fat	Maize silage and fodder beet feeding	Increase in fat %, but decrease with excess fodder beet feeding	Dalley et al., 2020
	Grass feeding	Increase in fat %	O'Callaghan, Hennessy, et al., 2016
	Concentrate feeding	Increase in fat yield	Gulati, Galvin, Lewis, et al., 2018; O'Callaghan, Hennessy, et al., 2016
	Overfeeding of fat or concentrates	Decrease in fat %	Broderick, 2003; Flachowsky et al., 2006
	Grass and clover feeding	Increase in polyunsaturated fatty acid and conjugated linoleic acid content	Chilliard et al., 2007; Couvreur et al., 2006; O'Callaghan, Hennessy, et al., 2016
	Concentrate feeding	Increase in palmitic acid content	
	Lipid supplementation	Increase in long-chain fatty acid content and decrease in de novo fatty acid content	Chilliard et al., 2009; Hoffmann et al., 2016; Shingfield et al., 2003
Lactose	Clover and concentrate feeding	Increase in lactose yield	Harris et al., 1998; O'Callaghan, Hennessy, et al., 2016; Panthi et al., 2019
	Concentrate feeding	Increase in lactose yield and %	Gulati, Galvin, Lewis, et al., 2018
Minerals	Concentrate feeding	Increase in zinc, copper and selenium concentrations	Gulati, Galvin, Lewis, et al., 2018; Rey-Crespo et al., 2013
	Grass feeding	Increase in calcium and phosphorus concentrations	Gulati, Galvin, Lewis, et al., 2018
	Fat supplementation	Increase in concentration of magnesium and decrease in concentration of phosphorus	Carroll et al., 2006
Vitamins	Organic production	Increase in riboflavin content	Poulsen et al., 2015
	Grass and clover feeding	Higher thiamine, riboflavin and biotin content	Magan et al., 2020
	Concentrate feeding	Higher nicotinic acid and nicotinamide content	
	Grass and grass silage feeding	Increase in retinol content	Agabriel et al., 2007
	Maize feeding	Increase in folate and cobalamin content	Chassaing et al., 2011

energy content promoted increased N synthesis by rumen microbiota, thus increasing milk protein content, although recent research has suggested that more specifically, this may be dependent on the supply of particular AA, which will be discussed in Section 3.1.3. A study by Zimmerman et al. (1991) also investigated the effect of dietary CP levels on milk production and compositional parameters, noting that total milk yield (kg/day) and milk CP yield increased with increasing dietary protein; however, it is important to note that the percentage of protein in the milk remained the same.

Overfeeding of dietary CP can, however, result in a decline in milk protein content. Leonardi et al. (2003) found that milk protein content decreased from 3.24% to 3.18% when dietary CP content was increased from 16.1% to 18.8% of dry matter (DM). Excess dietary protein was demonstrated by Colmenero and Broderick (2006) to limit the nitrogen conversion efficiency of rumen microflora, resulting in increased urinary excretion of nitrogen and, consequently, depressed nitrogen secretion in milk. The authors proposed a dietary CP content of 16.5% for optimal milk production and minimal urinary nitrogen losses. Notably, this study involved a high proportion of concentrate inclusion prior to the substitution of corn silage with soybean meal to increase protein content in the ration, which would suggest that the observed decrease in milk protein occurred in spite of the high level of energy intake attributable to the level of concentrates in the ration. An additional consideration for overfeeding of CP is the effect of excessive N excretion by the cow on subsequent water pollution (Kalscheur et al., 1999). This is an effect of considerable relevance in the efforts to move towards more sustainable farming and dairy production practices, particularly as global herd numbers increase.

The influence of varying dietary forage and forage:concentrate ratios on percentage milk protein has been more widely investigated in recent years (Couvreur et al., 2006; Gulati, Galvin, Lewis, et al., 2018; Harris et al., 1998; Mackle et al., 1999; McAuliffe et al., 2016; O'Callaghan, Hennessy, et al., 2016; Panthi et al., 2019). Harris et al. (1998) investigated the effect of varying the level of white clover in perennial ryegrass fed to cows housed indoors, and found milk protein concentrations did not change at 200, 500 and 800 g white clover per kg DM, both at restricted and ad libitum feeding levels, despite increased milk yields, which were attributed to the high nutritional value of white clover. Similarly, Mackle et al. (1999) found no significant differences in the CP content of milk derived from three groups of cows assigned to diets comprising pasture only, pasture supplemented with maize silage and pasture supplemented with maize and grass silage. Total casein content was also unaffected, but the β -casein level and casein:CP ratio increased significantly with maize

and grass silage supplementation. This effect would certainly seem to warrant investigation in further studies, as it is unusual that individual protein components would be directly affected by dietary changes. A curvilinear increase in milk yield and milk protein content was observed by Couvreur et al. (2006) as a function of increasing fresh grass (0%, 30%, 60%, and 100% DM) in place of corn silage, though each group also received varying ratios of soybean:cereal concentrate in a 3-kg supplemental mixture. The increase in percentage milk protein was significant from 0% to 30% DM but again, what seems of note is that this increase in milk protein content plateaued at the higher supplementation rates.

Several recent studies have been undertaken using milk derived from three specific feeding systems at the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Ireland. Cows were randomly assigned to the following feeding systems: Group 1 was housed indoors and fed a TMR diet (8.3 kg of concentrates, 7.15 kg of grass silage and 7.15 kg of maize silage on a DM basis), Group 2 was maintained outdoors at pasture consuming approximately 18 kg DM/day of perennial ryegrass (GRS) and Group 3 was also maintained outdoors consuming approximately 18 kg DM/day of perennial ryegrass/white clover pasture (CLV) with an average annual white clover content of 24%. The TMR formulation and chemical composition of the concentrate used by O'Callaghan, Hennessy, et al. (2016) is shown in Table 2. Table 3 shows the chemical composition of the grass silage and maize silage used in the TMR ration, and Table 4 shows the chemical composition of the GRS and CLV swards. The values presented in Tables 2, 3, and 4 are typical of the diets used in each of the studies based on the TMR, GRS and CLV feeding systems implemented at Teagasc Animal and Grassland Research and Innovation Centre. The TMR diet provided higher concentrations of CP and a higher DM intake, relative to the GRS and CLV systems, and the CLV system also provided higher DM intake and protein digestibility than the GRS-only diet. The research herds used in these studies represent best practice commercial milk supplies from different production systems; the GRS and CLV herds represent typical pasture-based systems, whereas the TMR herd represents a conventional indoor and concentrate-based system.

From the Teagasc research group, Gulati, Galvin, Lewis, et al. (2018), McAuliffe et al. (2016), O'Callaghan, Hennessy, et al. (2016) and Panthi et al. (2019) consistently observed significantly higher concentrations of total protein in milk produced from both the GRS and CLV systems when compared to the TMR system. O'Callaghan, Hennessy, et al. (2016) also observed the highest true protein content of the three diets in GRS-derived milk. Contrasting results were earlier found by Schroeder et al. (2003), where

TABLE 2 Typical ingredient formulation (% as fed) and chemical composition (%) of total mixed ration (TMR) diet (Megalac concentrate sourced from Volac Ireland, Co. Cavan, Ireland), adapted from O'Callaghan, Hennessy, et al. (2016)

TMR ingredient	(% of ration)
Soybean meal (48% crude protein)	30.00
Molassed beet pulp	15.50
Rolled barley	15.00
Maize	13.00
Maize distillers	12.00
Rapeseed meal	7.50
Megalac	3.30
Maize/beet mineral balancer	2.50
Acid buffer	0.70
Salt	0.50
Chemical composition (%)	(%)
Organic matter	93.50 ± 0.94
Dry matter	86.76 ± 0.75
Moisture	13.24 ± 0.75
Protein	23.73 ± 3.69
Fiber	7.77 ± 1.86
Starch	21.49 ± 1.93
Total sugar	9.62 ± 0.35
Ash	6.50 ± 0.94
Neutral cellulose plus gamanase digestibility	83.35 ± 1.15

the total protein content of milk from TMR-fed cows was significantly higher than two groups of pasture-fed cows supplemented with corn-based concentrate and a combination of corn-based concentrate with calcium salts of FAs, although the true protein content of each type of milk was not determined. In addition, O'Callaghan, Hennessy, et al. (2016) and Gulati, Galvin, Lewis, et al. (2018) found that milk yield and milk protein yield were higher for cows assigned to the TMR system than both the GRS and CLV systems. This may imply that milk yield and milk solids yield may be primarily influenced by the overall energy density of the feed, whereas percentage milk protein and quality is more dependent on levels of dietary roughage, higher proportions of which have been suggested by Couvreur et al. (2006) to increase propionic acid content in the rumen, thus modifying energy supply to the udder. The effect of lactation stage was also considered by O'Callaghan, Hennessy, et al. (2016) and Gulati, Galvin, Lewis, et al. (2018), who both found higher total protein concentrations in late-lactation milk than in early or mid-lactation, though, again, this may be augmented by variability in the quality of forage consumed throughout the lactation cycle.

The availability and quality of forage are quite variable depending on climatic and seasonal factors. Consumption of grasses which mature later in the growing season has been shown to significantly increase milk protein yield and milk protein concentration (Gowen et al., 2003). Likewise, white clover, which matures more slowly than perennial ryegrass and is more sensitive to low soil temperatures, can be included within a grass sward to increase milk yield and milk solids yield, although percentage milk protein is similar to that of grass-derived milk (Egan et al., 2015; Hennessy et al., 2018).

Overall, there has been a multitude of well-defined studies performed relating diet to milk CP; however, the exact biochemical mechanisms for how dietary factors translate to changes in milk CP still remain to be fully elucidated.

3.1.2 | Milk urea and non-protein nitrogen

Among the components of NPN in milk, urea is the most abundant, accounting for approximately 50% of NPN, which itself represents 3% to 5% of the total nitrogen content of milk. Creatine, free AAs, uric acid, phospholipids, peptides and organic acids constitute the remaining 50% of NPN. These nitrogenous compounds are transferred from the cow's blood to milk following protein metabolism (Ruska & Jonkus, 2014) and, despite representing a small proportion of the overall nitrogen content of milk, the concentration of milk NPN is an important indicator of milk protein quality.

O'Callaghan et al. (O'Callaghan et al., 2018; O'Callaghan, Hennessy, et al., 2016) and Magan, Tobin, et al. (2019) found higher concentrations of NPN and urea, respectively, in CLV-derived milk, when compared to GRS and TMR. Harris et al. (1998) also observed increased blood and milk urea in response to increasing white clover content in feed. This is a direct dietary effect arising from the uptake of soil N by N-fixing bacteria present in the root nodules of white clover. O'Callaghan, Hennessy, et al. (2016) and O'Callaghan et al. (2018) also observed lower concentrations of NPN and urea, respectively, in GRS-derived milk in comparison to TMR-derived milk, but contrasting results were reported by Mackle et al. (1999), who observed both higher NPN and urea contents in milk from cows fed pasture only, when compared to those receiving pasture supplemented with maize grain and pasture with maize grain and pasture silage. The group consuming pasture only received the highest CP and lowest metabolizable energy intakes in this case, which may indicate that increasing the energy intake rather than CP intake may have a more favourable effect in reducing milk NPN content. However, it is unusual that the true protein content (total protein – NPN) of

TABLE 3 Chemical composition (g/kg of DM; mean \pm SD) and nutritional content of silages from total mixed ration (TMR) diet (grass silage and maize silage) collected weekly throughout analyzed by near-infrared spectroscopy, adapted from O'Callaghan, Hennessy, et al. (2016)

Component	Grass silage	Maize silage
Dry matter	389.37 \pm 61.35	343.03 \pm 43.45
Organic matter	917.94 \pm 7.45	972.53 \pm 3.22
Acid detergent fiber	296.82 \pm 23.40	NA
Neutral detergent fiber	452.02 \pm 39.31	434.80 \pm 49.57
Crude protein	114.55 \pm 12.55	68.97 \pm 9.91
Starch	NA	285.37 \pm 28.81
Ash	82.06 \pm 6.75	27.47 \pm 3.22

Abbreviation: NA, not available.

TABLE 4 Chemical composition (g/kg of DM; mean \pm SD) and nutritional content of pasture systems (grass and clover) collected weekly throughout lactation, analyzed by near-infrared spectroscopy, adapted from O'Callaghan, Hennessy, et al. (2016)

Component	Grass	Clover
Organic matter	928.00 \pm 9.31	931.49 \pm 7.18
Organic matter digestibility	764.43 \pm 19.34	769.22 \pm 18.97
Acid detergent fiber	218.89 \pm 16.91	220.67 \pm 14.05
Neutral detergent fiber	427.62 \pm 23.83	423.46 \pm 18.94
Crude protein	210.90 \pm 23.71	220.67 \pm 14.05
Ash	72.00 \pm 9.31	68.51 \pm 7.18

milk did not vary between feeding systems in this study, considering the differences observed for NPN.

The milk urea nitrogen (MUN) content of milk from pasture-fed cows was also found to decrease with concentrate supplementation by Bargo et al. (2002), whereas conflicting results were again reported by Utama et al. (2018) and Magan, O'Callaghan, et al. (2019). In the former study, MUN content increased significantly with increasing concentrate:forage ratio and, in the latter, TMR-derived skim milk powder had significantly higher NPN content than that produced from GRS-derived milk. This variation in MUN between studies may be influenced by differences in the forage quality or pasture composition and specific composition of concentrate rations used in each study. Similar to for CP content, the relative levels of MUN and NPN appear to be influenced by the quality of dietary carbohydrate content and the metabolizable energy provided thereby. However, this also seems to be directly increased by increased N supply in the feed.

3.1.3 | AA composition

An effect of dietary factors on the AA profile of milk has not been conclusively determined. Rather, dietary alterations are understood to affect ruminal AA composition and flow of AA to the duodenum (Li et al., 2012; Overton

et al., 1995; Pacheco-Rios et al., 1999) and, consequently, availability of precursory AA to the mammary gland, influencing total milk protein yield and concentration (Li et al., 2012; O'Callaghan et al., 2018), but not the relative proportions of milk AA.

Methionine and lysine have been shown to be the primary limiting AA in milk protein production, and the effect of their dietary supplementation (particularly in rumen-protected form) on milk production has been widely investigated (Lee et al., 2015; Socha et al., 2005; Wang et al., 2010). The efficiency at which these limiting AA are utilized for milk protein production has been shown to increase with decreasing dietary availability of metabolizable protein (Lee et al., 2015; Socha et al., 2005), indicating a stabilizing effect in response to low CP in the diet. O'Callaghan et al. (2018) found increased concentrations of methionine and L-lysine in the rumen fluid of cows fed GRS and CLV diets in comparison to those fed a TMR diet, a relationship which reflects that of the milk protein content of these diets and is supported by the findings of Leonardi et al. (2003), who observed an increase in percentage milk protein with methionine supplementation at two levels of dietary CP. Schwab et al. (1976) and Hanigan et al. (2002) suggested that milk protein synthesis is dependent on the supply and interaction of multiple essential AA (cysteine, threonine, methionine, lysine, histidine, phenylalanine) to the udder, rather than individual limiting AA.

A limited effect of feeding system on the total AA composition of three types of whey powders was suggested by the results of a study by Magan, O'Callaghan, et al. (2019). Concentrations of cysteine, glycine, phenylalanine and valine were found to vary among GRS, CLV, and TMR feeding systems, though no clear explanation for why or how the diet may affect these AA was proposed. A compositional study carried out by Lindmark-Månsson et al. (2003) on milk collected from nine dairies across Sweden throughout the year also suggested a possible effect of feeding changes on milk AA profile. Although significant geographic variation was only observed for total concentrations of arginine and tyrosine, total concentrations of 16 AA were found to vary significantly by season. The authors suggested that feeding strategy and feed quality may vary by region, but are also likely to vary considerably between seasons, as cows are transitioned from outdoor grazing in summer to silage and concentrate feeding in winter housing. This would seem to imply that if geographical variation in AA profile due to differences in feeding practices occurs within one country, AA profiles would also vary considerably internationally. However, the geographic variation observed in this study may also be influenced by genotypic differences, as relative numbers of particular breeds on farms contributing to dairies throughout the country may be variable.

3.2 | Fat and FAs

3.2.1 | Milk fat yield/gross milk fat

Much like milk protein, the lipid fraction of milk displays significant variation between breeds (Woodford et al., 1986), seasons (Heck et al., 2009) and throughout lactation (Chilliard et al., 1991), and has also been demonstrated to be the milk component most susceptible to variation due to dietary interventions (Chilliard et al., 2007; Dewhurst et al., 2006; O'Callaghan, Hennessy, et al., 2016; Santos, 2002); for these reasons, it has been the most thoroughly investigated component. Variation in the forage:concentrate ratio has been identified as a major dietary consideration for influencing milk FA profile (Chilliard et al., 2007; Soita et al., 2005; Sterk et al., 2011), though various supplemental feedstuffs which can be easily introduced to the cow's diet have been shown to have an effect on the gross content of milk fat.

Whole cottonseed feeding was shown by DePeters et al. (1985) to result in decreased milk protein content, but resulted in increasing fat yield and milk fat content with increasing proportions of supplemental whole cottonseed. Dalley et al. (2020) also observed a high milk fat content of 5.3% for Holstein-Friesian cows when receiving supple-

mental maize silage. This was compared to fodder beet, which increased milk fat content to 5.54% when fed as a supplement to perennial ryegrass (25% of DM intake). Increasing the fodder beet supplementation rate to 40% resulted in a decrease in milk fat content to 5.07%, which is suggested to be due to the occurrence of sub-clinical acidosis in some of the cows assigned to this diet, whereby rumen pH was reduced due to the high starch content of the supplementary fodder beet.

Incidences of sub-clinical acidosis on high-producing conventional dairy farms are commonly attributed to overfeeding of high-starch, grain-based concentrates (Fiorentin et al., 2018; Nocek, 1997; Plaizier et al., 2014), which provide excessive levels of ruminally fermentable carbohydrate, leading to lactic acid and volatile FA accumulation and, consequently, reduced rumen pH (Broderick, 2003; Nocek, 1997). This effect is often associated with the occurrence of milk fat depression (MFD) (Krause & Oetzel, 2006; Stone, 2004) caused by inhibition of rumen microflora and, thus, reduced biohydrogenation of dietary FA, though this may be compounded by the behavioral effects of acidosis in cows, such as reduced feed intake and the resultant insufficient nutritional status (Plaizier et al., 2008). Cows in early lactation may be particularly at risk to sub-clinical acidosis for similar reasons, due to condition loss and the high energy requirement to maintain milk production relative to feed intake peripartum (Doepel et al., 2002).

The occurrence of MFD is multi-faceted, as a variety of dietary factors can lead to its development, from spring grazing (Rivero & Anrique, 2015) to low dietary roughage content (Gama et al., 2008), overfeeding of fat (Flachowsky et al., 2006) and high concentrate inclusion (Auldist, Marett, et al., 2016; Broderick, 2003). For pasture-based milk production systems, MFD in spring milk arises due to the lower proportion of fibrous material in comparison to leaves and the low concentrations of triglycerides in grass at an early developmental stage (Rivero & Anrique, 2015). Attempts to mitigate MFD typically involve the use of hay or concentrate supplementation to introduce additional dietary fiber, though balancing the ration is particularly important in avoiding concentrate overfeeding, which can exacerbate the issue by depressing ruminal pH, leading to sub-clinical acidosis and inhibiting ruminal fiber digestion (Broderick, 2003), ultimately further depressing milk fat content. Rumen function and MFD can also be influenced by levels of 'effective fiber', whereby the reduced particle size of ground or pelleted fiber present in concentrate rations reduces the effectiveness of ruminal fiber digestion (Grant et al., 1990).

In addition, direct supplementation of dietary fat or oils with high PUFA content can lead to MFD (Bauman & Grinari, 2001). The *trans-10 cis-12* isomer of CLA can inhibit

milk fat synthesis when present in high concentrations (Gama et al., 2008). Consideration of the level of FA saturation in supplemental dietary fat was also previously highlighted by Coppock and Wilks (1991). Reduced milk fat content may nonetheless be desirable to producers who receive payment on the basis of milk yield rather than milk fat or protein content, such as those contributing to the 'beverage product' class in the United States (Sneddon et al., 2013) or where preference is given to milk protein content over fat content.

The importance of dietary fiber levels in preventing MFD was also stated by Zimmerman et al. (1991), who observed a significant increase in both milk fat yield and percentage with increasing dietary CP in a high-fibre diet, which was attributed to increased synthesis of short-chain fatty acids (SCFA), with a less substantial effect observed for a low-fiber diet. It was also noted that the extent of MFD when transitioning from high-fiber to low-fiber diet was reduced in cows fed grass hay when compared to alfalfa hay, which may be influenced by the lower particle size of alfalfa hay relative to grass. Other efforts to influence milk composition by increasing CP intake have produced inconsistent results for milk fat. Broderick (2003) recorded a linear increase in milk fat yield (kg/day) but not milk fat percentage with increasing CP content, although the inverse was seen by Colmenero and Broderick (2006) and Wildman et al. (2007), whereas Leonardi et al. (2003) observed a significant increase in both fat yield and fat content in response to increased dietary CP.

Despite the consistent results seen for protein yield and concentration in milk derived from GRS, CLV and TMR feeding systems investigated by McAuliffe et al. (2016), O'Callaghan, Hennessy, et al. (2016), Gulati, Galvin, Lewis, et al. (2018) and Panthi et al. (2019), measurements for milk fat were more variable. Both McAuliffe et al. (2016) and O'Callaghan, Hennessy, et al. (2016) observed that milk fat concentrations were significantly higher for GRS than both CLV and TMR, whereas Gulati, Galvin, Lewis, et al. (2018) observed significantly higher fat content in GRS than in CLV only and Panthi et al. (2019) recorded no difference in fat content between the diets. The similarities in the former two studies and variation in the latter may indicate that milk fat content is more responsive to variation in feed composition or forage quality between years than milk protein content.

Harris et al. (1998) found that, on increasing white clover content from 500 to 800 g per kg DM, milk fat yield increased due to increased overall milk yield, which is typically observed with increasing white clover addition, but milk fat percentage was unaffected. Both O'Callaghan, Hennessy, et al. (2016) and Gulati, Galvin, Lewis, et al. (2018) also found that milk fat yield was consistently higher for TMR than both GRS and CLV and that minima and

maxima of milk fat percentage occurred in April and September, respectively, in all diets. This effect is more attributable to variation by lactation stage than to seasonal changes, as these diets were maintained throughout complete lactation cycles. However, the importance of changes in feeding regimes in the seasonal variations observed in other production systems has been shown by Heck et al. (2009), who determined that seasonal variation in Dutch milk composition was most likely due to dietary changes, as calving is evenly distributed throughout the year in the Netherlands. These dietary changes throughout the year would most likely constitute changes in forage availability and quality, along with decreasing forage:concentrate ratio, which would have a pronounced effect on the FA composition of milk.

3.2.2 | FA composition

Milk FA arise from two sources, depending on chain length; SCFA to medium-chain FA (C4:0 to C14:0) are derived from de novo synthesis in the epithelial cells of the mammary gland, whereas long-chain fatty acids (LCFA) are sourced from the diet of the cow or a combination of both diet and de novo synthesis in the case of palmitic acid (C16:0) (Knutsen et al., 2018). In addition to the overt effect of diet on LCFA content, the extent of de novo synthesis and, thus, SCFA content, may also be influenced by dietary factors, through modulation of rumen microflora based on levels of substrate available for biohydrogenation (Bauman & Griinari, 2003; Palmquist et al., 1993; Sterk et al., 2011). Relative levels of saturated (SFA) and unsaturated (UFA) FAs are significantly influenced by dietary factors, particularly depending on forage content (Chilliard et al., 2001). Indeed, apart from the supplementation of dietary fat, varying the level of pasture feeding is perhaps the most frequently practiced means of FA profile manipulation (Castillo et al., 2006).

Despite recent evidence to the contrary (Drouin-Chartier et al., 2016; Pereira, 2014), the widely held negative health associations between the SFA content of milk fat and increased risk of CVD are likely an influence on the broad interest in the potential for alteration or improvement of milk FA composition through relatively simple means at primary production level. Although the consensus is changing surrounding the health impact of milk fat on CVD, it remains of considerable interest to reduce the levels of lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids which have been shown to have adverse effects on low-density lipoprotein cholesterol levels, which is considered an important risk factor for CVD (Lordan et al., 2018). Thus, the effects of varying levels of pasture inclusion and pasture:concentrate ratios on milk FA

composition have been extensively studied (Bär et al., 2020; Benbrook et al., 2013; Chilliard et al., 2007; Couvreur et al., 2006; Kelly et al., 1998; O'Callaghan, Hennessy, et al., 2016), with generally consistent results and significant effects conclusively determined.

In comparing raw milk produced from GRS, CLV and TMR diets, O'Callaghan, Hennessy, et al. (2016) found that concentrations of a range of minor SFA (C11:0, C13:0, C15:0, C17:0, C23:0) and UFA (C18:2 n-6*trans*, C18:2 *cis-9trans-11*, C18:3 n-3, C20:1, C20:4 n-6) were significantly higher in both pasture-derived milks than in TMR, whereas several UFA (C18:2 n-6*c*, C18:3 n-6*c*, C22:1 n-9, C18:2 *cis-10trans-12*), along with the SFA C16:0 and C22:0, were found to be significantly higher in TMR milk than in both GRS and CLV. In addition, both pasture-based systems produced milk with significantly higher levels of omega-3 (n-3) FA and significantly lower levels of omega-6 (n-6) FA than the TMR system. Further studies on butter (O'Callaghan, Faulkner, et al., 2016) and full-fat Cheddar cheese (O'Callaghan et al., 2017) yielded similar results, with the largest variation between diets being amongst the medium-chain FA to LCFA, particularly PUFA content, n-3:n-6 ratio and concentrations of CLA isomers, α -linolenic acid (GRS and CLV > TMR), vaccenic acid (GRS and CLV > TMR) and behenic acid (TMR > GRS and CLV). Increased concentration of palmitic acid (C16:0), which has a high melting point (63 °C), in TMR-derived milk has a significant impact on the properties of butter and cheese, which will be discussed in Section 4.2.

The C18:2 *cis-9trans-11* (rumenic acid) isomer of CLA has been the subject of widespread interest in recent years for its potential anti-carcinogenic, anti-hypertensive, and adiposity-mediating physiological effects (Lehnen et al., 2015). Produced by ruminal biohydrogenation of UFA (principally vaccenic acid and α -linolenic acid), CLA isomers are present predominantly in ruminant-derived fats (Palmquist et al., 2005), making milk a major dietary source which has received extensive study. In agreement with the previously referenced studies by O'Callaghan et al. (2017), O'Callaghan, Faulkner, et al. (2016) and O'Callaghan, Hennessy, et al. (2016), various authors have seen a linear increase in milk CLA content with increasing pasture consumption (Couvreur et al., 2006; Kelly et al., 1998; Lock & Garnsworthy, 2003; Schroeder et al., 2005; Talpur et al., 2008; White et al., 2001), which has been attributed to the higher levels of rumen biohydrogenation intermediates such as vaccenic acid and α -linolenic acid in grass and other fresh forages than in concentrates, ensiled or lower quality, late season, forages.

The importance of forage type consideration in devising feeding systems for FA manipulation was discussed by Sterk et al. (2011), who found that transitioning from 80% grass silage to 80% corn silage (on a DM basis) resulted in

increased concentrations of linoleic acid (C18:2 n-6) and of *trans-10* C18:1, which is typically present in low concentrations in ruminant fats (Kuhnt et al., 2011), along with a decrease in α -linolenic acid and *trans-11*, *cis-15* C18:2 (a stearic acid intermediate). The high starch content of corn silage was also suggested to influence the relative abundance and function of rumen microflora, leading to the observed changes, particularly in *trans-10* C18:1 content.

Lourenco et al. (2008) compared the effects of feeding red clover, white clover and botanically diverse forages against ryegrass, finding a reduction in SCFA to medium-chain FA content (C6:0 to C18:0) and an increase in medium-chain FA to LCFA content (C18:1 to C18:3 n-3) when feeding a diverse forage diet. This diet also resulted in increased CLA *cis-9trans-11* in milk, associated with increased supply of vaccenic acid. Concentrations of α -linolenic acid were increased by red clover feeding, though neither red or white clover feeding had a substantial effect on the overall FA profile of milk when compared to grass. Lee et al. (2009) observed a greater effect of red clover and particularly chopped red clover feeding on milk FA profile; however, this was at a comparably high rate of red clover feeding.

Increased concentrations of CLA *cis-9trans-11* in summer milk, when forage quality is highest, also appear to be consistent between ryegrass (Stanton et al., 1997) and alfalfa pastures (Castillo et al., 2006). Comparison of fresh and ensiled grass has shown a nutritionally unfavourable trend in milk FA composition upon transition from grazing to silage feeding, which resulted in a linear increase in concentrations of myristic, palmitic, stearic and oleic acids and a linear decrease in vaccenic acid, total CLA and the proportion of rumenic acid to total CLA (Elgersma et al., 2004; Kelly et al., 1998). Consistent results for CLA, α -linolenic acid and oleic acid were seen by Larsen et al. (2014) when comparing milk from a grass-based organic dairy to two conventional dairies. A seasonal effect was also observed within the organic system, although the palmitic acid content and, consequently, melting point of the low-temperature melting fraction were increased in milk from this system. This result contrasted with those of other studies which determined higher palmitic acid content in concentrate-derived milk (Chilliard et al., 2007; Couvreur et al., 2006; O'Callaghan, Hennessy, et al., 2016).

Despite seeing similar overall results to other studies which compared the FA profiles of milks produced from diets of varying forage:concentrate ratios, Jaakamo et al. (2019) also recorded increased PUFA content in milk from cows fed a low (30:70) compared to high (70:30) forage:concentrate ratio, although the forage source used in these diets was grass silage administered in a TMR, rather than fresh grass forage. A 50:50 red clover:concentrate diet increased milk monounsaturated fatty acid (MUFA) con-

tent and further increased milk PUFA content over the low forage:concentrate diet, which has also been seen in previous studies (Moorby et al., 2009; Vanhatalo et al., 2007), though not at the high concentrate inclusion rate administered in the foregoing red clover:concentrate diet.

Efforts to alter milk FA composition through dietary lipid supplementation have utilized diverse sources, including fish oil (Shingfield et al., 2003), linseed and linseed extracted oil (Chilliard et al., 2009), soya oil (Agenäs et al., 2002), soy bean meal (Murphy et al., 1995) rapeseed (Hoffmann et al., 2016), tallow (Dunkley et al., 1977) and calcium salts of FAs (Castañeda-Gutiérrez et al., 2007). The general trend among these feeding trials was similar to that seen in pasture-feeding trials for concentrations of LCFA such as vaccenic acid, oleic acid, linoleic acid, and CLA, which tended to increase significantly. However, these diets appear to have a more pronounced depressive effect on concentrations of de novo-synthesized FA than interventions based on varying forage:concentrate ratios. Supplementation with crushed rapeseed or rapeseed oil (Chilliard et al., 2009) produced similar results to high-forage diets, as the FA profile tended towards increased LCFA content and increased UFA content (Chilliard et al., 2001; O'Callaghan, Hennessy, et al., 2016). High concentrations of omega-3 FA, such as eicosapentaenoic acid and docosahexaenoic acid, resulting from fish oil supplementation (Donovan et al., 2000) are of interest from a nutritional perspective, particularly for their beneficial effects on cardiovascular health, foetal development and cognitive performance in Alzheimer's patients (Stark et al., 2016).

In a study which examined the effect of various lipid sources, feed processing treatments, and heat treatments on the CLA content of milk, Chouinard et al. (2001) found significant increases in CLA content with supplementation of Ca salts of canola oil, linseed oil and soybean oil when compared to a basal diet consisting of a grass silage, corn silage and soybean meal ration. Increased CLA content was also observed with increasing soybean meal extrusion temperature, and CLA content increased significantly in response to fish oil supplementation. As an animal fat source, tallow is high in CLA; however, the effect of tallow supplementation in this study was minor when compared to the other oils used, particularly fish oil. The results of this comparison between tallow and fish oil are in agreement with the report of Jones et al. (2000). Dunkley et al. (1977) found that feeding tallow resulted in a reduction in de novo-synthesized FA, while also decreasing milk protein content, an effect which should be considered for all fat supplementation diets, depending on inclusion rates. However, feeding beef tallow to cows has seemingly fallen largely out of favour in recent years, possibly due to ethical concerns.

Levels of de novo-synthesized FA in milk may also be influenced by supplementation of individual FA. In addition to greater milk and fat yields (Mosley et al., 2007), increased de novo FA synthesis has also resulted from palmitic acid supplementation, modulated by increased synthesis of the de novo FA precursor butyrate in the mammary gland (Hansen & Knudsen, 1987). Other FA supplements were found to have neutral (lauric acid, oleic acid) or inhibitory (stearic acid, linoleic acid) effects on de novo FA concentrations. This may indicate a potential means of influencing SCFA to medium-chain FA composition, which is otherwise largely unresponsive to dietary variation. However, feeding supplements with high palmitic acid content has also been shown to have a limited effect on de novo FA synthesis and, rather, lead directly to considerably high palmitic acid deposition in milk (Lock et al., 2013), which would be a noteworthy concern for butter or cheese manufacturers with regard to product texture and hardness.

3.2.3 | Milk fat globule size

The differential between de novo-synthesized and diet-derived FA can also influence milk fat globule (MFG) size and secretion. The diameter of the MFG ranges from 0.1 to 15 μm , with an average of 2.5 to 4.6 μm (Fleming et al., 2017). Wiking et al. (2004) determined that variation in MFG size is influenced by the relative proportions of LCFA derived from the diet and not by SCFA (C4:0 to C14:0) arising from de novo synthesis. The authors noted that increasing MFG size was correlated with diurnal fat yield in this study, attributing this observation to a limitation in the availability of MFG membrane (MFGM) material with increased fat production. This may be due to the nature of the structure of the MFG as a core of bulk triglycerides surrounded by a membrane rich in polar lipids which, in turn, contain high LCFA content (Lopez et al., 2014). Increased dietary intake of LCFA may increase the polar lipid content of the MFGM, increasing the ratio of MFGM to bulk triglycerides, thus reducing MFG size. Feeding diets either naturally high or enriched in LCFA was also suggested by Logan et al. (2014) to have an influence on MFG size. Indeed, Carroll et al. (2006) showed that MFG size (D_{90} and D_{50}) increased linearly with increasing fat supplementation using yellow grease (vegetable oil), a fat source with particularly high LCFA content (Plascencia et al., 2003).

Couvreur et al. (2007) found several differences in MFG characteristics between the milk of cows fed pasture supplemented with cereal concentrates and those fed corn silage supplemented with soybean meal. The pasture treatment resulted in reduced Sauter diameter ($d_{3,2}$) (3.38 to 3.15 μm), volume mean diameter ($d_{4,3}$) (3.94 to 3.65 μm) and

size distribution range, with increased specific surface area (1.95 to 2.09 m²/g of fat). Conflicting results were reported by Argov-Argaman et al. (2014), who found that larger milk globules were secreted by cows assigned to a low-concentrate, high-forage diet (3.51 µm) compared to those on a high-concentrate, low-forage diet (3.3 µm), which was attributed to increased concentrations of phosphatidylserine, which reduces the interfacial surface tension between droplets, leading to fewer droplets of larger size. However, despite the association between feeding practice and MFG size made by the authors in the cited study, the observed values for average MFG diameter did not vary significantly ($P > 0.05$), rather the difference was reported as a tendency ($P = 0.1$) towards change in MFG size.

Jaakamo et al. (2019) also recorded increased volume mean diameter and decreased specific surface area in milk derived from a low-concentrate, high-forage diet (30:70), compared to high-concentrate, low-forage (70:30), but found that the application of a diet based on a 50:50 red clover:concentrate ratio resulted in increased PUFA content and a lower MFG size than a grass-based diet. The authors did, however, acknowledge that this variation may have been influenced by cow-to-cow variation in MFG secretion as much as any dietary effect. Overall, MFG size appears to be influenced by LCFA content, variation of which can influence both the nutritional profile and functional properties of milk (Logan et al., 2014), such as butter churning time (Avramis et al., 2003) and cheese yield and texture (O'Mahony et al., 2005).

3.3 | Water-soluble components and ash

3.3.1 | Carbohydrate content

In contrast to milk protein and fat content, concentrations of lactose are relatively constant and are not subject to significant variation outside of lactational changes (O'Callaghan, Hennessy, et al., 2016; Walker et al., 2004). However, feeding studies have previously indicated that lactose yield may increase with increasing dietary energy supply, as is often observed for protein content. As seen for protein and fat yield, the increased lactose yield observed by Harris et al. (1998) with increasing dietary white clover content was attributable only to increased milk yield, with percentage of lactose remaining unaffected. Mackle et al. (1999) reported minimal variation in lactose yield in milk from cows fed pasture, pasture supplemented with maize grain or pasture supplemented with maize grain and pasture silage, despite a significant increase in milk yield and the significantly higher levels of metabolizable energy in both supplemented diets. Notably, however, milk protein yield was also not significantly affected in this study.

Consistent reports of a greater lactose yield in CLV- and particularly TMR-derived milk over that of GRS were made by Panthi et al. (2019), Gulati, Galvin, Lewis, et al. (2018) and O'Callaghan, Hennessy, et al. (2016). Both O'Callaghan, Hennessy, et al. (2016) and Panthi et al. (2019) observed significantly higher lactose yield, but not lactose percentage in CLV and TMR milk than in GRS, whereas for Gulati, Galvin, Lewis, et al. (2018), TMR lactose yield was significantly greater than both CLV and GRS, with a significant effect also observed for lactose percentage between each diet, where TMR > CLV > GRS. These studies seem to substantiate the understanding that increased milk lactose yield is correlated with increased overall milk yield (Shahbazkia et al., 2010), attributable in this case to the increased dietary energy content of TMR and CLV diets over a GRS diet (Tables 2, 3, and 4).

3.3.2 | Mineral composition

The previously referenced investigations of varying pasture and concentrate feeding primarily focused on changes in gross composition and FA profile, with the micronutrient composition of milk receiving relatively limited focus. One study which did investigate this topic in detail is that of Gulati, Galvin, Lewis, et al. (2018), who, in comparing GRS, CLV and TMR feeding systems, found notable differences in mineral composition between milks produced from each system. The GRS system produced milk with significantly higher Ca and P levels than both the CLV and TMR systems, which was attributed to the greater casein content of the GRS milk, as the majority of Ca and P in milk is incorporated into the casein micelle as colloidal calcium phosphate. Concentrations of Mn were also significantly higher in GRS milk than in CLV milk.

Despite observed variation by lactational stage in the other macro-elements measured (Mg and Na), feeding system did not have a significant effect on their levels in milk. Along with Cl⁻, Na concentration is a primary influence on the osmotic balance of milk, increasing markedly in late-lactation milk, thus leading to reduced lactose content (Fox et al., 2015). Gulati, Galvin, Lewis, et al. (2018) observed differences in lactose yield and percentage among GRS, CLV and TMR samples in both mid- and late-lactation milk, which is therefore likely to be due in the part to the relative energy contents of the diets. Among the trace elements measured, greater concentrations of Zn, Cu and Se were present in TMR-derived milk than those of both pasture-based systems. This partition in the distribution of macro- and micro-elements between pasture-derived and concentrate-derived milk could be ascribed to uptake of Ca and P from the soil ingested during grazing

and the provision of supplemental trace elements in concentrate rations (Rey-Crespo et al., 2013).

Conflicting results were found by Gabryszuk et al. (2008) in a study comparing conventional indoor-feeding farms with organic farms. Concentrations of Ca, Mg and P were greater in milk produced in an intensive conventional farm feeding TMR than in milk from organic farms where cattle grazed from March to October. For this study, milk sampling took place in September, when declining forage quality may be responsible for reduced micronutrient content in grass (Nantapo & Muchenje, 2013). In addition, milk mineral profiles from organic farming systems may not be reflective of more widely practiced pasture-based systems, as restrictions on organic production may exclude the use of most mineral supplements (Blanco-Penedo et al., 2009), which can otherwise be used as required. In a meta-analysis which compared the mineral composition of milk produced from conventional and organic feeding systems in various countries, Zwierzchowski and Ametaj (2018) determined that milk from conventional farms was characterized by significantly higher concentrations of both macro elements (Ca, Mg, P, K, S) and trace elements (Cu, Mn, Se, Zn, I) than that produced on organic farms, citing the use of mineral supplementation in conventional systems as the most significant factor, while also noting that mineral composition varied considerably between different countries, where production factors also varied.

In agreement with Gulati, Galvin, Lewis, et al. (2018), Rey-Crespo et al. (2013) determined significantly higher concentrations of Zn, Cu and Se in conventionally than in organically produced milk, although the Fe content of organic milk was higher, attributed to soil consumption during grazing. Therefore, a noteworthy seasonal effect which this study also highlights is that of increasing adhesion of soil to forages and thus, soil ingestion, in autumn/winter, as the Fe content of organic milk increased significantly during this period.

Additional effects of increasing dietary fat supplementation reported by Carroll et al. (2006) were a linear decrease in P content and a linear increase in Mg content. However, the authors did not provide an explanation or suggest a mechanism for these findings. Supplemental dietary fats have previously been suggested to reduce Mg absorption in the bovine digestive tract by the formation of insoluble Mg soaps (Ramirez & Zinn, 2000). Overall, the variability of the mineral profile of milk from different feeding systems may be reflective of variation in several factors influencing the composition of the feed used, such as forage quality, soil type, levels of mineral fertilizers applied or concentrate formulation. Consideration of the mineral content of the feed provided in a given system therefore seems to offer a relatively responsive means of influencing the mineral composition of milk derived from that system.

Of particular note is that changes in the trace element composition of milk in relation to bovine diet are of significance to the IMF industry. The prevalent use of skim milk in IMF manufacture means that where significant quantities of concentrates are fed in the bovine diet, mineral specifications in IMF may be in excess of CODEX regulations (European Commission, 2006). This is often most significant for iodine, where supplementation in the feed can lead to substantial increases in milk (O'Brien et al., 2013).

3.3.3 | Vitamin composition

Despite the importance of milk as a source of fat- and water-soluble vitamins, there is a significant lack of information regarding the effects of varying feeding strategies on the overall vitamin profile of milk. The effect of dietary interventions on levels of vitamins and vitamin precursors in rumen fluid (Hayes et al., 1966; Miller et al., 1986; Santschi et al., 2005; Schwab et al., 2006) and the effects of vitamin supplementation on cow performance and health (Bergsten et al., 2003; Rosendo et al., 2004; Zimmerly & Weiss, 2001) have been well characterized, but relatively few studies have compared the final vitamin composition of milk produced from different feeding systems. In what is seemingly the first study to compare the vitamin B2 (riboflavin) content of milk from conventional and organic farms, Poulsen et al. (2015) found higher concentrations of this vitamin in organic milk and, interestingly, seasonal variation, which occurred only for the organic milk, resulted in higher riboflavin content in winter compared to summer milk. This seems quite unusual given the high proportion of riboflavin in fresh, leafy forage (Pinto & Zempeni, 2016). However, the authors suggested that this may have been due to increased ruminal synthesis of riboflavin in response to decreased intake of forage; however, the riboflavin content of the organic milk in this study was consistently higher than that produced from conventional farms, which utilized lower forage content all year-round.

Magan et al. (2020) compared the water-soluble B vitamin composition of skim milk, sweet whey, micellar casein whey and acid whey powders produced from mid-lactation milk derived from cows assigned to the GRS, CLV and TMR diets previously described. Concentrations of vitamin B1 (thiamine), riboflavin and vitamin B7 (biotin) were significantly higher in both GRS- and CLV-derived ingredients compared to those from TMR, which contained higher levels of vitamin B3 (nicotinic acid) and B3-amide (nicotinamide), with significant variation also present between ingredient types. The differences observed in riboflavin content, in particular, were likely to have been due to direct transfer from the proportion of fresh forage available in

the diet, but differences in biotin content were attributed to potential diet-induced modulation of rumen microbiota activity.

Within grazing systems, riboflavin content tends to be higher in milk produced from grass forage than from maize (Laverroux et al., 2014) or hay (Havemose et al., 2006), whereas comparison of grass and maize feeding has shown increased vitamin B9 (folate) in milk derived from grass and greater B12 (cobalamin) in milk produced from maize feeding (Chassaing et al., 2011). Concentrations of the fat-soluble vitamin A (retinol) precursor β -carotene and vitamin E have also been observed to increase with increasing grass or grass silage feeding (Adler et al., 2013; Agabriel et al., 2007; O'Callaghan, Faulkner, et al., 2016). Indeed, the characteristic yellow color of grass-derived milk and dairy products is attributable to their high β -carotene and riboflavin contents. Diet-based effects on the cobalamin content of milk have been observed to be marginal, with a slight positive correlation between percentage of chopped mixed silage in the diet and milk cobalamin concentrations being determined by Duplessis et al. (2019).

In addition to dietary intake, vitamin B synthesis occurring in the rumen provides sufficient levels to prevent deficiency (Schwab et al., 2006). However, by depression of rumen cellulolytic microflora, sub-clinical acidosis arising from concentrate over-feeding can lead to insufficient synthesis of thiamine (Pan et al., 2018) and biotin (Rosendo et al., 2003). The ability to influence the relative abundance of vitamins in milk through dietary means is an important nutritional consideration, as standard portions of milk and dairy products offer a significant proportion of the RDA for some vitamins (Magan et al., 2020).

4 | IMPACT OF MILK COMPOSITION ON DAIRY PRODUCT FUNCTIONALITY

4.1 | Liquid milk properties

Milk is subjected to various processing conditions during the manufacture of products such as powders, yogurt, cheese or milk-based beverages and must, therefore, be stable to the environmental stresses applied. As milk is most often pasteurized before sale, thermal stability is a particularly important property in dairy processing. Ultrahigh-temperature processing involves heating milk to over 135 °C for 1 to 8 s (Penfield & Campbell, 1990) and can result in whey protein denaturation, Maillard browning reactions, lactulose production and release of volatile compounds (Dursun et al., 2017), the extents of each depending on the specific thermal loads applied. Indeed, surface fouling or 'burn-on' can occur at various processing conditions, even at lower temperatures. The total protein and whey

protein contents of milk are, therefore, important factors determining its susceptibility to heat-induced aggregation and coagulation (Rosmaninho et al., 2007). A summary of the literature discussed in this section is shown in Table 5.

Increases in the NPN content of milk arising due to increased urea concentrations, such as those seen in clover-derived milk by Harris et al. (1998), O'Callaghan et al. (2018) and Magan, Tobin, et al. (2019), will markedly increase its heat coagulation time (HCT) and stability in thermal processing applications. Indeed, Magan, Tobin, et al. (2019) observed significantly higher HCT and NPN content in CLV-derived whole milk powder than that produced from TMR. Urea undergoes heat-induced decomposition to ammonia, resulting in a buffering effect against heat-induced acidification, which is also significantly influenced by the lactose concentration; HCT decreases with increasing lactose content (Hupertz, 2016). Increased acidity due to formic acid produced through heat-induced decomposition of lactose is primarily responsible for this trend (Singh, 2004). Hupertz (2016) suggested that lactose can, however, have a stabilisation effect on the HCT of milk through complex formation with homocitrulline, formed from the reaction of lysine residues with isothiocyanate, another heat-induced decomposition product of urea. Murphy et al. (2014) have previously shown a stabilizing effect of lactose in heat-treated IMF emulsions, suggesting that the presence of carbohydrates causes proteins to be preferentially hydrated, as interactions between carbohydrates and the hydrophobic regions of whey proteins are reduced at higher temperatures. As discussed in Section 3.1.2, the urea content of milk can vary substantially by diet, but lactose concentrations are generally less susceptible to dietary influence.

In addition to the improved thermal stability of milk with high NPN content, the contribution of NPN to overall CP content may appear to be beneficial to producers operating within a CP payment scheme; however, this will result in milk of reduced protein quality or true protein content, which would be of particular concern to cheese manufacturers, as cheese yield is positively correlated with the true protein and casein contents of milk and negatively impacted by increased levels of NPN (Amenu et al., 2006).

The HCT of milk is also negatively influenced by increasing levels of ionic calcium (Ca^{++}) (Sievanen et al., 2008), which represents approximately 10% of the total calcium content of milk (Akkerman et al., 2019). Akkerman et al. (2019) compared the calcium and citrate content of milk from two organic and one conventional dairy farm, finding significantly higher levels of total Ca^{++} and proportions of Ca^{++} relative to total Ca in milk from both organic farms when compared to that from the conventional farm, but noted that the differences observed were unlikely to be sufficient to lead to significant differences

TABLE 5 Summary of significant dietary effects on milk and dairy product functionality

Functionality	Dietary factor	Effect relative to comparison diets	References
Heat coagulation time (HCT)	Clover feeding	Increased HCT	Magan, Tobin, et al., 2019
	Organic production	Increased calcium ion activity	Akkerman et al., 2019
	Crude protein supplementation	Decreased HCT	Reid et al., 2015
Ethanol stability	Concentrate supplementation	Increased ethanol stability	O'Brien et al., 1999
Acid gelation	Grass and clover feeding	Increased gel strength	Magan, Tobin, et al., 2019
	Concentrate feeding	Increased gel strength	Jasińska et al., 2010
	Soy bean meal, palm kernel, beet pulp and standard concentrate feeding	Gel strength of soya-derived milk > palm kernel > beet pulp > standard concentrate	O'Callaghan et al., 2019
Butter properties	Linseed oil supplementation	Increased moisture content and decreased firmness	Hurtaud et al., 2010
	Fish oil supplementation	Improved spreadability	Avramis et al., 2003
	Pasture feeding	Lower melting temperature and hardness	Couvreur et al., 2006; Hurtaud et al., 2007; O'Callaghan, Faulkner, et al., 2016
	Concentrate feeding	Higher melting temperature and hardness	
	Pasture feeding	Improved spreadability	Couvreur et al., 2006
Rennet coagulation	Grass feeding	Lower rennet coagulation time and higher gel strength	Gulati, Galvin, et al., 2019
	Grass feeding	Higher flowability of Mozzarella cheese	Gulati, Galvin, Hennessy, et al., 2018
	Grass and clover feeding	Softer texture of Cheddar cheese	O'Callaghan et al., 2017
	Concentrate feeding	Higher firmness in Maasdam cheese	Panthi et al., 2019
	Partial mixed ration or formulated grain mix feeding	Increased cheddar cheese yield	Auldlist, Greenwood, et al., 2016
Sensory quality	Grass and clover feeding	Thicker texture in the mouth	Faulkner et al., 2018
	Clover feeding	Higher creaminess	Clarke et al., 2019
	Pasture feeding	Yellower color	Magan, Tobin, et al., 2019; Martin et al., 2005; O'Callaghan, Faulkner, et al., 2016
	Concentrate feeding	Whiter color	
	Grass and clover feeding	'Cooked milk' flavor and 'barnyard' aroma	Clarke et al., 2019; Faulkner et al., 2018
	Concentrate feeding	'Hay-like' flavor	
Traceability	Pasture feeding	Lutein, β -carotene, toluene, p-cresol and dimethyl sulfone biomarkers	Clarke et al., 2019; Faulkner et al., 2018; Glover et al., 2012; Nozière et al., 2006; O'Callaghan et al., 2017; Panthi et al., 2019
	Concentrate feeding	2,3-Butanediol, citrate and 2-pentanone biomarkers	
	Pasture and concentrate feeding	Differentiation by fatty acid profiling	O'Callaghan et al., 2017; Pagano & Marcella, 2010
	Pasture and concentrate feeding	Differentiation by nuclear magnetic resonance	O'Callaghan et al., 2018; Panthi et al., 2019
	Pasture and concentrate feeding	Differentiation by Fourier-transform infrared spectroscopy	Capuano et al., 2014
	Pasture and concentrate feeding	Differentiation by liquid chromatography–mass spectrometry	Magan, O'Callaghan, et al., 2019
	Pasture and concentrate feeding	Differentiation by Raman spectroscopy	Gómez-Mascaraque et al., 2020

in milk processability. Levels of Ca^{++} were also observed to increase significantly as the season progressed, which had also been previously observed by Chen et al. (2014) in milk from a year-round calving herd, indicating that the seasonal variation recorded by Akkerman et al. (2019) may have occurred independent of lactational changes, which were likely to have occurred in both organic systems.

Gulati, Hennessy, et al. (2019) found higher levels of Ca^{++} in reconstituted low-heat skim milk powder produced from GRS, than those produced from CLV and TMR, but did not observe any significant variation in HCT between the samples, suggesting that the increased protein and Ca^{++} content of the GRS sample may be offset by its reduced lactose content. Reid et al. (2015) observed decreasing HCT of milk with increasing dietary CP supplied through concentrate supplementation, which was attributed to increased total protein content, but also correlated increased HCT with increasing NPN content. Magan, Tobin, et al. (2019) observed the highest and lowest Ca^{++} in TMR- and CLV-derived whole milk powder, respectively, which was inversely associated with the trend observed in HCT between the samples.

Another common and more rapid method for determining raw milk quality is the ethanol stability test. Ethanol-induced aggregation and precipitation in milk occurs due to inhibition of the κ -casein 'hairy layer' on the exterior of the casein micelle, reducing the steric barrier and thus promoting micelle flocculation (Horne, 2016). The ethanol stability of GRS, CLV and TMR-derived skim milk was compared by Gulati, Hennessy, et al. (2019), who did not observe any significant differences in stability between the samples. Similarly, Machado et al. (2014) and Grimley et al. (2009) did not observe significant variation in ethanol stability in milk from cows fed with varying forage:concentrate ratios or when turning out to pasture from indoor housing, although O'Brien et al. (1999) observed increased ethanol stability of milk with concentrate supplementation in a grazing system.

Potential effects of diet-induced compositional variation on the functional properties (e.g. solubility, wettability, bulk density, flowability) of milk powder products have not yet been investigated, but are unlikely to be significant, as powder properties are primarily dependent on processing parameters such as dryer temperatures and the type of atomizer used (Sharma et al., 2012).

4.2 | Yogurt, butter and cheese

The functional characteristics of concentrated end products of milk processing such as butter or cheese tend to experience greater variation due to compositional variation than those of liquid milk and acidified milk products

such as yogurt, in which the major components (fat and protein) are not concentrated with respect to the milk used for their production. The production of yogurt, fermented dairy beverages or acid cheese involves acid coagulation of heat-treated liquid milk, typically through the addition of lactic acid-producing bacterial cultures such as *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (Hill & Kethireddipalli, 2013). The application of a high thermal load to milk for use in yogurt production has two critical functions: (1) the removal of spoilage microorganisms which are potentially competitive to the starter cultures used and (2) extensive denaturation of whey proteins, thus exposing sulphhydryl groups to allow complex formation of covalent bonds with κ -caseins. As the pH of the acidified milk declines to the isoelectric point of casein (pH 4.6), the surface charge of the casein micelle is reduced, causing the κ -casein 'hairy layer' to flatten, reducing the steric and electrostatic repulsion between the micelles and facilitating micelle aggregation and the subsequent formation of a three-dimensional gel matrix (Lee & Lucey, 2010). In research studies, acidification is often achieved through the addition of glucono- δ -lactone to milk (Gastaldi et al., 2003).

Yogurt gel formation is influenced by a variety of factors such as the extent of heat treatment applied, homogenisation pressure and the fat, protein and mineral composition and casein:whey ratio of milk (Lee & Lucey, 2010). Kamal et al. (2017) showed that increasing the Ca content of cows' milk through the addition of calcium chloride (CaCl_2) at two concentrations (10 and 20 mM) resulted in a significant decrease in gelation time and significant increase in gel firmness with increasing Ca concentration. However, increasing P content through the addition of hydrogen phosphate dehydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) resulted in a significant increase in gelation time, a significant delay in the onset of gelation and a significant decrease in gel firmness, only at a concentration of 20 mM. The differences in compositional factors due to diet which have been previously discussed would, therefore, be expected to influence yogurt gel formation and gel strength. The higher protein, casein, Ca and P contents of GRS- compared to CLV- and TMR-derived milk observed by Gulati, Galvin, Lewis, et al. (2018) would imply that it is the most suitable for this purpose. However, in spite of these compositional differences, Gulati, Hennessy, et al. (2019) did not observe significant differences in the gelation time or gel strength (storage modulus) of yogurts produced from reconstituted skim milk powder made from milk from each feeding system, suggesting that the differences in protein content were insubstantial relative to the natural variation in starter culture activity and fermentation rates between replicates. However, Magan, Tobin, et al. (2019) observed significantly higher gel strength and textural firmness in yogurts

produced from both GRS- and CLV-derived reconstituted whole milk powder than those from TMR, though gelation time was unaffected. The FA distribution of each whole milk powder would be expected to have an inverse effect on gel strength to that observed, as concentrations of the high-melting point FA palmitic acid are typically significantly higher in TMR-derived milk fat than that derived from pasture (Couvreur et al., 2006; O'Callaghan, Faulkner, et al., 2016).

Jasińska et al. (2010) compared the textural properties of yogurts produced from the milk of cows grazing pasture supplemented with concentrates and with those fed a conventional indoor TMR, observing significantly higher gel firmness values for TMR-fed cows, although this was not correlated to differences in the protein content of milk from each diet, and a consistent significant effect on gel firmness was not observed throughout the entire lactation period. O'Callaghan et al. (2019) observed more substantial differences in acid gels produced from the milks of four groups of cows fed different supplementary feed types (16% CP parlour concentrate, palm kernel plus parlour concentrate, soya hulls plus parlour concentrate and molassed beet pulp plus parlour concentrate) in a pasture-based system. The storage moduli and loss moduli of feed type differed significantly, in the order: soya feed > palm kernel > beet pulp > standard parlour concentrate. Increasing gel strength was also inversely correlated with decreasing casein micelle size in these samples, indicating that gel strength may have increased with increasing casein micelle surface area availability for aggregation.

The production of butter involves concentration of the fat content of cream which has been allowed to partially coalesce, following centrifugal separation from heat-treated milk (Buldo et al., 2013). The shearing forces applied during the churning process destabilize the cream emulsion and disrupt the MFGM enclosing the fat globule, inverting the oil-in-water emulsion to a water-in-oil emulsion as the aqueous phase (buttermilk) is released (Vanderghem et al., 2010). The efficiency of this process is determined by the percent recovery of fat in the solid phase, which has been shown to be reduced when using cream with lower MFG size (Hurtaud et al., 2010). Hurtaud et al. (2010) reported that increasing levels of supplementation of extruded linseed oil (2.1% and 4.3% of a TMR) compared to a control ration caused a linear decrease in MFG size (from 4.18 to 4.07 μm at 2.1% and 3.49 μm at 4.3%) and increase in UFA content in milk. The decrease in MFG size also caused an increase in the moisture content of butter as the proportion of fat lost in buttermilk increased. These changes resulted in reduced firmness values for butter and, consequently, increased spreadability. A similar effect was recorded by Avramis et al. (2003), who observed decreasing MFG size (2.31 to 1.84 μm) and

improved spreadability of butter produced from the milk of cows supplemented with fish oil in comparison to those fed a corn-based TMR control diet. The authors also stated that the improved spreadability was influenced by the presence of lower-melting-point FA, but that this was unlikely to be the primary determinant, as the iodine values of the butter samples did not vary significantly. The iodine value of a fat measures the degree of unsaturation of its constituent FA (Sanders, 2003) and is an important indicator of the susceptibility of butter to oxidation. The potential for reduced oxidative stability due to increasing UFA content arising from pasture feeding, as observed by Couvreur et al. (2006), O'Callaghan, Faulkner, et al. (2016) and O'Callaghan, Hennessy, et al. (2016), may be counterbalanced by the increased levels of the antioxidant species β -carotene and α -tocopherol present in milk derived from these systems (Butler et al., 2008; Nozière et al., 2006).

In contrast to the results of Avramis et al. (2003), differences in the FA composition of milk have been shown to significantly influence the hardness and spreadability of butter (Couvreur et al., 2006; Hurtaud & Peyraud, 2007; Hurtaud et al., 2007; O'Callaghan, Faulkner, et al., 2016). Both Couvreur et al. (2006) and O'Callaghan, Faulkner, et al. (2016) observed lower butter hardness and melting temperatures and improved texture in pasture-derived butter, when compared to concentrate-derived butter, attributing these differences to the increased UFA content of pasture-derived milk and, in particular, the increased concentrations of the high-melting-point FA palmitic acid (C16:0) in concentrate-derived milk. Significant variation in spreadability scores was observed by Couvreur et al. (2006), but not by O'Callaghan, Faulkner, et al. (2016), despite the differences in textural and melting properties. On balance, it should be accepted that the texture and spreadability of butter is influenced by variation in FA melting points. This is most likely the primary cause for the improved spreadability observed by Avramis et al. (2003), despite the recorded iodine values.

The final major aspect of milk processability which will be discussed is rennet-induced coagulation and cheese formation. The cheese-making properties of milk are highly susceptible to variation due to compositional changes, as influenced by seasonal and lactational factors (Coulon et al., 1998; Sapru et al., 1997). Rennet coagulation of milk requires destabilisation of the casein micelle and is achieved through the addition of rennet, a blend of mucosal enzymes present in the abomasum of ruminants which contains the protease chymosin, which selectively hydrolyses κ -casein at the surface of the casein micelle (Anema et al., 2007). This facilitates coagulation in two ways: proteolysis of the κ -casein layer removes the steric barrier between casein micelles and, in addition, the loss of the negatively charged glycomacropeptide region on

the κ -caseins reduces electrostatic repulsion between the micelles, allowing calcium-induced micelle aggregation and the formation of an ordered three-dimensional matrix to occur.

Heat treatment of milk prior to rennet addition has been used as a means of increasing cheese yield (Anema et al., 2007) by incorporating denatured whey proteins, which would otherwise be removed when cutting the curd, into the gel matrix by binding to κ -casein via disulphide linkages. However, the level of whey protein denaturation is dependent on the heat load applied during pasteurisation, which is negligible at standard high temperature–short time conditions of 72 °C × 15 s (van Lieshout et al., 2020). More severe heat treatments (80 to 140 °C × 4 s) have been shown to result in increased rennet coagulation times (RCT) when more than 60% of β -lactoglobulin has been denatured and reduced gel strength at more than 10% denatured β -lactoglobulin (Waungana et al., 1996). Excessive complex formation between denatured whey proteins and κ -casein inhibits the action of chymosin, inhibiting micelle flocculation and subsequent curd formation (Salih & Abdalla, 2020). Furthermore, the decrease in syneresis which would result from whey protein incorporation would be undesirable in most cheese production applications. As NPN is present in milk serum and not involved in gel formation, increased proportions of NPN relative to total protein and, thus, a lower true protein content in milk will be linked to decreased cheese yield (Panthi et al., 2019).

The coagulation time and gel strength of rennet gels are highly variable due to the influence of a variety of factors, including the level of pre-heat treatment of milk, concentration of rennet used, temperature during coagulation, total protein and casein content, casein micelle size, distribution of casein fractions, milk pH, and levels of ionic and colloidal calcium (Avramis et al., 2003; Corredig & Salvatore, 2016; Martin et al., 2008). The effect of dietary factors on the rennet coagulation properties of milk was determined by Gulati, Galvin, et al. (2019) and Gulati, Hennessy, et al. (2019), who investigated the effect of varying daily herbage allowance (DHA) on the processability of milk throughout lactation (Gulati, Galvin, et al., 2019) and the effect of pasture and concentrate feeding on the processability of reconstituted low-heat skim milk powder (Gulati, Hennessy, et al., 2019). In agreement with a similar study by O'Brien et al. (1997), rennet coagulation properties did not vary significantly according to DHA in early-, mid- or late-lactation milk, which corresponded to the lack of significant compositional variation (total and soluble casein content and ratios of Ca and P to casein) in milk produced at each level of DHA. Interestingly, when compared to mid- and early-lactation milk, significantly reduced RCT and increased gel strength were observed in late-lactation milk (Gulati, Galvin, et al., 2019), when cheese-making proper-

ties are typically negatively affected by changes in the protein and mineral profiles of milk (Lucey & Fox, 1992). The authors suggested that the significantly increased casein content was sufficient to counteract the lower Ca and P to casein ratios and increased level of soluble casein which occurred in late lactation.

Further evidence for the effect of increased protein content in milk on RCT and gel strength was provided by Gulati, Hennessy, et al. (2019) using reconstituted skim milk powder. Milk derived from GRS displayed significantly lower RCT and significantly higher gel strength than that of TMR, with intermediate values observed for CLV for both variables. This was correlated with the significantly higher protein content of the GRS sample. In addition, the same lactational effect as was previously observed by Gulati, Galvin, et al. (2019) also occurred in this study. Further to these studies, Gulati, Galvin, Hennessy, et al. (2018) measured the functional properties of mozzarella cheese produced from GRS, CLV and TMR diets. Compositional variation between the raw milk samples from each feeding system was standardized in the resultant cheese samples, with the exception of the levels of I, Se and Cu, which were significantly higher in TMR-derived milk than in both GRS and CLV. Yield, extent of proteolysis, water-holding capacity and textural properties were unaffected by feeding system. Despite this, the flowability and loss tangent (ratio of storage modulus to loss modulus) of GRS-derived cheese were significantly higher at room temperature than that from TMR, indicating that grass feeding results in a more fluidic mozzarella product that melts at a lower temperature, which is a notable consideration for mozzarella functionality. Although FA profiling was not carried out on the samples used in this study, the difference in fat liquefaction was attributed to the significantly higher proportion of palmitic acid measured previously in TMR samples (O'Callaghan, Hennessy, et al., 2016) derived from the same feeding systems used in this study. This may also have been due to increased proportions of UFA relative to SFA in the GRS sample, as has previously been observed in pasture-derived milk (Couvreur et al., 2006; O'Callaghan, Faulkner, et al., 2016).

A more pronounced effect of feeding system on the textural properties of full-fat Cheddar cheese was reported by O'Callaghan et al. (2017), despite a lack of significant variation in proteolysis between samples. Both GRS- and CLV-derived cheese samples were significantly softer at room temperature than TMR samples, although no significant differences in firmness were observed at refrigeration temperature. This variation was attributed to the considerable variation in FA composition between the cheese samples, as has been observed for milk samples from these feeding systems. As reported for butter, the greater palmitic acid content of TMR-derived cheese is likely the most

substantial contributor to its higher firmness values at room temperature. Panthi et al. (2019) also reported no significant variation in the yield or gross composition of standardized Maasdam cheeses produced from GRS, CLV and TMR feeding systems. Significant differences in sensory attributes were observed, however, including textural qualities which correspond to those observed by O'Callaghan et al. (2017), as TMR-derived cheese scored highest for hardness and rubbery texture in the mouth. Provision of corn grain and canola meal in a partial mixed ration or formulated grain mix to grazing cows receiving standard parlor meal and alfalfa hay was observed to have a beneficial effect on Cheddar cheese yield by Auldust, Greenwood, et al. (2016), with no changes to coagulation properties, cheese composition, or sensory acceptability.

4.3 | Sensory quality

Sensory evaluation of dairy products encompasses all aspects of organoleptic perception, although olfactory stimuli are perhaps the most significant, as aromatic compounds are generally considered to account for a considerable majority of flavor perception, relative to gustatory stimuli. Flavor chemistry techniques can quantify volatile compounds present to elucidate differences in perception identified through sensory evaluation. Compositional variation due to feeding system has notable effects on the appearance and texture of dairy products and the relative abundance of numerous volatile aromatic compounds has also been seen to be associated with feeding practices (Clarke et al., 2019; Faulkner et al., 2018; Panthi et al., 2019). Volatile compounds are transferred into milk by direct and indirect means, the former being direct inhalation of airborne volatiles which are diffused into the bloodstream through the lungs and ultimately to the mammary gland and the latter being absorption during digestion and subsequent diffusion through the blood supply to the mammary gland (Faulkner et al., 2018).

As discussed in Section 4.2, the substantial variation due to diet in milk FA composition results in variation in the textural properties of milk and products such as butter and cheese. This is primarily due to variation in the ratio of high- (palmitic acid) to low-melting point (oleic acid) FA and fat globule size, although perceptible textural differences are not limited to products of high fat content. In a comprehensive study combining volatile analysis with hedonic sensory acceptance and ranked descriptive analysis, Faulkner et al. (2018) compared pasteurized samples of the whole milk collected from the GRS, CLV, and TMR feeding systems by O'Callaghan, Hennessy, et al. (2016). Milk derived from the GRS system was scored significantly higher for the 'viscosity' attribute than both

CLV- and TMR-derived milks by an Irish consumer panel, despite the higher palmitic acid content of TMR milk. The observed effect may be due to the higher gross fat content of GRS-derived milk relative to the other samples (O'Callaghan, Hennessy, et al., 2016) or possibly due to variation in free fatty acid content between samples, which can influence the surface tension of milk (Kamath et al., 2008). The GRS sample also scored significantly higher for 'liking of texture' and 'overall liking' than both CLV and TMR samples in hedonic ranking, indicating that the panel consisting of Irish consumers seemed to assign the highest ranking to the sample with which they were most familiar. The perceived difference in viscosity may, therefore, be linked to the overall preference for the texture of the familiar sample.

The abundance of free SCFAs, which are present in greater concentrations in TMR-derived milk, can also influence the flavor of cheese (Kilcawley, 2017). Likewise, increased LCFA content, as observed for pasture-derived milk, provides greater levels of substrate for oxidation or reduction to odor-active compounds (Villeneuve et al., 2013). As stated in Section 4.2, the higher PUFA content resulting from pasture feeding also increases the susceptibility of milk to oxidation (Hedegaard et al., 2006), though this may be counteracted by the presence of antioxidants such as β -carotene, as perceptible sensory differences due to lipid oxidation have not been observed between milks produced from pasture and concentrate (Kilcawley et al., 2018).

Visual differences between pasture- and concentrate-derived products are generally dependent on lightness (L^*) and yellowness (b^*) values (as determined by CIE LAB color space), whereby pasture-derived products have a characteristic 'golden hue' of increasing intensity in higher-fat products, with concentrate-derived products displaying a whiter appearance (Faulkner et al., 2018; Magan, Tobin, et al., 2019; Martin et al., 2005; Nozière et al., 2006; O'Callaghan, Faulkner, et al., 2016). This can be attributed to the higher carotenoid (chiefly β -carotene) and riboflavin contents of grass and other leafy forages, transferring a yellow pigmentation to milk and other products manufactured therefrom (Magan, Tobin, et al., 2019; O'Callaghan, Faulkner, et al., 2016).

Milk flavor is influenced by a range of classes of volatile compounds, including ketones, sulphur compounds, acids, alcohols, esters, phenols, aldehydes and lactones (Kilcawley et al., 2018), primarily arising from rumen AA metabolism (Faulkner et al., 2018). Dimethyl-sulfone is frequently determined to be present in significantly higher concentrations in pasture-derived milk (Clarke et al., 2019; Coppa et al., 2011; Faulkner et al., 2018; Villeneuve et al., 2013) and cheese (Carpino et al., 2004; Panthi et al., 2019) and is an important compound

in sensory evaluation due to its low odor-threshold (Kilcawley et al., 2018) and 'cooked milk' flavor (Clarke et al., 2019). Notably, release of volatile sulphur compounds is strongly associated with heating of milk (Al Attabi et al., 2009), though Villeneuve et al. (2013), Faulkner et al. (2018) and Clarke et al. (2019) all applied low-heat pasteurisation treatments (approximately 72 °C × 15 s) to their milk samples, whereas Coppa et al. (2011) utilized unpasteurized milk. Increased concentrations of dimethyl-sulfone have been associated with increased methionine metabolism due to the higher protein content of pasture-derived milk, which provides greater substrate for deamination, resulting higher levels of volatile sulphur compounds (Faulkner et al., 2018).

Volatile analysis carried out by Clarke et al. (2019) identified sulphur compounds and hydrocarbons as being associated with GRS and CLV feeding, whereas TMR feeding was correlated with increased concentrations of aldehydes and esters of SCFA, which corresponds to the increased SCFA content previously identified in TMR-derived milk. Faulkner et al. (2018) also observed increased concentrations of carbohydrate esters in TMR milk samples, and attributed this to alcohols produced through fermentation of the high carbohydrate content of the ration.

Levels of the hydrocarbon toluene in milk vary considerably by level of pasture feeding. Toluene is primarily a breakdown product of β -carotene metabolism and is present in correspondingly high concentrations in pasture-derived products (Faulkner et al., 2018; Panthi et al., 2019; Villeneuve et al., 2013), though its odor threshold is comparatively high. The phenolic compound *p*-cresol is an important odor-active compound in comparing milk and cheese derived from pasture- and concentrate-based feeding systems. The sensory evaluation carried out by Faulkner et al. (2018) identified a 'barnyard' aroma, alongside color and viscosity, as the most significant discriminating factors among GRS-, CLV-, and TMR-derived milk samples. This trait scored higher for both the GRS and CLV samples, in comparison to TMR, and was associated with the concomitant high levels of *p*-cresol identified in these samples. Like toluene, *p*-cresol is derived from rumen degradation of β -carotene, in addition to degradation of tryptophan, tyrosine, and isoflavones present in white clover (Faulkner et al., 2018). A similar association between *p*-cresol and pasture feeding was observed by Lopez and Lindsay (1993) and O'Callaghan et al. (2018). Moio et al. (1996) also showed evidence for direct transfer of *p*-cresol arising from lignin degradation in forage to milk.

Terpenoids are another major class of odor-active compounds in milk which undergo variation due to feed type. They are secondary plant metabolites with concentrations in milk which are directly correlated to their concentra-

tions in the feed consumed by the cow (Bugaud et al., 2001), varying considerably between different forage types. The terpenoid content of dicotyledonous forages is particularly high relative to monocotyledons (Agabriel et al., 2007; Tornambé et al., 2006) and, similarly, diverse forages contain substantially greater levels than monocultures (Faulkner et al., 2018; O'Callaghan, Hennessy, et al., 2016). Despite the diversity of feeds supplied in indoor TMR systems, pasture-derived milk has demonstrated greater diversity in terpenoid content, though this is also dependent on forage diversity (Fernandez et al., 2003). Sensory discrimination between milk and dairy products from different feeding systems based on terpenoid content may be limited, however, by their high odor threshold and the development of terpenoid compounds during cheese ripening (Kilcawley et al., 2018).

Using a trained descriptive sensory panel, Clarke et al. (2019) compared pasteurized milk samples from GRS, CLV and TMR feeding systems, which could be clearly discriminated. The CLV samples scored significantly higher than GRS for creaminess, and TMR samples scored highest for white color, with both GRS and CLV samples scoring higher for creamy color and TMR scoring significantly higher than both GRS and CLV for hay-like flavor. Hedonic sensory analysis was carried out on pasteurized milk samples from these feeding systems by Faulkner et al. (2018) using an Irish consumer panel. The GRS sample scored highest for all preferential attributes and scored statistically higher than both CLV and TMR for liking of texture, liking of flavor and overall acceptability. The CLV sample scored lowest for all attributes other than liking of texture, which is surprising, given the unfamiliarity that Irish consumers are likely to have with TMR-derived milk.

Similar results were found by O'Callaghan, Faulkner, et al. (2016) for hedonic and ranked descriptive analysis of sweet-cream butter produced from GRS, CLV, and TMR milk, where an untrained consumer panel preferred GRS samples over both CLV and TMR samples, but did not determine significant descriptive differences between GRS and CLV samples. In this study, GRS butter scored highest for liking of appearance and flavor and scored significantly higher than TMR for color, diacetyl aroma, diacetyl flavor and cream flavor. The CLV-derived butter scored significantly higher for texture than TMR, as seen by Faulkner et al. (2018) for pasteurized milk. However, a study by Croissant et al. (2007) found that a trained descriptive panel was capable of discriminating between pasture- and concentrate-derived pasteurized milks, where an untrained consumer panel was not. Where consumers can differentiate between products derived from different feeding systems, preference and overall acceptance are likely to reflect their familiarity with products from a given system. American consumers are likely to prefer products

manufactured from TMR milk, whereas Irish consumers will favor those from GRS or CLV (Cheng et al., 2020). Analysis of cross-cultural differences is therefore an area which is particularly appropriate for further research.

4.4 | Dairy product traceability

The differences in milk composition arising from types of feeding practices also offer potential means for verification of milk and dairy product origin, which will be increasingly important as 'grass-fed' product labelling becomes more widespread. Certain carotenoids and volatile compounds which are particularly associated with pasture- and concentrate-based feeding have been identified as potential biomarkers for products derived from these systems. The significantly higher concentrations of the carotenoids 'lutein' and ' β -carotene' in pasture-derived milk are frequently cited as being applicable for this purpose (Faulkner et al., 2018; Mirzad et al., 2018; Nozière et al., 2006; O'Callaghan et al. 2017; O'Callaghan, Hennessy, et al., 2016), along with the volatile degradation products of β -carotene metabolism, toluene and p-cresol (Clarke et al., 2019; Faulkner et al., 2018; O'Callaghan et al. 2017). Other volatiles correlated with particular feeding systems include 2,3-butanediol in TMR-derived cheese (O'Callaghan et al. 2017), dimethyl-sulfone and pentanal in pasture-derived milks (Clarke et al., 2019; Coppa et al., 2011; Faulkner et al., 2018) and 2-pentanone in concentrate-derived milk (Glover et al., 2012). Volatile analysis offers an effective method of determination of product origin, although the evaluation made by the consumer, while subjective, can be determined with reasonable certainty based on the perceptible color difference in fat-containing products arising from the relative levels of β -carotene.

Multivariate statistical analysis has also been used in conjunction with FA (O'Callaghan et al., 2017; Pagano & Marcella, 2010) and metabolomics profiling (Magan, O'Callaghan, et al., 2019; O'Callaghan et al., 2018; Panthi et al., 2019) and spectroscopic methods (Capuano et al., 2014; Coppa et al., 2012; Gómez-Mascaraque et al., 2020; Pagano & Marcella, 2010) to clearly discriminate between pasture- and concentrate-derived dairy products. Using Fourier-transform infrared spectroscopy, Capuano et al. (2014) distinguished between grass-fed and conventional milk, though this method could not as effectively discern between grazed grass and grass silage fed indoors or between organic and conventional milk. The substantial effect of feeding system on the FA profile of milk also presents an effective means of differentiation, as demonstrated by Pagano and Marcella (2010) in milk and by O'Callaghan et al. (2017) for Cheddar cheese. Using quantitative nuclear magnetic resonance (1H-NMR), consis-

tent effects of pasture and concentrate feeding on the metabolome of milk and Maasdam cheese were reported by O'Callaghan et al. (2018) and Panthi et al. (2019), respectively, showing clear separation between both the GRS and CLV diets and the TMR diet. O'Callaghan et al. (2018) identified particular correlations with urea, dimethyl-sulfone and p-cresol and the CLV system, whereas hippuric acid was indicative of pasture feeding in general and particularly of GRS feeding. Panthi et al. (2019), also utilizing gas chromatography-mass spectrometry (GC-MS), similarly identified toluene, dimethyl sulphide and citrate as clear biomarkers for the GRS, CLV and TMR feeding systems, respectively. The separation between GRS, CLV and TMR feeding systems was also supported by the results of Magan, O'Callaghan, et al. (2019), using liquid chromatography-mass spectrometry (LC-MS) to distinguish skim milk powder and whey protein ingredients derived from each system.

The application of 1H-NMR, GC-MS or LC-MS is useful for resolution of a large number of metabolite compounds, however, these methods are expensive and require relatively long sample run times. A recent application of Raman spectroscopy in verification of butter samples may offer a rapid and cost-effective alternative to the foregoing methods. Using Raman spectroscopy, Gómez-Mascaraque et al. (2020) determined a clear distinction between pasture and TMR-derived butter on the basis of FA and micronutrient quantification, providing further insight into the nutritional quality of butter originating from different feeding systems, which would be of particular interest to the consumer making a selection on the basis of 'grass-fed' labelling.

5 | CONCLUSION

As new trends in the consumption of dairy products have evolved in recent years, a substantial body of work has been developed to characterize the effect of commonly practiced pasture- and concentrate-based feeding systems on milk and dairy product quality and processability. Considerable differences in the compositional, functional, and organoleptic properties of milk derived from these systems have already been determined, but the opportunity remains to establish further knowledge on the effect of dietary factors and milk powder functionality, protein and micro-composition and, ultimately, possible nutritional benefits of milk produced from a given system. The practice of seasonal, pasture-based feeding systems is largely determined by economic and environmental factors, but evidence for potential nutritional benefits associated with dairy commodities produced therefrom could also provide valuable considerations in this regard.

AUTHOR CONTRIBUTIONS

All authors conceptualized the manuscript. Jonathan B. Magan developed the original draft of the manuscript. Noel A. McCarthy, Tom F. O'Callaghan and Alan L. Kelly proof-read and edited the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Article

Impact of Bovine Diet on Metabolomic Profile of Skim Milk and Whey Protein Ingredients

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Abstract: The influence of bovine diet on the metabolome of reconstituted skim milk powder (SMP) and protein ingredients produced from the milk of cows fed on pasture or concentrate-based diets was investigated. Cows were randomly assigned to diets consisting of perennial ryegrass only (GRS), perennial ryegrass/white clover sward (CLV), or indoor total mixed ration (TMR) for an entire lactation. Raw milk obtained from each group was processed at pilot scale, to produce SMP and sweet whey, and SMP was further processed at laboratory scale, to yield ideal whey and acid whey. The total amino acid composition and metabolome of each sample were analyzed, using high-performance cation exchange and a targeted combination of direct-injection mass spectrometry and reverse-phase liquid chromatography–tandem mass spectrometry (LC–MS/MS), respectively. The nitrogen composition of the products from each of the diets was similar, with one exception being the significantly higher nonprotein nitrogen content in TMR-derived skim milk powder than that from the GRS system. Total amino acid analysis showed significantly higher concentrations of glycine in GRS- and CLV-derived sweet whey and acid whey than in those from TMR. The cysteine contents of CLV-derived ideal whey and acid whey were significantly higher than for TMR, while the valine content of GRS-derived acid whey was significantly higher than TMR. The phenylalanine content of GRS-derived ideal whey was significantly higher than that from CLV. Metabolomic analysis showed significantly higher concentrations of the metabolites glutamine, valine, and phosphocreatine in each ingredient type derived from TMR than those from GRS or CLV, while the serine content of each GRS-derived ingredient type was significantly higher than that in TMR-derived ingredients. These results demonstrate that the type of bovine feeding system used can have a significant effect on the amino acid composition and metabolome of skim milk and whey powders and may aid in the selection of raw materials for product manufacture, while the clear separation between the samples gives further evidence for distinguishing milk products produced from different feeding systems based on LC–MS/MS.

Keywords: bovine diet; metabolome; amino acids; skim milk; sweet whey; acid whey; ideal whey

1. Introduction

Skim milk powder (SMP) and whey typically form the basis of various dairy product formulations, particularly in infant milk formula (IMF) manufacture. Whey is obtained from the side-streams of other milk processing applications, such as cheese manufacture [1] or directly from skim milk by membrane microfiltration [2]. All essential amino acids (EAA) are present in whey [3], including branched-chain amino acids (BCAA), with high bioavailability relative to other dietary protein sources [4]. It is widely accepted that the concentration of amino acids (AA) in bovine milk is primarily influenced by genetic factors [5]. While previous studies have investigated the effect of bovine dietary supplementation of individual AAs on milk gross composition [6,7] and protein synthesis [8], little information exists on the potential effect of standard bovine feeding systems on the overall AA composition of milk. Recently, consumer interest has increasingly been focused on dairy products derived from what is perceived as a healthier [9], natural, or more sustainable, origin [10]. While previous research has demonstrated the effect of diet on the fat fraction of milk, the micronutrient composition of various protein ingredients is an important consideration in the manufacture of dairy products which aim to meet this demand. The relative abundance of low-molecular-weight metabolites may also provide an insight into verification methods for milk from different systems. The increasing prevalence of “grass-fed” product labeling will necessitate such verification methods.

The production of milk from cows fed outdoors on pasture is generally regarded by consumers as a more environmentally sustainable method, with distinct welfare advantages for cows free to forage naturally [11,12]. Despite this developing consumer interest, pasture-based production systems are estimated to represent only 10% of the global milk supply [13]. The dominance of this production system in Ireland and New Zealand is primarily attributable to their mild, temperate climates, with plentiful rainfall enabling long, consistent grass-growing seasons. Larger, more-intensive milk-producing industries in the Americas or Central Asia are almost exclusively based on indoor, concentrate-based feeding systems, allowing for more independence from climatic variances [12]. The extent of pasture grazing for milk production in the USA has been observed to decrease substantially with increasing herd size [14].

The application of quantitative nuclear magnetic resonance (¹H-NMR)-based metabolomics to distinguish between rumen fluid and raw milk from pasture-based and total-mixed-ration-based feeding systems has previously been reported [15]. This nontargeted method has proven to be suitable for the purpose of verification of milk product origin, particularly in reference to claims of “pasture-fed” provenance. Reverse-phase liquid chromatography–tandem mass spectrometry (LC–MS/MS) is a highly sensitive analysis technique which utilizes liquid chromatography for the separation of compounds within a sample and subsequent compound analysis, using mass spectrometry. In targeted format, this method can be used for the detection and quantification of known compounds or metabolites, which can be identified from the established metabolome database for that sample type (e.g., milk metabolome). The potential to now extensively analyze the metabolome of dairy ingredients allows for greater understanding of the potential health effects from their consumption. Metabolomics has been demonstrated to offer potential as a mechanism for the verification of milk product origin claims [15].

The objectives of this study were to determine (1) the influence of perennial ryegrass (*Lolium perenne* L.), perennial ryegrass/white clover (*Trifolium repens* L.), and indoor total-mixed-ration-based feeding systems on the metabolome of SMP and whey protein ingredients and (2) the potential of LC–MS-based metabolomics for differentiating between milk and dairy product samples derived from different feeding systems.

2. Results and Discussion

2.1. Nitrogen Composition

Average concentrations of total nitrogen and nonprotein nitrogen (NPN) for SMP and whey powder samples are shown in Table 1. The total protein content of SMP and acid whey samples derived

from both pasture-based systems were higher than those from the TMR system, while TMR-derived sweet whey and ideal whey samples contained higher total protein levels than samples derived from either pasture-based system. Nonprotein nitrogen content was highest in all TMR-derived ingredients and lowest in all samples derived from GRS. Indeed, the NPN content of TMR-derived SMP was significantly higher than that produced from the GRS-derived milk (Table 1). Increased NPN content has previously been observed in raw milk [16] and whole milk powder [17] derived from a CLV feeding system. Urea is the primary component of NPN [18] and products derived from the CLV system exhibit increased urea levels, resulting from the inclusion of nitrogen-fixing white clover in this system [15]. The increased concentrations of NPN observed in the TMR sample may arise from greater levels of dietary crude protein in the concentrate ration, which does not necessarily correlate with increased milk true protein content, but rather increased milk urea [19]. A study which analyzed the rumen fluid of cattle fed varying proportions of roughage in a total mixed ration diet found decreasing NPN content with increasing roughage content relative to concentrate content, though these figures did not differ significantly [20]. In the present study, increased average total protein content was observed in sweet whey samples derived from each feeding system, relative to native whey and acid whey, both of which were equivalent. The higher level of protein in sweet whey is likely attributable to the presence of soluble glycomacropeptide (GMP) resulting from the renneting process. Sweet whey derived from cheese manufacture contains 20–25% glycomacropeptide [21].

Table 1. Average total nitrogen and nonprotein nitrogen content of skim milk powder, rennet whey, ideal whey, and acid whey, as determined by Kjeldahl analysis.

Sample Type	Total Protein (% w/w)			Nonprotein Nitrogen (% w/w)		
	GRS	CLV	TMR	GRS	CLV	TMR
Skim milk powder	37.2 (± 0.57) ^b	37.5 (± 0.06) ^d	36.1 (± 0.61) ^c	0.27 (± 0.01) ^{b,A}	0.32 (± 0.06) ^{b,A,B}	0.37 (± 0.00) ^{c,B}
Sweet whey powder	9.27 (± 0.29) ^a	9.41 (± 0.68) ^{b,c}	9.64 (± 0.17) ^b	0.25 (± 0.01) ^{a,b}	0.27 (± 0.04) ^{a,b}	0.32 (± 0.01) ^{a,b,c}
Ideal whey powder	8.07 (± 0.45) ^a	6.98 (± 0.61) ^a	8.23 (± 0.86) ^{a,b}	0.21 (± 0.01) ^{a,b}	0.23 (± 0.04) ^a	0.28 (± 0.03) ^{a,b}
Acid whey powder	7.64 (± 0.17) ^a	7.98 (± 0.17) ^{a,b}	7.50 (± 0.47) ^a	0.19 (± 0.01) ^a	0.21 (± 0.04) ^a	0.25 (± 0.01) ^a

GRS—cows fed perennial ryegrass only. CLV—cows fed perennial ryegrass/20% white clover sward. TMR—cows fed indoor total mixed ration *ad-libitum*. ^{a,b,c,d} indicates values within a column not sharing a common lower-case superscript letter differed significantly ($p < 0.05$). ^{A,B} indicates values within a row not sharing a common upper-case superscript letter differed significantly ($p < 0.05$).

2.2. Total Amino Acid Composition

Average concentrations (g per kg total protein) for nineteen AAs in each ingredient type from each feeding system are shown in Table 2. The feeding system had a significant effect on concentrations of glycine, cysteine, valine, and phenylalanine in sweet whey, ideal whey, and acid whey samples. Concentrations of glycine in GRS-derived acid whey were significantly higher than those from TMR, while concentrations were significantly higher in sweet whey derived from both pasture-based systems when compared to TMR. Glycine is a nonessential proteinogenic AA, primarily utilized in the synthesis of collagen, with limited function in the synthesis of other proteins and metabolic pathways [22]. Previous work by Meléndez-Hevia and de Paz-Lugo [23] suggests that a restriction in the stoichiometry of the glycine synthesis reaction may lead to insufficient glycine production relative to metabolic demand, making glycine an essential or conditionally essential AA.

The CLV feeding system produced both ideal whey and acid whey with significantly higher concentrations of cysteine than the TMR system. Cysteine is a nonessential proteinogenic AA which, uniquely amongst the AAs, contains a thiol group. Cysteine also has a function in energy metabolism, along with some antioxidant capacity, owing to the affinity for redox reactions due to the presence of the thiol group [24]. Acid whey derived from GRS feeding had a significantly higher valine content than that from the TMR system. Valine, alongside leucine and isoleucine, is one of the three essential BCAAs [25]. It plays a structural role in the synthesis of globular proteins, where it forms the nonpolar center surrounded by polar residues [26], along with other metabolic functions, such as insulin secretion [27]. The only significant difference between both pasture-based feeding systems

was that of the phenylalanine content of ideal whey, with $GRS > CLV$. Phenylalanine is an EAA which acts as a precursor for the synthesis of the nonessential AA tyrosine in the body. Both phenylalanine and tyrosine are utilized in the synthesis of amine-based hormones, such as dopamine, tyramine, and adrenaline, released within the body in stress responses [28].

Table 2. Total amino acid composition of skim milk powder, sweet whey, ideal whey, and acid whey, determined by high-performance cation exchange.

TAA g/kg Total Protein	Skim Milk Powder			Sweet Whey Powder			Ideal Whey Powder			Acid Whey Powder		
	GRS	CLV	TMR	GRS	CLV	TMR	GRS	CLV	TMR	GRS	CLV	TMR
Cysteic acid	11.2	11.8	10.6	29.4	29.0	28.9	34.1	31.4	32.9	34.6	32.9	34.0
Taurine	9.52	8.97	9.78	32.4	36.2	35.7	38.3	50.8	35.5	35.7	41.7	41.4
Methionine Sulfone	33.3	34.3	34.6	21.0	21.3	20.0	23.8	21.0	21.2	20.0	20.4	20.3
Asparagine	73.3	73.9	74.1	97.0	96.4	94.0	99.8	93.7	96.9	101	98.1	98.6
Threonine	41.1	41.0	41.2	62.0	60.4	57.5	41.5	37.7	40.1	41.4	40.2	39.2
Serine	50.1	49.9	49.9	41.2	39.7	37.8	35.4	31.0	33.8	34.6	33.1	32.1
Glutamine	190	193	189	159	155	142	155	138	143	159	153	139
Glycine	16.3	16.7	15.6	20.2 ^b	18.9 ^b	15.8 ^a	20.6	20.0	18.2	19.6 ^b	18.6 ^{a,b}	16.4 ^a
Alanine	28.4	28.0	27.9	39.6	41.2	36.9	38.6	32.0	31.4	38.0	35.6	34.8
Cysteine	7.13	8.14	7.88	20.7	24.4	21.5	25.7 ^{a,b}	34.6 ^b	21.6 ^a	22.2 ^{a,b}	30.4 ^b	15.4 ^a
Valine	55.3	57.8	56.2	54.6	52.9	52.0	51.8	46.6	43.3	51.3 ^b	48.9 ^{a,b}	36.7 ^a
Isoleucine	43.7	45.6	44.5	53.8	51.5	48.4	44.1	38.1	40.9	46.6	44.0	42.7
Leucine	93.5	95.5	94.7	88.1	88.4	82.1	98.5	91.5	92.5	103	100	93.7
Tyrosine	30.2	34.1	32.7	8.92	9.63	12.0	13.7	12.5	13.2	8.87	10.5	8.77
Phenylalanine	42.4	42.2	40.8	24.3	22.8	23.2	31.1 ^b	24.5 ^a	28.4 ^{a,b}	30.0	30.0	26.9
Histidine	35.7	35.4	35.7	25.3	28.2	25.6	33.6	35.7	31.7	40.2	41.2	35.7
Lysine	72.6	73.1	73.7	75.7	74.5	70.8	77.8	68.4	71.5	82.0	77.8	74.7
Arginine	31.4	32.0	31.5	19.8	19.5	20.0	24.1	23.3	24.9	25.3	24.2	23.0
Proline	84.2	86.1	88.7	44.0	48.0	35.0	27.2	24.2	32.9	30.3	25.1	21.9

GRS—cows fed perennial ryegrass only. CLV—cows fed perennial ryegrass/20% white clover sward. TMR—cows fed indoor total mixed ration *ad-libitum*. ^{a,b} indicates values within a row for each ingredient not sharing a common superscript letter differed significantly ($p < 0.05$).

The overall average values for each diet (combining each protein ingredient type) indicate a significant effect of feeding system over a wider range of AAs than in each ingredient type alone. These differences can be briefly summarized as follows: $GRS > TMR$ for glutamine, alanine, and isoleucine; GRS and $CLV > TMR$ for glycine and valine; $CLV > GRS$ and TMR for cysteine; $GRS > CLV$ and TMR for phenylalanine ($p < 0.05$). These effects are noteworthy, given the dominant influence of genetic factors in the total AA distribution of milk and the randomized distribution of cows into each feeding system in this study. Vanhatalo et al. [29] observed a significant effect of forage-feed type on plasma AA concentrations, with generally increased concentrations recorded for cows fed red clover silage in comparison to those fed grass.

Variations in the AA composition of each whey ingredient type are apparent when expressed as a combined average of the three feeding systems (Table 3). When compared to ideal whey and acid whey, sweet whey samples contained a significantly higher average concentration of the AAs most associated with the GMP component of cheese: isoleucine, proline, serine, valine, and threonine. Threonine, in particular, is an EAA present in high concentrations within GMP. Significantly higher concentrations of glycine, cysteine, and tyrosine were present in ideal whey, when compared to sweet whey and acid whey, while the histidine content of acid whey was significantly higher than both other whey types. While the total protein content of the SMP samples is markedly higher than that of each whey type, increased values for cysteic acid, taurine, aspartic acid, glycine, alanine, and cysteine were observed for each whey type when compared to SMP, owing to concentration of these AAs in the whey protein fraction of milk protein. Gorissen and Witard [30] reported similarly increased relative concentrations of glycine, cysteine, alanine, and aspartic acid in whey protein, when compared to skimmed milk and particularly casein. The protein used in the manufacture of IMF is selected on the basis of the amino acid profile that best mimics that of human milk. Variations due to feeding in the amino acid profile of skim milk powder and whey-protein powders, which typically form the primary ingredients for conventional IMF production, would be a noteworthy consideration for IMF manufacturers.

Table 3. Average values for total amino acid composition of sweet whey, ideal whey, and acid whey, determined by high-performance cation exchange.

TAA g/kg Total Protein	Sweet Whey Powder	Ideal Whey Powder	Acid Whey Powder
Cysteic acid	29.1 (± 0.26) ^a	32.8 (± 1.34) ^b	33.8 (± 0.89) ^b
Taurine	34.8 (± 2.06)	41.5 (± 8.16)	39.6 (± 3.39)
Methionine Sulfone	20.8 (± 0.65)	22.0 (± 1.55)	20.2 (± 0.24)
Asparagine	95.8 (± 1.57)	96.8 (± 3.04)	99.3 (± 1.69)
Threonine	60.0 (± 2.32) ^b	39.8 (± 1.94) ^a	40.3 (± 1.08) ^a
Serine	39.6 (± 1.67) ^b	33.4 (± 2.22) ^a	33.3 (± 1.24) ^a
Glutamine	152 (± 8.50)	145 (± 8.61)	150 (± 10.2)
Glycine	18.3 (± 2.30) ^a	19.6 (± 1.23) ^b	18.2 (± 1.65) ^a
Alanine	39.2 (± 2.13) ^b	34.0 (± 3.97) ^a	36.1 (± 1.63) ^{a,b}
Cysteine	22.2 (± 1.98) ^a	27.3 (± 6.61) ^b	22.7 (± 7.52) ^a
Valine	53.2 (± 1.36) ^b	47.2 (± 4.30) ^a	45.7 (± 7.83) ^a
Isoleucine	51.2 (± 2.72) ^b	41.0 (± 3.02) ^a	44.5 (± 1.98) ^a
Leucine	86.2 (± 3.57) ^a	94.2 (± 3.77) ^b	99.4 (± 5.23) ^b
Tyrosine	10.2 (± 1.63) ^a	13.2 (± 0.64) ^b	9.38 (± 0.97) ^a
Phenylalanine	23.4 (± 0.77) ^a	28.0 (± 3.35) ^b	29.0 (± 1.80) ^b
Histidine	26.4 (± 1.57) ^a	33.7 (± 1.98) ^b	39.0 (± 2.96) ^c
Lysine	73.7 (± 2.51)	72.6 (± 4.81)	78.2 (± 3.63)
Arginine	19.8 (± 0.26) ^a	24.1 (± 0.82) ^b	24.2 (± 1.13) ^b
Proline	42.3 (± 6.69) ^b	28.1 (± 4.42) ^a	25.8 (± 4.25) ^a

GRS—cows fed perennial ryegrass only. CLV—cows fed perennial ryegrass/20% white clover sward. TMR—cows fed indoor total mixed ration *ad-libitum*. ^{a,b,c} indicates values within a row not sharing a common superscript letter differed significantly ($p < 0.05$).

2.3. Metabolomic Profiles of Protein Ingredients

LC-MS analysis identified 46 individual metabolite compounds (47 in total) in SMP and whey protein samples, 25 of which were free AAs, including 19 of the 20 standard proteinogenic AAs. The average concentration of each metabolite is shown for SMP, sweet whey, ideal whey, and acid whey in Supplementary data Tables S1–S4, respectively, and as an overall average by feeding system in Table S5. Glutamic acid was the most abundant free AA in all samples from each feeding system. Glutamic acid has previously been shown to be the free AA present in the highest concentration in milk [31,32].

Four compounds were found to be significantly different between diets across the four ingredient types and are shown in abbreviated form in Table 4. In each of the four ingredient types, concentrations of glutamine in the TMR sample were significantly higher than both the GRS and CLV samples. Glutamine is generally regarded as a nonessential AA, although it has recently been suggested to be considered conditionally essential, following investigation of stress-response requirements [33]. It is primarily utilized in the biosynthesis of proteins, with additional functions in glycogen synthesis and the maintenance of the intestinal mucous membrane [33]. Concentrations of serine in GRS-derived SMP, sweet whey, and ideal whey samples were significantly higher than in those derived from TMR. Serine is regarded as a conditionally essential AA, which, like most other AAs, plays a role in protein synthesis, with additional functions in cell proliferation, hepatic gluconeogenesis [34], and immune response [35]. Similarly, significantly higher concentrations of phosphocreatine were observed in the TMR sample in each of these three ingredient types. Phosphorylation of the endogenous AA creatine occurs within muscle tissue by the action of creatine kinase [36]. Phosphocreatine is integral to adenosine triphosphate generation within the muscle and subsequent control of muscle contraction [37]. Concentrations of valine were significantly higher in TMR-derived SMP, ideal whey, and acid whey than both pasture-based systems. This is notable, given the lower concentrations of valine in TMR samples in the aforementioned total AA analysis, which may imply a greater proportion of total valine in the GRS and CLV samples is present in the bound form. While the concentrations of most total AAs were lower in the TMR-derived samples than both the GRS and CLV samples (Table 2), the inverse can be seen for the concentrations of most free AAs in the metabolome analysis

(Supplementary data Tables S1–S5). As free AAs are constituents of the nonprotein nitrogen component of milk [38], the overall increased concentrations of free AAs in the TMR samples in each product may be correlated to their increased nonprotein nitrogen contents (Table 1), which represent a greater proportion of the total nitrogen of these samples than those from GRS or CLV. This trend may also suggest that, while the quaternary structure of milk proteins assembled from amino acids transferred to the bovine mammary gland may be genetically determined, the concentrations of free amino acids in the serum phase of milk (i.e., aqueous phase containing whey proteins) may be influenced by dietary interventions. In comparison to the other protein ingredients, acid whey samples exhibited greater variation in the average concentrations of a number of metabolites. Average concentrations of acetyloronithine, alpha-amino adipic acid, and leucine in acid whey were 3 to 4 times higher than the other ingredient types, while concentrations of tyrosine were extremely low or absent in comparison to the other products.

Table 4. Average concentrations (μM) of metabolites which showed significant differences between feeding systems for skim milk powder, sweet whey, ideal whey, and acid whey, determined by LC–MS/MS.

Ingredient	Metabolite (μM)	GRS	CLV	TMR
Skim milk powder	Glutamine	4.38 (± 0.54) ^a	4.95 (± 1.24) ^a	12.4 (± 0.28) ^b
	Phosphocreatine	8.05 (± 1.70) ^{a,b}	6.32 (± 0.56) ^a	16.3 (± 4.45) ^b
	Serine	22.1 (± 0.78) ^b	18.3 (± 1.20) ^{a,b}	10.0 (± 0.55) ^a
	Valine	7.49 (± 0.12) ^a	7.27 (± 1.09) ^a	11.3 (± 1.34) ^b
Sweet whey powder	Glutamine	1.36 ^{*a}	0.187 (± 0.02) ^a	7.02 (± 3.20) ^b
	Phosphocreatine	9.65 (± 0.93) ^a	6.49 (± 0.70) ^a	22.6 (± 6.01) ^b
	Serine	25.3 (± 3.11) ^b	18.7 (± 2.33) ^{a,b}	9.31 (± 0.64) ^a
Ideal whey powder	Glutamine	4.04 (± 0.23) ^a	3.19 (± 1.77) ^a	11.5 (± 2.64) ^b
	Phosphocreatine	7.21 (± 1.64) ^a	5.37 (± 0.61) ^a	17.1 (± 1.41) ^b
	Serine	21.9 (± 1.13) ^b	18.8 (± 1.27) ^{a,b}	9.62 (± 1.10) ^a
	Valine	7.00 (± 0.74) ^a	7.92 (± 1.43) ^{a,b}	10.6 (± 0.07) ^b
Acid whey powder	Glutamine	2.39 (± 1.74) ^a	2.97 (± 1.07) ^a	12.3 (± 0.42) ^b
	Valine	6.80 (± 1.97) ^a	7.29 (± 0.69) ^a	12.0 (± 1.63) ^b

GRS—cows fed perennial ryegrass only. CLV—cows fed perennial ryegrass/20% white clover sward. TMR—cows fed indoor total mixed ration *ad-libitum*. ^{a,b} indicates values within a row not sharing a common superscript letter differed significantly ($p < 0.05$). * Denotes where a replicate was below the limit of detection or limit of quantification.

Metabolomic analysis of raw milk produced from cows on the three feeding systems used in this study, using 1H-NMR (O’Callaghan et al.) [15], identified 11 metabolites in common with this study: aspartic acid, betaine, choline, creatine, creatinine, isoleucine, glutamic acid, leucine, proline, tyrosine, and valine. Among these compounds, a significant effect of feeding system was found for concentrations of betaine, choline, creatinine, proline, tyrosine, and valine (Supplementary Data, Table S5) in both studies. Of all metabolites identified in the present study, choline was present in the highest concentrations in all samples, though average concentrations for GRS samples were significantly higher than those from TMR (Supplementary Data, Table S5). A similar result was observed for raw milk by O’Callaghan et al. [15], although the inverse was found in rumen fluid samples with significantly higher choline concentrations observed in TMR samples than in both GRS and CLV samples. Choline is considered an essential nutrient both in bovine [39] and human [40] nutrition, wherein it has a number of important functions, such as phospholipid biosynthesis and neurotransmitter synthesis via conversion to acetylcholine [40]. In cattle, dietary choline is susceptible to extensive ruminal degradation and must be supplemented in a rumen-protected form [41], while the majority of choline secreted into milk is the product of *de novo* synthesis of phosphatidylcholine [42]. It is therefore unlikely that the differences in choline content observed between milk and whey produced from cows from each feeding system can be attributed to direct absorption and transfer of choline from each feed

type. Rather, it may infer that the differences observed are attributable to modulation due to diet of the rumen microflora (in this case, protozoa), as was previously suggested by O'Callaghan et al. [15], leading to variation in rates of de novo choline synthesis. José, Santos & Lima [42] suggest that a high-concentrate diet may reduce rumen protozoa numbers, leading to a decrease in the availability of choline to the cow.

In the present study, the overall metabolome of the protein ingredients was shown to be significantly influenced by the type of feeding system when analyzed by principal component analysis (Supplementary Materials Figure S1) of the metabolomics analysis data. While both pasture-based systems show similar distributions, the distribution of the TMR system is narrow and distinct from both GRS and CLV, displaying low axial variance. Partial-least-square discriminant analysis (Figure 1) shows the significant overlap between the samples from the GRS and CLV systems and the pronounced separation between both of these systems and that of TMR. The overlap between the GRS and CLV samples is to be expected, given the similarity of these diets. This trend can also be shown clearly by using hierarchical clustering analysis (Figure 2), which represents the degree of positive or negative correlation of each metabolite to each feeding system. A clear separation between the TMR and pasture-based samples was also demonstrated by this analysis, offering evidence to support the applicability of LC–MS-based metabolomics for differentiation between dairy products derived from different feeding systems. This method may be appropriate for implementation into a milk-quality-determination laboratory setting, where the preparatory equipment used is typically readily available, and operators may already be trained in chromatographic and mass spectroscopy methods. However, in comparison to NMR, this method requires more extensive sample preparation (e.g., derivatization) and longer sample run times, though LC–MS offers greater sensitivity and greater potential for resolution of a larger number of compounds.

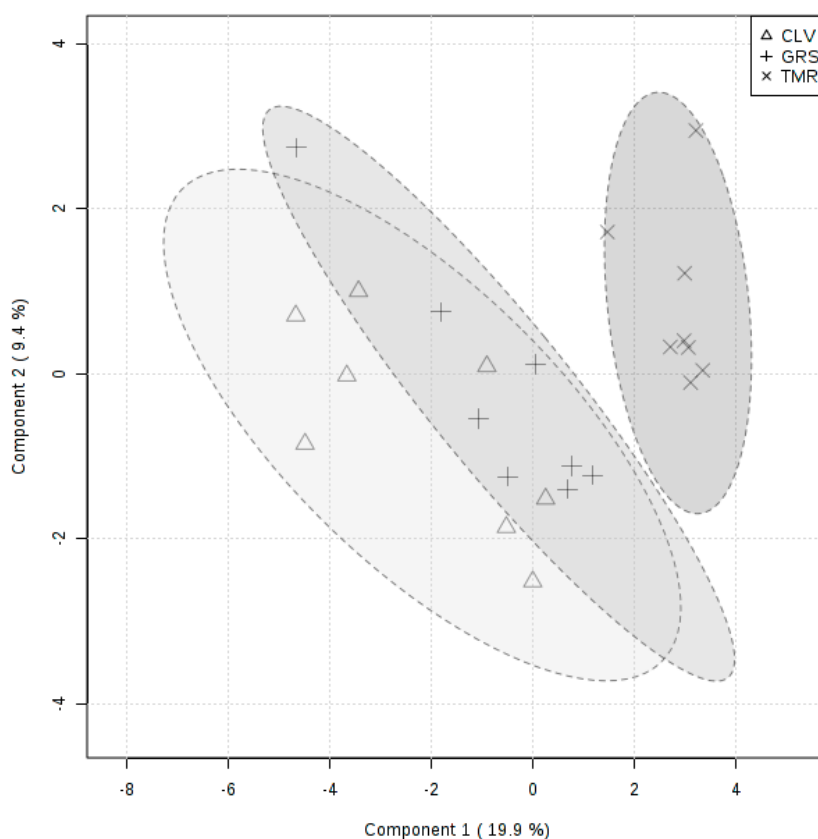


Figure 1. Partial-least-square discriminant analysis (PLS-DA) score plot for protein ingredient metabolome from milk of cows fed perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), and total mixed ration (TMR) feeding systems, determined by LC–MS/MS.

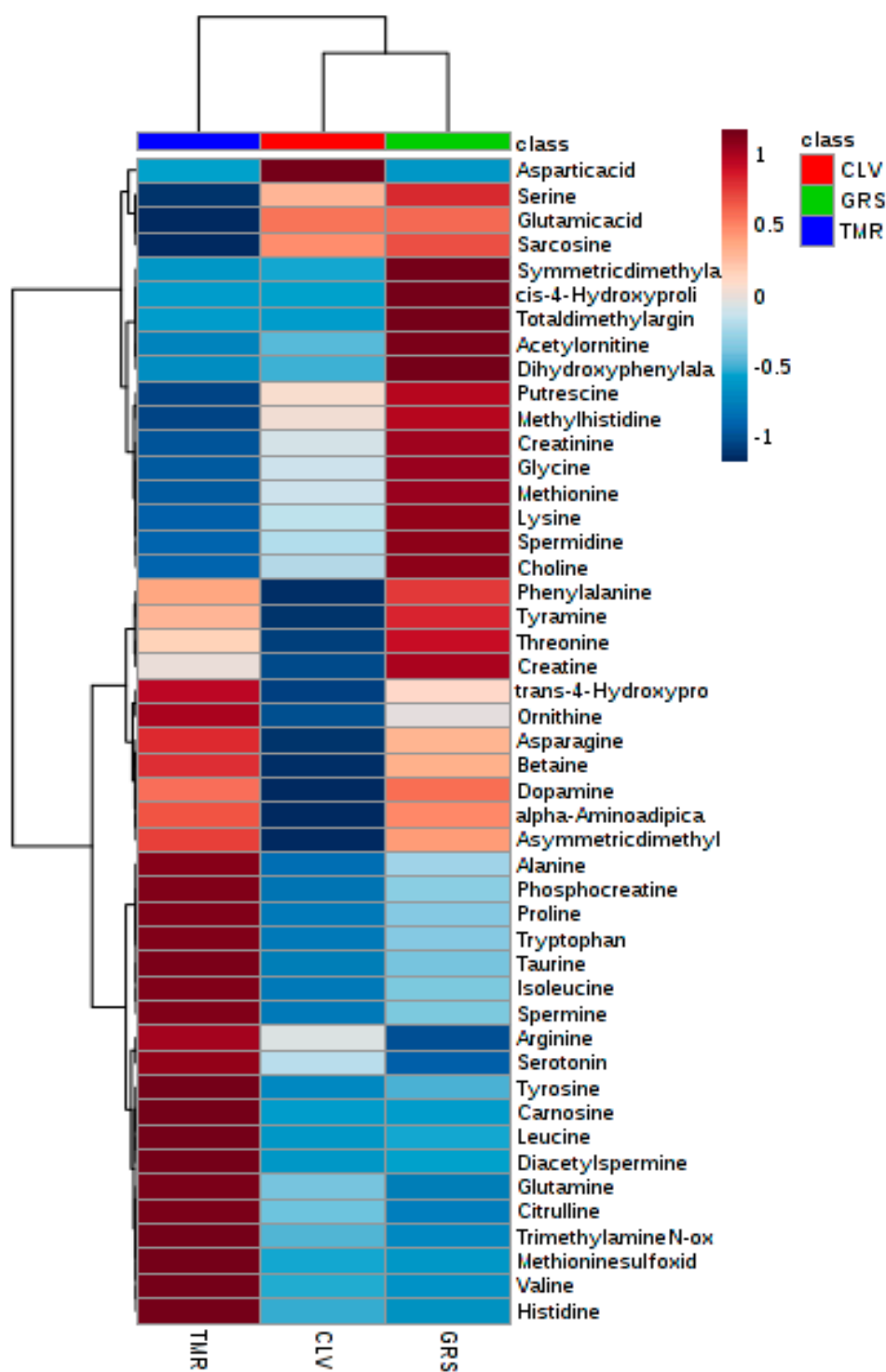


Figure 2. Heatmap showing average SMP and why ingredient metabolites from cows fed on perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), or total mixed ration (TMR) feeding systems, determined by LC-MS/MS. Degree of positive and negative correlation between metabolite and diet is indicated by +1 (red) to -1 (blue).

3. Materials and Methods

3.1. Materials

Raw milk was obtained from Teagasc Animal and Grassland Research and Innovation Centre (Moorepark, Fermoy, Co. Cork, Ireland). Hydrochloric acid and sodium hydroxide used for acid whey production were sourced from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

3.2. Experimental Design

The experimental design for this study was the same as that previously described in studies which investigated the quality of butter [43], cheddar cheese [44], and raw milks [15,16] from these feeding systems. Briefly, fifty-four spring calving Friesian cows were randomly allocated to three groups ($n = 18$) at the Teagasc Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland. Group 1 was housed indoors and fed a TMR diet, and 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]. For further information on the allocation of pasture-based dry matter using estimates of pre-grazing herbage mass and daily post grazing sward heights, see Egan et al. [45]. Milk was collected from each of the groups in the trial for milk powder manufacture on two separate occasions over a two-week period in July 2017, to produce 2 independent batches of skim milk powder (SMP) from each feeding system at pilot plant scale. All of the milk powders within each batch were manufactured on the same day at Moorepark Technology Ltd. (Moorepark, Fermoy, Co. Cork, Ireland).

3.3. Ingredient Manufacture

3.3.1. Skim Milk Preparation

Raw whole milk (approximately 1000 kg) was obtained from bulk milk tanks designated to each dietary treatment. This milk was preheated to 50 °C in an APV plate heat exchanger (SPX Flow Technology, Crawley, West Sussex, UK), followed by separation in a Westfalia centrifugal disk separator (GEA Westfalia, Oelde, Germany), and then it was pasteurized at 72 °C, for 15 s. The pasteurized skim milk was preheated to approximately 78 °C and concentrated to ~43% total solids (TS) in a Niro three-effect falling film evaporator (GEA Niro A/S, Soeborg, Denmark), at sequential effect temperatures of 73, 64, and 55 °C, respectively. The concentrate feed was then spray-dried, using a Niro Tall-Form Anhydro three-stage spray dryer (air inlet and outlet temperatures were set at 180 and 85 °C, respectively), using a high-pressure atomization system. External first and second fluid bed temperatures were set at 74 and 24 °C, respectively. All fines were returned from the cyclone to the second fluid bed, yielding a low-heat non-agglomerated skim milk powder (SMP) (~3% moisture).

3.3.2. Sweet Whey Preparation

Raw whole milk from each dietary treatment was set aside from the bulk collection described in Section 3.3.1 and pasteurized at 72 °C, for 15 s, using a Microthermics™ tubular heat-exchanger (Microthermics Inc., Raleigh, North Carolina, USA), and then stored in sterilized containers. Milk samples (~10 kg) were then added to a laboratory scale jacketed cheese-production vessel and preheated to 33 °C. Chymosin (Chy-Max Plus, 200 IMCU mL⁻¹; Chr Hansen Ireland Ltd., Cork, Ireland) was diluted in 30 mL of deionized water and added to the milk (0.272 mL L⁻¹), followed by controlled stirring for 3 min. The stirring paddles were then removed from the vessel and replaced with cutters. An aliquot (17 g) of the inoculated milk was weighed into a concentric cylinder in a Discovery HR-1 Hybrid Rheometer (TA Instruments, New Castle, Delaware, USA). After ~35 min, at an elastic modulus (G') reading of 30 Pa, curd cutting was carried out for 1 h, at 45 °C. The whey was then separated from the curd, using cheesecloth, and stored at -80 °C. The whey was later filtered, using Whatman No. 1

filter paper, and clarified, using a 0.1 µm Sartocoon Slice polyethersulfone cassette membrane (Sartorius AG, Göttingen, Germany), before being freeze-dried in a Labconco stoppering tray dryer equipped with a Freezone 12 plus vacuum collector/refrigerator unit (Labconco, Kansas City, Missouri, USA).

3.3.3. Acid Whey Preparation

Skim milk powder from each dietary treatment was reconstituted to 9% TS and maintained at 20 °C in a water bath. Hydrochloric acid (HCl) (2 M) was added to the milk in order to decrease the pH to the isoelectric point of pH 4.6, and the precipitated casein curd was then removed from the whey, using cheesecloth. The pH of the whey was then readjusted to pH 6.7, using sodium hydroxide (NaOH), and it was later filtered, clarified, and freeze-dried, as described in Section 3.3.2.

3.3.4. Ideal Whey Preparation

Skim milk powder from each dietary treatment was reconstituted to 9% TS and filtered through a 0.1 µm Sartocoon Slice polyethersulfone cassette membrane, at approximately 2.0 bar under recirculation mode (30 °C) in order to separate micellar casein and whey protein. Both micellar casein from the retentate and the whey permeate stream were then freeze-dried, as described in Section 3.3.2.

3.4. Determination of Nitrogen Content

The total nitrogen content of each sample was determined through the Kjeldahl method, as described in ISO 8968-1 (2001), using a nitrogen-to-milk protein conversion factor of 6.38. Nonprotein nitrogen content was determined by precipitation of the protein component of each sample, using trichloroacetic acid (15% *w/v*). The precipitate was removed from the mixture by using Whatman No. 1 filter paper, and a Kjeldahl determination was then carried out on the filtrate, as described above.

3.5. Total Amino Acid Analysis

Acid hydrolysis was carried out to break complete proteins and peptides down to individual AAs. Proteins were hydrolyzed with 6 N HCl at 110 °C, for 23 h, using a Gals-Col combo mantle (Gals-Col, Terre Haute, USA) for the determination of all AAs except sulfur AAs and tryptophan. Methionine and cysteine were oxidized with performic acid to methionine sulfone and cysteic acid, respectively, and then hydrolyzed with HCl. The resulting hydrolysates were then diluted 1 in 2 with the internal standard, norleucine, to give a final concentration of 125 nm/mL. Amino acids were quantified by using a Jeol JLC-500/V amino acid analyzer (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na⁺ high-performance cation-exchange column. The analyzer uses an ion-exchange column with post-column online reactor derivatization with ninhydrin.

3.6. Liquid Chromatography–Mass Spectrometry (LC–MS)

Metabolite analysis was carried out at The Metabolomics Innovation Centre (University of Alberta, Edmonton, Alberta, Canada). A targeted quantitative metabolomics approach was used to analyze the milk samples, using a combination of direct injection mass spectrometry (DI-MS) with a reverse-phase LC–MS/MS assay. The method used combines the derivatization and extraction of analytes and the selective mass-spectrometric detection, using multiple reaction monitoring (MRM) pairs. Isotope-labeled internal standards were used for metabolite quantification. All the milk samples were thawed on ice and were vortexed and centrifuged at 13,000× *g*. Ten µL of each milk sample was loaded and dried in a stream of nitrogen, and 50 µL of a 5% solution of phenyl-isothiocyanate was then added for derivatization. After incubation, samples were dried again, using a nitrogen evaporator. Extraction of the metabolites was then achieved by adding 300 µL of methanol containing 5 mM of ammonium acetate. Extracts (150 µL) were diluted in a 1:1 ratio with water for LC–MS/MS analysis of AAs and biogenic amines. The remaining 150 µL of extracts was mixed with 400 µL of running solvent for DI-MS analysis of lipids and carnitines.

Mass spectrometric analysis was performed on a 4000 QTrap[®] tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, California, USA) equipped with an Agilent 1100 LC system. The samples were delivered to the mass spectrometer by an LC method, followed by a direct injection (DI) method. Data analysis was performed, and concentrations were calculated by using Analyst software 1.6.2.

3.7. Statistical Analysis

Statistical analysis was performed by using Genstat v18.1 (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK). Datasets were analyzed for normality, using the Shapiro–Wilk’s test. Data were deemed normally distributed, and the analysis was carried out by using a 4 × 3 factorial ANOVA with post hoc Tukey test. The *p*-values < 0.05 were considered significant. Multivariate statistical analysis was carried out by using Metaboanalyst 3.0 [46] software, from which the figures were also generated.

4. Conclusions

The data presented in this study contribute to the overall characterization of the composition of milk products derived from the milk of cows fed via three widely practiced feeding systems. This lends an insight into the effect of these diets on the AA composition of milk, which has received limited attention to date. The significant differences observed in the total AA analysis suggest that the AA composition of milk may be more responsive to variation due to diet than previously assumed. Significant variation was observed in the average AA composition between each whey type (acid, sweet, and ideal), which may be an important consideration for nutritional formulations. The greater overall concentrations of free AAs in TMR-derived samples may be linked to the increased nonprotein nitrogen content of these milks. The significant effect of the type of feeding system on the metabolome of each ingredient type supports previous work examining the metabolome of pasture and concentrate-derived milk. As such, this implies that LC–MS-based metabolomics may be a suitable method for the differentiation of milks and whey products from different feeding systems.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2218-1989/9/12/305/s1>, Table S1: Average concentrations of total metabolites (μM) for skim milk powder derived from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS., Table S2: Average concentrations of total metabolites (μM) for sweet whey powder derived from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS., Table S3: Average concentrations of total metabolites (μM) for ideal whey powder derived from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS., Table S4: Average concentrations of total metabolites (μM) for acid whey powder derived from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS., Table S5: Average concentrations of total metabolites (μM) from skim milk powder, sweet whey powder, ideal whey powder and acid whey powder derived from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS., Figure S1: Principal component analysis (PCA) score plot for metabolomics analysis of protein ingredients from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

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Article

Effect of Diet on the Vitamin B Profile of Bovine Milk-Based Protein Ingredients

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Abstract: The influence of diet on the water-soluble vitamin composition of skim milk powder and whey protein ingredients produced from the milk of cows fed pasture or concentrate-based diets was examined. Fifty-one Holstein-Friesian cows were randomly assigned into three diets ($n = 17$) consisting of outdoor grazing of perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), or indoor feeding of total mixed ration (TMR) for an entire lactation. Raw mid-lactation milk from each group was processed into skim milk powder and further processed to yield micellar casein whey and acid whey. Sweet whey was also produced by renneting of pasteurised whole milk from each system. The water-soluble vitamin profile of each sample was analysed using a combination of direct injection mass spectrometry and reverse-phase liquid chromatography–mass spectrometry. Vitamin B3 and B3-amide concentrations were significantly higher ($p < 0.05$) in TMR-derived samples than in those from CLV and GRS, respectively. Vitamin B1, B2, and B7 concentrations were significantly higher in GRS and CLV-derived samples than those from TMR. Significant differences in vitamins B1, B2, and B3-amide were also observed between protein ingredient types. This study indicates that bovine feeding systems have a significant effect on B vitamin composition across a range of protein ingredient types.

Keywords: Bovine diet; B vitamin composition; skim milk; sweet whey; acid whey; micellar casein whey

1. Introduction

Dairy protein commodities such as skim milk powder (SMP) and derivatives thereof (e.g., whey produced from milk acidification or membrane filtration) are widely used as the primary materials in dairy-based product formulations, particularly in the manufacture of infant milk formula, providing much of the protein, lactose and water-soluble micronutrients required for such formulations. Due to the high value of milk fat in comparison to skim milk, fat-filled milk powders can be produced at low cost by blending vegetable oils with SMP. However, with studies on the potential health benefits of milk fatty acids becoming increasingly positive [1,2], applications for milk fat in added-value nutritional

beverages may become more widespread. This may contribute to reducing dependency on palm oil, the production of which is widely recognised as a major environmental issue [3].

Consumer awareness of sustainable food production is increasing [4,5], along with professional interest in communicating the concept of high nutrient-density foods to the consumer [6]. Milk and dairy products provide a good dietary source of water-soluble B vitamins, particularly vitamins B2 (riboflavin), B5 (pantothenic acid), and B12 (cobalamin), each with comparatively high bioavailability [7–9]. Milk is also a particularly good source of cobalamin as it is exclusively produced by soil bacteria and archaea [9], which can be consumed by grazing ruminants or by ruminal synthesis through the uptake of precursory cobalt from the soil [10]. Cobalamin cannot be acquired by humans from most dietary plant sources [11]. Previously, Jensen (1995) reported the standard quantities of water-soluble [12] and fat-soluble [13] vitamins in milk.

Consumer perceptions of healthier milk and dairy products increasingly tend towards pasture-based production systems [14]. Rising interest in pasture-derived milk is reflected by the increasing prevalence of dairy products marketed on the basis of claims of “pasture-fed” provenance. However, pasture-based milk production systems are climate-dependent and represent only a minor proportion of overall global milk production [15]. These systems are the most widely practiced in New Zealand and Ireland, where recent marketing efforts have been increasingly focused on the potential environmental [16] and health [17] benefits of their use. “Grass-fed” marketing claims have also become widespread among the protein supplement industry in the USA, where milk production is almost exclusively based on indoor, concentrate feeding-based systems [18]. Indeed, the vast majority of international milk suppliers use a concentrate-based system. For the consumer, concerns often emerge regarding animal welfare and the environmental impact of these systems [19]. These changing considerations have resulted in renewed interest in pasture-based milk production in the USA, where research into their economic merit is on-going.

A significant effect of feeding system on the fatty acid profile of milk and dairy products has previously been shown [20,21]. While the effect of pasture or concentrate feeding on levels of fat-soluble vitamins such as retinol (vitamin A) and alpha-tocopherol (vitamin E) has been investigated in meat [22], neither the fat-soluble nor water-soluble vitamin profiles have been established for the milk of cows fed on these systems. Numerous studies [23–25] have investigated the effect of dietary supplementation of water-soluble vitamins (particularly biotin) on cow performance and health, though not on the subsequent vitamin composition in milk. Similarly, studies [26,27] have determined the effect of grass and concentrate feeding on water-soluble vitamin levels in rumen fluid and muscle tissue, but not in milk or milk-derived ingredients such as whey. Nonetheless, the importance of milk and dairy products as major sources of water soluble vitamins in human nutrition merits investigation of the variation in vitamin composition between milks derived from cows fed on different commonly practiced feeding systems.

Bovine milk is a source of water-soluble vitamins which are present in quantities that can contribute substantially to the minimum recommended daily allowance for children or adults. Skim milk powder also typically forms the nutritional base for infant milk formula (IMF) manufacture, where it is combined with lactose, demineralised whey, or whey protein concentrate to achieve an overall composition similar to that of human breast milk. Therefore, potential effects of ruminant feeding systems on the vitamin B content of skim milk and whey ingredients may be important considerations for formulation design and nutritional quality, particularly in relation to IMF production.

With this considered, the objective of this study was to determine the influence of perennial ryegrass (*Lolium perenne* L.), perennial ryegrass/white clover (*Trifolium repens* L.), and indoor total mixed ration-based feeding systems on the water-soluble vitamin composition of SMP and whey protein ingredients.

2. Materials and Methods

2.1. Materials

Raw milk was obtained from Teagasc Animal and Grassland Research and Innovation Centre (Moorepark, Fermoy, Co. Cork, Ireland). Hydrochloric acid and sodium hydroxide used for acid whey production were sourced from Sigma Aldrich (Merck KGaA, Darmstadt, Germany).

2.2. Experimental Design

This study utilised the same SMP and whey powders used by Magan et al. [28] and, as such, all experimental design conditions and powder production methods are as previously described. For detailed descriptions of the feeding system experimental design and the chemical composition of the feeds used in this study, see Egan et al. [29] and O'Callaghan et al. [30], respectively. Briefly, 54, 51, and 51 spring-calving Holstein-Friesian, with some Holstein-Friesian × Jersey cross-bred cows from the Teagasc Moorepark dairy herd were selected in 2015, 2016, and 2017, respectively, and randomly assigned to three groups ($n = 18$ in 2015; $n = 17$ in 2016 and 2017) with separate feeding systems. Cows were randomised by breed, calving date (mean 17 February; ± 16 days), parity (2.45) and milk yield (23.7 kg) and milk solids (fat+protein) yield (2.04 kg) for the first 2 weeks post-calving. Mean body weight at the beginning of the experimental period was 519 kg. Group 1 was fed a total mixed ration (TMR) diet and housed indoors, Group 2 was maintained outdoors on perennial ryegrass only pasture (GRS), and Group 3 was also maintained outdoors on a perennial ryegrass/white clover pasture (CLV) with an average annual sward white clover content of 24%. On a dry matter (DM) basis, the TMR diet consisted of 8.3 kg of concentrates, 7.15 kg of grass silage, and 7.15 kg of maize silage. Individual electronically controlled Griffith Elder Mealmaster feed bins (Griffith Elder and Company Ltd., Suffolk, England) were used to administer ad libitum feed to the cows within the TMR system at 08:30 h daily. Both groups of pasture based cows consumed ~ 18 kg DM/day. This was allocated using estimates of pre-grazing herbage mass and daily post grazing sward heights as described by Egan et al. [29]. Each group of cows was milked twice daily at 07:30 and 15:30 h. Cows from each of the three feeding systems were milked separately and their milk was segregated into designated 5000 L refrigerated tanks. Tanks were maintained at 4 °C with morning and evening milk being added together and agitated prior to sample collection.

The raw milk of each of the three groups of 17 cows was bulked into three designated bulk tanks over 5 consecutive milkings (3 morning milkings, 2 evening milkings) and collected on two separate occasions over a two-week period in July 2017 (mean days in milk 143) and processed into two batches of skim milk powder (SMP) from each feeding system at Moorepark Technology Ltd. (Moorepark, Fermoy, Co. Cork, Ireland). All of the milk powders within each batch were manufactured on the same day.

2.3. Protein Ingredient Manufacture

Each SMP, sweet whey, micellar casein whey, and acid whey powder was produced as described in detail in Magan et al. [28]. Briefly, low-heat non-agglomerated SMP was produced from pasteurised, separated, and spray-dried raw whole milk from each group of cows at pilot plant scale. Sweet whey powder was produced at laboratory scale by the addition of chymosin (Chy-Max Plus, 200 IMCU mL⁻¹; Chr Hansen Ireland Ltd., Cork, Ireland) to pasteurised whole milk and subsequent curd cutting and whey drainage. Acid whey was produced at laboratory scale by acidification of reconstituted SMP from each feeding system, followed by curd cutting and whey drainage. Both sweet whey and acid whey were filtered using Whatman No. 1 filter paper and clarified using a 0.1- μ m Sartocon Slice polyethersulfone cassette membrane (Sartorius AG, Göttingen, Germany). Micellar casein whey was produced at laboratory scale by filtering reconstituted SMP from each feeding system using this membrane. Each whey type was then freeze-dried in a Labconco stoppering tray dryer equipped

with a Freezone 12 plus vacuum collector/refrigerator unit (Labconco, Kansas City, MO, USA) to yield whey powder.

2.4. Liquid Chromatography–Mass Spectrometry (LC-MS/MS)

2.4.1. Sample Preparation

Water-soluble vitamin (B1, B2, B3, B3-amide, B5, B6-Pyridoxine, and B7) analysis was carried out at The Metabolomics Innovation Centre (University of Alberta, Edmonton, Alberta, Canada) with a targeted mass spectrometry (multiple reaction monitoring) method using a reverse-phase LC-MS/MS assay. Standard solutions, internal standard solution, and quality control solutions were all diluted using 0.1% formic acid in deionised water. Internal standard solution (10 µL) was first added to 0.6 mL Eppendorf tubes. Calibration standard solutions (50 µL), quality control standard solutions (50 µL), and reconstituted skim milk/whey samples (50 µL) were then added to their corresponding Eppendorf tubes. Protein was precipitated by the addition of 60 µL of trichloroacetic acid (50 mg mL⁻¹) to each tube, after which each sample was vortexed for 30 s. All tubes were stored on ice for 4 h, followed by centrifugation at 13,000 rpm for 15 min. The supernatant from each tube was then transferred into a 96 deep-well collection plate and sealed with a pre-slit mat.

2.4.2. Operating Conditions

The aqueous phase (solvent A) consisted of 5 mM ammonium formate and 0.1% formic acid in water, while the organic phase (solvent B) consisted of 5 mM ammonium formate and 0.1% formic acid in methanol. Samples were separated using an Agilent reversed-phase Zorbax Eclipse XDB C18 (3.0 mm × 100 mm, 3.5 µm particle size, 80 Å pore size) column (Agilent Technologies, Santa Clara, CA, USA) at a column temperature of 40 °C. Samples were injected at a volume of 10 µL at an auto-sampler temperature of 4 °C. Flow rate was 400 µL min⁻¹ and run time was 9.5 min. Mass spectrometric analysis was performed on an AB SciexQTrap[®] 4000 tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA). Multiple reaction monitoring was carried out in positive ionisation mode at a temperature of 450 °C and an ion spray voltage of 5500 V. Curtain gas, gas stream 1, and gas stream 2 pressures were 20, 40, and 60 psi, respectively. All 48 samples were analysed together in the same run (24 samples in duplicate). Validation parameters (calibration range, calibration regression coefficient, concentrations (µM) of quality control solutions, % accuracy, precision and recovery, limit of detection, and limit of quantitation) for each vitamin in the assay are shown in Supplementary Materials Table S1. Quality control samples were run in triplicate to calculate average accuracy (80%–120%) and precision (within 20%) percentages. Unspiked samples, together with low, medium, and high-spiked samples were measured in triplicate to calculate recovery percentages (80%–120%). Data analysis and calculations of vitamin concentrations were carried out using Analyst Software 1.6.2.

2.5. Nephelometry

The vitamin B12 content of each SMP sample was determined by nephelometry by an external laboratory (Eurofins Food Testing Ireland—EFTI Cork, Glanmire Industrial Estate, Glanmire, Co. Cork) using the AOAC 952.20 microbiological assay method [31]. Briefly, vitamin B12 was extracted from the sample in an autoclave using a buffered solution. After dilution with basal medium (containing all required growth nutrients except cobalamin), the growth response of *Lactobacillus leichmanii* (ATCC 7830) to extracted cobalamin was measured turbidimetrically and compared to calibration solutions of known concentrations. Vitamin B12 concentrations were measured only in the SMP samples.

2.6. Statistical Analysis

Statistical analysis was performed using Genstat v18.1 (VSN International Ltd., Hemel Hempstead, Hertfordshire, UK). The mean of two replicates was used for each sample value. Datasets were analysed

for normality using the Shapiro–Wilk’s test. Data was deemed normally distributed and analysis was carried out using a 4×3 factorial ANOVA with post hoc Tukey test. The effect of ingredient type (4 levels: SMP, sweet whey, MCW, and acid whey) and the effect of cow diet (3 levels: GRS, CLV, and TMR) were considered in the ANOVA. p -values < 0.05 were considered significant. Multivariate analysis of the vitamin profiles was also performed. A supervised multivariate model was built using partial-least-square discriminant analysis (PLS-DA). To validate the model, a permutation test with 2000 repetitions was performed to check that the model differed from a random model ($p < 0.05$). In addition, the R^2 and Q^2 parameters were obtained to assess the performance of the model using 10-fold cross validation approach. The variables which have the greater influence on the latent variables of the built model were determined using a variable importance plot (VIP). Unsupervised hierarchical clustering analysis (HCA) was performed to observe patterns in the data, and is shown as a heatmap. Each of these tests and generation of subsequent figures were carried out using Metaboanalyst 3.0 software (www.metaboanalyst.ca) [32].

3. Results and Discussion

3.1. Overall Distribution

Table 1 shows the average concentration of each water-soluble vitamin expressed as μg per g of protein in each ingredient type. Average concentrations (μM) for each vitamin in SMP at 9.5% total solids and each whey type at 6.5% total solids from each feeding system are shown in Supplementary Materials Table S2. Table 2 compares the average concentration of each water-soluble vitamin in the sweet whey, micellar casein whey (MCW), and acid whey samples, expressed as μg per g of protein. Average concentrations (μM) for each vitamin in each whey type at 6.5% total solids are shown in Supplementary Materials Table S3. Table S4 in Supplementary Materials shows average concentrations ($\mu\text{g/g}$ protein) for each vitamin in sweet whey, micellar casein whey, and acid whey powders derived from each feeding system. The overall significant effect of feeding system on vitamin profile can be shown using partial-least-square discriminant analysis (PLS-DA) (Figure 1, panel A) and hierarchical clustering analysis (Figure 2). Figure 1A shows a substantial overlap in the distribution of GRS and CLV samples and a more pronounced separation between both and the TMR samples. In contrast to the clustered variables of the GRS and CLV systems, each TMR variable was distinct from those of the other two systems. In this PLS-DA, $R^2 = 0.80$ and $Q^2 = 0.71$, indicating a close fit to predicted variation, with 83% of the observed variance also being explained by the model. Figure 1B shows the variable importance plot (VIP) generated from this PLS-DA, which determines the variables which contribute most to the observed variance in the model and indicates that vitamins B7, B3, and B2 contribute most to the discrimination between classes. The degree of positive or negative correlation of each vitamin to a particular feeding system is shown in Figure 2, which shows the notably positive correlation between the level of vitamin B3 complex and the TMR feeding system and the respective negative correlations of nicotinamide with the GRS system and nicotinic acid to the CLV system. The positive correlation of riboflavin and biotin to both the GRS and CLV systems is also apparent from Figure 2, while these vitamins exhibit a strongly negative correlation with the TMR system. The relative abundance of particular vitamins in milk from different feeding systems may thus be used as an effective means of differentiation between products derived from these different systems. The potential to distinguish between pasture-derived and concentrate-derived milks based on the metabolomics profile has also previously been determined in raw milk [33], SMP, and whey ingredients [28] using quantitative nuclear magnetic resonance ($^1\text{H-NMR}$) and reverse-phase liquid chromatography–mass spectrometry (LC-MS/MS), respectively.

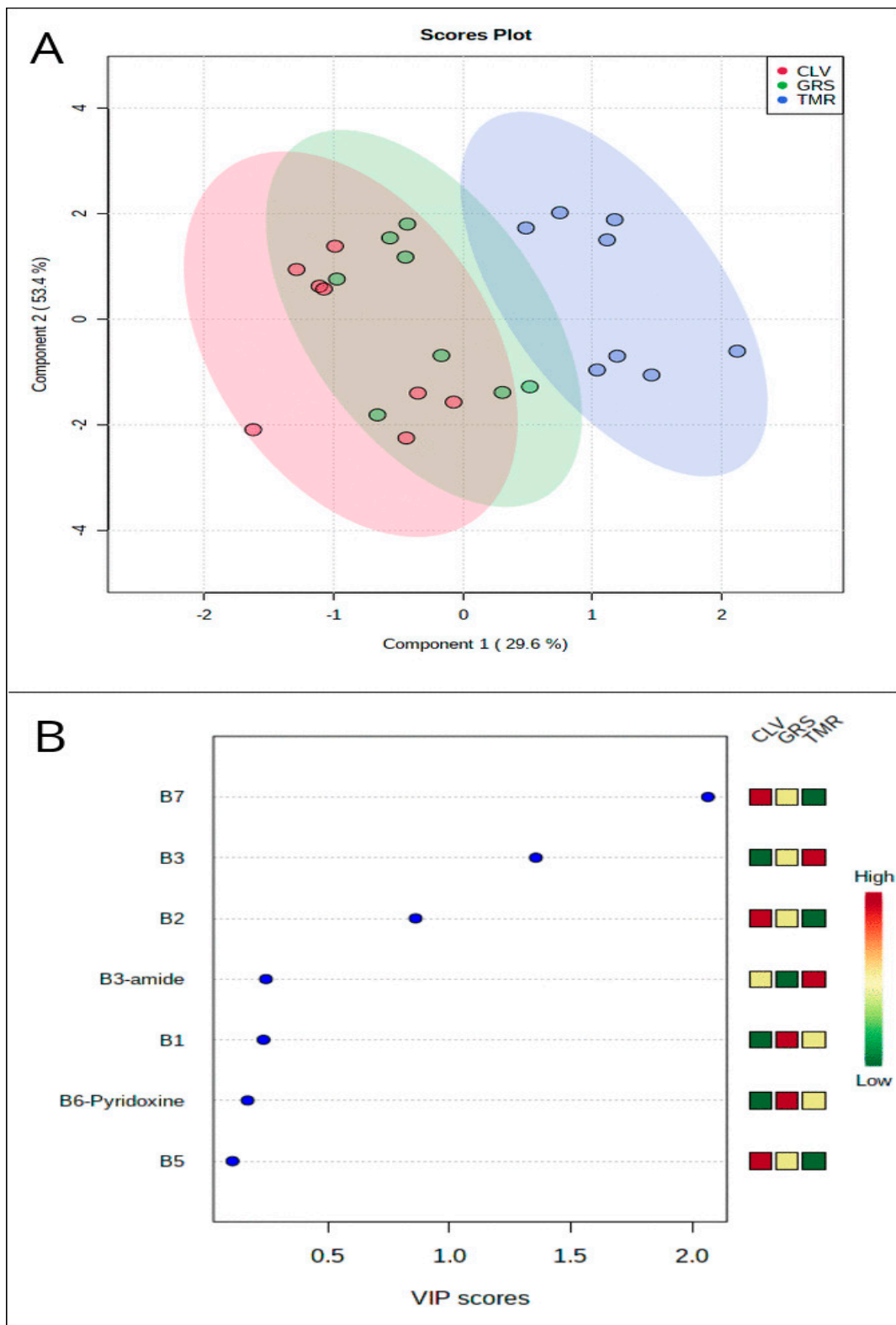


Figure 1. Panel (A): partial-least-square discriminant analysis (PLS-DA) score plot for water-soluble vitamins in skim milk powder and whey ingredients from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), and total mixed ration (TMR) feeding systems, determined by LC-MS/MS ($R^2 = 0.80$, $Q^2 = 0.71$). Panel (B): variable importance plot of vitamins most responsible for separation observed in PLS-DA.

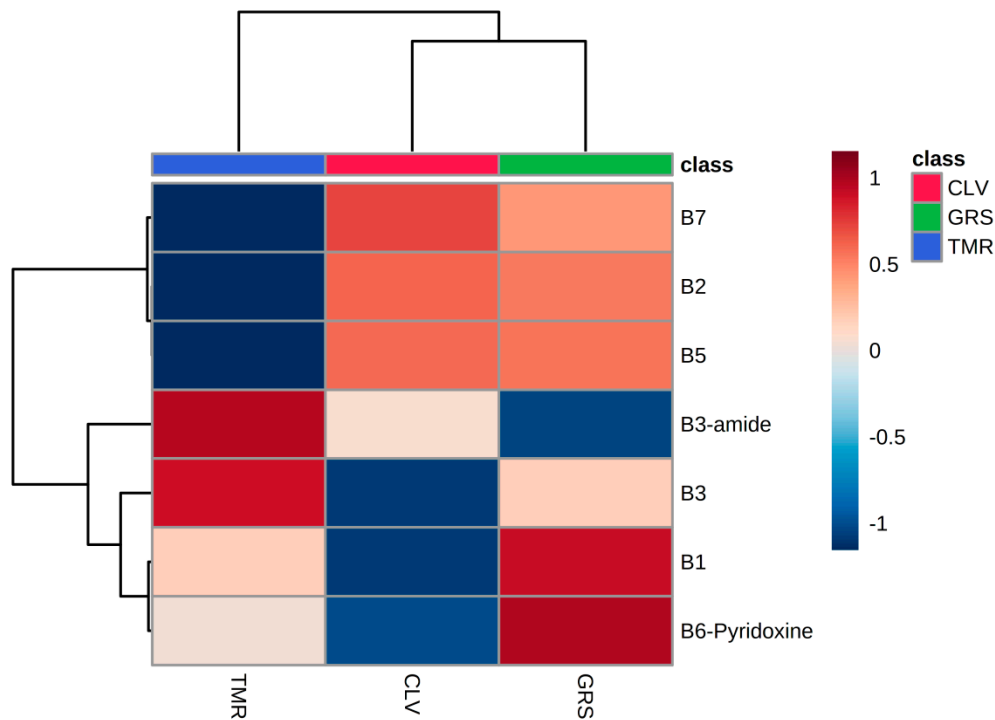


Figure 2. Hierarchical clustering analysis of average skim milk powder and whey ingredient vitamins from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), or total mixed ration (TMR) feeding systems, determined by LC-MS/MS. Degree of positive and negative correlation between vitamin and diet is indicated by +1 (red) to −1 (blue).

Table 1. Average concentrations (µg/g protein) of water-soluble vitamins for skim milk, sweet whey, micellar casein whey, and acid whey powders derived from the milk of Holstein-Friesian cows assigned to perennial ryegrass (GRS), perennial ryegrass/white clover (CLV), and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

Sample Type	Water-Soluble Vitamin (µg/g Protein)	GRS	CLV	TMR
Skim milk powder	B1	5.47 ^b	5.44 ^b	4.31 ^a
	B2	422 ^b	432 ^b	250 ^a
	B3	0.86	0.63	0.94
	B3-amide	15.4	15.4	18.0
	B5	112	117	125
	B6-Pyridoxine	0.13	0.11	0.13
	B7	0.46	0.53	0.25
Sweet whey powder	B1	29.5	27.9	36.3
	B2	1489 ^b	1400 ^b	636 ^a
	B3	4.60 ^a	3.37 ^a	5.41 ^b
	B3-amide	74.9	82.0	81.9
	B5	738	695	675
	B6-Pyridoxine	0.71	0.77	0.77
	B7	3.04 ^{a,b}	3.12 ^b	1.23 ^a
Micellar casein whey powder	B1	23.2	22.1	20.9
	B2	191 ^b	232 ^b	85.2 ^a
	B3	4.48	4.51	4.91
	B3-amide	117	150	137
	B5	869	1067	811
	B6-Pyridoxine	0.77	0.86	0.74
	B7	3.97 ^b	5.65 ^b	1.77 ^a

Table 1. Cont.

Sample Type	Water-Soluble Vitamin ($\mu\text{g/g}$ Protein)	GRS	CLV	TMR
Micellar casein whey powder	B1	23.2	22.1	20.9
	B2	191 ^b	232 ^b	85.2 ^a
	B3	4.48	4.51	4.91
	B3-amide	117	150	137
	B5	869	1067	811
	B6-Pyridoxine	0.77	0.86	0.74
	B7	3.97 ^b	5.65 ^b	1.77 ^a
Acid whey powder	B1	30.9	22.4	29.4
	B2	81.7	83.8	49.6
	B3	5.89	4.03	8.03
	B3-amide	110 ^a	115 ^{a,b}	129 ^b
	B5	929	843	933
	B6-Pyridoxine	1.03	0.77	0.80
	B7	3.55 ^b	3.75 ^b	1.95 ^a

Values are presented as the average of duplicate samples. GRS—Cows fed perennial ryegrass only. CLV—Cows fed perennial ryegrass/white clover. TMR—Cows fed total mixed ration ad libitum. Vitamins: B1—Thiamine, B2—Riboflavin, B3—Nicotinic acid, B3-amide—Nicotinamide, B5—Pantothenic acid, B7—Biotin. Note: Only the pyridoxine form of vitamin B6 is represented in the data. ^{a,b} Different superscripts within a row indicate significant differences ($p < 0.05$).

Table 2. Average concentrations ($\mu\text{g/g}$ protein) of total water-soluble vitamins for sweet whey, micellar casein whey, and acid whey powders derived from the milk of Holstein-Friesian cows assigned to each feeding system, determined by LC-MS/MS.

Water-Soluble Vitamin ($\mu\text{g/g}$ Protein)	Sweet Whey Powder	Micellar Casein Whey Powder	Acid Whey Powder
B1	31.2 ^b	22.1 ^a	27.6 ^a
B2	1175 ^b	170 ^a	71.7 ^a
B3	4.46	4.64	5.98
B3-amide	79.6 ^a	135 ^b	118 ^b
B5	703	916	902
B6-Pyridoxine	0.75	0.79	0.87
B7	2.46	3.80	3.08

Values are presented as the average of data from duplicate samples. Vitamins: B1—Thiamine, B2—Riboflavin, B3—Nicotinic acid, B3-amide—Nicotinamide, B5—Pantothenic acid, B7—Biotin. Note: Only the pyridoxine form of vitamin B6 is represented in the data. ^{a,b} Different superscripts within a row indicate significant differences ($p < 0.05$).

3.2. Vitamin Composition

3.2.1. Vitamin B1 (Thiamine)

Concentrations of thiamine in GRS and CLV-derived SMP samples were significantly higher ($p < 0.05$) than those derived from TMR (Table 1), despite higher concentrations of thiamine typically being present in the germ and seed of cereal grains than other plant sources [34]. Differences between diet were not observed in any of the whey samples, however, Pan et al. [35] suggested that ruminal thiamine production may be reduced in low rumen pH caused by subacute ruminal acidosis arising from feeding of high levels of concentrates. A previous study by Duckett et al. [36] also found thiamine concentrations to be three times greater in the muscle tissue of grass-fed bulls compared to those fed on a high-concentrate diet, while Shingfield et al. [37] found no significant variation in the thiamine content of milk derived from cows fed varying levels of concentrates. Thiamine is, however, stored in particularly high concentrations in animal muscle tissue [38]. Thiamine content was also shown to be significantly different between whey ingredient types in the present study (Table 2). Average concentrations of thiamine in sweet whey (31.2 $\mu\text{g/g}$ protein) were significantly higher ($p < 0.05$) than

for both MCW (22.3 µg/g protein) and acid whey (27.3 µg/g protein). Thiamine content is associated with protein content, as serum thiamine is primarily bound to albumin [39]. The SMP and whey powders used in this study are the same as those previously used by Magan et al. [28], where the total protein content of the powders was determined, with sweet whey exhibiting higher average total protein content (9.44%) than MCW (7.76%) and acid whey (7.71%).

3.2.2. Vitamin B2 (Riboflavin)

Average concentrations of riboflavin were significantly ($p < 0.05$) higher in both GRS and CLV samples when compared to the TMR sample (Table 1). Bovine dietary riboflavin is primarily sourced from green, leafy forage, though riboflavin synthesis also occurs in the rumen [40]. Riboflavin provides pigmentation in leaves [41], conferring a yellow colour similar to β -carotene, the relative abundance of which is primarily responsible for the intensity of yellow colour in fat-containing dairy systems [30,42]. Although each of the ingredient types in the present study are derived from non-fat systems and β -carotene is fat-soluble, visible differences in yellowness were observed between samples derived from each feeding system. Riboflavin is present in considerably lower concentrations in cereal grains, compared to fresh leafy forage (i.e., grass) [43].

Previous studies by Hayes et al. [44] and Duckett et al. [36] reported increased riboflavin concentrations in the rumen fluid of bulls receiving increased dietary roughage (i.e., hay) content and in the muscle tissue of bulls assigned to a pasture-based, rather than high concentrate-based finishing system, respectively. Conflicting results were found in a study by Santschi et al. [26], where increased riboflavin content was recorded in the rumen fluid of cows fed at a high concentrate to forage ratio. Poulsen et al. [45] compared the riboflavin content of bulk milk from three dairies in Denmark, recording higher riboflavin concentrations in milk from an organic dairy derived from high dietary proportions of grass and legume-based forage, when compared to the milk from two conventional dairies. The average riboflavin concentration of sweet whey (1169 µg/g protein) was significantly higher ($p < 0.05$) than both MCW (166 µg/g protein) and acid whey (71.9 µg/g protein) (Table 2). Previously, Mavropoulou and Kosikowski [46] examined commercially produced spray dried whey samples and found higher concentrations of riboflavin in sweet whey than in acid whey powders (data presented on a g per kg powder basis). Glass and Hedrick [47] reported similarly increased riboflavin content in commercial dried sweet whey compared to acid whey (data presented on a mg per 100 g powder basis). However, the differences observed in both of the previous studies are significantly lower than those for the present study. The exact reason for the present finding is still not fully understood.

3.2.3. Vitamin B3 (Nicotinic Acid)

The vitamin B3 complex comprises two common forms; nicotinic acid, nicotinamide, and a third recently discovered form; nicotinamide riboside [48]. Both nicotinic acid and nicotinamide are identified in the LC-MS/MS analysis used in the present study, though nicotinamide is present in substantially higher concentrations in bovine milk than nicotinic acid as the latter is converted to the amide form in the rumen [49]. The average concentration of nicotinic acid in ingredients derived from TMR was significantly higher ($p < 0.05$) than those from CLV, while the nicotinamide concentration of TMR-derived ingredients was significantly higher than those derived from GRS (Table 1). The bioavailability of both forms of vitamin B3 is equivalent [50].

Primary bovine dietary sources of nicotinic acid are cereal grains, although, similar to riboflavin, synthesis of nicotinic acid also occurs in the rumen [51]. The high inclusion rate of cereal-derived concentrates in the TMR feeding system is likely the most significant contributor to the increased nicotinic acid content in these samples. Hayes et al. [41] showed increased levels of nicotinic acid in the rumen fluid of bulls fed a concentrate-based diet. Nicotinic acid is synthesised from the essential amino acid tryptophan [52], though previous analysis of the tryptophan content of the samples used in the present study did not reveal significant differences between the diets [28]. The lack of significant

variation in tryptophan content between the diets relative to nicotinic acid content may be explained by the primary utilisation of tryptophan for protein synthesis [53] and the relative inefficiency of the synthesis of nicotinic acid from tryptophan [54]. Contrary to riboflavin, average nicotinamide concentrations of MCW (134 µg/g protein) and acid whey (138 µg/g protein) were significantly higher than those of sweet whey (79.6 µg/g protein) (Table 2).

3.2.4. Vitamin B5 (Pantothenic Acid) and B6 (Pyridoxine)

While concentrations of pantothenic acid were high in all samples, particularly when compared to the low concentrations of pyridoxine (Table 1), both vitamin B5 and pyridoxine were not found to be significantly different between feeding systems or ingredient types. However, the forage:grain ratio of foodstuffs consumed by the cow is generally regarded as an influence on pantothenic acid synthesis [55]. The effect of varying this ratio on the levels of pantothenic acid transferred into the milk of the cow has not previously been investigated. Sources of vitamins B5 and B6 are consistent in the bovine diet, with relatively similar concentrations present in leafy forages and cereal grains [55,56]. A study comparing the vitamin B6 content of commercial milk with that of milk produced from cows fed a diet restricted in B6 found consistent levels of the vitamin between both milk sources, indicating that the concentrations of vitamin B6 present in milk may be independent of diet and it may instead be entirely supplied through ruminal synthesis [57].

The vitamin B6 complex consists of six vitamers: pyridoxine, pyridoxal, pyridoxamine, and the phosphate ester form of each; pyridoxine phosphate, pyridoxal phosphate, and pyridoxamine phosphate [58]. The multiple reaction monitoring assay carried out in the present study exclusively measured the pyridoxine vitamer, while the predominant vitamer present in bovine milk is the active form pyridoxal phosphate [59]. Consequently, concentrations for vitamin B6 observed in this study were negligible in comparison to average values for total vitamin B6 observed for milk in the literature [12,60,61].

3.2.5. Vitamin B7 (Biotin)

The average biotin concentrations of CLV and GRS were significantly higher ($p < 0.05$) than that of TMR (Table 1). Biotin is present in low concentrations in milk [62], though milk remains a good dietary source as requirements for the vitamin are comparatively low [63]. As with the other B-complex vitamins, biotin is a product of rumen metabolism [24], though conflicting information exists on the relationship between dietary intake, net ruminal synthesis, and duodenal flow of biotin [64–66]. However, the analysis carried out herein suggests that biotin content was significantly affected by feeding system.

The role of nutrition in biotin synthesis has been widely investigated. Briggs et al. [67] suggested that feeding of dietary urea may increase rumen biotin synthesis, which corresponds to the increased concentration of the vitamin in the CLV sample in the present study. Increased urea content has previously been observed in samples derived from this system [33], while increasing dietary biotin supplementation was found to significantly increase milk biotin concentrations [24]. Other studies have outlined a decrease in rumen biotin content with increasing grain content [68] or decreasing dietary forage content [26] and an increase in rumen acidity with increasing grain supplementation, leading to inhibition of cellulolytic rumen microflora [69], as previously suggested for thiamine content in Section 3.2.1. This is supported by O'Callaghan et al. [33], who showed no significant effect of bovine feeding system on the overall composition of rumen microflora, but rather rumen microflora functionality. The results of the present study support this indirect effect of the feeding system, whereby the functionality of rumen microflora and, hence, efficiency of rumen biotin synthesis may be increased or decreased depending on the substrate derived from the type of feed consumed by the cow. However, this is a notable contrast to pantothenic acid and pyridoxine (Section 3.2.4), which are primarily products of rumen metabolism, but do not display a significant effect of the feeding system on their concentrations in the SMP and whey samples.

3.2.6. Vitamin B12 (Cobalamin)

Cobalamin concentrations did not differ significantly ($p > 0.05$) between SMP samples. The highest concentrations were present in CLV-derived SMP ($38.6 \mu\text{g kg}^{-1}$), followed by TMR ($37.2 \mu\text{g kg}^{-1}$) and GRS ($34.3 \mu\text{g kg}^{-1}$). This is likely due to increased levels of cobalt present in the concentrate used in the TMR ration and in the root nodules of the CLV sward. Leguminous plants such as *Trifolium repens* L. exhibit a high affinity for the concentration of cobalt [70], which is required by nitrogen-fixing microflora present in the root nodule [71] and forms the central constituent in cobalamin synthesis [72]. The lower concentrations observed in GRS-derived SMP may therefore be due to the absence of cobalt from the leaves of the perennial ryegrass exclusively consumed by the cows assigned to this feeding system.

3.3. Relationship between Skim Milk and Recommended Daily Allowances

Table 3 shows the recommended daily allowance (RDA) and adequate intake (AI) values for each water-soluble vitamin for mature females and males (aged 14+) [63], along with the mass of each vitamin present in a 200-mL serving of skim milk derived from each feeding system and the percentage which they contribute to the RDA for each vitamin. Skim milk from each diet offers a low proportion of the RDAs for vitamins B1 and B3, though the stated allowance for vitamin B3 represents “niacin equivalent”, a figure which incorporates the typical daily intake of tryptophan, the mass of which is not included in the skim milk sample values in the table. As discussed in Section 3.2.4, the proportion of vitamin B6 in each SMP refers to the pyridoxine form only and is, therefore, omitted from Table 3, as an approximate comparison to the full RDA for vitamin B6 cannot be made for the individual vitamin. The vitamin B5 content of skim milk from each feeding system would account for 16% to 17% of its RDA, while, notably, almost one third of the average RDA (RDA based on recommendations of US Institute of Medicine [63]) for vitamin B12 would be provided from a 200-mL serving of skim milk from each feeding system (Table 3). As previously described, concentrations of vitamins B2 and B7 were approximately twice as high in GRS and CLV-derived samples with respect to TMR-derived samples. Thus, approximately 10% of the RDA of vitamin B7 may be provided by a skim milk serving from either pasture-derived sample, with approximately 5% available from TMR-derived milk. While this ratio is similar for vitamin B2, the riboflavin content of a 200-mL serving of skim milk from each feeding system would provide substantially higher than the average RDA for the vitamin.

Table 3. Recommended daily allowances (US Institute of Medicine, 1998) for females and males (aged 14+) and average mass (mg) of water-soluble vitamins present in skim milk powder reconstituted at 9.5% total solids derived from Holstein-Friesian cows assigned to each feeding system.

Water-Soluble Vitamin	Recommended Daily Allowance (mg)		Mass (mg) in 200 mL Skim Milk	% of Adult Human RDA		Mass (mg) in 200 mL Skim Milk	% of Adult Human RDA		Mass (mg) in 200 mL Skim Milk	% of Adult Human RDA	
	Female	Male	GRS	Female	Male	CLV	Female	Male	TMR	Female	Male
B1	1.1	1.2	0.038	3.5	3.2	0.038	3.5	3.2	0.029	2.6	2.4
B2	1.1	1.3	2.964	269	228	3.049	277	235	1.697	154	131
B3 complex	14	16	0.114	0.8	0.7	0.113	0.8	0.7	0.128	0.9	0.8
B5	5.0 *	5.0 *	0.789	16	16	0.829	17	17	0.846	17	17
B7	0.03 *	0.03 *	0.0033	11	11	0.0037	12	12	0.0017	5.7	5.7
B12	0.0024	0.0024	0.0006	27	27	0.0007	30	30	0.0007	29	29

Skim milk values are presented as the average of duplicate samples. GRS—Cows fed perennial ryegrass only. CLV—Cows fed perennial ryegrass/white clover. TMR—Cows fed total mixed ration ad libitum. Vitamins: B1—Thiamine, B2—Riboflavin, B3 complex—Nicotinic acid and Nicotinamide, B5—Pantothenic acid, B7—Biotin, B12—Cobalamin. * Indicates adequate intake values where recommended daily allowance values have not been derived. Note: Recommended daily allowance values for the vitamin B3 complex are expressed as “niacin equivalent”, comprising vitamin B3, B3-amide, and tryptophan. Values for the vitamin B3 complex are expressed as the sum of vitamin B3 and B3-amide.

3.4. Vitamin Content of Skim Milk for Use in Infant Milk Formula Manufacture

Table 4 shows the B vitamin contribution of SMP in an IMF (1.4%, *w/w*, protein), if 50% (*w/w*) of the total protein is obtained from SMP. In addition, shown are the minimum amount of each vitamin required (mg/100 mL), based on the recommendations of the Codex Alimentarius standard 72 [73] for IMF with an energy density of 65 kcal/100 mL. While further quantities of water-soluble vitamins would be introduced to an IMF mixture through the addition of whey protein ingredients or by supplementation, the SMP base from any of the three feeding systems would provide substantially more riboflavin than the minimum required level. Similarly, the required cobalamin content would be achieved using CLV (0.000068 mg/100 mL) or TMR-derived SMP (0.000065 mg/100 mL) alone, with GRS-derived SMP only slightly lower at 0.000060 mg/100 mL. The use of SMP derived from GRS, CLV, and TMR would provide 30%, 35%, and 16% of the required biotin content, respectively, while each SMP type would provide approximately 30% of the pantothenic acid requirement. Quantitative differences in thiamine, nicotinic acid, and pyridoxine content between the three feeding systems are unsubstantial when compared to the high requirements for these vitamins. This necessitates the use of vitamin concentrate pre-mixes to supplement the required levels for IMF. As discussed in Section 3.2.4, the predominant form of vitamin B6 in bovine milk is pyridoxal phosphate, whereas in human milk, pyridoxal is the dominant form, followed by pyridoxal phosphate, with the other vitamers present in very low concentrations [58]. Fortification of most foods, including IMF, is most commonly achieved through the addition of pyridoxine alone in the form of the salt pyridoxine hydrochloride [74].

Table 4. B-vitamin contribution (mg) of skim milk powder derived from each feeding system in infant milk formula (65 kcal per 100 mL).

Water-Soluble Vitamin	Mass (mg/100 mL)			Minimum Requirement (CODEX STAN 72, 1981)
	GRS	CLV	TMR	
B1	0.004	0.004	0.003	0.039
B2	0.279	0.287	0.160	0.052
B3 complex	0.011	0.011	0.012	0.195
B5	0.074	0.078	0.080	0.260
B6-Pyridoxine	0.000088	0.000075	0.000086	0.023
B7	0.0003	0.0003	0.0002	0.001
B12	0.000060	0.000068	0.000065	0.000065

SMP values are presented as the average of duplicate samples. GRS—Cows fed perennial ryegrass only. CLV—Cows fed perennial ryegrass / white clover. TMR—Cows fed total mixed ration ad libitum. Vitamins: B1—Thiamine, B2—Riboflavin, B3 complex—Nicotinic acid and Nicotinamide, B5—Pantothenic acid, B7—Biotin, B12—Cobalamin. Note: Only the pyridoxine form of vitamin B6 is represented in the data. Values for the vitamin B3 complex are expressed as the sum of vitamin B3 and B3-amide.

4. Conclusions

The utilisation of pasture or concentrate-based bovine feeding systems significantly affected the relative concentrations of a limited number of water-soluble vitamins in the skim milk and whey protein powder ingredients used in this study and varied across ingredient types. Significant differences in thiamine (B1) content were observed only in SMP, while significantly higher riboflavin (B2) content was apparent in GRS and CLV-derived SMP, sweet whey, and MCW, when compared to TMR. This may be primarily attributable to the forage content of the GRS and CLV diets which contain high concentrations of riboflavin. Despite the higher proportions of the nicotinic acid/amide complex in cereal grains, significantly higher concentrations of vitamin B3 and B3-amide were observed only in TMR-derived sweet whey and acid whey, respectively. Variations in the concentration of biotin (B7) may, however, be indirectly affected by bovine diet, through substrate-based modulation of rumen microflora and subsequent variation in the relative efficiency of rumen biotin synthesis. The PLS-DA, VIP, and HCA plots provide a visual representation of the distinguishable difference in milk protein

ingredients derived from each feeding system based on their vitamin profile, which offers potential as a means of milk product verification. Riboflavin and biotin exhibited the most biologically significant differences in SMP when compared to average recommended daily allowances and requirements for infant milk formulation. The data presented also suggest that significant differences in vitamin content may arise due to the type of whey production method used, independent of dietary effects.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2304-8158/9/5/578/s1>, Table S1: Validation parameters for water-soluble vitamin determination by LC-MS/MS, Table S2: Average concentrations (μM) of water-soluble vitamins for reconstituted skim milk (9.5% total solids), sweet whey (6.5% total solids), micellar casein whey (6.5% total solids) and acid whey (6.5% total solids) powders derived from the milk of Holstein-Friesian cows assigned to perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS, Table S3: Average concentrations (μM) of total water-soluble vitamins for reconstituted rennet whey (6.5% total solids), micellar casein whey (6.5% total solids) and acid whey (6.5% total solids) powders derived from the milk of Holstein-Friesian cows assigned to each feeding system, determined by LC-MS/MS. Table S4: Average concentrations ($\mu\text{g/g}$ protein) of water-soluble vitamins for sweet whey, micellar casein whey and acid whey powders derived from the milk of Holstein-Friesian cows assigned to from perennial ryegrass (GRS), perennial ryegrass/white clover (CLV) and total mixed ration (TMR) feeding systems, determined by LC-MS/MS.

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Physicochemical properties of whole milk powder derived from cows fed pasture or total mixed ration diets

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ABSTRACT

This study examined the effect of dietary factors on compositional and functional properties of whole milk powder (WMP) produced from bovine milk. Raw milk samples were obtained from 3 groups of 18 Holstein Friesian spring-calving cows randomly assigned to diets based on perennial ryegrass (GRS), perennial ryegrass/white clover sward (CLV), and total mixed ration (TMR). Raw milks obtained in late lactation were subsequently standardized for fat, heat-treated (90°C for 30 s), evaporated, and homogenized before spray drying. The WMP produced from each diet were analyzed to determine differences in color, particle size distribution, heat coagulation time, yogurt gelation, texture profile, and protein profile due to each diet. Significant differences in heat coagulation time were observed between the CLV and TMR samples, whereas color values were significantly different between GRS and TMR samples. No significant differences in gross composition, protein profile, or whey protein nitrogen index were found between the 3 WMP samples. Average D_{90} values (the particle size at which 90% of the particles were smaller than the specified size) for fat globules were significantly lower in the TMR sample compared with the GRS and CLV samples. Yogurts produced from GRS- and CLV-derived WMP had significantly higher elastic moduli (G') than those produced from TMR-derived WMP. Similarly, texture profile analysis revealed significantly higher firmness values in yogurt samples derived from CLV compared with TMR samples. Our data characterize the effect of these diets on the composition and functional properties of fat-standardized WMP, suggesting better yogurt functionality and thermal stability in WMP derived from pasture-based bovine diets.

Key words: whole milk powder, pasture, total mixed ration, yogurt gelation, heat coagulation time

INTRODUCTION

Whole milk powder (WMP) is a high-value dairy commodity produced through processing and spray drying of standardized whole milk. Although WMP retains the inherent fat composition of the whole milk from which it is produced, it typically undergoes the same centrifugal separation process as skim milk to yield skim and cream, which are subsequently recombined to standardize the fat content of the whole milk (Kelly et al., 2002). Whole milk powder is used extensively in the production of chocolate and ice cream, but it is also frequently rehydrated as a source of whole milk or for use as the base ingredient in yogurt production.

The temperate climate of countries such as Ireland and New Zealand is most suited to pasture-based milk production systems, the utilization of which has been shown to result in a range of compositional differences in milk compared with that from concentrate-based production systems. The latter systems are widely implemented in the Americas and parts of Europe (Chilliard et al., 2001; Couvreur et al., 2006). Previous studies have demonstrated greater levels of carotenoids (particularly β -carotene), greater unsaturated fatty acid content, most notably CLA (Kelly et al., 1998), and greater true protein content in pasture-derived milk compared with TMR-derived milk (O’Callaghan et al., 2016b). Because of the increased energy density of high-protein TMR diets, their application has been shown to result in an increase in milk yield but not milk protein content (Kolver et al., 2000). Indeed, over-feeding of dietary CP may not necessarily lead to an increase in milk protein content, because excess nitrogen is excreted as urinary nitrogen (Broderick, 2003). Similarly, increasing dietary oil supplements and reducing dietary fiber content can affect rumen biohydrogenation pathways, producing inhibitory fatty acid

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intermediates that limit milk fat synthesis (Bauman and Griinari, 2003). Conversely, pasture-based diets that are higher in roughage content tend to promote the action of cellulolytic rumen microflora, leading to increased milk fat biosynthesis and ultimately higher milk fat content (Bauman and Griinari, 2003).

Differences in milk composition due to dietary variation may have a significant effect on the thermal stability and processability of products derived from WMP. Gel strength, viscosity, and textural qualities of acidified reconstituted WMP are properties of significant importance in determining the behavior of set-style yogurts. Gelation can be influenced by various factors, such as milk fat and protein (particularly casein) content, heat treatment, and homogenization pressure (Lee and Lucey, 2010). Given the various heating processes involved in WMP production, thermal stability is an important functional parameter (Singh and Creamer, 1991). Heat-induced changes are primarily caused by the denaturation or aggregation of whey protein fractions (particularly β -LG) or the interaction of κ -casein and whey proteins (Anema and Li, 2003). The whey protein nitrogen index (WPNI) quantifies the level of undenatured whey protein in dairy commodities, indicating the level of heat treatment a dairy powder has received during processing. These WPNI values are of particular importance for predicting the gel strength of yogurts.

Although the effects of pasture-based and concentrate-based bovine feeding systems on the composition of raw milk have been reported, limited information exists to characterize their effect on the composition of standardized milk powder products. The objective of this study was to characterize the influence of perennial ryegrass (*Lolium perenne* L.), perennial ryegrass/white clover (*Trifolium repens* L.), and TMR-based feeding systems on the color, thermal stability, particle size distribution, and protein profile of standardized WMP produced from raw milk derived from each system and the subsequent gelation and textural properties of yogurt produced from the WMP.

MATERIALS AND METHODS

Materials

Raw milk was obtained from the Teagasc Animal and Grassland Research and Innovation Centre dairy unit (Moorepark, Fermoy, Co. Cork, Ireland). The mesophilic starter culture MO1, used for yogurt production (a mixture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*), was sourced from Chr. Hansen (Cork, Ireland).

Experimental Design

The experimental design for the bovine feeding systems was similar to that described previously for butter (O'Callaghan et al., 2016a), Cheddar cheese (O'Callaghan et al., 2017), and raw milk (O'Callaghan et al., 2016b, 2018; Faulkner et al., 2018). Fifty-four spring-calving Friesian cows were allocated to 3 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 February 19, 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); and group 3 was maintained outdoors on a perennial ryegrass/white clover pasture (CLV). For further information on the chemical and nutritional values of each of the feeding systems, see O'Callaghan et al. (2016b). Briefly, the TMR system consisted of, on a DM basis, 7.15 kg of grass silage, 7.15 kg of maize silage, and 8.3 kg of concentrates. Cows within the TMR system were fed at 0830 h daily into electronically controlled individual feed bins (Mealmaster; Griffith Elder and Company Ltd., Suffolk, UK) and the TMR was available ad libitum. Both groups of pasture-based cows consumed ~ 18 kg of DM/d, allocated using estimates of pregrazing herbage mass and daily postgrazing sward heights, as described by Egan et al. (2017). The CLV sward contained 20% (wt/wt) white clover. Milking took place at 0730 and 1530 h daily. To obtain a representative sample of milk, the cows in each of the 3 feeding systems were milked separately into designated 5,000-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 before sample collection. Milk was collected from each of the groups in the trial for milk powder manufacture on 3 separate occasions over a 3-wk period in September 2015 to produce 3 batches of WMP from each feeding system, when cows were 184 ± 7 d in lactation on their respective diets.

WMP Production

All milk powder production was carried out at the Moorepark Technology Limited pilot-plant facility (Fermoy, Co. Cork, Ireland). Raw whole milk ($\sim 1,000$ kg) was heated to 50°C in an APV plate heat-exchanger (SPX Flow Technology, Crawley, UK) before being separated into skim milk and cream in a centrifugal disk separator (GEA Westfalia, Oelde, Germany). The separate skim and cream fractions were then re-

combined to produce a whole milk of standardized fat content (3.5%, wt/wt). This standardized whole milk was then pasteurized at 90°C for 30 s using the APV plate heat-exchanger, before being evaporated to ~52% TS in a Niro 3-effect falling film evaporator (GEA Niro A/S, Soeborg, Denmark). The pasteurized concentrate was heated to 65°C in the APV plate heat-exchanger and homogenized using a 2-stage homogenizer (APV Gaulin, Lake Mills, WI) at first- and second-stage pressures of 15,000 and 5,000 kPa, respectively. The homogenized whole milk concentrate was then dried using a Niro Tall-Form Anhydro 3-stage spray dryer (air inlet temperature 180°C; air outlet temperature 80°C). First and second fluid bed temperatures were set at 65°C and 25°C, respectively. Fines were returned from the second fluid bed and the cyclone to the top of the spray dryer to yield an agglomerated WMP of approximately 97% TS. The WMP production was carried out in triplicate from 3 independent raw milk collections.

Compositional Analysis of Powder

Determination of Nitrogen and Fat Contents.

The total nitrogen content of each powder was determined using the Kjeldahl method, as described in ISO 8968-1 (ISO, 2001). A nitrogen-to-milk protein conversion factor of 6.38 was used. The fat content of each powder was determined using the Röse-Gottlieb gravimetric method, as described by the International Dairy Federation (1996). The ash content of each powder was determined by ashing approximately 3 g of each sample in a Carbolite muffle furnace (Carbolite Gero Ltd., Hope, Sheffield, UK) overnight. The moisture content of each powder was determined using a HR83 Halogen rapid moisture analyzer (Mettler Toledo, Columbus, OH). The lactose content of each powder was calculated by difference.

Noncasein nitrogen (NCN) content was determined by precipitation of the casein component of a whole milk sample. The sample was diluted with deionized water at 40°C and acidified to pH 4.6 by addition of acetic acid and sodium acetate. The mixture was cooled to 20°C and allowed to settle before filtration using Whatman No. 1 filter paper. A Kjeldahl determination was then carried out on the filtrate as described above. The NPN content was determined by precipitation of the protein component of a whole milk sample using trichloroacetic acid (15% wt/vol). The precipitate was removed from the mixture using Whatman No. 1 filter paper and a Kjeldahl determination was then carried out on the filtrate as described above.

HPLC. The protein profile of each WMP was determined using reversed-phase HPLC as described by

Mounsey and O’Kennedy (2009). The aqueous (phase A) and organic (phase B) phases consisted of acetonitrile, HPLC-grade water, and trifluoroacetic acid (TFA) in ratios of 100:900:0.1 (vol/vol/vol) and 900:100:0.1 (vol/vol/vol), respectively. Samples at a dilution factor of 1:20 sample:buffer were filtered through a 0.2- μ m filter and separated using an Agilent 300SB Poroshell (2.1 \times 75mm) column (Agilent Technologies, Santa Clara, CA) at 35°C. Detection wavelength was 214 nm, injection volume was 5 μ L, and flow rate was 0.5 mL/min.

Color Measurements

The CIE L*a*b* method was used to measure the color of each WMP. Lightness (L*), red/green color (a*), and yellow/blue color (b*) values were determined using a Konica Minolta CR-400 Chroma Meter (Chiyoda, Tokyo, Japan). Powdered and reconstituted (13% wt/wt) samples of each WMP were placed into plastic cuvettes and measured in triplicate.

Whey Protein Nitrogen Index

The WPNI was determined using the GEA Niro Method No. A21a, modified from the Harland-Ashworth method (Kuramoto et al., 1959).

Heat Coagulation Time

The heat coagulation time (HCT) of raw whole milk and reconstituted powders was determined using an Elbanton Oil Bath (Hettich Benelux Laboratory Equipment, Geldermalsen, the Netherlands). Each WMP sample was reconstituted to 1.5% (wt/wt) protein using deionized water. Milk samples were divided into a further 13 aliquots (~30 mL each), the pH values of which were adjusted in pH increments of 0.1 between pH 6.2, and 7.4 using 0.1 N HCl or NaOH. Following approximately 2 h of equilibration and final pH adjustment, where necessary, ~3.4-g aliquots of each sample were pipetted into 4-mL glass tubes, stoppered, and inserted into the oil bath rack. The rack was then inserted into the temperature-controlled oil bath at 140°C and rocked at 8 oscillations/min. The time taken (in minutes) for visible coagulation of each sample was recorded.

Particle Size of Rehydrated WMP

The particle size distribution of 15% (wt/wt) WMP dispersions was measured in triplicate by static light scattering using a Malvern Mastersizer laser-light dif-

fraction unit (Hydro MV, Mastersizer 3000, Malvern Instruments Ltd., Malvern, UK) equipped with a 300 RF lens. Refractive indices were set at 1.462 and 1.33 for the particle and dispersant (water), respectively. Size measurements were determined as the median (D_{50}) and cumulative diameters D_{90} and D_{10} , in which 50, 90, and 10% of the volume of particles were smaller than the specified size, respectively. Size distributions were determined by polydisperse analysis, and measurements were taken when laser obscuration reached ~3%.

Rheological Properties of Yogurt

Yogurt Production. Whole milk powder from each group of cows was reconstituted to 15% TS in deionized water and refrigerated overnight at 4°C to ensure complete hydration. Dispersions (1 L) were then tempered at 30°C before inoculation in a laminar flow hood. Freeze-dried pellets of the starter culture (0.2 g) were then added to ~20-mL aliquots of the tempered milk before being added back into the dispersions and mixed thoroughly. Sub-samples (100 mL) not intended for rheological measurements were added to sealed cups and placed in an incubator at 30°C (temperature chosen based on supplier recommendation). The pH of the inoculated milk was constantly monitored until a pH of 4.6 was reached. At pH 4.6, the incubated samples were immediately steeped in an ice bath to halt starter culture activity. Upon reaching a temperature of 10°C, these samples were refrigerated overnight.

Low-amplitude oscillation measurements were carried out using a Discovery HR-1 hybrid rheometer (TA Instruments, New Castle, DE), equipped with a concentric cylinder, maintained at 30°C. An aliquot (17 g) of the freshly inoculated milk was immediately weighed into the concentric cylinder. A time sweep was initiated using the following conditions: 5-s temperature equilibration at 30°C, 15-s pre-shear at a shear rate of 50 s⁻¹, and 10-s equilibration. The sample was then oscillated at 1% strain and a frequency of 1 Hz over a 10-s sampling interval until pH 4.6 was reached. This was monitored by measuring the pH of parallel-incubated samples of inoculated milk, which were maintained at the same temperature as the sample in the rheometer for the same duration. Once the pH reached pH 4.6, the time sweep was stopped. This was followed immediately by a logarithmic frequency sweep from 1 to 63.1 Hz at a constant strain of 1%.

Texture Profile Analysis. Following overnight refrigerated storage, set yogurt samples (100 mL) were analyzed at 4°C using a 35-mm flat-disk backward extrusion rig on a Texture Expert Exceed system (Stable Microsystems, Godalming, UK). Probe force was cali-

brated using a 2-kg weight mounted on a 5-kg load cell. Trigger force was set at 2 g. The probe penetrated the sample to a depth of 25 mm and returned to the starting point. Pretest, test, and posttest probe speeds were set at 1 mm/s. The sample firmness, consistency, cohesiveness, and index of viscosity were recorded. Yogurt gels from each WMP sample were also tested at 30°C to determine the influence of variation in fatty acid melting points between the samples.

Statistical Analysis

All analyses were carried out on WMP from 3 independent trials from each dietary treatment. Statistical analysis was performed using SPSS v18.0 (IBM Statistics Inc., Armonk, NY). Data sets were analyzed for normality using the Shapiro-Wilk test. Data were deemed normally distributed, and analysis was carried out using one-way ANOVA with post hoc Tukey test. *P*-values < 0.05 were considered significant.

RESULTS AND DISCUSSION

WMP Composition

Total nitrogen, total fat, lactose, NPN, and NCN contents of WMP samples are shown in Table 1. The highest mean protein content was present in the GRS sample (31.5%, wt/wt), followed by CLV (30.7%, wt/wt), and TMR (30.3%, wt/wt), although these values were not significantly different (*P* > 0.05). Similarly, no significant difference in mean fat content was found between the powders, with mean fat contents of 25.8, 25.7, and 25.5% (wt/wt) for CLV, TMR, and GRS milk samples, respectively. No significant differences in lactose, ash, or free moisture contents were found between the samples. However, NPN and NCN values for CLV were significantly higher (*P* < 0.05) than those from TMR. Increased NPN content may be attributed to increased urea content arising from the inclusion of white clover in the CLV feeding system. Significantly higher levels of urea have previously been reported in milk from this system, compared with milk from a TMR system (O'Callaghan et al., 2018). Significant differences in protein and fat content in pasture-derived raw unstandardized whole milk, relative to concentrate-derived raw whole milk, have been observed previously (O'Callaghan et al., 2016b); however, no information exists on the effect of these feeding systems on the composition of standardized WMP. Another study that used these feeding systems recorded significant differences in total protein and casein content between pasture-derived and concentrate-derived raw whole milks,

Table 1. Compositional analysis (% wt/wt) data for whole milk powders (mean values \pm SD)

Sample ¹	Total protein	Total fat	Lactose	NPN	Noncasein N	Ash	Free moisture
GRS	31.5 \pm 0.99 ^a	25.5 \pm 0.78 ^a	35.6 \pm 0.52 ^a	0.32 \pm 0.05 ^{ab}	0.99 \pm 0.02 ^{ab}	5.42 \pm 0.54 ^a	1.94 \pm 0.29 ^a
CLV	30.7 \pm 0.53 ^a	25.8 \pm 0.63 ^a	36.2 \pm 0.79 ^a	0.37 \pm 0.02 ^a	1.03 \pm 0.02 ^a	5.57 \pm 0.13 ^a	1.83 \pm 0.02 ^a
TMR	30.3 \pm 1.35 ^a	25.7 \pm 0.48 ^a	36.4 \pm 1.22 ^a	0.29 \pm 0.01 ^b	0.97 \pm 0.02 ^b	5.72 \pm 0.03 ^a	1.84 \pm 0.12 ^a

^{a,b}Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

¹Whole milk powders were from cows fed perennial ryegrass only (GRS), perennial ryegrass/20% white clover sward (CLV), or an indoor TMR ad libitum (TMR).

although a significant difference in total fat was only observed between the 2 pasture-derived diets (Gulati et al., 2018a).

The mass of protein fractions present in each WMP dispersion (13% wt/wt) are shown in Table 2. No significant differences ($P > 0.05$) were observed in protein profile between the WMP samples. Slightly higher amounts of κ -CN, α_{S2} -CN, α_{S1} -CN, β -CN, β -LG-a, and β -LG-b were observed in the GRS sample than in the CLV and TMR samples. A previous study by Mackle et al. (1999) investigated the effect of feeding cows on ryegrass-white clover pasture, pasture supplemented with maize grain, or pasture supplemented with a combination of maize grain and pasture silage. The above study, using SDS-PAGE, identified a significantly lower proportion of β -CN in milk from cows supplemented with maize grain only and higher β -CN content in milk from cows fed on pasture only. This trend for β -CN appears to be similar to that in the present study; however, the lack of significance ($P > 0.05$) could be related to reduced sample size ($n = 3$) in the present study.

Average WPNI values for the CLV, GRS, and TMR powder samples were 4.2 ± 0.19 , 3.8 ± 0.11 , and 4.0 ± 0.05 mg of undenatured whey protein per gram of powder, respectively. Excessive heat treatment will lead to increased whey protein denaturation, as indicated by a low WPNI value of <1.5 (Harland and Ashworth, 1947); in contrast, a high WPNI value (>6.0) indicates a high level of native whey protein in dairy-based powders (Sikand et al., 2008). All WMP samples exhibited WPNI values in the medium heat treatment range (>1.5 to <6.0), indicating a consistent heat treatment process and no significant difference in the level of denatured whey protein content between the samples.

Color Analysis of WMP

Color measurements of WMP samples are shown in Table 3. Lightness (**L***) indicates the degree of whiteness of a sample, ranging from 0 (black) to 100 (white). Positive values on the red/green (**a***) component indicate redness and negative values indicate greenness. Similarly, positive values on the yellow/blue (**b***) component indicate sample yellowness and negative values indicate blueness.

Differences in **a*** and **L*** values were not significant between milk powders from each of the diets, although significant differences ($P < 0.05$) were observed in **b*** values. These differences were apparent between the GRS and CLV samples and the TMR sample. The powder samples exhibited higher overall color intensity and **L*** values than the rehydrated powder samples. Maximum powder **L*** values were observed in the TMR sample, followed by CLV and GRS. The highest **L*** value of the rehydrated WMP samples was observed in the CLV sample, followed by TMR and GRS. The highest **a*** values in milk powder and rehydrated WMP samples were observed in the GRS sample, followed by CLV and TMR, respectively. However, there were no significant differences in **L*** or **a*** values between the milk powders or between the rehydrated samples. The GRS powder **b*** values were significantly higher ($P < 0.05$) than those of the TMR sample. The CLV **b*** values were also higher than TMR **b*** values, albeit not significantly ($P > 0.05$). Table 3 includes ΔE^* values for each sample, denoting the difference between each sample that can be visibly determined. A difference of 3.21 in ΔE^* value between the TMR and GRS samples indicates that these samples were visibly distinct. Dif-

Table 2. Mass of protein fractions (mg/mL) present in whole milk powder dispersions (13% wt/wt), determined by reversed-phase HPLC (mean values \pm SD)

Sample ¹	κ -CN	α_{S2} -CN	α_{S1} -CN	β -CN	α -LA	β -LGa	β -LGb
GRS	3.4 \pm 0.32	2.9 \pm 0.08	12.4 \pm 0.20	12.1 \pm 0.48	0.4 \pm 0.10	1.5 \pm 0.26	1.4 \pm 0.51
CLV	3.1 \pm 0.44	2.5 \pm 0.26	11.4 \pm 0.44	11.3 \pm 0.49	0.5 \pm 0.15	1.4 \pm 0.09	1.3 \pm 0.39
TMR	2.6 \pm 0.69	2.5 \pm 0.56	10.9 \pm 1.47	10.8 \pm 1.23	0.6 \pm 0.15	1.4 \pm 0.43	1.4 \pm 0.30

¹Whole milk powder dispersions were from cows fed perennial ryegrass only (GRS), perennial ryegrass/20% white clover sward (CLV), or an indoor TMR ad libitum (TMR).

Table 3. Average lightness (L^*), red/green color (a^*), and yellow/blue color (b^*) values for whole milk powders and whole milk powder dispersions (15% wt/wt; mean values \pm SD)

Sample ¹	Powder			Dispersion			ΔE^*
	L^*	a^*	b^*	L^*	a^*	b^*	
GRS	93.0 \pm 0.71 ^a	-5.44 \pm 0.56 ^a	18.5 \pm 1.40 ^a	85.0 \pm 1.64 ^a	-4.58 \pm 0.86 ^a	11.0 \pm 1.67 ^a	2.78 ^(GRS-CLV)
CLV	93.3 \pm 0.75 ^a	-5.24 \pm 0.63 ^a	17.5 \pm 1.13 ^{ab}	86.2 \pm 0.27 ^a	-3.69 \pm 0.10 ^a	8.66 \pm 0.49 ^a	0.86 ^(CLV-TMR)
TMR	93.5 \pm 0.85 ^a	-4.65 \pm 0.70 ^a	15.0 \pm 1.15 ^b	85.7 \pm 1.53 ^a	-3.84 \pm 1.00 ^a	7.96 \pm 1.72 ^a	3.21 ^(GRS-TMR)

^{a,b}Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

¹Whole milk powders and dispersions were from cows fed perennial ryegrass only (GRS), perennial ryegrass/20% white clover sward (CLV), or an indoor TMR ad libitum (TMR).

² ΔE^* denotes total color difference between samples that can be visibly determined. Values <1 are imperceptible; values between 1 and 3 are visibly perceptible; values >3 are visually distinct.

ferences between GRS and CLV were visibly perceptible, whereas differences between the TMR and CLV samples were imperceptible.

Similar differences in the color of milk and milk products derived from TMR or pasture-based diets have been identified previously (Hurtaud et al., 2002). The slightly lower L^* and more negative a^* values exhibited by the GRS sample, along with the more positive b^* values exhibited by the GRS and CLV samples, may be attributed to increased β -carotene content in both pasture-based samples (Nozière et al., 2006; O'Callaghan et al., 2016a). Present in high concentrations in grass, β -carotene imparts a red/orange pigmentation and acts as a precursor compound to retinol (vitamin A) synthesis in the liver (Darwish et al., 2016). As vitamin A is fat-soluble, the quantity of β -carotene in various dietary fat sources has been widely investigated. Studies that investigated the effect of pasture and concentrate-based feeding systems on the composition of beef muscle (Duckett et al., 2009), butter (O'Callaghan et al., 2016a), and Cheddar cheese (O'Callaghan et al., 2017) have identified significantly higher concentrations of β -carotene in pasture-derived samples than in concentrate-derived samples.

HCT of Reconstituted WMP

All raw whole milk samples exhibited typical “type A” HCT–pH profiles, characterized by clear HCT maxima and minima and a sharp decline in HCT to a local minimum at pH 6.9 (O'Sullivan et al., 2001). Figure 1A shows typical HCT–pH profiles for raw milk samples from each feeding system. The trend of HCT increasing as a function of pH to a local maximum at pH 6.7 and then decreasing before further increasing is typically attributed to heat-induced dissociation of κ -CN from the casein micelle and subsequent complex formation with β -LG at pH >6.6 (Singh and Fox, 1985; Anema, 2008). The κ -CN-depleted casein micelle is then susceptible to calcium-induced aggregation, leading to protein precipitation (McSweeney et al., 2004). This is followed by increased HCT above pH 6.9, as the charge of the casein micelle increases due to the loss of κ -CN (Panda, 2011).

In contrast, all WMP samples exhibited unusual HCT–pH profiles, whereby HCT increased as a function of pH (type B profile); however, a slight decrease in HCT was observed at pH 7.1 for both the TMR and CLV samples and at 7.2 for the GRS sample, followed by HCT maxima at pH 7.4 in all samples (Figure 1B). The highest overall HCT was observed in the CLV sample, followed by GRS and TMR samples, respectively. Visible coagulation occurred after approximately 26, 19, and 18 min at pH 7.4 and after 19, 15, and 14

min at pH 7.2 for the CLV, GRS, and TMR samples, respectively. We detected significant differences ($P < 0.05$) between the CLV and TMR samples at each pH increment between pH 6.6 and 7.4. The HCT of the GRS sample was significantly higher than that of the

TMR sample at pH 7.1 ($P < 0.05$), whereas the HCT of the CLV sample was significantly higher than that of the GRS sample at pH 6.7, 7.3, and 7.4 ($P < 0.05$). These samples, however, did not exhibit a decrease in HCT between pH 6.7 and 6.9.

Similar type-B HCT–pH profiles have previously been observed in low-heat ($72^{\circ}\text{C} \times 15 \text{ s}$) skim milk concentrates heated at 120°C (Lin et al., 2018). Skim milk powder samples reconstituted at low concentration (9.4% TS) showed trends of steadily increasing HCT with increasing pH, followed by a slight decline at $\sim\text{pH } 7.0$, whereas HCT decreased significantly at higher pH values in samples reconstituted at higher TS concentrations (Lin et al., 2018). In the present study, standardization of reconstituted WMP samples at 1.5% total protein content resulted in a TS content of approximately 5%. This reduced concentration may contribute to the type-B HCT–pH profile and lack of decline in HCT at pH 6.9 observed in all 3 samples. The significant differences in HCT between feeding systems is notable because a previous study on mid-lactation skim milk produced from these same feeding systems did not show a similar trend of significant variation of HCT (Gulati et al., 2018b).

Results for calcium ion activity (Ca^{2+}) showed a higher average concentration of ionic calcium in TMR-based WMP (2.26 mM), compared with the GRS (2.21 mM) and CLV (2.12 mM) samples, although these did not differ significantly ($P > 0.05$). Although Ca^{2+} level decreases with increasing pH (Tsioulpas et al., 2007), milk HCT decreases with increasing Ca^{2+} (Sievanen et al., 2008). The significant differences in HCT observed between the CLV and TMR samples may be attributable to increased NPN content in the CLV sample, arising from increased urea levels (Huppertz, 2016) because of the white clover content of the diet (Harris et al., 1998). The addition of high concentrations of urea to unconcentrated milk has been shown to increase milk HCT (Muir and Sweetsur, 1976). Heat-induced decomposition of urea to ammonia reduces the susceptibility of milk to heat-induced acidification, leading to increased heat-induced dissociation of $\kappa\text{-CN}$ and a decrease in Ca^{2+} level (Huppertz, 2016). As previously discussed, significantly higher concentrations of urea have been detected in milk derived from the CLV feeding system compared with milk derived from the TMR system (O’Callaghan et al., 2018).

The lower HCT values in the TMR sample may also have been due to reduced fat particle size. The D_{90} values for TMR WMP dispersions were significantly lower than those for both GRS and CLV dispersions (Figure 4). Decreases in HCT due to homogenization and consequently reduced fat particle size have been previously described (McCrae, 1999).

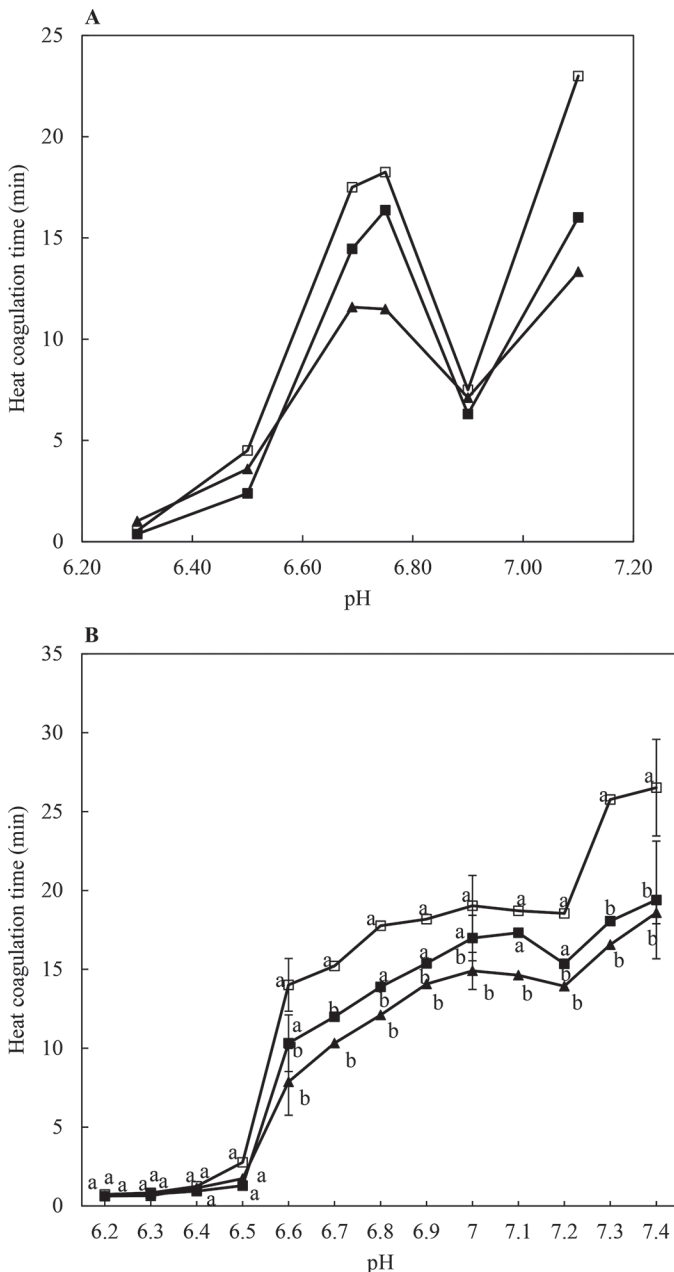


Figure 1. Heat coagulation time of (A) raw whole milk, and (B) whole milk powder dispersions (1.5%, wt/wt, protein) heated at 140°C at 8 oscillations per minute obtained from cows fed grass (■), grass/clover (□), or TMR (▲). Values in A represent a single measurement, and values in B are the means of data from triplicate trials and duplicate analysis. Error bars represent standard deviations. Data points with different letters (a, b) differed significantly ($P < 0.05$).

Rheological Properties of Yogurt

Rheological values showing the onset of gelation of yogurts produced from each WMP sample are shown in Table 4 and Figures 2 and 3. We found no significant differences in the time and pH values at which the elastic modulus (G') values of each sample exceeded 1 Pa or the time at which G' values exceeded viscous modulus (G'') values. At pH 4.6, the TMR sample yielded an average maximum G' of 51.5 Pa after 514 min. In contrast, the GRS and CLV samples yielded average G' maxima of 92.6 and 94.3 Pa after 525 and 541 min, respectively. The G' and G'' values were significantly ($P < 0.05$) higher in the CLV and GRS samples compared with the TMR sample. A previous study investigating characteristics of mid-lactation milk from these feeding systems reported significantly higher G' values and gel-firming rates in rennet gels from GRS-derived milk than in those from TMR-derived milk, although no significant differences were found between CLV and TMR rennet gels (Gulati et al., 2018b). No significant differences were found between the other gelation properties in the present study (Table 4).

Logarithmic frequency sweeps recorded typical viscoelastic behavior in each set yogurt sample up to a frequency of 63.1 Hz (Figure 3). At this frequency, the highest average G' value was observed in the GRS sample (236 Pa), followed by CLV (152 Pa) and TMR (105 Pa). The highest average G' values for the CLV (165 Pa) and TMR (109 Pa) samples were observed at 25.1 and 39.8 Hz, respectively. All samples exhibited overall thixotropic (time-dependent shear-thinning) behavior, characterized by a substantial decrease in complex viscosity (η^*) as a function of increasing frequency, along with a decrease in G' and concomitant increase in G'' at 63.1 Hz (Figure 3). The GRS sample, however, did not exhibit a decrease in G' at this frequency, despite an increase in G'' . The highest average η^* value at 1 Hz was observed in the GRS sample ($18.2 \text{ Pa}\cdot\text{s}^{-1}$), followed by CLV ($16.0 \text{ Pa}\cdot\text{s}^{-1}$) and TMR ($9.37 \text{ Pa}\cdot\text{s}^{-1}$). The η^* value of the GRS sample was significantly higher ($P < 0.05$) than that of the TMR sample. The higher G' , G'' ,

and η^* values observed in the GRS and CLV samples, compared with those from the TMR sample, indicate the formation of stronger, more cohesive gel matrices, which are more resistant to deformation.

Standardization of yogurt samples at 4% true protein content did not result in significant differences in gel strength in yogurt samples from the same dietary treatments standardized at 15% TS. This implies that variations in the gel strength of yogurts between dietary treatments were not attributable to variations in their true protein content. The significantly higher G' values observed in both pasture-based samples relative to the TMR sample may be attributable, therefore, to variation in the composition and structure of their fat components.

The fat particle size distribution results indicated the relative abundance of fat globules of varying size in WMP dispersions. No significant difference in volume mean diameter ($D_{[4,3]}$) was observed among the samples (Figure 4); however, the TMR sample had a significantly lower ($P < 0.05$) D_{90} value compared with both the GRS and CLV samples. This indicated a lower abundance of large fat globules in the TMR sample, which may be caused by increased fat globule flocculation or coalescence in the GRS and CLV samples due to the presence of lower-melting-point fatty acids (O'Callaghan et al., 2016a). The degree of electrostatic repulsion between protein-adsorbed fat globules in milk at the onset of gelation may be an influencing factor in the gel strength of the final gel matrix.

Texture Profile Analysis

Average texture profile data for each yogurt gel are shown in Table 5 and Figure 5. Firmness, cohesiveness, and consistency are defined as the force required to penetrate the gel structure, the degree of deformation the gel matrix can withstand before it ruptures (Rawson and Marshall, 1997), and the resistance of the gel matrix to deformation (do Espírito Santo et al., 2012), respectively. The highest gel firmness (192.8 g) and consistency ($4,218 \text{ g}\cdot\text{s}^{-1}$) were exhibited by the CLV

Table 4. Gelation properties (elastic modulus, G' ; and viscous modulus, G'') of yogurt gels produced from whole milk powder dispersions (15%, wt/wt, TS) inoculated with 0.2 g of mesophilic starter culture (mean values \pm SD)

Sample ¹	Time at which $G' = 1 \text{ Pa}$ (min)	pH at which $G' = 1 \text{ Pa}$	Time at which $G' > G''$ (min)	Time at which pH = 4.6 (min)	Final G' value at pH 4.6 (Pa)
GRS	469 \pm 10 ^a	4.77 \pm 0.09 ^a	462 \pm 10 ^a	525 \pm 13 ^a	92.6 \pm 10 ^a
CLV	473 \pm 20 ^a	4.76 \pm 0.04 ^a	471 \pm 20 ^a	541 \pm 23 ^a	94.3 \pm 5 ^a
TMR	467 \pm 31 ^a	4.77 \pm 0.02 ^a	463 \pm 27 ^a	514 \pm 29 ^a	51.5 \pm 12 ^b

^{a,b}Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

¹Whole milk powder dispersions were from cows fed perennial ryegrass only (GRS), perennial ryegrass/20% white clover sward (CLV), or an indoor TMR ad libitum (TMR).

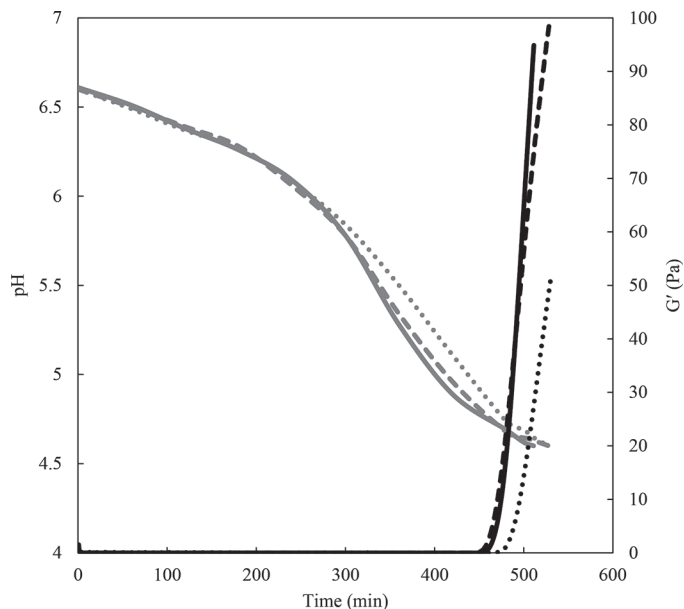


Figure 2. Typical elastic modulus, G' (black; Pa) and pH profile (gray) of yogurt gels produced from whole milk powder dispersions (15% wt/wt) for grass (solid line), grass/clover (dashed line), and TMR (dotted line) diets as a function of incubation time at 30°C. Measurements were ceased at pH 4.6.

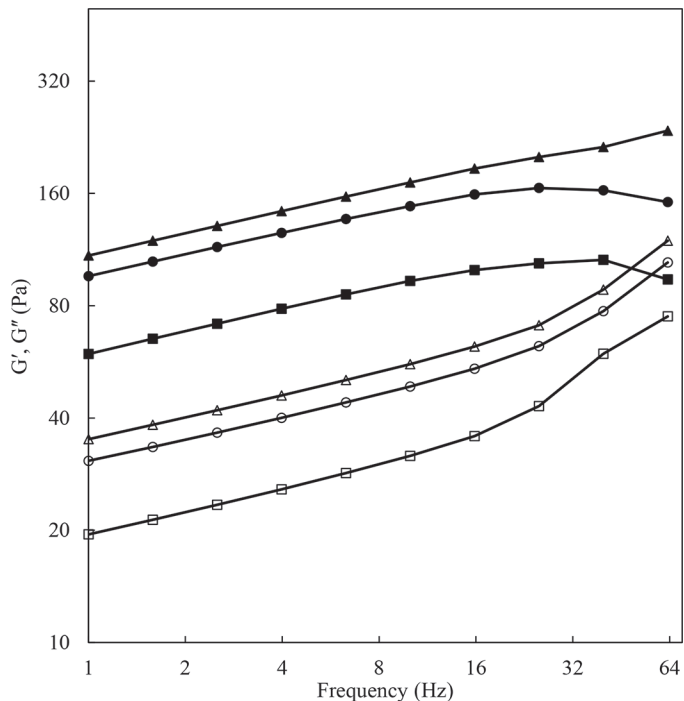


Figure 3. Average elastic (G' , filled symbols; Pa) and viscous (G'' , open symbols; Pa) moduli of yogurt gels produced from whole milk powder dispersions (15% wt/wt) for grass (▲, △), grass/clover (●, ○), and TMR (■, □) diets as a function of oscillation frequency (1–63.1 Hz).

yogurt sample. The CLV-derived yogurt gel yielded significantly higher ($P < 0.05$) firmness values than did the TMR-derived sample. Maximum gel cohesiveness (-60.9 g) and index of viscosity (-940 g·s $^{-1}$) were exhibited by the GRS sample. Lower values were observed in the TMR sample for all texture profile components. The WMP from the CLV feeding system produced the thickest and firmest yogurt samples overall, whereas WMP from the GRS system produced a more cohesive, elastic gel.

Warming yogurt samples to 30°C before texture profile analysis resulted in an approximately 30% decrease in firmness and consistency and an approximately 50% decrease in cohesiveness and index of viscosity across all samples. This implies that the textural variations identified between yogurts produced from WMP from each diet were not attributable to variations in their fatty acid melting points. The effect of seasonality must be noted as a source of variation in WMP composition and functionality. The characteristic behavior of the late-lactation milk processed and analyzed in this study may differ substantially from that of milk produced at a different stage of lactation or in a separate lactation cycle. A previous study by Gulati et al. (2018a) noted higher concentrations of total protein, fat, and casein in late-lactation milk from these feeding systems compared with that of mid-lactation milk.

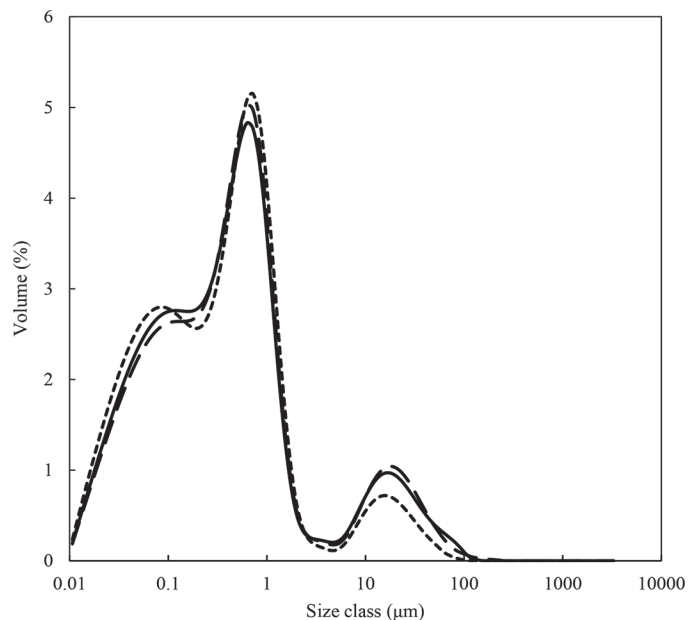


Figure 4. Particle size distribution of whole milk powder dispersions (15% wt/wt, total solids), for grass (solid line), grass/clover (long-dashed line), and TMR (short-dashed line) diets represented as a function of triplicate trials.

Table 5. Textural properties of yogurts produced from whole milk powder dispersions (15%, wt/wt, TS) inoculated with 0.2 g of mesophilic starter culture (mean values \pm SD)

Sample ¹	Firmness (g)	Consistency (g·s ⁻¹)	Cohesiveness (g)	Index of viscosity (g·s ⁻¹)
GRS	175 \pm 36.1 ^{ab}	4,089 \pm 805 ^a	-60.9 \pm 12.3 ^a	-940 \pm 172 ^a
CLV	192 \pm 20.9 ^a	4,218 \pm 477 ^a	-57.3 \pm 8.87 ^a	-878 \pm 123 ^a
TMR	130 \pm 6.96 ^b	3,095 \pm 194 ^a	-40.4 \pm 2.95 ^a	-644 \pm 32.7 ^a

^{a,b}Values within a column not sharing a common superscript differed significantly ($P < 0.05$).

¹Whole milk powder dispersions were from cows fed perennial ryegrass only (GRS), perennial ryegrass/20% white clover sward (CLV), or an indoor TMR ad libitum (TMR).

CONCLUSIONS

Pasture-based feeding of cows compared with a TMR diet resulted in some significant differences in WMP functionality, characterized by increased thermal stability of WMP (particularly CLV) and significantly higher yogurt gel strength and firmness. However, the WMP samples were not found to differ significantly over a range of characteristics, including WPNI and gross composition (powders were standardized for fat content), although NPN values did differ between samples. The differences in minor compositional constituents and behavior of the WMP samples used in this study may be attributed to the feeding system used. The sig-

nificantly higher b* values observed in both WMP and reconstituted WMP produced from the GRS feeding system compared with the CLV and TMR systems supports previous studies on the color profile of pasture-derived milk products. Overall, our results suggest that application of pasture-based dietary treatments confers increased thermal stability in WMP and increased gel strength in yogurt derived from these systems, indicating increased functionality when used as a base material for set-style yogurt manufacture.

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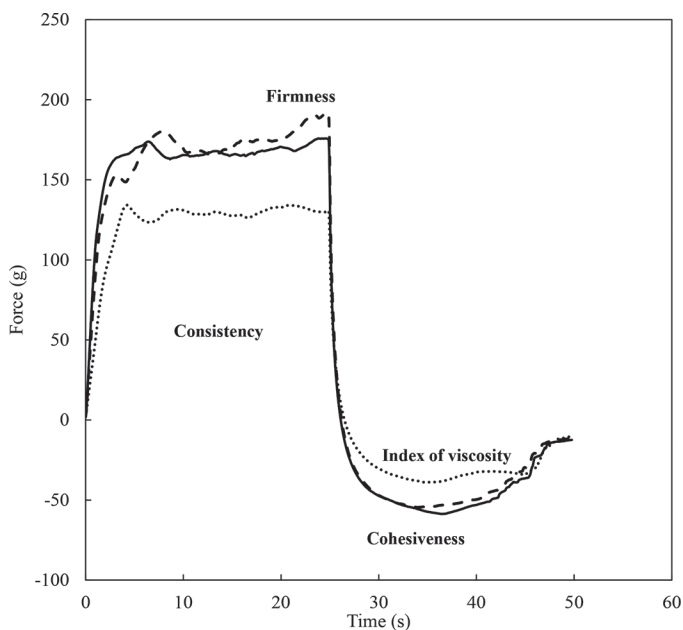


Figure 5. Average texture profile of yogurt gels produced from whole milk powder dispersions (15% wt/wt) for grass (solid line), grass/clover (dashed line), and TMR (dotted line) diets. Firmness indicates maximum force (g) recorded on initial probe stroke. Consistency (area) indicates the force per second (g·s⁻¹) on initial probe stroke. Cohesiveness indicates maximum negative force (g) recorded on probe return stroke. Index of viscosity (area) indicates the force per second (g·s⁻¹) on probe return stroke.

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