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Implications of changes to primary production and management practices in pasture-based dairy systems, on the compositional and processing properties of milk and Cheddar cheese quality

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

by

Richard M. Page

Submitted: October 2025

Research supervisors: Dr Prabin Lamichhane, Dr Tom O'Callaghan, Prof. Seamus (James) A. O'Mahony

Head of School: Prof. Alan L. Kelly

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 - Eurofins Food Testing Ireland determined the cheese β-carotene content.

Chapter 2 - The Rose-Gottlieb method was completed by Vijaya Lakshmi Chirumamilla.

Chapters 2, 3 and 5 - Somatic cell counts were determined by Jimmy Flynn.

Chapters 2, 3, 4 and 5 - Analysis of free fatty acids, volatile organic compounds and total fatty acids were completed by David Mannion and Iwona Skibinska.

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Chapters 6 and 7 – Milk rennet coagulation behaviour determined by Digvijay Digvijay.

Chapters 6 and 7 - Casein micelle size, zeta potential, and fat globule size determinations completed by Prashant Shelke.

Chapter 7 - The RP-HPLC procedure was completed by Bernard Corrigan.

Signed:  Date: 9th October 2025

Richard Page student number: 121127442

Dedicated to Victoria, Isaac and Elijah

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On leaving school, I attended UCL where I read Genetics. I stayed at home, close to where Charles Darwin once lived and worked, and commuted to Gower Street, where Darwin once lived and worked. This well-known scientist had an impact, but I more so need to acknowledge the inspirational lecturing of Steve Jones, whose books I continued to read even when working outside of science, and Jane Sowden and Faisal Idrees for my first taste of real research as I completed a final year dissertation at ICH. I now skip ahead, brushing over a business career, this is not a business thesis! I returned to science by attending UCD, where I completed the Food, Nutrition and Health programme, and here I must acknowledge Fiona Lalor because the mentorship and support given to me by Fiona was an essential part of getting me to the point of pursuing this PhD. Also, from my time at UCD, I would like to thank Aifric O'Sullivan, Briege McNulty and Eileen Gibney, who each made time for me as I planned my future career.

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It is useful to understand what it is like being 2-0 up only to go on to lose. In football, life, and the lab, it is not really about the wins, but about the sticking with. Sometimes you might get a result too.

Supervisors are important for getting a thesis done, but getting through a PhD requires navigating all the rest of life too.

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May all your desires be fulfilled except for one, so you always have something to strive for...

Abstract

This thesis considers the effects of some key primary production and management practices on the compositional, technological and ripening properties of Cheddar cheese. In Ireland's predominantly pasture-based dairy system, about a third of all milk is used for cheese production. As dairy farming practices evolve (e.g., adoption of practices to enhance sustainability of primary milk production), the composition of milk can change, with implications for dairy products made therefrom. Developing a deeper understanding of how farm-level decisions influence cheese quality is therefore of scientific and commercial relevance.

The impacts of dairy production practices on the processing of milk into cheese were initially explored through a review of the literature. Various practices, both intrinsic and extrinsic to the dairy animal, were considered in-turn. Due consideration was also given to interactions taking place within the dairy food system, because primary production practices impact on each other, making it a highly complex system. Substantial evidence is available to suggest that some changes in production practices can be made without adverse impacts on cheese quality. The experimental chapters of this thesis then contribute to this evidence-base by examining pertinent research gaps, improving our understanding of the influence of milking frequency, pasture type, and cow breeding and genetics on cheese biochemistry.

Twice-a-day (TAD) milking is most commonly used within Irish pasture-based systems, and the impact of milking frequency on cheese production was explored by contrasting TAD with once-a-day (OAD) milking. Most notably, OAD milking yielded significantly more cheese from a given volume of milk compared to TAD, suggesting a possible increased economic value for OAD milk within cheese manufacturing. The OAD strategy also resulted in cheese that was significantly more yellow, a difference confirmed to be associated with significantly higher β-carotene content, β-carotene being both a pigment and pro-vitamin A. Consumers often favour more yellow dairy

products, and increased β-carotene concentrations could provide nutritional benefits (supporting iron metabolism, skin and mucous membrane function, vision and the immune system).

Pasture-type was investigated through comparisons of milk and cheese derived from multispecies swards (MSS) and perennial ryegrass (PRG) diets. Milk compositions were found to be broadly similar, but MSS milks had significantly higher protein content. Significantly higher actual cheese yields (Ya) resulted from MSS grazing as compared to PRG, at 11.13 and 10.58%, respectively. For many investigated attributes, cheese obtained from the differing pasture diets were broadly comparable. These novel findings provide assurances that a biodiverse MSS strategy can be adopted while maintaining Cheddar cheese quality. The increased cheesemaking efficiency suggested through increased cheese yields could also offer an opportunity for manufacturers.

Investigations into crossbreeding compared differences arising when using Jersey-Holstein-Friesian (JFX) milk compared to Holstein-Friesian (HF) milk in the production of Cheddar cheese. The JFX milk had higher protein and fat content, but no statistically significant differences in set-to-cut time or cheese yield resulted. Cheese produced from both breeds was broadly comparable, but JFX derived cheese was significantly more yellow in colour. These intrinsic aspects of primary production were then explored further through investigations into genetic polymorphism of κ-CN, using milk collected according to the following treatment groups: HF with AA κ-CN (HF-AA), HF with AB κ-CN (HF-AB), and JFX with AB κ-CN (JFX-AB). On average, HF-AA milks had lower fat, protein and casein contents, smaller fat globule size, higher pH and larger casein micelle size compared to HF-AB and JFX-AB milks. The HF-AA treatment was associated with poorer rennet coagulation properties and lower cheese yields compared to HF-AB and JFX-AB. The highest cheese yields and fastest coagulation times were associated with the JFX-AB treatment. These findings suggest

crossbreeding can be combined with the selection of appropriate genetic variants to achieve improved production trait outcomes.

Considered collectively, these findings advance our understanding of how decisions on the farm can influence the processing performance of milk obtained from Irish pasture-based systems, and the key quality attributes of cheese made therefrom. When milking frequency, grazing system and breeding selection strategies are applied appropriately on the farm, milk can be processed into Cheddar cheese without adverse impacts. Additionally, some areas have been highlighted where targeted use of these primary production strategies can enhance cheesemaking efficiency and product quality. The findings contained herein offer potential future benefits for stakeholders within Irish dairy food systems, in particular for processors and dairy consumers.

Abbreviations

AA, AB, BB	Genotypes of κ-casein
A_1A_1, A_1A_2, A_2A_2	Genotypes of β-casein
ANOVA	Analysis of Variance
CN	Casein protein
CLA	Conjugated Linoleic Acid
EBI	Economic Breeding Index
FFA	Free Fatty Acids
FTIR	Fourier Transform Infrared Spectroscopy
G' (G prime)	Storage modulus, a measure of gel firmness in rheology
GC-FID	Gas Chromatography – Flame Ionization Detection
GC-MS	Gas Chromatography – Mass Spectrometry
GHG	Greenhouse Gas
GLM	General Linear Model
HF	Holstein-Friesian (dairy cow breed)
HF-AA / HF-AB	Holstein-Friesian cows with AA or AB κ-casein genotype
HPLC	High-Performance Liquid Chromatography
HS-SPME	Headspace Solid-Phase Microextraction
HTST	High-Temperature Short-Time (pasteurisation)
IDF	International Dairy Federation
IMCU	International Milk Clotting Units
ISO	International Organization for Standardization
JFX	Jersey-Holstein-Friesian (crossbred cows)

JFX-AB	Jersey-Holstein-Friesian cows with AB κ-casein genotype
MFG	Milk Fat Globule
MSS	Multispecies Swards (pasture type)
NCN	Non-Casein Nitrogen
NPN	Non-Protein Nitrogen
OAD	Once-a-Day milking
ODP	Optimized Descriptive Profiling (sensory analysis)
pH	Potential of Hydrogen (acidity/alkalinity)
PRG	Perennial Ryegrass (pasture type)
PUFA	Polyunsaturated Fatty Acids
Q–Q Plot	Quantile–Quantile Plot (statistical test for normality)
R604	Commercial cheese culture supplied by Chr. Hansen
SCC	Somatic Cell Count
SOL	Stage of Lactation
SPSS	Statistical Package for the Social Sciences
TAD	Twice-a-Day milking
TFA	Total Fatty Acids
TMR	Total Mixed Ration
TS	Total Solids
UHT	Ultra-High Temperature (milk processing protocol)
VOC	Volatile Organic Compounds
Y_a	Actual Cheese Yield
Y_{ma}	Moisture-Adjusted Cheese Yield
Y_n	Moisture-Adjusted Cheese Yield with Fat and Protein Adjusted to Reference Levels

Publications, Conference Presentations and Awards

Publications in peer-reviewed journals

Page, R M, Tobin, J T, Kilcawley, K N, et al. (2025b) The impacts of a typical dairy cow crossbreeding strategy on Cheddar cheesemaking efficiency, nutrition and quality. International Journal of Dairy Technology 78 e70013

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Page, R M, Magan, J B, Mannion, D T, et al. (2024) The impacts of milking frequency on nutrient composition and functional characteristics of Cheddar cheese. International Journal of Dairy Technology, 77: 630-644.

Daniloski, D, Page, R M, Lamichhane, P, et al. (2024) Cheddar cheese production, structure and in-vitro semi-dynamic gastric digestion: The role of β-casein phenotype. Food Research International 196 115008.

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Page, R M, Digvijay D, Shelke P et al. The implications of k-CN polymorphism and breed background on milk composition and processing attributes

Page, R M, Digvijay D, Shelke P et al. The cheesemaking efficiency, quality and ripening characteristics of Cheddar cheese manufactured from standardised milks of Holstein-Friesian and Jersey-Holstein-Friesian cows of differing κ-casein phenotypes

Other publications

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Page, R (2024) Milking cows less often might benefit farmers (and possibly cheese). The Boolean: Snapshots of Doctoral Research at University College Cork 32-36.

Page, R. M. (2024). Is milking cows once-a-day a potential game-changer for farmers? RTE Brainstorm, 2 May 2024. https://www.rte.ie/brainstorm/2024/0502/1446960-milking-frequency-research-cheddar-cheese-farmers-quality-of-life/

Page, R. M., & Lamichhane, P. (2024). Once-a-day milking: the impacts on Cheddar cheese. Teagasc Daily, 10 May 2024. https://www.teagasc.ie/news--events/daily/dairy/once-a-day-milking-the-impacts-on-cheddar-cheese.php

Conference presentations

Poster presentation at the 50th Anniversary Institute of Food Science and Technology Ireland Conference (2022).

Oral presentation at the 51st Institute of Food Science and Technology Ireland Conference (2023).

Flash oral presentation at the 4th International GreenFoodTech Conference (2025)

Poster presentation at the 4th International GreenFoodTech Conference (2025)

Awards

The Andrew Wilbey Award. A one-off award given to the dairy science student in Ireland and the UK who best demonstrated effective engagement with the literature of the Society of Dairy Technology.

Editor's Choice Article awarded to "The impacts of milking frequency on nutrient composition and functional characteristics of Cheddar cheese" within the August 2024 issue of the International Journal of Dairy Technology. Also later highlighted by the publisher Wiley as a Top Viewed Article.

Shortlisted presentation within the 2023 International Dairy Federation Irish National Committee student prize.

Winner of the Society of Chemical Industry sponsored "Best Food Science and Technology" Poster at the 2022 (50th Anniversary) Institute of Food Science and Technology Ireland Conference.

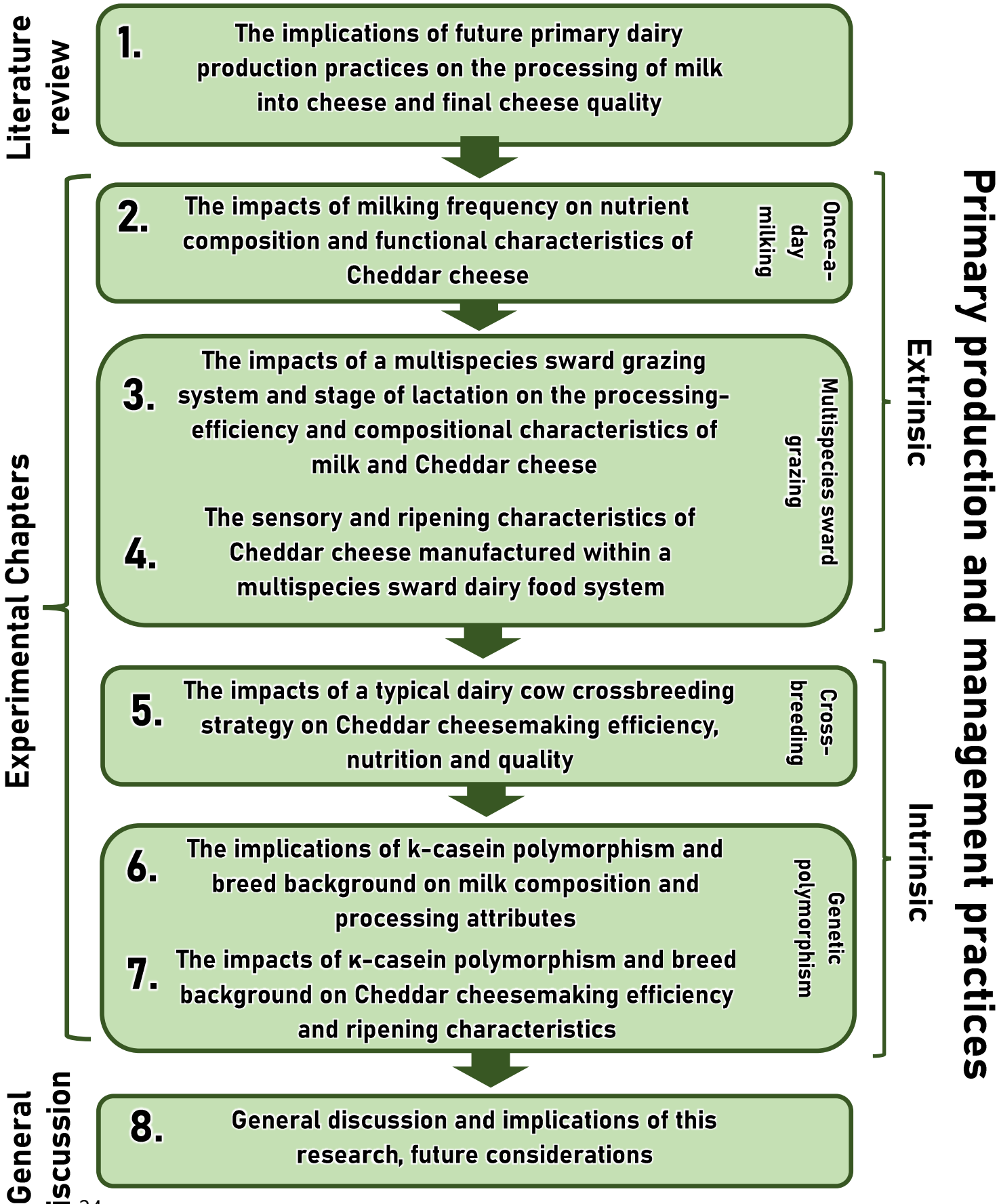
A winning entry within the Teagasc 2022 Vision of Research and Innovation Exhibition Awards (Joint entry with Prabin Lamichhane and with the support of Alann Jezequel and Brendan Horan). The entry, which featured at the exhibition, is shown below,



Transforming green to gold: the alchemy of Irish cheese

In Ireland, cows generally graze upon ryegrass monocultures. Multispecies swards offer benefits by increasing biodiversity and reducing costly chemical inputs through natural nitrogen fixation. This research investigates the nutritional and techno-functional consequences within cheese when applying multispecies grazing. Potential differences in cheese made from multispecies systems need to be better understood in terms of dairy processing and potential health impacts.

Schematic overview of the thesis structure



Chapter 1

The implications of future primary dairy production practices on the processing of milk into cheese and final cheese quality

1.1. Abstract

While primary dairy production systems are generally modified in response to environmental, policy, and market considerations, it must be appreciated that farming practices influence milk quality and composition, which can have significant implications for final product quality, functionality and overall consumer acceptability. Such issues are pertinent to cheese as a high value commodity dairy product. Therefore, this review explores the issues and challenges arising in this key product category from changes to primary production practices. Evidence suggests that strategies like crossbreeding, pasture diversity and altered milking frequency might be adopted without practical negative impacts on cheese quality. Studies have determined that some explicit changes to these primary production factors can be achieved without adverse variations in cheese composition, and without significant differences to ripening behaviour. However, some evidence gaps are also still notable. For example, research has resulted in large databases of candidate genes that might alter the expression of milk proteins, and this offers unrealised potential within milk-processing and cheese production. Additionally, novel dairy cow diets targeted towards making the dairy food system more sustainable still need to be investigated in terms of impacts to final products like cheese. Consequently, further research into alterations in product quality and functionality arising from on-farm changes, and appropriate due diligence, will still be warranted in the future.

1.2. Introduction

Within primary dairy production, strategic decisions are required to maximise production efficiency and sustainability while adapting to external factors such as climate change and the demands of global markets. These include, but are not limited to, the choice of cow breed, which production traits to select for, how to support herd fertility, how to manage disease and herd wellbeing, which feeding strategies to follow, how to manage farm operations and farm labour, the role of the farm as an ecosystem within the rural environment, the economic and market conditions for milk and milk products, and the many facets of regulation, policy and financial incentives. As new scientific evidence is disseminated, novel on-farm approaches come to prominence, and adaptations within primary production strategies on the farm may then need to be considered. Recently, a policy focus towards increased agricultural sustainability has been a driver of change at the farm-level (Amruddin *et al.*, 2024; Commission, 2024), and this is also supported by rising consumer demand for sustainability driven change (Peterson *et al.*, 2021; Matzembacher and Meira, 2019; Antonides, 2017; Schiano *et al.*, 2020; Vermeir and Verbeke, 2006). However, the implications of changes to production strategies beyond sustainability measures needs to be given consideration, in particular the impact these changes can have on the quality, processability and acceptability of milk and dairy products. This provides the focus of this review; the compositional and biochemical properties of milk and cheese as new farm practices are considered and implemented.

1.3. Cheese, milk quality and primary production

1.3.1. On the consideration of cheese

It is possible that the milk of the future might practically differ in composition and function from the milk of now due to changes within primary production practices. When the composition and function of milk is altered this can have implications for key dairy products, and this has particular relevance to cheese (Alothman *et al.*, 2019; Timlin *et al.*, 2023; O'Callaghan *et al.*, 2017; Page *et al.*, 2024). Cheese is an important dairy commodity product, with a third of all milk in Ireland utilised for cheese production and similar proportions also seen in other geographies such as France, Germany and the United Kingdom (Fox *et al.*, 2017). In 2025, the world produced about 23 million metric tons of cheese (USDA, 2026). Given the volume of cheese produced, changes in cheese characteristics can have far reaching impacts to public health (Feeney *et al.*, 2016; Lamichhane *et al.*, 2018), consumer satisfaction (Kilcawley *et al.*, 2007) and for dairy business (Politis and Ng-Kwai-Hang, 1988). When considering changes to a dairy food system, thought should be given to all stages of the production chain; from the soil (Ikoyi et al., 2023) and swards (Jezequel et al., 2024), the cows (Kennedy et al., 2021), resultant milk (Timlin et al., 2023), milk processing (Keogh et al., 1982), and final product quality (Kilcawley et al., 2018). The major focus of this review is whether milk and cheese quality can be sufficiently maintained, or even improved, when new on-farm approaches are applied. The impacts to cheese can also exemplify the issues faced more broadly within dairy production.

1.3.2. The biochemistry of milk and cheese when practices change on the farm

Primary production practices can influence the concentration of fat, protein and lactose in milk, alter the profile of fatty acids and amino acids, and change milk micronutrient levels (Kostovska *et al.*, 2025; Kelly *et al.*, 2025a; Keogh *et al.*, 1982; Timlin *et al.*, 2023; Alothman *et al.*, 2019). This has implications for cheese because the composition and function of milk entering into production impacts how that milk performs during processing, and influences the composition and attributes of the final product (Skeie, 2007; Lamichhane *et al.*, 2018). Within cheese manufacturing, milk coagulation properties (such as coagulation time, set-to-cut time, gel strength), syneresis, and fat and protein losses into whey are important processing considerations (Lamichhane *et al.*, 2018). Cheese is a complex dairy food matrix, it is made up of proteins, water and fats and possesses a complex micro-composition of vitamins and minerals. When milk chemistry is changed, the total amount of cheese yielded from a given amount of milk can be impacted, the macro- and micro-composition of the cheese can be altered (Page *et al.*, 2024; Page *et al.*, 2025a; Skeie, 2007; Emmons and Modler, 2010; Lamichhane *et al.*, 2018; O'Callaghan *et al.*, 2017). However, cheesemilk standardisation provides a practical processing response to farm-level associated variations in fat, protein and casein composition (Guinee et al., 2006). Standardising to a particular protein, or casein, to fat ratio can also improve vat-to-vat consistency when milk composition is altered (Guinee et al., 2006; Emmons and Modler, 2010).

Some cheese types also undergo a maturation period, and this also warrants consideration. Hard cheeses, such as Cheddar, undergo ripening periods, typically from three to twenty-four months (Fox & Cogan, 2004), and the final quality of ripened cheese is influenced by the biochemical processes that occur during maturation. Flavour and the formation of volatile organic compounds (VOC) and free fatty acids (FFA), colour, texture, and the overall sensory experience, can all be impacted by practices implemented at the farm (Kilcawley et al., 2018). Primary production decisions can flow through the entire production chain from the farm to consumer, from initial milk quality right through to the eventual sensory attributes and consumer acceptance of the product. For example, cheese texture development during ripening can be influenced by the concentration and profile of fats that are present (Kilcawley et al., 2018). Saturated fats are more solid at room temperature, while unsaturated fats are more fluid. The relative proportion of saturated and unsaturated fats found within a given cheese will have arisen from the raw milk, and this fatty acid profile can be influenced by practices implemented at the farm, such as the cows diet (O'Callaghan *et al.*, 2017). The specific profile of fats present in the cheese is then also subject to the extent of lipolysis that takes place during the ripening period, with implications for cheese texture and flavour (Frétin *et al.*, 2019). This is just one example of how cheese function can be impacted, with quality of the initial raw milk ultimately having implications for final sensory quality and consumer acceptance (Fox & Cogan, 2004).

1.3.3. Differing types of primary dairy production factors

A distinction is sometimes made within primary dairy production research between intrinsic and extrinsic factors. Intrinsic factors are inherent to the milk itself; in simplistic terms they can be thought of as the nature side within a simplified nature and nurture paradigm (Moore, 2013). Intrinsic factors include animal physiology and health, the breed of cow, genetics, stage-of-lactation and parity (Table 1.1). Extrinsic factors are those that have externally influenced the animal, and through that, the milk. Extrinsic factors include animal diet, farm hygiene, outdoor or confinement rearing, the milking system, the geographical location of the farm and seasonality (Table 1.2). While such categories can be helpful, these factors do not operate in isolation and interactions take place between them. However, as a method of providing structure within this review, different primary production approaches, and their associated potential impacts to milk and cheese quality, will be examined under these headings.

1.4. Intrinsic primary production factor impacts on milk and cheese

1.4.1. Animal physiology and health

Scientific research that supports dairy cow health can also have impacts on milk and cheese quality. A key animal health example of relevance to dairy product quality is mastitis risk, closely monitored on dairy farms through milk somatic cell count (SCC) (Schukken *et al.*, 2003). Differences in SCC levels represent differences in immune response, and this correlates with infections like mastitis (Schukken *et al.*, 2003). During

mastitis infections, the integrity of the blood milk barrier can be compromised, allowing immune cells, antibodies and blood derived proteins like serum albumin and immunoglobulins to transfer from the blood into the milk (Wellnitz and Bruckmaier, 2021). From a dairy product perspective, high SCC levels can impact on milk yields, milk composition, product yields and product quality (O'Brien *et al.*, 2001; Politis and Ng-Kwai-Hang, 1988). Elevated SCC levels increase the proteolytic and lipolytic enzyme activity of milk (Moradi *et al.*, 2021). The plasmin/plasminogen system can be activated, increasing milk proteolytic activity (Kelly *et al.*, 1998). Additionally, lysis of somatic cells results in numerous enzymes being released into milk, including lipases (such as, lipoprotein lipase), oxidases (such as, catalase and lactoperoxidase), glycosidases (such as, lysozyme), and proteases (such as, cathepsins, elastase, and collagenase) (Li *et al.*, 2014). Proteolytic enzymes break casein down into smaller peptides and free amino acids, resulting in significantly higher protein losses into the whey during cheese manufacturing, and a reduced ability to incorporate protein into the curd (Politis and Ng-Kwai-Hang, 1988). High SCC levels can significantly reduce casein content, but casein micelle stability is also impacted, and both lead to poor coagulation properties and reduced cheese yields (Olives *et al.*, 2015). Elevated SCC values also alter milk mineral content, and milk with high SCC values is associated with a lower phosphorus and potassium content and higher concentration of sodium (Summer *et al.*, 2009). High milk SCC values also impact on the sensory properties of cheese (Coulon *et al.*, 2004; Moradi *et al.*, 2021). Moradi *et al.* (2021) described how the increased proteolytic and lipolytic activity associated with high SCCs can result in

off-flavours in cheese; rancid flavours resulting from the lipolysis of fats, and bitter flavours derived from products of proteolysis. Indeed, increased proteolytic activity is also associated with increased plasmin activity (Kelly et al. 1998). Overall, when mastitis risk is appropriately managed on farms the milk produced has more consistent functionality within processing (O'Brien *et al.*, 2001), and allows for better control of final cheese quality (Moradi *et al.*, 2021). Within the EU, milk used for cheesemaking will be subject to a general raw cow's milk SCC standard of ≤400,000 cells/mL, (Regulation (EC) No 853/2004). However, this threshold should be interpreted as a regulatory minimum; milk below this value may still differ in processing performance (O'Brien et al., 2001; Kelly et al., 1998). Indeed, O'Brien *et al.* (2001) suggested that SCC values below 300,000 cells/mL do not negatively impact composition or processability of fresh milk, but did also note that milk below this threshold may still decline in quality when stored for longer than 72 h.

The appropriate management of animal health, as exemplified here through mastitis risk, provides a pertinent example of how innovation in support of animal health might also simultaneously support more consistent product quality (Cai *et al.*, 2024). When SCCs are lower, animals are healthier and dairy product quality is more likely to be assured. A further similar example is regarding parity. Higher-parity cows produce more milk than cows in their first lactation, but older animals can be more prone to health risks. Consequently, innovation in support of longer-term health for higher-parity cows might result in impacts for cheese quality. In particular, cow parity can impact milk coagulation properties (Bittante *et al.*, 2015). Bittante *et al.* (2015) found

that milk from primiparous cows had a higher curd firming rate and decreased milk protein content as the number of lactations increased. Consequently, if average herd parity increases, impacts to milk processing quality might result, and such changes would need to be managed by cheese manufacturers.

This section considered some aspects of dairy cow health and the associated impacts on cheese, but the health and resilience of dairy animals is also extensively influenced by breeding and genetics. Oltenacu and Broom (2010) described how animal health is supported by breeding practices., Cai *et al.* (2024) noted that the identification of candidate genes within breeding programmes can support healthier and more sustainable animal production. Breed and genetics also impact on production traits relevant to dairy product quality. These factors are considered within the next sections.

1.4.2. Dairy cow breeding practices and production traits

Global milk production has more than doubled over the past fifty years, and this has also coincided with a reduction in the number of cows required (Brito *et al.*, 2021). Modern-day dairy cows represent many generations of selective breeding, and the production traits observed within current herds are the combined results of historical and more recent breeding efforts (Scheu *et al.*, 2015; Young, 1784; Darwin, 1868). Darwin (1868) specifically discussed dairy cow inherited characteristics, noting how some breeds produce milk better suited to dairy products, while others excel in milk yield. Indeed, until recently, breeding approaches tended to align with what Darwin (1868) observed, focusing efforts towards breeds that excel in milk quantity. One milk

for all purposes was typical, with a focus towards increasing solids yields within specific breeds such as Holsteins (HO) and Holstein-Friesians (HF) (Evers *et al.*, 2021; Brito *et al.*, 2021). However, when giving consideration to future primary dairy production practices, the negative aspects of selective breeding become increasingly pertinent (Brito *et al.*, 2021; Fikse and Philipsson, 2007; Hansen, 2006). Genetic diversity within the global dairy cow population has reduced dramatically, fewer cow breeds are used and within-breed diversity has declined (Brito *et al.*, 2021). Intense selection of sires has resulted in increased inbreeding rates (Ducrocq and Wiggans, 2015). Inbreeding has brought fertility issues, increased stillbirth rates, susceptibility to mastitis and other diseases, modification of animal behaviours and declines in animal welfare (Fikse and Philipsson, 2007; Hansen, 2006). Additionally, Hansen (2006) noted how female fertility, calving ease, calf mortality, health, and survival have not been supported within HO and HF breeding selections until relatively recently. Typically, these animal related intrinsic factors have been managed by making changes to extrinsic factors; for example, with the living environment being adjusted to better support afflicted animals. However, under new constraints imposed by improved sustainability practices, this previously available latitude within dairy cow management might no longer be available. Future primary production scenarios will likely require improved levels of genetic diversity within herds so that animals are sufficiently adaptable and resilient to differing farming systems (Brito *et al.*, 2021).

Some farms are choosing to address inbreeding issues by continuing to use the same breed while implementing breeding practices that increase within-breed diversity

(Fikse and Philipsson, 2007; Brito *et al.*, 2021). Alternatively, the breed being used can be changed entirely, or increased genetic diversity can be introduced through crossbreeding programmes (Ducrocq, 2010). New Zealand has embraced crossbreeding as a solution, with a national herd population that is sixty percent Jersey-Holstein-Friesian (JFX) crossbred cows (DairyNZ, 2022). Coffey *et al.* (2016) found that crossbreeding resulted in superior animal performance and greater expected profitability compared to purebreds. However, Ireland has typically focused on breeding programmes for HF cows, and the economic breeding index (EBI) is the regularly applied metric when making dairy cow breeding decisions (Coffey *et al.*, 2016). Fikse and Philipsson (2007) and Brito *et al.* (2021) described the use of selection indexes that can refine breeding decisions. Increased genetic diversity directly supports more sustainable dairy systems, but selective breeding can also offer the opportunity to directly tackle some future challenges (Brito *et al.*, 2021; Nguyen *et al.*, 2023). Brito *et al.* (2021) described how greater environmental efficiency could be achieved by increasing the weighting of traits such as methane emissions and feed efficiency within breeding indexes. Nguyen *et al.* (2023) developed a specific selection index for Australian dairy cows to target emissions, and they concluded that this revised index could be incorporated into primary production with only minimal impacts on profit.

The future prospects for cheese quality must be considered against this backdrop of potential change within animal breeding practices. It has long been known that different dairy cow breeds produce milk with differing compositions, with implications

for dairy products (Darwin, 1868). New breeding practices within typical HF herds, crossbreeding, and increased farming of alternative breeds, could result in dairy herds with differing production traits to today. Benefits can arise when using specific breeds for selected production purposes; for example, substantial evidence is available regarding the use of Jersey milk within cheese manufacturing (Auldist *et al.*, 2004; Bland, 2015; Yoo *et al.*, 2019; Kristensen *et al.*, 2015). Auldist et al. (2004) increased cheese yields by ~10% when using JE milk in Cheddar manufacturing as compared to purebred Friesian milk, and this was attributed to JE milk's higher solids content. Similarly, Bland (2015) found that JE milk resulted in higher Cheddar cheese yields compared to HF milk, albeit when using non-standardised milks. Yoo et al. (2019) compared JE milk to HO milk within cheese manufacturing, and the JE derived cheese had increased micronutrient content and improved colour, taste, flavour and texture scores, particularly in ripened cheese. However, within Ireland the use of HF cows with an EBI focus, and in some circumstances crossbred JFX cows, are more typical approaches. Both systems have been shown to offer benefits when applied appropriately, such as increased milk production efficiency (Evers *et al.*, 2021; O'Sullivan *et al.*, 2019). For example, O'Sullivan *et al.* (2019) highlighted the success of the EBI in achieving the targeted production goals of higher fat and protein content milk. However, breeding decisions are not only made with production goals in mind, and within the EBI production traits represent only a third of the contribution to the metric. Contrasting this with crossbreeding approaches, the relative contribution of

hybridisation has been reported as 0-10% for milk production traits, as compared to 5-25% for fertility related traits (Coffey *et al.*, 2016).

A number of studies have investigated the cheesemaking potential of milks derived from differing approaches to dairy cow crossbreeding (Page *et al.*, 2025b; Sneddon, 2015; Saha *et al.*, 2020; Puppel *et al.*, 2018). Saha *et al.* (2020) investigated the cheesemaking potential of milks obtained from cows in a three-breed system that utilised Viking Red (VR), Montbéliarde (MO), and HO animals raised for producing Grana Padano and Parmigiano Reggiano cheese. Various crossbreeding combinations were investigated and, regardless of combination, it was found that HO cows produced significantly more milk per day than crossbreds. They also observed that milk obtained from crossbred cows had significantly higher protein and casein (CN) content, and lower lactose content. Milk from crossbred cows had similar rennet coagulation times compared as that of purebred HO cows, but significant differences in gelation properties were observed. Crossbred milk took less time to reach a curd firmness (k20) value of 20 mm, and had greater curd firmness at 30, 45, and 60 min from rennet addition; in addition, cheese yields were comparable between each of the investigated groups. Malchiodi *et al.* (2014) investigated milk obtained from the progeny of crosses of HO cows with bulls from Nordic and Alpine European breeds (Swedish Red, Montbéliarde, and Brown Swiss). Crossbred cows were found to produce lower milk volumes, with lower lactose content, higher fat and protein content, and a tendency for higher CN content. Milk derived from crossbred cows can have significantly faster curd-firming rates and increased curd firmness compared to that of HO cows. Puppel

et al. (2018) compared the production traits of crossbred progeny obtained from pairings of Polish HF cows with bulls of Norman, Norwegian Red, Danish Red, Brown Swiss, Montbéliarde, and Simmental breeds. The authors stated that crossbred cows were associated with a higher quantity and quality of milk. The milk of crossbred cows was also described by the authors as being *better* from a health perspective, because of lower saturated fatty acid levels compared to HF milk. The whey protein content of milk derived from crosses of Polish HF and Norwegian Red cows was ~19% higher than that of purebred HO cows. Considering these issues in relation to Cheddar cheese production, Page *et al.* (2025b) (Chapter 5) investigated a JFX crossbreeding strategy, as contrasted with an HF system. This Irish study did not observe evidence of adverse impacts to Cheddar quality arising from this crossbreeding approach and observed that cheese manufactured from JFX milk was significantly more yellow. Yellower cheese is generally favoured by consumers and can also be indicative of an increased vitamin A content (Page *et al.*, 2024; O'Callaghan *et al.*, 2017). A previous study conducted in New Zealand had suggested that significantly increased cheese yields might be achieved when using JFX milk within cheese production (Sneddon, 2015). However, the findings of Sneddon (2015) were based on predicted cheese yields, and this can be contrasted with the findings of Page *et al.* (2025b) who used JFX milk of a standardised protein to fat ratio in actual cheese manufacturing trials. Page *et al.* (2025b) found actual yield values were ~3% higher for JFX milk compared to HF, but these differences were not of sufficient magnitude to be statistically significant.

Crossbred cows typically produce lower milk volumes, but often with higher fat and protein concentrations. The lower milk volumes obtained from crossbred cows might create logistical challenges; processing is organised around the volume of milk supplied (Coffey et al., 2016). However, for cheesemaking, lower milk volumes are less problematic when milks solids yields are maintained, and the overall suitability of crossbred milk for processing into cheese is mostly a function of overall component recovery, casein content, and subsequent cheese yield, rather than milk volume in isolation (Emmons and Modler, 2010).

1.4.3. Genetic polymorphism and production traits

Going beyond broad breed categories, increased gene-to-phenotype knowledge of dairy animals has improved our understanding of the intrinsic biochemical mechanisms that mediate production traits, and this can further revolutionise how breeding decisions are made. The previous discussion centred on breed groups, and cows of the same breed often possess common traits, but breed categories only provide an over-simplified macroscopic descriptor. Common characteristics shared by animals of the same breed result from numerous genes transcribed and translated into proteins. Intron, exon and regulatory sequences held in common between animals of the same breed will result in observations of similar production traits between these animals, this being the output of similar genetics presenting phenotypically. However, within phenotypically defined breed groupings, individual animals do still differ in their underlying genes. Indeed, Page *et al.* (2025b) (Chapter 5) describes how broad breed

categories might by insufficient when focusing on specific production objectives, noting that specific genetic markers can offer a more targeted approach. The following discussion considers production trait specific gene sequences, how they influence milk function and processability, and the associated differences that can result in cheese attributes.

Genes code proteins, and it is the protein fraction of milk that is most likely to vary due to an animal possessing a different version of a particular gene. However, individual proteins operate as part of a complex system, and how these proteins interact within this overall wider system also leads to much further complexity. Arguably, the proteins most relevant to dairy production are the six main ruminant milk proteins. These are the two whey proteins, α-lactalbumin and β-lactoglobulin, and the four CN proteins, namely α_{s1}, α_{s2}, β- and κ-CN (Farrell *et al.*, 2004). Each of these proteins have various alleles, also termed genetic variants, and it is this polymorphism that leads to many of the differences observed within the protein fraction of milk. These differences have implications for processing and relevance for final products like cheese (Gai *et al.*, 2024; Daniloski *et al.*, 2024; Kelly *et al.*, 2025b). However, before moving on to discuss the impacts of these specific genetic variants in greater detail, it is worth considering how much additional complexity exists. One area of additional complexity is regarding the rate of expression of milk protein genes. Rates of expression can vary through additional polymorphism within the intron and exon sequences of the transcription unit, through polymorphism within regulatory elements and mutations causing premature stop codons that result in decreased levels of transcripts (Martin *et al.*,

2002). Indeed, Martin *et al.* (2002) stated that this additional polymorphism might impact the functional attributes of milk with potential impacts to cheese yields and cheese organoleptic characteristics. Such considerations do still, however, remain under-explored in the literature. The situation is not as simple as just the six major milk proteins, and many researchers have contributed to databases of additional candidate genes and genetic markers that could have relevance to milk production (Ogorevc *et al.*, 2009; Križanac *et al.*, 2025; Cai *et al.*, 2024). Vast amounts of genetic data have been obtained, particularly over the last twenty to thirty years, and while progress has been made, much genetic data have yet to be translated into functional knowledge or practical, meaningful applications (van den Berg *et al.*, 2020; Wiggans *et al.*, 2017; Križanac *et al.*, 2025). Notably, Križanac *et al.* (2025) recognised that knowledge on the causal relationships between traits of economic importance and their genetic background is still scarce; a better understanding of this still needs to be developed. Within dairy research, some notable gene-function knowledge gaps include the impact of genetic variants on the allergenic properties of milk, and the genetics of minor serum milk proteins such as immunoglobulins, blood serum albumin and lactoferrin (Kelly *et al.*, 2025b). Essentially, many research areas within this domain are not yet sufficiently developed to be of practical relevance to on-farm primary production decisions. However, this wider discussion is still worth noting. In time, this additional complexity might be untangled and might be a source of improved outcomes for future generations of dairy farmers.

Considering this discussion in terms of what is possible now, and in the not-so-distant future, opportunities for improved process efficiency, enhanced sustainability and better product quality might already be possible within cheese manufacturing by making targeted selections of alleles within the six major bovine milk proteins. Around 80% of bovine milk protein is CN protein, and this includes α_{s1}-CN (38%), β-CN (36%), κ-CN (13%), and α_{s2}-CN (10%) (Davies and Law, 1980). Genetic polymorphism results in differing amino acid sequences within these proteins, and differences in phosphorylation and glycosylation (O'Regan *et al.*, 2009). This can result in changes to hydrophilicity and metal binding, and such changes can impact milk protein behaviour during gelation, and this can have implications for cheese manufacturing (O'Regan *et al.*, 2009). The α_{s1}-CN protein has a role within micelle structure; the protein has amphiphilic properties that facilitate interaction with other CNs and calcium (O'Regan *et al.*, 2009). This protein has multiple identified variants, but in bovine milk the polymorphism is limited to two, categorised as A and B (Farrell *et al.*, 2004). The B variant has been associated with higher overall protein and CN content in milk (Farrell *et al.*, 2004). Milk obtained from cows possessing the B variant tends to have better coagulation properties and typically results in higher cheese yields, while softer curd has been associated with the A variant (Creamer *et al.*, 1982; Farrell *et al.*, 2004). The α_{s2}-CN protein has a role in micelle stability and calcium binding (O'Regan *et al.*, 2009), which suggests a possibility of impacts on the cheesemaking process, but other studies have described αs2-CN as having only a limited influence on cheesemaking (Cipolat-Gotet et al., 2018). Indeed, while various genetic variants of αs2-CN have been

identified, commercial bovine breeds typically only possess the same A variant (Farrell *et al.*, 2004). The β-CN variants have been associated with impacts to milk digestibility and cheese texture (Gai *et al.*, 2024). Multiple variants have been identified, but two bovine variants are typical and are known as A_1 and A_2 (Farrell *et al.*, 2004). In particular, A_2 milk has been associated with easier digestion and benefits for people with milk intolerances. Daniloski *et al.* (2024) described how there was less protein breakdown during *in vitro* gastric digestion of A_2A_2 milk compared to A_1A_1 and A_1A_2 variants. Milk obtained from A_2A_2 cows has also been found to have poorer rennet coagulation properties (Gai *et al.*, 2024; Daniloski *et al.*, 2024). Daniloski *et al.* (2024) noted that the time required for curd cutting in cheese manufacturing was delayed for milk derived from A_2A_2 cows compared to milk obtained from A_1A_1 and A_1A_2 variants, Gai *et al.* (2024) reported that A_1A_1 derived cheese was the softest, and that A_2A_2 cheese was the most fracturable. Daniloski *et al.* (2024) reported that A_2A_2 derived cheese had higher levels of hardness, fracturability, and cohesiveness as compared to A_1A_2 and A_1A_1 variants. The κ-CN protein has a role in stabilising CN micelles and is important for successful milk-coagulation, curd formation and moisture retention in cheese (Farrell *et al.*, 2004; O'Regan *et al.*, 2009; Fox and Brodkorb, 2008). In dairy cows it is typically found in two common variants known as A and B, and is less frequently found as a third E variant (Farrell *et al.*, 2004). The B variant of κ-CN is strongly linked to improved milk coagulation properties, greater curd firmness and higher cheese yields; it has been associated with higher levels of casein in milk, smaller casein micelle size, and more efficient chymosin cleavage (Guggisberg *et al.*, 2024; Walsh *et al.*, 1998;

Lavon *et al.*, 2023). Conversely, variant A has more moderate coagulation properties, and the rarer E variant is associated with poor coagulation properties, lower cheese quality and lower cheese yields (Guggisberg *et al.*, 2024; Farrell *et al.*, 2004; Lavon *et al.*, 2023). Moisture retention in cheese can also be indirectly influenced by κ-CN variant through impacts on casein micelle stability, rennet gel formation and gel permeability, because these in-turn impact on syneresis and whey expulsion during manufacture (Walsh *et al.,* 1999). Regarding the two major whey proteins, β-lactoglobulin and α-lactalbumin, they have limited relevance to cheese because they are largely lost into the whey during manufacturing (Farrell *et al.*, 2004). However, β-lactoglobulin has a role in cheese texture, moisture-retention, and flavour in whey cheeses such as ricotta (Jelen, 2011), and the two typical bovine variants are named A and B (Farrell *et al.*, 2004). Regarding α-lactalbumin, this has been noted as providing nutritional value to whey and contributes to whey protein functionality, but limited functional differences have been recognised between the two typical A and B variants (Nicoleta and Rapeanu, 2010; Korhonen, 2011). A further discussion on major milk protein genetic variants is provided by Kelly *et al.* (2025b), and the authors concluded that appropriate selection of genetic variants could be used to create more nutritious milk, and could bring benefits to the production of various dairy products including cheese.

Current levels of understanding within dairy cow genetics can already offer opportunities to make more targeted breeding choices on the farm. As knowledge develops, further opportunities will likely materialise to offer even more chances to

enhance dairy production efficiency and product quality. However, it should be noted that a key potential barrier to this has been recognised; farmers might not choose to make investments in improved milk properties through appropriate genetic selections if the payments received for that milk do not also acknowledge those enhanced properties (Cole *et al.*, 2023). Presently, the main incentives for dairy farmers are milk volume and the total protein and fat yielded (Geary et al., 2010), so incentives might need to be structured differently in the future if the full benefits of future genetic selections that support product quality are to be realised.

1.4.4. Summary of intrinsic factor impacts

Table 1.1: The various impacts of changes within intrinsic primary production practices on milk and cheese

Factor	References	Evidence observed in milk and cheese associated with the primary production factor
Health and somatic cell count (SCC)	Schukken et al., 2003; O'Brien et al., 2001; Politis & Ng-Kwai-Hang, 1988; Coulon et al., 2004; Moradi et al., 2021	Increased SCC levels can have implications for milk yield, milk composition, product yields and product quality. Mastitis can result in negative impacts on cheese sensory properties. SCCs can act as an indicator of increased proteolytic and lipolytic activity, which can result in off-flavours. Elevated SCCs are associated with higher protein losses into whey and lower total solids in cheese.
Parity	Bittante et al., 2015	Cow parity can impact milk coagulation properties. Milk from primiparous cows had a higher curd firming rate and decreased milk protein content as the number of lactations increased.
Selective breeding	Brito et al., 2021; Fikse & Philipsson, 2007; Hansen, 2006	Genetic diversity within the global dairy cow population has reduced dramatically. Inbreeding has brought fertility issues, increased stillbirth rates, susceptibility to mastitis and other diseases, modification of animal behaviours and declines in animal welfare.

Crossbreeding	Coffey et al., 2016; Malchiodi et al., 2014; Puppel et al., 2018; Saha et al., 2020; Page et al., 2025b; Sneddon, 2015	Milk obtained from crossbred cows had significantly higher protein and casein content, and lower lactose content. Crossbred milk showed differences in gelation properties and greater curd firmness. Crossbred cows produce lower milk volumes but higher milk solids. Cheese yields were comparable between crossbreds and non-crossbreds in some studies and differed in others. Some crossbreeds have milk with higher whey protein content and lower saturated fatty acid levels. Cheese from JFX crossbred milk was more yellow, had higher β-carotene content, and no adverse impacts in quality compared to HF cheese.
α_{s1}-CN	Creamer et al., 1982; Farrell et al., 2004	The B variant has been associated with higher protein and CN content in milk. Milk from cows of the B variant tends to have better coagulation properties and result in higher cheese yields. Softer curd has been associated with the A variant.
β-CN	Farrell et al., 2004; Gai et al., 2024; Daniloski et al., 2024	A_2 milk has been associated with easier digestion. Milk obtained from A_2A_2 cows has poorer rennet coagulation properties. The set-to-cut time was slower for A_2A_2 milk. A_1A_1 derived cheese was the softest, and A_2A_2 cheese was the most fracturable, A_2A_2 derived cheese had higher levels of hardness, fracturability, and cohesiveness.

κ-CN	Farrell et al., 2004; Walsh et al., 1998; Lavon et al., 2023; Guggisberg et al., 2024	The B variant of κ-CN is linked to improved milk coagulation properties, greater curd firmness and higher cheese yields. Variant A has more moderate coagulation properties. Variant E is rarer, and is associated with poor coagulation properties, lower cheese quality and lower cheese yields.
β-lactoglobulin	Farrell et al., 2004; Jelen, 2011	Has a role in cheese texture, moisture-retention, and flavour in whey cheeses. The two typical bovine variants are A and B.
α-lactalbumin	Nicoleta & Rapeanu, 2010; Korhonen, 2011	Provides nutritional value to whey and contributes to whey protein functionality. Limited functional differences have been recognised between the two typical A and B variants.

1.5. Extrinsic primary production factor impacts on milk and cheese

1.5.1 The implications of dairy cow diets

Good dairy cow nutrition is essential to support and maintain animal health, maximise milk-yield, milk-quality and milk-processing attributes, factors essential for successful cheese production. Decisions on which diet to use within a primary production system are typically associated with the geographical location of the farm. Temperate regions tend to utilise pasture grazing within seasonal production systems, while non-temperate locations tend to utilise indoor confinement. Changing dairy cow diets can impact both the macro- and micro composition of milk, with implications for final dairy

product quality (Alothman *et al.*, 2019). Alothman *et al.* (2019) acknowledged that many factors contribute to milk composition, but recognised diet as a key mechanism for altering the nutrition of milk and its processing attributes. For example, considering micro-composition, Dunshea *et al.* (2019) described how feeding practices can be used to reduce variation in milk mineral levels. Confinement systems can be argued to offer greater control over milk composition compared to pasture systems (White *et al.*, 2002), and this consistency can be beneficial within dairy processing and cheese production. However, pasture-systems benefit from lower feed costs, lower labour costs, higher fertility rates, lower culling rates and easier forage management (White *et al.*, 2002). Pasture feeding has been associated with better milk nutrition (Alothman *et al.*, 2019), and pasture derived milk products have been described as having improved quality relative to non-pasture derived milks (Elgersma, 2015; Alothman *et al.*, 2019; Timlin *et al.*, 2023; O'Callaghan *et al.*, 2017). The modified rumen lipid metabolism resulting from diets of fresh forage have been associated with nutritionally favourable milk fatty acid profiles (Elgersma, 2015; Alothman et al., 2019). Fresh herbage can also increase the transfer of carotenoids and tocopherols into milk fat, contributing to the colour, nutritional profile and sensory distinctiveness of pasture-derived dairy products (Nozière et al., 2006). Given this reviews focus on cheese, it is noteworthy that O'Callaghan *et al.* (2017) described higher levels of beneficial fatty acids in Cheddar manufactured from grass-fed milk; demonstrating that the nutritional gains achieved in milk through pasture grazing are transferred to cheese.

It seems likely that grazing practices should continue to be favoured in the future, especially under a paradigm of increasing sustainability. Alothman *et al.* (2019) noted that the nutritional profile of "*Grass-Fed"* dairy resonates well with consumers and their desire for sustainable dairy products. However, despite the benefits of grazing, confinement systems can still end up being favoured in certain circumstances (Arnott *et al.*, 2015). Arnott *et al.* (2015) stated that confinement will be chosen over pasture-grazing when it is necessary to meet the nutritional needs of high-yielding cows, because of herd size exceeding grazing capacity, due to fragmented land on farms, and the desire to utilise robotic milking systems. Indeed, it seems likely that the scenarios described by Arnott *et al.* (2015) will have relevance within future primary production systems; confinement will still be favoured over pasture in many circumstances. A distinction should also be recognised between grass-fed and outdoor grazing. For example, *'Zero-Grazing'* systems are an alternative approach whereby grass is harvested and fed to housed cows, and *"partial confinement systems"*, that combine confinement and grazing, also provide a further alternative approach (Arnott *et al.*, 2015). These systems offer some of the nutritional benefits associated with milk derived from a pasture diet within a confinement system. Furthermore, it must also be recognised that current approaches to pasture grazing are associated with some challenging environmental consequences. Monoculture systems are typical in Ireland, and tend to utilise perennial ryegrass (PRG), and such systems reduce biodiversity and require regular replenishment of lost nitrogen. The fertilisers used to replace lost nitrogen can result in soil-acidification, nutrient run-off, eutrophication of waterways,

and increased emissions (Walling and Vaneeckhaute, 2022). Establishing pastures where cows graze on multiple plant species simultaneously provides a potential solution. Referred to in the literature under the term multispecies swards (MSS), such pastures have been found to result in soil with a greater diversity of ecologically relevant indicator species (Ikoyi *et al.*, 2023), and can increase biodiversity by acting as important habitats (Beye *et al.*, 2022; Cooledge, 2022). Improved soil health and soil structure, and enhanced soil-carbon sequestration are associated with the growth habits and root structures of MSS (Cooledge, 2022; Buzhdygan *et al.*, 2020; Isbell *et al.*, 2017). Leguminous species within MSS replenish lost soil-nitrogen, and have been associated with reduced nitrogen-leaching (Pijlman, 2024; Walling and Vaneeckhaute, 2022). Biodiverse grazing systems such as MSS might be increasingly utilised under a paradigm of enhanced environmental sustainability. As changing dairy cow diets is known to impact milk and final product quality (Elgersma, 2015; Alothman *et al.*, 2019; O'Callaghan *et al.*, 2017; Timlin *et al.*, 2023), consideration needs to be given to how this dietary change might impact milk and cheese. Until recently, investigations into this had been limited in scope, focusing on factors such as milk-yield and macro-composition (Bryant *et al.*, 2017; McCarthy *et al.*, 2023; Minneé *et al.*, 2017). Bryant *et al.* (2017) observed that MSS grazing did not compromise milk-yield. Minneé *et al.* (2017) noted that including chicory and plantain in pasture had little impact on milk-composition, and milk-yield and total-solids (TS) yields also remained similar. McCarthy *et al.* (2023) described significantly higher milk-yields and TS in MSS derived milk. A recent study by Kostovska *et al.* (2024) compared milks derived from PRG and

MSS systems over an entire lactation, and found significant impacts arising from cow diet; the MSS diet increased total monthly yields of fat, TS and protein. Furthermore, Kostovska *et al.* (2024) noted that MSS milk might have implications for dairy processing and product quality. Milk fatty acid profile was impacted by diet, cow breed, and stage of lactation within this study; an MSS diet resulted in higher levels of polyunsaturated fatty acids (PUFA), whereas milk derived from a PRG diet was higher in trans fatty acids and branched chain fatty acids. Such changes in fatty acids were noted as potentially having implications for milk-processing. However, a more recent study by Page *et al.* (2025a) determined that the macro-composition and fatty acid profiles of MSS and PRG milks were broadly equivalent. The MSS-derived milk did have significantly lower SCC levels compared to PRG-derived milks, and significantly higher protein content. Within this study, MSS and PRG milks were used to manufacture Cheddar cheese, and significantly increased cheese yields resulted when using MSS milk. Interestingly, these yield difference remained significant even when adjustment was made for the differing moisture, fat and protein contents of the milks. This suggests the possibility that MSS-derived milk might have an increased economic value within cheese manufacturing. Furthermore, no significant differences in rennet coagulation properties were observed, and the MSS-derived cheese had a macro-composition that was typical for Cheddar cheese.

Outdoor systems are often generally perceived as being beneficial compared to confinement, but one noted area where evidence is conflicted is regarding greenhouse gas emissions (GHG) (Arnott *et al.*, 2015). Reviewing the literature, Arnott *et al.* (2015)

described how the carbon footprints of grazed and confinement systems can be similar. It was noted that some studies found similar GHG levels resulted from grazed and confinement systems, others reported higher emissions from confinement, and one study found higher carbon emissions resulted from grazing. However, despite such discrepancies, a general consensus has been reached on GHG within dairy more generally that regardless of the system applied, emissions arising from dairy production are too high with adverse impacts for environmental sustainability. Strategies based on changing dairy cow diets have been suggested to be an effective approach within the ruminant breeding sector to reduce GHG levels (Marin *et al.*, 2020). While such dietary changes might be supported on sustainability grounds, it must also be recognised that this dietary change might have implications for milk and cheese quality. Research is lacking in this area at this time, so this should become an area of consideration for future investigators. However, referring back to the previous discussion on MSS, MSS systems have already been recognised as being associated with reduced emissions (Pijlman, 2024). Giving consideration to the work of Page *et al.* (2025a) and Kostovska *et al.* (2024) it appears that a change towards increased use of MSS grazing might be achieved without adverse impacts for milk and cheese quality, and might even bring some benefits.

1.5.2. Management practices in support of dairy cow health

Dairy cow health supports product quality, and both result from good management practices. Animal welfare has been defined as the avoidance of undue suffering, and optimisation of health and vigour, through the provision of fair and reasonable

conditions (Jago *et al.*, 2000). Management practices can impact on consumer acceptance of dairy products (Cui *et al.*, 2021; Oltenacu and Broom, 2010). Suboptimal animal welfare has even been found to detrimentally impact consumer perceptions of product quality regardless of any actual measured quality impacts (Oltenacu and Broom, 2010; Jago *et al.*, 2000). Poor management practices, such as inappropriate confinement and improper handling, can directly impact on the physical and psychological wellbeing of dairy cows (Belay, 2024; Linstädt *et al.*, 2024). Belay (2024) stated that housing design, quality of bedding, and appropriate access to resources like feed, water and space are important priorities when assuring dairy cow welfare. Linstädt *et al.* (2024) determined that pasture-based systems can support improved welfare because they result in reduced dairy cow lameness and improved animal behaviour. Regarding the impacts of this on milk and milk products, Jago *et al.* (2000) noted better dairy product quality, based on both nutritional and organoleptic criteria, when good animal welfare conditions were maintained. Poor management practices result in physiological changes within dairy cows that also influence milk quality. Animal stress increases animal maintenance requirements, and this results in reductions in the synthesis of milk components (Razzaghi *et al.*, 2023). Synthesis of milk fat depends on availability of dietary substrates for lipid synthesis, ruminal fermentation and adipose tissue stores and these are altered under stress conditions with implications for milk composition and milk yields (Razzaghi *et al.*, 2023). In-turn, these stress related changes in milk can impact on cheese yields and quality. Boonkum *et al.* (2024) found that heat stress significantly reduced milk yields and altered the

ratio of milk fat-to-protein. In a study on Grana Padano cheese, Bernabucci *et al.* (2015) found that dairy cows subjected to heat stress had milk with an altered protein profile and poorer milk coagulation behaviour. In a study that investigated stress resistance in Ukrainian red dairy cows, Chernenko *et al.* (2021) found that dairy cows with low resistance to stress produced milk unsuitable for rennet cheese production because of poor coagulation properties and a bitter taste and rubbery texture within the final cheese.

Earlier within this review it was noted that potential advances in primary production practices that support dairy cow health might also offer chances to maximise dairy farm productivity and product quality (Cai *et al.*, 2024). Animal health is supported by good management practices and consequently relates back to this previous discussion. Indeed, primary production factors do not typically operate in isolation. Good dairy cow wellbeing results from the combined impacts of intrinsic animal factors and their interaction with correct management practices on the farm. Likewise, the final quality of milk and cheese is also a function of these interactions between intrinsic and extrinsic factors within this complex system.

1.5.3. Milking equipment and technology

That product quality and animal welfare go hand-in-hand is also seen through choices of milking equipment. Suitable milking equipment assists in the efficient removal of milk, stimulates further milk production, improves animal hygiene and facilitates udder health management (Zigo *et al.*, 2021). Well maintained milking equipment, with

suitable cleaning regimes, facilitate lower levels of microbial contamination, preventing compromises within milk safety and improved milk quality (Kathiriya *et al.*, 2025). A further pertinent consideration for primary production decision makers is whether to adopt automatic milking systems (AMS) (Bhoj *et al.*, 2022). The use of AMS can reduce labour requirements, improve feed conversion to milk, enhance milk quality through lower SCCs, improve cow productivity and provide data in support of farm management (Simões Filho *et al.*, 2020). The use of AMS can also support animal welfare through robotic processes such as automatic teat-cleaning and milking cup attachment which can result in improved udder health (Bhoj *et al.*, 2022). However, Simões Filho *et al.* (2020) also highlighted limitations of AMS including high investment costs, unwanted changes in milk composition (including increased somatic cell counts, reduced fat content and elevated free fatty acids) and increased risk of ketosis in cows. However, in a Canadian survey of farms utilising AMS, milk producers reported increased milk yields with limited impact on milk quality (Tse *et al.*, 2018). Tse *et al.* (2018) reported a mean daily milk yield per cow from AMS farms of 32.6 kg, with a median milk fat of 4.0% and median milk protein of 3.3%, and median bulk tank somatic cell count levels of 180,000 cells/ml. Such compositions would be suitable, and typical, for use within cheese production.

1.5.4. Milking frequency

Milking-frequency provides a key example of an extrinsic management practice that can also be considered holistically and in tandem with the other aspects of the dairy primary production system (Jayawardana *et al.*, 2023). Most dairy farms in Ireland

practice twice-a-day (TAD) milking, but alternative milking frequencies can be used. Intensive systems might milk as much as 4 times a day (Stelwagen *et al.*, 2013), but pasture-based systems might reduce milking frequency to just once-a-day (OAD) (Lopez-Villalobos *et al.*, 2023; Stelwagen *et al.*, 2013). An explanation of why TAD milking is generally favoured again links back to how incentives are structured; dairy farm profitability is linked to the volume of milk solids produced (Beukes *et al.*, 2005; Armstrong and Ho, 2009), and OAD systems typically result in lower milk solids yields than TAD (Carruthers *et al.*, 1993; O'Brien *et al.*, 2009; Murphy *et al.*, 2023). During a three year experiment Murphy *et al.* (2023) found that OAD milking reduced the overall cumulative milk yield by 26%, and milk solids yields by 21%, compared to cows milked TAD. However, OAD milking has also been found to reduce milk solids yields by as much as 40% in early-lactation (Rémond *et al.*, 1999), or by as little as 7% in late-lactation (Stelwagen *et al.*, 1994).

Despite milk solids yield reductions, OAD strategies are now increasingly coming into focus because of other benefits (Page *et al.*, 2024; Page, 2024; Murphy *et al.*, 2023). Reduced milking time is associated with OAD strategies, and this can facilitate improved farmer wellbeing, opportunities for better work-life balance and increased job satisfaction (Tipples and Verwoerd, 2007; Ferris *et al.*, 2008; Page, 2024; Kennedy *et al.*, 2021). Labour savings can be realised (Armstrong and Ho, 2009), along with more conventional working hours for employees (Edwards, 2020). The practice can also create additional time for alternative business activities (Armstrong and Ho, 2009). The practice of OAD milking has also been associated with positive animal health impacts;

fertility and reproductive health have been reported as being positively impacted by OAD milking (Dalley *et al.*, 2007; O'Brien *et al.*, 2009) and animals milked OAD have shown better body condition scores (Sanjayaranj *et al.*, 2022), but somatic cell counts do tend to be higher for OAD milked animals (O'Brien *et al.*, 2009; Page *et al.*, 2024). The extent to which a change in milking frequency has an impact will also depend on interactions with other primary production factors. Stelwagen *et al.* (2013) described how the extent of impact on milk resulting from a change in milking frequency also depends on the interaction of the milking frequency strategy with other management practices (extrinsic factors) and animal breeding, genetics and animal physiology (intrinsic factors).

Considering further the impact of changes in milking frequency practice on milk quality, it has already been mentioned that OAD milking typically increases the fat and protein content of milk (Rémond *et al.*, 2004; O'Brien *et al.*, 2009). Changes in milk composition associated with reduced milking frequency practices have the potential to impact on dairy product quality (Stelwagen et al. 2013). Such changes might even potentially benefit cheesemaking, especially given that OAD practices have also been associated with increased CN content in milk (Rémond *et al.*, 2004). However, negative impacts to coagulation time and curd firmness have been demonstrated in some research (Pomiès et al. 2007). While other research has reported that coagulation properties appear unaffected (Page *et al.*, 2024; O'Brien *et al.*, 2002). As mentioned previously, higher SCCs are associated with reduced milking frequencies (O'Brien *et al.*, 2009; Page *et al.*, 2024). Therefore, the general potential negative impacts of increased

SCCs on product quality do need to be noted (as discussed earlier within this review). However, regarding a specific study into SCC levels in OAD milk, proteolytic activity was shown to be higher in this milk, with the activity linked to activation of the plasmin/plasminogen system (Kelly et al. 1998). Page *et al.* (2024) also noted significantly higher SCC levels in OAD milk as compared to TAD milk but found no meaningful experimentally determined adverse quality impacts between OAD and TAD cheese. Furthermore, Page *et al.* (2024) noted that OAD milking resulted in significantly higher cheese yields, with cheese that was significantly more yellow and this was associated with a significantly increased β-carotene content. Such findings have relevance to farmers considering reduced milking frequency practices due to the aforementioned benefits, to processors considering the use of OAD milk to produce cheese, and to consumers through the sensory appearance and nutritional value of the food (Page, 2024).

1.5.5. Summary of extrinsic factor impacts

Table 1.2: The various impacts of changes within extrinsic primary production practices on milk and cheese

Primary Production Factor	References	Evidence observed in milk and cheese associated with the primary production factor
Diet – Pasture and non-pasture	Alothman et al., 2019; Elgersma, 2015; O'Callaghan et al., 2017; Timlin et al., 2023	Pasture derived milk and milk products have been described as having improved quality relative to non-pasture derived milks. Improved fatty-acid profiles have been observed. Cheddar manufactured from grass-fed milk had higher levels of beneficial fatty acids.
Diet - Multispecies swards (MSS)	Kostovska et al., 2024; Page et al., 2025a	MSS-derived milk had significantly lower SCC levels, significantly higher protein content and significantly increased cheese yields. No significant differences in gelation properties were observed. Cheese made from MSS-derived milk had a macro-composition typical for Cheddar cheese.
Welfare	Jago et al., 2000; Oltenacu & Broom, 2010	Suboptimal animal welfare has been found to detrimentally impact consumer perceptions of product quality, regardless of any actual measured quality impacts. However, better dairy product quality is noted when good animal welfare conditions are maintained.
Stress	Bernabucci et al., 2015; Chernenko et al., 2021; Boonkum et al., 2024; Razzaghi et al., 2023	Heat stress significantly reduced milk yields and altered the ratio of milk fat-to-protein. Dairy cows subjected to heat stress had milk with an altered protein profile and poorer

		milk coagulation behaviour. In some research cows with low resistance to stress produced milk unsuitable for rennet cheese production with bitter taste and rubbery texture.
Equipment	Zigo et al., 2021; Kathiriya et al., 2025	Well maintained milking equipment facilitates lower levels of microbial contamination, preventing compromises within milk safety and improved milk quality. Suitable milking equipment improves animal hygiene and facilitates udder health management.
Automated milking systems (AMS)	Simões Filho et al., 2020; Bhoj et al., 2022; Tse et al., 2018	The use of AMS can reduce labour requirements, improve feed conversion to milk, enhance milk quality through lower SCCs, improve cow productivity and provide data in support of farm management. Limitations include high investment costs and unwanted changes in milk composition. However, in farm surveys, AMS farms reported increased milk yields with limited impacts on milk quality.
Milking frequency - Once-a-day (OAD)	Rémond et al., 2004; O'Brien et al., 2009; Page et al., 2024	OAD milking can offer benefits for some farms through reduced milking times and labour savings. The practice reduces overall solids yields but typically increases the fat and protein content of milk, and has been associated with increased CN content. Higher SCCs are associated with OAD milk. Cheese yields can be significantly higher, cheese is significantly more yellow, with higher β-carotene content. significantly more yellow, with higher β-carotene content.

1.6. Conclusions

The evidence-base suggests that farming practices will change in the future. The extent of change is debatable but change occurring is probably indisputable. Consideration was given to cheese, and this complex food matrix also exemplified issues relevant to dairy processing, nutrition and consumer acceptability more broadly. Numerous interactions take place between primary production factors, and a key theme has been the complex system that results. Some farm-level changes can be implemented without adverse impacts on cheese, but complacency must be avoided. It is affirmed that investigations need to continue, and some evidence gaps have been highlighted. This includes, converting genetic data into useful functional understanding, and gaining improved knowledge on milk quality impacts arising from novel cow diets. Findings need to be disseminated effectively, and it is anticipated that this review can support strategic decision making as future challenges are confronted.

Change within the dairy industry has in-part resulted from policy and business conditions, particularly regarding sustainability driven change. This review has highlighted opportunities for dairy food systems; examples such as improved genetic selections, welfare focused herd management and cow diets that align with environmental objectives. It is asserted that dairy product quality and nutrition should be front and centre within decision making, and that policymakers and dairy business have a responsibility. Incentives should be aligned, preferably targeted, towards achieving positive future outcomes for dairy products, proactively supporting product quality, public health and the commercial opportunities offered to many economies.

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Chapter 2

The impacts of milking frequency on nutrient composition and functional characteristics of Cheddar cheese

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2.1. Abstract

This study investigated the effects of once-a-day (OAD) and twice-a-day (TAD) milking strategies on Cheddar cheese properties, within an Irish pasture-based system. Cheese derived from OAD milk was more yellow, with significantly higher b* values and a significantly higher β-carotene content. A significantly higher cheese yield also resulted from OAD milk. Compositional data were largely consistent with previously reported results, and no meaningful differences were found in texture, proteolysis, pH, volatile organic compounds, and free-fatty-acid profile. Indications are that OAD milking does not negatively impact Cheddar quality, providing valuable insights for those considering milking frequency impacts upon this key dairy product.

2.2. Introduction

The majority of dairy farms in Ireland practice twice-a-day (TAD) milking, but, alternative milking frequencies can sometimes better meet the objectives of a farming business. In intensive and automated dairy systems, milkings can be 3 or 4 times daily with extremes of 5 or 6 milkings a day (Stelwagen *et al.*, 2013). Conversely, reduced milking frequencies are used in regions applying low-input pasture-based systems, examples include once-a-day (OAD) milking and three milkings in two days (Lopez-Villalobos *et al.*, 2023; Stelwagen *et al.*, 2013). In New Zealand, 10% of farmers practice OAD milking for the whole lactation, 55% apply TAD milking and 35% apply mixed systems (Lopez-Villalobos *et al.*, 2023). In comparison, approximately 200 dairy farms

in Ireland are operating full-time OAD milking, about 1% of Irish farms, but, this is expected to increase (Teagasc, 2021). A small number of TAD Irish farms utilise OAD strategies as a management tool for shorter periods within the milking season, such as during the early and late periods of lactation (Kennedy *et al.*, 2021). Typically, OAD systems result in lower milk yield and milk solids yield than TAD (Carruthers *et al.*, 1993; O'Brien *et al.*, 2009). With OAD systems, milk solids yield can be reduced by up to 40% in early lactation (Rémond *et al.*, 1999), or, by as little as 7% in late lactation (Stelwagen *et al.*, 1994). The impact of OAD milking varies across the milking season, but, the overall association of OAD milking with lower milk solids helps to explain why TAD milking is generally favoured, dairy farm profitability is closely linked to the volume of milk solids produced (Beukes *et al.*, 2005; Armstrong and Ho, 2009).

Typically, a OAD milking strategy will result in reduced milking income, but the approach provides specific benefits that can sometimes offset these lost earnings. On a case study farm in Ireland, OAD milking reduced profits by €34,000 in the first year and six years later the profit reduction was €18,000 per year, importantly working hours were also reduced by 2.5 hours/day for 250 days of the year (Ramsbottom *et al.*, 2018). Other research has similarly reported reduced milking times of as much as 35% with OAD milking systems (Kennedy *et al.*, 2021). Consequently, OAD strategies are associated with improved farmer wellbeing, opportunities for better work-life balance and increased job satisfaction (Tipples and Verwoerd, 2007; Ferris *et al.*, 2008). Financially successful OAD systems utilise labour related savings effectively (Armstrong and Ho, 2009) and time savings can provide more conventional working hours for

employees (Edwards, 2020). Furthermore, the time savings associated with OAD milking can allow additional business activity that could generate greater profits than the second milking (Armstrong and Ho, 2009). It is also important to consider milking frequency impacts upon animal wellbeing. Somatic cell counts tend to be higher for OAD milked animals, and this is particularly observed at late lactation (O'Brien *et al.*, 2009). It has also been reported that OAD milked cows suffer impaired locomotion (Gleeson *et al.*, 2007). Positive animal health impacts have also been observed with OAD practises. Fertility and reproductive health have been reported as being positively impacted by OAD milking (Dalley *et al.*, 2007; O'Brien *et al.*, 2009) and animals milked OAD have shown better body condition scores (Sanjayaranj *et al.*, 2022).

Applying a reduced milking frequency is known to impact milk composition (Stelwagen *et al.*, 2013; Lopez-Villalobos *et al.*, 2023). The greatest known impact is on fat and protein contents, and these have repeatedly been shown to increase in percentage terms with OAD strategies (Rémond *et al.*, 2004; Boyle *et al.*, 2005; O'Brien *et al.*, 2009), but, not sufficiently to compensate for, and be explained by, the lower overall milk volume (Clark *et al.*, 2006). Some investigations have suggested that fat and protein profiles are not altered with milking frequency (Bobe *et al.*, 2007), but, other research has found lower saturated fatty acids within milk obtained from cows milked OAD (Sanjayaranj *et al.*, 2022). Such compositional changes can impact dairy product quality (Stelwagen *et al.*, 2013). For example, lower levels of free fatty acids have been reported in OAD milk which could be favourable to butter and cream producers (Pomiès *et al.*, 2007). Some investigations have demonstrated increases in the total casein content of

milk obtained from cows milked OAD (Rémond *et al.*, 2004), and this could be regarded as beneficial for cheese making. Proteolytic activity has been shown to be higher in milk from cows milked OAD in late lactation and was linked to increased SCC and activation of the plasmin/plasminogen system (Kelly *et al.*, 1998). Also, less desirable whey protein to casein ratios have been observed in OAD milk (Rémond *et al.*, 2004; Davis *et al.*, 1999). Negative impacts to rennet coagulation time and curd firmness have also been demonstrated (Pomiès *et al.*, 2007), but other research has reported these rennet coagulation properties as being unchanged (O'Brien *et al.*, 2002).

Many studies have considered the effects of milking frequency on milk, with a focus on variables such as milk yield, milk composition and milk coagulation properties. Whilst some investigations have also been carried out on the effects of milking frequency on dairy products, few have focused directly on overall cheese quality. Particularly, within Irish pasture-based seasonal systems. Increasing the evidence base in this area could provide additional confidence to farmers and manufacturers considering OAD strategies. Cheese is an important dairy product, around a third of Irish milk is used for cheese production (Fox *et al.*, 2017), and changes in cheese properties can impact upon the dairy industry. As a concentrated dairy matrix, nutritional changes can also bring health consequences. It is within this context that this study aimed to investigate the effects of milking frequency upon the nutritional, compositional, yield and quality characteristics of Cheddar cheese produced from an Irish dairy production system.

2.3. Materials and methods

2.3.1 Milking systems and milk collection

The milk used in this study was supplied by the Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark. The work utilised late-lactation milk collected on two occasions in November 2021. Cows were grazed in two herds, one herd milked OAD and the other TAD. The treatment groups comprised of Holstein-Friesian (HF) and Holstein-Friesian × Jersey (JFX) crossbred cows, reflecting a typical structure of Irish pasture-based herds, where HF cows and JFX cows can both be commercially relevant (Coffey et al., 2016). Each treatment group comprised of 20 cows randomised for age, breed, yield and parity. The OAD cows were milked daily at 6.30 am and TAD cows at 6.30 am and 4.30 pm. Both herds were adjacent to one another, separated using a temporary electric fence to ensure diets of similar quality were offered. Pre-grazing yield was similar between treatments 1407 ± 324.4 kg (DM/ha >4cm). Sward composition was similar between treatments, 28.9 % ± 1.20% (% crude protein), while the dry matter digestibility was 81.2 % ± 1.55 % and water-soluble carbohydrates were 11.6 % ± 1.86 %. The cows were offered 16 kg dry matter per day of a grazed pasture (*Lolium perenne* L.; <80%) diet. The TAD group received 1 kg of concentrate at each milking (overall 2 kg/d) and OAD animals received 2 kg of concentrate during milking.

This study was completed at late lactation due to logistical considerations (including, the availability of researchers and the availability of cows of the appropriate treatment groups to provide milk for cheese production). It is acknowledged that a full lactation

study could provide further insights. However, this late lactation approach does also have particular scientific relevance to Irish pasture-based systems because OAD milking is sometimes used as a short-term seasonal management strategy during this period (Kennedy et al., 2021). Further details of the management system can be found in Kennedy *et al.* (2021), and the milk utilised within this study was obtained from this same group of cows according to the same milking system. Raw pooled milks derived from the OAD and TAD treatment groups were collected from separate stirred bulk tanks between 8:30 AM and 9:30 AM on the same day. This raw milk was then stored at 4°C, ready for the preparation of the milk for cheese production. For simplicity, throughout this study, cows following the OAD and TAD milking regimens described above will be referred to as OAD cows or TAD cows, respectively. Likewise, the milk obtained from each of these treatment groups will be referred to as OAD milk or TAD milk, respectively.

2.3.2. Cheese manufacture

A portion of the collected raw milk was separated into cream and skim milk at 50°C using a benchtop centrifuge (Armfield FT15 Disc Bowl Centrifuge**,** Armfield Limited, Hampshire, UK). Milk from each milking system was standardised to a protein-to-fat ratio of 0.95 using skim milk, raw whole milk or cream, pasteurised at 72°C for 15 s, and stored overnight at 4°C. Throughout this study, standardised and pasteurised milk will be referred to as cheesemilk. Cheese manufactured from OAD milk or TAD milk (as defined previously) will be referred to as OAD cheese or TAD cheese, respectively. On the day following pasteurisation, Cheddar cheese was manufactured at the

Biofunctional Food Engineering Facility, Teagasc Food Research Centre, Moorepark, Fermoy, Co Cork, Ireland as described by Xia et al. (2020). Briefly, cheeses were made utilising pilot-scale production vats (10 L; Pierre Guerin Technologies, Mauze, France). For each of the milking treatments 12 kg of cheesemilk was used. The milks were heated to 32°C using a re-circulating water bath (Grant Y28; Grant Instruments Ltd., Cambridge, UK). The pH of the cheese milk was adjusted to 6.55 with a 4% (v/v) lactic acid solution. After pH adjustment, a frozen starter culture comprising *Lactococcus lactis subsp. cremoris* and *Lactococcus lactis subsp. lactis* was immediately added to the cheesemilk (2.2 g per vat; R604, Chr. Hansen Ireland Ltd., Co. Cork, Ireland). After a 20 min incubation period, coagulant was added to each vat (0.18 mL per kg of milk, diluted 1:10 prior to addition). Fermentation-produced calf chymosin was used (CHY-MAX, 200 international milk clotting units (IMCU) mL^{-1}; Chr. Hansen Ireland Ltd., Co. Cork, Ireland). All gels were cut at a constant firmness (G′) value of 35 Pa (as measured using a small-amplitude oscillatory rheometer, Anton Paar, Graz, Austria). Further details are provided in the below section on rennet coagulation properties. Curds were cooked to 38°C at a rate of 0.25°C/min. Curds were drained at pH 6.15, milled at pH 5.35, salted at 2.7% (w/w), mellowed for 20 min, moulded and pressed overnight. The cheese was then vacuum packed and ripened at 8°C for 6 months (180 d). Cheesemaking trials were completed in duplicate.

2.3.3. Rennet coagulation properties

Samples were taken to determine the coagulation properties of OAD and TAD cheesemilks according to the method described by Lamichhane *et al.* (2018a). Briefly,

milk was removed from cheese vats 3 min after rennet addition and stirring. Samples were then placed into the cell of a small amplitude oscillatory rheometer (AR 2000ex, TA Instruments), utilising a concentric-cylinder measuring geometry. The dynamic changes in rheological properties during the coagulation process were monitored using a dynamic time sweep analysis with an angular frequency of 1.0 Hz, and a strain of 0.01 at 32°C, within the linear viscoelastic region (strain <0.03) reported for rennet milk gels. Data was recorded for analysis and the time from rennet addition to reaching a G′ value of 35 Pa was used as the point to cut gels within the cheese manufacturing process. Rennet coagulation time (RCT) was calculated by measuring the time taken from rennet addition until a storage modulus of 1 Pa was reached. The maximum curd firming rate (CFR_{max}) was determined as the maximum slope of the curve plotted for G′/time.

2.3.4. Composition of raw milk, cheesemilk, whey and cheese

The cheesemilk and bulk whey samples were analysed for total solids using the oven drying method (IDF, 2010), (bulk whey defined as the wheys collected during whey drainage and curd cheddaring (Guinee *et al.*, 2006)). Fat, protein and lactose contents in raw milk were determined within the milk standardisation process through a milk analyser using FTIR spectroscopy (Bentley Instruments DairySpec FT, Bentley Instruments Inc, PO BOX 150, Chaska, Minnesota, USA). Fat and total protein contents in cheesemilk and whey were determined with the Rose-Gottlieb method (IDF, 1987) and Kjeldahl method (IDF, 2014), respectively. A nitrogen to milk protein conversion factor of 6.38 was used. Non-protein nitrogen (NPN) and non-casein nitrogen (NCN)

were determined as described by Xia *et al.* (2020). Somatic cell counts were measured (SCC, cells/mL) using a fluoro-opto-electronic counter (Fossomatic FC, Foss, Hillerød, Denmark).

The concentration of Ca, Fe, K, Mg, Mn, Na, P and Zn were determined in milk, bulk whey and cheese using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES; Agilent 5110, Santa Clara, California, USA). The determination of mineral content was carried out using standard International Dairy Federation (IDF) methodology as described in IDF (ISO/IDF229:2018, 2018)/Agilent application note 15151 (Agilent, 2023). Samples were digested using a microwave digestion system (CEM Mars 6, CEM Corporation, Matthews, North Carolina, USA) as described by Deshwal *et al.* (2023).

Grated cheese samples were analysed in duplicate at 20 d of ripening for moisture, fat and protein as described by Xia *et al.* (2020). The β-carotene content was determined by Eurofins Food Testing Ireland Ltd (Dublin, Ireland) utilising a Thermo Fisher Ultimate HPLC including Diode Array Detector (LC-DAD).

2.3.5. Cheese yield

Actual cheese yield (Ya), moisture-adjusted cheese yield (Yma), and moisture-adjusted cheese yield with fat and protein adjusted to reference levels (Ymafpam) were calculated as described by Guinee et al. (2006). The reference moisture for determination of moisture-adjusted cheese yield (Yma) was 38.5% (w/w).

2.3.6. Analysis of volatiles and free fatty acids

The volatile compounds in cheese were determined at 180 d of ripening using gas-chromatography–mass-spectrometry (GC-MS) as described by Lamichhane *et al.* (2018a). The free fatty acid profile of cheese samples was determined according to the methods of De Jong and Badings (1990) with modifications as outlined in Mannion *et al.* (2016).

2.3.7. Texture profile analysis

Analysis was performed using a Stable Micro Systems, TA.XTplus Texture Analyser (Goldalming, Surrey, UK) equipped with a 70 mm (diameter) compression plate and a 50 kg load cell. Three cube-shaped cheese samples were removed from the centre of each cheese type with a wire cutter, tightly wrapped in aluminium foil and brought to a stable temperature of 4°C, similar to as described by O'Callaghan *et al.* (2017). More specifically, the cheese were allowed to equilibriate for at least five hours, sufficient time to make certain that the internal cheese temperature had dropped from around 8°C to 4°C. This analysis was repeated at 4, 20, 60, 100 and 180 d over-ripening. The texture parameters were determined using Texture Expert Exceed software.

2.3.8. Colour analysis

Colorimetric analysis was performed according to the Commission Internationale de l'Eclairage, or, CIELAB colorspace approach; this enabled quantification of lightness to darkness (L*) green to red (a*) and blue to yellow (b*) colour differences.

Measurements were made on the internal surfaces of the cheese with freshly cut samples taken at 4, 20, 60, 100 and 180 d of ripening.

2.3.9. pH and proteolysis

The pH was measured in cheese samples at 4, 20, 60, 100 and 180 d of ripening. A cheese slurry was prepared with 20 g of fresh ground cheese and 12g of de-ionised water. The pH was recorded using a bench pH meter (Mettler Toledo SevenCompact™ pH meter S210, Mettler-Toledo Ltd. 64 Boston Road, Beaumont Leys, Leicester, UK). To measure the relative extent of casein degradation between OAD and TAD cheeses, the levels of nitrogen soluble (expressed as % of total nitrogen) at pH 4.6 (pH 4.6-SN/TN) were measured after 4, 20, 60, 100 and 180 d as described by Fenelon and Guinee (2000).

2.3.10. Statistical analysis

All statistical analysis of data was performed using Microsoft Excel 64 bit, 2016 Version with Analysis ToolPak (Microsoft Corporation, Redmond, Washington, USA). When comparing for statistically significant differences in single factors a two-Sample T-Test was carried out for the comparison of means. Repeated measures ANOVA was used to assess for statistical significance in data related to changes in cheese during the ripening period. For all statistical analyses, P values < 0.05 were considered significant.

2.4. Results and discussion

2.4.1. Raw milk and cheesemilk composition

The raw milk and cheesemilk compositions are shown in Table 2.1. The OAD milks had numerically higher levels of protein (total and true protein), fat and total solids compared to TAD milks, and TAD milks exhibited higher levels of lactose, but these differences were not statistically significant ($P > 0.05$). Consequently, these results show no significant effects on milk composition resulting from the milking frequency treatment ($P > 0.05$). However, this study was completed in duplicate, and it is acknowledged that further replicates may possibly have uncovered significant effects not observed here; absence of evidence is not evidence of absence. Likewise, no significant effects of milking frequency were observed for NPN, casein content and casein number ($P > 0.05$). The relatively higher (albeit not significantly higher) protein, fat and total solids contents observed in OAD milk in this study are consistent with previous findings and may be due to concentration effects that result from the overall reduced milk volume (Stelwagen *et al.*, 2013).

The OAD cheesemilk had significantly higher levels of calcium, magnesium and sodium compared to TAD cheesemilk ($P < 0.05$) (Table 2.1). Increased levels of magnesium and calcium in milk have been linked to higher protein content (Gulati *et al.*, 2018), with large proportions of calcium and magnesium being associated with the casein micelle. The protein content, and casein content and casein number, in OAD milk were also higher within this work compared to TAD, albeit not significantly ($P > 0.05$). The TAD

cheesemilk had significantly higher levels of potassium ($P < 0.05$). Previous work has also described decreases in potassium concentration within OAD milk, and this has been explained as having resulted from increased permeability of mammary tight junctions. These changes cause an increase in serum proteins in the milk and a decrease in lactose and potassium (Davis *et al.*, 1999). Higher Na in OAD milk could reflect increased mammary epithelial permeability and may also be associated with elevated SCC values, both reflect impaired mammary barrier integrity (Harmon, 1994). Regarding all other minerals tested in cheesemilk (iron, manganese, phosphorous and zinc), no further significant differences were observed ($P > 0.05$).

Overall, the values obtained here for mineral content in milk are comparable to that already reported in the literature, but it should be noted that all values are at the higher end of the range of previously reported values. This study was conducted during late lactation, and it is important to consider these results within that context, the late stage of lactation is likely to have had an impact. Gulati *et al.* (2018) explained how higher mineral content values are generally reported at the end of the milking season, and how others have also previously observed these effects (Keogh *et al.*, 1982; O'Brien *et al.*, 1999). However, it is observed here within this work that OAD milking results in additional significant increases in magnesium, calcium and sodium as compared to TAD ($P < 0.05$), increases that are resulting over and above the seasonal impact.

The OAD milk had significantly higher Somatic Cell Counts (SCC) than TAD milk ($P < 0.05$) (Table 2.1). It is noteworthy that SCC levels were high across all samples. The

findings of this research support evidence obtained within other work, that SCC levels tend to be higher for OAD milk and also tend to be higher late in the milking season, both can often be associated with higher incidence of mastitis (O'Brien *et al.*, 2009), an important consideration for those thinking of adopting OAD strategies.

Table 2.1: Raw milk and standardised cheesemilk composition under different milking frequency treatments of once-a-day (OAD) and twice-a-day (TAD) (n=2)

	OAD Milk	TAD Milk	P-Value
Raw Milk Composition			
Total Protein (% wt/wt)	4.46 ± 0.11	4.17 ± 0.03	NS
Fat (% wt/wt)	5.40 ± 0.06	5.26 ± 0.11	NS
Lactose (% wt/wt)	4.41 ± 0.01	4.49 ± 0.09	NS
Cheesemilk Composition			
Total Protein (% wt/wt)	4.55 ± 0.09	4.28 ± 0.04	NS
True Protein (% wt/wt)	4.33 ± 0.08	4.07 ± 0.02	NS
Fat (% wt/wt)	4.76 ± 0.11	4.48 ± 0.02	NS
Lactose (% wt/wt)	4.45 ± 0.02	4.54 ± 0.09	NS
Total Solids (%)	14.44 ± 0.47	12.76 ± 1.48	NS
Non-Protein Nitrogen (%wt/wt)	4.74 ± 0.22	4.81 ± 0.46	NS
Casein Number (% wt/wt)	77.09 ± 2.49	77.65 ± 2.32	NS
Casein Content (% wt/wt)	3.50 ± 0.18	3.32 ± 0.13	NS
Ca (mg/kg)	1476 ± 6	1393 ± 11	*
Fe (mg/kg)	0.415 ± 0.02	0.375 ± 0.05	NS
K (mg/kg)	1195 ± 21	1391 ± 9	*
Mg (mg/kg)	143 ± 2	133 ± 3	*
Mn (mg/kg)	0.05 ± 0.01	0.04 ± 0	NS
Na (mg/kg)	552 ± 22	417 ± 11	*
P (mg/kg)	1054 ± 9	1049 ± 6.00	NS
Zn (mg/kg)	4.76 ± 0.17	5.19 ± 0.47	NS
SCC (cells/mL)(x1000)	494 ± 91	203 ± 21	*

*$P < 0.05$, $^{NS}P > 0.05$

Data are presented as mean ± standard deviation

2.4.2. Rheology of coagulated cheesemilks

Some differences in rheological properties were observed between OAD and TAD coagulated cheesemilks (Figure 2.1). The set-to-cut time for OAD milk was on average 51.5 ± 3.67 min, compared to a slower 62.5 ± 4.45 min for TAD milk. The rennet coagulation time (RCT) was on average 22 ± 0.5 min for OAD milk, compared to 24 ± 0.33 min for TAD milk. The maximum curd firming rate was 1.5 Pa/min for OAD and 1.05 Pa/min for TAD. The average 2 min difference in RCT between treatment groups suggests that milking frequency had only a limited effect on the primary stage of coagulation, the enzymatic cleavage of kappa-casein. However, the secondary stage of rennet coagulation, the aggregation of para-casein, does seem to be impacted in a more meaningful way. The set-to-cut time was on average 11 min faster for OAD milk compared to TAD milk. It is possible that this faster set-to-cut time might offer a commercial advantage through improved cheese processing efficiency, and this might be of interest to the cheese making industry. However, it should be noted that converse to the findings within this work, it has previously been reported that rennet coagulation time and curd firmness are negatively impacted by OAD strategies, and this was explained by differences in casein content between OAD and TAD milk (Pomiès *et al.*, 2007). Other research has reported coagulation properties as being unaffected by milking frequency, and here casein contents were reported as being similar between OAD and TAD milks (O'Brien *et al.*, 2002). Regarding this study, the casein content was found to be higher in OAD milk, albeit not significantly ($P > 0.05$), but, the amount of calcium and magnesium, minerals associated with the casein micelle, were significantly

higher in OAD milk (P <0.05). Differences in casein content and the minerals associated with casein, might suggest a possible explanation for the rheology of OAD milk.

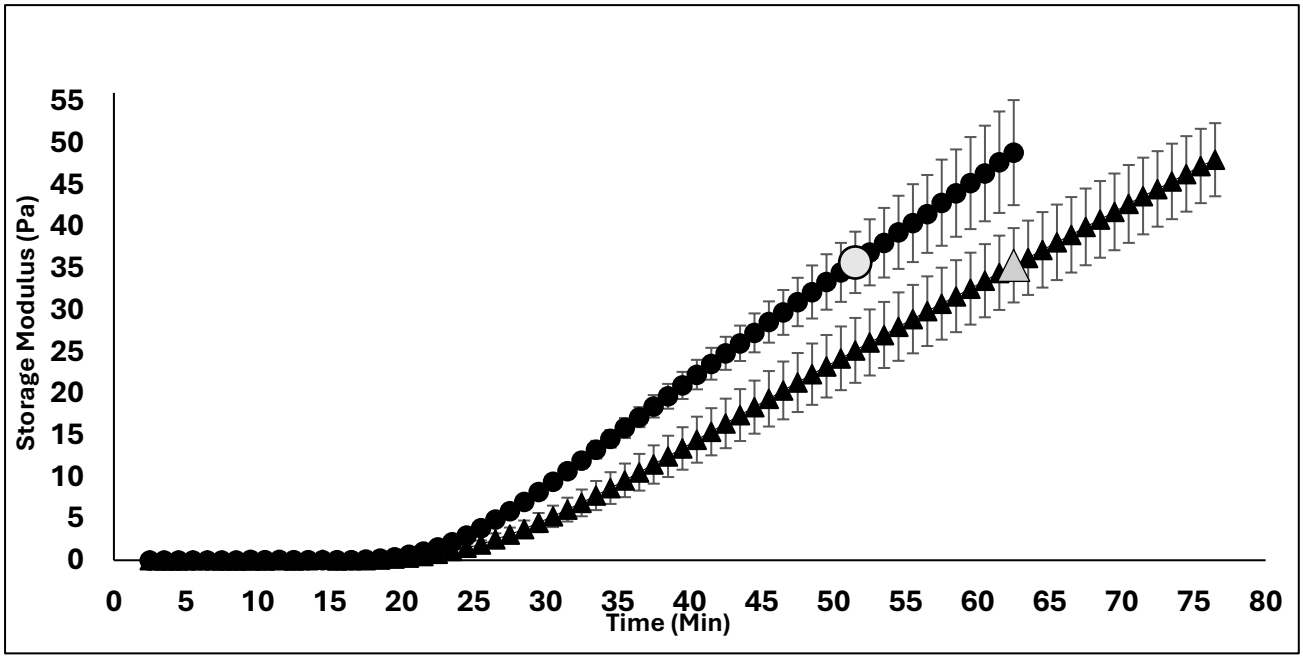


Fig. 2.1. Effect of once-a-day (OAD, ●) and twice-a-day (TAD, ▲) milking on the storage modulus of the rennet-treated milk; the bigger symbols indicate the value of storage modulus at cut. Presented values are the means from 2 replicate cheese trials; error bars show standard deviations of the means.

2.4.3. Whey composition

No significant differences in protein, fat, total solids and mineral content were observed in whey in response to the OAD and TAD treatments (P > 0.05) (Table 2.2).

Table 2.2: Mean composition of bulk whey (± standard deviation) under milking frequency treatments of once-a-day (OAD) and twice-a-day (TAD) (n=2)

	OAD	TAD	P-Value
Protein (% wt/wt)	1.31 ± 0.03	1.17 ± 0.01	NS
Fat (% wt/wt)	0.37 ± 0.02	0.35 ± 0.01	NS
Total Solids (%)	7.07 ± 0.11	7.08 ± 0.05	NS
Ca (mg/kg)	479 ± 1	495 ± 84	NS
Fe (mg/kg)	0.27 ± 0.02	0.33 ± 0.11	NS
K (mg/kg)	1295 ± 4	1687 ± 165	NS
Mg (mg/kg)	111 ± 2	115 ± 16	NS
Mn (mg/kg)	0.01 ± 0	0.005 ± 0.007	NS
Na (mg/kg)	627 ± 57	547 ± 51	NS
P (mg/kg)	406 ± 3	484 ± 64	NS
Zn (mg/kg)	0.38 ± 0.05	0.31 ± 0.05	NS

$^{NS}P > 0.05$

2.4.4. Cheese composition

The compositional results for OAD and TAD cheese (Table 2.3) are broadly consistent with that which has previously been reported for standard full-fat Cheddar cheese (Feeney *et al.*, 2021). No significant effects of milking frequency were observed on protein, fat, moisture, fat-in-dry matter (FDM) and moisture in non-fat substance (MNFS) ($P > 0.05$). However, TAD cheeses had significantly higher salt levels and salt-in-moisture contents than OAD cheeses ($P < 0.05$). Although the specific reasons for such observations are unknown, the levels are within the range typical of cheddar cheese (Feeney *et al.*, 2021). It is possible that this result is due to differences in curd microstructures before salting. The cheese curd microstructure was not measured

before salting, but differences in the gelation behaviour of cheesemilk can give rise to variances in curd microstructure, providing a possible explanation (Lamichhane *et al.*, 2018b). Such differences would lead to varying rates of salt diffusion within the curd during the salting stage. In addition, the manual salting technique used within the manufacturing process could also account for this observed variation.

On average, the OAD treatment resulted in 4.1% higher cheese yield than TAD. The actual yield (Y_a) and moisture-adjusted cheese yield (Y_{ma}) were significantly ($P < 0.05$) higher in OAD cheese than TAD. However, there is no significant effect of milking frequency on moisture-adjusted cheese yield adjusted for protein and fat (Y_n) ($P > 0.05$), suggesting that the higher actual yield and moisture-adjusted yield is due to the higher protein and fat content in OAD cheesemilk. Putting these results into a real-world context, the higher cheese yield associated with OAD could benefit the cheese industry, as processors could obtain more cheese from a given volume of cheesemilk. If these increased cheese yield attributes are appropriately compensated for through financial incentives, this might offset some of OAD farms lost revenue from the removal of the second milking. However, cheese yield is just one factor amongst a complex set of variables determining which milking frequency strategy to adopt. Overall, the results suggest a possibility of increased economic value for OAD cheesemilk, but, further investigation would be necessary to determine whether commercial utilisation of these characteristics is actually possible.

There was no significant effect of treatment on the mineral composition of cheese, except for sodium and potassium (Table 2.3); TAD cheese had significantly higher levels of sodium and potassium than OAD cheese ($P < 0.05$). The potassium content of TAD cheese was on average 14% higher than in OAD cheese. Changes in potassium levels in milk as a result of milking frequency have been explained as being the result of changes in mammary permeability (Davis *et al.*, 1999). It appears that these consequences might also be translated from the milk into cheese, the TAD cheesemilk also had significantly higher levels of potassium ($P < 0.05$). Regarding sodium, these differences should be taken within the context that salt is added to Cheddar as part of the manufacturing process, the salt content is not just a function of the milk composition. Also, the result for sodium in cheese is contrary to the mineral observations made in cheesemilk, in cheesemilk sodium levels in TAD cheese were significantly lower ($P < 0.05$). The minerals in cheese as compared to minerals in milk were contrary to each other in a number of instances. The OAD cheese had lower levels of calcium and magnesium than TAD cheese (albeit not significantly ($P > 0.05$)) whilst OAD cheesemilk had significantly higher levels of calcium and magnesium ($P < 0.05$). Regarding calcium, iron, manganese, magnesium, phosphorous and zinc, no significant differences were observed in response to milking frequency ($P > 0.05$).

Applying a OAD milking frequency significantly impacted the β-carotene content in cheese ($P < 0.01$) (Table 2.3). The OAD cheese within these trials had an average increase in β-carotene content of 31% compared to TAD cheese. β-carotene is a provitamin, as a plant pigment/carotenoid that the body converts to Vitamin A the

real-world impacts of these results should be considered through the importance of Vitamin A to health. Within an Irish context, the European Food Safety Authority (EFSA) makes evidence-based decisions on which health claims for foods are authorised, information is available through the 'EU register of nutrition and health claims made on foods'. With reference to this resource, approved health claims for Vitamin A (and β-carotene) include it supporting the metabolism of iron, maintenance of normal skin and mucous membranes, maintenance of normal vision and maintenance of the normal function of the immune system (EFSA Panel on Dietetic Products & Allergies, 2010), Commission Regulation (EU) 432/2012 of 16/05/2012. Cheese is a widely consumed food, and so OAD cheese having an increased pro-vitamin A content might have positive impacts for the above areas of health. However, it must be recognised that it is not possible to make this health claim for OAD cheese without conducting additional work.

Table 2.3: Mean composition of cheeses (± standard deviation) under different milking frequency treatment types of once-a-day (OAD) and twice-a-day (TAD) (n=2)

	OAD	TAD	P-Value
Protein (% wt/wt)	27.77 ± 0.15	26.13 ± 0.61	NS
Fat (% wt/wt)	34.32 ± 0.56	33.96 ± 0.26	NS
Moisture (% wt/wt)	35.49 ± 0.66	35.51 ± 0.63	NS
FDM (% wt/wt)	53.19 ± 0.32	52.65 ± 0.11	NS
MNFS (% wt/wt)	54.03 ± 0.54	53.76 ± 0.74	NS
Salt (% wt/wt)	1.58 ± 0.04	1.77 ± 0.02	*
S/M (% wt/wt)	4.47 ± 0.21	5.00 ± 0.51	*
Actual Yield (Ya)	13.16 ± 0.38	12.62 ± 0.31	*
MACY (Yma)	13.87 ± 0.18	13.15 ± 0.12	*
MACYPFAM (Yn)	9.99 ± 0.02	10.07 ± 0.07	NS
Beta Carotene (mg/kg)	3.81 ± 0.15	2.91 ± 0.05	*
Calcium (mg/kg)	8067 ± 343	8326 ± 247	NS
Phosphorous (mg/kg)	5262 ± 213	5450 ± 87	NS
Iron (mg/kg)	2.97 ± 0.47	2.165 ± 0.45	NS
Potassium (mg/kg)	754 ± 87	858 ± 74	*
Magnesium (mg/kg)	379 ± 21	388 ± 11	NS
Manganese (mg/kg)	0.335 ± 0.005	0.325 ± 0.035	NS
Sodium (mg/kg)	5869 ± 315	6394 ± 349	*
Zinc (mg/kg)	36.59 ± 1.13	38.34 ± 2.16	NS

***P < 0.05, ^{NS}P > 0.05**

Abbreviations: FDM, Fat in Dry Matter, MNFS; Moisture in Non-Fat Substance, S/M; Salt in Moisture, MACY; Moisture Adjusted Cheese Yield, MACYPFAM; Moisture Adjusted Cheese Yield Adjusted for Protein and Fat

2.4.5. pH

No significant differences in pH values were observed in response to the experimental treatment at any of the ripening time points (P > 0.05) (Table 2.4). The pH values are in the range of 5.10-5.23, which is typical of Cheddar cheese. Overall, the pH of cheese increased slightly as it ripened, albeit not significantly (P > 0.05). Change in pH with

ripening time is to be expected and is likely due to a number of factors, which include solubilisation of calcium phosphate associated with the paracasein during ripening, the proteolytic liberation of ammonia, free basic amino acids and amines (Lamichhane et al., 2018a).

Table 2.4: Mean pH of cheeses over ripening for the different milking frequency treatments (± standard deviation); Once-A-Day (OAD), Twice-A-Day (TAD) (n=2)

Days	OAD	TAD	P-VALUE
4	5.10 ± 0.08	5.14 ± 0.10	NS
20	5.15 ± 0.05	5.14 ± 0.09	NS
60	5.19 ± 0.01	5.17 ± 0.07	NS
100	5.22 ± 0.00	5.17 ± 0.07	NS
180	5.23 ± 0.02	5.18 ± 0.07	NS

$^{NS}P > 0.05$

2.4.6. Free fatty acid profile

No significant differences in free fatty acid (FFA) profile were observed between OAD and TAD cheese at 180 d of ripening. This result provides an interesting comparison with work already completed on FFAs and milking frequency, (Sanjayaranj *et al.*, 2022). Most notably, Sanjayranj (2022) observed differences in saturated fatty acid and *de novo* synthesised fatty acids between OAD and TAD, but, it is essential to recognise

that animal diet was not controlled within that work; indeed animal diet has already been shown to impact FFA profile in dairy products (O'Callaghan *et al.*, 2017). Within the experimental design for this research OAD and TAD animals were all grazed on perennial ryegrass pastures and offered diets of similar quality to control this potentially confounding variable (Kennedy *et al.*, 2021). It is therefore interesting that under a controlled diet no significant differences in total fatty acids (TFAs) were observed within OAD and TAD cheese, different to that described by Sanjayaranj (2022). Comparing these two pieces of work suggests the possibility that previously made observations about differences in FFAs in response to milking frequency could actually be attributable to dietary differences.

2.4.7. Texture

Milking frequency did not have a significant impact on the instrumentally obtained values for hardness, fracturability, chewiness, springiness, cohesiveness and resilience of cheese ($P>0.05$) (Table 2.5). Small differences were observed in fracturability and hardness during the early ripening period, but, they were not statistically significant and were no longer apparent as ripening progressed further. Interpretation of texture data early in the ripening period should be approached with caution. Salt equilibration may not be complete early within ripening, and texture values may reflect localised salt and moisture gradients, with cheese on average becoming more homogenous with time. However, the hardness, fracturability, chewiness, cohesiveness and resilience values of both OAD and TAD cheese decreased significantly over ripening ($P < 0.05$). This can be attributed to ripening-related changes within cheese matrices, such as

proteolysis and solubilisation of colloidal calcium. Similar results have been reported previously in other studies (Lamichhane et al., 2018a; O'Callaghan et al., 2017).

Table 2.5: Texture profile attributes of cheeses over ripening when applying different milking frequencies (± standard deviation) of once-a-day (OAD) and twice-a-day (TAD) (n=2)

	4 d		20 d		60 d		100 d		180 d				
	OAD	**TAD**	**OAD**	**TAD**	**OAD**	**TAD**	**OAD**	**TAD**	**OAD**	**TAD**	**Treat-ment**	**Time P-Value**	**Inter-action**
Fracturability (N)	323 ± 16	384 ± 38	274 ± 58	290 ± 95	229 ± 40	232 ± 45	196 ± 25	199 ± 21	183 ± 3	185 ± 15	NS	**	NS
Hardness (N)	344 ± 21	380 ± 37	314 ± 19	330 ± 44	279 ± 14	278 ± 27	240 ± 20	237 ± 12	238 ± 8	242 ± 9	NS	***	NS
Resilience	8.07 ± 0.57	7.25 ± 1.74	7.17 ± 1.22	7.27 ± 2.27	6.4 ± 0.29	6.39 ± 0.94	5.66 ± 0.13	5.63 ± 0.57	4.63 ± 0.21	4.61 ± 0.09	NS	*	NS
Cohesiveness	0.23 ± 0.01	0.19 ± 0.05	0.25 ± 0.01	0.23 ± 0.01	0.24 ± 0.01	0.23 ± 0.02	0.22 ± 0.00	0.22 ± 0.01	0.18 ± 0.00	0.17 ± 0.00	NS	**	NS
Springiness	60.0 ± 5.5	55.2 ± 12.6	72.5 ± 38.1	71.5 ± 37.4	40.8 ± 2.7	44.9 ± 1.0	42.22 ± 4.2	37.60 ± 3.6	31.24 ± 3.9	31.42 ± 8.1	NS	NS	NS

Statistical analysis determined by repeated measures ANOVA

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $^{NS}P > 0.05$

2.4.8. Proteolysis

The level of primary proteolysis in cheese is shown in Table 2.6. The results are consistent with that which has already been described for Cheddar cheese as it matures. Primary proteolysis increased significantly with time for both treatment types ($P < 0.05$), which is attributed to the action of residual chymosin, endogenous milk enzymes (e.g. plasmin) and enzymes from starter and non-starter bacteria. In addition, OAD cheese had a significantly greater extent of proteolysis compared to TAD cheese ($P < 0.05$), and the effect appears to be present across all ripening time points. It has previously been reported that the proteolytic activity of milk increases with OAD due to the activation of the plasmin-plasminogen system (Stelwagen *et al.*, 2013). Proteolysis is one of the important biochemical events for cheese as it matures, playing an important role in texture and flavour development (Fox, 1989). The significant differences observed here are interesting, but, it should be recognised that the magnitude of the difference between the milking frequency treatment types is not particularly large. This perhaps suggests that these differences should not be of concern from a dairy industry and consumer perspective, and indeed, no significant differences were observed in cheese texture within this study. Other work has also already reported that OAD does not impact the sensory properties of cheese (Martin *et al.*, 2009). Future investigations could focus on consumer perception of flavour and textural differences in Cheddar in response to milking frequency. A sensory panel could provide a more complete assessment, something that was beyond the scope of this particular work.

Table 2.6: Proteolysis of cheese applying different milking frequency treatments (± standard deviation) of once-a-day (OAD), twice-a-day (TAD) (n=2)

	4 d		20 d		60 d		100 d		180 d				
	OAD	**TAD**	**OAD**	**TAD**	**OAD**	**TAD**	**OAD**	**TAD**	**OAD**	**TAD**	**Treatment P-Value**	**Time P-Value**	**Time x treat P-Value**
pH 4.6 SN/TN (%)	5.25 ± 0.95	4.92 ± 0.35	7.13 ± 0.17	6.88 ± 0.81	11.55 ± 0.06	11.02 ± 0.98	14.15 ± 0.42	13.38 ± 0.61	18.91 ± 1.90	18.36 ± 1.10	***	***	NS

Statistical analysis determined by repeated measures analysis of variance (ANOVA)

[1] Abbreviation: pH 4.6 SN/TN, nitrogen soluble at pH 4.6 (expressed as % of total nitrogen)

*****P < 0.001 , [NS]P > 0.05**

2.4.9. Volatile compounds

Volatile compounds influence the odour and sensory characteristics of cheese in ways that can be both positive and negative to consumer enjoyment of the food. All together 38 volatile compounds were identified in cheese samples analysed at 180 d of ripening. These consisted of 3 acids, 12 alcohols, 4 aldehydes, 4 alkanes, 1 benzene, 3 esters, 9 ketones and 2 sulphur-compounds (Table S2.1). The relative abundance of each of these is the result of proteolysis, lipolysis and glycolysis as the cheese matures (Lamichhane et al., 2018c). Most volatile compounds showed no significant difference ($P > 0.05$) in response to the milking frequency treatments, except for four volatile compounds, i.e., butanoic acid, 1-hexanol, acetone, and 2-pentanone (Table S2.1).

The OAD cheese showed significantly lower levels of butanoic acid compared to TAD cheese ($P<0.01$). Although butanoic acid at low levels contributes positively to the aroma of cheese, it gives an undesirable rancid note at high concentration (Lamichhane *et al.*, 2018a). The presence of this volatile compound is typically associated with the extent of lipolysis in the curd at the start of ripening, lipolytic activity of the starter culture during ripening or can occasionally result from microbial contamination by non-starter lactic acid bacteria (Hickey *et al.*, 2006). The OAD cheese also had significantly lower levels of acetone ($P < 0.05$), a compound with a solvent, sour milk or apple odour. The comparable results obtained for acetone and butanoic acid could be related, it is possible that more free butanoate was available in TAD cheese to subsequently undergo degradation to acetone. It should be noted that whilst the difference in acetone was statistically significant the actual magnitude of the

difference appears to be relatively small. Furthermore, it is noteworthy that butanoic acid did not show a significant difference within the free fatty acid analysis. The statistical approach should also be considered here, with the study being completed in duplicate it should be recognised that further replicates might have further refined these results. OAD cheese also showed significantly higher levels of 1-hexanol ($P < 0.05$) which is associated with green and floral aromas and had significantly higher levels of 2-pentanone ($P < 0.05$), a compound connected to sweet, fruity, ethereal, fermented and winey aroma.

When differences in volatile profiles in Cheddar cheese are observed it is often because of varying extents of lipolysis resulting from differences in fatty acid levels. As no significant differences were observed between fatty acid profiles in this work ($P > 0.05$) it is possibly not surprising that 34 out of the 38 volatiles observed also showed no significant differences ($P > 0.05$). These results suggest that milking frequency has little impact on volatile compound levels, and it is suggested that consumers should not notice any change. However, sensory panel investigations could still expand further upon these findings and could be a focus for future studies. A sensory panel might detect differences not observed by GC-MS, with sensory perception depending on individual odour thresholds and the complex system of interactions that can take place between volatile compounds (Lawless and Heymann, 2010).

2.4.10. Colour

Colorimetric readings were taken over six months of ripening and milking frequency had a significant effect on the colour attributes of Cheddar cheese (Table 2.7). In particular, OAD cheese had significantly higher b* values ($P<0.05$) in all individual ripening periods apart from the 100 d measurement. The statistical significance of the higher b* values of OAD cheese can also be seen across the entire ripening time through repeated measures ANOVA ($P<0.05$). The b* parameter references blue to yellow colour and the significantly higher b* values indicate that OAD cheese is more yellow in colour than TAD cheese. OAD cheese samples also appeared visually more yellow in colour compared to TAD. The L* and a* values did not show a significant difference in response to the reduced milking frequency treatment.

Further assessment of the overall visible colour difference was also achieved by calculating the average total colour difference, Delta E (ΔE), as described by Divine *et al.* (2012). The authors described a colour difference of 1.0 as corresponding to the smallest possible visual perception of a change in colour by the human eye, and the values of ΔE calculated here (For example, $\Delta E=4.41$ at 20 d of ripening) seem consistent with the differences in colour observed when taking measurements. The overall colour difference obtained within the ΔE calculation is mostly attributable to differences within b* values.

From a sensory perspective, yellow colour is generally seen as a favourable attribute for cheese, and within the Irish market yellow colour is positively associated with

animals reared on pasture (O'Callaghan *et al.*, 2017). Yellow colour in milk and cheese has also been linked to positive nutritional qualities through an association with the presence of β-carotene (O'Callaghan *et al.*, 2017). The authors described how b* values within CIELAB colorimetry correlate highly with β-carotene content ($P < 0.001$, Pearson $r = 0.948$). However, whilst yellow colour is generally accepted as preferable, it should be noted that consumer preferences for cheese colour, and sensory attributes more generally, can be different in certain geographical markets (Ouyang *et al.*, 2021).

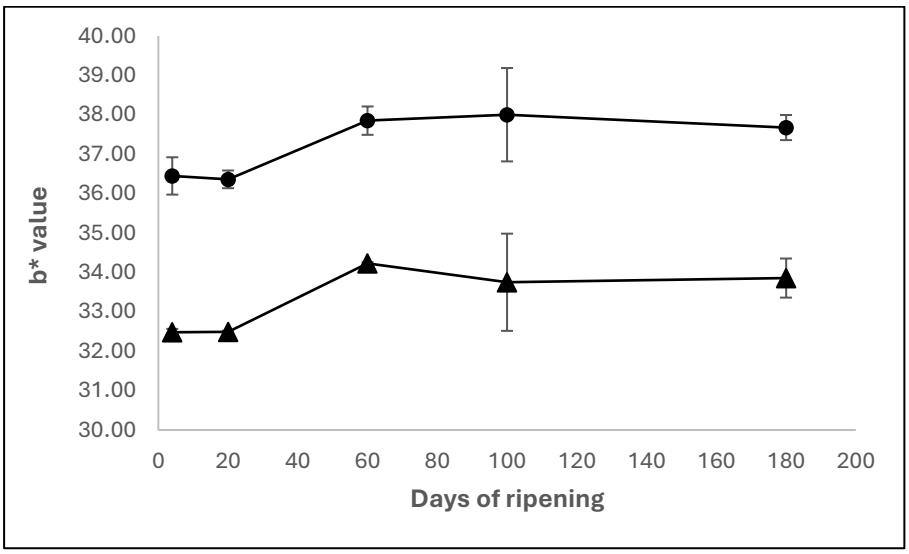


Fig. 2.2. Effect of once-a-day (OAD, ●) and twice-a-day (TAD, ▲) milking on the b* values of cheeses during ripening. Presented values are the means from 2 replicate cheese trials; error bars show standard deviations of the means.

Ripening time also had a significant effect on the colour of cheese ($P<0.05$) (Table 2.7). The L* values for both treatments decreased significantly during ripening, this parameter describing how the cheese got darker as it matured. The decreasing L* values observed with time may reflect changes in cheese microstructure during ripening that alter light scattering. The b* values also increased significantly with ripening time for both milking frequency treatments, particularly during the first 60 days of the ripening period.

Table 2.7: Colorimetry of cheeses over ripening when applying different milking frequency treatments (± standard deviation) of once-a-day (OAD) and twice-a-day (TAD) (n=2)

	4 d		20 d		60 d		100 d		180 d		Treatment P-Value	Time P-Value	Interaction P-Value
	OAD	**TAD**	**OAD**	**TAD**	**OAD**	**TAD**	**OAD**	**TAD**	**OAD**	**TAD**			
L*	82.57 ± 0.16	82.53 ±0.54	80.27 ± 0.51	78.16 ± 0.58	80.32 ± 0.87	80.44 ± 1.85	79.43 ± 0.94	78.29 ± 3.68	78.73 ± 1.29	80.42 ± 0.69	NS	*	NS
	(P=0.45^{NS})		*(P=0.01*)*		*(P=0.45^{NS})*		*(P=0.33^{NS})*		*(P=0.22^{NS})*				
a*	-4.56 ± 0.19	-4.8 ± 0.02	-4.66 ± 0.11	-4.78 ± 0.07	-4.79 ± 0.11	-5.01 ± 0.22	-4.81 ± 0.20	-4.8 ± 0.06	-4.89 ± 0.46	-4.92 ± 0.11	NS	NS	NS
	(P=0.19^{NS})		*(P=0.26^{NS})*		*(P=0.26^{NS})*		*(P=0.46^{NS})*		*(P=0.46^{NS})*				
b*	36.45 ± 0.47	32.48 ± 0.09	36.36 ± 0.22	32.49 ± 0.03	37.85 ± 0.36	34.23 ± 0.03	38 ± 1.19	33.75 ± 1.23	37.68 ± 0.32	33.86 ± 0.50	***	**	NS
	(P=0.03)*		*(P=0.01*)*		*(P=0.02*)*		*(P=0.12^{NS})*		*(P=0.01*)*				
ΔE	3.98		4.41		3.63		4.4		4.18				

Statistical analysis determined by repeated measures analysis of variance (ANOVA) or by t-test as appropriate, values in parentheses show significance at each individual time point

***P < 0.05, **P < 0.01, ***P <0.001, ^{NS}P > 0.05**

2.5. Conclusions

No significant compositional impacts were observed in OAD Cheddar as compared to TAD Cheddar. Furthermore, OAD milk resulted in a significantly increased cheese yield. If more cheese can be obtained from a given volume of milk it suggests a possible increased economic value for that milk within cheese production. The reduced milking frequency treatment resulted in cheese that was significantly more yellow, a colour difference that is generally regarded as preferable to consumers. Also associated with this colour difference was an increased β-carotene content for OAD cheese, and the prospect that OAD cheese could bring health benefits.

The observations made within this research could offer commercial opportunities to the dairy industry and should also be of interest to nutritional researchers. It is anticipated that additional investigations within this area should now be forthcoming. All scientific work needs to be considered within the constraints and limits of the experimental design. This study was completed in duplicate, and it is acknowledged that additional replicates might have improved the analysis and interpretation of data. This was a late lactation study, and these findings need to be considered within that context, future work could look to investigate these impacts across an entire milking season. The findings made here in relation to sensory attributes could be expanded upon further in future investigations that utilise a sensory panel. A large amount of work has already been carried out on milking frequency. However, it should be recognised that some investigators, including these authors, would want to consider

opportunities for future research. Most specifically, the authors believe further work could be carried out on consumer acceptance and dairy industry impacts of milking frequency upon cheese. Less research is also available in relation to this area within the context of Irish seasonal pasture grazing systems. Building upon this specific research domain by carrying out similar analyses on different types of cheese and alternative dairy products also offers a great potential opportunity to advance the evidence base. It is hoped that this particular research contributes to the current body of evidence and informs any such future investigations. Within this study, Cheddar cheese appears to not be negatively impacted by OAD milking, and it is exciting to consider that new opportunities within cheese manufacturing and nutrition might eventually be made possible, as our understanding of milking frequency science improves.

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Chapter 3

The impacts of a multispecies sward grazing system and stage of lactation on the processing-efficiency and compositional characteristics of milk and Cheddar cheese

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3.1. Abstract

Milk chemistry can be altered by dairy cow diet, with implications for processing and product quality. This study investigated Cheddar manufacturing, using milks derived from biodiverse multispecies swards (MSS) or perennial ryegrass (PRG) monocultures, at mid- and late-lactation. The macro-composition and fatty-acid-profiles of milks were broadly equivalent, but MSS-derived milks had significantly higher protein and phosphorus contents, and significantly lower somatic cell counts, compared to PRG-derived milks. No significant differences in rennet coagulation properties were observed, and MSS-derived cheese had a macro-composition typical for Cheddar. Significantly increased cheese yields resulted from MSS compared to PRG; actual yields of 11.13% and 10.58% respectively, and moisture-adjusted cheese yields adjusted for fat and protein of 18.78% and 18.25% respectively. As agri-food industries adapt to maximise efficiency and sustainability, these investigations provide assurances; within this study, MSS grazing did not appear to adversely impact the processing-efficiency of milk into Cheddar, a high-value dairy commodity.

3.2. Introduction

In temperate regions dairy-farming typically utilises pasture-grazing within seasonal-production systems. In economic terms, regions suited to pasture-grazing possess a cost-based comparative-advantage over those utilising indoor confinement; pasture-systems benefit from lower feed costs, lower labour costs, higher fertility rates, lower

culling rates and easier forage management (White *et al.*, 2002). Pasture systems are also recognised as providing a more sustainable approach to dairy-farming compared to high-input systems (Alan Rotz *et al.*, 2009; Hennessy *et al.*, 2020; Moscovici Joubran *et al.*, 2021), and can result in dairy products with improved nutrient profiles compared to non-pasture alternatives (Elgersma, 2015; Alothman *et al.*, 2019; O'Callaghan *et al.*, 2017; Timlin *et al.*, 2023; Magan *et al.*, 2021). Enhanced fatty-acid profiles have been associated with pasture derived milks and milk products (Elgersma, 2015; Alothman *et al.*, 2019; Timlin *et al.*, 2023; O'Callaghan *et al.*, 2017). In particular, O'Callaghan *et al.* (2017) described higher levels of beneficial fatty-acids in Cheddar cheese manufactured from grass-fed milk.

However, typical approaches to pasture grazing can be associated with some challenging environmental consequences. Current pasture-systems rely heavily on perennial ryegrass monocultures (PRG), which reduce biodiversity and require use of nitrogen-based fertilizers. One suggested strategy for improvement is establishing biodiverse pastures where cows graze on multiple plant species simultaneously, known as multispecies swards (MSS). Pastures of MSS can provide ecologically important habitats (Beye *et al.*, 2022; Cooledge, 2022), with soil containing a greater diversity of indicator species (Ikoyi *et al.*, 2023). The varied growth habits and root structures of MSS improve soil health (Isbell *et al.*, 2017), soil-carbon sequestration and soil-structure (Cooledge, 2022; Buzhdygan *et al.*, 2020). Furthermore, PRG systems can require regular replenishment of lost nitrogen, and the nitrogen-fixing properties of leguminous species within MSS are particularly advantageous by comparison (Walling

and Vaneeckhaute, 2022). Excessive fertilizer use can result in soil acidification, nutrient run-off, eutrophication of waterways, and increased greenhouse gas emissions (Walling and Vaneeckhaute, 2022), while MSS have been associated with reduced nitrogen-leaching and reduced emissions (Pijlman, 2024). Chemical nitrogen costs need to be carefully managed (Buckley *et al.*, 2020), and these costs have been particularly pertinent on farms during times of commodity price inflation and price volatility. Adoption of MSS grazing may be constrained by establishment costs and the difficulty of maintaining botanical diversity over time (Cooledge et al., 2022; Isbell et al., 2017). The adoption of MSS systems depends not only on environmental benefits, but also on whether productive and persistent swards can be maintained under commercial grazing conditions (Cooledge et al., 2022). A criticism levied against MSS grazing is the difficulty in maintaining complex swards; simpler binary mixes of clover and ryegrass do offer an alternative solution (Dineen *et al.*, 2018; Fitzpatrick *et al.*, 2022), but do not necessarily provide the full benefits associated with MSS. However, the evidence-base linking MSS grazing to improved sustainability is now relatively well-established, and the possibility of increased utilisation of MSS within future dairy systems would align well with sustainable policy objectives. Within a policy context, the European Union Green deal aims to support improved environmental and economic sustainability

As noted by Magan *et al.* (2021), pasture-grazing is generally utilised because of economic and environmental factors, but a growing body of evidence suggests that the nutritional benefits associated with pasture-grazing should be more widely

considered. Changing the diets of dairy cows is known to impact milk composition (Elgersma, 2015; Alothman *et al.*, 2019; O'Callaghan *et al.*, 2017; Timlin *et al.*, 2023), and dairy-product quality (O'Callaghan *et al.*, 2017). From a due diligence perspective, it is of paramount importance to determine whether MSS grazing results in negative, positive or neutral impacts to milk and commodity milk-products. Greater consideration might then be given to increasing the utilisation of biodiverse pastures at a national and international level. Indeed, some knowledge has already been obtained on the influence of MSS grazing on milk, but investigations so far have tended to be limited in scope, focusing on factors such as milk-yield and/or macro-composition (Bryant *et al.*, 2017; McCarthy *et al.*, 2023; Minneé *et al.*, 2017). However, Bryant *et al.* (2017) observed that MSS grazing did not compromise milk-yield. Minneé *et al.* (2017) described how including chicory and plantain in pasture had little impact on milk-composition, and that milk-yield and total-solids (TS) yields remained similar. McCarthy *et al.* (2023) described significantly higher milk-yield and TS in MSS derived milk compared to PRG or PRG plus white-clover. Although, McCarthy et al. (2023) utilised a zero-grazing, cut-and-carry, feeding system which could increase specific intakes compared to grazing studies, where selective grazing can occur. Until recently, no comprehensive studies have been completed on MSS impacts on milk. However, a recent study by Kostovska *et al.* (2024) compared milks derived from PRG and MSS systems over an entire lactation, and found significant effects arising from cow diet. The MSS diet increased total monthly yields of fat, TS and protein, and the authors

noted that compositional differences might also have important implications for dairy-processing and dairy-product quality.

Overall, a paucity of evidence is available regarding detailed evaluations of milk composition and the quality of dairy products when utilising MSS grazing. Most notably, a lack of evidence is available on the influence of MSS on milk-processability and final product composition. These are factors most pertinent within cheese production, and Cheddar cheese is a highly popular and economically important food, that is also subject to substantial research as a model dairy product. Consequently, this research aimed to investigate some of the main processing-efficiency, nutritional and techno-functional consequences relevant to Cheddar when applying MSS grazing within an Irish seasonal pasture-based system.

3.3. Materials and methods

3.3.1 Experimental design

The milk used in this study was supplied by the Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark. The work utilised milk collected from a grazing farm systems experiment undertaken by Jezequel *et al.* (2024), with milk being collected for cheese manufacture on three occasions in June 2022 and three occasions in October 2022. The cows were grazed in two herds grazing different sward types initially established during 2020 and 2021: a monoculture of *Lolium perenne L.* (perennial ryegrass – PRG) and an eight species multispecies sward (MSS). The MSS

sward was composed of PRG, meadow fescue *(Festuca pratensis L.),* timothy *(Phleum pratense L.),* white clover *(Trifolium repens L.),* red clover *(Trifolium pratense L.),* hybrid clover *(Trifolium hybridum L.),* plantain *(Plantago lanceolate L.) and* chicory *(Chicorium intybus L.).* The treatment groups comprised of both Holstein-Friesian and Holstein-Friesian × Jersey crossbred animals, with 25 cows per group, randomised at the beginning of lactation for age, breed, yield and parity. All animals were milked daily at 6.30 am and 3.30 pm. Both herds were adjacent to one another, separated using a temporary electric fence to ensure diets of similar quality were offered. Pre-grazing yield, daily herbage allocation and post grazing residual heights were similar between treatments (1650 ± 28.9 kg (DM/ha >4cm), 15.5 kg DM/ha, 40 mm, respectively). Sward composition was similar between treatments, 20.5 % ± 1.50% (% crude protein), while the dry matter digestibility was 80.1 and 78.8 % ± 3.3 % for PRG and MSS, respectively (Jezequel *et al.*, 2024)**.** The cows were also offered 2 kg per day of concentrate. Further details can be found in Jezequel *et al.* (2024), which describes the detailed grazing characteristics and general animal performances during the study period.

3.3.2 Seasonality considerations

Milk quality within the middle of the milking season is typically most relevant to cheese production, and research has noted interactions between dairy cow diets and stage of lactation (SOL) (Elgersma, 2015). In order to take into consideration SOL effects, triplicate cheesemaking studies were completed in both June 2022 and October 2022. Timlin *et al.* (2023) defined the mid-lactation period as falling 14-27 weeks after the

average calving date, and the late-lactation period as falling 28-38 weeks after the average calving date. Consequently, for the purposes of this study, milk collected for cheesemaking in June 2022 is referred to as mid-lactation milk, while milk collected for cheesemaking in October 2022 is referred to as late-lactation milk.

3.3.3 Cheese manufacture

A portion of the collected raw milk was separated into cream and skim milk at 50°C using a benchtop centrifuge (FT15 Disc Bowl Centrifuge, Armfield Limited, Hampshire, UK). Milk from each dietary treatment, MSS and PRG, was then standardised to a protein-to-fat ratio of 0.95 using skim milk, raw whole milk or cream. These standardised milks were then pasteurised at 72°C for 15s (HTST/UHT System HT122, OMVE, Scgalkwijk, the Netherlands) and stored overnight at 4°C. Milk that has been standardised and pasteurised will also be referred to as cheesemilk within this study. On the day following pasteurisation, Cheddar cheese was manufactured using pilot-scale production vats (10 L; Pierre Guerin Technologies, Mauze, France) at the Biofunctional Food Engineering Facility, Teagasc Food Research Centre, Moorepark, Fermoy, Co Cork, Ireland, as previously described by Page *et al.* (2024) (Chapter 2). After manufacturing, all cheeses were vacuum-packed and ripened at 8°C for 6 months (180 d). The end-to-end cheesemaking process, including milk collection and standardisation, was performed in full on three separate occasions during three separate weeks within June 2022 and three weeks in October 2022, resulting in a total of six independent cheesemaking trials (n = 6). Within this study, cheese from the June

2022 triplicate trials will be referred to as mid-lactation cheese, whilst cheese from the October 2022 triplicate trials will be referred to as late-lactation cheese.

3.3.4 Rennet coagulation properties

Samples were taken to determine the coagulation properties of MSS and PRG cheesemilks, at both mid- and late-lactation, according to the method described by Page *et al.* (2024) (Chapter 2).

3.3.5 Composition of raw milk, cheesemilk, bulk whey and cheese

These attributes were analysed similarly to methods described in Chapter 2.

3.3.6 Cheese yield

Determined according to the method described in Chapter 2.

3.3.7 Analysis of total-fatty-acids

The total-fatty-acid (TFA) profiles of cheesemilks were determined by gas chromatography flame ionization detection (GC-FID) according to the methods of De Jong and Badings (1990) with modifications as described in O'Callaghan *et al.* (2020). Briefly, lipid extraction was carried out by adding 10 mL heptane to a 1g sample, vortexing for 30s, and clarifying by centrifugation at 3000 g for 5 min. After centrifugation, the top layer of supernatant was transferred to 50 mL tubes and the bottom layer of supernatant was further extracted with heptane and centrifuged. These steps were repeated in total three times with the collected extracts pooled and converted to methyl esters for GC-FID analysis.

3.3.8 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 27 (IBM Statistics Inc.), with some processing and formatting of data carried in Microsoft Excel 64-bit, 2016 Version (Microsoft Corporation Inc.). The reported data are averaged results of six separate independent cheesemaking trials (n=6); three cheese manufacturing trials completed within a mid-lactation study, and three additional cheesemaking trials completed within a late-lactation study. Results are presented as the mean average ± standard deviation. Univariate ANOVA were used for the comparison of means in single factors in SPSS. When comparing multiple related variables, the between-subjects effects of diet, seasonality and their interactions were interpreted through a general linear model (GLM) analysis of variance (ANOVA) multivariate procedure in SPSS. When applying this multivariate GLM model, Levene's test was also applied to confirm homogeneity of variance. The normality of residuals was confirmed through the visual inspection of Q-Q plots to take into consideration smaller sample sizes. For all statistical analyses, P-values < 0.05 were considered significant. Effect sizes were also calculated as partial eta squared ($\eta 2$), a $\eta 2 < 0.06$ was considered a small effect size, $0.06 \leq \eta 2 < 0.14$ was considered a medium effect size, and $\eta 2 \geq 0.14$ considered a large effect size.

3.4. Results and discussion

3.4.1 Raw milk composition

The macro-compositions of MSS and PRG raw milk, and mid- and late-lactation raw milk, are shown in Table 3.1.

The MSS diet treatment resulted in no significant differences in raw milk fat and lactose contents as compared to the PRG treatment ($P > 0.05$). However, the MSS diet treatment was associated with significantly higher protein content in raw milk as compared to the PRG treatment ($P < 0.05$); on average, the protein content of MSS raw milk was 3.83% compared to 3.70% for PRG milk. The grazing systems experiment that supplied milk for this analysis provides further insights (Jezequel et al., 2024). Jezequel *et al.* (2024) reported that sward type had a significant effect on daily milk yield; the MSS diet resulted in an average daily milk yield of 18.1kg per cow compared to 17.1kg for the PRG treatment. Consequently, the increased protein content observed in MSS milk within this current study likely occurred alongside an increased milk volume, and this suggests a potential genuine improvement in milk protein synthesis for the MSS treatment as compared to PRG.

While statistically significant, the practical impact of an overall average 3.4% higher level of protein in MSS milk is open to interpretation. However, this magnitude of protein content difference should not have an adverse practical impact. Indeed, Geary *et al.* (2010) noted that Irish farmers are paid for total protein and fat yielded with penalties for milk-volume, so, this increased protein content could be interpreted as

beneficial. While significantly higher protein content in MSS milk has been demonstrated within these investigations, conclusions should probably be drawn tentatively at this time given mixed evidence provided by other studies. Bryant *et al.* (2017) found that protein in milk was unaffected when comparing between binary-swards and MSS grazing. McCarthy *et al.* (2023) observed significantly lower protein content in milk derived from MSS compared to PRG, and Minneé *et al.* (2017) observed reductions in milk protein content as the amount of forbs was increased in dairy cow diets.

Table 3.1. Mean macro-composition of raw milks obtained from diet and stage of lactation treatments (± standard deviation)

	Treatment[1]				Statistical interpretation					
	Diet PRG	Diet MSS	SOL MID	SOL LATE	Diet P-Value	η2	SOL P-Value	η2	SOL x Diet P-Value	η2
Protein (% wt/wt)	3.7	3.83	3.39	4.15	*	0.425	***	0.963	NS	0.065
±	0.45	0.41	0.11	0.11						
Fat (% wt/wt)	5.1	5.21	4.8	5.51	NS	0.054	***	0.732	NS	0.104
±	0.56	0.34	0.34	0.12						
Lactose (% wt/wt)	4.67	4.66	4.74	4.58	NS	0.006	***	0.767	NS	0
±	0.09	0.10	0.06	0.02						
SCC (cells/mL)(x1000)	173	116	126	162	***	0.909	**	0.799	NS	0.539
±	34	15	26	44						

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

[1]Abbreviations: PRG, perennial ryegrass; MSS, multispecies swards; SOL, stage-of-lactation; η2, effect-size, SCC; Somatic cell count.

The diet-associated determinants of protein content in milk are complex, resulting from factors such as available rumen ammonia and peptides, type of dietary amino acids consumed by the dairy animal, and their ease of intestinal absorption (Schwab and Broderick, 2017). Furthermore, the possibility of interactions between breed and diet also requires consideration (Kostovska *et al.*, 2024; Page *et al.*, 2025). Epigenetics, post-transcriptional modifications, and gene-regulation are also processes relevant to this discussion (Qian and Zhao, 2014; Singh *et al.*, 2010). Singh *et al.* (2010) argued that substantial variability in mammary epithelial cell function might be due to epigenetic regulation arising from environmental cues such as management practices like nutrition. Overall, it is plausible that one or more of the above factors have varied through the MSS grazing treatment as compared to PRG, providing multiple possible explanations for the significant protein differences seen between these milks. Differences in dry matter intake may also have arisen between the cow groups, but sward compositions were similar between the treatments, with 20.5 % ± 1.50% (% crude protein), and roughly comparable dry matter digestibilities of 80.1 and 78.8 % ± 3.3 % for PRG and MSS, respectively (Jezequel *et al.*, 2024). Reaching an overall final conclusion on how these protein content differences in milk have arisen is beyond the scope of this work, and further exploration of these possibilities could provide an interesting area of consideration for future investigators.

The stage of lactation (SOL) was also associated with significant impacts on the macro-composition of raw milk ($P < 0.05$), but no significant interaction was found between SOL and these pasture diets ($P > 0.05$). The late-lactation period resulted in significant

increases in protein, fat and TS levels, while lactose content decreased significantly ($P < 0.05$) as compared to the mid-lactation period. Mid-lactation milk averaged 3.39% protein, 4.80% fat and 4.74% lactose. Late-lactation milk averaged 4.15% protein, 5.51% fat and 4.58% lactose. Such relationships between SOL and milk composition are well-known and have been reported previously (Timlin *et al.*, 2021). Indeed, it has long been known that the late-lactation period is associated with lower milk volumes and higher component concentrations as compared to mid-lactation (Wood, 1967). It is likely that the SOL differences observed in this study are attributable to concentration effects; declining milk yield in late-lactation resulting in higher observed fat, protein, and total solids concentrations. Overall, within this analysis, the impact of SOL on milk macro-composition appears to be more pronounced than differences arising between these pasture diets. Particularly regarding protein content where SOL and diet both had significant effects, but the magnitude of these effects were different (DIET, $P<0.05$, $\eta2 =0.425$; SOL, $P<0.001$, $\eta2 = 0.963$).

3.4.2 Raw milk somatic cell count

The MSS diet was associated with a significantly lower SCC in raw milk compared to the PRG diet, the average SCC in MSS raw milk averaged 116,000 cells/mL, whilst SCC levels within PRG raw milk averaged 173,000 cells/mL ($P<0.001$, $\eta2 = 0.909$; Table 3.1). No significant interaction was found between the diet treatment and SOL ($P > 0.05$). Differences in SCC levels represent a difference in immune response that correlates with mastitis infections, with the potential for negative impacts to dairy herd wellbeing

and reductions in profitability of dairy production. O'Brien et al. (2001) suggested that SCC values up to 300,000 cells/mL do not negatively impact the composition or processability of fresh milk, and all milk in this study remained below this threshold. They did also note that milk quality may still decline if stored for more than 72 hours.

It is possible that the reduced SCCs associated with an MSS diet might be attributable to some beneficial aspect of the botanical make-up of MSS as compared to PRG. Some plant plants species provide antimicrobial properties, described in the literature under the term phytogenics or phytobiotics, and they have been suggested as feed additives or alternatives to antibiotics within animal husbandry (Górniak et al., 2022; Diaz-Sanchez et al., 2015). Studies have suggested phytobiotic supplementation can lower SCCs and improve dairy performance (Hashemzadeh-Cigari et al., 2014). Similar mechanisms might be contributing to lower SCCs with MSS, although further research would be needed to explore these ideas further.

3.4.3 Cheesemilk macro-composition

The macro-compositions of MSS and PRG cheesemilk, and mid- and late-lactation cheesemilk, are shown in Table 3.2.

Within cheesemilk, protein and fat levels were significantly higher with the MSS diet ($P < 0.05$). The higher protein content of MSS cheesemilk results from the increased protein levels in MSS raw milk, while the elevated fat content is due to the process of milk-fat standardisation. No significant effects were observed for lactose, TS content, NPN, casein content and casein number as a result of the diet treatments ($P > 0.05$).

During the late-lactation period protein, fat, NPN, casein content, casein number and TS levels were all significantly higher, and lactose contents were significantly lower, than during the mid-lactation period ($P < 0.05$).

Table 3.2. Mean macro-composition of cheesemilks obtained from MSS and PRG diet, and stage of lactation, treatments (± standard deviation)

	Treatment[1]				Statistical interpretation					
	Diet PRG	**Diet MSS**	**SOL MID**	**SOL LATE**	**Diet P-Value**	**η2**	**SOL P-value**	**η2**	**SOL x Diet P-value**	**η2**
Composition										
Total Protein (% wt/wt)	3.74	3.88	3.42	4.2	*	0.42	***	0.96	NS	0.06
±	0.46	0.42	0.12	0.12						
Fat (% wt/wt)	3.91	4.1	3.59	4.42	*	0.52	***	0.95	NS	0.21
±	0.51	0.42	0.18	0.13						
Lactose (% wt/wt)	4.67	4.69	4.76	4.6	NS	0.03	***	0.66	NS	0
±	0.11	0.11	0.09	0.02						
Total solids (%)	13.54	13.56	12.62	14.47	NS	0	***	0.88	NS	0.06
±	0.11	0.01	0.12	0.02						
Casein content (% wt/wt)	3.04	3.12	2.70	3.46	NS	0.23	***	0.92	NS	0.05
±	0.43	0.42	0.09	0.11						
Casein number (% wt/wt)	81.22	80.38	79.09	82.51	NS	0.05	*	0.52	NS	0.02
±	1.79	2.56	1.63	0.71						
NPN	3.89	3.62	3.14	4.37	NS	0.03	NS	0.37	NS	0
±	0.87	1.33	0.66	1.1						

Rheology										
Set-to-cut (mins)	29.82	32.09	34.23	27.68	NS	0.16	***	0.62	NS	0.02
±	5.04	4.08	3.71	2.39						
RCT (mins)	15.79	18.7	19.32	15.17	NS	0.19	NS	0.32	NS	0.08
±	5.3	2.54	2.04	5.02						
Minerals										
Calcium (mg kg^{-1})	1348	1387	1267	1484	NS	0.27	***	0.83	NS	0.02
±	141	124	74	48						
Potassium (mg kg^{-1})	1493	1520	1542	1461	NS	0.06	***	0.64	NS	0.22
±	33	79	49	23						
Magnesium (mg kg^{-1})	123	122	108	141	NS	0.05	***	0.97	NS	0.37
±	21	16	4	5						
Sodium (mg kg^{-1})	380	358	342	403	NS	0.39	***	0.85	NS	0.47
±	44	25	23	23						
Phosphorus (mg kg^{-1})	945	1017	912	1057	**	0.79	***	0.93	NS	0.17
±	97	76	63	39						
Zinc (mg kg^{-1})	4.62	4.59	4.16	5.14	NS	0	NS	0.44	NS	0.08
±	0.79	0.52	0.47	0.35						

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

[1]Abbreviations: PRG, perennial ryegrass; MSS, multispecies swards; SOL, stage-of-lactation; η2, effect-size, RCT; Rennet coagulation time.

3.4.4 Cheesemilk mineral composition

Milk mineral content is of interest from a nutritional perspective (Feeney *et al.*, 2016) and differences in mineral levels can impact functional properties of milk relevant to processing and final cheese quality (Lamichhane *et al.*, 2018b). Regarding the two

investigated pasture diets, MSS grazing was associated with significantly higher levels of phosphorus in cheesemilk as compared to the PRG diet ($P < 0.01$) (Table 3.2). This difference was observed at both mid- and late-lactation. No significant differences were seen in calcium, potassium, magnesium, sodium or zinc content as a result of the diet treatments ($P > 0.05$). Regarding the impact of these mineral observations on milk function, phosphorus content was significantly higher in MSS derived cheesemilk and phosphorus does have a role within milk-processing that can impact final cheese quality (Lamichhane *et al.*, 2018b). Typically, milk with higher levels of protein also contains higher levels of phosphorus, and the mineral is associated with a structural role within the casein micelle (Gulati *et al.*, 2018). Indeed, MSS milk was also found to have a significantly higher protein content, making it reasonable to expect that it also contains higher levels of minerals associated with casein protein. Calcium is also associated with casein, and calcium levels were also slightly higher in cheesemilk derived from MSS grazing, albeit not significantly ($P>0.05$). Furthermore, the casein content of MSS derived milk was on average higher than PRG milk, albeit again not significantly ($P > 0.05$), and the analysis measured total calcium, and these results cannot distinguish between serum calcium and the colloidal calcium associated with casein micelles. Higher levels of casein in milk have been associated with benefits to cheese manufacturing, however, such differences cannot in isolation be taken to confirm or deny functional changes within the milk. Further consideration is given to milk processability within the rheology section.

It is well-reported that levels of certain minerals increase in milk later in the milking season (Keogh *et al.*, 1982; O'Brien *et al.*, 1999; Page *et al.*, 2024; Gulati *et al.*, 2018). Indeed, within this research, the late-lactation study showed significantly higher levels of calcium, magnesium, sodium and phosphorous in milk as compared to the levels seen at mid-lactation ($P<0.05$). As lactation advances, milk composition alters, milk yield declines and mammary physiology changes, and the higher sodium content of late-lactation milk may reflect changes in mammary epithelial permeability (Davis et al., 1999). Late-lactation milk having higher levels of protein is a well-reported phenomenon, and calcium, phosphorous and magnesium are all associated with casein. Indeed, within this research the late-lactation milks also contained significantly higher levels of protein, casein content and casein number ($P < 0.05$). Conversely, potassium levels were significantly lower at late-lactation than at mid-lactation ($P < 0.05$). Potassium is more-so associated with the aqueous phase of milk (Gaucheron, 2010), and is for the most part lost into the whey during manufacturing. No significant difference was observed for zinc ($P > 0.05$); levels of zinc were consistent across all trials and treatments.

Other research has also been completed on the mineral contents of milks derived from MSS and PRG diets while giving consideration to SOL (Boudon *et al.*, 2022). Boudon *et al.* (2022) found that individual mineral levels varied strongly with the month in the milking season, and some interactions were found between grazing system, SOL and the individual minerals investigated.

3.4.5 Cheesemilk total-fatty-acid profile

The total-fatty-acid (TFA) profile of MSS and PRG cheesemilk, and mid- and late-lactation cheesemilk, are shown in Table 3.3.

The TFA profiles of milks can have implications for health (Feeney et al., 2016), processing-efficiency (Lamichhane *et al.*, 2018b) and dairy product quality (O'Callaghan *et al.*, 2017). Within this research, most TFAs in cheesemilk showed no significant differences between the PRG and MSS treatments ($P>0.05$). However, the cheesemilk derived from the MSS treatment was associated with significantly lower levels of saturated-fatty-acids (SFA) C4:0 (butanoic acid) and C6:0 (caproic acid). Also, significantly lower levels of the less abundant SFAs C21:0 (heneicosanoic acid), C22:0 (behenic acid) and C24:0 (lignoceric acid) were found in MSS milk ($P<0.05$). Milk derived from MSS grazing was also associated with significantly higher levels of C16:1 (palmitoleic acid), and significantly higher levels of less abundant C17:1 (heptadecenoic acid) ($P<0.05$).

Previously, SFAs have been scrutinised for having potential negative consequences for health (Keys, 1953), but current evidence does not tend to support these past negative associations (Hamley, 2017; Lovegrove and Givens, 2016; Rooney *et al.*, 2025). Health outcomes appear more dependent on the overall dietary context of SFA intake, particularly regarding dairy foods, and the specific type of SFA present (Mohan *et al.*, 2021; Gómez-Cortés *et al.*, 2018). Consequently, the significantly lower levels of some specific SFAs within MSS derived milk (C4:0, C6:0 and C21:0, C22:0) should not be

regarded as beneficial, although, some research has also suggested potential health benefits associated with their consumption (Gómez-Cortés *et al.*, 2018). The paradox of SFA intakes and health within a dairy context has been acknowledged in the literature (Givens, 2017), and this debate is still ongoing. Milk derived from MSS grazing was also associated with significantly higher levels of C16:1 and C17:1. Consumption of these monounsaturated fatty acids (MUFA) has been suggested as being positive for health (Nunes and Rafacho, 2017), but it must be recognised that while statistically significant differences have been measured here, the magnitude of difference in absolute terms is very small, and this amount of difference is unlikely to have any real and meaningful impact to the consumer.

Table 3.3. Mean total-fatty-acid profile of cheesemilks obtained from the MSS and PRG diet, and stage of lactation, treatments (± standard deviation)

	Treatment[1]				Statistical interpretation					
	PRG	MSS	MID	LATE	SOL P-VALUE	η2	Diet P-VALUE	η2	SOL x Diet P-Value	η2
C4:0	5.04 ± 0.74	4.19 ± 0.68	4.19 ± 0.88	5.04 ± 0.48	*	0.454	*	0.445	NS	0.087
C6:0	2.57 ± 0.2	2.31 ± 0.14	2.37 ± 0.23	2.51 ± 0.19	NS	0.192	*	0.438	NS	0.005
C8:0	1.43 ± 0.08	1.4 ± 0.09	1.45 ± 0.08	1.39 ± 0.07	NS	0.178	NS	0.049	NS	0.195
C10:0	3.19 ± 0.19	3.24 ± 0.39	3.41 ± 0.25	3.03 ± 0.21	*	0.576	NS	0.026	NS	0.394
C11:0	0.07 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	0.06 ± 0.02	NS	0.111	NS	0.014	NS	0.258
C12:0	3.58 ± 0.27	3.71 ± 0.52	3.89 ± 0.35	3.39 ± 0.28	*	0.512	NS	0.067	NS	0.262
C13:0	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.02	0.09 ± 0.02	NS	0.167	NS	0.048	NS	0.238
C14:0	10.87 ± 0.67	11.08 ± 0.98	11.56 ± 0.54	10.39 ± 0.57	**	0.621	NS	0.054	NS	0.145
C14:1	0.95 ± 0.07	1 ± 0.11	0.97 ± 0.05	0.98 ± 0.13	NS	0.008	NS	0.091	NS	0.271
C15:0	1.17 ± 0.14	1.22 ± 0.1	1.16 ± 0.12	1.23 ± 0.12	NS	0.102	NS	0.052	NS	0.184
C16:0	30.94 ± 2.5	29.93 ± 2.15	31.45 ± 2.55	29.42 ± 1.59	NS	0.228	NS	0.068	NS	0.01
C16:1	1.66 ± 0.1	1.77 ± 0.11	1.7 ± 0.04	1.73 ± 0.17	NS	0.039	*	0.42	*	0.513
C17:0	0.61 ± 0.04	0.62 ± 0.04	0.63 ± 0.05	0.59 ± 0.02	NS	0.229	NS	0.045	NS	0.194
C17:1	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.04 ± 0	***	0.834	*	0.51	*	0.51
C18:0	9.36 ± 0.84	9.04 ± 0.52	9.14 ± 0.85	9.26 ± 0.55	NS	0.01	NS	0.072	NS	0.172
C18:1 TFA	2.87 ± 0.51	3.02 ± 0.57	2.65 ± 0.47	3.24 ± 0.4	NS	0.358	NS	0.031	NS	0.002
C18:1n9c	17.1 ± 1.74	17.73 ± 1.19	17.35 ± 1.75	17.48 ± 1.27	NS	0.002	NS	0.055	NS	0.078
C18:1n9t	0.15 ± 0.03	0.15 ± 0.04	0.13 ± 0.04	0.17 ± 0.01	*	0.414	NS	0.005	NS	0.194
C18:2 TFA	1.43 ± 0.56	1.92 ± 0.78	1.21 ± 0.43	2.14 ± 0.59	*	0.569	NS	0.263	NS	0.008
C18:2n6c	0.94 ± 0.07	0.98 ± 0.2	0.99 ± 0.2	0.93 ± 0.07	NS	0.042	NS	0.025	NS	0.001

C18:2n6t	0.09 ± 0.04		0.1 ± 0.03		0.09 ± 0.04		0.1 ± 0.02		NS	0.042	NS	0.042	NS	0.001
CLA c9t11	0.86 ± 0.16		0.86 ± 0.18		0.79 ± 0.16		0.93 ± 0.14		NS	0.282	NS	0	NS	0.339
C18:3 TFA	0.29 ± 0.13		0.42 ± 0.22		0.23 ± 0.08		0.48 ± 0.18		*	0.531	NS	0.244	NS	0.008
C18:3n3	0.8 ± 0.07		0.79 ± 0.09		0.78 ± 0.08		0.8 ± 0.08		NS	0.008	NS	0.005	NS	0.018
C20:0	0.13 ± 0.01		0.12 ± 0.02		0.13 ± 0.01		0.12 ± 0.02		NS	0.229	NS	0.143	NS	0.069
C20:1	0.04 ± 0.01		0.03 ± 0.02		0.03 ± 0.02		0.03 ± 0.01		NS	0.017	NS	0.134	NS	0.134
C20:3n6	0.03 ± 0.01		0.03 ± 0.02		0.02 ± 0.02		0.04 ± 0		NS	0.309	NS	0.067	NS	0.067
C20:4n6	0.05 ± 0		0.03 ± 0.02		0.04 ± 0.02		0.05 ± 0.01		NS	0.065	NS	0.383	NS	0
C20:5	0.1 ± 0		0.09 ± 0.01		0.1 ± 0.01		0.1 ± 0.01		NS	0.014	NS	0.111	NS	0.014
C21:0	0.04 ± 0.01		0.03 ± 0.02		0.03 ± 0.02		0.05 ± 0		*	0.563	*	0.562	NS	0.364
C22:0	0.06 ± 0.01		0.04 ± 0.02		0.04 ± 0.02		0.06 ± 0.01		*	0.55	*	0.479	NS	0.21
C22:1	0.07 ± 0.01		0.06 ± 0.03		0.06 ± 0.03		0.07 ± 0		NS	0.247	NS	0.127	NS	0.076
C23:0	0.03 ± 0.01		0.02 ± 0.01		0.02 ± 0.02		0.03 ± 0		NS	0.243	NS	0.243	NS	0.034
C24:0	0.04 ± 0.01		0.02 ± 0.02		0.03 ± 0.02		0.03 ± 0		NS	0	*	0.474	NS	0.385
Dietary Indices														
Atherogenic	3.83 ± 0.53		3.68 ± 0.45		3.94 ± 0.49		3.57 ± 0.41		NS	NS	NS	NS	NS	NS
Thrombo-genic	4.07 ± 0.48		3.89 ± 0.35		4.12 ± 0.43		3.84 ± 0.39		NS	NS	NS	NS	NS	NS
Health Promoting	0.24 ± 0.03		0.25 ± 0.03		0.24 ± 0.03		0.26 ± 0.03		NS	NS	NS	NS	NS	NS
Desaturase	0.38 ± 0.05		0.4 ± 0.04		0.38 ± 0.05		0.4 ± 0.04		NS	NS	NS	NS	NS	NS

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

Overall, some statistically significant TFA differences have been observed between MSS- and PRG- derived milks, but the magnitude of difference seems unlikely to be practically problematic. Furthermore, regarding any potential real-world health impacts arising from these differences, the calculated atherogenic, thrombogenic, health promoting and desaturase indices of these milks did not show significant differences between the two pasture types ($P>0.05$). Indeed, while a small number of fatty-acids showed some differences, it must be recognised that most fatty-acids present in these milks did not deviate significantly between the MSS and PRG treatments. Studies that have investigated milk fatty-acid-profile differences between pasture and non-pasture diets have found more meaningful differences, but, PRG and MSS are both pasture diets, and the differences in TFA profiles of milks obtained from these two pasture systems appear broadly comparable. The fat profiles of typical PRG derived milks are widely recognised in the literature as being beneficial to health (Elgersma, 2015; Alothman *et al.*, 2019; Timlin *et al.*, 2023; O'Callaghan *et al.*, 2017), so, it is perhaps reassuring that MSS derived milk does not show many significant deviations in fat profile from these PRG systems.

Regarding the impacts of SOL to milk TFA profiles, more differences were associated with seasonality than with diet. On average, mid-lactation milks contained significantly lower levels of C4:0, C18:1n9t, C18:2 trans-fatty-acid, C18:3 trans-fatty-acid, and C21:0 and C22:0 ($P<0.05$), whilst the late-lactation milks averaged lower levels of C10:0, C12:0, C14:0, C17:1 ($P<0.05$). Additionally, a significant statistical interaction was found between diet and SOL for the C16:1 and C17:1 content of cheesemilk ($P<0.05$), but no

other significant interactions were observed (P>0.05). Despite some differences, in terms of potential health impacts, SOL was not associated with any significant differences in any of the calculated health indices (P>0.05), likewise, no statistical interactions were found between SOL, pasture type and any of the health indices.

3.4.6 Whey composition

No significant differences were observed between the fat, protein and TS contents of PRG and MSS bulk whey (P > 0.05). The fat content of PRG bulk whey was numerically higher than MSS bulk whey, but these were not statistically significant differences (P > 0.05). The high degree of variance in results between trials meant that these differences did not meet the threshold for statistical significance. Overall, the investigated cow diets did not significantly impact the components lost to the whey during the Cheddar cheese manufacturing process. Regarding SOL, significantly higher TS levels were observed in whey during the late-lactation period (P < 0.05).

3.4.7 Rheology

No statistically significant differences were observed between the rheological properties of PRG and MSS cheesemilks (P > 0.05). A repeated pattern of slightly slower gelation behaviour was observed for MSS milk, but the magnitude of this difference should not be practically meaningful, and did not meet the threshold for statistical significance (P > 0.05). The average set-to-cut time of PRG milk was 29.82 ± 5.04 min, compared to 32.09 ± 4.08 min for MSS milk. Likewise, the RCT was also consistently faster for PRG milk. The average RCT of PRG milk was 15.79 ± 5.30 min, compared to

18.70 ± 2.54 min for MSS milk. Overall, no significant rheological differences were observed, and this should provide reassurance to the cheese manufacturing industry. However, it is still worth acknowledging that a pattern of slower milk gelation behaviour was observed for milk obtained from a MSS diet. In particular, this difference also needs to be considered within the context of MSS milk having a significantly higher protein content. The casein content of MSS milk was also higher, albeit not significantly ($P > 0.05$). Increased protein and casein content in milk is generally associated with faster milk coagulation times; however, within this study MSS milk still gelled at a slower rate even with a significantly higher protein content. The significantly higher protein level of MSS milk was therefore insufficient to bring about a significant improvement in the gelation behaviour of the MSS cheesemilk ($P > 0.05$).

Regarding seasonality impacts, some clear and significant differences are observable. The set-to-cut time was significantly faster at late-lactation than at mid-lactation ($P < 0.001$, $\eta 2 = 0.620$). The RCT was also faster at late-lactation than at mid-lactation, but not significantly faster ($P > 0.05$). Referring to compositional data in Table 3.2, the significantly higher protein and casein contents observed at late-lactation do probably provide an explanation for these seasonality related differences. No significant statistical interaction was found between the diet treatments and the timing of the study within the milking season ($P > 0.05$).

3.4.8 Cheese composition

The percentages of protein, fat and moisture measured in cheese were all consistent with ranges previously reported for full-fat Cheddar (Feeney *et al.*, 2021) (Table 3.4). No significant differences were observed between the fat and moisture levels of cheese produced from the PRG and MSS diet treatments ($P > 0.05$), but the amount of protein present in MSS cheese was significantly higher than that of PRG cheese ($P < 0.05$). Comparing the cheese by SOL, late-lactation cheese had significantly lower moisture levels than cheese from the mid-lactation period ($P < 0.05$), while fat and protein levels in cheese were not significantly impacted by SOL ($P > 0.05$). The lower moisture content of late-lactation cheese may reflect the higher protein, casein and total solids content of late-lactation milk, which can result in denser curd, and increased whey expulsion, reducing final cheese moisture. No significant differences were seen in fat-in-dry matter (FDM) and moisture in non-fat substance (MNFS) ($P > 0.05$).

3.4.9 Cheese yield

The MSS diet treatment resulted in significantly more cheese being produced compared to PRG ($P<0.05$). The actual yield (Ya) was on average 10.58% for PRG compared to 11.13% for MSS. The Ya was on average 5.24% higher for MSS compared to PRG; in absolute terms significantly more cheese was obtained when using MSS milk to make Cheddar ($P<0.001$, $\eta2=0.591$). Additionally, the moisture-adjusted cheese yield (Yma) was significantly impacted by MSS grazing ($P<0.05$, $\eta2=0.534$). The Yma was 11.09% for PRG compared to 11.82% for MSS. So, the MSS diet treatment resulted

in an on average 6.58% higher Yma, and practical increases in the amount of cheese produced have still occurred even when adjusting for moisture content. Furthermore, the higher cheese yields associated with MSS have not just resulted from a higher protein and fat content in the milk. Statistically significant differences in the moisture-adjusted-cheese-yield-adjusted-for-protein-and-fat (Y_n) were also seen between the two pasture diet treatments ($P<0.05$, $\eta 2=0.534$). The average Y_n was 18.25% for PRG compared to 18.78% for MSS. So, an on average 2.96% higher Y_n was associated with Cheddar production with MSS derived milk compared to PRG milk. It can therefore be seen that significant and meaningful increases in the amount of cheese produced have occurred over and above the differences arising due to differing protein and fat contents of MSS and PRG cheesemilk ($P<0.05$, $\eta 2=0.524$).

Timing within the milking season is already known to impact cheese yield (Galli *et al.*, 2023; Banks and Tamime, 1987), and within these investigations, mid-lactation Ya was on average 9.89% compared to 11.82% at late-lactation. So, when comparing absolute differences in cheese yield between the mid- and late-lactation periods, highly significant impacts can be seen due to seasonality ($P < 0.001$, $\eta 2=0.946$). It is already generally accepted that more cheese can be obtained from a given volume of milk during the late-lactation period (Galli *et al.*, 2023; Banks and Tamime, 1987). No statistical interaction was found between diet and lactation stage ($P > 0.05$).

When only considering Ya as the measure of yield, SOL had a far larger impact upon cheese yield than the diet factor. However, when adjusting the cheese yield to consider

moisture, protein and fat content, the extent of yield difference between the mid- and late-lactation periods diminished. Regarding the diet factor, a significant difference in cheese yield remained even when adjusting for moisture, fat and protein ($P<0.05$); the MSS and PRG associated Y_n were significantly different from each other ($P<0.05$). The significant yield differences associated with MSS are in part explained by protein, fat and moisture content differences, but this does not provide a complete explanation. Factors that contribute to increased cheese yields beyond fat, protein and moisture include casein protein function/variants, mineral content, curd microstructure, fines losses and whey drainage during manufacturing. In these investigations, cheese yield differences associated with SOL really just provide verification of already known impacts of milk-composition on cheese production, but the observations in relation to MSS diet and cheese yield are more novel.

3.4.10 Mineral content in cheese

Regarding the diet treatments, no significant differences were found between the mineral contents of MSS and PRG cheese ($P > 0.05$; Table 3.4). Significantly higher levels of phosphorus were measured in MSS milk, but this difference did not translate significantly into the cheese manufactured from that milk. Overall, a lack of evidence is available in the literature regarding these cow diets, SOL and cheese mineral content levels; it is therefore not possible to compare these findings with other comparable cheese related studies at this time.

The SOL had a significant impact on the mineral content of cheese ($P < 0.05$). In particular, the late-lactation period was associated with cheese that contained significantly lower levels of potassium ($P < 0.05$, $\eta 2=0.580$), and significantly higher levels of magnesium ($P < 0.01$, $\eta 2=0.687$). This is the same relationship as described for the milk that made this cheese; with reference to Table 3.1, potassium levels were also significantly lower in late-lactation milk, and magnesium levels were significantly higher in late-lactation milk ($P < 0.05$). Not all significant differences in milk translated into significant differences in cheese. In cheese, levels of calcium, sodium and phosphorus were all higher at late-lactation than at mid-lactation, but not at levels that met the threshold for statistical significance ($P > 0.05$). In milk, levels of calcium, sodium and phosphorous were significantly higher at late-lactation ($P < 0.05$). It is notable that calcium and phosphorus differences, both in cheese and milk, are differences found within the minerals associated with casein protein.

Table 3.4. Mean macro-composition of cheese obtained from MSS and PRG diet and stage of lactation treatments (± standard deviation)

	Treatment[1]				Statistical interpretation					
	Diet PRG	Diet MSS	SOL MID	SOL LATE	Diet P-Value	η2	SOL P-value	η2	SOL x Diet P-value	η2
Composition										
Protein (% wt/wt)	26.77	28.48	27.51	27.74	*	0.525	NS	0.019	0.52	0.053
±	0.90	0.94	1.37	1.25						
Fat (% wt/wt)	33.41	33.50	33.26	33.64	NS	0.002	NS	0.041	0.37	0.100
±	1.30	0.79	1.24	0.83						
Moisture (% wt/wt)	35.62	34.76	35.87	34.51	NS	0.209	NS	0.399	0.51	0.057
±	1.31	1.07	1.17	0.91						
FDM (% wt/wt)	51.89	51.37	51.86	51.40	NS	0.032	NS	0.025	NS	0.043
±	1.40	1.82	1.32	1.89						
MNFS (% wt/wt)	53.49	52.29	53.74	52.03	NS	0.174	NS	0.300	NS	0.003
±	1.34	2.02	1.11	1.93						
Salt (% wt/wt)	1.51	1.53	1.39	1.64	NS	0.007	*	0.463	NS	0.028
±	0.20	0.20	0.19	0.09						
S/M (% wt/wt)	4.24	4.41	3.89	4.75	NS	0.035	*	0.498	NS	0.035
±	0.67	0.68	0.60	0.35						
Actual yield (Y_a)	10.58	11.13	9.89	11.82	***	0.591	***	0.946	NS	0.004
±	1.09	1.08	0.37	0.42						

MACY (Y_{ma})	11.09	11.82	10.32	12.59	*	0.534	***	0.917	NS	0.014
±	1.32	1.28	0.53	0.56						
MACYPFAM (Y_n)	18.25	18.78	18.43	18.60	*	0.524	NS	0.088	NS	0.002
±	0.24	0.34	0.39	0.43						
Calcium (mg kg^{-1})	8770	8792	8651	8911	NS	0.000	0.61	0.035	NS	0.007
±	726	798	551	906						
Potassium (mg kg^{-1})	760	742	812	690	NS	0.028	*	0.580	NS	0.061
±	101	76	34	78						
Magnesium (mg kg^{-1})	343	348	310	381	NS	0.010	**	0.687	NS	0.003
±	42	52	13	35						
Sodium (mg kg^{-1})	5679	5716	5360	6035	NS	0.001	NS	0.336	NS	0.027
±	639	649	352	352						
Phosphorous (mg kg^{-1})	5717	5832	5681	5868	NS	0.019	NS	0.047	NS	0.005
±	450	487	329	565						

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

1Abbreviations: PRG, perennial ryegrass; MSS, multispecies swards; SOL, stage-of-lactation; η2, effect-size, partial ETA2, RCT; Rennet coagulation time; FDM, Fat in Dry Matter, MNFS; Moisture in Non-Fat Substance, S/M; Salt in Moisture, MACY; Moisture Adjusted Cheese Yield, MACYPFAM; Moisture Adjusted Cheese Yield Adjusted for Protein and Fat

3.5. Conclusions

Within dairy production, pasture systems are often recognised for supporting environmental sustainability, and MSS grazing might offer additional sustainability benefits compared to typical PRG grazing. This research compared Cheddar derived from an MSS system to Cheddar derived from a PRG system, and for many of the investigated attributes the cheese obtained from these two differing pasture diets appears to be broadly comparable.

Within this study, MSS-derived milks had a significantly higher protein content, and significantly lower somatic cell count compared to PRG-derived milk. Furthermore, significantly increased cheese yields resulted when using MSS-derived milk in Cheddar production. These significant cheese yield differences were not only observable for actual yield, but remained when adjustment was made for moisture, protein and fat content. Consequently, it is suggested that MSS milk might have an increased economic value to cheese manufacturers. This study only investigated Cheddar cheese, and the extent to which these results apply beyond Cheddar will depend on the specific cheese type and manufacturing process, particularly regarding high-moisture, acid-coagulated or long-ripened cheeses where milk composition may influence yield and quality differently. Further research is still required, and conclusions should be drawn tentatively at this time. In particular, all results from this study could be further validated at commercial-scale.

Overall, these investigations have not indicated that practical adverse impacts result within milk composition and milk-processability when practicing MSS grazing. From a due-diligence perspective, it is hoped that these findings can contribute to an evidence-base that will support a sustainable future for all stakeholders within grazed-dairy food-systems.

3.6. References

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Chapter 4

The sensory and ripening characteristics of Cheddar cheese manufactured within a multispecies sward dairy food system

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4.1. Abstract

The implications of multispecies sward (MSS) grazing on sensory and quality characteristics of dairy products are inadequately understood. This study compared Cheddar cheese derived from MSS and perennial ryegrass (PRG) grazing, analysing for differences in ripening characteristics and sensory attributes. In this spring-calving production system, seasonality was considered by completing investigations at mid- and late-lactation. No meaningful differences in ripening attributes, free fatty acid profile, and nearly all volatile compounds, were associated with pasture type. Significantly higher toluene abundance was associated with MSS-derived cheese, which might provide an opportunity as an authentication biomarker. Pasture type was associated with some significant fatty acid profile differences, but greater differences resulted from seasonality. Consumer assessors found no significant differences between MSS and PRG-derived cheese, while semi-trained judges found significant differences in some sensory attributes. Within this study, MSS- and PRG-derived Cheddar appear practically equivalent, providing assurance that this more sustainable pasture type might be adopted without adverse impacts to this high-value commodity.

4.2. Introduction

Pasture-based dairy production is typically practiced in temperate regions with plentiful rainfall, such as Ireland and New Zealand, but global demand for more sustainable dairy production has also brought grazing into focus within countries that

historically practice confinement-based systems (Wilkinson *et al.*, 2020). Enhanced environmental sustainability and improved dairy product quality have been associated with pasture-grazing as compared to non-pasture alternatives (Wilkinson *et al.*, 2020; Elgersma, 2015; O'Callaghan *et al.*, 2017; Timlin *et al.*, 2023; Jaramillo *et al.*, 2021). However, current grazing practices typically rely on monoculture systems that reduce biodiversity and require inputs of nitrogen-based fertilisers (Walling and Vaneeckhaute, 2022). A potential solution to these challenges of pasture-grazing is the adoption of multispecies swards (MSS). Within MSS systems, dairy cows feed on mixtures of grasses, legumes and herbs simultaneously, and such sward mixes can enhance biodiversity (Beye *et al.*, 2022; Cooledge, 2022), improve soil health (Ikoyi *et al.*, 2023; Isbell *et al.*, 2017), and reduce chemical fertiliser use (Pijlman, 2024). A robust evidence-base for environmental benefits associated with MSS has been established, but research on how MSS may influence the quality of dairy products, and their acceptance by consumers, is more limited. This potential for dietary-induced change resulting from differing pasture compositions is an important issue that warrants further attention.

In 2023, Ireland produced 284.9 metric tonnes of cheese (CSO, 2024), primarily Cheddar; it is a widely consumed and economically important dairy product, and is consequently the main focus of this study. Cheddar undergoes a ripening period before sale, typically anywhere from three to twenty four months, and longer in some instances (Fox and Cogan, 2004). Fox and Cogan (2004) described how most of the characteristic differences between numerous varieties of rennet-coagulated cheeses,

such as Cheddar, develop during the ripening period. Important biochemical processes, such as proteolysis, lipolysis, and the formation of volatile organic compounds (VOC) and free fatty acids (FFAs) can impact the taste, texture and overall sensory acceptability of cheese (Fox and Cogan, 2004). Proteolysis plays an important role in developing cheese texture, and the hydrolysis of caseins into smaller peptides and free amino acids also directly contributes to the taste of cheese, with these molecules acting as precursors for the VOC that are highly relevant to cheese aroma (McSweeney and Maria José, 2000; Kilcawley *et al.*, 2018). However, Kilcawley *et al.* (2018) did note that the impact of cow diets on VOC levels in milk and cheese is only really practically meaningful to sensory perception above certain concentrations and odour activities. In addition, lipolysis is also an essential part of cheese flavour development, with flavour compounds occurring with the liberation of FFAs (McSweeney and Maria José, 2000), especially short- and intermediate-chain FFAs (Collins *et al.*, 2003).

Changes to dairy cow diets affect ripening-related pathways, altering the taste, aroma and textural profile of cheese (Kilcawley *et al.*, 2018; O'Callaghan *et al.*, 2017), but differences in cheese texture also result from the profile of fats that are present (Kilcawley et al., 2018). Milk derived from pasture-fed cows tends to be higher in monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), and cows fed on total mixed ration (TMR) diets produce milk higher in saturated fatty acids (SFA) (Rooney *et al.*, 2025; Timlin *et al.*, 2023). Typically, higher levels of SFAs present in cheese result in harder cheese, and harder cheese has been reported in investigations

into cheese derived from pasture systems (Coppa et al., 2011; Uzun et al., 2018). Additionally, differences in cheese texture have been associated with good quality, nutrient-rich pasture as compared to poorer quality pasture (Bovolenta et al., 2014; Aprea et al., 2016).

Colour is also an important attribute for consumer acceptance of dairy products (O'Callaghan *et al.*, 2017). Many consumers associate deeper yellow coloured cheese with enhanced cheese quality (Coppa *et al.*, 2011; O'Callaghan *et al.*, 2017). Coppa *et al.* (2011) found that pasture-derived cheese had a more intense yellow-red colour than cheese produced from hay and concentrates. O'Callaghan *et al.* (2017) observed yellower cheese from the milk of cows reared on typical PRG pasture diets as compared to a TMR diet. Yellowness is particularly associated with pasture-derived Cheddar, because it has an association with beta-carotene, both a plant derived pigment and pro-vitamin (O'Callaghan *et al.*, 2017; Page *et al.*, 2024; Timlin *et al.*, 2024; Page *et al.*, 2025b).

Within laboratory-based studies, individual characteristics of cheese tend to be determined in isolation, and the full complexity of human taste perception is not fully captured by analytically determined data. The full hedonic experience of cheese occurs through myriad interactions within a highly complex food matrix, and each consumer then experiences this through their own set of personal and cultural perceptions. This justifies a more holistic evaluation, and is the aim of sensory panel investigations. When comparing non-pasture and pasture grazing systems, O'Callaghan *et al.* (2017)

described how feeding system, and maturation period, both had a significant effect on Cheddar sensory profile. Sensory analyses can use both trained and untrained judges, each providing differing insights into acceptability (Kilcawley *et al.*, 2018). Comparing cheese derived from pasture and non-pasture diets, Kilcawley *et al.* (2018) suggested that the sensory differences in cheese arising through cow dietary change can be subtle. Furthermore, general cheese consumers are more likely to notice changes within cheese texture and visual appearance, such as cheese colour, while trained judges are more readily able to recognise differences within individual flavour attributes (Kilcawley *et al.*, 2018).

Changing dairy cow diets can influence multiple sensory and ripening related characteristics of cheese, but the full extent to which such impacts differ between MSS- and PRG-pasture types is unknown. Given current trends of consumer preference for pasture-derived products, addressing this research gap has become a pertinent consideration for the dairy industry. Consequently, this research utilised both laboratory- and sensory panel-based methods to compare MSS-derived and PRG-derived Cheddar. Stage of lactation (SOL) was also considered, within this spring-calving Irish pasture system, by investigating these factors during both mid- and late-lactation.

4.3. Materials and methods

4.3.1. Experimental design and cheese manufacturing

The cheese used within this study was produced as part of a separate research study which has been reported previously (Page *et al.*, 2025a), Chapter 3. Details on the compositional and processing attributes of these milks can also be found in Page *et al.* (2025a), Chapter 3.

4.3.2. pH

The pH was measured in cheese as described in Chapter 2. Measurements were taken at 1, 21, 90, and 180 d during ripening.

4.3.3. Colour analysis

Colourimetric analysis was performed according to the Commission Internationale de l'Eclairage, or CIELAB colorspace approach, described in Chapter 2. Measurements were taken at 1, 21, 90 and 180 d of ripening.

4.3.4. Texture profile analysis

Texture profile analysis was performed as described in Chapter 2. Measurements were taken at 1, 21, 90, and 180 d during ripening.

4.3.5. Proteolysis

The relative extent of primary proteolysis was determined between MSS- and PRG-derived cheeses, as described in Chapter 2, with measurements taken at 1, 21, 90, and 180 d during ripening.

4.3.6. Analysis of total fatty acids, free fatty acids and volatile compounds

The total fatty acid (TFA), free fatty acid (FFA) and volatile compound levels were determined in cheese at 180 d of ripening. Within this study, the TFA profiles of cheese were determined by gas chromatography flame ionization detection (GC-FID) according to the methods of De Jong and Badings (1990) with modifications as outlined in Mannion *et al.* (2016), and also similar to as described in O'Callaghan *et al.* (2020). The TFA profiles of the cheesemilks used to produce this cheese can be found in Page *et al.* (2025a), Chapter 3, and this determination also followed the same methodology. Cheese fatty acids data were also utilised for the determination of dietary indices. The atherogenic, thrombogenic, health promoting and desaturase indices were calculated as described by Timlin et al. (2023b), with Timlin et al. (2023b) referencing Ulbricht and Southgate (1991) and the calculation method of O'Callaghan et al. (2016) for the atherogenic and thrombogenic indices, and Chen et al. (2004) and (Kay et al., 2005) for the calculation of the health promoting and desaturase indices, respectively. Multivariate analysis of the cheese sample fatty acid profiles were carried out using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/) (Pang et al., 2024). Data was initially uploaded, log transformed and auto-scaled and principal component analysis (PCA) conducted on the processed data matrix with scores plots generated for PC1 and PC2. Group confidence ellipses were displayed at the 95 % confidence level. The volatile compounds were determined using headspace solid phase micro extraction (HS SPME), followed by gas-chromatography-mass-spectrometry analysis (GC-MS), as described in Chapter 2.

4.3.7. Sensory profile analysis

Sensory acceptance testing of mid-lactation cheese was completed at the end of the 180d maturation period (Stone and Sidel, 2004; Stone *et al.*, 2012). The analysis was completed under White light (1000 Lux) using 25 untrained assessors recruited at University College Cork (16 male, 9 female, aged 20-55 years). All individuals described themselves as being Cheddar cheese consumers.

The investigated hedonic attributes were appearance, aroma, flavour, texture and overall acceptance. The analysis was carried out in panel booths, conforming to international standards (ISO 8589: 2007). Samples were stored at 4˚C until required, before presentation to the assessors at ambient temperatures (21°C) and coded with a randomly selected three digit code. Each assessor was provided with deionised water and were instructed to cleanse their palates between tastings. Assessors indicated their degree of liking on a 10 cm line scale ranging from 0 (extremely dislike) at the left to 10 (extremely like) at the right, with a rating subsequently scored in cm from left. The order of the presentation of all test samples was randomized to prevent first order and carryover effects (MacFie *et al.,* 1989).

In addition, an optimized descriptive profiling (ODP) method was applied; obtaining sensory descriptions through the utilisation of semi-trained judges (da Silva et al. 2012, 2013). The reported results are the mean data obtained from 25 assessors, with this assessment having been completed in triplicate. Assessors were presented with samples simultaneously, but in a randomised order to prevent first order and carry-

over effects (MacFie et al. 1989). Assessors used a consensus list of sensory descriptors, measured on a 10 cm line scale with the term "none" used at the 0 cm end of the scale and "extreme" for the 10 cm end of the scale. For this study, training and the use of a consensus sensory lexicon were used as similarly described by Cheng et al. (2023). Sensory terms, which were the main sensory dimensions, were pre-selected using an expert sensory panel (n = 10). Assessors evaluated the intensity of each attribute for each sample on the scales. Samples were also prepared in the same manner as within the previously described consumer analysis.

4.3.8. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 27 (IBM Statistics Inc.), with some processing and formatting of data carried out in Microsoft Excel 64 bit, 2016 Version (Microsoft Corporation Inc.) and GraphPad Prism version 9 (GraphPad Software Inc.). The reported data are the overall averages of results obtained from three separate cheesemaking trials completed within a mid-lactation study, and three additional cheesemaking trials completed within a late-lactation study (n=6). Results are presented as the mean average ± standard deviation. Univariate ANOVA were used for the comparison of means in single factors. For each cheese ripening attribute, at each time-point during the cheese ripening process (1 d, 21 d, 90 d and 180 d), the between-subjects effects of diet, seasonality and their interactions were interpreted through a general linear model (GLM) analysis of variance (ANOVA) procedure in SPSS. When applying this statistical model, Levene's test was also applied to confirm

homogeneity of data. Additionally, a repeated-measures GLM ANOVA model was performed in SPSS to interpret statistical relationships between changes in cheese attributes over the ripening period with the treatment factors of diet and season. Tests of within-subjects effects were utilised to determine whether significant ripening related changes had occurred for that given attribute across the ripening period, and to determine whether any significant effects arose on that dependent variable from interactions with the independent variables of diet and season. Sphericity of data was also confirmed within this model through Mauchly's Test, and when sphericity of data was violated Greenhouse-Geisser corrected values were instead used. For all statistical analyses, P-values < 0.05 were considered significant, and effect sizes were also calculated as partial eta squared ($\eta 2$). A $\eta 2 < 0.06$ was considered a small effect size, $0.06 \leq \eta 2 < 0.14$ was considered a medium effect size, and $\eta 2 \geq 0.14$ considered a large effect size.

4.4. Results and discussion

4.4.1. The pH changes in cheese during the maturation period

Throughout ripening, all cheese pH values were within the range of 5.00-5.35, which are typical of Cheddar cheese (Table 4.1). The pH of cheese increased significantly as it ripened with a large effect size ($P < 0.001$, $\eta 2 = 0.913$), and such ripening-related changes in cheese are to be expected (Lamichhane *et al.*, 2018a). Additionally, timing within the milking season and pasture type did have some statistically significant

impacts on the pH of cheese ($P < 0.05$). Regarding the investigated diets, a repeated pattern of higher pH values can be seen for MSS cheese as compared to PRG cheese at each ripening time point, but the effect of diet on cheese pH was only statistically significant when measured in isolation at 180 d of ripening ($P < 0.05$, $\eta 2 = 0.551$). Additionally, when interpreting this data via a repeated measures ANOVA model, over the entire ripening period, no significant interaction between diet type and ripening was observed ($P > 0.05$); the diet variable did not contribute significantly to the ripening related changes in pH ($P > 0.05$). Late-lactation cheese consistently showed significantly higher pH values than mid-lactation cheese when analysed at each individual ripening time point ($P < 0.01$). However, when comparing the overall within-subjects effects through repeated measures ANOVA, no significant difference was observed between the mid-lactation and late-lactation periods ($P > 0.05$). The repeated-measures ANOVA suggests that the independent variable of season does not significantly alter how pH develops with time, but the analysis of between-subject-effects shows that the pH of late-lactation cheese is significantly higher at every ripening time point. Essentially, late-lactation manufactured cheese had a pH that was initially higher from the point of production, and at the beginning of the ripening period, and pH then remains higher by an equivalent amount at each ripening time point (the initial pH is significantly impacted by seasonality, but, the rate of change of pH during ripening does not appear to be significantly impacted by seasonality). No significant statistical interactions were found between the investigated independent variables of diet and season ($P > 0.05$). It should be recognised that whilst some

statistically significant differences in pH were observed during these investigations, it is unlikely that these outliers will be of adverse practical consequence to cheese as a foodstuff; all pH values were within the range of acceptable values for Cheddar cheese, and the magnitude of any differences measured were small.

Table 4.1: The development of pH in Cheddar cheese over 180 days of ripening; cheese obtained from multispecies sward (MSS) and perennial ryegrass (PRG) diets, manufactured at both mid-lactation and late-lactation

	Mid-lactation						Late-lactation						Effect of Diet		Effect of Season		Diet x Season	
	PRG			MSS			PRG			MSS			P	η2	P	η2	P	η2
1 d	5.00	±	0.07	5.04	±	0.04	5.16	±	0.07	5.16	±	0.05	NS	0.045	**	0.707	NS	0.03
21 d	5.14	±	0.02	5.15	±	0.02	5.23	±	0.02	5.28	±	0.03	NS	0.399	***	0.899	NS	0.23
90 d	5.20	±	0.02	5.21	±	0.01	5.27	±	0.02	5.29	±	0.02	NS	0.258	***	0.871	NS	0.11
180 d	5.23	±	0.01	5.25	±	0.01	5.32	±	0.02	5.35	±	0.03	*	0.551	***	0.927	NS	0.06

*** P < 0.05, ** P < 0.01, *** P < 0.001 (Significant), NSP > 0.05 (non-significant)**
Effect size measured as the partial eta squared (η2)

4.4.2. Cheese colour development during the maturation period

Ripening time had a significant effect on the colour attributes of Cheddar cheese ($P < 0.05$) (Table 4.2). The average L* values observed in these experiments ranged between ~77 and 88, the a* values were in the range ~-4 to -5.5, and b* values ranged ~27 to 33. Similar colourimetry values have been reported previously for Cheddar cheese derived from pasture diets, and all of these measurements fall within the range of expected values for Cheddar (Page *et al.*, 2024; O'Callaghan *et al.*, 2017; Page *et al.*, 2025b). No significant differences in colour parameters were observed between MSS and PRG cheese at any of the ripening time points ($P > 0.05$) (Table 4.2). Regarding

SOL, some significant differences in cheese colour were observed for specific measurements within the ripening period ($P < 0.05$), but most measurements showed no significant differences ($P > 0.05$). No significant statistical interactions were observed between pasture type and SOL ($P > 0.05$).

Regarding change in cheese colour during maturation, most of these ripening-related changes occurred within the first 21 d, with only more limited changes in colour occurring during the remainder of the ripening period. Overall, the within-subjects repeated measured ANOVA statistical model showed that the L*, a*, b* parameters all changed significantly during the ripening period ($P<0.001$) (Table 4.2). The L* parameter decreased significantly during ripening ($P < 0.001$, $\eta2= 0.884$).; cheese developed a darker appearance as it matured, particularly during early ripening. The a* parameter also changed significantly with ripening time ($P < 0.001$, $\eta2 = 0.895$), indicating an increase in red tone as the cheese matured. Whilst this difference was highly statistically significant, the magnitude of the change was only very slight. The b* values also increased significantly with ripening time ($P < 0.001$, $\eta2 = 0.932$), and the most substantial rise in yellow colouration was observed between 1 d and 21 d. Overall, during ripening, the main observable trend within colour for all cheeses was an increase in yellowness and a darkening of the cheese. Previous studies have also reported similar ripening trends for cheese colour (Page *et al.*, 2024; O'Callaghan *et al.*, 2017).

Table 4.2: The development of colour in Cheddar cheese over 180 days of ripening; cheese obtained from multispecies sward (MSS) and perennial ryegrass (PRG) cow diets, manufactured at both mid-lactation and late-lactation

		Mid-lactation		Late-lactation		Effect of Diet		Effect of Season		Diet*Season Interaction	
		PRG	MSS	PRG	MSS	P	η2	P	η2	P	η2
L*	1 d	88.01 ± 0.72	87.59 ± 1.13	83.80 ± 3.10	85.40 ± 3.21	NS	0.024	*	0.421	NS	0.067
	21 d	80.13 ± 1.33	77.57 ± 2.52	77.81 ± 0.71	77.56 ± 0.52	NS	0.342	NS	0.262	NS	0.26
	90 d	77.65 ± 4.13	77.00 ± 4.08	78.00 ± 0.77	79.02 ± 2.86	NS	0.002	NS	0.058	NS	0.03
	180 d	78.28 ± 1.39	79.16 ± 0.50	77.78 ± 0.85	79.52 ± 1.33	NS	0.356	NS	0.002	NS	0.055
a*	1 d	-4.07 ± 0.18	-4.35 ± 0.20	-4.26 ± 0.35	-4.36 ± 0.43	NS	0.128	NS	0.04	NS	0.03
	21 d	-5.19 ± 0.12	-5.18 ± 0.14	-5.37 ± 0.26	-5.40 ± 0.05	NS	0	NS	0.357	NS	0.005
	90 d	-4.13 ± 0.24	-4.08 ± 0.03	-5.00 ± 0.08	-4.93 ± 0.18	NS	0.002	NS	0.054	NS	0.053
	180 d	-4.56 ± 0.19	-4.82 ± 0.39	-4.97 ± 0.15	-5.06 ± 0.01	NS	0.174	*	0.429	NS	0.053
b*	1 d	27.00 ± 0.73	27.62 ± 0.12	28.20 ± 0.52	27.45 ± 0.89	NS	0.004	NS	0.196	NS	0.304
	21 d	32.64 ± 0.99	31.32 ± 0.75	32.28 ± 1.43	32.18 ± 1.20	NS	0.13	NS	0.019	NS	0.1
	90 d	31.26 ± 1.07	30.83 ± 0.57	32.58 ± 0.81	32.47 ± 0.71	NS	0.04	*	0.555	NS	0.014
	180 d	30.73 ± 1.48	30.98 ± 0.25	31.12 ± 1.07	31.31 ± 1.11	NS	0.016	NS	0.04	NS	0

*** P < 0.05, ** P < 0.01, *** P < 0.001 (Significant), NSP > 0.05 (Non-significant)**

Effect size measured as the partial eta squared (η2)

Regarding the effect of seasonality on cheese colour, the day one measurement of the L*parameter was significantly lower for late-lactation cheese, the a* parameter was significantly lower for late-lactation cheese at 180 d of ripening, and the b* parameter was significantly higher for late-lactation cheese at 90 d of ripening ($P<0.05$). However, in each of these examples the cheese colour differences appear to be one off differences within the ripening period, and although statistically significant they are also of small magnitude. No other significant differences in colour were observed as a result of seasonality at any of the other times measured in the maturation period ($P<0.05$). When analysed through a within-subjects repeated measured ANOVA model, seasonality did not contribute significantly to the ripening related changes in the colour attributes ($P>0.05$) (Table 4.2).

Regarding the impact of cow diet on cheese colour, the MSS diet treatment did not have a significant impact on the colour attributes (L*, a* and b*) of Cheddar cheese at any individual ripening time point ($P > 0.05$), and when analysed through a within-subjects repeated measured ANOVA model, the diet variable did not contribute significantly to the ripening related changes observed in any of the colour attributes ($P>0.05$). Colour is an important quality characteristic in cheese; it can be relevant to taste perception, can influence consumer purchasing decisions, and previous research had shown that differences in cheese colour can be associated with differences in dairy cow diets (O'Callaghan *et al.*, 2017; Kilcawley *et al.*, 2018). Previously, O'Callaghan *et al.* (2017) had observed colour differences in cheese when it was derived from TMR diets as compared to pasture. Within this study, no significant difference in colour was

seen as a result of these differing pasture compositions, and the yellow colouring of dairy products does have a particular association with pasture-fed milk. Yellow colour is also regarded as positive from a nutrition and health perspective, especially when associated with increased levels of β-carotene which is both a pigment and a pro-vitamin (O'Callaghan *et al.*, 2017; Page *et al.*, 2024). Cheese colour has an important role in consumer perception and acceptance of dairy products (Kilcawley *et al.*, 2018), and, because MSS and PRG derived Cheddar did not differ significantly in colour attributes, it appears that the favoured colour attributes associated with cheese derived from typical grazing systems of PRG are still maintained in cheese manufactured from a MSS system.

4.4.3. Cheese texture development during the maturation period

The texture parameters of fracturability, hardness, resilience, cohesiveness and springiness all decreased significantly during ripening ($P < 0.05$) (Table 4.3). These ripening-related changes within cheese are expected, and arise because of proteolysis, pH changes and solubilisation of colloidal calcium, they are typical and have been reported previously (Lamichhane *et al.*, 2018a; O'Callaghan *et al.*, 2017; Lamichhane *et al.*, 2019).

The overall impact of pasture type on cheese texture was quite limited (Table 4.3). A pattern of harder texture values for MSS derived Cheddar as compared to PRG Cheddar is observable, but these differences did not meet the threshold for statistical significance ($P > 0.05$). Similar results were also obtained for nearly all comparisons of

fracturability between MSS and PRG cheese ($P > 0.05$). The resilience value of MSS Cheddar at 1 d was significantly higher than that of PRG Cheddar with a large effect size ($P < 0.05$, $\eta 2 = 0.473$); however, all measurements of resilience taken later in the ripening period showed no significant difference between the pasture types ($P > 0.05$). As Cheddar cheese would not be consumed within the first days after production this early ripening difference should not be of practical consequence. Additionally, any texture data obtained so early in the ripening period should be approached with caution, and measurements at this stage will likely not accurately reflect the true texture profile of the cheese (salt equilibration is unlikely to be complete so early within cheese ripening, and texture values may reflect localised salt and moisture gradients). No significant difference was seen in the cohesiveness and springiness parameters between the two pasture treatments ($P > 0.05$). Seasonality did not significantly impact any of the measured texture parameters ($P > 0.05$), and no significant interactions were observed between diet and seasonality ($P > 0.05$). Additionally, when these texture values were analysed through the alternative repeated-measures within-subjects statistical model, no significant differences were observed ($P > 0.05$). No interaction is suggested to be taking place between the texture ripening characteristics and the variables of diet and season, and diet and season do not significantly contribute to any changes to the rate of ripening for any of the measured texture parameters ($P > 0.05$).

Table 4.3: The change in Cheddar cheese texture over 180 days of ripening; cheese derived from multispecies sward (MSS) and perennial ryegrass (PRG) diets at both mid-lactation and late-lactation

	Mid-lactation		Late-lactation		Diet		Season		Interaction	
	PRG	MSS	PRG	MSS	P	η2	P	η2	P	η2
Fracturability (g)										
1 d	31820 ± 15199	38552 ± 11265	36404 ± 10483	38071 ± 7352	NS	0.048	NS	0.012	NS	0.018
21 d	26758 ± 5542	29987 ± 3604	30098 ± 2819	34808 ± 3995	NS	0.259	NS	0.27	NS	0.012
90 d	21311 ± 4489	24768 ± 4578	26576 ± 3243	28533 ± 5585	NS	0.117	NS	0.27	NS	0.01
180 d	20212 ± 4415	26189 ± 3311	25271 ± 3994	23222 ± 2736	NS	0.097	NS	0.03	NS	0.31
Hardness (g)										
1 d	42277 ± 10650	44916 ± 13149	38236 ± 10105	38794 ± 6889	NS	0.009	NS	0.082	NS	0.004
21 d	28538 ± 3698	30484 ± 3182	31466 ± 2285	35647 ± 3786	NS	0.245	NS	0.361	NS	0.041
90 d	26426 ± 4827	27567 ± 6183	31803 ± 3383	33230 ± 6628	NS	0.021	NS	0.281	NS	0
180 d	26751 ± 6825	29885 ± 5338	28813 ± 2530	29446 ± 3229	NS	0.055	NS	0.011	NS	0.025
Resilience										
1 d	10.69 ± 0.92	13.08 ± 1.63	10.53 ± 1.23	14.35 ± 3.33	*	0.473	NS	0.028	NS	0.046

21 d	7.99 ± 1.12		8.35 ± 1.17		7.64 ± 0.04		9.58 ± 1.81		NS	0.25	NS	0.047	NS	0.137
90 d	5.37 ± 0.45		5.65 ± 0.64		5.05 ± 0.02		5.41 ± 0.64		NS	0.13	NS	0.102	NS	0.003
180 d	4.30 ± 0.96		4.18 ± 0.44		4.22 ± 0.40		4.36 ± 0.45		NS	0	NS	0.003	NS	0.016
Cohesiveness														
1 d	0.25 ± 0.02		0.28 ± 0.03		0.24 ± 0.01		0.30 ± 0.05		NS	0.383	NS	0.009	NS	0.144
21 d	0.24 ± 0.02		0.24 ± 0.04		0.21 ± 0.01		0.24 ± 0.03		NS	0.133	NS	0.093	NS	0.176
90 d	0.18 ± 0.01		0.18 ± 0.01		0.18 ± 0.01		0.21 ± 0.02		NS	0.226	NS	0.13	NS	0.325
180 d	0.14 ± 0.03		0.15 ± 0.02		0.15 ± 0.01		0.17 ± 0.01		NS	0.121	NS	0.177	NS	0.121
Springiness (%)														
1 d	97.60 ± 45.02		72.73 ± 1.14		72.45 ± 6.12		72.97 ± 4.65		NS	0.096	NS	0.1	NS	0.104
21 d	52.95 ± 12.54		55.90 ± 5.08		54.63 ± 6.92		55.85 ± 2.00		NS	0.027	NS	0.004	NS	0.005
90 d	37.25 ± 7.10		45.27 ± 7.63		44.34 ± 3.80		43.43 ± 4.64		NS	0.116	NS	0.067	NS	0.171
180 d	28.13 ± 6.33		35.23 ± 9.96		37.90 ± 3.21		35.53 ± 0.88		NS	0.053	NS	0.202	NS	0.183

*** P < 0.05, ** P < 0.01, *** P < 0.001 (Significant), NSP > 0.05 (non-significant)**

Effect size measured as the partial eta squared (η2)

Overall, some small magnitude differences in cheese texture were observed to result from the MSS pasture diet, but only the resilience value measured at 1 d met the threshold for being a statistically significant difference. Overall, in the context of practical functional applications for cheese, the texture attributes of MSS and PRG Cheddar do appear to be broadly comparable.

4.4.4. Proteolysis in cheese during the maturation period

Primary proteolysis is expressed as the percentage of total nitrogen soluble at pH 4.6 (pH4.6-SN/TN), Table 4.4. Proteolysis increased significantly with time ($P < 0.05$), and this is attributed to the action of residual coagulant (e.g. chymosin), indigenous milk proteases (e.g. plasmin) and proteolytic enzymes from starter lactic acid bacteria (e.g. intracellular peptidases). However, no significant differences in primary proteolysis were observed as a result of SOL or cow diet ($P > 0.05$), and the extent of primary proteolysis within all cheese investigated was consistent with previously reported ranges of results; the values are typical for Cheddar cheese (Fenelon and Guinee, 2000). Fenelon and Guinee (2000) reported that levels of intact casein in cheese decrease with ripening time at about 0.022g per 100g of cheese per day. Proteolysis has an important role in cheese texture and flavour development (Fox, 1989), and these proteolysis results seem to align well with the previous discussion of cheese texture within this study, providing further validation of the overall findings.

Table 4.4: The change in primary proteolysis of Cheddar cheese over 180 days of ripening; cheese obtained from multispecies sward (MSS) and perennial ryegrass (PRG) cow diets, manufactured at both mid-lactation and late-lactation

	Mid-lactation		Late-lactation		Effect of Diet		Effect of Season		Diet x Season Interaction	
	PRG	MSS	PRG	MSS	P	η2	P	η2	P	η2
Day 1	2.23 ± 0.21	2.69 ± 0.49	2.47 ± 0.08	2.50 ± 0.19	NS	0.22	NS	0.003	NS	0.17
Day 21	5.81 ± 0.48	7.12 ± 2.81	4.93 ± 0.28	5.79 ± 0.91	NS	0.163	NS	0.169	NS	0.008
Day 90	12.64 ± 0.68	15.52 ± 3.34	11.45 ± 0.83	11.86 ± 1.02	NS	0.232	NS	0.398	NS	0.146
Day 180	16.98 ± 1.56	20.59 ± 3.20	16.85 ± 1.40	17.55 ± 1.55	NS	0.291	NS	0.181	NS	0.157

*** P < 0.05, ** P < 0.01, *** P < 0.001 (Significant), NSP > 0.05 (Non-significant)**
Effect size measured as the partial eta squared (η2)

4.4.5. The volatile organic compounds in cheese after a 180d maturation period

The VOC profile of cheese was measured after 180 d of ripening, reflective of a typical maturation period prior to consumption. Taste and odour are important sensory characteristics of cheese, and the profile of volatile compounds present contributes to the quality of the cheese and sensory experience of the consumer. In this study, 45 volatile organic compounds were identified in Cheddar cheese after 180 d maturation. These consisted of 6 acids, 6 alcohols, 7 aldehydes, 5 benzenes, 1 diol, 7 esters, 1 ether, 9 ketones, 2 pyrazines, 1 sulfur compound and 1 terpene (Table S4.1)

Most volatile compounds in MSS and PRG cheese were not impacted significantly by the different pasture type ($P > 0.05$). Regarding this diet factor, only toluene showed a significant difference, the detected toluene abundances are reported in Table S4.1, and a significant increase in toluene was found to be associated with the MSS diet ($P < 0.001$, $\eta2=0.860$). Higher levels of toluene have already been reported in cheese produced from grass and clover derived diets as compared to milk resulting from a TMR diet (O'Callaghan *et al.*, 2017). Toluene was described by O'Callaghan *et al.* (2017) as being present in cheese as a product of β-carotene oxidation. Toluene has also been

identified as a possible biomarker for dairy products derived from pasture systems (Kilcawley *et al.*, 2018), so, it is noteworthy that within this research, toluene is present at higher levels within the MSS cheese as compared to PRG cheese. However, while other work has attributed the presence of toluene in cheese to the oxidation of β-carotene derived from forage, it should be noted that β-carotene content was not measured directly in cheese in this current study.

All other volatile compounds recorded showed no significant difference associated with an MSS diet as compared to PRG ($P > 0.05$). Overall, an MSS cow diet had only a very limited impact on volatile compound levels, apart from toluene which was detected at higher abundance in MSS cheddar as compared to PRG, but still at levels that were overall relatively low. Toluene has been described as having only a limited impact upon sensory perception because of a high odour threshold, but it does have a nutty, bitter almond or plastic odour at high levels (Kilcawley *et al.*, 2018). An overabundance of this odour could have some sensory impact in cheese, but sensory panel investigations were also carried out within this work, and the insights obtained within those investigations provide an additional direct assessment of the consumer sensory experience of these cheeses. Additionally, a significant interaction was found between diet and SOL for toluene abundance ($P < 0.05$, $\eta 2 = 0.58$).

Further to the diet factor, most volatile compounds were also not significantly impacted by SOL ($P > 0.05$). The compounds 1-butanol and 2-hexanone were only detected at mid-lactation, while 1-hexanol was only detected at late-lactation. The

abundance of pentanal and 2,3-butanedione were significantly higher at late-lactation as compared to mid-lactation ($P < 0.05$), while toluene, butanoic acid, decanoic acid, ethanol and acetone were detected at significantly higher abundance in mid-lactation Cheddar ($P < 0.05$). Pentanal, 2, 3 butanadione and buatnoic acid are important flavour compounds with relatively low odour thresholds that can impact the sensory quality of cheese. Of particular relevance are butanoic acid, with cheesy and rancid notes, and 2,3 butanedione with buttery and creamy notes. Due to availability of resources, sensory testing was only performed on mid-lactation cheese; the period typically most relevant within cheese production. Consequently, these experimentally determined differences in volatile compounds associated with SOL could not be corroborated through the sensory profile analysis.

Overall, in conclusion, regarding levels of VOCs in cheese within this study, it appears that SOL has a greater relevance to the volatile profile of Cheddar than any differences resulting between the two different pasture types.

4.4.6. Cheese FFA profile after a 180d maturation period

Cheese flavour is influenced by the profile of FFAs that are present, and differences in FFAs between cheese samples can provide indications of differences in dairy product quality. The FFA profile of cheese was determined after 180d of ripening to reflect a typical maturation period prior to consumption (Table S4.2). No significant differences in FFA levels were observed between the MSS and PRG diets ($P > 0.05$). Some significant differences in FFAs were associated with SOL, in particular significantly

higher levels of C10:0 and C12:0 were observed at late-lactation ($P < 0.05$). Other FFAs were also lower at mid-lactation than at late-lactation, but these differences did not meet the threshold for statistical significance ($P > 0.05$). It should be noted that the variance of results between individual trials was high. For all cheese investigated, the range of observed values for FFAs were within the expected range for Cheddar cheese (Kilcawley *et al.*, 2007).

4.4.7. Cheese total fatty acid profile after the 180 d maturation period

The TFA profile of MSS and PRG derived cheddar, and mid- and late-lactation Cheddar, are shown in Table 4.5. The TFA composition of the cheese was measured after 180 d of ripening, reflective of a typical industrially-relevant maturation period applied to Cheddar prior to consumption. The profile of fatty acids present in cheese has relevance to both final product quality and the nutritional value of the food (Feeney *et al.*, 2016; O'Callaghan *et al.*, 2017). A small number of, mostly low magnitude, significant differences in cheese TFA profile were found to be associated with pasture type, while a greater number of significant differences were found to be associated with SOL ($P < 0.05$). A small number of statistically significant interactions were also observed between SOL and diet ($P < 0.05$).

Regarding seasonality, mid-lactation Cheddar on average contained significantly higher levels of C14:0, C16:0, C17:0, C17:1, and C18:1 TFA ($P < 0.05$); saturated or monounsaturated longer chain fatty acids. Cheese derived from late-lactation milk exhibited significantly higher levels of C18:2n6c, C18:2n6t, C18:3, C18:3n3, C20:3n6,

and C20:4n6 ($P < 0.05$). So, cheese manufactured later in the milking season seems to be associated with higher levels of polyunsaturated fatty acids (PUFA). Health-related indices were also calculated, and these were significantly impacted by SOL. The desaturase index is a ratio used to estimate the activity of desaturase enzymes, the atherogenic and thrombogenic indices are measures of a foods propensity to promote or protect against coronary heart disease, and the health-promoting index is the inverse of the atherogenic index (Ulbricht and Southgate, 1991; Timlin et al., 2023; Chen et al., 2004). The atherogenic, thrombogenic, health promoting, and desaturase index values all differed significantly between mid- and late-lactation cheeses ($P<0.05$). Late-lactation cheese showed more favourable values; lower atherogenic and thrombogenic indices, and higher health promoting and desaturase indices. Although late-lactation cheese showed more favourable fatty-acid index values, this alone does not justify a general recommendation to preferentially use late-lactation milk for cheesemaking. Cheesemakers must balance nutritional differences against practical considerations such as milk volume, seasonal composition and coagulation behaviour (Banks and Tamime, 1987; Timlin et al., 2023).

The impact of pasture type on TFA profile was far less pronounced than SOL, but several significant differences were still noted. MSS-derived cheese showed significantly higher levels of C18:2n6c, C20:3n6 and C18:3n3 ($P < 0.05$); these are omega-6 and omega-3 fatty acids. Significantly higher levels of C22:1, C23:0 and C18:1 conjugated fatty acids (CFA) were also observed to be associated with the MSS diet. However, it should be recognised that while the C22:1 and C23:0 differences were

calculated as significant, these fats are only actually present at very low levels, and the result does not appear to be practically meaningful. Regarding the calculated health indices, the desaturase index values were very similar between cheese obtained from the two diet treatments. However, a significantly lower thrombogenic index value was found for MSS derived cheese ($P<0.05$), suggesting the possibility of some limited nutritional difference between cheese produced from the two pasture systems. Likewise, MSS-derived cheese was also associated with a lower atherogenic index and a higher health promoting index, but these differences did not meet the threshold for statistical significance.

Regarding statistically determined interaction effects, a significant interaction was found between SOL and pasture type for C14:0, C18:3n3 and C18:1 CFA content ($P < 0.05$). Additionally, a significant interaction was found between SOL and pasture type for two of the dietary indices, i.e., the thrombogenic and health promoting indices ($P < 0.05$). On average, an MSS diet resulted in more favourable dietary index values for cheese, but these impacts mostly occurred later in the milking season. For example, at mid-lactation PRG and MSS Cheddar showed similar health promoting index values of 0.23 and 0.22, respectively, but at late-lactation these values differed, and were 0.25 and 0.31 respectively. Likewise, regarding the thrombogenic index, with PRG and MSS Cheddar showing values of 4.28 and 4.30 respectively at mid-lactation, but at late-lactation PRG Cheddar had a thrombogenic index value of 3.89 compared to 2.99 for MSS derived Cheddar.

A further analysis of this fatty-acid-profile data was completed through a principal component analysis (PCA), Fig. 4.1. The presented scores plot provides further visual confirmation regarding the previous discussion on this data. A clearer separation can be seen based on SOL than by pasture type. The mid- and late-lactation treatments are clearly separated within the PRG and MSS groupings by the second principal component (PC2), and this separation indicates a consistent SOL impact on fatty-acid-profiles for each cow diet group. Regarding pasture type, a substantial overlap is present for the MSS and PRG groupings, suggesting that the impacts of this dietary difference on the overall fatty-acid-profile of this cheese is less pronounced. It is less possible to differentiate between the groups based on dietary influence on fatty-acids-profiles, particularly as compared to the relative differences associated with SOL.

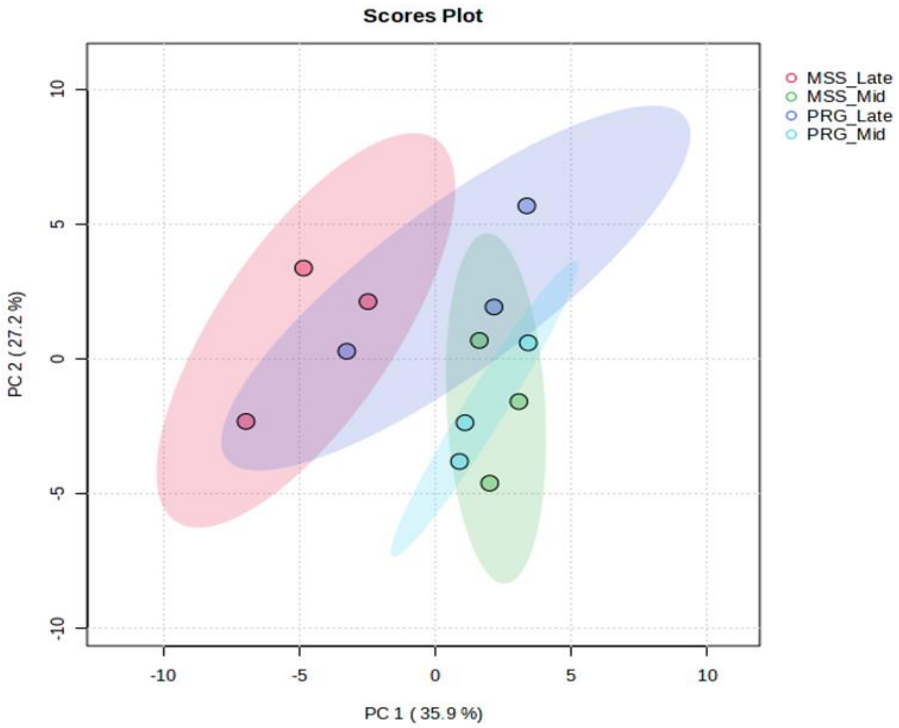


Fig. 4.1. Effect of diet and stage of lactation on the total fatty acid profile of Cheddar cheese at 180d of ripening. The principal components are obtained from the averaged data from 3 replicate cheese trials completed at mid- and late- lactation.

In a previous study, O'Callaghan *et al.* (2017) described significant differences in TFA profile between pasture-derived Cheddar and that derived from a total-mixed-ration (TMR) diet. In the current study, some significant differences in fatty acid profile have been observed between the differing pasture types of MSS and PRG, but these differences are much less meaningful than those recognised between PRG and TMR by O'Callaghan *et al.* (2017). Indeed, the overriding conclusion is that the TFA profile of cheese derived from the two pasture diets of MSS and PRG are broadly comparable. Given that PRG-derived fatty acids profiles have already been noted for their benefits to health (O'Callaghan *et al.*, 2017; Elgersma, 2015), it is perhaps encouraging that MSS-derived cheese does not differ significantly from PRG in its composition for most of these fatty acids. Additionally, the results of this study can be compared back to the previous investigations completed on the cheesemilk used to produce this cheese (Page *et al.*, 2025a). Similarly, this previous Page *et al.* (2025a) study found very limited differences in TFA profile between PRG and MSS derived milks. This provides further assurance that the impacts of these two grazing systems on product quality are in practical terms relatively limited. Indeed, within these investigations SOL was found to result in more significant impacts to TFA profile than diet, and in some cases, SOL also showed an interaction with diet ($P<0.05$). This interaction is particularly notable for the thrombogenic index and health promoting index, and through these measures, a more favourable fatty acid profile appears to be obtained for MSS, as compared to PRG, under the specific circumstances of an MSS grazing system being applied at late-lactation.

Table 4.5: Mean total-fatty-acid profile of Cheddar cheese obtained from MSS and PRG diet, and stage of lactation, treatments (± standard deviation)

	Treatment[1] (g/100 g fat)				Statistical Interpretation					
	PRG	MSS	MID	LATE	SOL P-VALUE	η2	Diet P-VALUE	η2	SOL x Diet P-Value	η2
C4:0	4.55 ± 0.83	4.42 ± 0.72	4.26 ± 0.97	4.72 ± 0.40	NS	0.105	NS	0.009	NS	0.02
C6:0	2.48 ± 0.43	2.34 ± 0.34	2.26 ± 0.44	2.56 ± 0.24	NS	0.179	NS	0.042	NS	0.011
C8:0	1.48 ± 0.23	1.38 ± 0.15	1.36 ± 0.16	1.50 ± 0.21	NS	0.173	NS	0.092	NS	0.097
C10:0	3.44 ± 0.52	3.21 ± 0.31	3.24 ± 0.27	3.40 ± 0.56	NS	0.05	NS	0.104	NS	0.224
C11:0	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	NS	0	NS	0.011	NS	0.095
C12:0	3.96 ± 0.44	3.73 ± 0.39	3.86 ± 0.21	3.83 ± 0.58	NS	0.002	NS	0.113	NS	0.299
C13:0	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	NS	0.029	NS	0.003	NS	0.21
C14:0	11.61 ± 0.61	11.10 ± 1.34	11.88 ± 0.60	10.83 ± 1.14	*	0.47	NS	0.17	*	0.511
C14:1	1.07 ± 0.11	1.01 ± 0.17	0.98 ± 0.04	1.11 ± 0.18	NS	0.25	NS	0.071	NS	0.029
C15:0	1.15 ± 0.08	1.20 ± 0.16	1.17 ± 0.11	1.17 ± 0.15	NS	0	NS	0.076	NS	0.304
C16:0	30.72 ± 2.53	30.09 ± 2.68	32.40 ± 1.81	28.40 ± 0.96	**	0.727	NS	0.062	NS	0.088
C16:1	1.65 ± 0.34	1.72 ± 0.22	1.57 ± 0.30	1.80 ± 0.21	NS	0.195	NS	0.02	NS	0.009
C17:0	0.58 ± 0.06	0.59 ± 0.07	0.63 ± 0.05	0.54 ± 0.03	**	0.647	NS	0.009	NS	0.036
C17:1	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	***	0.783	NS	0.286	NS	0.091
C18:0	9.12 ± 1.14	9.07 ± 1.30	9.01 ± 0.70	9.17 ± 1.58	NS	0.005	NS	0	NS	0.002
C18:1 TFA	2.75 ± 0.61	2.88 ± 0.62	2.45 ± 0.36	3.17 ± 0.58	*	0.423	NS	0.025	NS	0.073
C18:1n9c	17.57 ± 1.53	17.97 ± 1.70	17.01 ± 1.60	18.54 ± 1.17	NS	0.318	NS	0.031	NS	0.217
C18:1n9t	0.15 ± 0.03	0.17 ± 0.05	0.13 ± 0.02	0.19 ± 0.02	***	0.812	NS	0.122	NS	0.39
C18:2 TFA	0.81 ± 0.21	1.15 ± 0.35	0.75 ± 0.16	1.21 ± 0.30	***	0.816	**	0.703	NS	0.399
C18:2n6c	0.98 ± 0.18	1.32 ± 0.41	0.95 ± 0.19	1.36 ± 0.36	*	0.538	*	0.44	NS	0.165
C18:2n6t	0.07 ± 0.05	0.10 ± 0.05	0.04 ± 0.03	0.13 ± 0.03	***	0.805	NS	0.281	NS	0.006
CLA c9t11	0.93 ± 0.27	0.98 ± 0.31	0.77 ± 0.12	1.14 ± 0.26	*	0.525	NS	0.018	NS	0.062
C18:3 TFA	0.14 ± 0.03	0.14 ± 0.02	0.16 ± 0.02	0.12 ± 0.02	*	0.58	NS	0.027	NS	0.072

C18:3n3	0.74 ± 0.06		1.02 ± 0.28		0.77 ± 0.08		1.00 ± 0.30		*	0.502	**	0.606	*	0.415
C20:0	0.11 ± 0.02		0.12 ± 0.02		0.11 ± 0.01		0.12 ± 0.02		NS	0.013	NS	0.106	NS	0.321
C20:1	0.04 ± 0.01		0.04 ± 0.01		0.04 ± 0.01		0.04 ± 0.01		NS	0.077	NS	0	NS	0.077
C20:3n6	0.03 ± 0.02		0.04 ± 0.03		0.02 ± 0.01		0.05 ± 0.02		*	0.523	NS	0.157	NS	0.157
C20:4n6	0.05 ± 0.01		0.06 ± 0.02		0.04 ± 0.01		0.06 ± 0.01		**	0.6	NS	0.04	NS	0.04
C20:5	0.08 ± 0.01		0.09 ± 0.02		0.09 ± 0.01		0.09 ± 0.02		NS	0.026	NS	0.296	NS	0.397
C21:0	0.03 ± 0.01		0.04 ± 0.01		0.03 ± 0.01		0.04 ± 0.01		NS	0.167	NS	0.167	NS	0.167
C22:0	0.04 ± 0.01		0.05 ± 0.02		0.05 ± 0.01		0.05 ± 0.02		NS	0.047	NS	0.12	NS	0.21
C22:1	0.06 ± 0.01		0.07 ± 0.02		0.06 ± 0.01		0.07 ± 0.02		*	0.472	*	0.472	NS	0.243
C23:0	0.02 ± 0.01		0.03 ± 0.00		0.03 ± 0.01		0.03 ± 0.01		NS	0.018	*	0.467	NS	0.018
C24:0	0.03 ± 0.01		0.04 ± 0.01		0.04 ± 0.01		0.03 ± 0.01		NS	0.014	NS	0.258	NS	0.111
C18:1 CFA	1.14 ± 0.10		1.31 ± 0.27		1.11 ± 0.09		1.34 ± 0.25		*	0.552	*	0.404	*	0.5
Dietary Indices														
Atherogenic	3.89 ± 0.46		3.61 ± 0.72		4.15 ± 0.41		3.35 ± 0.49		**	0.628	NS	0.18	NS	0.357
Thrombo-genic	4.09 ± 0.36		3.65 ± 0.76		4.30 ± 0.30		3.44 ± 0.55		***	0.746	*	0.442	*	0.461
Health Promoting	0.24 ± 0.02		0.27 ± 0.06		0.23 ± 0.02		0.28 ± 0.05		**	0.624	NS	0.255	*	0.488
Desaturase	0.39 ± 0.04		0.41 ± 0.06		0.36 ± 0.04		0.43 ± 0.03		**	0.675	NS	0.11	NS	0.311

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

[1]Abbreviations: PRG, perennial ryegrass; MSS, multispecies swards; SOL, stage-of-lactation; η2, effect-size, partial ETA[2;] TFA, Trans-fatty-acid

Dietary indices calculated as described by Timlin et al. (2023b): *Atherogenic index = [C12:0 + (4 × C14:0) + C16: 0]/ ΣUFA. Thrombogenic index = (C14:0 + C16:0 + C18:0)/ [(0.5 × ΣMUFA) + (0.5 × Σn-6 PUFA) + (3 × n-3 PUFA) + (n-3/n-6)]. Health-promoting index = ΣUFA/ [C12:0 + (4 × C14:0) + C16:0] Desaturase index = (C14:1 + C16:1 + C18:1n-9 cis + CLA cis-9,trans-11)/(C14:0 + C14:1 + C16:0 + C16:1 + C18:0 + ΣC18:1 + CLA cis-9, trans-11).*

4.4.8. Sensory profile analysis after a 180 d maturation period

To validate the laboratory-based analyses completed within these investigations, within the context of real-world product acceptability, sensory analysis was also performed on mid-lactation Cheddar cheese. This analysis was completed by panellists representative of general cheese consumers, and also by semi-trained panellists

Within the consumer centred sensory analysis, no significant differences were observed between MSS and PRG-derived cheese ($P > 0.05$), Fig. 4.2. The PRG cheese did score slightly higher for all hedonic attributes than MSS cheese (appearance, aroma, flavour, texture and overall acceptance), but in all cases, the magnitude of these differences were very small and of insufficient magnitude to be statistically significant ($P>0.05$). Therefore, in terms of absolute size of difference, and lack of statistical significance, no meaningful or practical difference is seen in sensory quality between MSS and PRG cheese as determined by this group of Cheddar consumers.

Through ODP analysis, 5/16 attributes showed significant differences between PRG and MSS derived cheese ($P<0.05$), Fig. 4.3. The PRG treatment scored significantly higher for ivory colour, Cheddar aroma, firmness in the mouth and Cheddar flavour, and the MSS treatment scored significantly higher for astringent aftertaste ($P < 0.05$). Therefore, PRG cheese does appear to be marginally favourable in sensory quality compared to MSS cheese based on this ODP assessment. However, in order to consider the full practical meaning of these results, the magnitude of these differences must be considered. Indeed, all differences seen here are relatively small in magnitude.

The greatest difference found was for dairy sweet flavour with an average score of ~ 3.2 for MSS cheese compared to ~ 4 for PRG cheese, and in this example the variance was sufficiently large that it resulted in no statistical significance ($P>0.05$). In practical terms, it is perhaps arguable that the small magnitude differences observed within this ODP assessment are unlikely to be noticeable within a real-world consumer acceptability context. Indeed, this is also what was suggested through the naïve consumer assessment. Similarly, Uzun et al. (2018) investigated consumer acceptability of mozzarella produced from varied levels of fresh forage in buffalo diets, and they also found that consumers were unable to detect subtle differences in sensory profile reported by a trained panel. As noted by Kilcawley et al. (2018), trained judges are more capable of recognising differences within the individual flavour attributes of cheese than general cheese consumers.

Within these investigations, sensory acceptance testing performed by untrained cheese consumers did not find significant differences between MSS and PRG cheese, while the ODP method did find some significant, albeit low magnitude, differences. A consumer panel size of 25 panellists could be a limitation, and a larger panel would be preferable, increasing the statistical power of the analysis (Stone et al., 2012). The most pertinent finding from these investigations is that consumer acceptance of cheese produced from a MSS system should be likely. These sensory panel results also appear to align appropriately with the many laboratory-obtained experimental results completed throughout the rest of this study (i.e., colourimetry, texture, volatile organic compounds, fat profile).

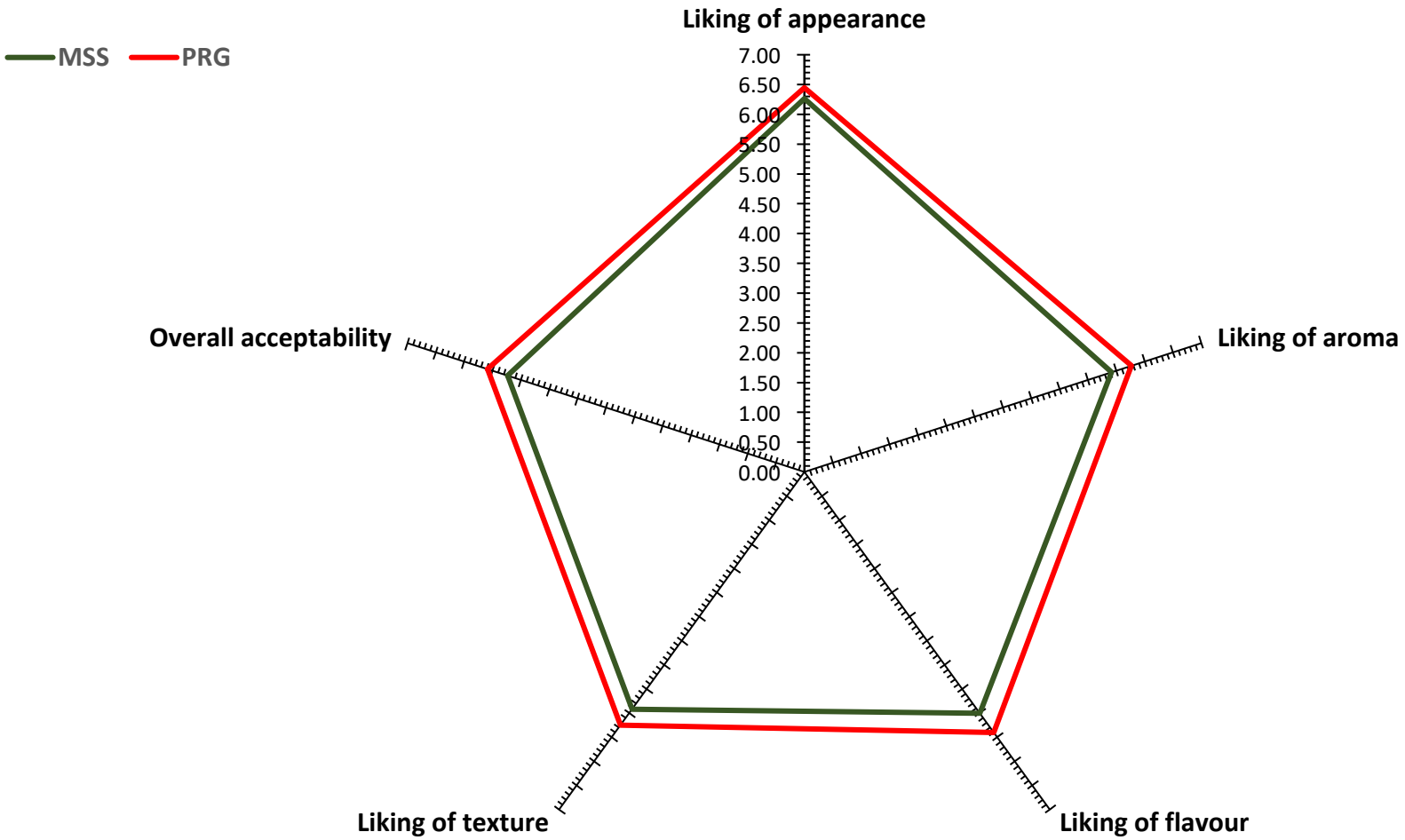


Fig. 4.2. Effect of diet on the sensory profile of Cheddar cheese.
Analysis completed at 180 d of ripening, data obtained from 25 untrained panellists representing general cheese consumers.

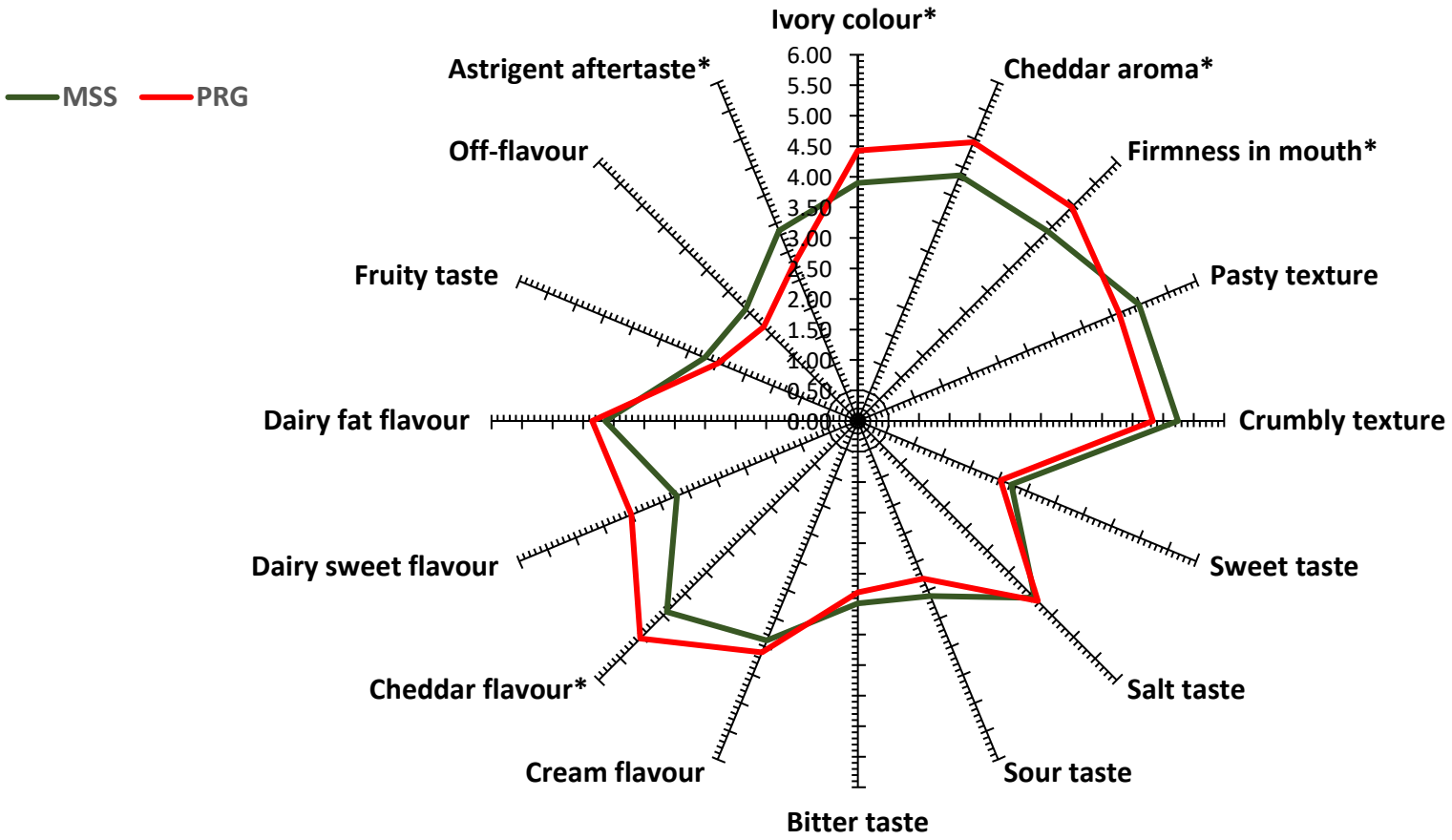


Fig. 4.3. Effect of diet on the sensory profile of Cheddar cheese. Analysis completed at 180 d of ripening by an ODP panel.

***Some statistically significant differences (P < 0.05) were observed between the cheese types.**

4.5. Conclusions

Within this study, laboratory-based measures of product quality were broadly similar when comparing Cheddar cheese obtained from PRG and MSS grazing systems. Additionally, sensory panel investigations further validated the main themes uncovered within these laboratory-based experiments. In practical terms, and industrially relevant contexts, MSS and PRG derived Cheddar appear to be largely equivalent, particularly regarding general consumer acceptability. Some dairy consumers favour pasture-derived products over non-pasture alternatives. When chosen, pasture-derived products are often recognised for their association with environmental sustainability, or because of their favourable nutritional and quality profile. As agri-food industries target their future sustainability needs, it is vital that a focus is maintained on final product quality. It is expected that the new findings provided by this study can provide assurance that greater sustainability might be achievable within grazed dairy systems without causing practical adverse impacts to high-value commodity foods such as Cheddar cheese.

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Chapter 5

The impacts of a typical dairy cow crossbreeding strategy on Cheddar cheesemaking efficiency, nutrition and quality

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5.1. Abstract

Crossbreeding can be utilised in animal husbandry to increase genetic diversity and improve health and fertility in the next generation. In dairy herd management, a common strategy involves crossbreeding Jersey (JE) cows with Holstein-Friesians (HF), resulting in Jersey-Holstein-Friesian (JFX) progeny. This study investigated the impact of these breeds on processing efficiency and product quality within Cheddar manufacturing. Raw JFX cow's milk had significantly higher protein (+ 5.68%) and fat (+13.20%) compared to HF cows, but no significant differences in coagulation properties or cheese yield resulted. All cheese manufactured had macro-compositions within the range of expected values for Cheddar, texture, proteolysis, pH, volatile organic compounds and fat profile values were similar. From a due-diligence perspective, it was not evident from any of the analyses performed, that JFX phenotypes cause practical negative impacts to the cheesemaking ability of milk or adversely influence final cheese quality. Furthermore, cheese derived from JFX milk was more yellow in colour, with significantly higher b* values, which is indicative of preferable sensory and nutritional quality.

5.2. Introduction

In Ireland, the national dairy herd is predominantly comprised of Holstein-Friesian (HF) cows, a breed established through careful selective breeding of Holstein (HO) and Friesian (FR) animals (Evers *et al.*, 2021). The breed is highly specialised, producing high

volumes of milk with sufficient levels of protein and fat to maximise total solids yields (Coffey *et al.*, 2016; Palladino *et al.*, 2010). Milk-fat plus protein is more important than milk-volume in Ireland and Irish farmers are paid for total protein and fat yielded with a penalty for milk-volume (Geary *et al.*, 2010). European dairy systems have typically focused selective breeding efforts towards increased productivity and efficiency within the HF population (Brito *et al.*, 2021). The approach has brought benefits, but a problematic consequence has been increased rates of inbreeding depression (Teagasc, 2016; Brito *et al.*, 2021). Breeding strategies that increase genetic diversity are therefore coming into greater focus.

Dairy cows inherit two copies of each gene, one from each parent, and inbreeding increases the likelihood that two matching gene-copies will be present in the next generation. This is desirable for *good-genes*, but over time an increase in deleterious-recessive-gene-pairs also occurs (Cassell, 2007). Repeated generations of inbreeding negatively impacts herd health, causing reduced conception rates, increased stillbirth rates, and reduced disease resistance (Hansen, 2006). Crossbreeding can reverse inbreeding-related-reductions in animal-health and improve herd-productivity (Buckley *et al.*, 2014; Saha *et al.*, 2020), because the frequency of different gene-copies is increased in the next generation (Sørensen *et al.*, 2008). Increased heterozygosity increases genetic diversity and brings hybrid-vigour (Shull, 1948; Teagasc, 2016). The amount of hybrid-vigour resulting in crossbred progeny is positively correlated with the genetic distance between parental breeds (Mäki-Tanila, 2007; Ducrocq and Wiggans, 2015). Coffey *et al.* (2016) described greater hybrid-vigour for JFX offspring

than HF offspring, resulting from a greater genetic distance between parental breeds. Teagasc (2012) found that JFX cows are better at maintaining body condition, have superior reproductive efficiency and longer milking durations than HF cows. Crossbreeding has already grown in popularity in some countries. Sixty percent of New Zealand's dairy herd are JFX crossbreeds, 25% HF, and the remainder comprises JE and other breeds (DairyNZ, 2022). In Ireland, HF cows dominate, and JFX cows represent around 5% of the Irish herd (Coffey *et al.*, 2016).

Crossbreeding can improve herd health, but due consideration must also be given to the impact of crossbreeding on milk production traits and milk composition arising from such animals. Some previous research has associated crossbreeding with lower milk volumes (Anderson *et al.*, 2007), other work suggests a neutral or positive impact on production traits (Bryant *et al.*, 2007; Coffey *et al.*, 2016; Anderson *et al.*, 2007; Teagasc, 2012; Vance *et al.*, 2012). Coffey *et al.* (2016) found crossbreeds exceeded their respective parents in milk-solids production, and the greatest milk-solids were observed in JFX milk. Similarly, Anderson *et al.* (2007) and Bryant *et al.* (2007) found JFX milk had higher fat and protein contents than purebred HO milk. A Teagasc (2012) study reported lower milk-volumes from JFX cows compared to HF but with higher milk-solids. Vance *et al.* (2012) found HF milking performance could be improved relative to JFX by increasing dietary concentrate levels, an interaction between genetics and diet. It should be noted that whilst hybrid-vigour is relevant to milk production, heterotic impacts have been reported as being only 0-10% for milk production traits, compared to 5-25% for fertility traits (Coffey *et al.*, 2016). Regarding the phenotypic

presentation of parental genetics within progeny, this can be additive or synergistic, synergism suggesting hybrid-vigour. Heterosis is observed when offspring exceed the average performance of their parents for a given trait (Ducrocq and Wiggans, 2015). Palladino *et al.* (2010) found that JFX offspring had both protein and fat yields that exceeded parental averages; JFX milk yielded 0.69 kg/d protein, above the parental average of 0.665 kg/d, and yielded 0.81 kg/d fat which exceeded the parental average of 0.775 kg/d.

Further to milk composition, thought should be given to milk processing attributes; there is a paucity of information on the impacts of crossbreeding on milk processability. Purebred JE milk can provide specific benefits for cheesemaking (Auldist *et al.*, 2004; Bland, 2015; Yoo *et al.*, 2019; Kristensen *et al.*, 2015). Auldist *et al.* (2004) demonstrated better curd formation and a 10% greater cheese yield from JE milk compared to FR milk, attributed to higher milk solids content. Similarly, using non-standardised milk, Bland (2015) found that JE milk resulted in a higher yield of Cheddar cheese compared to HF milk. Yoo *et al.* (2019) found that cheese made from JE milk had higher calcium and phosphorous contents, and improved colour, taste, flavour and texture scores, particularly in ripened cheese, compared to HO milk. Consideration should also be given to the cheese manufacturing qualities of crossbred cow's milk, and whether JFX milk shares techno-functional properties with JE milk (Saha *et al.*, 2020; Sanjayaranj *et al.*, 2023; Sneddon, 2015). Saha *et al.* (2020) found higher casein contents and slightly improved coagulation and curd-firming properties in milk from crossbred cows compared to purebreds. Sanjayaranj *et al.* (2023) found a positive

association between milk coagulation properties and crossbreeding, but acknowledged interpretation of these results was limited by sample size, and some confounding variables also limited the usefulness of this study, particularly farm management practices such as milking frequency. Sneddon (2015) observed significant positive heterosis for cheese yield, they reported that JFX cows should yield 25.4 kg more cheese over a lactation than HF cows.

Teagasc (2012) stated that JFX cows could increase profits on Irish dairy farms by €20,000/year within a 40-hectare unit. This potential for improved economic sustainability, and other aforementioned evidence, provide justification to consider additional use of crossbreeding within dairy food systems. Approximately one-third of Irish milk is destined for cheese production (Fox *et al.*, 2017), and investigations into cheese quality utilising JFX milk are under-reported in the literature. Consequently, this study aimed to investigate the effects of JFX crossbreeding on Cheddar cheese produced from an Irish pasture-grazing system.

5.3. Materials and methods

5.3.1. Milking systems and milk collection

The milk used within this study was supplied by the Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark. The work utilised mid-lactation milk collected on three occasions in August 2022, from a grazing farm systems experiment undertaken by Jezequel *et al.* (2024)**.** Cows were grazed in two herds on a typical Irish

pasture diet of monoculture swards of *Lolium perenne* (perennial ryegrass (PRG)). The treatment groups comprised of Holstein-Friesian (HF) and Holstein-Friesian × Jersey (JFX) crossbred animals, with 25 cows per group randomised for age, breed, yield and parity. The average economic breeding index (EBI), milk, fertility, calving, beef, maintenance, management, and health sub-indices of the HF cows was €205, 63, 103, 33, -12, 15, 2, and -1, respectively, and €198, 68, 89, 30, -24, 32, 3, and -1, respectively, for the JFX cows. The average EBI of all cows ranked them in the top 1% of the national herd during the experimental period. All animals were milked twice daily at 6.30 am and 4.30 pm. Both herds grazed on a rotational basis adjacent to one another, separated using a temporary electric fence to ensure diets of similar quality were offered. Pre-grazing yield, daily herbage allocation and post-grazing residual heights were similar between treatment groups (1650 ± 28.9 kg (DM/ha >4cm), 15.5 kg DM/ha, 40 mm, respectively). Sward composition was 20.5 % ± 1.50% (% crude protein) with dry matter digestibility of 80.1 ± 3.3 % (Jezequel *et al.*, 2024)**.** The cows were offered 1 kg of concentrate at each milking (overall 2 kg/d). Further details can be found in Jezequel *et al.* (2024) which describes the detailed grazing characteristics and general animal performances during the study period. Raw milk for both treatment groups was collected on the same day between 8:30 am and 9:00 am. This raw milk was then stored overnight at 4°C, ready for the preparation of the milk for cheese production, beginning the next day.

5.3.2. Preparation of cheesemilk

On the day following milk collection, the standardisation and pasteurisation of milks were completed for both treatment types; all milks within a given trial underwent preparation for cheesemaking at the same time and under the same conditions. A portion of the collected raw milk, ~ 5kg, was separated into cream and skim milk at 50°C using a benchtop centrifuge operating at 10,000 revolutions per min (FT15 Disc Bowl Centrifuge, Armfield Limited, Hampshire, UK). Milk from each crossbreeding treatment was then standardised to a total protein-to-fat ratio (Guinee *et al.*, 2006) of 0.95 using skim milk, raw whole milk or cream. These standardised milks were then pasteurised at 72°C for 15 s (HTST/UHT System HT122, OMVE, Scgalkwijk, the Netherlands). Milk that has been standardised and pasteurised will be referred to as cheesemilk within this study. All cheesemilks were then stored overnight at 4°C, ready for the Cheddar cheese manufacturing process which would commence the following morning.

5.3.3. Cheese manufacturing

Within a given cheese manufacturing trial, all Cheddar produced within that trial, for both treatment types, was manufactured side-by-side, and under the same production conditions, on the same day. On the day following standardisation and pasteurisation, Cheddar cheese was manufactured utilising pilot-scale production vats (10 L; Pierre Guerin Technologies, Mauze, France) at the Biofunctional Food Engineering Facility, Teagasc Food Research Centre, Moorepark, Fermoy, Co Cork, Ireland, as described by

Page *et al.* (2024). Briefly, 12 kg of cheesemilk per vat was heated to 32°C and the pH was adjusted to 6.55 using a 4% (v/v) lactic acid solution. A frozen starter culture, comprising *Lactococcus lactis subsp. cremoris* and *Lactococcus lactis subsp. lactis*, was added at a level of 2.2 g per 12 kg cheesemilk (R604, Chr. Hansen Ireland Ltd., Co. Cork, Ireland). After 20 min, fermentation-produced calf chymosin was added to each vat (0.18 mL per kg of milk, diluted 1:10 prior to addition, CHY-MAX, 200 international milk clotting units (IMCU)/mL; Chr. Hansen Ireland Ltd., Co. Cork, Ireland). Gels were then cut at a constant firmness (G′) value of 35 Pa (Anton Paar, Graz, Austria). Curds were cooked to 38°C at a rate of 0.25°C/min, drained at pH 6.15, milled at pH 5.35, salted at 2.7% (w/w), mellowed for 20 min, moulded and pressed overnight. After manufacturing, cheeses were vacuum packed and ripened at 8°C for 6 months (180 d).

Cheddar cheese manufacturing trials were completed in triplicate in August 2022; this means that the entire end-to-end cheesemaking process, and process of milk collection and standardisation described above, were each performed in full on three separate occasions.

5.3.4. Rennet coagulation properties

Samples were taken to determine the coagulation properties of HF and JFX cheesemilks according to the method described in Chapter 2.

5.3.5. Composition of raw milk, cheesemilk, whey and cheese

Analyses completed as described in Chapter 2. Fat lost in whey was calculated as the mass of fat recovered in whey divided by the mass of fat present in cheesemilk, multiplied by 100.

5.3.6. Cheese yield

Determined as described in Chapter 2

5.3.7. Analysis of total-fatty-acids, free-fatty-acids and volatile compounds

Analyses completed as described in Chapter 3

5.3.8. Texture profile analysis

Analysis completed as described in Chapter 3.

5.3.9. Colour analysis

Analysis completed as described in Chapter 4.

5.3.10. pH and proteolysis

Analysis completed as described in Chapter 4.

5.1.11. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 27 (IBM Statistics Inc.), with some processing and formatting of data carried in Microsoft Excel 64 bit, 2016 Version (Microsoft Corporation Inc.) and GraphPad Prism version 9 (GraphPad Software Inc.). Univariate ANOVA was used for the comparison of means in single factors and repeated measures ANOVA was used to assess for statistical significance in data related to changes in cheese during the ripening period. For all statistical analyses, P values < 0.05 were considered significant. The reported data is the average

of data taken from three separate cheesemaking trials, with results presented as the mean ± standard deviation.

5.4. Results and discussion

5.4.1. Raw milk, cheesemilk and whey composition

The raw milk and cheesemilk compositions of HF and JFX milk are shown in Table 5.1. The JFX raw milk had significantly higher levels of protein and fat compared to HF raw milk ($P < 0.05$). Similar results for increased fat and protein in JFX milk have been reported previously, milk solids are already known to be higher in milks derived from JFX cows, resulting from a lower overall milk volume and concentration effects associated with the breed (Bryant *et al.*, 2007; Anderson *et al.*, 2007; Teagasc, 2012; Coffey *et al.*, 2016). No significant effects resulting from the crossbreeding factor were observed for lactose content ($P > 0.05$). Similar to raw milk, JFX cheesemilk had significantly higher levels of protein and fat compared to HF milk ($P < 0.05$). No significant effects resulting from the crossbreeding factor were observed in cheesemilk for total solids, lactose content, non-protein-nitrogen, casein-number and casein-content ($P > 0.05$). The significantly higher total and true protein contents in JFX cheesemilk were not accompanied by a statistically significant increase in casein content, suggesting that the breed effect may have involved both casein and non-casein protein fractions.

Table 5.1: Raw milk, cheesemilk, and whey compositions, Holstein-Friesian (HF) and Jersey-Holstein-Friesian (JFX)

	HF			JFX			P-Value
Raw Milk Composition							
Total Protein (% wt/wt)	3.52	±	0.11	3.72	±	0.03	*
Fat (% wt/wt)	4.62	±	0.22	5.23	±	0.32	*
Lactose (% wt/wt)	4.68	±	0.11	4.57	±	0.16	NS
Cheesemilk Composition							
Total Protein (% wt/wt)	3.55	±	0.13	3.78	±	0.02	*
True Protein (% wt/wt)	3.38	±	0.12	3.61	±	0.02	*
Fat (% wt/wt)	3.74	±	0.13	3.99	±	0.04	*
Lactose (% wt/wt)	4.67	±	0.10	4.59	±	0.18	NS
Total Solids (%)	12.94	±	0.43	13.79	±	0.42	NS
Non-Protein Nitrogen (%wt/wt)	4.75	±	0.64	4.51	±	0.10	NS
Casein Number (% wt/wt)	79.00	±	0.69	78.28	±	0.40	NS
Casein Content (% wt/wt)	2.80	±	0.12	2.96	±	0.01	NS
Calcium (mg/kg)	1515	±	128	1421	±	87	NS
Potassium (mg/kg)	1524	±	58	1489	±	64	NS
Magnesium (mg/kg)	110	±	16	128	±	10	NS
Sodium (mg/kg)	418	±	64	382	±	31	NS
Phosphorous (mg/kg)	1117	±	119	1028	±	75	NS
Zinc (mg/kg)	5.09	±	0.29	4.74	±	0.28	NS
SCC (cells/mL)(x1000)	131	±	67	222	±	136	NS
Whey Composition							
Weight of whey (Kg)	10.41	±	0.02	10.26	±	0.04	*
Total Solids (%)	7.20	±	0.22	7.10	±	0.29	NS
Protein (% wt/wt)	0.95	±	0.04	1.12	±	0.12	NS
Fat (% wt/wt)	0.40	±	0.04	0.38	±	0.01	NS
Fat lost in whey (%)	9.33	±	1.56	8.20	±	0.22	NS

***P < 0.05, ^{NS}P > 0.05 (non-significant)**
Data are presented as mean ± standard deviation
Fat lost in whey (%) = [(whey mass × whey fat concentration) / (cheesemilk mass × cheesemilk fat concentration)] × 100

The mineral contents of both cheesemilks were within the range of expected values; they were similar to previously reported data from other investigations. Additionally,

no statistically significant differences were found in milk mineral composition as a result of the crossbreeding treatment ($P > 0.05$). Levels of calcium, potassium, sodium, phosphorous and zinc were marginally higher in HF milk, and levels of magnesium were slightly higher in JFX milk, but none of these differences were statistically significant ($P > 0.05$). The mineral content of milk is important nutritionally and in terms of functional properties. From a micronutrient perspective, dairy products provide meaningful contributions of minerals to Irish diets (Feeney *et al.*, 2016). Mineral levels can impact the functional performance of milk during processing with relevance to the cheese manufacturing process (Lamichhane *et al.*, 2018b). Lamichhane *et al.* (2018b) described how the mineral contents of milk, particularly calcium and phosphorous, can impact upon myriad aspects of final cheese quality. Indeed, the calcium and phosphorous associated with casein have a structural role in Cheddar cheese and deviations in these minerals can impact cheese texture (O'Mahony *et al.*, 2005). Overall, no significant differences in mineral composition between the treatment groups was found, and this suggests that crossbreeding might be applied without any adverse mineral-related nutritional and processing impacts.

This research found no significant association between SCC and crossbreeding. The SCC values were higher in JFX milk, albeit not significantly ($P > 0.05$), and the high standard deviations in SCC results indicate substantial variability among replicate milk collections and reduce confidence in interpreting these SCC differences. Previous work has found significantly higher SCC for JFX milk (Coffey *et al.*, 2016). In other work,

reduced SCCs associated with the crossbreeding of dairy cows has been described as being the result of hybrid-vigour related effects (Saha *et al.*, 2020).

Regarding the whey obtained during cheese manufacturing, the JFX treatment did result in a significantly lower amount of bulk whey compared to HF ($P < 0.05$), 10.26kg compared to 10.41 kg respectively, and this difference in whey volume is consistent with the greater amount of cheese yielded from the JFX crossbreeding treatment. This may partly reflect the more concentrated nature of JFX milk, because higher fat and protein concentrations can increase curd solids retention and reduce the relative amount of whey separated during cheesemaking (Guinee et al., 2006). Regarding the composition of bulk whey, any differences observed between the fat, protein and total solids contents were not significant differences ($P > 0.05$) (Table 5.1). The percentage of fat from cheesemilk lost into the whey was greater for HF compared to JFX, but this difference was not statistically significant ($P>0.05$). Overall, it is not evident through this study that the crossbreeding factor significantly impacts the components lost into the whey.

5.4.2. Cheese composition and cheese yield

The protein, fat and moisture percentages in cheese were consistent with previously reported observations for standard full-fat Cheddar cheese (Feeney *et al.*, 2021) (Table 5.2). In addition, no statistically significant differences were observed between HF and JFX Cheddar for protein, fat and moisture. Likewise, fat-in-dry matter (FDM), moisture

in non-fat substance (MNFS), salt content and salt-in-moisture (S/M) also showed no significant differences between the crossbreeding treatments ($P > 0.05$).

Regarding cheesemaking efficiency, no statistically significant differences were observed in actual yield (Ya), moisture-adjusted cheese yield (Yma) or moisture-adjusted cheese yield adjusted for protein and fat (Yn) ($P > 0.05$). At the very least, cheese manufacturers should be reassured by this evidence; within this work, the use of JFX milk did not reduce cheese yield, and the macro-composition was not adversely impacted by crossbreeding. It should, however, be noted that JFX milk did on average result in a higher Ya of 10.36 compared to 10.05 for HF milk (Table 5.2), a 3.14 % higher yield. This higher cheese yield from JFX cheesemilk, compared to HF cheesemilk, is attributed to its significantly higher protein content (Table 5.1). The casein content of JFX cheesemilk was also 5.71% higher than that of HF cheesemilk, but this was not a statistically significant difference ($P<0.05$) (Table 5.1). Although JFX cheesemilk had significantly higher total and true protein content, casein content did not differ significantly, and neither did Ya; the compositional differences observed in this study were not large enough to produce statistically confirmed yield differences. Cheese yield is influenced by recoverable casein and fat rather than total protein alone, and JFX casein content was only numerically higher than HF milk (Guinee et al., 2006; Emmons and Modler, 2010). When moisture is considered, the cheese yield differences between the JFX and HF breeds are much less apparent; the Yma values for JFX and HF were 10.82 and 10.72, respectively. Previous work has suggested the possibility of improved cheese yield from JFX milk. This included milk with significantly increased

protein and fat yield from crossbreeds (Coffey *et al.*, 2016) and JFX milk having a significantly increased milk solids yield (Teagasc, 2012). Indeed, significantly higher protein and fat levels were also found in JFX milk within this work, and previous studies have shown that milk with higher protein and fat levels typically results in a higher cheese yield (Guinee *et al.*, 2006; Page *et al.*, 2024). In a New Zealand focused study, JFX milk was suggested to yield significantly more cheese than HF milk (Sneddon, 2015), JFX milk was suggested to yield 6.5% more cheese than HF milk, with average yields of 12.4% and 11.64%, respectively. However, the work of Sneddon (2015) was completed on data analysis in New Zealand, and many differences could be present within a study focused within a different location, so, care does need to be taken when comparing between studies. Indeed, crossbreeding effects are known to be mediated by interactions with the environment, the exact diet the cows are given, and the farming system applied (Vance *et al.*, 2012). In addition, experimental methods differed substantially; this current study was a triplicate cheese manufacturing study completed at mid-lactation, whilst the significant differences in cheese yield suggested by Sneddon (2015) utilised data from a mass-balance milk-processing model applied over an entire lactation; Sneddon (2015) relied on predicted cheese yields obtained from compositional data, allowing investigation of a much large data set, but without having actually conducted any cheesemaking trials.

Table 5.2: Composition of Cheddar cheese, Holstein-Friesian (HF) and Jersey-Holstein-Friesian (JFX)

	HF	JFX	P-Value
Protein (% wt/wt)	26.87 ± 0.68	27.27 ± 0.61	NS
Fat (% wt/wt)	33.83 ± 0.57	32.65 ± 0.58	NS
Moisture (% wt/wt)	34.42 ± 1.14	35.79 ± 0.37	NS
[1]FDM (% wt/wt)	51.59 ± 0.2	50.84 ± 0.61	NS
[2]MNFS (% wt/wt)	52.02 ± 1.29	53.14 ± 0.13	NS
Salt (% wt/wt)	1.63 ± 0.19	1.64 ± 0.08	NS
[3]S/M (% wt/wt)	4.74 ± 0.54	4.58 ± 0.22	NS
Actual Yield (Ya)	10.05 ± 0.43	10.36 ± 0.35	NS
[4]MACY (Yma)	10.72 ± 0.51	10.82 ± 0.4	NS
[5]MACYPFAM (Yn)	9.85 ± 0.13	9.34 ± 0.42	NS
Calcium (mg/kg)	8977 ± 818	8381 ± 569	NS
Potassium (mg/kg)	732 ± 49	678 ± 38	NS
Magnesium (mg/kg)	347 ± 45	342 ± 29	NS
Phosphorous (mg/kg)	5880 ± 416	5564 ± 325	NS
Sodium (mg/kg)	6179 ± 498	6364 ± 570	NS
Zinc (mg/kg)	42 ± 3	39 ± 1.1	NS

[NS]P > 0.05 (non-significant)
Data are presented as mean ± standard deviation
[1]Fat in Dry Matter, [2]Moisture in Non-Fat Substance, [3]Salt in Moisture [4]Moisture Adjusted Cheese Yield, [5]Moisture Adjusted Cheese Yield Adjusted for Protein and Fat (Reference moisture 38.5% (w/w))

Regarding the mineral composition of cheese, no significant effects were observed as a result of the crossbreeding treatment (P > 0.05). Similar to cheesemilk mineral data, calcium, potassium, zinc and phosphorous contents were also higher in HF cheese, but again these differences were not statistically significant (P > 0.05). The magnesium content of HF milk was slightly higher than JFX milk, but the magnesium content was higher in JFX cheese. These differences in magnesium are unlikely to be meaningful; they were not statistically significant, and the magnitude of the differences was also

particularly small. Regarding mineral content, the overriding suggestion is that mineral composition appears to be largely unaffected in cheese manufactured from JFX milk compared to HF milk. However, some low-magnitude and non-significant differences are present, and within these differences HF milk and cheese did repeatedly tend to show a pattern of higher mineral levels.

5.4.3. Rheological properties of coagulated cheesemilk

No significant differences in rheological properties were observed between JFX and HF cheesemilks ($P > 0.05$). The average set-to-cut time was 28.97 ± 3.5 min for JFX cheesemilk compared to a slower 33.63 ± 3.8 min for HF cheesemilk, but with a high degree of variation in this property, these differences were not statistically significant ($P > 0.05$). Future work might consider an increased number of cheesemaking trials. The protein content of milk impacts upon rennet gelation properties, and the faster set-to-cut time of JFX cheesemilk was most likely due to this milk having a slightly higher protein content compared to HF cheesemilk (Table 5.1). The casein content of JFX milk was also higher than HF milk, albeit not significantly ($P > 0.05$). The average rennet coagulation time (RCT) was also not significantly different between the two breed types ($P > 0.05$) at 17.37 ± 2.1 min for JFX cheesemilk and 18.43 ± 0.6 min for HF cheesemilk.

Overall, milk gelation properties appear to be not adversely impacted by this crossbreeding strategy. The average set-to-cut time was ~5 min faster for JFX milk, and whilst this was not a statistically significant result, this observation might still

indicate a pattern for JFX milk compared to HF milk. It is worth noting that in commercial operations with automated vat systems, slight adjustments to the cutting time could be required when transitioning from HF milk to JFX milk. Consequently, additional research into the rheological properties of JFX milk might be a consideration for future investigators, in particular, giving consideration to this property at a commercial-scale, given this study was completed at pilot-scale. If this magnitude of rheological difference was repeatable in a larger study, it could be practically meaningful to manufacturers.

In this study, milk was standardised by protein-to-fat ratio, which does not necessarily equalise casein content, and this study does not consider casein variant functionality; both of these factors can influence milk gelation properties (Lucey et al., 2003; Farrell, 2004). Milk standardisation based on casein-to-fat rather than protein-to-fat, and a consideration of specific casein genetic variants and their functionality could provide a focus for future investigations. Indeed, it is noteworthy that some other research studies have observed statistically significant associations between milk coagulation and curd-firming properties when utilising milk obtained from crossbred dairy cows, and have described the link between these factors and increased protein or casein contents (Saha *et al.*, 2020; Sanjayaranj *et al.*, 2023).

5.4.4. pH of cheese during the ripening period

No significant differences in cheese pH values were observed during the ripening period in response to the crossbreeding factor (Table 5.3). The pH values are in the

range of 5.10-5.37, which is typical of Cheddar cheese. Overall, the pH of all cheese increased significantly as it ripened ($P < 0.05$). Change in pH with time is likely due to a number of factors, which include the proteolytic liberation of ammonia, free basic amino acids and amines (Lamichhane *et al.*, 2018a), and the solubilisation of calcium associated with casein (Hassan *et al.*, 2004).

5.4.5. Proteolysis of cheese during the ripening period

The extent of primary proteolysis within all cheese investigated was consistent with previously reported ranges of results (Fenelon and Guinee, 2000; Page *et al.*, 2024). Primary proteolysis is expressed as a percentage of soluble nitrogen at pH 4.6 as a percentage of total nitrogen (pH4.6-SN/TN) (Table 5.3). Primary proteolysis increased significantly with ripening time ($P < 0.05$), attributed to the action of residual chymosin, plasmin and other enzymes present from starter and non-starter bacteria. Importantly, no significant differences in primary proteolysis were observed between the breed treatment types ($P > 0.05$), and proteolysis has an important role within the texture development of cheese (Fox, 1989).

5.4.6. Cheese texture during the ripening period

The data obtained within the texture development experiments (Table 5.3) are supported by the previously discussed primary proteolysis analysis; the results obtained by these different experimental approaches provides further validation of the findings. No significant impacts were observed between HF and JFX cheese for the texture attributes of fracturability, hardness, resilience, cohesiveness and springiness

(P>0.05) (Table 5.3). All of the investigated texture parameters decreased significantly over ripening (P < 0.05). This type of ripening-related change within cheese is to be expected, resulting from factors such as proteolysis, pH and solubilisation of colloidal calcium, and similar results have already been reported previously (Lamichhane *et al.*, 2018a; O'Callaghan *et al.*, 2017; Lamichhane *et al.*, 2019).

5.4.7. Cheese colour during the ripening period

The crossbreeding factor had a significant impact on the colour attributes of Cheddar cheese, specifically through significantly higher b* values (P < 0.05), whilst L* and a* values did not show significant differences (Table 5.3) (Figure 1). The b*parameter references blue to yellow colour, and the significantly higher b* values indicate that JFX cheese is more yellow in colour than HF cheese. The JFX cheese also appeared visually more yellow in colour when compared to HF cheese. Yellow colouring of dairy products has an association with pasture-grazing, and a positive correlation between colour scores awarded by sensory panellists and b* values of Cheddar has already been described in previous studies (O'Callaghan *et al.*, 2017). However, it should also be acknowledged that sensory preferences can differ between geographical locations (Ouyang *et al.*, 2021). Further to consumer choice, yellow colouring of milk and cheese can stem from a legitimate nutritional and health association, particularly regarding pasture derived milks where yellow colouring results from β-carotene which is both a pigment and a pro-vitamin (O'Callaghan *et al.*, 2017; Page *et al.*, 2024). Considering the β-carotene content of Abondance, Tomme de Savoie, Cantalet, Salers and

Rocamadour cheeses, Lucas *et al.* (2006) found that β-carotene is transferred efficiently from milk to cheese during manufacturing, at an average rate of 950 g/kg. Consequently, when differences in β-carotene arise in milk these differences should also be apparent in the resultant cheese. Within this work, significant differences in yellow colour were seen between JFX and HF Cheddar, and other investigators have also described how β-carotene concentration in milk can vary substantially between dairy cow breeds (Jensen *et al.*, 1999; Nozière *et al.*, 2006; Morris *et al.*, 2002). Jensen *et al.* (1999) suggested that β-carotene transfers from the blood of the dairy cow to milk through an active transport mechanism and, as a consequence of this mechanism, only a genetically pre-determined maximum daily limit of β-carotene can be secreted into the milk. Morris *et al.* (2002) described a positive correlation between the amount of carotenoids present in milk and the ratio of JE genes compared to FR genes possessed by dairy cows. It is also possible that the breed related differences in carotenoid concentrations between these milks are linked with breed related milk fat content differences. Overall, based on this discussion of the evidence, it seems likely that the higher b* values observed here for JFX Cheddar are probably indicative of a higher β-carotene content, and that this higher β-carotene content probably arises as a result of JE associated genes present in JFX cows that are either not present, or present at a lower frequency, in HF cows.

Ripening time also had a significant effect on cheese colour ($P<0.05$) (Table 5.3). The L* parameter decreased significantly during ripening, this parameter describing how the cheese got darker as it matured. The parameter a* also changed significantly with time, this indicates a slight increase in red tone as the cheese matured. The b* values also increased significantly with ripening time (Figure 1); overall, all cheese became visually more yellow as it ripened. These trends mainly took place earlier in the ripening period, with early proteolysis and lipolysis influencing light scattering properties (Ferraz et al., 2024), after 90 d of ripening a slight decrease in yellow colour, red colour and darkness occurred.

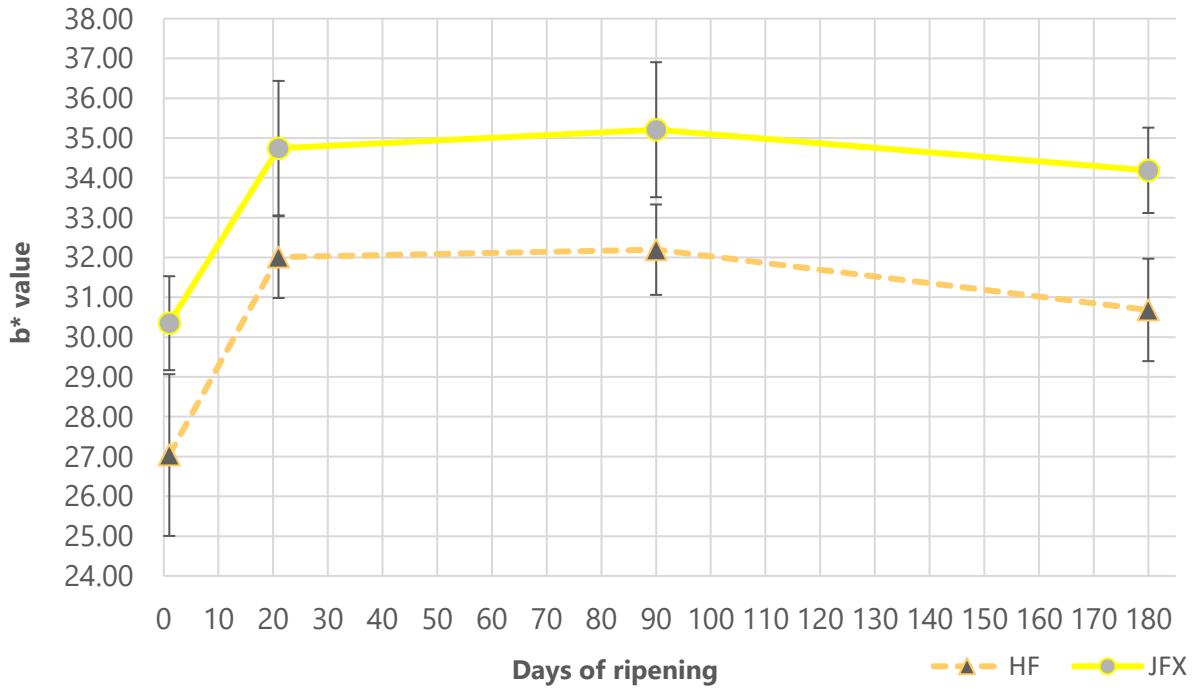


Fig. 5.1. The effect of Holstein-Friesian (▲, HF) and Jersey-Holstein-Friesian Crossbreeding (●, JFX) on the b* values of cheeses during ripening. Presented values are the means from 3 replicate

Table 5.3: Maturation-related changes in Cheddar cheese over 180 days of ripening; Holstein-Friesian (HF) and Jersey-Holstein-Friesian (JFX)

	1 d		21 d		90 d		180 d			
	HF	**JFX**	**HF**	**JFX**	**HF**	**JFX**	**HF**	**JFX**	**Treatment P-Value**	**Time P-Value**
pH Development										
pH	5.10 ± 0.04	5.09 ± 0.04	5.21 ± 0.06	5.22 ± 0.04	5.30 ± 0.03	5.33 ± 0.02	5.28 ± 0.03	5.37 ± 0.05	NS	*
Texture Profile Analysis										
Fracturability (N)	415 ± 30	434 ± 55	314 ± 33	327 ± 32	259 ± 32	264 ± 11	222 ± 17	239 ± 23	NS	*
Hardness (N)	373 ± 11	365 ± 36	319 ± 84	344 ± 22	310 ± 19	335 ± 33	284 ± 13	286 ± 20	NS	*
Resilience (%)	8.33 ± 0.85	6.81 ± 2.52	6.60 ± 0.18	6.76 ± 0.80	5.92 ± 0.25	6.40 ± 1.12	5.10 ± 0.48	4.69 ± 0.37	NS	*
Cohesiveness (%)	0.210 ± 0.03	0.192 ± 0.04	0.200 ± 0.02	0.201 ± 0.02	0.199 ± 0.01	0.213 ± 0.02	0.169 ± 0.01	0.158 ± 0.01	NS	*

Springiness (%)	85.31 ± 27.61	78.92 ± 8.80	51.74 ± 5.51	51.27 ± 3.47	40.17 ± 4.58	38.9 ± 8.67	35.16 ± 7.16	35.52 ± 7.24	NS	*
Proteolysis										
[1]pH 4.6 SN/TN	2.44 ± 0.10	2.26 ± 0.08	4.85 ± 0.58	5.09 ± 0.59	10.78 ± 0.36	11.17 ± 0.38	14.83 ± 1.45	15.33 ± 0.79	NS	*
Colour										
L*	86.53 ± 1.93	87.89 ± 1.22	78.65 ± 0.54	76.56 ± 1.60	77.64 ± 1.59	77.37 ± 1.16	78.76 ± 1.81	78.01 ± 1.03	NS	*
a*	-4.36 ± 0.35	-4.43 ± 0.46	-5.25 ± 0.23	-5.36 ± 0.27	-5.15 ± 0.40	-5.33 ± 0.43	-4.70 ± 0.27	-5.03 ± 0.17	NS	*
b*	27.04 ± 2.03	30.35 ± 1.18	32.01 ± 1.03	34.75 ± 1.69	32.20 ± 1.14	35.21 ± 1.70	30.68 ± 1.29	34.19 ± 1.07	*	*

*** P < 0.05 (Significant), [NS]P > 0.05 (non-significant)**
[1]Nitrogen soluble at pH 4.6 as a percentage of total nitrogen
No significant treatment × time interactions were observed

5.4.8. Milk and cheese total-fatty-acid profile

The fats present in dairy products provide a meaningful contribution to diets, and the specific profile of fats that are present in milk and cheese has relevance to public health (Feeney *et al.*, 2016). Within this research, no significant differences in total-fatty-acid (TFA) profile were observed between HF and JFX cheesemilk ($P > 0.05$) (Table S5.1). As this crossbreeding strategy did not significantly impact the types of fatty-acid present in milk, it might also be suggested that the nutrition and health implications associated with fatty-acid-profile are also not meaningfully changed by crossbreeding. However, other research has found significant links between crossbreeding strategies and the fatty-acid-profiles of milk and dairy products (Palladino *et al.*, 2010; Vance *et al.*, 2012; White *et al.*, 2001), but, a lot of variation and contradiction can be seen when comparing between studies. For example, Vance *et al.* (2012) reported that HF milk had significantly higher C14:1, C16:1, and C18:1 and higher conjugated linoleic acid (CLA) content than JFX milk, whilst JFX milk had greater levels of C18:0. They also found short-chain fatty-acids (C4–C14) and saturated-fatty-acid (SFA) C16:0 to be unaffected by breed. Vance *et al.* (2012) qualified their interpretation of their findings by acknowledging and describing multiple contradictory results from other studies. Taking just one example, Vance *et al.* (2012) described how the work of White *et al.* (2001) was in agreement with their results regarding a lack of significant differences in C16:0 content, but, the results of Palladino *et al.* (2010) contradicted this with a significantly higher C16:0 content in JFX milk. Such conflicting results are problematic, and have real-world relevance, given considerable interest in dietary SFA from a

nutrition and health perspective. For example, Feeney *et al.* (2016) described how dairy products provide a significant proportion of dietary SFA, with possible implications for cardiovascular health. Ultimately, Vance *et al.* (2012) conclude that variation between individual animals might be of greater importance to TFA profiles than associations linked to breed categories. Such an argument is probably relevant here too. The lack of observance of any statistically significant differences within these investigations seems reasonable within a paradigm where variation at the level of the individual cow is significantly large that it is more influential than any variation present at the level of breed.

Animal diet is also a known confounder, both in isolation and through interactions that occur between specific diets and specific genetics (Vance *et al.*, 2012). Diet has not been controlled within all studies, and is certainly not controlled between studies, adding further complexity. Within this study, animal diet was controlled and under these controlled conditions no significant differences were observed in fatty-acid-profiles. Comparing this study to investigations where diet was not controlled, it should be considered that conclusions made about fatty-acid-profiles in response to crossbreeding might actually be attributable to dietary differences rather than to breed.

As with the milk investigated in this study, no significant differences in fatty-acid-profile were observed between HF and JFX cheese ($P > 0.05$). The previous discussion in relation to milk related nutrition, public health and TFA profiles can therefore also

be considered within the context of cheese, this aspect of milk quality, and lack of differences also being transferred to, and represented within, the Cheddar manufactured from the milk.

5.4.9. Cheese free-fatty-acid profile

Differences in FFAs can be used as an indicator of dairy product quality, and are an important flavour component in Cheddar cheese. Arising from lipolysis, and contributing directly to Cheddar flavour development, at appropriate concentrations FFAs contribute to desirable cheese flavours, but excessive lipolysis can generate rancid and sharp notes that reduce product quality (Kilcawley et al., 2007). No significant differences in FFA levels were observed at 180 d between the JFX and HF treatment groups ($P > 0.05$) (Table S5.2). Overall, for both treatments, the FFA results observed in this study are within a comparable range of typical results for full-fat Cheddar cheese (Kilcawley *et al.*, 2007).

5.10.1. Volatile organic compounds in cheese

Volatile organic-compound levels can impact the sensory properties of cheese with both positive and negative results for consumer enjoyment of the food. The volatile compounds identified within all cheese consisted of 6 acids, 8 alcohols, 5 aldehydes, 3 alkanes, 1 alkene, 8 benzenes, 1 diol, 7 esters, 1 ether, 8 ketones, 2 pyrazines and 1 sulphur compound (Table S5.3). In total, 51 volatile compounds were identified, but amongst all of these compounds, no significant differences were observed in response to the crossbreeding factor ($P > 0.05$). The variances were noticeably high, although

high variances are not atypical within this type of analysis, and similar ranges of values have been obtained previously within Cheddar research (O'Callaghan *et al.*, 2017; Page *et al.*, 2024). Overall, these results might be explained through trial related impacts to volatile profile, which appear to be more consequential to this attribute within this study, as opposed to any differences related to the crossbreeding variable under consideration.

5.5. Conclusions

In terms of protein and fat content, the compositions of HF and JFX milk were significantly different from each other, but the compositions of HF and JFX Cheddar did not differ significantly. Manufacturing Cheddar with JFX milk resulted in an average ~ 5 min faster set-to-cut time and actual yield increase of ~3%, these differences were not statistically significant but could warrant future investigation. Indeed, all results from this study could be further validated at commercial-scale, this study was completed at pilot-scale. No meaningful differences were found in TFA profile, texture, proteolysis, pH, volatile-organic-compounds and FFA profile between HF and JFX Cheddar; results tended to show either a lack of statistically significant differences, or the values obtained in analyses suggested practical equivalence between the treatments. Overall, it is not evident that this crossbreeding strategy results in negative effects on the quality and nutritional attributes of Cheddar. Indeed, the significantly increased yellow colour, and likely increased β-carotene content, should align

favourably with nutritional and quality preferences, although β-carotene content was not determined in this study but inferred with reference to the literature. To acknowledge some further limitations of this work, when pondering consumer choices, the price paid by the consumer to buy the product also needs to be considered, and an economic analysis of the relative costs required to manufacture JFX Cheddar as compared to HF Cheddar did not form any part of this study. Likewise, sensory panel studies were not performed within these investigations, and any consideration on possible consumer choices are acknowledged as being limited to conjecture based on the interpretation of results from laboratory analyses, backed-up by a reading of the literature. The study utilised sample groups of 25 cows per treatment, in one research-farm system, to obtain milk for triplicate cheesemaking trials at one point within the milking season; this experimental context must be acknowledged when considering the results and their broader interpretation.

Crossbreeding is broadly accepted as a way of improving genetic diversity, herd-health and fertility. This research suggests that a commonly practised crossbreeding strategy can be applied without adverse impacts on milk and Cheddar quality. However, the lack of significant impacts also suggests attempts to improve cheese quality through crossbreeding might not work; when targeting certain production phenotypes, dairy breeding strategies might need to focus on individual genetic markers for production traits as opposed to breed categories. Crossbreeding is already commonplace in New Zealand, and it could become more readily applied in other international locations. Overall, this research suggests that the quality of Cheddar cheese is not negatively

impacted when manufactured with JFX milk, a valuable suggestion for all who might consider using this milk within dairy processing.

5.6. References

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Chapter 6

The implications of k-casein polymorphism and breed background on milk composition and processing attributes

6.1. Abstract

This study compared selected composition and processing attributes of milks with different κ-casein phenotypes, A and B, in individual Holstein-Friesian (HF) and Jersey-Holstein-Friesian (JFX) cows. Milks were collected from three groups of genotyped cows: HF with AA κ-casein (HF-AA), HF with AB κ-casein (HF-AB), and JFX with AB κ-casein (JFX-AB). Some significant differences in milk attributes were observed at an individual cow-level, both within and between treatment groups. However, on average, the HF-AA treatment resulted in milks with lower fat, protein and casein content, smaller fat globule size, higher pH and larger casein micelle size, compared with the HF-AB and JFX-AB derived milks. Breed background impacted the concentration of protein and casein in milks, and breed-associated impacts were more compositional in nature. The κ-casein genotype influenced casein micelle size and impacts associated with genotype were more functional in nature. The HF-AA milks had poorer rennet coagulation properties than the HF-AB and JFX-AB treatments. The shortest set-to-cut times were measured for the JFX-AB group, significantly faster than HF-AB. The phenotypic impact of κ-CN B on milk functionality did not arise in isolation and was moderated by breed and cow-level factors. It is anticipated that these insights provide valuable information for cow breeding programmes and for dairy manufacturing.

6.2. Introduction

Breeding selections on dairy farms can impact milk composition and functionality, with important implications for processability and final product quality. Such issues are pertinent within the manufacturing of key dairy products like cheese (McSweeney *et al.*, 2017; Lamichhane *et al.*, 2018). Certain breeds and genotypes are associated with increased protein and fat content in milk, clotting properties during cheesemaking and final cheese yield (Guggisberg *et al.*, 2024; Li and Gaunt, 1972; Tsiaras *et al.*, 2005). Consequently, dairy breeding programmes can target desirable production traits at the breed scale (Sneddon, 2015; Page *et al.*, 2025; Bland, 2015), and more specifically by selecting cows that possess particular genetic variants (Daniloski *et al.*, 2024; Walsh *et al.*, 1998; Guggisberg *et al.*, 2024). Breed and genetics are of course interrelated, and some breeds are associated with certain genes and traits (Auldist *et al.*, 2004; Li and Gaunt, 1972; Czerniewicz *et al.*, 2006). Most Irish dairy herds comprise of Holstein-Friesian (HF) cows, whose breeding has been targeted towards maximising protein and fat production (Coffey *et al.*, 2016; Palladino *et al.*, 2010). However, alternate breed selections can be made for specific production objectives; for example, Jersey (JE) cows can provide benefits for cheese production, through improved curd formation and higher cheese yields (Auldist *et al.*, 2004; Bland, 2015). Breed selection is also used to improve herd health and fertility, and crossbreeding strategies, such as those resulting in Jersey-Holstein-Friesian (JFX) crossbred progeny, support this (Buckley *et al.*, 2014; Saha *et al.*, 2020; Page *et al.*, 2025).

Genes code proteins, with the protein fraction of milk being directly impacted by breeding and genetics. The six main ruminant milk proteins are the two whey proteins, α-lactalbumin and β-lactoglobulin, and the four casein proteins, α_{s1}, α_{s2}, β-casein (β-CN) and κ-casein (κ-CN)) (Li and Gaunt, 1972; Farrell *et al.*, 2004). Casein proteins are particularly important to cheese production, and polymorphism of the κ-CN (CSN3) gene can impact on milk function and processability (Farrell et al., 2004; O'Regan et al., 2009; Fox and Brodkorb, 2008). In dairy cows, two allelic variants of κ-CN are typical: A and B (Farrell et al., 2004). Diploid organisms, such as dairy cows, possess two copies of each allele, with three resultant genotypes: AA, AB, and BB. The B variant is associated with higher levels of protein and casein in milk, smaller casein micelle size, more efficient chymosin cleavage, improved milk coagulation properties and increased cheese yields (Guggisberg *et al.*, 2024; Tsiaras *et al.*, 2005; Lavon *et al.*, 2023). Cows with a single B allele possess phenotypically favourable production traits relative to A homozygotes, so the B allele is considered dominant to A. However, the B allele has also been considered co-dominant because the phenotypic impact can be stronger for BB homozygotes than AB heterozygotes (Walsh *et al.*, 1998). Walsh (1999) found only ~2 % of HF cows had the BB genotype. The B allele is present in HF herds, but exists at higher frequencies in JE and JFX herds (Gurses *et al.*, 2018; Ren *et al.*, 2011). Ren *et al.* (2011) described the B allele as being present in JE cows at a frequency of 0.88, and in Holstein cows at a frequency of 0.33. Milk derived from AA and AB κ-CN genotypes is most typical within dairy production, but the overall trend is seemingly towards increased frequency of the B allele (Lavon *et al.*, 2023; Kamiński *et al.*, 2023).

Glantz *et al.* (2010) stated that further studies are needed on genes related to production traits, particularly with regards to the genetics underpinning casein micelle size and milk pH, which have specific relevance to cheese production. Kruchinin *et al.* (2023) emphasised the need for further research into milk coagulation properties associated with κ-CN variants. More generally, Križanac *et al.* (2025) described how knowledge of relationships between economically important production traits and genetic background is still scarce, and Kelly *et al.* (2025) described a need for further studies on gene-function relationships related to bovine milk proteins. Additionally, it is noteworthy that previous investigations into production traits have tended to focus on bulk milk analyses, but the importance of phenotypic variation at an individual cow-level has been noted by some investigators (Vance *et al.*, 2012; Couvreur and Hurtaud, 2007). Couvreur and Hurtaud (2007) reported that individual cow-level variability had a greater impact on milk fat globule (MFG) size than breed, parity or lactation stage. Vance *et al.* (2012) concluded that variation between individual cows might be of greater importance to some milk attributes than associations linked to breed.

Many differences conferred by B κ-CN on the processing performance of milk are well-understood, but an explicit lack of evidence exists for the relative production trait implications of κ-CN genotypes, when considered between breed groups and individual cows. A complex system of interactions takes place from genes to product; individual genetic variants are possessed by individual cows that constitute herds and breeds, individual cows produce individual milks, but these milks are typically pooled, considered in bulk and then processed. The aim of this experimental design was to

compare milk attributes on a breed (HF and JFX) and genotype (AA or AB κ-CN) basis, while also gaining insights into the extent of individual cow-level variation. The hypotheses of this study are as follows. Firstly, genetic factors tend not to operate in isolation but in combination, and it is hypothesised that the relative phenotypic presentation of the B κ-CN allele might vary by breed, i.e., HF versus JFX. Secondly, because genetic factors tend not to operate in isolation, it is hypothesised that individual cow-level factors will also impact on the investigated attributes. In addition, validation is required regarding known differences in production traits that arise when cows possess different variants of κ-CN within the HF breed, considering this now within the context of current Irish pasture grazing systems.

6.3. Materials and methods

6.3.1 Cow selections based on genotype and breed

Twelve individual cows were selected from a database of previously genotyped animals. Within this group of twelve cows, three treatment groups were established of four cows each, with breed and κ-CN genotype acting as variables. Cows 1-4 were Holstein-Friesian cows (HF) of an AA κ-CN genotype and are referred to as the HF-AA group within this study. Cows 5-8 were also HF cows, but of an AB κ-CN genotype, and are denoted the HF-AB group within this study. Cows 9-12 differed by being Jersey-Holstein-Friesian crossbred (JFX) cows but were also confirmed to possess an AB κ-CN genotype, and this group is denoted JFX-AB within this study. The B κ-CN

allele is generally considered dominant to A κ-CN, and the AB κ-CN genotype, and its phenotypes, exist at a greater frequency in typical herds compared to BB κ-CN. Consequently, the AB κ-CN genotype was chosen for consideration in this work, rather than BB κ-CN, due to its practical relevance to Irish dairy farms. It is envisaged that this will further support the translational impact of the findings.

The study considered individual cow level factors alongside genotype and breed, and this provided logistical challenges. This resulted in three treatment groups of four cows each, and it is recognised that the sample sizes used reduce the power of some statistical analyses. This is an accepted limitation of the work that also enabled the broader objectives of the work to be achieved within resource constraints, and the availability of animals meeting the requirements for the study. Indeed, polymorphism of the β-CN genotype had the potential to act as a confounder in this study, so to prevent this issue, all cows within the study were selected to be of the same β-CN genotype (A_2A_2), limiting the number of available cows. Controlling of the β-lactoglobulin genotype would also have been desirable, but given the already specific nature of this study, this was not possible. This is an additional acknowledged limitation, and the β-lactoglobulin genotypes of each individual cow used in this study are described in Table S6.1 so due consideration can be given to these differences, as required.

6.3.2. Milk collection

The milk for this study was collected on three occasions in June 2024, from the Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark. All cows had been grazed on a typical Irish pasture diet of monoculture swards of *Lolium perenne* (perennial ryegrass (PRG)). Cows were milked twice daily at 6.30 am and 4.30 pm, while the milk used within this study was obtained only from the morning milkings. The entire morning milking was taken for each cow, and this milk was then mixed thoroughly and divided into sub-samples for analysis. The reason for only using morning milk was logistical; the approach supported controlled management of the complexity of the study with the constraints of the resources that were available. It is acknowledged that this is a limitation of the study.

6.3.3. Chemical composition analysis

Fat, protein and lactose contents were determined for each individual cow raw milk sample using an FTIR spectroscopy-based milk analyser (DairySpec FT, Bentley Instruments Inc, Minnesota, USA). A routine calibration was completed for this instrument using commercial milk standards (Actalia, Cecalait, France), with thirteen milks of known value analysed and compared against obtained data. The instrument also undergoes weekly validation against internal milks standards of known composition, obtained using reference methods. The total solids were determined using the oven drying method (IDF, 2010). Non-protein nitrogen (NPN) and non-casein nitrogen (NCN) concentrations were determined for each milk using the method

described by Xia et al. (2020) and were in-turn used to determine casein content and casein number values, together with a nitrogen-to-milk protein conversion factor of 6.38. The concentrations of calcium, potassium, magnesium, sodium and phosphorous were determined using Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES; Agilent 5110, California, USA). The determination of mineral content was performed using standard International Dairy Federation (IDF) methodology as described in IDF (ISO/IDF229:2018, 2018) and Agilent application note 15151 (Agilent, 2023). Samples were digested using a microwave digestion system (CEM Mars 6, CEM Corporation, NC, USA) as described by Deshwal et al. (2023). The ICP-OES system undergoes instrument calibration runs once a month using a Wave Calibration Standard from Agilent; a powder reference material of known mineral content is acid digested and run alongside tested samples to validate that all sample measurements are in range. The pH of each individual raw milk was recorded on the day of collection using a bench pH meter (Mettler Toledo SevenCompact™ pH meter S210, Mettler-Toledo Ltd. 64 Boston Road, Beaumont Leys, Leicester, UK). The pH measurements were all completed at room temperatures of ~18°C.

6.3.4. Rennet coagulation properties

Samples were taken to determine the coagulation properties of individual raw milks, with analysis completed according to the method described by Page *et al.* (2024). Raw milk (20 ml) was pipetted into a concentric cylinder measuring cell of a Physica MCR 302 rheometer (Anton Paar, Graz, Austria) and equilibrated to 32°C. The samples were

added with 40 µl of diluted (1:10) rennet solution (CHY-MAX® Plus, Chr. Hansen, Ireland; declared activity: 200 IMCU.ml^{-1}) followed by mixing for 30 s at a shear rate of 600 s^{-1}. The rennet gelation was measured every 6 s (total 500 time points) by recording G′ (Pa) during small amplitude oscillatory shear analysis, conducted at a constant strain of 0.1%, at a constant frequency of 1 Hz. The time from rennet addition to reaching a G′ value of 35 Pa was considered as a measure of set-to-cut time for the cheese manufacturing process (Lamichhane *et al.*, 2018).

6.3.5. Casein micelle size and zeta potential

Casein micelle size and zeta potential were determined using a Zetasizer Pro analyser (Malvern Panalytical Ltd., Malvern, UK), following the method of Gai *et al.* (2023) with slight modifications. For casein micelle size, milk from each individual cow was centrifuged twice at 4500 g for 20 min at 4 °C to remove fat, and the resulting skim milk was filtered through a 1.2 µm syringe filter. The filtrate was then diluted 1:50 (v/v) in ultrapure water (18.2 MΩ·cm resistivity), and measurements were performed in Malvern DTS0012 disposable cuvettes, filled to approximately three-quarters of their volume. Particle size was measured in backscatter detection mode. For zeta potential determination, whole milk was diluted 1:25 (v/v) in ultrapure water, and measurements were conducted under auto-optimised field conditions using disposable folded capillary zeta cells (DTS1070, Malvern). All measurements were performed in automatic mode, with 10–20 runs per sample, conducted in triplicate at 25 °C following 120 s

equilibration. All data were recorded and analysed using ZS Xplorer software (version 4.0.0).

6.3.6. Fat globule size

Particle size distribution was determined using a Mastersizer 3000 (Malvern Analytical, UK) equipped with a Hydro MV wet dispersion unit, following a method adapted from Kostovska *et al.* (2024). The Hydro MV unit was first primed with deionized water (dH_2O) as the dispersant, and a background measurement was conducted to establish baseline scattering. Refractive indices of 1.33 and 1.462 were used for water and milk fat globules (MFG), respectively. Milk was then added dropwise into the stirred dispersant (2260 rpm) until an obscuration level of 3–7% was achieved, ensuring optimal light scattering while avoiding multiple-scattering artefacts. Particle size measurements were performed in triplicate, with each run lasting 20 s, while maintaining constant stirring at 2260 rpm to ensure sample homogeneity. Data acquisition and analysis were carried out using the Mastersizer 3000 software (version 3.81).

6.3.7. The relative expression of A and B κ-CN protein

The protein fractions, including κ-CN, were analysed by reversed phase high performance liquid chromatography (RP-HPLC) according to the procedure described by Reid et al. (2015).

Three independent replicate milk collections had been completed, and aliquots of 100 ml of raw milk per individual cow sample per trial were used in this analysis. The milk

samples and standards were prepared according to Guyomarc'h et al. (2007) and diluted volumetrically. Standards were supplied by Sigma-Aldrich (Sigma-Aldrich, Ireland, LTD). The separation of milk protein fractions was achieved using an Agilent 1200s with quaternary pump and multi-wavelength detector with an Agilent Poroshell 300SB C18 column (2.1 mm × 75 mm; Agilent Technologies UK Ltd.).

On the chromatograms, three peaks were relevant to κ-CN; one for each protein variant, A and B, with a further peak representing the glycosylated isoform of both κ-CN variants (Visser et al., 1991). For each sample, the relative expression of κ-CN protein was determined from the chromatograms by measuring the integrated peak area under the curve (AUC), indexed to the total peak area associated with κ-CN.

6.3.8. Statistical Data Analysis

Statistical analysis was performed using IBM SPSS Statistics version 27 (IBM Statistics Inc.), with some processing and formatting of data in Microsoft Excel 64-bit, 2016 Version (Microsoft Corporation Inc.). Results are presented as the mean ± standard deviation across independent replicate trials. Reported data for each of the twelve individual cow's milks is averaged data taken from milks collected on three different occasions (n=3). Data was also averaged and interpreted by treatment group, HF-AA, HF-AB and JFX-AB.

Univariate ANOVA were used for the comparison of means in single factors using SPSS. When comparing some related variables, a general linear model (GLM) analysis of variance (ANOVA) multivariate procedure was applied in SPSS. Levene's test was also

applied as needed to confirm homogeneity of variance, and Welches ANOVA was used when homogeneity was violated. When significant differences were observed between more than two sampling groups (such as when considering the twelve individual cow's milks, or three treatment groups HF-AA, HF-AB and JFX-AB) a post-hoc Tukey multiple comparisons test was performed in SPSS to confirm where significant differences were arising. For all statistical analyses, P-values < 0.05 were considered significant. Effect sizes were also calculated as partial eta squared (η2), a η2 < 0.06 was considered a small effect size, 0.06 ≤ η2 < 0.14 was considered a medium effect size, and η2 ≥ 0.14 considered a large effect size.

6.4. Results and discussion

6.4.1. Milk macro-compositions, considered individually and by treatment type

Some individual cow's milks differed significantly from each other in protein, fat, and total solids (TS) content (P<0.05) (Table S6.2).

The milk TS values of these individual milks ranged from 11.26 to 16.04%. As expected, the lowest TS value was observed for milk obtained from a cow of the HF-AA group and the highest value was from a cow of the JFX group. Individual milks of the HF-AA group tended to have lower overall fat content, followed by HF-AB, and JFX-AB milks had the highest fat content. The milks that differed significantly in fat content varied by both breed *and* κ-CN genetics, some HF-AA milks compared to JFX-AB (P < 0.05). However, it is also noteworthy that some individual milks of the HF-AA and JFX-AB groups did not differ significantly from each other in fat content (P > 0.05). Regarding

milk protein content, cows of the HF-AA group tended to have lower protein content milk, followed by HF-AB, and JFX-AB milks had the highest milk protein content. The protein content of all HF milks were broadly comparable, even when they differed by κ-CN genotype, and no significant differences in protein content were observed between the individual HF-AA and HF-AB milks ($P > 0.05$). As with milk fat content, some individual milks that differed by breed *and* κ-CN genotype also differed significantly in protein content, but some individual milks of the HF-AA and JFX-AB groups did not differ significantly from each other ($P > 0.05$) (Fig. 6.1.).

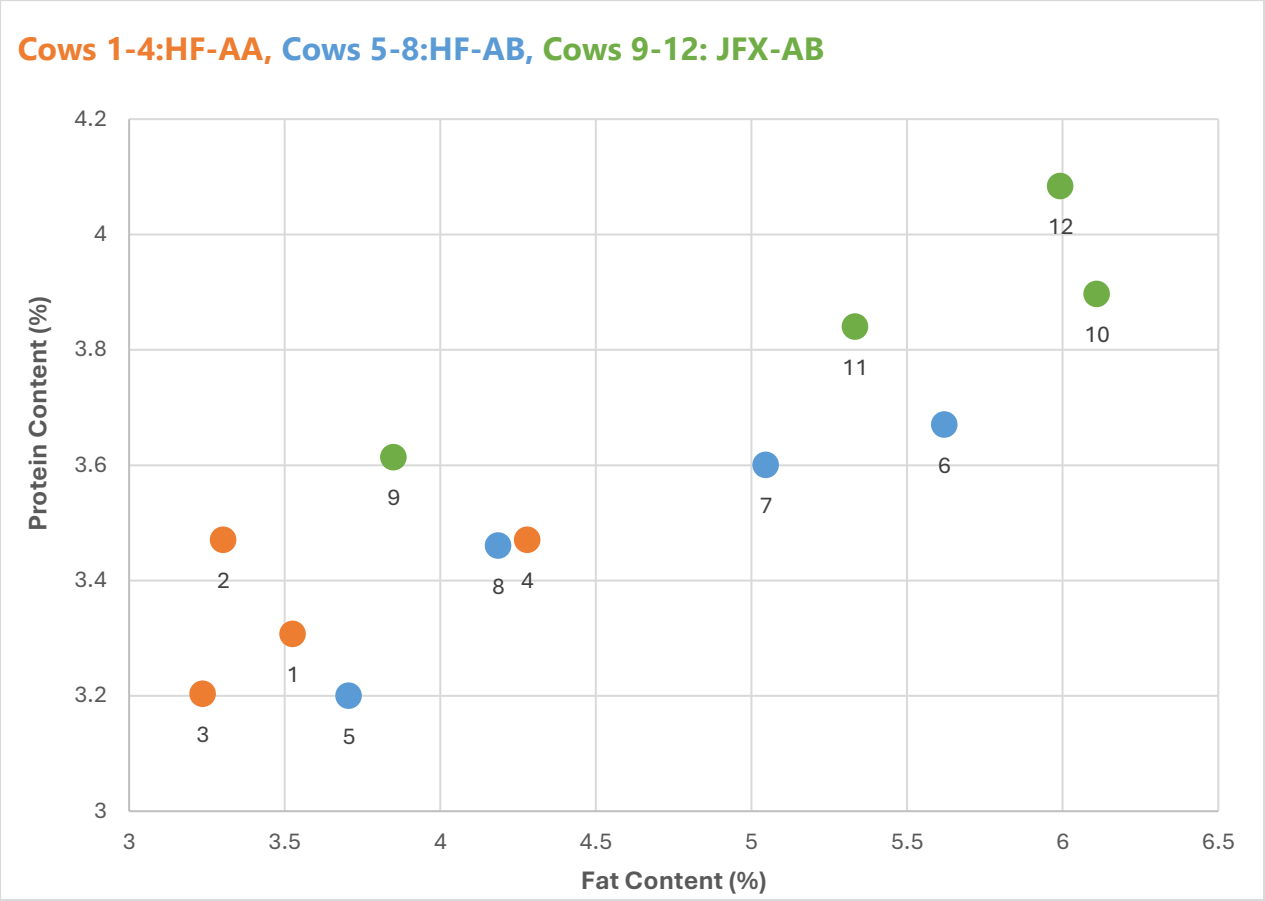


Fig. 6.1. The fat (*x*) and protein (*y*) content of individual raw cow's milks, of the treatment types, Holstein-Friesian κ-CN AA (HF-AA) (orange), Holstein-Friesian κ-CN AB (HF-AB) (blue) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB) (green). Data are the average of three independent milk collections.

The overall impact of the HF-AA, HF-AB and JFX-AB treatment groups on milk macro-composition can be seen in Table 6.1. Regarding TS content values, the milk of HF-AA, HF-AB and JFX-AB cows averaged 12.04, 13.62 and 14.99%, respectively. The average TS of HF-AB milk did not differ significantly from that of either HF-AA or JFX-AB milk ($P > 0.05$), but the TS of HF-AA milk did differ significantly from that of JFX-AB ($P < 0.05$). Regarding fat content, the HF-AA, HF-AB and JFX-AB milks averaged 3.59, 4.64 and 5.32% fat, respectively. The HF-AB milk did not differ significantly in fat content from HF-AA or JFX-AB ($P > 0.05$), but HF-AA and JFX-AB milks did differ significantly from each other in fat content ($P < 0.05$). Consequently, the averaged TS and fat content values of the treatments only differed significantly when they varied both by breed *and* k-CN genotype ($P < 0.05$). Regarding protein content the relationship was different. The JFX-AB milk had significantly higher protein content than HF-AB and HF-AA milk ($P < 0.05$), while HF-AA and HF-AB milks did not differ significantly from each other ($P > 0.05$). The HF-AA, HF-AB and JFX-AB groups averaged 3.36, 3.48 and 3.86% protein, respectively.

Past studies have also found that significant differences in milk macro-composition do not result from κ-CN polymorphism within the HF breed (Walsh *et al.*, 1998). The breed related differences in this study are interesting; indeed, within this study, all cows were of an A_2A_2 genotype, so β-CN polymorphism should not be implicated in causing these significant differences. Potential extrinsic confounding factors, such as diet, were also controlled within this study. It therefore seems plausible that some other intrinsic aspect of the JFX animals contributed to these higher average protein content values

for milks derived from JFX cows, which could be attributed to breed-associated genetic background effects. These comparisons between HF and JFX macro-composition also broadly align with other investigations into compositional differences between JFX and HF derived milks, albeit not from the same controlled genotypes (Teagasc, 2012; Coffey *et al.*, 2016; Page *et al.*, 2025).

Table 6.1: Composition of raw milks considered by treatment group

	HF-AA	HF-AB	JFX-AB	P-Value	η2
Fat (% wt/wt)	3.59[a] ± 0.55	4.64[ab] ± 1.13	5.32[b] ± 1.26	*	0.499
Total Protein (% wt/wt)	3.36[a] ± 0.15	3.48[a] ± 0.21	3.86[b] ± 0.21	**	0.644
Total Solids (% wt/wt)	12.05[a] ± 0.97	13.62[ab] ± 1.53	14.99[b] ± 1.77	*	0.530
Casein Number (% wt/wt)	81.27[a] ± 1.83	79.34[a] ± 2.07	80.20[a] ± 2.58	NS	0.138
Non-protein-Nitrogen (% wt/wt)	5.13[a] ± 0.91	5.34[a] ± 1.23	5.98[a] ± 1.29	NS	0.097
Casein content (% wt/wt)	2.73[a] ± 0.16	2.77[a] ± 0.20	3.05[b] ± 0.18	***	0.393
Lactose (% wt/wt)	4.72[a] ± 0.10	4.69[a] ± 0.06	4.69[a] ± 0.04	NS	0.021
Calcium (mg/kg)	1148[a] ± 96	1247[ab] ± 110	1286[b] ± 138	*	0.210
Potassium (mg/kg)	1525[a] ± 88	1495[a] ± 150	1523[a] ± 162	NS	0.011
Magnesium (mg/kg)	99[a] ± 4	106[ab] ± 8	113[b] ± 14	**	0.304

Sodium (mg/kg)	319[a] ± 38	378[b] ± 52	354[ab] ± 39	*	0.244
Phosphorous (mg/kg)	863[a] ± 50	927[b] ± 77	937[ab] ± 99	NS	0.156

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05; η2, effect-size, partial ETA2**
Holstein-Friesian breed, κ-CN AA genotype (HF-AA), Holstein-Friesian breed, κ-CN AB genotype (HF-AB), Jersey-Holstein-Friesian crossbreed, κ-CN AB genotype (JFX-AB)
Mean values of individual cow's milks, based on three replicate trials, ± standard deviation
Values within rows not sharing a common superscript were significantly different

6.4.2. Milk mineral content

The individual cow's milks were all broadly equivalent to each other for all investigated minerals, with none of the individual milks differing significantly from each other in calcium, potassium, sodium, magnesium or phosphorus content (P > 0.05) (Table S6.3). However, when grouped by HF-AA, HF-AB and JFX-AB treatment group, some significant differences were apparent between the averaged data (P < 0.05) (Table 6.1). On average, HF-AA milk had significantly lower calcium and magnesium content than JFX-AB milk, and HF-AA milk also had significantly lower sodium content than HF-AB milk (P < 0.05). The average calcium content of HF-AA, HF-AB and JFX-AB milks were 1148, 1247 and 1286 mg/kg, respectively. The average magnesium content of HF-AA, HF-AB and JFX-A milks were 99, 106 and 113 mg/kg, respectively. The average sodium content of HF-AA, HF-AB and JFX-AB milks were 319, 378 and 354 mg/kg, respectively. Given the small magnitude of differences observed, the practical relevance of these differences should not be overstated. However, higher levels of magnesium and calcium in milk have been linked to milk of a higher protein and casein content, with these minerals being structurally associated with the casein micelle (Gulati et al., 2018). Indeed, on average, the JFX-AB milks had significantly higher levels of these minerals

and also a significantly higher protein and significantly casein content, than the HF-AA milks ($P < 0.05$).

6.4.3. Casein number, non-protein-nitrogen, and casein content

The casein numbers of milks ranged from 77.89 to 82.89, and no significant differences in casein number were found between the individual milks ($P > 0.05$) (Table S6.2). Additionally, when grouping these milks by the HF-AA, HF-AB or JFX-AB treatment groups, no significant differences in casein number were observed ($P>0.05$) (Table 6.1). Likewise, these milks did not differ significantly in NPN content ($P > 0.05$).

Significant differences in casein content were observed ($P < 0.05$). The individual cow's milk casein content values ranged from 2.47 to 3.24%, and when grouping casein content data by the HF-AA, HF-AB and JFX-AB treatment groups, JFX-AB milk had a significantly higher casein content compared to HF-AA and HF-AB milks ($P < 0.001$). The average HF-AA and HF-AB milk casein content values did not differ significantly from each other ($P > 0.05$).

Comparisons between individual milks also provided additional confirmation of this pattern for milk casein content, and some outliers also provided insights into the extent of individual cow variation that is possible for this attribute. When individual cow's milk casein content values are considered, some JFX-AB milks differed significantly from some HF-AA milks (differing AA κ-CN), and some JFX-AB milks also differed significantly from some HF-AB cow's milks (same AB κ-CN). Additionally, two cows of the same HF-AB group differed significantly from each other in milk casein

content ($P < 0.05$). Another potential outlier was regarding the lowest observed casein content milk, which was obtained from a cow of the B κ-CN phenotype, the phenotype that has for a long time been associated with higher milk casein content values (Ng-Kwai-Hang *et al.*, 1984; Walsh *et al.*, 1998). These examples highlight that individual cow-level variation in casein content can be significant, despite the overall trends discussed.

In this study, breed category was more influential on milk casein content than κ-CN variant. However, it must be acknowledged that these results must only be interpreted within the context of the actual experimental design applied. In particular, consideration should be given to the sample sizes used, and conclusions should be interpreted with caution. Within these treatment samples, the casein content of milks of the HF-AB group were broadly aligned with those of the HF-AA group, and JFX-AB milks differed significantly from HF-AB and HF-AA milks. These results can be contrasted with Chapter 5, where JFX and HF milks were compared for casein content and did not differ significantly from each other, averaging 2.80 and 2.96%, respectively. However, these milks were different, in Chapter 5 the κ-CN and β-CN genotypes were not determined, and the analysis was also performed on bulk milks that had been standardised and pasteurised prior to cheese manufacturing.

6.4.4. Milk pH values

Milk pH values ranged from 6.57 to 6.74 (Table 6.2) and these values were typical for fresh raw milks (Tsioulpas *et al.*, 2007). The HF-AA milk pH values differed significantly

from both HF-AB and JFX-AB milks ($P < 0.001$), and HF-AB and JFX-AB milk pH values were not significantly different from each other ($P > 0.05$). The HF-AA milks had the highest pH values, HF-AB milks had intermediate pH values and JFX-AB milks had the lowest pH values, averaging 6.72, 6.64 and 6.59 respectively. The implications of these differences in pH values are now also further discussed in the following sections on rheological properties of coagulated raw milks and casein micelle size.

Table 6.2: Functional properties of raw milks considered by treatment group

	HF-AA	HF-AB	JFX-AB	P-Value	Partial ETA2
pH	6.72[a] ± 0.05	6.64[b] ± 0.05	6.59[b] ± 0.06	**	0.505
Set-to-cut time (min)	failed to gel	32.68[a] ± 9.1	25.99[b] ± 6.2	*	0.167
Casein micelle size (nm)	188[a] ± 16	156[b] ± 10	151[b] ± 6.0	***	0.686
Zeta potential (mV)	-27.66[a] ± 1.00	-26.44[b] ± 0.78	-27.03[ab] ± 1.53	*	0.210

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CNA B (JFX-AB)

6.4.5. Rheological properties of coagulated raw milks

The set-to-cut times of individual coagulated raw milks were determined and compared by treatment group (Table 6.2) (Fig. 6.2). The milks of the HF-AA group

demonstrated poor gelation behaviour and failed to reach the 35 Pa storage modulus required within this experimental design. Consequently, no set-to-cut time values were determined for these milks. Previous studies indicate that κ-CN B milks generally show more favourable rennet coagulation properties than κ-CN A milks, however, the actual rate of rennet coagulation results from the combined effects of κ-CN cleavage, micelle size, casein content, calcium balance and pH (Walsh et al., 1998; Lavon et al., 2023). Therefore, it is acknowledged that the poorer coagulation of AA κ-CN milk compared to AB κ-CN milk in this study should also be interpreted as a function of the entire milk system, and not just κ-CN genetics.

However, in this study, it is noteworthy that only milks obtained from cows possessing the B variant of κ-CN reached the required storage modulus of 35 Pa. Additionally, significantly faster average set-to-cut times were associated with JFX-AB milks as compared to HF-AB milks ($P < 0.05$), Fig. 6.2. Some additional insights into the extent of cow-level variations in set-to-cut time were also obtained. Significant differences in gelation behaviour were observed between some of the individual milks obtained from the HF-AB group, so milks of the same HF breed and B κ-CN phenotype differed significantly in set-to-cut time. Conversely, within the JFX-AB group, none of the individual milks differed significantly from each other in set-to-cut time ($P > 0.05$). The HF-AA milks demonstrated by far the poorest gelation behaviour. However, it should be recognised that many cows in typical herds are HF cows of an AA κ-CN genotype, and the milks of these cows do gel sufficiently well within industrial cheese manufacturing. Higher milk pH values are associated with poorer gelation (Ong *et al.*,

2012), and the HF-AA milks had the highest pH values. In real-world cheese production, a starter culture is added to milk, lowering the pH, accelerating gelation behaviour, and in some industry settings calcium chloride is also added to aid gelation. Higher casein content in milk is also generally known to result in faster gelation times (Lamichhane *et al.*, 2018) and the HF-AA milks tended to have lower casein content values. The JFX-AB milks tended to gel the fastest, and the casein content values of JFX-AB milks were on average significantly higher than those of HF-AB and HF-AA milks ($P < 0.05$). Additionally, the pH of JFX-AB milk was on average the lowest, followed by HF-AB, and HF-AA milks had the highest pH values.

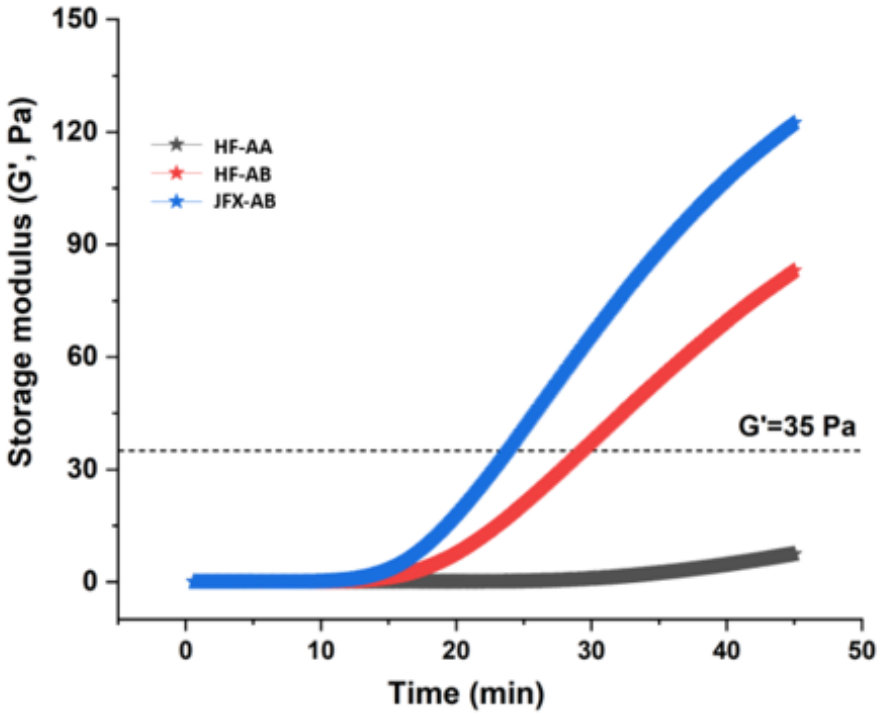


Fig. 6.2. The average storage modulus of raw milks considered by treatment group Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

A previous study had noted that the relative gelation behaviour of JFX and HF milk could warrant further investigation (Page *et al.*, 2025) (Chapter 5). Page *et al.* (2025) described milk that had been pasteurised and standardised within the Cheddar cheese manufacturing process and noted that JFX milk had an average ~5 min faster set-to-cut time compared to HF milk, but the difference was not statistically significant. It is interesting comparing between this current study and that past study, while at the same time noting the differing experimental designs. Within this current study, it was successfully demonstrated that significant differences in set-to-cut time can arise between JFX and HF raw milks, when they are of the same κ-CN and β-CN genotype. Treatment group HF-AB and JFX-AB both possessed AB κ-CN, and all investigated cows had A_2A_2 β-CN, yet they still differed significantly in gelation behaviour ($P > 0.05$). The study described in Chapter 5, Page *et al.* (2025), used bulk milk confirmed to be from HF or JFX cows, but without genotyping, and this bulk milk contained a mixture of κ-CN and β-CN phenotypes, so the coagulation properties represented a mixture of phenotypes.

The genetics of κ-CN is not all that describes milk coagulation properties, and β-CN polymorphism also impacts milk gelation (Daniloski *et al.*, 2024). The proportion of CN present as κ-CN was assessed by RP-HPLC in this study as a relative integrated peak area within the total κ-CN chromatographic peak (not as an absolute concentration in milk). However, this analysis does provide some further insights in relation to rheological properties, and these results are discussed in section 6.4.9.

Daniloski *et al.* (2024) determined that bulk milks and A_1A_1 milks had similar set-to-cut times of ~32 min, A_1A_2 milks had set-to-cut times of ~36 min, and A_2A_2 milks had much slower set-to-cut times of ~56 min. In contrast to Daniloski *et al.* (2024), the current study selected cows known to have A_2A_2 β-CN. In pooled bulk milk (i.e., the milk typically used in industrial production situations) many genotypes are present, including those with better gelation properties. As described by Daniloski *et al.* (2024), bulk milks can perform similarly to genotypes with the best set-to-cut times (in the work of Daniloski *et al.* (2024) the bulk milks had similar gelation behaviour to the fastest gelling A_1A_1 genotype). The gelation behaviour of the individual raw milks used in this study are acknowledged to behave differently to typical pooled bulk milks, but this precise controlled scenario was intentional. Through this experimental design, insight was also gained into the gelation behaviour of A_2A_2 β-CN milk, in addition to κ-CN gelation behaviour. This current study has demonstrated that it is possible for A_2A_2 β-CN milk to have significantly faster set-to-cut times when that A_2A_2 milk is obtained from cows that also possess a B κ-CN phenotype. The impact was even greater still regarding the use of A_2A_2 JFX milk of a confirmed B κ-CN phenotype. These insights might be of relevance to those with an interest in A_2A_2 milk, a type of milk that has come to prominence due to evidence for its easier digestibility, benefits for people with milk intolerances and differences in technological properties such as foaming (Gai *et al.*, 2024; Daniloski *et al.*, 2024; Borș *et al.*, 2024).

6.4.6. Casein micelle size

The average casein micelle size of HF-AA, HF-AB and JFX-AB treatment milks, were 188, 156 and 151 nm, respectively (Fig. 6.3). The casein micelle size results obtained for all milks in this study were within the typical range of expected values (Fox and Brodkorb, 2008). However, the average casein micelle size of HF-AA milk was significantly larger than that of the HF-AB and JFX-AB treatments ($P < 0.05$). The average casein micelle size of HF-AB and JFX-AB milks were not significantly different ($P > 0.05$). The casein micelle size data for each individual cow's milk is shown in Fig. 6.3, and some significant differences are apparent ($P > 0.05$). However, the casein micelle sizes of individual milks of the κ-CN B phenotype tended not to differ significantly from each other. The similarities in casein micelle size for most of the κ-CN B phenotype milks can also be observed visually in Fig. 6.3. The relatively larger casein micelle size of the HF-AA group is also clearly apparent through this visual representation of the data. All individual HF-AA milks had larger casein micelle sizes, but one HF-AA cow had a notably larger casein micelle size still. Indeed, post hoc analysis determined that the casein micelle size associated with this cow's milk was significantly higher than that of all other HF-AA milks, in addition to being significantly higher than that of all other milks analysed in this study ($P < 0.05$). Overall, this analysis has demonstrated some clear patterns. Individual milks of the same κ-CN genotype tended to have similar casein micelle size, with κ-CN AA milks associated with larger micelles than κ-CN AB. However, individual cow-level variation was also demonstrated,

with some significant differences observed between individual cows, even when they were of the same κ-CN genotype and breed.

These findings for casein micelle size should also be considered alongside other analyses completed within this current study, and alongside evidence obtained from previous research. Within this range of milk pH values, previous studies have shown that higher pH values are associated with larger casein micelle sizes, the more alkaline conditions result in greater electrostatic repulsion between casein molecules that loosen/swell the micelle structure with increased binding of water (Sinaga *et al.*, 2017). Within this study, HF-AA milks had the highest pH values, and the largest average casein micelle size. Casein micelle size and pH are both known to influence gelation behaviour (Glantz *et al.*, 2010; Sinaga *et al.*, 2017; Walsh *et al.*, 1998). Indeed, Glantz *et al.* (2010) stated that breeding selections for smaller casein micelle size and lower milk pH could offer improvements for the processing of milk into cheese. Smaller casein micelles have also been associated with stronger milk gels and faster curd firming rates (Glantz *et al.*, 2010; Logan *et al.*, 2015). Additionally, smaller casein micelle sizes have been associated with obtaining higher cheese yields during Cheddar production (Walsh *et al.*, 1998).

Walsh *et al.* (1998) specifically focused on κ-CN polymorphism and casein micelle size. Like this current study, Walsh *et al.* (1998) noted that κ-CN B phenotypes were associated with significantly smaller average casein micelle sizes, and improved rennet coagulation properties, compared to the κ-CN A phenotype. However, this current

study differs in design to that of Walsh *et al.* (1998), providing some additional interesting insights. Walsh *et al.* (1998) compared AA κ-CN production traits to BB κ-CN, whereas this current study compared AA κ-CN to AB κ-CN. The AB κ-CN genotype exists at a greater frequency in typical herds compared to the BB κ-CN genotype, so the findings of this current study might offer some additional practical relevance. This current study has demonstrated that the presence of a single B allele still results in significantly smaller casein micelle sizes, compared to AA κ-CN. Further insights are also offered here through a consideration of a breed-related factor, with Walsh *et al.* (1998) only considering the HF breed. Within this current study, the casein micelle sizes associated with the JFX-AB and HF-AB treatments, both of an AB κ-CN genotype, were not significantly different ($P > 0.05$). Therefore, while the gelation behaviours of JFX-AB and HF-AB milks differed, it seems likely that these differences were not due to casein micelle size, but due to differing casein content and pH levels of the milks. The larger casein micelle sizes of HF-AA milks might however be implicated as a possible contributing factor towards the poorer gelation behaviour observed for these milks.

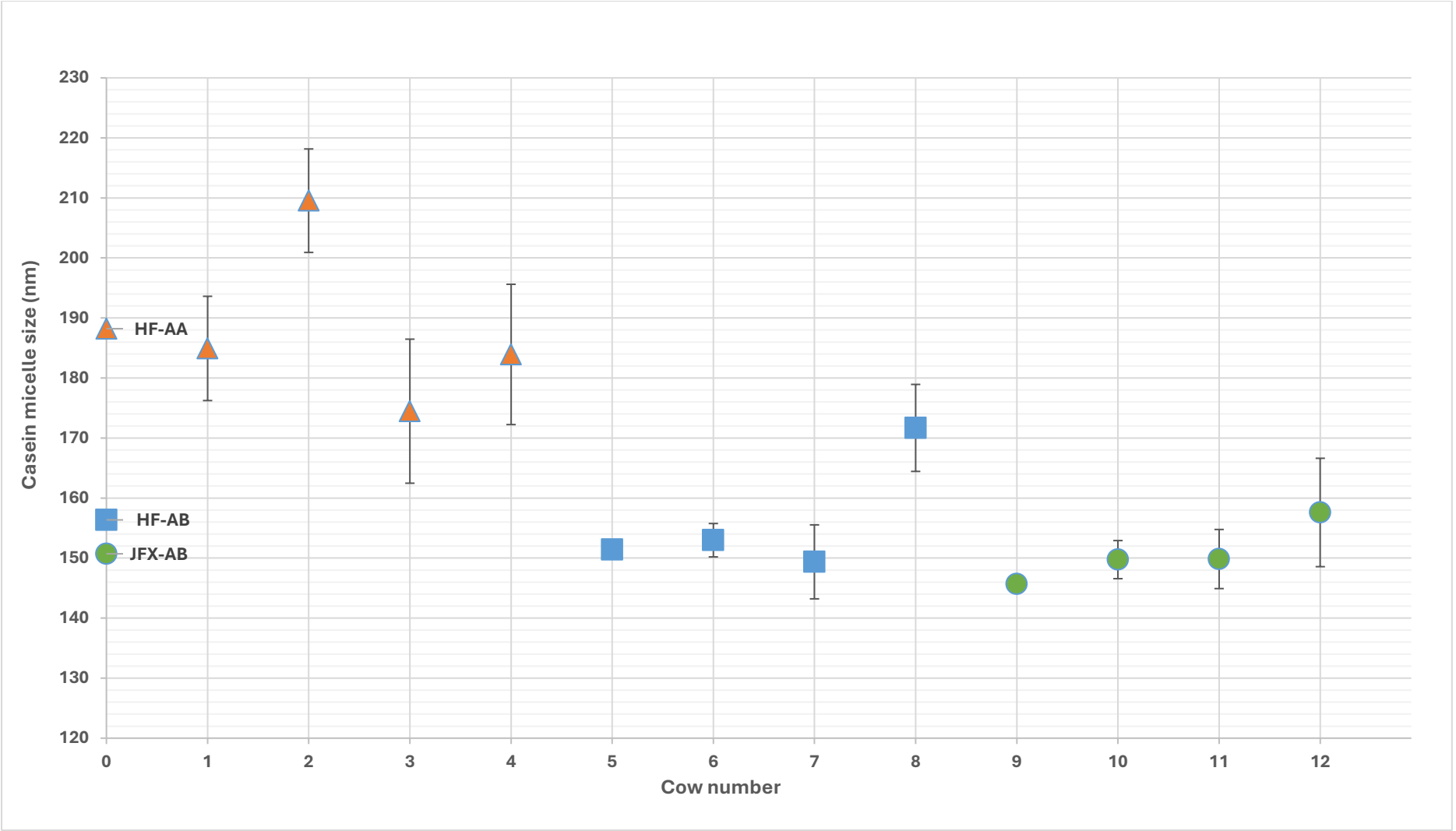


Fig. 6.3. The average casein micelle size (nm) variability of individual raw cow's milks, with treatment group averages shown on the y-axis, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CNA B (JFX-AB)

6.4.7 Zeta potential

The zeta potential values for all milks were very similar, with no significant differences observed between any of the individual samples ($P > 0.05$). Grouping this data by treatment type, the averaged zeta potentials for the HF-AA, HF-AB and JFX-AB treatments were -27.66, -26.44 and -27.03 mV, respectively, with these values being typical for raw bovine milk samples. Despite these values being similar, when completing a statistical analysis of the grouped data, the averaged HF-AA and HF-AB values did differ significantly from each other ($P < 0.05$). However, the stability of these milks, as described by these similar values, is suggested to be functionally comparable. Zeta potential values provide indications of the electrostatic stability of the milk system, and values below a certain threshold suggest that aggregation will occur (Walstra, 1990). The values obtained in this study are sufficiently far from that threshold to suggest an appropriate degree of colloidal stability for these milks.

6.4.8. Fat globule size

The parameters Dx10, Dx50, and Dx90 denote the milk fat globule (MFG) diameters below which 10, 50 and 90% of the cumulative volume distribution of MFG, respectively, are contained. A visual representation of these MFG size distributions for each individual cow's milk can be seen in Fig. 6.4. Milks of the HF-AA group had the smallest Dx90 values, ranging from 4.91 to 6.40 μm, while HF-AB milks showed intermediate values, ranging from 5.97 to 6.30 μm, and JFX-AB milks had the largest values for Dx90, of 6.58 to 9.17 μm. The averaged Dx90 values for JFX-AB milk were

significantly higher than HF-AB and HF-AA milk Dx90 values ($P < 0.05$), these values were 7.93, 6.10 and 5.59 µm, respectively. The Dx90 values of the HF-AB and HF-AA milks did not differ significantly from each other ($P > 0.05$). Regarding Dx50 values, HF-AA cows milks ranged 2.92–3.56 µm, HF-AB ranged 3.44–3.67 µm, and JFX-AB ranged 3.68–4.64 µm. These median percentile values were all significantly different from each other ($P < 0.05$), and the average HF-AA, HF-AB and JFX-AB group values were 3.20, 3.57 and 4.19 µm, respectively. Likewise, for Dx10 values, the HF-AA cows had the lowest values, ranging from 0.90 to 1.37 µm, with HF-AB cows ranging from 1.70 to 1.98 µm, and JFX-AB values ranging from 1.76 to 2.11 µm. Regarding Dx10 values, the average HF-AA milk values differed significantly from both HF-AB and JFX-AB milks, and these values were 1.13, 1.80 and 1.94 µm, respectively. The average Dx10 values of the HF-AB and JFX-AB milks did not differ significantly ($P > 0.05$). All MFG size values were within the typical range of expected values for bovine MFG size (Logan *et al.*, 2014).

With reference to Fig. 6.4, it should also be recognised that some notable variation is apparent at the individual cow-level. Other research has also provided evidence on individual cow-level variation and MFG size (Couvreur and Hurtaud, 2007). Indeed, Couvreur and Hurtaud (2007) reported that individual cow level variation in MFG size was sufficiently large that breed had little impact by comparison. Overall, within the current study, it is contended that distinct relationships are apparent between MFG size and the investigated breed and κ-CN groups. The B κ-CN phenotype was associated with larger MFG sizes compared to A κ-CN. The JFX-AB milks had larger

MFG sizes across all distribution percentiles, and significantly higher Dx50 values compared to the HF-AA and HF-AB groups ($P < 0.05$). It should be noted that breed-related impacts on MFG size have been reported previously (Fleming *et al.*, 2017; Carroll *et al.*, 2006). However, investigations that diminish the impact of breed-related associations on MFG size are also duly noted (Couvreur and Hurtaud, 2007).

The practical relevance of these findings warrants consideration with a number of potential impacts arising for cheese manufacturers when using milks of differing MFG size (O'Mahony *et al.*, 2005; Michalski *et al.*, 2003; Logan *et al.*, 2017). O'Mahony *et al.* (2005) and Logan *et al.* (2017) both noted that milks of differing MFG size can offer opportunities when developing differentiated cheese products. O'Mahony *et al.* (2005) found that use of milk with smaller MFG size resulted in lower fat content Cheddar cheese compared to that produced with larger MFG size milk. Logan *et al.* (2017) produced Cheddar cheese using milk that had been separated into large and small MFG size and observed differences in texture, microstructure and lipolysis. Michalski *et al.* (2003) demonstrated significant impacts on the functional and sensory properties of Camembert cheese due to MFG size. In particular, Michalski *et al.* (2003) determined that smaller MFG sizes were associated with softer cheese textures and faster rates of proteolysis. However, a causal link between MFG size and rate of proteolysis was not suggested. Michalski *et al.* (2003) also demonstrated that in Camembert cheese smaller fat globule size can increase fat recovery within cheese production. Conversely, O'Mahony et al., 2005 noted that milk with smaller MFG size can lead to lower fat

retention in Cheddar cheese. Lower fat retention in Cheddar cheese could reduce cheese yields because fat recovery is an important determinant of yield.

The potential impacts of MFG size described in other research suggests differences observed in this study, due to differing breed and genotype combinations, might need to be given further consideration by dairy breeders, manufacturers and other industry stakeholders, with impacts differing depending on production objectives such as the type of cheese being produced.

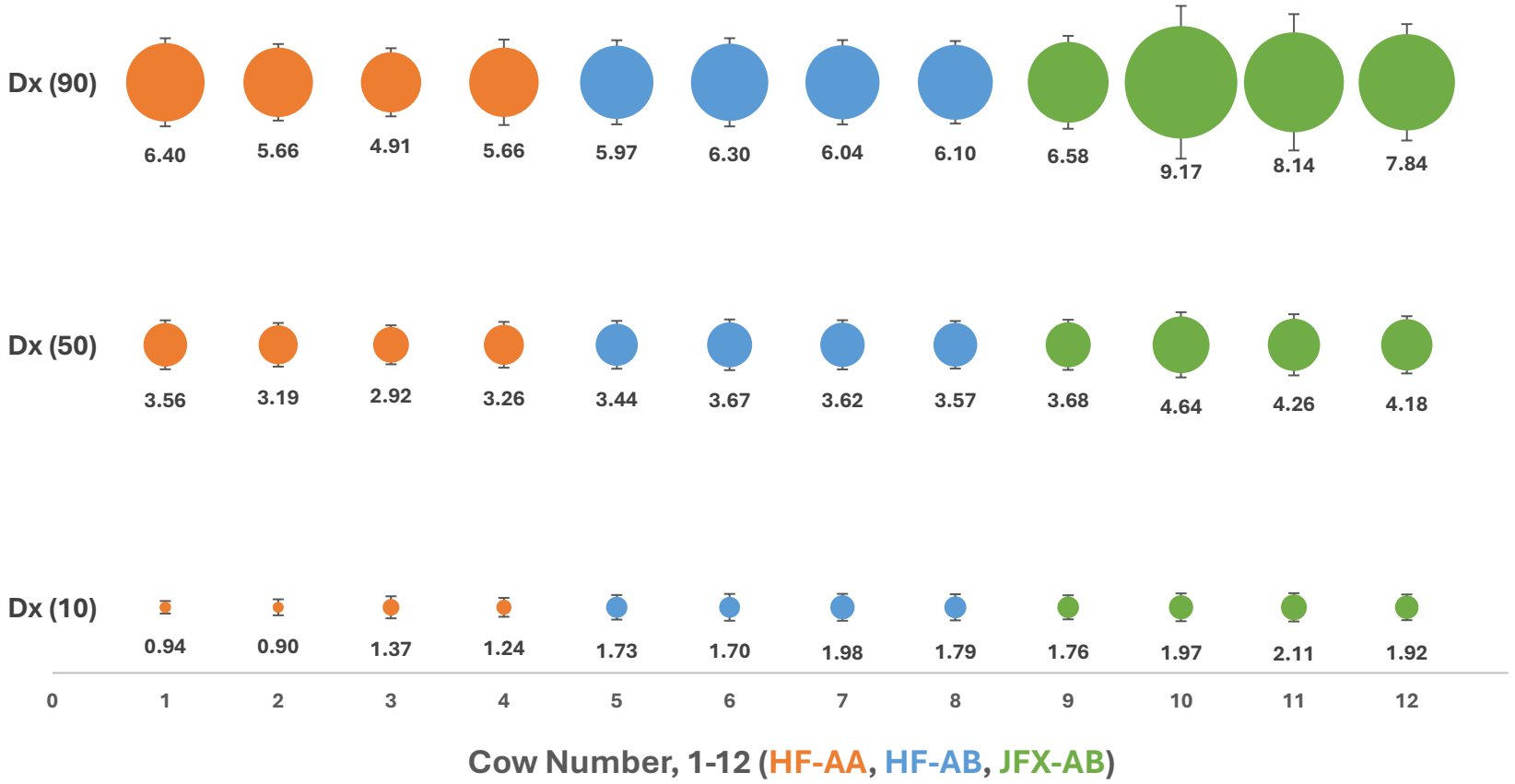


Fig. 6.4. The average milk fat globule size (μm) distribution of individual raw cow's milks, within the treatment groups Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CNA B (JFX-AB)

6.4.9. The relative expression of A and B κ-CN protein

The relative abundance of each κ-CN protein variant was determined for all milks using RP-HPLC, and this is reported as the integrated peak area under the curve (AUC) indexed to the total peak area associated with κ-CN. On the chromatograms, three peaks were associated with κ-CN; one for each protein variant, A and B, and a further peak that represents the glycosylated isoform of both κ-CN variants (Visser *et al.*, 1991) (Fig. 6.5). With reference to Fig. 6.5, the chromatogram peaks appropriately reflect the genotypes of the cows in this study. For HF-AA milks, the A κ-CN peak was present, and the B κ-CN peak was not present. For HF-AB and JFX-AB milks, peaks representing both A and B κ-CN variants were visible. For the HF-AA group, the peak associated with A κ-CN on average contributed 0.68 of the total integrated peak area, and ranged from 0.64 to 0.72. For the HF-AB and JFX-AB milks, the A κ-CN peak represented on average 0.29 and 0.30 of the total integrated peak area, respectively, with values ranging from 0.27 to 0.31, and the B κ-CN peak area represented on average 0.42 and 0.41 of the total integrated peak area, respectively, with values ranging from 0.39 to 0.43.

The milks derived from AB κ-CN genotypes, HF-AB and JFX-AB, are suggested to have very similar relative expression of A κ-CN and B κ-CN protein at ~0.30 and ~0.42, respectively. In simplistic terms, a cow possessing an AB κ-CN genotype might be assumed to express the A and B alleles equally, with A and B protein polymorphs then present in milk in equal proportions. However, as observed here, the rates of

expression of each allelic variant can vary, and is influenced by factors outside of the coding sequence (Keating *et al.*, 2007; Debeljak *et al.*, 2000). Debeljak *et al.* (2000) quantified allele specific mRNA transcripts in bovine mammary tissue and on average found 13.4% more B κ-CN allele specific transcripts than A κ-CN. Keating *et al.* (2007) identified two distinct promoter regions of potential importance within the regulation of κ-CN transcription, with one promotor resulting in stronger κ-CN expression than the other. Additionally, not all κ-CN protein was accounted for within the main A and B peaks. The remaining proportion of the total integrated peak area was similar for all treatment groups and is contained within the glycosylated k-CN peak (Fig. 6.5). Glycosylated isoforms represent one class of post-translational modified proteins, and the ~0.30 proportion of glycosylated k-CN protein observed in this study is consistent with other research findings (Bonfatti *et al.*, 2014). The HF-AA milks, derived from an AA κ-CN genotype, had glycosylation peaks representative of only glycosylated A κ-CN protein. Regarding the HF-AB and JFX-AB milks, these glycosylation peaks contain both A and B post-translational variants, and it is not possible to discern the relative proportions of each variant. For these milks, it seems plausible that a larger proportion of the glycosylated fraction might be made up of glycosylated A, rather than B, partially explaining the difference observed between the main A and B κ-CN peaks. Indeed, Farrell *et al.* (2004) described how A κ-CN does tend to have a great extent of glycosylation that B κ-CN. However, it is acknowledged that this study is limited by the method applied; a method utilising targeted LC-MS could be used to provide further detail on patterns of phosphorylation and glycosylation (Poulsen et al., 2016).

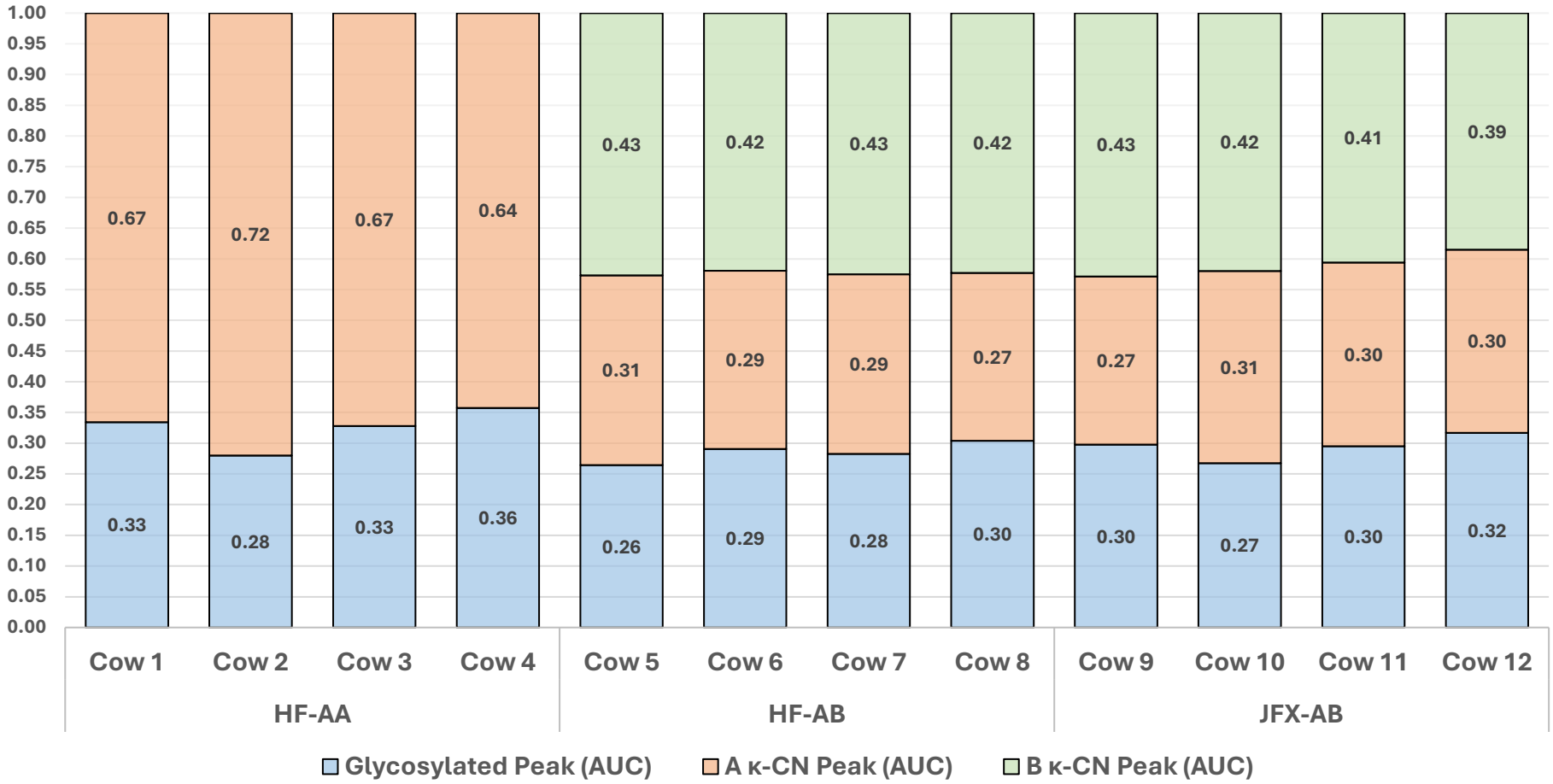
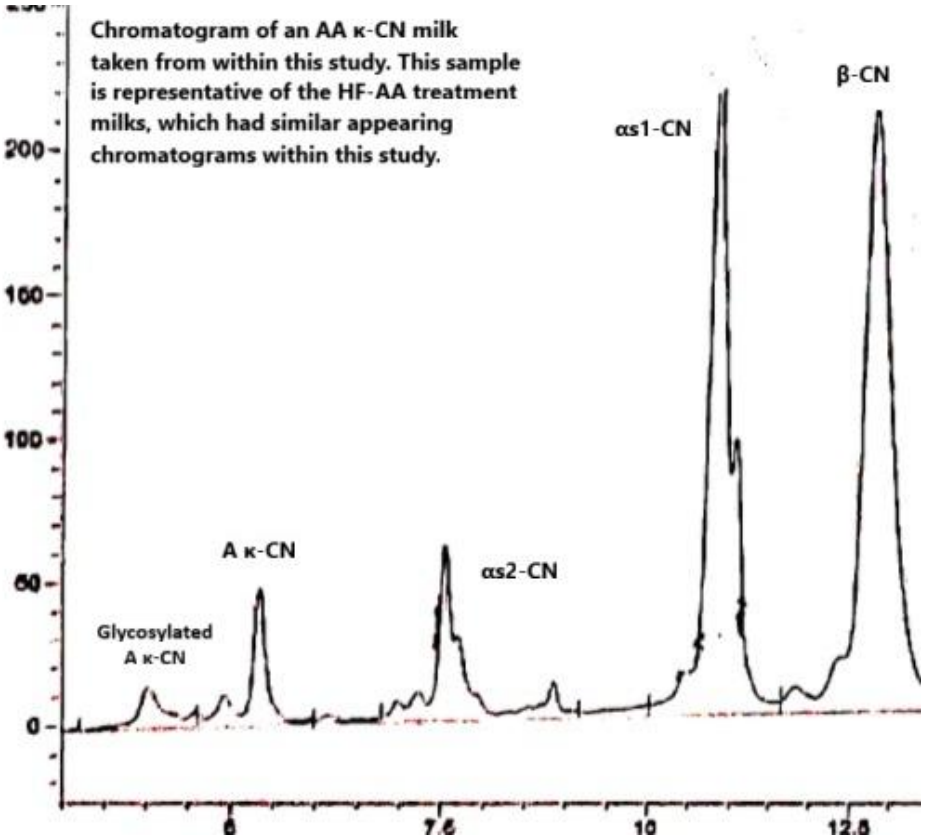
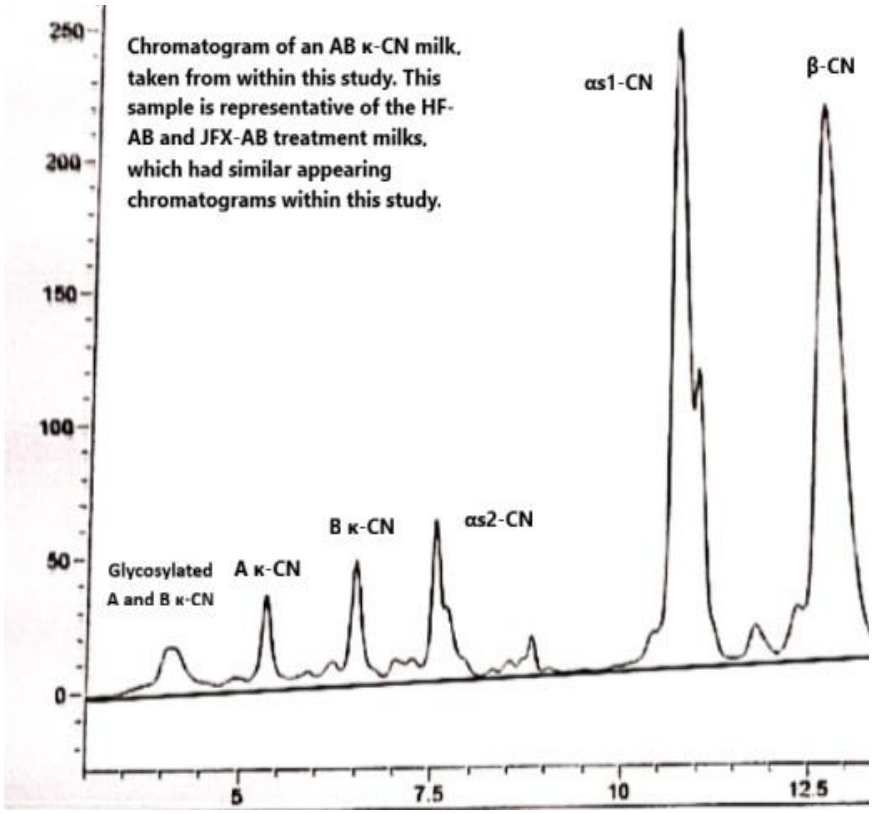


Fig. 6.5. The average relative expression of A and B κ-CN protein in milk by each individual cow of each treatment group, reported as the integrated peak area under the curve (AUC) indexed to the total peak area associated with κ-CN. Three peaks were associated with κ-CN on the chromatograms, one for each protein variant, A and B, with a further peak representing the glycosylated isoform of both κ-CN variants. Data presented are the average of three independent trials.

Fig. 6.6. Sample chromatograms representing the two patterns of expression of κ-CN observed in this study.





In this study, the glycosylated peak on average contributed ~0.30 of the total integrated peak area for all milks investigated and, by this measure, the relative extent of post-translational modification might also be suggested to be similar. Post-translational modification of k-CN proteins can impact casein micelle stability and size, zeta potential and rennet coagulation properties (Poulsen *et al.*, 2016). Poulsen *et al.* (2016) determined that breed-related factors can impact the extent of glycosylation, with Holstein milk possessing a higher relative abundance of glycosylated k-CN than Jersey milk, with values of 0.24 and 0.20, respectively. However, Poulsen *et al.* (2016) also found large variations between individual cows, with glycosylated k-CN ranging from 0.09-0.46 for Holstein cows and 0.10-0.60 for JE cows. Within this current study, the extent of glycosylation was similar between HF and JFX breeds, and across all analysed milks, the proportion of glycosylated κ-CN only ranged from 0.26 to 0.33. Regarding the manifestation of breed-related differences, candidate genes have been identified for the post-translational modification of proteins which can impact milk coagulation attributes and are linked to particular cow breeds (Poulsen *et al.*, 2016; Tyrisevä *et al.*, 2008). However, while significant differences in milk coagulation properties were observed between breeds within this current study, the relative extent of κ-CN glycosylation is unlikely to be a contributing factor given the similar amounts of glycosylation observed. Phosphorylation is a further type of post-translational modification, but within this study these isoforms were not resolved into distinct peaks on the chromatograms, and the phosphorylated isoform is included as a small fraction within the peak area of the main A and B allelic peaks.

Within this analysis, the relative expression profile of κ-CN protein variants was observed to be similar between all cows of the same κ-CN genotype, even when they differed by breed background. Additionally, the relative extent of glycosylation was observed to be similar between all investigated milks. This discussion has compared the relative expression of each κ-CN variant; indexed values for the proportion of each κ-CN protein contained within the overall κ-CN fraction. The proportions of A, B and glycosylated κ-CN protein were very similar between the AB κ-CN genotype milks, but as noted in section 6.4.4., the casein content of these milks did differ significantly. It has long been suggested that B κ-CN is associated with higher overall levels of casein in milk (Ng-Kwai-Hang *et al.*, 1984; Walsh *et al.*, 1998). Within this study, the presence versus absence of the B allele might be implicated in causing differences in casein content between HF-AA milks and HF-AB and JFX-AB. However, it is not necessarily possible to implicate differences in rates of expression of the B variant as being responsible for differences in casein content between the HF-AB and JFX-AB groups; the relative abundance described by the B variant peak appeared similar between these groups. Consequently, this evidence might support the possibility that some factor other than relative expression of B κ-CN, is influencing the higher casein content of JFX-AB milks as compared to HF-AB. As discussed, breed related genetic background effects and regulatory regions independent to the κ-CN locus might provide possible explanation of these differences. Studies like Keating *et al.* (2007) hint at this, with it being well-known that rates of κ-CN transcription are controlled by many factors, including regulatory sequences found outside of the main κ-CN coding region.

6.4.10. Treatment group specific clustering of results

Within this study, an overall clustering pattern is apparent for the production traits associated with the protein fraction of milk, Fig 6.7. Fig. 6.7 provides a visual representation through a simple min-max scaling of the data (Obaid et al., 2019), comparing protein related compositional and functional results obtained for the HF-AA, HF-AB and JFX-AB treatment groups. Notably, the protein related compositional data (casein and protein content) cluster on a by breed basis, whereas functional data (pattern of gene expression, casein micelle size, gelation behaviour) cluster by genotype.

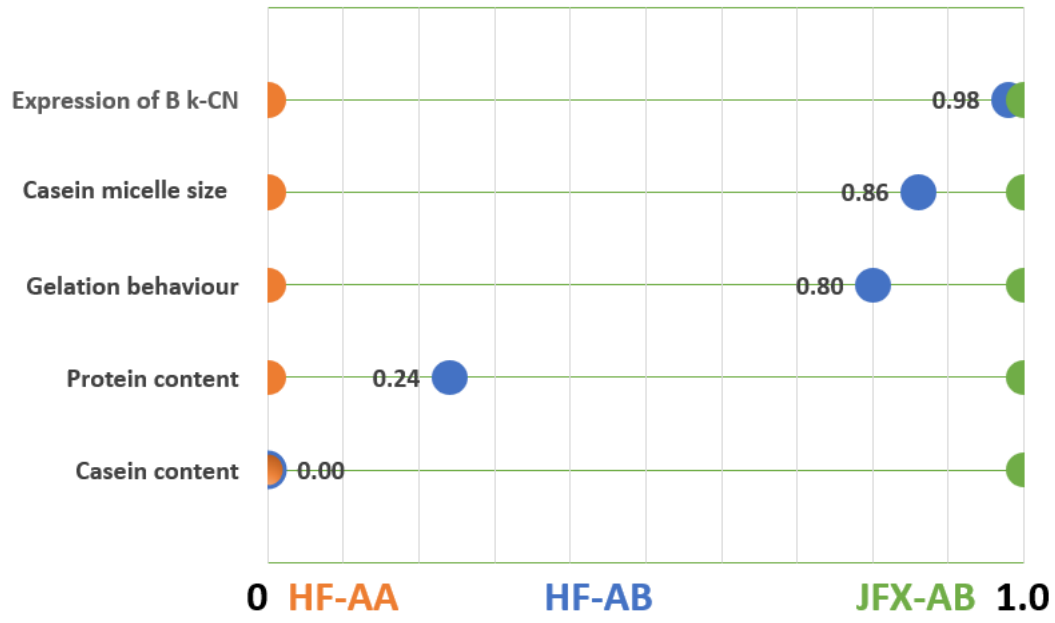


Fig. 6.7. Protein related data indexed by a simple min-max scaling method (Obaid et al., 2019): Scaled Value = (Value – Minimum) / (Maximum – Minimum)

Treatment groups; Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CNA B (JFX-AB). Scaling; HF-AA values =0.00 and JFX-AB values=1.00, HF-AB values intermediate from 0.00 to 1.00 based on the min-max scaling method

6.5. Conclusions

Milk attributes differed significantly with κ-CN polymorphism and breed background, but meaningful differences also arose at an individual cow-level, both between treatment groups and within them, emphasising the challenge for dairy breeding selections.

As anticipated, the expression pattern of κ-CN protein was similar between HF-AB and JFX-AB treatments, and these patterns differed from HF-AA milks. Regarding milk functionality, the clustering of results followed this pattern of gene expression; HF-AB and JFX-AB milks were functionally aligned by significantly smaller casein micelle size, lower pH values and significantly faster gelation behaviour compared to HF-AA milks.

All milks were of an A_2A_2 β-CN genotype, a genotype associated with benefits for dietary intolerances but also poorer gelation behaviour. In this study, faster set-to-cut times resulted for A_2A_2 β-CN milks when AB κ-CN was present, with the fastest gelation times observed for this genotype combination within the JFX-AB treatment.

The impact of the κ-CN genotype appears to be moderated by breed, JFX-AB milks had smaller casein micelles, lower pH and even faster gelation behaviour than HF-AB. Compositionally, HF-AA and HF-AB milks did not differ significantly in protein and casein content, despite differing κ-CN genotypes, and both HF treatments were associated with significantly lower protein and casein content milk compared to JFX-AB. Indeed, while the HF-AB and JFX-AB milks were functionally aligned by factors such as κ-CN expression and casein micelle size, it does also need to be acknowledged that

the JFX-AB and HF-AB milks used in this study were also compositionally different, in particular, the JFX-AB milk had significantly higher casein and calcium content compared to HF-AB milk.

Overall, the findings contained herein provide practical insights to guide future milk production phenotype research, supporting breeding programmes and other dairy industry stakeholders.

6.6. References

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Chapter 7

The impacts of κ-casein polymorphism and breed background on Cheddar cheesemaking efficiency and ripening characteristics

7.1. Abstract

This study compared the compositional and ripening characteristics of Cheddar cheese produced from protein-to-fat ratio-standardised milks derived from A and B κ-CN phenotypes of Holstein-Friesian (HF) and Jersey-Holstein-Friesian (JFX) cows. Specifically, three treatment groups were established: HF cows of an AA κ-CN genotype (HF-AA), HF cows of an AB κ-CN genotype (HF-AB), and JFX cows of an AB κ-CN genotype (JFX-AB). The resultant HF-AA and HF-AB cheesemilk did not differ significantly in macro-composition, while JFX-AB cheesemilk had significantly higher protein, fat and total solids content. The casein content of HF-AA, HF-AB and JFX-AB cheesemilks averaged 2.81%, 2.95% and 3.27%, respectively, and these milks yielded 9.83%, 10.36% and 11.31% of Cheddar, respectively. The casein content of HF-AA and HF-AB cheesemilks were not significantly different, but JFX-AB cheesemilk had a significantly higher casein content. The HF-AA, HF-AB and JFX-AB cheese yields were all significantly different from each other. Ripening attributes were equivalent across a 210 d maturation period, but JFX Cheddar was significantly more yellow, a generally favoured attribute for European consumers. It is anticipated that the evidence provided by this study furthers understanding of gene-to-phenotype production trait outcomes and can support dairy breeding programmes. Importantly, it has been demonstrated that crossbreeding can be combined with the selection of appropriate genetic variants to achieve significantly increased cheese yields, and this can have notable effects on efficiency within Cheddar manufacturing.

7.2. Introduction

Casein protein represents the major protein fraction in bovine milk and underpins the production of several commodity high value dairy ingredients such as cheese and yoghurt. Within the domain of dairy cow genetics the κ-casein (κ-CN) CSN3 gene is of particular interest; polymorphism at this locus has been demonstrated to have implications for the processing efficiency and characteristics of milk in cheese manufacturing and on final cheese quality (Farrell *et al.*, 2004; O'Regan *et al.*, 2009; Fox and Brodkorb, 2008). The three major κ-CN genotypes present in dairy cows are AA, AB, and BB, although other variants have been identified (Farrell *et al.*, 2004). The B allele of κ-CN has been associated with higher protein and casein content, improved curd firmness, and increased cheese yield compared to the A allele (Walsh *et al.*, 1998; Tsiaras *et al.*, 2005; Guggisberg *et al.*, 2024). Furthermore, certain allelic variants are present at higher frequencies within particular breeds, and in most commercial herds the A allele dominates (Walsh, 1999; Bangar *et al.*, 2021; Tsiaras *et al.*, 2005; Ren *et al.*, 2011). A meta-analysis by Bangar *et al.* (2021) described the A allele as being present at an overall frequency of 0.71 across multiple cow breeds. Within this analysis, Bangar *et al.* (2021) also noted that the A allele was present at a frequency of 0.94 in Holstein cows (Tsiaras *et al.*, 2005), and at a frequency of 0.11 in Jersey (JE) cows (Ren *et al.*, 2011). Ren *et al.* (2011) also described the B allele as being present in JE cows at a frequency of 0.88, and in Holstein cows at a frequency of 0.33. Consequently, the B allele is more typically associated with JE cows than HF and, due to this association, it is also present at a higher rate in crossbred, Jersey-Holstein-Friesian (JFX), herds than

HF herds (Walsh, 1999; Gurses *et al.*, 2018). However, an overall trend towards increased frequency of the B allele is taking place, with breeding programmes tending to favour selection of the B phenotype for its associated positive production traits (Kamiński *et al.*, 2023).

Most dairy herds in Ireland typically comprise of HF cows, and increased production efficiency has been achieved within the HF breed through selective breeding approaches (Coffey *et al.*, 2016; Palladino *et al.*, 2010), however, the impact of crossbreeding strategies on key dairy products also warrants attention (Buckley *et al.*, 2014; Saha *et al.*, 2020). In Chapter 5, a typical JFX crossbreeding strategy resulted in no adverse impacts to Cheddar quality as compared to HF, and it was also noted that selective breeding based on breed categories alone would likely be insufficient when targeting specific production objectives. Genetic selections for specific genetic markers for production traits, such as κ-CN genotype, might offer a more advantageous approach. In typical breeding strategies the consequences of such genotypes on milk processability are still not readily considered.

The implications of κ-CN polymorphism on milk composition, milk coagulation properties, casein content and casein micelle size are well-understood, and the implications of κ-CN polymorphism on cheese manufacturing has already been considered (Guggisberg *et al.*, 2024; Walsh *et al.*, 1998; Lavon *et al.*, 2023). However, such factors have not been considered recently within Irish pasture-grazing systems, and the relative impacts on production traits when different breeds possess different

κ-CN variants is an under-explored research area. Essentially, a research gap has been identified regarding genetic background effects (the rest of a genome influencing how genes are expressed and present phenotypically). One consideration is that the phenotypic presentation of κ-CN might be altered through interactions with polymorphism of the other major milk proteins. Additionally, rates of gene expression can vary due to polymorphism within exon sequences, polymorphism of regulatory elements and due to other mutations that cause premature stop codons and decreased levels of transcripts (Martin *et al.*, 2002). Martin *et al.* (2002) stated that this additional polymorphism should be recognised for its impact on the functional attributes of milk and possible implications for cheese yields and cheese quality. Going further still, previous investigators have contributed to vast amounts of data describing potential candidate genes and genetic markers that might have relevance to milk production traits (Ogorevc *et al.*, 2009; Križanac *et al.*, 2025; Cai *et al.*, 2024). These as yet not fully understood regions of genetic variation introduce further complexity to how milk protein genes present phenotypically.

Genetic factors tend not to operate in isolation, but in combination, and this study attempts to acknowledge this consideration. It is proposed here that the relative phenotypic manifestation of B κ-CN might vary for cheese production traits according to the breed related genome within which that variant resides. Specifically, it is hypothesised that differences might arise in cheesemilk and cheese composition, cheese yield and cheese ripening attributes when κ-CN polymorphism is considered in combination with HF and JFX breed backgrounds. Specifically, the aim of this study

was to compare Cheddar cheese derived from HF cows with AB κ-CN (HF-AB), JFX cows with AB κ-CN (JFX-AB), and HF cows with AA κ-CN (HF-AA).

7.3. Materials and methods

7.3.1. Experimental design

The milk used for cheese production within this study was collected as a part of a separate research study reported previously, Chapter 6. The milk was supplied by the Animal and Grassland Research and Innovation Centre, Teagasc, Moorepark, Fermoy, Co. Cork, Ireland. Full details can be found in Chapter 6. Both the milk collections and cheese manufacturing trials were completed in July and August 2024, with each trial separated by two weeks. The entire end-to-end cheesemaking process, including the process of milk collection and standardisation described, were each performed in full on three separate occasions providing three independent replicate trials.

7.3.2. Preparation of cheesemilk

Raw milks from twelve individual cows (selected based on specific breed and genotype combinations) were collected as a part of the Chapter 6 study. Within the milk collection procedure for this study, the full morning milking was taken for each individual cow, mixed thoroughly and aliquots were then taken.

Regarding the twelve individual cow's milks collected, cows 1-4 were HF cows of a confirmed AA k-CN genotype (HF-AA), cows 5-8 were HF cows of a confirmed AB k-CN genotype (HF-AB) and cows 9-12 were JFX cows of a confirmed AB k-CN genotype (JFX-AB). Consequently, the four relevant individual milks were combined in equal

proportions by weight in the ratio 1:1:1:1 according to the treatment groups described above.

The standardisation and pasteurisation, in preparation for cheesemaking, was then completed for each of these three combined milks, HF-AA, HF-AB and JFX-AB. Three independent cheesemaking trials were completed in July and August 2024. For each trial the milks were obtained from the same precise cow groups that had been maintained under the same conditions. For all milks within each trial the milk undergoing preparation for cheesemaking was also standardised and pasteurised at the same time and under the same conditions. For each combined treatment type milk, HF-AA, HF-AB and JFX-AB, a portion of the raw milk, ~ 5kg, was separated into cream and skim milk at 50°C using a benchtop centrifuge operating at 10,000 revolutions per min (FT15 Disc Bowl Centrifuge, Armfield Limited, Hampshire, UK). Milk from each treatment was then standardised to a total protein-to-fat ratio of 0.95 using skim milk, raw whole milk or cream (Guinee *et al.*, 2006). These standardised milks were then pasteurised at 72°C for 15 s (HTST/UHT System HT122, OMVE, Scgalkwijk, the Netherlands). The standardised and pasteurised milk is referred to as cheesemilk within this study. All cheesemilks were stored overnight at 4°C, and Cheddar cheese manufacturing took place the following morning.

7.3.3. Cheese manufacturing

On the day following standardisation and pasteurisation, Cheddar was manufactured utilising pilot-scale production vats (10 L; Pierre Guerin Technologies, Mauze, France)

at the Biofunctional Food Engineering Facility, Teagasc Food Research Centre, Moorepark, Fermoy, Co Cork, Ireland, as described by Page *et al.* (2024) (Chapter 2).

7.3.4. Composition of cheesemilk, whey and cheese

Analyses completed as described in Chapter 2

7.3.5. Zeta potential, casein micelle size and fat globule size of cheesemilk

These attributes of cheesemilks were analysed similarly to methods described in Chapter 6.

7.3.6. Cheese yield

Determination completed as described in Chapter 2

7.3.7. Analyses performed during cheese ripening

Measurements of pH, colour, cheese texture and primary proteolysis were completed as described in Chapter 2, but with measurements taken in cheese samples at 1 d, 40 d, 110 d, and 210 d of ripening.

7.3.8. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 27 (IBM Statistics Inc.), with some processing and formatting of data carried out in Microsoft Excel 64 bit, 2016 Version (Microsoft Corporation Inc.). The reported data are the averages of results obtained from three separate cheesemaking trials (n=3). Results are presented as the mean average ± standard deviation. Univariate ANOVA were used for the comparison of means in single factors. A repeated-measures GLM ANOVA model was

performed in SPSS to interpret statistical relationships between changes in cheese attributes over the ripening period. Tests of within-subjects effects were utilised to determine whether significant ripening related changes had occurred during the ripening period, tests of between-subjects effects described significant differences between the treatment groups. Sphericity of data was confirmed through Mauchly's Test, and when sphericity of data was violated Greenhouse-Geisser corrected values were used. For all statistical analyses, p-values < 0.05 were considered significant. Effect sizes were also calculated where appropriate; partial eta squared ($\eta 2$). A $\eta 2 < 0.06$ was considered a small effect size, $0.06 \leq \eta 2 < 0.14$ was considered a medium effect size, and $\eta 2 \geq 0.14$ considered a large effect size.

7.4. Results and discussion

7.4.1. Cheesemilk macro-composition

The macro-compositions of HF-AA, HF-AB and JFX-AB cheesemilk are shown in Table 7.1. The macro-compositions of HF-AA and HF-AB cheesemilk were not significantly different from each other ($P > 0.05$). The HF-AA cheesemilk averaged 3.66% protein, 3.48% fat, 12.34% total solids (TS) and 4.77% lactose, while the HF-AB cheesemilk averaged 3.86% protein, 3.62% fat, 12.76% TS and 4.73% lactose. Conversely, the JFX-AB cheesemilk was significantly higher in protein, fat and TS content as compared to both HF-AA and HF-AB milks ($P < 0.05$); the JFX-AB cheesemilk averaged 4.29% protein, 4.06% fat, 13.80% TS and 4.78% lactose. No significant effect of treatment was

observed for lactose content (P > 0.05). Significantly higher levels of protein, fat and TS have been reported previously in JFX raw milk and cheesemilk as compared to HF, albeit not using the same controlled genotypes (Teagasc, 2012; Coffey *et al.*, 2016; Page *et al.*, 2025).

Table 7.1: The macro-compositions of cheesemilks, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

	HF-AA	HF-AB	JFX-AB	P-Value	η2
Fat (% wt/wt)	3.66^{a}	3.86^{a}	4.29^{b}	***	0.924
±	0.03	0.12	0.10		
Total Protein (% wt/wt)	3.48^{a}	3.62^{a}	4.06^{b}	***	0.900
±	0.10	0.10	0.11		
Total Solids (% wt/wt)	12.34^{a}	12.76^{a}	13.80^{b}	*	0.777
±	0.48	0.43	0.27		
Lactose (% wt/wt)	4.77^{a}	4.73^{a}	4.78^{a}	NS	0.083
±	0.11	0.07	0.09		

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

A general trend is apparent between these cheesemilks regarding fat, protein and TS values; HF-AA milk had the lowest average amounts of these macro-constituents, followed by HF-AB, and JFX-AB milk had significantly higher fat and protein values. This has relevance for cheese production because the amount of cheese yielded from a given volume of milk is positively correlated with the milk's fat and casein protein

content (Lamichhane *et al.*, 2018b; Page *et al.*, 2024). Genes code proteins and in past studies the B variant of κ-CN has been associated with higher levels of protein more generally and casein protein in milk (Lavon *et al.*, 2023). Indeed, within this current study, the milk obtained from cows of the A κ-CN phenotype, group HF-AA, had lower average protein content compared to both B κ-CN phenotype groups, HF-AB and JFX-AB. However, it is noteworthy that the protein content of HF-AA milk was not significantly lower than that of HF-AB milk in this study ($P > 0.05$). So, cheesemilk derived from differing AB κ-CN and AA κ-CN genotypes did not differ significantly in protein content when that milk was obtained from the same HF breed. While macro-compositions provide overall context, casein protein is the most relevant protein to cheese production, and the casein contents of HF-AA and HF-AB cheesemilks are considered in section 7.4.2.

The hypothesis of this study was that breed related background might mediate the phenotypic presentation of production traits associated with B κ-CN. Regarding the JFX-AB milk used in this study, this milk was obtained from animals of a known κ-CN genotype, the same heterozygous AB κ-CN genotype as that of the HF-AB group, and these cheesemilks did differ significantly in protein content, despite being of the same AB κ-CN genotype. So, it seems likely that breed related genetic background is contributing to the significantly increased protein content observed in JFX-AB milk as compared to HF-AB milk. Overall, regarding the analysis of macro-composition performed within this study, breed related background appears to be more impactful on cheesemilk protein, fat and TS content than κ-CN genotype in isolation ($P < 0.05$).

However, casein protein is the most relevant protein to cheese production and the influence of the AB κ-casein genotype and this breed related factor on milk casein content are discussed in the next section.

7.4.2. Cheesemilk casein number, non-protein-nitrogen and casein content

Casein number and non-protein nitrogen (NPN) content did not differ significantly between the HF-AA, HF-AB and JFX-AB cheesemilks ($P > 0.05$), but regarding casein content, some significant differences were observed ($P < 0.05$) (Table 7.2).

The average casein content values of the HF-AA, HF-AB and JFX-AB cheesemilks were 2.81%, 2.95% and 3.27%, respectively. The casein content of HF-AA and HF-AB cheesemilks did not differ significantly ($P > 0.05$), whereas JFX-AB cheesemilk had a significantly higher casein content than both HF-AA and HF-AB ($P < 0.001$).

The JFX-AB treatment appears to have a greater influence on milk casein content than the presence of the B k-CN variant in isolation within the HF groups. Again, JFX-AB cows are, by virtue of the experimental design, confirmed to be of an AB k-CN genotype. However, the AB k-CN genotype did not in itself result in significantly higher casein content values. The JFX-AB and HF-AB treatment groups were both defined to contain only animals of the same AB k-CN genotype, and under this scenario milk derived from JFX cows had significantly higher casein content than that of HF cows ($P < 0.05$). Significant differences in casein content were not observed between the HF-AA and HF-AB cheesemilks despite differing AA versus AB genotypes ($P > 0.05$).

Within this study efforts were made to control potential confounders, and the animals of the differing treatment groups were maintained under the same extrinsic conditions, such as being given the same diets. It is therefore suggested that some other aspect intrinsic to these JFX cows is resulting in these casein content differences. Within the experimental design, all cows were confirmed through genotyping to be of the same A_2A_2 β-CN genotype, so β-CN polymorphism should not be implicated in causing these significant differences in casein content. It is suggested that some as yet unknown, or undefined, aspect of JFX background might be the cause of these observed differences.

These findings for casein content also reflect that which was previously reported in chapter 6, the investigations carried out into the individual raw milks that relate to this current study. While the implications of individual cow variations on milk components should be considered (Couvreur and Hurtaud, 2007; Vance et al., 2012), the impacts of this factor seem to be far less practically meaningful when milks are pooled for cheesemaking. The separate study described in Chapter 5 observed average casein content values for HF and JFX milks of 2.80 and 2.96, respectively; values that were not statistically significant. The analysis of casein content in milk within the Chapter 5 study was also performed on cheesemilk prepared for Cheddar manufacturing. However, this previous study's findings cannot be directly compared to this current study, because crossbreeding was investigated more broadly in Chapter 5, with cheese manufactured from bulk milks, and the defining of k-CN and β-CN genetics was not a part of that studies design. It is therefore interesting to contrast the results of this current study

with Chapter 5. Within this current study significant differences in casein content were observed between HF and JFX cheesemilks when specific genetic variants were present.

Table 7.2: Cheesemilk casein content, casein number and non-protein nitrogen content, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

		HF-AA	HF-AB	JFX-AB	P-Value	η2
Casein Content (% wt/wt)		2.81[a]	2.95[a]	3.27[b]	**	0.848
	±	0.10	0.11	0.10		
Casein Number (% wt/wt)		80.71[a]	81.33[a]	80.62[a]	NS	0.240
	±	0.69	0.83	0.53		
Non-Protein Nitrogen (% wt/wt)		3.88[a]	5.52[a]	5.05[a]	NS	0.368
	±	0.64	1.24	0.48		

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

7.4.3. Mineral content of cheesemilk

The investigated cheesemilks were all broadly equivalent to each other in mineral content with no significant differences in calcium, potassium, sodium, magnesium or phosphorus content (P > 0.05) (Table 7.3).

The lack of differences observed in calcium content might be considered unexpected given differences seen in casein content (Table 7.2, Table 7.3). However, total calcium

does not correlate perfectly with casein content. The total calcium measure includes the calcium associated with casein, colloidal calcium, and the calcium present in the serum phase (Gaucheron, 2005). Calcium partitioning was not measured in this study.

Table 7.3: Cheesemilk mineral content, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

	HF-AA	HF-AB	JFX-AB	P-Value	η^2
Calcium (mg/kg)	1166	1138	1223	NS	0.040
±	152	265	202		
Potassium (mg/kg)	1512	1396	1458	NS	0.061
±	134	266	257		
Magnesium (mg/kg)	99	97	109	NS	0.143
±	10	19	17		
Sodium (mg/kg)	313	353	339	NS	0.116
±	12	75	60		
Phosphorous (mg/kg)	862	832	895	NS	0.048
±	92	171	149		

^{NS}P > 0.05 (non-significant)

7.4.4. Zeta potential of cheesemilk

The average zeta potentials for the HF-AA, HF-AB and JFX-AB treatment cheesemilks were comparable at -29.27 mV, -27.46 mV, and -28.41 mV, respectively, and were within the range of typical expected values. The HF-AA and HF-AB values were

significantly different from each other ($P < 0.05$), but these values are sufficiently close together that in practical terms they can probably be considered as about equivalent.

7.4.5. Casein micelle size of cheesemilk

The average casein micelle sizes of cheesemilks derived from the HF-AA, HF-AB and JFX-AB treatments were 185 nm, 151 nm and 147 nm, respectively. These values fall within the typical range of expected values for casein micelle size (Fox and Brodkorb, 2008). The HF-AA treatment resulted in casein micelles that were on average significantly larger than those of the HF-AB and JFX-AB treatments ($P < 0.05$). The average casein micelle size of HF-AB and JFX-AB cheesemilks were comparable and did not differ significantly from each other ($P > 0.05$), Fig. 7.1.

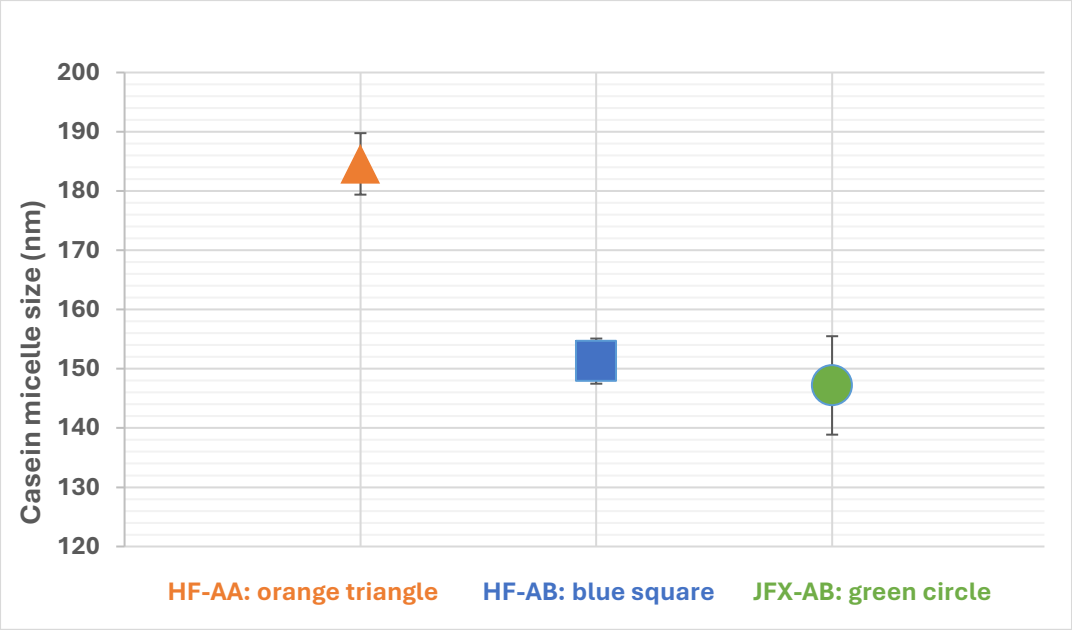


Fig.7.1. Casein micelle size (nm), Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB). The values are the average of 3 independent trials ±standard deviation.

These results are similar to the results determined for the raw milks used to create these combined cheesemilks (Chapter 6). The cheesemilks in this current study differ from the previous Chapter 6 study by having undergone pasteurisation and standardisation. These processing steps, typical to Cheddar manufacturing, were not anticipated to substantially alter casein micelle size, and the results of this analysis confirm this and also align with similar observations made within other studies (Anema, 2021). However, it is noteworthy that other processing methods such as heat treatment, pH adjustment and calcium addition can influence casein micelle structure through changes in mineral equilibrium and changes to protein interactions (Gaucheron, 2005; Anema, 2021).

7.4.6. Fat globule size of cheesemilk

The Dx10 values of the HF-AA, HF-AB and JFX-AB cheesemilks were 0.87 µm, 1.76 µm and 1.83 µm, respectively. The Dx10 values of the HF-AA cheesemilks differed significantly from those of HF-AB and JFX-AB ($P < 0.05$), while HF-AB and JFX-AB values were comparable and did not differ significantly from each other ($P > 0.05$). Regarding Dx50 values, the HF-AA cheesemilks differed significantly from those of JFX-AB ($P < 0.05$), and the values for the HF-AA, HF-AB and JFX-AB cheesemilks were 3.29 µm, 3.54 µm and 3.90 µm, respectively. The Dx90 values of the HF-AA, HF-AB and JFX-AB cheesemilks did not differ significantly from each other, and they were 5.96 µm, 6.07 µm and 6.70 µm, respectively ($P > 0.05$), Fig.7.2.



Fig. 7.2. The average milk fat globule size (µm) distribution of cheesemilks, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CNA B (JFX-AB). The values are the average of 3 independent trials ±standard deviation.

With reference to Chapter 6, it is notable that the fat globule distributions of cheesemilks had less differences between them than the raw milks used to produce them. In particular, the cheesemilk showed no significant differences for the largest Dx90 fat globule size group, while the raw milks described in Chapter 6 had significant differences between the treatments for the Dx90 group. This provides information on the relative attributes of milk which has undergone the processing steps of standardisation and pasteurisation, as compared to raw milks. It seems likely that the processes of heating and centrifugation during standardisation and pasteurisation have impacted on fat globules within the cheesemilk. Processing steps involving heat

treatments, mechanical disruption or shear, such as homogenisation, cream separation and recombination are known to alter milk fat globule size distribution, while pH adjustment and calcium addition are more relevant to casein micelle structure but can still have some indirect impacts on fat globules (Hayes and Kelly, 2003; Gaucheron, 2005; Anema, 2021).

7.4.7. Whey composition

Some significant differences in bulk whey components were observed between the HF-AA, HF-AB and JFX-AB treatment groups ($P < 0.05$) (Table 7.4). The HF-AA treatment resulted in the greatest average fat content in whey, followed by JFX-AB, and then HF-AB; these values were 0.49%, 0.43%, and 0.37%, respectively. The average fat content values of HF-AA and HF-AB bulk whey samples were significantly different from each other ($P < 0.05$). However, the average fat content of HF-AA and HF-AB whey samples were not significantly different from JFX whey ($P > 0.05$). The JFX-AB whey had a significantly higher total protein content (1.37%) than HF-AA (1.06%) and HF-AB (1.05%) ($P < 0.05$), while HF-AA and HF-AB did not differ significantly ($P > 0.05$). The JFX-AB treatment resulted in a average whey total protein content that was significantly higher than that of both HF-AB and HF-AA whey ($P < 0.05$). The JFX-AB whey had an average total protein content of 1.37%, while the HF-AA and HF-AB treatments were associated with whey total protein content values that were not significantly different from each other, 1.06% and 1.05% respectively. Similarly, the JFX-AB whey had a significantly higher TS content (7.22%) than HF-AA (6.98%) and HF-AB

(6.87%) ($P < 0.05$), while HF-AA and HF-AB did not differ significantly ($P > 0.05$).The HF-AA whey had the highest numerical levels of curd fines, followed by HF-AB, and JFX-AB whey had the least curd fines, but none of these curd fine values were significantly different from each other ($P > 0.05$).

The higher whey fat content observed for the HF-AA treatment could reflect less efficient fat retention during curd formation, and fat losses into whey are influenced by milk fat globule size. In this study, the Dx10 values of the HF-AA cheesemilks were significantly smaller than those of HF-AB and JFX-AB ($P < 0.05$), and regarding Dx50 values, the HF-AA cheesemilks had milk fat globule sizes which were significantly smaller than those of the JFX-AB cheesemilks ($P < 0.05$). These differences in milk fat globule size distribution might provide some of the explanation for the differences observed in whey fat content. Additionally, when protein and fat content levels are higher there is a greater tendency for losses of both components into the whey. Indeed, the whey protein content levels observed do broadly reflect the protein content of the milks; JFX-AB milk had both the highest cheesemilk protein content and the highest whey protein content. However, regarding whey fat content, the HF-AA derived whey had the highest value fat content but the HF-AA cheesemilk was significantly lower in fat than HF-AB and JFX-AB cheesemilk. Consequently, HF-AA whey had a higher fat content than HF-AB and JFX-AB despite being derived from cheesemilk that had a lower fat content as compared to HF-AB and JFX-AB treatments.

The significant differences in protein and TS in whey follow the same pattern of significant differences that were observed in milk composition between the JFX-AB and the two HF treatment groups. Within this study, JFX-AB milk and whey both had significantly higher protein and TS content compared to HF-AA and HF-AB ($P < 0.05$).

Table 7.4: Composition of bulk whey, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

	HF-AA	HF-AB	JFX-AB	P-Value	η^2
Fat (% wt/wt)	0.49^{a} 0.10	0.37^{b} 0.02	0.43^{ab} 0.04	*	0.376
Protein (% wt/wt)	1.06^{a} 0.09	1.05^{a} 0.10	1.37^{b} 0.07	**	0.833
Total Solids (% wt/wt)	6.98^{a} 0.11	6.87^{a} 0.15	7.22^{b} 0.10	***	0.644
Curd Fines (mg/kg)	245^{a} 135	192^{a} 87	170^{a} 141	NS	0.090

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

7.4.8. Cheese composition

Some statistically significant differences in cheese macro-constituents were observed between the HF-AA, HF-AB and JFX-AB treatment groups ($P < 0.05$) (Table 7.5). The fat content values of HF-AA, HF-AB and JFX-AB cheese were 32.50%, 35.32% and 36.24%, respectively. The HF-AA cheese had significantly lower fat content than both HF-AB and JFX-AB derived cheese ($P < 0.05$), but the fat content values for HF-AB and JFX-

AB cheese were not statistically different from each other (P > 0.05). The moisture content values of HF-AA, HF-AB and JFX-AB cheese were 36.69%, 34.88% and 34.25%, respectively. The moisture content of JFX-AB cheese was significantly lower than that of HF-AA cheese (P <0.05). The moisture content of HF-AB cheese was not significantly different from that of both HF-AA and JFX-AB derived cheese (P > 0.05). The protein content of HF-AA, HF-AB and JFX-AB cheese were 25.87, 25.66, and 25.75%, respectively. These protein content values were not significantly different from each other (P > 0.05).

Table 7.5: Composition of Cheddar cheese derived from treatments, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

	HF-AA	HF-AB	JFX-AB	P-Value	η2
Fat (% wt/wt)	32.50^{a}	35.32^{b}	36.24^{b}	**	0.820
±	0.98	0.66	1.05		
Protein (% wt/wt)	25.87a	25.66a	25.75a	NS	0.020
±	0.88	0.59	0.78		
Moisture (% wt/wt)	36.69^{a}	34.88^{ab}	34.25^{b}	*	0.742
±	0.59	0.33	1.11		

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

It should be noted that none of these observed compositional differences should pose practical concerns. The protein, fat and moisture percentages observed here for all cheese treatment groups were consistent with previously reported observations for standard full-fat Cheddar cheese (Feeney et al., 2021).

7.4.9. Cheese yield

Significant differences in cheese yield were observed between the HF-AA, HF-AB and JFX-AB treatments ($P < 0.05$) (Table 7.6). Using HF-AB cheesemilk to manufacture Cheddar resulted in an average 5.43% higher actual yield (Y_a) than HF-AA, and the JFX-AB treatment resulted in an average 15.18% higher Y_a than HF-AA. The JFX-AB treatment also yielded significantly more cheese than HF-AB, with an on average 9.25% higher Y_a value ($P < 0.05$). The averaged Y_a values for the HF-AA, HF-AB and JFX-AB treatments were 9.82, 10.36 and 11.32%, respectively. Similarly, moisture-adjusted cheese yield (Y_{ma}) was significantly impacted by κ-CN polymorphism and breed ($P < 0.05$). The averaged Y_{ma} values of the HF-AA, HF-AB and JFX-AB treatments were 10.11%, 10.97% and 12.10%, respectively. However, the moisture adjusted cheese yield adjusted for-protein and fat (Y_n), did not differ significantly between the treatments ($P > 0.05$). The higher cheese yields associated with the κ-CN B variant and JFX breed both appear to have resulted from differences in protein (mainly casein) and fat content between these cheesemilks.

It is apparent within this study that an AB κ-CN genotype increases cheese yields relative to AA κ-CN, validating previous knowledge on these genotypes within a current Irish pasture-based seasonal production system. However, the greatest amount of Cheddar produced resulted from the JFX-AB treatment, the cheesemilk with the highest casein content. Regarding JFX-AB cheesemilk, the AB κ-CN genotype is a likely contributing factor towards these increased cheese yields, but some other aspect

of JFX breed background has also acted to increase cheese yields further. The experimental design enabled comparisons between breed groups by controlling κ-CN genetics, and the study was also approached with an awareness towards impacts arising from other genetic variants. Marziali and Ng-Kwai-Hang (1986) previously associated β-CN A_1 phenotypes with improved cheese yields, although Daniloski *et al.* (2024) later stated that β-CN polymorphism had no effect on cheese yields. To avoid potential issues with β-CN acting as a possible confounder, all cows within this study were selected to have an A_2A_2 β-CN genotype. Consequently, β-CN polymorphism should not be considered as contributing towards the observed cheese yield differences. Milk solids are known to be higher in milks derived from JE and JFX cows, resulting from a lower overall milk volume and concentration effects associated with these breeds. The genetic determinants of reduced milk volumes associated with the JFX breed, in addition to other differing aspects of JFX breed background are not fully understood. With reference to the earlier discussion of the literature in this manuscript, Martin *et al.* (2002) noted that rates of gene expression can vary due to additional polymorphism within regulatory elements with potential implications for cheese yields. Breed associated differences in regulatory regions that control rates of transcription could provide a possible explanation for these observations. Furthermore, the large amounts of additional genetic data that have yet to been interpreted into functional understanding provides a further rich source of genetic variation that could contribute to describing these observations (Ogorevc *et al.*, 2009; Križanac *et al.*, 2025; Cai *et al.*, 2024). As far as the authors are aware, these specific findings on differing relative

cheese yield performance of AB k-CN on a by breed basis for JFX and HF cows is a novel observation. Further investigations into JFX and JE genetic background effects could clarify this and elucidate the specific causes of these differences. Studies such as Keating *et al.* (2007); Debeljak *et al.* (2000); Gerencsér *et al.* (2002) have investigated regulatory regions outside of the κ-CN coding sequence that impact on rates of k-CN transcription and gene expression, and hint at how such differences might be resulting between these breeds. However, based on this current study, all that can be concluded at this time is that meaningful significant differences in milk composition and cheese yields are associated with JFX-AB milks as compared to HF-AB, differences that appear to have resulted over and above that which can be explained by k-CN and β-CN polymorphism.

Table 7.6: Average cheese yields, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

	HF-AA	HF-AB	JFX-AB	P-Value	η2
Actual Yield (Y_a)	9.82^a 0.11	10.36^b 0.20	11.32^c 0.22	***	0.946
[1]MACY (Y_{ma})	10.11^a 0.16	10.97^b 0.20	12.10^c 0.04	***	0.978
[2]MACYPFAM (Y_n)	9.50^a 0.19	9.83^a 0.46	9.72^a 0.25	NS	0.214

*** P < 0.01**, P < 0.05, ^{NS}P > 0.05 (non-significant)**

[1]Moisture Adjusted Cheese Yield, [2]Moisture Adjusted Cheese Yield Adjusted for Protein and Fat. (Reference moisture 38.5% (w/w))

7.4.10. Cheese colour during the maturation period

The development of colour during a 210 d ripening period in HF-AA, HF-AB and JFX-AB cheese are shown in Table 7.7.

The HF-AA and HF-AB cheese did not differ significantly from each other in colour parameters ($P > 0.05$). The JFX-AB treatment resulted in significantly higher b* values than HF-AA and HF-AB, indicating that JFX-AB cheese was significantly more yellow in colour than cheese obtained from either of the HF breed groups ($P < 0.05$). However, while no significant difference in colour was determined between HF-AA and HF-AB cheese, an overall pattern in yellowness was suggested. The lowest b* values were seen for HF-AA cheese, followed by HF-AB, and JFX-AB cheese had the highest b* values, Fig.7.3. No significant differences in the L* and a* parameters were observed as a result of the HF-AA, HF-AB and JFX-AB treatment types ($P > 0.05$).

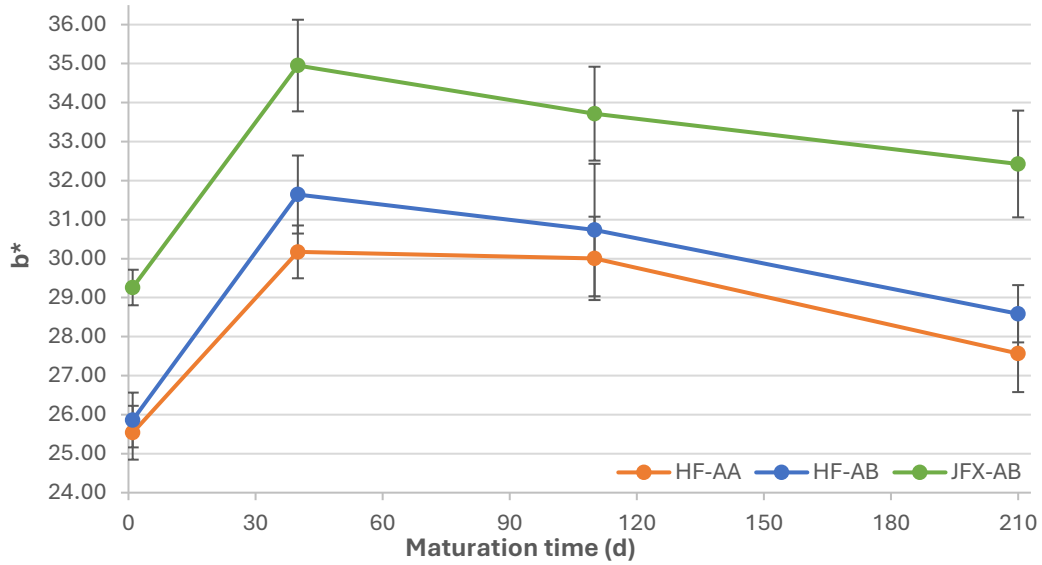


Fig 7.3 Cheese colour development, b* parameter, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CNA B (JFX-AB). The values are the average of 3 independent trials ±standard deviation.

Maturation time had a significant effect on Cheddar cheese colour attributes ($P < 0.05$) (Table 7.7). The L* parameter decreased significantly during ripening ($P < 0.05$). Increased darkening of cheese during ripening, signified here by decreasing L* values, can suggest changes in cheese microstructure that alter light scattering. Previous studies have also reported a darkening of cheese during ripening (Page et al., 2024; O'Callaghan et al., 2017), particularly earlier in the ripening period, with moisture redistribution, early proteolysis and lipolysis more readily influencing light scattering properties (Ferraz et al., 2024). The a* parameter also changed significantly with time, but while this change was statistically significant, the magnitude of difference was relatively small. Overall, the b* parameter increased significantly with maturation time ($P < 0.05$), but the increase took place early in the ripening period. All cheese became overall more yellow as it ripened, with this change occurring during 1 d to 40 d of ripening. A decrease in yellow colour then took place from the 110 d measurement until the end of the ripening period at 210 d. Similar patterns in ripening related colour change in Cheddar cheese have been reported previously (Page *et al.*, 2025).

Cheese colour is an important sensory attribute for consumer acceptability, but the optimal ripening time for the consumer does not only depend on colour. This will depend on multiple factors, indeed, the combined development of appearance, flavour and texture during ripening all influence cheese quality and decide the optimum ripening period. However, yellow coloured dairy products are often regarded positively

due to an association with the nutritional and environmental benefits of pasture-grazing, the colouring typically resulting from increased β-carotene levels and β-carotene being both a pigment and a pro-vitamin (O'Callaghan *et al.*, 2017; Page *et al.*, 2024; McDermott *et al.*, 2024). McDermott *et al.* (2024) described how breed had a significant effect on colostrum colour, with JFX cows producing colostrum with higher b* values compared to HF cows. Significantly higher b* values for JFX Cheddar have also been reported by past investigators. In the Chapter 5 study Cheddar cheese produced from JFX milk had higher b* values compared to HF milk derived cheese, and this was acknowledged as likely having resulted from higher levels of β-carotene. This association is discussed further in Chapter 5. Briefly, other investigators have described substantial differences in milk β-Carotene concentration between dairy cow breeds (Jensen *et al.*, 1999; Nozière *et al.*, 2006; Morris *et al.*, 2002). Morris *et al.* (2002) described a positive correlation between carotenoids in milk and the presence of JE genes. Jensen *et al.* (1999) determined that β-carotene is efficiently transferred from milk to cheese. The higher b* values associated with JFX-AB cheese in this study might be regarded as a positive sensory attribute. Previous research on Cheddar cheese colour in Ireland has linked increased b* values to the awarding of higher scores given by sensory panellists (O'Callaghan *et al.*, 2017). It should however be noted that sensory preferences may vary between different geographical locations (Ouyang *et al.*, 2021).

Table 7.7: Analysis of cheese colour over 210 days of ripening (± standard deviation), cheese derived from treatment groups, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

	1 d			40 d			110 d			210 d							
	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	Treatment P-Value	η2	Time P-Value	η2	Time x Treatment	η2
L*	89.65	89.69	88.22	80.33	82.44	81.11	80.39	82.19	81.11	80.10	82.34	81.56	NS	0.536	***	0.952	NS	0.275
	0.46	0.70	0.89	0.74	1.34	1.19	2.03	0.76	1.45	1.60	0.63	1.57						
a*	-4.57	-4.40	-4.74	-5.53	-5.32	-5.35	-5.08	-4.90	-5.08	-4.45	-4.50	-4.73	NS	0.091	***	0.569	NS	0.059
	0.06	0.10	0.23	0.28	0.07	0.10	0.36	0.33	0.35	0.92	0.81	0.90						
b*	25.54	25.86	29.26	30.17	31.64	34.95	30.01	30.73	33.72	27.57	28.59	32.43	***	0.975	***	0.873	NS	0.092
	0.69	0.70	0.46	0.68	1.00	1.17	1.07	1.70	1.20	0.99	0.73	1.37						

Statistical analysis determined by repeated measures ANOVA; η2, effect-size, partial ETA2
***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

7.4.11. The pH development in cheese during the maturation period

The cheese manufactured from HF-AA milk had significantly lower pH values than both the HF-AB and JFX-AB derived cheese ($P < 0.05$) (Table 7.8). Cheddar cheese derived from cows of the same B κ-CN phenotype, the HF-AB and JFX-AB groups, did not differ significantly in pH from each other during ripening ($P > 0.05$). The pH values of all cheese ranged between ~ 5.07-5.39 during the ripening period, and these are typical values for Cheddar cheese. The pH of all cheese increased significantly as it ripened with a large effect size ($P < 0.001$, $\eta 2 = 0.904$), and ripening-related increases in the pH of Cheddar cheese are to be expected (Lamichhane *et al.*, 2018a). All pH values observed during this study were within an acceptable range for Cheddar cheese, and although some significant differences in cheese pH were determined, it should be acknowledged that the magnitude of any differences seen here were relatively small.

It is noteworthy that the lactose content values of the cheesemilks used in this study did not differ significantly, so the lower pH of HF-AA cheese is unlikely to be explained by differences in milk lactose concentration. Consequently, it is suggested that the higher moisture content of HF-AA cheese might have influenced acid distribution, buffering residual lactose metabolism. However, lactic acid and residual lactose were not measured in this study, so the cause of a lower pH in HF-AA cheese cannot be confirmed. Future work could consider including analyses on lactic acid, residual lactose, salt-in-moisture and buffering-capacity.

Table 7.8: Analysis of cheese pH over 210 days of ripening (± standard deviation), cheese derived from treatment groups, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

	1 d			40 d			110 d			210 d								
	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	Treatment P-Value	η2	Time P-Value	η2	Time x Treatment	η2
pH	5.08	5.07	5.08	5.16	5.20	5.18	5.23	5.31	5.27	5.27	5.32	5.39	*	0.775	***	0.904	NS	0.260
±	0.07	0.07	0.03	0.00	0.02	0.03	0.01	0.02	0.01	0.03	0.03	0.12						

Statistical analysis determined by repeated measures ANOVA; η2, effect-size, partial ETA2

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

7.4.12. The texture development in cheese during the maturation period

The development of cheese texture parameters during a 210 d ripening period are shown in Table 7.9. All texture parameters decreased significantly during ripening ($P < 0.05$), which is typical, arising through increases in proteolysis and pH during ripening (Lamichhane et al., 2018a; O'Callaghan et al., 2017; Lamichhane et al., 2019).

The impact of the HF-AA, HF-AB and JFX-AB treatments on cheese texture were limited, and no significant differences were observed for the parameters of resilience, cohesiveness and springiness ($P > 0.05$). Significant differences in fracturability and hardness were observed, but only when comparing JFX-AB Cheddar to HF-AA Cheddar; JFX-AB Cheddar was significantly harder and had significantly higher fracturability values than HF-AA Cheddar ($P < 0.05$). The greater hardness of JFX-AB cheese could be associated with its lower moisture content, with lower moisture content in cheese generally increasing the density and firmness of the cheese matrix (Lucey et al., 2003; Lamichhane et al., 2018a). Consequently, the lower moisture content of JFX-AB cheese compared to HF-AA cheese might be a contributing factor to the higher observed hardness and fracturability values of JFX-AB Cheddar. No significant effect of treatment was seen between the HF-AA and HF-AB cheese for any measured texture parameters ($P > 0.05$), and no significant effect was seen between JFX-AB and HF-AB cheese for any measured texture parameters ($P > 0.05$).

These texture measurements are representative of typical results for Cheddar cheese during ripening. Sensory profile testing was not part of these investigations, and this

would be necessary before making further conclusions on consumer acceptability. With an acceptance of the limitations of the methods applied, no practical adverse differences in cheese texture were observed to result through these treatments. However, some limited significant textural differences were observed in cheese that was derived from milks that differed both by breed *and* k-CN genotype.

Table 7.9: Analysis of cheese texture over 210 days of ripening (± standard deviation), cheese derived from treatment groups, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

	1 d			40 d			110 d			210 d								
	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	Treatment P-Value	η2	Time P-Value	η2	Time x Treatment	η2
Fracturability (N)	345	343	428	288	285	336	260	294	333	269	265	304	*	0.267	**	0.388	NS	0.052
	24	31	47	97	41	56	52	54	84	35	37	47						
Hardness (N)	330	343	419	328	321	347	328	358	380	293	301	331	*	0.262	*	0.268	NS	0.102
	33	22	49	91	36	52	34	31	60	23	13	15						
Resilience (%)	8.44	7.65	8.33	6.54	6.24	6.64	5.66	5.52	5.56	5.00	5.02	4.87	NS	0.019	***	0.62	NS	0.030
	1.21	1.04	2.96	0.65	0.73	1.12	0.99	0.66	0.23	0.58	0.45	0.63						
Cohesiveness (%)	0.19	0.19	0.23	0.20	0.20	0.20	0.17	0.18	0.18	0.16	0.15	0.15	NS	0.129	***	0.62	NS	0.229
	0.03	0.01	0.03	0.02	0.03	0.02	0.01	0.02	0.02	0.02	0.02	0.03						
Springiness (%)	62.16	60.40	58.73	44.05	42.86	60.69	34.51	32.76	37.38	35.96	39.57	42.08	NS	0.061	***	0.448	NS	0.098
	7.18	15.06	17.93	7.23	8.34	30.17	5.25	1.31	6.68	9.12	8.04	12.23						

Statistical analysis determined by repeated measures ANOVA; η2, effect-size, partial ETA2
***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

7.4.13. The extent of primary proteolysis during the maturation period

Primary proteolysis was determined as the percentage of total nitrogen soluble at pH 4.6 (pH4.6-SN/TN) (Table 7.10). No significant differences in primary proteolysis were observed between the cheese derived from the HF-AA, HF-AB and JFX-AB treatments ($P > 0.05$). The overall pattern of development of primary proteolysis was also consistent with previously reported findings for Cheddar cheese, and the values obtained were within the range of typically determined values (Page *et al.*, 2025; Fenelon and Guinee, 2000). Proteolysis did increase significantly during the maturation period, which is to be expected, and is associated with enzymes present from both the starter and non-starter bacteria ($P < 0.05$). Proteolysis has an important role within cheese texture development (Fox, 1989), and only limited impacts on texture were observed in these investigations. Consequently, the lack of significant differences observed within this analysis of proteolysis is further validated by data obtained within the texture profile analysis.

Table 7.10: Analysis of proteolysis over 210 days of ripening (± standard deviation), cheese derived from treatment groups, Holstein-Friesian κ-CN AA (HF-AA), Holstein-Friesian κ-CN AB (HF-AB) and Jersey-Holstein-Friesian κ-CN AB (JFX-AB)

	1 d			40 d			110 d			210 d								
	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	HF -AA	HF -AB	JFX -AB	Treatment P-Value	η2	Time P-Value	η2	Time x Treatment	η2
Proteolysis																		
pH 4.6 SN/TN	2.15	2.68	2.45	6.99	7.73	6.82	10.45	10.28	10.11	15.77	18.56	17.19	NS	0.199	**	0.986	NS	0.435
±	0.04	0.26	0.47	1.45	1.37	2.33	0.20	0.24	0.95	2.69	1.88	2.15						

Statistical analysis determined by repeated measures ANOVA; η2, effect-size, partial ETA2
***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**

7.5. Conclusions

This study has demonstrated that crossbreeding can be combined with the selection of appropriate genetic variants to achieve improved production trait outcomes, and this could bring benefits to Cheddar cheese manufacturing. The HF and JFX breeds have relevance to the dairy industry through typical use and have different relative frequencies of A and B allelic variants of κ-CN in typical populations. The B κ-CN variant and JFX breed both have reasons to increase in frequency within dairy cow breeding programmes.

However, conclusions must be considered within the constraints and limits of the experimental design. In this study, the combined treatment groups (HF-AA, HF-AB and JFX-AB) represent specific breed factor and κ-CN genotypes combinations. This contrasts with a complete factorial breed × genotype model covering all combinations. The experimental design was developed to manage logistics within a complex study, within available resources. It allowed for targeted contrasts between HF-AA and HF-AB treatments (a specific genotype within the HF breed) and comparisons between HF-AB and JFX-AB (differing breed background within a controlled AB κ-CN genotype). Consequently, not all breed-genotype combinations are represented and full breed × genotype statistical interactions were not calculated. This is an accepted limitation of the study, and interpretation of the results must take this under consideration. Conclusions should also be interpreted while recognising the specific pilot-scale

design of three independent cheesemaking trials and manufacturing Cheddar with pooled cheesemilks derived from the morning milks of four cows per treatment group.

However, within this study the following potential opportunities are suggested for cheese manufacturers. Relative to typical HF breeds possessing both AA and AB κ-CN, JFX milk of an AB κ-CN genotype resulted in cheesemilk of a significantly higher protein, fat and casein content. Significantly higher cheese yields resulted, and this cheese was also significantly more yellow, an attribute generally favoured by consumers. Other ripening related attributes were broadly equivalent between the treatments during a 210 d maturation period. The evidence contained herein suggests that targeted crossbreeding efforts that are combined with the selection of appropriate genetic variants could provide a method to increase production efficiency. It is anticipated that the specific findings of this study, on selections for B κ-CN variants made in combination with JFX crossbred cows, can act as a valuable insight for dairy breeding programmes at a time when increased genetic diversity and improved production efficiency are being pursued within Irish dairy food systems.

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Chapter 8

General discussion

Blessed are the cheesemakers (Cleese et al., 1979)

8.1. General discussion and implications of this research

Within Ireland, cheese production accounts for about one third of all milk utilisation. In 2023, Ireland produced ~285,000 metric tonnes of cheese (CSO, 2024), with Cheddar representing the single largest variety. As a key commodity of great commercial importance, Cheddar cheese is a well-researched dairy product, but gaps do still exist in our scientific understanding of this product. This thesis considers the impact of selected primary production and management practices, within Irish pasture-based systems, on the compositional and processing attributes of milk, and the quality attributes of Cheddar cheese produced from that milk.

The first chapter reviewed the literature, and the likely impacts on cheese arising from possible future changes within primary dairy production practices were explored. Through this discussion, the complexity of the dairy value chain, with respect to cheese, became increasingly apparent. Dairy production practices were considered individually, but the repeated theme was that interactions take place between individual primary production factors. For example, poor herd health can negatively impact on milk quality, and consideration might then need to be given to how to best manage a herd's extrinsic environment to support health. However, how these dairy animals respond to that environment will be moderated by how adaptable they are, and consideration might then also need to be given to the herds breeding and genetics. In this example, the challenge of maintaining herd health, and with it, dairy product quality, could be approached through multiple routes; management of the

cow's environment, creating more resilient animals through breeding programmes, or perhaps both. Consequently, a key conclusion of this chapter was that the findings of individual studies within this domain, whilst useful, should always be considered holistically, with collective consideration given to the broader implications of such changes. However, a further theme through this reviewing of the literature (and through the experimental chapters of this thesis) was the robustness of the Cheddar cheese manufacturing process. In many instances, if new practices are implemented appropriately on the farm, a lack of negative impacts on cheese can be achieved. In these instances, it is of great practical significance that a key dairy commodity can be produced without practical adverse consequences arising due to that changed approach. Changes to primary production and management practices can be highly topical, and the experimental chapters of this thesis consider this idea further by investigating the implications of differing milking frequencies, pasture types, breeds and genetics on milk and Cheddar cheese quality.

The management practice of once-a-day (OAD) milking was explored for its impacts on cheese because reduced milking frequency strategies can provide opportunities for dairy farmers. Dairy cow welfare must be considered, and the suitability of OAD milking depends on stage of lactation, udder capacity and overall herd health. For high-performing cows, an abrupt transition to OAD can result in milk leakage, discomfort and yield loss and increased mastitis risk (Stelwagen et al., 2013). Reduced milking frequencies generally result in reduced incomes compared to the more typical twice-a-day (TAD) approach, but OAD milking also reduces milking times by as much as 35%

(Kennedy et al., 2021). The time savings associated with OAD strategies can result in cheaper costs of employment on the farm, more conventional working hours for farm employees, enhanced farmer wellbeing, opportunities for farm business diversification, better work-life balance and increased job satisfaction (Tipples and Verwoerd, 2007; Ferris *et al.*, 2008; Armstrong and Ho, 2009; Edwards, 2020). Some farms might consider these benefits sufficient to consider adapting from a TAD to a OAD strategy, and so the impacts of such a change on key dairy products should be considered.

Dairy cow diets were investigated because grazing practices have implications for environmental sustainability. Typically, in Ireland, cows graze on monoculture pastures of perennial ryegrass (PRG) and such pastures reduce biodiversity and require inputs of nitrogen-based fertilisers (Walling and Vaneeckhaute, 2022). The adoption of multispecies swards (MSS) has been suggested as a potential solution to these challenges. Within an MSS system, dairy cows graze on mixtures of grasses, legumes and herbs simultaneously, enhancing biodiversity (Beye *et al.*, 2022; Cooledge, 2022), improving soil health (Ikoyi *et al.*, 2023; Isbell *et al.*, 2017), and reducing chemical fertiliser use (Pijlman, 2024). Consequently, the impacts of MSS on cheese quality were explored to understand if this alternate pasture type can be used without negative impacts arising in the food.

The intrinsic aspects of primary production were researched through studies into dairy cow crossbreeding. Current breeding programmes have been implicated in reducing genetic diversity, also termed inbreeding depression. Breeding strategies that increase

genetic diversity have come into greater focus, and crossbreeding can be used as a method to improve herd health and fertility (Teagasc, 2016; Brito et al., 2021). A common strategy involves crossing Jersey (JE) cows with Holstein-Friesians (HF), resulting in Jersey-Holstein-Friesian (JFX) progeny. This study considered the implications of using JFX versus HF milks within Cheddar cheese production. The intrinsic aspects of primary dairy production were then explored further through investigations into impacts arising on Cheddar cheese from genetic polymorphism of κ-CN. As agri-food industries adapt to maximise production efficiency, the appropriate selection of genetic variants is likely to become a more regularly applied strategy to enhance production traits, bringing with it benefits for dairy product manufacturers (Kelly *et al.*, 2025).

This thesis has a theme of due diligence running throughout; in several instances, valuable assurance has been provided that can support multiple dairy industry stakeholders as they implement programmes of change. Within these investigations, it has been suggested that OAD milking, MSS grazing and JFX crossbreeding can all be utilised as strategies on farms without adverse impacts arising within Cheddar cheese composition, quality and ripening attributes, as compared to the more typically applied approaches. Additionally, in some instances, beneficial opportunities have also been suggested, examples whereby the manufacturing of Cheddar might be improved through efficiency gains, or better-quality Cheddar produced. Consequently, the findings contained herein provide insights on potential future advancements within

primary dairy production, with the following sections providing a more detailed discussion of these opportunities.

The value of milk in Ireland is closely associated with protein and fat content (Geary *et al.*, 2010). Within this thesis, some primary production approaches were determined to result in increased concentrations of milk solids. Investigations into milking frequency determined that OAD milk had significantly higher protein content than TAD milk. While OAD milking is acknowledged to reduce overall milk volume compared to TAD, the higher solids concentrations of OAD milk might go some way towards offsetting these losses. This observation could be particularly meaningful to farmers when considered holistically and in combination with time saving benefits offered by OAD milking. The utilisation of MSS grazing significantly increased the protein content of milk compared to typical PRG. This observation suggests that the economic value of milk might be increased in tandem with the adoption of a more biodiverse pasture type. Crossbreeding investigations validated previous knowledge regarding JFX breed impacts on milk solids. The JFX milk had significantly higher protein and fat content compared to HF milk, suggesting that increased milk solids concentrations can be achieved at the same time as utilising crossbreeding to improve genetic diversity. Similarly, investigations into genetic polymorphism validated previous evidence that the B variant of κ-CN is associated with increased protein content of milk. However, within the HF breed, AA and AB κ-CN milk (HF-AA and HF-AB treatments, respectively in this study) did not differ significantly in protein, fat or overall solids content. The HF-AB milk had higher milk solids values numerically compared to HF-AA, but these values

did not meet the threshold for statistical significance. Within this study, JFX milk of an AB κ-CN genotype (JFX-AB) had significantly higher protein, fat and TS values compared to HF-AA. The study suggests that the selection of κ-CN variants, in combination with breed type, can be used as a strategy to target increased protein content in milk.

The focus of this thesis is Cheddar cheese, and altered milk compositions arising from changes to on-farm practices could offer opportunities for cheese producers; higher cheese yields are associated with increased casein protein and fat concentrations. Within investigations into milking-frequency, the OAD treatment resulted in a 4.1% higher actual yield (Y_a) than TAD. When cheese yields were adjusted to consider protein, fat and moisture content (Y_n) the significant differences were no longer present. The significantly higher cheese yields associated with OAD milk were likely associated with the higher protein content of this milk. Regarding the cheesemaking potential of MSS milk, the average Y_a was 5.24% higher for this milk compared to milk derived from PRG. While MSS milk had significantly higher protein content, this attribute did not fully explain the increase in cheese yield. The significant increases in cheese yield associated with the MSS diet compared to PRG still remained, even when cheese yields were adjusted to consider protein, fat and moisture content (Yn). Regarding the use of JFX milk within Cheddar production, no significant differences in cheese yield were observed between the JFX and HF treatments within the initial crossbreeding study. The JFX treatments Y_a values were on average ~3% higher than HF, but this did not meet the threshold for statistical significance. However, within

investigations into κ-CN, which also considered breed-related effects, the average Y_a values of the HF-AA, HF-AB and JFX-AB treatments were 9.83, 10.36 and 11.31%, respectively. Significant increases in cheese yield resulted for the B κ-CN variant within the HF breed, but significantly higher yields still resulted for the JFX-AB group, JFX-AB milk had the highest fat and casein content. When adjusted Y_n yields were applied, no significant differences were apparent between the treatments, and the yield differences were indeed associated with the significant differences in protein and fat in the milks.

The gelation behaviour of milk was also altered by some of the investigated primary production practices, and this might be of interest for cheese manufacturers. Faster milk coagulation times can improve efficiency, but can also create challenges in large-scale operations that cut curds based on time rather than measured curd firmness. If coagulation occurs too rapidly, curd can become too firm before cutting, altering syneresis and increasing component losses into whey. Manufacturers can optimise coagulation behaviour by pre-acidifying milk, adding calcium chloride and adjusting temperature (Lucey et al., 2003). Within the milking frequency study, OAD milk was associated with set-to-cut times that were on average 11 min faster compared to TAD milk. It is possible that this faster set-to-cut time might offer a commercial advantage for some cheese producers. Regarding the study into dairy cow diets, no statistically significant differences were observed between the rheological properties of PRG and MSS cheesemilks, and this is interesting given that MSS milk had a significantly higher protein content. Increased protein content in milk is generally associated with faster

milk coagulation times, particularly casein protein, but the higher protein and casein content of MSS milk was not sufficient to bring about significant improvement in the gelation behaviour of MSS cheesemilk. No significant differences in gelation behaviour were observed between the JFX and HF cheesemilks within the crossbreeding study. However, within the κ-CN study, the fastest gelation times were associated with the JFX-AB group, significantly faster than the HF-AB group, and the HF-AA raw milks had particularly poor gelation behaviour. Selection for B κ-CN within breeding programmes could be used as a method to achieve faster milk coagulation times, but when B κ-CN is found in combination with the JFX breed, even faster gelation times can be achieved. These differences in speed of gelation were associated with differing functional properties of the milks. The HF-AA milks had significantly larger casein micelle sizes and higher pH values, and increased milk clotting times were observed for the HF-AA milks. The JFX-AB milk also had a significantly higher casein content compared to HF-AA and HF-AB, and JFX-AB treatment had the fastest milk clotting time. Additionally, all milks within the κ-CN polymorphism study were selected to be of an of A_2A_2 β-CN genotype. Consequently, findings from this study might also have relevance to those with an interest in A_2A_2 β-CN milk, a milk that has been associated with benefits for individuals with dietary intolerances, but that is also known to have poorer gelation behaviour. The presence of B κ-CN resulted in faster set-to-cut times for A_2A_2 milk, and even faster gelation times still were achieved when these genotypes were found in combination with the JFX breed.

The investigations completed within this thesis have also provided observations that might interest Cheddar cheese consumers. The investigated primary production factors tended to not result in significant impacts to the ripening behaviour of cheese, suggesting a lack of potential adverse impacts on its enjoyment. However, one area where cheese did differ significantly in some of these studies was regarding colour. The OAD milking treatment was noted to result in significantly higher b* values during colourimetric analysis, and yellow colouring is generally a favoured attribute for Cheddar cheese consumers in Ireland (O'Callaghan *et al.*, 2017). Increases in yellowness in cheese can partly be due to a higher fat content; β-carotene is associated with the milk fat globule membrane (MFGM). However, levels of β-carotene were also quantified within the milking frequency study, and OAD cheese had on average 31% higher β-carotene content compared to TAD cheese. While further investigations would be required before any dietary claim could be made, β-carotene is pro-vitamin A and this result demonstrates the possibility of a nutritional benefit associated with OAD milk. Vitamin A supports iron metabolism, skin and mucous membrane function, vision and the immune system. Cheese colour was also found to be impacted by breed. Within all studies utilising milk from cows of the JFX breed, this JFX derived cheese had significantly higher b* values compared to that of HF cows. It is likely that these differences are again associated with differences in β-carotene, although determination of β-carotene was not performed in these studies, and was inferred. If primary production decision makers want to target more yellow Cheddar cheese this might be achieved using OAD milking or by selecting cows of the JFX breed. Regarding

comparisons of MSS- and PRG-derived cheese, no significant differences in colour attributes were observed, but MSS- and PRG- derived cheese did undergo sensory panel investigations with some interesting results. Through this sensory analysis, semi-trained judges did note subtle differences in some sensory descriptors, but the consumer centred assessment found no significant differences between MSS- and PRG-derived Cheddar. Also, of interest within this dairy cow diet study, it was notable that MSS-derived Cheddar had significantly higher levels of toluene, suggesting the possibility of using this compound as an authentication biomarker for MSS production systems, should that be a requirement in the future. However, toluene's use as a biomarker should perhaps be considered cautiously. The implementation of testing might require in-line processor changes or third-party laboratory analysis, with protocols likely validated by methods such as GC-MS; from an industrial perspective adoption could pose challenges.

Overall, this thesis has provided new experimental evidence demonstrating that certain changes to primary production and management practices can be applied without adverse impacts on a key dairy commodity product, Cheddar cheese. The findings of this thesis send a strong signal in support of evidence-based change within primary production systems. The evidence generated, opportunities identified, and context discussed above would have relevance for multiple stakeholders. For farmers, the milking frequency study demonstrates that a milking strategy that brings with it benefits of time savings can be implemented without loss of cheese quality, while improving manufacturing efficiency and product appeal. Farmers considering business

diversification by utilising the time saved by OAD milking might consider this product centred evidence in support of taking such action. For farmers looking to increase genetic diversity within herds, it has been demonstrated that JFX herds can offer at least equivalent, and in some scenarios improved, milk processing outcomes. This evidence on impacts to production traits might have relevance for farmers considering such a crossbreeding programme. For processors, evidence has been provided on several approaches that can increase cheese yield. The practice of OAD milking, MSS grazing and breeding selections for B κ-CN can all increase the amount of cheese yielded from a given amount of milk, so each of these strategies could support efficiency gains at the factory. The increased yellowness and β-carotene content of OAD and JFX cheese aligns well with established consumer sensory and nutritional preferences. From a policy perspective, the findings on MSS grazing are particularly timely. They demonstrate that MSS grazing can be applied in support of biodiversity and sustainability goals without compromising product quality and at the same time as increasing production efficiency. This evidence supports current policy measures and is likely to encourage the wider adoption of such systems.

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8.2. Future considerations

These investigations have provided new insights into the impact of various primary production and management practices on the key dairy commodity product of Cheddar cheese. Future investigators might look to build on these findings through studies into the below possibilities:

1. Converting databases of bovine genomic data into useful, functional understanding should be a key research goal. This should include gaining a better understanding of polymorphism within regulatory elements that impact on milk protein transcription. This thesis suggests that genetic background effects arising at the cow- and breed-level have a meaningful influence on milk attributes. Studies into the genetics underpinning production trait differences between JFX and HF breeds could provide benefits to the dairy industry. An overall improved understanding of gene-to-phenotype relationships is needed to support the targeting of production traits through breeding programmes.

2. Novel dairy cow diets are being developed continuously, driven particularly by a focus on improved environmental sustainability. Such diets should be analysed for potential downstream impacts on dairy product quality. In particular, knowledge is lacking on impacts to products arising from specialist cow diets targeted towards reduced greenhouse gas emissions.

3. A paucity of evidence is available regarding the suitability of automated milking systems to produce dairy products. The limited evidence does appear positive, but research is still lacking in this area, particularly in Ireland.

4. Farmers might not choose to invest in improved milk properties when the payments received fail to acknowledge those enhanced properties. Presently, dairy farmers are paid for the total protein and fat yielded, and incentives might need to be structured differently in the future if the full benefits that can be achieved from changes on the farm are to be realised.

5. Significant increases in β-carotene were noted in cheese derived from OAD milking and JFX crossbred cows. This should be investigated further to determine whether these differences are of practical nutritional significance. Additionally, the cheese described in Chapters 5 and 7 did not undergo β-carotene determination (the attribute was indicated by colour). Determination of β-carotene content in cheese of differing breed and genotype combinations could be an area for future investigation.

6. Milk derived from MSS grazing had significantly higher protein content compared to that of a PRG system. Attempts to understand this were made with reference to the literature, but the actual cause of this remains unknown. Determining how these milk protein content differences arose would be a

worthwhile endeavour for future investigators given the opportunities this could bring for manufacturing efficiency.

7. The detection of significantly higher levels of toluene in milk derived from MSS as compared to PRG warrants further assessment to establish its reliability as an authentication tool. It is possible that this could act as a biomarker for milk derived from different diet types.

8. Cheese derived from MSS and PRG grazing underwent sensory panel testing, but this analysis was not performed in the milking frequency, crossbreeding and κ-CN studies. Sensory studies on cheese derived from these treatments could bring valuable consumer insights and should be a focus of future work.

9. Cheddar cheese can in a limited way exemplify biochemical impacts more generally within dairy products, but further insights could be gained though investigations into alternate cheese types and alternate products. Additionally, full seasonality studies for the factors considered would provide information on the impacts of these treatments at different stages of the milking season.

10. The findings of this thesis suggest an increased potential value for OAD, MSS, JFX and k-CN B derived milks for processors. Investigations completed at a larger commercial scale could enable the potential efficiency and product benefits identified here to be realised by manufacturers.

Appendix

Supplementary material

Table S2.1: Volatile compounds in cheeses determined by GC-MS (± standard deviation) under different milking frequency treatment types of once-a-day (OAD) and twice-a-day (TAD)

Name	CAS	RI	RI Ref	Reference retention odour	OAD (AUC)	TAD (AUC)	P-Value
				Acid			
Acetic acid	64-19-7	691	690	pungent, vinegar, sour	41659 ± 9132	61794 ± 15476	0.23^{NS}
Butanoic acid	107-92-6	864	864	rancid, cheesy, strong, sweaty	289902 ± 131738	422236 ± 123471	0.01*
Hexanoic acid	142-62-1	1052	1052	acidic, sweaty, cheesy, sharp, goaty	21542 ± 17396	15794 ± 9201	0.41^{NS}
				Alcohol			
Ethanol	64-17-5	505	506	sweet, alcoholic, medicinal	675916 ± 41546	705676 ± 92246	0.28^{NS}
Isopropyl Alcohol	67-63-0	541	539	alcoholic, musty, woody	16208 ± 22922	8323 ± 11770	0.25^{NS}
1-Propanol	71-23-8	611	612	sweet (candy), fruity	1468 ± 2075	3233 ± 4572	0.25^{NS}

2-Methyl-1propanol	78-83-1	678	678	ethereal, winey	3734	±	5280	2467	±	3489	0.25^{NS}
1-Butanol	71-36-3	711	716	banana-like, fruity, green, medicinal	14267	±	12031	12314	±	15095	0.47^{NS}
2-Pentanol	6032-29-7	744		green, fusel, oil, fermented, alcoholic, sweet, winey, banana, apple	114746	±	63022	58704	±	26642	0.14^{NS}
3-Methyl-1-butanol	123-51-3	784	784	whiskey, fusel, alcoholic, fruity, banana	30643	±	6119	20357	±	14123	0.16^{NS}
1-Pentanol	71-41-0	815	815	fermented, yeasty, balsamic, fusel, winey (fruity, alcoholic, green, woody)	5042	±	1146	6048	±	1967	0.17^{NS}
2,3-Butanediol	513-85-9	877	880	fruity, creamy, buttery	103451	±	74492	79360	±	22452	0.39^{NS}
Diacetone alcohol	123-42-2	912		odourless	60764	±	67412	36020	±	27077	0.39^{NS}
1-Hexanol	111-27-3	916	916	green, floral	15598	±	5357	9205	±	6025	0.02*
2-Heptanol	543-49-7	943		fresh, lemon grass, herbal, sweet, floral, fruity, green	63248	±	37725	83020	±	44995	0.08^{NS}

Aldehyde											
3-Methyl-butanal	590-86-3	692	692	malty, cheese, green, dark chocolate, cocoa	3215	±	4547	3986	±	5636	0.25^{NS}
Hexanal	66-25-1	839	839	green, grassy, herbal, lemon, tallow	3795	±	5367	8213	±	112	0.23^{NS}
Benzaldehyde	100-52-7	1030	1032	bitter almond, sweet cherry	5086	±	4633	2862	±	1447	0.25^{NS}
Nonanal	124-19-6	1149	1150	green, citrus, fatty, floral	2347	±	254	2079	±	284	0.30^{NS}
Alkane											
Pentane	109-66-0	501	500	gasoline-like	57628	±	65427	44883	±	39877	0.30^{NS}
n-Hexane	110-54-3	600		gasoline-like	26815	±	11683	20299	±	5740	0.18^{NS}
Heptane	142-82-5	700		sweet, ethereal	86757	±	45675	52475	±	889	0.24^{NS}
Octane	111-65-9	799		gasoline-like	28840	±	15971	25263	±	2309	0.41^{NS}
Benzene											

Toluene	108-88-3	793	794	nutty, bitter, almond, plastic	29618	±	25568	32637	±	14823	0.38^{NS}
				Ester							
Ethyl acetate	141-78-6	642	642	fruity, pineapple, apples, weedy, green	10987	±	15537	4881	±	6903	0.38^{NS}
Ethyl butanoate	105-54-4	825	826	fruity, pineapple	18233	±	1894	31575	±	4709	0.11^{NS}
Ethyl hexanoate	123-66-0	1023	1024	fruity, pineapple, waxy, fatty, banana, fresh	3466	±	1085	3471	±	528	0.50^{NS}
				Ketone							
Acetone	67-64-1	532	533	solvent, ethereal, sour milk, apple	124181	±	29147	129052	±	29867	0.03*
2-Butanone	78-93-3	638	639	buttery, sour milk, etheric	21781	±	7503	29764	±	132	0.18^{NS}
2-Pentanone	107-87-9	728	730	sweet, fruity, ethereal, fermented, winey (>1%)	2284618	±	1008759	1804799	±	1065370	0.03*
Acetoin	513-86-0	776	778	buttery, creamy, dairy, milky, fatty	127091	±	27698	233203	±	38696	0.13^{NS}
2-Hexanone	591-78-6	832	834	floral, fruity	37258	±	31557	48457	±	45114	0.23^{NS}

2-Heptanone	110-43-0	934	936	fruity, spicy, sweet, herbal, woody (cheesy, blue cheese, Roquefort)	1986857	±	1496220	2327033	±	2364231	0.34^{NS}
2-Octanone	111-13-7	1036		earthy, weedy, natural, woody, herbal	26155	±	21806	36000	±	36170	0.25^{NS}
2-Nonanone	821-55-6	1139		fresh, sweet, green, weedy, earthy, herbal	407963	±	244665	275826	±	90960	0.22^{NS}
2-Undecanone	112-12-9	1344	1353	waxy, fruity, creamy, fatty, orris, floral	83452	±	92490	32348	±	36943	0.21^{NS}
				Sulfur							
Dimethyl sulfide	75-18-3	537	538	sufurous, onion, cabbage, tomato, vegetable	1187	±	1678	958	±	108	0.44^{NS}
Carbon disulfide	75-15-0	545	546	cabbage, sulphur, fruity, burnt	333064	±	265441	175831	±	57050	0.24^{NS}

***P < 0.05, ^{NS}P > 0.05**

Table S4.1: Volatile organic compounds in Cheddar cheese determined by GC-MS (± standard deviation) at 180 days of ripening; cheese obtained from multispecies sward (MSS) and perennial ryegrass (PRG) cow diets, manufactured at both mid-lactation and late-lactation

	Treatment (AUC)												Diet		SOL		Diet x SOL	
	MSS			PRG			MID			LATE			P	η2	P	η2	P	η2
Acetic acid	802454	±	4.E+05	642030	±	1.E+05	801944	±	4.E+05	642541	±	9.E+04	NS	0.092	NS	0.091	NS	0.003
Butanoic acid	3306452	±	7.E+05	3504498	±	1.E+06	4046377	±	1.E+06	2764573	±	3.E+05	NS	0.019	NS	0.454	NS	0.019
Hexanoic acid	941142	±	3.E+05	1102009	±	8.E+05	1294250	±	7.E+05	748901	±	2.E+05	NS	0.030	NS	0.26	NS	0.053
Octanoic acid	100259	±	5.E+04	92253	±	5.E+04	121037	±	5.E+04	71475	±	2.E+04	NS	0.011	NS	0.302	NS	0.002
Decanoic acid	10681	±	4.E+03	9066	±	2.E+03	12192	±	4.E+03	7556	±	6.E+02	NS	0.114	*	0.514	NS	0.072
Ethanol	1053936	±	5.E+05	1409720	±	8.E+05	1613983	±	7.E+05	849672	±	2.E+05	NS	0.135	*	0.418	NS	0.034
1-Butanol	26986	±	3.E+04	160061	±	3.E+05	187047	±	3.E+05	0	±	0.E+00	NS	0.120	NS	0.212	NS	0.12
1-Butanol, 3-methyl-	37808	±	3.E+04	60294	±	4.E+04	68482	±	4.E+04	29619	±	2.E+04	NS	0.183	*	0.402	NS	0.206
1-Pentanol	27877	±	1.E+04	28962	±	1.E+04	32490	±	1.E+04	24349	±	8.E+03	NS	0.004	NS	0.182	NS	0.017
1-Hexanol	4747	±	1.E+04	18028	±	3.E+04	0	±	0.E+00	22775	±	3.E+04	NS	0.181	NS	0.394	NS	0.181
1-Hexanol, 2-ethyl-	20329	±	5.E+03	19696	±	3.E+03	20121	±	5.E+03	19904	±	4.E+03	NS	0.009	NS	0.001	NS	0.246
1-Octanol	3589	±	3.E+03	3426	±	2.E+03	2176	±	3.E+03	4839	±	1.E+03	NS	0.002	NS	0.312	NS	0.033
Butanal, 3-methyl-	112525	±	1.E+05	82175	±	3.E+04	118457	±	1.E+05	76242	±	1.E+04	NS	0.049	NS	0.091	NS	0.054
Pentanal	15565	±	2.E+04	19451	±	2.E+04	1165	±	3.E+03	33850	±	7.E+03	NS	0.153	***	0.928	NS	0.028
Hexanal	12166	±	1.E+04	15095	±	1.E+04	16450	±	1.E+04	10811	±	4.E+03	NS	0.026	NS	0.089	NS	0.002
Heptanal	13528	±	4.E+03	11124	±	6.E+03	10957	±	5.E+03	13695	±	4.E+03	NS	0.079	NS	0.1	NS	0.023
Benzaldehyde	15295	±	6.E+03	11823	±	4.E+03	12643	±	4.E+03	14475	±	6.E+03	NS	0.167	NS	0.053	NS	0.224
Benzeneacetaldehyde	19970	±	8.E+03	17073	±	2.E+03	19959	±	7.E+03	17085	±	4.E+03	NS	0.076	NS	0.075	NS	0.008
Nonanal	17111	±	5.E+03	15071	±	4.E+03	14820	±	5.E+03	17362	±	4.E+03	NS	0.064	NS	0.095	NS	0.012
Toluene	261575	±	2.E+05	91585	±	7.E+04	73091	±	6.E+04	280070	±	1.E+05	***	0.860	***	0.901	*	0.58
Ethylbenzene	6145	±	2.E+03	6292	±	2.E+03	5309	±	2.E+03	7129	±	1.E+03	NS	0.002	NS	0.233	NS	0.209

p-Xylene	14512	±	6.E+03	14897	±	8.E+03	11402	±	6.E+03	18007	±	6.E+03	NS	0.002	NS	0.325	NS	0.274
o-xylene	6334	±	3.E+03	8760	±	7.E+03	5141	±	2.E+03	9953	±	6.E+03	NS	0.096	NS	0.294	NS	0.118
Styrene	15644	±	1.E+04	8671	±	8.E+03	10659	±	1.E+04	13655	±	1.E+04	NS	0.123	NS	0.025	NS	0.162
2,3-Butanediol	292098	±	2.E+05	448537	±	4.E+05	293867	±	3.E+05	446768	±	3.E+05	NS	0.078	NS	0.074	NS	0.021
Ethyl acetate	63599	±	5.E+04	127083	±	4.E+04	106246	±	6.E+04	84435	±	6.E+04	NS	0.352	NS	0.06	NS	0.015
Methyl butanoate	25317	±	1.E+04	30266	±	1.E+04	22055	±	1.E+04	33528	±	1.E+04	NS	0.054	NS	0.234	NS	0
Ethyl butanoate	133908	±	7.E+04	215659	±	2.E+05	206906	±	2.E+05	142661	±	5.E+04	NS	0.127	NS	0.083	NS	0.005
Methyl hexanoate	6977	±	1.E+04	6191	±	1.E+04	3588	±	9.E+03	9580	±	1.E+04	NS	0.002	NS	0.1	NS	0.164
Ethyl hexanoate	33588	±	2.E+04	56371	±	3.E+04	45972	±	4.E+04	43987	±	2.E+04	NS	0.173	NS	0.002	NS	0.036
Ethyl octanoate	6048	±	3.E+03	8735	±	4.E+03	7039	±	5.E+03	7744	±	4.E+03	NS	0.127	NS	0.01	NS	0.009
Ethyl decanoate	4253	±	4.E+03	7054	±	3.E+03	6940	±	5.E+03	4367	±	2.E+03	NS	0.175	NS	0.152	NS	0.034
Ethyl ether	40945	±	3.E+04	52363	±	5.E+04	29684	±	3.E+04	63624	±	5.E+04	NS	0.026	NS	0.188	NS	0.035
Acetone	236547	±	6.E+04	251817	±	7.E+04	282903	±	5.E+04	205461	±	6.E+04	NS	0.031	*	0.45	NS	0.171
2,3-Butanedione	213464	±	1.E+05	238066	±	8.E+04	151070	±	9.E+04	300459	±	7.E+04	NS	0.035	*	0.571	NS	0.225
2-Butanone	148315	±	3.E+04	157506	±	4.E+04	148906	±	4.E+04	156915	±	3.E+04	NS	0.018	NS	0.014	NS	0.098
2-Pentanone	884787	±	5.E+05	604767	±	2.E+05	655690	±	2.E+05	833864	±	5.E+05	NS	0.165	NS	0.074	NS	0.055
Acetoin	5785380	±	2.E+06	5271711	±	2.E+06	5528545	±	2.E+06	5528545	±	2.E+06	NS	0.016	NS	0	NS	0
MethylIsobutyl Ketone	1626426	±	3.E+05	1499235	±	1.E+05	1562003	±	3.E+05	1563658	±	2.E+05	NS	0.075	NS	0	NS	0.062
2-Hexanone	2285	±	6.E+03	5606	±	1.E+04	7891	±	1.E+04	0	±	0.E+00	NS	0.036	NS	0.175	NS	0.036
2-Heptanone	590128	±	2.E+05	659240	±	6.E+05	749615	±	5.E+05	499753	±	2.E+05	NS	0.010	NS	0.112	NS	0.094
Pyrazine, 2,5dimethyl-	39026	±	4.E+04	42937	±	4.E+04	39117	±	3.E+04	42845	±	5.E+04	NS	0.003	NS	0.003	NS	0.047
Pyrazine, trimethyl-	6170	±	1.E+04	3641	±	7.E+03	1602	±	4.E+03	8209	±	1.E+04	NS	0.027	NS	0.157	NS	0.002
Carbon disulfide	147446	±	2.E+05	132891	±	1.E+05	210242	±	2.E+05	70095	±	2.E+04	NS	0.003	NS	0.201	NS	0.009
α-Pinene	10887	±	4.E+03	11643	±	3.E+03	11219	±	4.E+03	11311	±	3.E+03	NS	0.013	NS	0	NS	0.003

*** P < 0.05, ** P < 0.01, *** P < 0.001 (Significant), NSP > 0.05 (non-significant)**

Between-subjects effects obtained using a general linear model (GLM) analysis of variance (ANOVA)

Effect size measured as the partial eta squared (η2), AUC, Area Under Curve

Table S4.2: Free fatty acids in Cheddar cheese determined by GC-MS (g/100 g) (± standard deviation) at 180 days of ripening; cheese obtained from multispecies sward (MSS) and perennial ryegrass (PRG) cow diets, manufactured at both mid-lactation and late-lactation

	Treatment				Diet		SOL		Diet x SOL	
	MSS	PRG	MID	LATE	P	η2	P	η2	P	η2
C4:0	91 ± 21	113 ± 71	78 ± 15	126 ± 64	NS	0.066	NS	0.264	NS	0.048
C6:0	29 ± 7	39 ± 34	26 ± 7	43 ± 32	NS	0.06	NS	0.15	NS	0.063
C8:0	45 ± 16	44 ± 24	37 ± 12	52 ± 24	NS	0.002	NS	0.152	NS	0.001
C10:0	121 ± 53	106 ± 41	86 ± 18	141 ± 50	NS	0.057	*	0.429	NS	0.099
C12:0	125 ± 47	114 ± 37	95 ± 18	143 ± 44	NS	0.037	*	0.419	NS	0.096
C14:0	294 ± 106	272 ± 84	233 ± 46	332 ± 103	NS	0.026	NS	0.343	NS	0.093
C16:0	673 ± 205	639 ± 167	555 ± 107	757 ± 187	NS	0.016	NS	0.37	NS	0.093
C18:0	207 ± 54	208 ± 67	188 ± 50	227 ± 63	NS	0	NS	0.13	NS	0.028
C18:1	465 ± 123	467 ± 266	402 ± 143	530 ± 236	NS	0	NS	0.114	NS	0.001
C18:2	53 ± 17	46 ± 16	53 ± 19	46 ± 13	NS	0.058	NS	0.058	NS	0.012
C18:3	42 ± 14	36 ± 14	38 ± 16	41 ± 13	NS	0.052	NS	0.011	NS	0.011

*** P < 0.05, ** P < 0.01, *** P < 0.001 (Significant), NSP > 0.05 (non-significant)**
Between-subjects effects obtained using a general linear model (GLM) analysis of variance (ANOVA)
Effect size measured as the partial eta squared (η2)

Table S5.1: Total-fatty-acid determination. Mean fatty-acid-methyl-ester (FAME) results of milk and cheese at 180 days of ripening (g/100 g of milk fat ± SD), Holstein-Friesian (HF) and Jersey-Holstein-Friesian (JFX)

Milk Total Fatty Acids							Cheese Total Fatty Acids						
	HF			JFX		P-Value		HF			JFX		P-Value
C4:0	3.557	±	0.301	2.843	± 0.321	NS	**C4:0**	4.223	± 0.413	4.870	± 0.250	NS
C6:0	2.040	±	0.024	1.857	± 0.053	NS	**C6:0**	2.817	± 0.330	2.930	± 0.401	NS
C8:0	1.283	±	0.074	1.240	± 0.042	NS	**C8:0**	1.820	± 0.190	1.780	± 0.356	NS
C10:0	3.037	±	0.284	3.030	± 0.202	NS	**C10:0**	4.237	± 0.306	4.090	± 0.894	NS
C11:0	0.067	±	0.012	0.060	± 0.008	NS	**C11:0**	0.073	± 0.012	0.087	± 0.033	NS
C12:0	3.633	±	0.446	3.640	± 0.291	NS	**C12:0**	4.740	± 0.254	4.493	± 0.930	NS
C13:0	0.100	±	0.022	0.090	± 0.014	NS	**C13:0**	0.113	± 0.012	0.123	± 0.034	NS
C14:0	10.833	±	1.007	11.097	± 0.520	NS	**C14:0**	13.073	± 0.414	12.350	± 1.205	NS
C14:1	1.057	±	0.175	1.047	± 0.154	NS	**C14:1**	1.303	± 0.156	1.067	± 0.165	NS
C15:0	1.260	±	0.201	1.227	± 0.126	NS	**C15:0**	1.330	± 0.094	1.323	± 0.138	NS
C16:0	29.307	±	1.824	29.743	± 1.138	NS	**C16:0**	30.580	± 0.296	31.190	± 0.439	NS
C16:1	1.737	±	0.159	1.587	± 0.307	NS	**C16:1**	1.807	± 0.082	1.583	± 0.091	NS
C17:0	0.563	±	0.033	0.557	± 0.026	NS	**C17:0**	0.573	± 0.038	0.580	± 0.050	NS
C17:1	0.043	±	0.005	0.030	± 0.022	NS	**C17:1**	0.037	± 0.005	0.040	± 0.008	NS
C18:0	9.677	±	1.240	9.637	± 1.108	NS	**C18:0**	8.210	± 0.679	9.043	± 1.163	NS
C18:1 TFA	3.497	±	0.365	3.503	± 0.340	NS	**C18:1 TFA**	2.400	± 0.382	2.333	± 0.078	NS
C18:1n9c	18.553	±	1.372	18.460	± 0.738	NS	**C18:1n9c**	16.153	± 0.996	15.287	± 1.833	NS
C18:1n9t	0.193	±	0.005	0.167	± 0.024	NS	**C18:1n9t**	0.140	± 0.014	0.140	± 0.022	NS
C18:2 TFA	1.803	±	0.477	1.537	± 0.266	NS	**C18:2 TFA**	0.820	± 0.147	0.797	± 0.045	NS
C18:2n6c	1.263	±	0.017	1.427	± 0.290	NS	**C18:2n6c**	0.817	± 0.042	0.810	± 0.112	NS
C18:2n6t	0.123	±	0.017	0.290	± 0.335	NS	**C18:2n6t**	0.080	± 0.042	0.103	± 0.029	NS
CLA c9t11	1.177	±	0.261	1.093	± 0.232	NS	**CLA c9t11**	0.863	± 0.107	0.733	± 0.025	NS

C18:3 TFA	0.317	±	0.097	0.317	±	0.097	NS	**C18:3 TFA**	0.107	±	0.019	0.123	±	0.026	NS
C18:3n3	0.877	±	0.093	1.037	±	0.246	NS	**C18:3n3**	0.683	±	0.061	0.717	±	0.045	NS
C20:0	0.123	±	0.017	0.133	±	0.012	NS	**C20:0**	0.090	±	0.008	0.113	±	0.040	NS
C20:1	0.037	±	0.005	0.023	±	0.017	NS	**C20:1**	0.030	±	0.000	0.030	±	0.008	NS
C20:3n6	0.063	±	0.005	0.043	±	0.033	NS	**C20:3n6**	0.030	±	0.000	0.020	±	0.008	NS
C20:4n6	0.073	±	0.009	0.057	±	0.017	NS	**C20:4n6**	0.033	±	0.005	0.057	±	0.024	NS
C20:5	0.087	±	0.009	0.087	±	0.005	NS	**C20:5**	0.070	±	0.000	0.077	±	0.009	NS
C21:0	0.050	±	0.008	0.023	±	0.026	NS	**C21:0**	0.030	±	0.000	0.037	±	0.009	NS
C22:0	0.063	±	0.005	0.040	±	0.029	NS	**C22:0**	0.043	±	0.005	0.053	±	0.019	NS
C22:1	0.087	±	0.005	0.053	±	0.039	NS	**C22:1**	0.050	±	0.000	0.060	±	0.014	NS
C23:0	0.030	±	0.000	0.023	±	0.017	NS	**C23:0**	0.020	±	0.000	0.027	±	0.009	NS
C24:0	0.037	±	0.005	0.027	±	0.019	NS	**C24:0**	0.023	±	0.005	0.033	±	0.012	NS

^{NS}P > 0.05

Table S5.2: Free-fatty-acid (FFA) Profile of cheeses at 180 d of ripening in mg/kg (± standard deviation), Holstein-Friesian (HF) and Jersey-Holstein-Friesian (JFX)

	HF	JFX	P-VALUE
C4	169 ± 105	129 ± 36	NS
C6	70 ± 62	57 ± 28	NS
C8	73 ± 58	64 ± 27	NS
C10	168 ± 111	151 ± 51	NS
C12	164 ± 91	146 ± 42	NS
C14	392 ± 190	346 ± 107	NS
C16	928 ± 403	803 ± 224	NS
C18	300 ± 152	254 ± 64	NS
C18:1	882 ± 638	634 ± 304	NS
C18:2	61 ± 35	55 ± 21	NS
C18:3	54 ± 34	48 ± 17	NS

^{NS}P > 0.05 (non-significant)

Table S5.3: Volatile-organic-compounds in Cheddar cheese determined by GC-MS (± standard deviation) at 180 days of ripening; Holstein-Friesian (HF), Jersey x Holstein-Friesian (JFX)

Name	CAS	RI	Ref RI	Reference Retention odour	HF	JFX	P-value
Acids							
Acetic acid	64-19-7	691	690	Pungent, vinegar, sour	518453 ± 318930	449856 ± 165056	NS
Butanoic acid	107-92-6	866	864	Rancid, cheesy, strong, sweaty	4512978 ± 1556788	3813551 ± 241689	NS
Butanoic acid, 3methyl-	503-74-2	917	917	Cheesy, sweaty, rancid, goat, rotten fruit	77682 ± 134550	40896 ± 70835	NS
Hexanoic acid	142-62-1	1052	1049	Acidic, sweaty, cheesy, sharp, goaty	1651510 ± 1071109	1524076 ± 320945	NS
Octanoic acid	124-07-2	1244	1242	Waxy, soapy, cheesy, rancid, pungent, sweat	135077 ± 103406	142085 ± 32923	NS
Decanoic acid	334-48-5	1440	1450	Rancid, fatty	12039 ± 7698	14089 ± 3567	NS
Alcohols							
Ethanol	64-17-5	510	506	Sweet, alcoholic, medicinal	1114802 ± 69334	1047470 ± 130162	NS
1-Propanol, 2-methyl-	78-83-1	680	678	Ethereal, winey	112499 ± 194855	15141 ± 26225	NS

1-Butanol	71-36-3	716	716	Banana-like, fruity, green, medicinal	195376	±	201308	108890	±	74985	NS
1-Butanol, 3-methyl-	123-51-3	785	784	Whiskey, fusel, alcoholic, fruity, banana	457304	±	672093	138162	±	142507	NS
1-Pentanol	71-41-0	806	815	Fermented, yeasty, balsamic, fusel, winey	20910	±	15970	27226	±	7861	NS
1-Hexanol	111-27-3	917	915	Green, floral	2850	±	4936	35251	±	54754	NS
1-Hexanol, 2-ethyl-	104-76-7	1080	1077	Citrus, floral, green, fresh	16557	±	1813	25065	±	4675	NS
1-Octanol	111-87-5	1118	1118	Waxy, green, citrus, floral, sweet, fatty	549	±	951	2420	±	2789	NS
Aldehydes											
Butanal, 3-methyl-	590-86-3	693	692	Malty, cheese, green, dark chocolate, cocoa	61016	±	11354	72040	±	35335	NS
Heptanal	111-71-7	943	943	Fatty/oily, green, citrus, rancid	21199	±	6807	15440	±	1985	NS
Benzaldehyde	100-52-7	1030	1031	Bitter almond, sweet cherry	24895	±	16482	17285	±	2742	NS
Benzeneacetaldehyde	122-78-1	1119	1120	Green, sweet, floral, clover, honey, cocoa	15909	±	2733	17111	±	2276	NS
Nonanal	124-19-6	1150	1150	Green, citrus, fatty, floral	13164	±	948	12837	±	1625	NS
Benzenes											

Toluene	108-88-3	794	794	Nutty, bitter, almond, plastic	115848	±	52097	140351	±	42550	NS
Ethylbenzene	100-41-4	891	890	Heavy, floral	1421	±	1469	5024	±	3468	NS
p-Xylene	106-42-3	899	898	Grainy, sweet	7713	±	1399	11539	±	4433	NS
o-xylene	95-47-6	928	929	Geranium	2043	±	2145	3146	±	4301	NS
Styrene	100-42-5	929	929	Plastic	21283	±	19037	30054	±	24525	NS
				Diols							
2,3-Butanediol, [R-(R*,R*)]- *	24347-58-8	877		-	741312	±	296111	933611	±	401563	NS
				Esters							
Ethyl acetate	141-78-6	644	642	Fruity, pineapple, apples, weedy, green	43336	±	64418	23737	±	41114	NS
Methyl butanoate	623-42-7	750	754	Fruity	49940	±	18517	44280	±	15103	NS
Ethyl butanoate	105-54-4	826	826	Fruity, pineapple	213130	±	96354	140655	±	53852	NS
Methyl hexanoate	106-70-7	951	949	Fruity, pineapple, tropical	14395	±	16560	13075	±	15058	NS
Ethyl hexanoate	123-66-0	1023	1024	Fruity, pineapple, waxy, fatty, banana, fresh	56468	±	34118	42715	±	11563	NS

Ethyl octanoate	106-32-1	1222	1222	Fruity, green, orange, winey, pineapple, apricot	7952	±	4232	8730	±	3094	NS
Ethyl decanoate	110-38-3	1423	1422	Fruity, grape, Cognac, waxy	7076	±	5460	7027	±	7966	NS
Ethers											
Ethyl ether	60-29-7	519	515	Pungent, etheral	4166	±	7216	5915	±	10246	NS
Ketones											
Acetone	67-64-1	536	533	Solvent, ethereal, sour milk, apple	760526	±	722845	366586	±	117955	NS
2,3-Butanedione	431-03-8	633	631	Buttery, creamy, sweet, pungent	265437	±	96492	289236	±	35711	NS
2-Butanone	78-93-3	640	639	Buttery, sour milk, etheric	168136	±	38258	1130367	±	1588118	NS
2-Pentanone	107-87-9	730	730	Sweet, fruity, ethereal, fermented ,winey	2715697	±	1180350	1504752	±	1055426	NS
Acetoin	513-86-0	777	778	Buttery, creamy, dairy, milky, fatty	5271711	±	2420657	5785380	±	2482237	NS
Methyl Isobutyl Ketone	108-10-1	781	784	Sharp, solvent, green, herbal, fruity, dairy, spicy	1269050	±	18108	1330384	±	57524	NS
2-Hexanone	591-78-6	833	834	Floral, fruity	32093	±	27815	30045	±	27241	NS
2-Heptanone	110-43-0	935	936	Fruity, spicy, sweet, herbal, woody	965556	±	167362	878376	±	258900	NS

Pyrazines										
Pyrazine, 2,5dimethyl-	123-32-0	951	951	Nutty, musty, roasted, cocoa, earthy, woody	23205	±	36343	14187	±	24572	NS
Pyrazine, trimethyl-	14667-55-1	1042	1039	Roasted, chocolate, earthy, musty	13497	±	19648	6583	±	7140	NS
Sulfurs										
Carbon disulfide	75-15-0	550	546	Cabbage, sulphur, fruity, burnt	318006	±	23634	412858	±	131773	NS
Terpene										
α-Pinene	80-56-8	955	951	Pine, camphoreous, earthy, woody	12013	±	835	12036	±	3118	NS

^{NS}P > 0.05 (non-significant)

Table S6.1: Breed and genotype data of the investigated individual cows

Moorepark Farm Reference	Study designation			Breed (%)				Genotype		
	Cow number	Breed group	κ-casein variant	HO (%)	FR (%)	JE (%)	Other (%)	β-casein	β-lactoglobulin	κ-casein
77	1	HF	-AA	66	28	6	0	A_2A_2	AB	AA
258	2	HF	-AA	69	31	0	0	A_2A_2	AB	AA
299	3	HF	-AA	72	22	0	6	A_2A_2	AB	AA
9402	4	HF	-AA	69	31	0	0	A_2A_2	AB	AA
58	5	HF	-AB	84	16	0	0	A_2A_2	AB	AB
1271	6	HF	-AB	84	16	0	0	A_2A_2	BB	AB
8938	7	HF	-AB	78	19	0	3	A_2A_2	AA	AB
9185	8	HF	-AB	78	22	0	0	A_2A_2	AB	AB
314	9	JFX	-AB	47	22	31	0	A_2A_2	AA	AB
387	10	JFX	-AB	25	13	50	12	A_2A_2	AA	AB
405	11	JFX	-AB	38	9	50	3	A_2A_2	AB	AB
438	12	JFX	-AB	41	9	50	0	A_2A_2	AA	AB

Table S6.2. Composition of individual raw cow's milks, 1 to 12 (± standard deviation). Cows 1-4; Holstein-Friesian κ-CN AA (HF-AA), Cows 5-8; Holstein-Friesian κ-CN AB (HF-AB), Cows 9-12; Jersey-Holstein-Friesian κ-CN AB (JFX-AB). Data are the average of three independent milk collections.

Cow ID	1	2	3	4	5	6	7	8	9	10	11	12	P-Value	η2
Fat (% wt/wt)	3.53	3.30	3.24	4.28	3.71	5.62	5.05	4.19	3.85	6.11	5.33	5.99	***	0.693
±	0.53	0.46	0.30	0.17	0.08	1.87	0.37	0.27	0.56	1.51	0.55	0.98		
Protein (% wt/wt)	3.31	3.47	3.20	3.47	3.20	3.67	3.60	3.46	3.61	3.90	3.84	4.08	***	0.881
±	0.08	0.07	0.18	0.04	0.11	0.09	0.07	0.14	0.03	0.09	0.18	0.19		
Lactose (% wt/wt)	4.81	4.69	4.65	4.72	4.72	4.75	4.48	4.82	4.58	4.80	4.72	4.64	*	0.529
±	0.07	0.25	0.04	0.07	0.03	0.12	0.15	0.04	0.04	0.13	0.07	0.08		
Total Solids (% wt/wt)	11.94	11.78	11.26	13.19	12.05	15.15	14.17	13.13	12.68	16.28	14.98	16.04	***	0.804
±	0.80	1.02	0.44	0.55	0.09	2.08	0.65	0.18	0.90	1.41	0.96	1.18		
Casein Content (% wt/wt)	2.70	2.83	2.58	2.83	2.47	2.97	2.82	2.70	2.89	3.08	3.10	3.24	***	0.791
±	0.16	0.01	0.22	0.06	0.04	0.11	0.09	0.11	0.12	0.02	0.05	0.28		
Casein Number (% wt/wt)	81.55	81.63	80.31	81.58	78.24	81.02	78.25	79.54	80.05	77.89	82.89	80.06	NS	0.428
±	2.75	1.61	2.39	0.90	4.30	1.01	1.09	1.00	2.97	0.37	2.61	1.89		

Non-Protein-Nitrogen(% wt/wt)	4.81	5.60	5.18	4.92	4.66	4.76	6.50	5.14	6.22	6.80	5.84	4.93	NS	0.370
±	0.068	0.155	0.045	0.095	0.069	0.100	0.149	0.083	0.121	0.009	0.068	0.250		
SCC (cells/mL)(x1000)	14	14	19	19	121	275	23	14	20	18	45	36	***	0.763
±	11	10	8	7	149	84	7	2	6	9	13	10		

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05, η2, effect-size, partial ETA2**

Table S6.3. Mineral composition of individual raw cow's milks, 1 to 12 (± standard deviation). Cows 1-4; Holstein-Friesian κ-CN AA (HF-AA), Cows 5-8; Holstein-Friesian κ-CN AB (HF-AB), Cows 9-12; Jersey-Holstein-Friesian κ-CN AB (JFX-AB). Data are the average of three independent milk collections.

Cow	1	2	3	4	5	6	7	8	9	10	11	12	P-Value	η2
Calcium (mg/kg)	1196	1140	1030	1241	1186	1263	1233	1306	1212	1330	1280	1320	NS	0.04
±	64	74	32	31	50	157	107	128	50	191	144	177		
Potassium (mg/kg)	1478	1484	1599	1523	1585	1326	1573	1494	1636	1514	1505	1436	NS	0.061
±	20	118	76	74	64	173	103	120	63	171	223	173		
Magnesium (mg/kg)	96	102	97	99	102	112	102	109	112	125	107	110	NS	0.143
±	1	8	3	2	2	10	8	7	8	15	11	18		
Sodium (mg/kg)	268	307	340	346	340	429	367	374	373	343	365	334	NS	0.116
±	13	10	39	26	28	67	14	57	20	56	47	31		
Phosphorus (mg/kg)	832	917	815	876	947	994	912	857	902	1005	934	906	NS	0.048
±	22	49	22	17	23	105	63	51	29	121	127	112		

***P < 0.05, **P < 0.01, ***P < 0.001, NS = P > 0.05**