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The impact of genetic variation on the functional, technological and digestive properties of β-lactoglobulin

Thesis presented by

Liam M. Kelly

For the degree of

Doctor of Philosophy

in

Food Science and Technology

2025

Dedicated to the life and memory of my uncle and namesake,

Michael Wood

Table of Contents

Declaration i
Acknowledgements ii
Abstract iv
Abbreviations viii
List of publications and conference contributions xi
Statement of research objectives xiv

Chapter 1- Genetic variation in bovine milk proteins: Implications for functional and nutritional properties 1
Abstract 2
1.1. Introduction 3
1.2. History of milk protein variants 4
1.2.1. β-lactoglobulin 4
1.2.2. α-lactalbumin 5
1.2.3. β-casein 6
1.2.4. α-casein 8
1.2.5. κ-casein 9
1.3. Protein modification due to genetic variation 10
1.3.1. DNA mutation 10
1.3.2. Phosphorylation 11
1.3.3. Glycosylation ..... **Error! Bookmark not defined.**
1.4. Genotype frequencies 13
1.4.1. Holstein Friesian 14
1.4.2. Jersey 15
1.4.3. Guernsey 16
1.4.4. Rare variants 16
1.5. Impact of milk protein variants on nutrition 17
1.5.1. A1 and A2 β-casein 18
1.5.2. Allergic response to milk protein variants 20
1.6. Functional properties of milk protein variants 21
1.6.1. Heat stability 21
1.6.2. Milk coagulation 24
1.6.3. Foaming and emulsifying 28
1.7. Conclusion 30

1.8. References 31

Chapter 2 - Factors influencing the variation in macro chemical composition and protein profile in milk among individual cows 47

Abstract 48

2.1 Introduction 49

2.2 Materials and methods 51

2.2.1 Sample collection 51

2.2.2 Compositional analysis 51

2.2.3 Nitrogen fractions 51

2.2.4 Protein profile analysis 52

2.2.5 Statistical analysis 53

2.3 Results and Discussion 53

2.3.1. Macro Chemical Composition 53

2.3.2 Protein composition 60

2.3.3 Protein profile analysis 66

2.3.4 Impact of β-casein variants on protein content 72

2.3.5 κ-casein 75

2.3.6 β-lactoglobulin 76

2.4 Conclusion 77

2.5 References 78

Chapter 3 - Impact of genetic variants on the separation efficiency of β-lactoglobulin during microfilation of skim milk 83

Abstract 84

3.1 Introduction 85

3.2 Materials and methods 87

3.2.1 Feed material 87

3.3.2 Membrane processing 88

3.2.3 Sample preparation and compositional analysis 90

3.2.4 Dry matter content 90

3.2.5 Analysis of nitrogen fractions 90

3.2.6 Protein profile analysis 91

3.2.7 Calculations 92

3.2.8 Statistical data analysis 93

3.3 Results and discussion 93
3.3.1 Fouling 95
3.3.2 Mass balance and whey protein partitioning 98
3.4 Conclusion 104
3.5 References 105

Chapter 4 - The impact of genetic variation on the functional properties of β-lactoglobulin 109
Abstract 110
4.1 Introduction 111
4.2 Materials and methods 114
4.2.1 Assembly and processing of milk 114
4.2.2 Membrane processing 114
4.2.3 Spray drying 115
4.2.4 Sample preparation 115
4.2.5 Powder composition 116
4.2.6 Protein profile analysis by liquid chromatography 117
4.2.7 Protein secondary structure analysis by Fourier Transform Infrared Spectroscopy 117
4.2.8 Heat-induced gelation 118
4.2.9 Heat stability 118
4.2.10 Differential scanning calorimetry 119
4.2.11 Statistical Analysis 119
4.3 Results and discussion 120
4.3.1 Compositional analysis 120
4.3.2 Protein structure 124
4.3.3 Differential scanning calorimetry 126
4.3.4 Gelation properties 128
4.3.5 Heat stability 132
4.4. Conclusion 134
4.5 References 135

Chapter 5 - Influence of heat treatment and genetic variation of β-lactoglobulin on the digestive characteristics of a model infant formula 140
Abstract 141

5.1 Introduction 142
5.2 Materials and methods 144
5.1.1 Infant milk formula production 146
5.2.2 Compositional analysis 148
5.2.3 In vitro semi-dynamic digestion 149
5.2.4 Gastric phase 152
5.2.5 Protein content in digested samples 152
5.2.6 Confocal laser scanning microscopy 153
5.2.7 Electrophoretic analysis of protein profile 153
5.2.8 Primary amine analysis 153
5.2.9 Size exclusion chromatography 154
5.2.10 Statistical data analysis 154
5.3 Results and discussion 155
5.3.1 Structural changes during in vitro digestion 155
5.3.2 Identification of proteins in infant milk formula using SDS-PAGE 157
5.3.3 Protein hydrolysis 160
5.3.4 Peptide distribution 162
5.4 Conclusion 164
5.5 References 165

Chapter 6 - General discussion and suggestions for future work 170
6.1 Summary of key findings 171
6.2 Scientific and industrial impact of the research 174
6.3 Suggestions for future research 177
6.4 References 180

Appendix (Part 1) 181
Appendix (Part 2) 181

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.

Chapter 3: William McCarthy and Surabhi Subhir assisted in the operation of the pilot-scale membrane filtration equipment. William McCarthy also contributed to the protein profile analysis by SDS-PAGE. Brendan Horan contributed to the organisation of the milk collection prior to membrane filtration.

Chapter 4: William McCarthy and Surabhi Subhir assisted in the membrane filtration work during the manufacture of whey protein isolates. Alan Jezequel contributed to the collection of milk for the manufacturing of the whey protein isolates.

Chapter 5: Conor Fitzpatrick contributed to the *in vitro* digestion analysis.

Signed: Date: 24th December 2024

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Abstract

Genetic variations of milk proteins have been studied since their discovery in 1946. Since then, over 50 variants have been found among the four caseins (α_{s1}-, α_{s2}-, β-, and κ-casein) and the two main whey proteins, β-lactoglobulin (β-lg) and α-lactalbumin. In the early 1990's, it was speculated that certain genetic variants of β-casein could be associated with negative health effects, including heart disease, diabetes and schizophrenia. While no scientific evidence has since been published in support of these health claims, these reports did stimulate a renewed scientific and commercial interest in genetic variants of milk proteins, with some companies even structuring their business models around them, such as the a2 Milk Company (Auckland, New Zealand). Undoubtedly, there is potential for genetic variation among the milk proteins to alter functional, technological and possibly nutritional and digestive properties of dairy products. Thus, research is required to define potential beneficial and deleterious effects of specific protein variants on the processing and nutritional quality of milk.

Irish herds generally do not select for specific genetic variants as part of the national breeding programme, resulting in a diverse pool of variants among Irish cows. Initially, to support understanding of the distribution of genetic variants within an Irish herd, the protein profiles of 48 cows was assessed; of these cows, 17 genetic variants were identified among β-casein, κ-casein and β-lg. The A2A2 β-casein genotype had the highest frequency in Holstein Friesian (HF) cows; however, the A1I and the A2B genotypes were associated with higher levels of true protein and casein. In addition, it has also been found that milk with the A variant of β-lg was associated with higher levels of total whey proteins compared to milk with the B variant. This difference in β-lg concentration has been linked to the DNA mutations that cause the genetic

variation being located close to the promoter region for gene expression. The differences in the macro composition of milk as a result of genetic variation could be of benefit to both the farmer and milk processor, in terms of farm gate milk price and differentiated added value products, respectively. Additionally there may also be a benefit to consumers who seek milk products with designated milk genetic variants.

Identification of the genetic variants among 48 cows, using high performance liquid chromatography analysis, indicated that the AA, AB and BB genotypes of β-lg were commonly distributed among the cows tested. As β-lg is the principal whey protein on a mass basis, it determines the nutritional and functional quality of subsequent whey protein ingredients, such as concentrates and isolates. These whey protein ingredients, produced from pre-treated cheese whey, or isolated directly from milk, through a combination of microfiltration (MF), ultrafiltration (UF)/nanofiltration and spray drying, often find application in high value, life-stage nutritional products. Thus, the primary whey protein β-lg, its genetic variants and ingredients/products produced therefrom, are the primary research focus of this thesis.

The primary structural differences between the A and B variants of β-lg are due to amino acid substitutions that occur at positions 64 and 118. At position 64, the A variant has an aspartic acid, whereas the B variant has a glycine. Aspartic acid contains a charged functional group, while the glycine functional group is not charged. This substitution in particular causes the A variant of β-lg to have a greater net negative charge. The substitution that occurs at position 118, in which the A variant has a valine and the B variant has an alanine is not thought to significantly impact the structure, and thus functionality, of β-lg.

Most studies to date have assessed the functional properties of β-lg in milk, a complex system in which other components may present confounding factors which mask differences in functionality attributable to the protein variant. By purifying the whey protein fraction of milk into a whey protein isolate (WPI) containing either the A or B variant of β-lg, the true effects of genetic variation can be assessed with limited interference from other components of milk. As this thesis had already identified cows that produced only the homozygous AA or BB genotype of β-lg, their milk was segregated for separation of the individual β-lg variants using a combination of MF and UF. MF is widely used in the manufacture of WPIs from pre-treated cheese whey, and also in the separation of caseins and whey proteins from skim milk. While separation and purification of the β-lg variants was the primary goal, their impact on the efficiency of whey protein separation was also assessed. The results of this work demonstrated that the accumulation of reversible and irreversible foulants, rejection of whey proteins and overall process efficiency were determined by the β-lg variant present in the skim milk. This is not surprising, considering that fouling of the membrane plays a critical role in both flux decline, and by proxy, process efficiency, with β-lg reported to be a major contributor to fouling. It was found that transmission of whey proteins during MF of skim milk was higher for the skim milk containing the A variant than the B variant of β-lg, and while the skim milk containing the A variant had higher accumulation of both total and reversible fouling than the skim milk with the B variant, it was not rate limiting relative to whey protein permeation. These results indicate that skim milk with the A variant of β-lg could be advantageous to whey processors in terms of process efficiency and yield.

Analysis of the WPIs produced by a combination of MF and UF described in this thesis identified unique functional properties specific to the β-lg variants. The thermal

denaturation and gelation onset temperatures were higher for the WPI with the B variant of β-lg. However, despite the A variant having a lower denaturation/gelation temperature, it had better stability against heat-induced gel formation in the pH range 6.6-7.2. These differences in functionality, specifically in terms of denaturation and aggregation processes, presented additional research questions surrounding behaviour of the proteins in more complex products.

By manufacturing WPI containing either β-lg A or B genetic variants, and observing unique functional properties between the variants, the research question was proposed as to whether the differences between the variants would also be evident in a more complex dairy product, and whether the variants had a significant effect on *in vitro* digestion behaviour. Infant milk formula (IMF) was thus chosen as a suitable system to evaluate the β-lg variants, as it constitutes approximately 50% of the protein in a 0-6 month formula. By creating IMF with different heat treatments of either high temperature short time (HTST) or ultra-high temperature (UHT), containing either the A or B variants of β-lg, the impact of the genetic variation of β-lg on digestion was assessed using a semi-dynamic infant digestion model. While the effect of heat treatment between the samples was very clear, surprisingly, there was little difference between the β-lg variants during simulated digestion, in terms of curd formation, protein breakdown and free amine concentrations at the end of digestion.

Overall, the original research presented in this thesis provides new and important insights into the impact of genetic variation on the functional, technological and digestive properties of β-lg, by producing purified whey ingredients for evaluation at semi-commercial scale. Overall, there does appear to be a case for selection of the A variant of β-lg in the dairy herd, especially for dairy processors who specialize in whey protein ingredients.

Abbreviations

°C	Degrees Celsius
α-lac	Alpha lactalbumin
α_{s1}-CN	Alpha S1 casein
α_{s2}-CN	Alpha S2 casein
β-CN	Beta casein
β-lg	Beta lactoglobulin
κ-CN	Kappa casein
µL	Microlitre
ANOVA	Analysis of variance
AU	Absorbance units
BSA	Bovine serum albumin
CLSM	Confocal laser scanning microscopy
CP	Crude protein
DNA	Deoxyribonucleic acid
DM	Dry matter
DSC	Differential scanning calorimetry
FA	Free amides
FTIR	Fourier transform infrared
g	Gram
G′	Elastic modulus
G″	Viscous modulus
GE	Gastric emptying point
HCl	Hydrochloric acid
HCT	Heat coagulation time
HF	Holstein Friesian
HTST	High temperature short time
Hz	Hertz
h	Hour
IDF	International Dairy Federation
IgG	Immunoglobulin

IMF	Infant milk formula
J	Flux
JFX	Jersey Friesian
kcal	Kilocalories
kDa	Kilodaltons
kg	Kilograms
kPa	Kilopascal
L	Litres
M	Molar
MF	Microfiltration
mg	Milligrams
min	Minutes
mL	Millilitre
mM	Millimolar
MSS	Multi species swards
n	Number of samples
NaOH	Sodium Hydroxide
NCN	Non-casein nitrogen
NPN	Non-protein nitrogen
OPA	*o*-phthaldialdehyde
Pa.s	Pascal seconds
ppm	Parts per million
PRG	Perennial ryegrass
PRGWC	Perennial ryegrass and white clover
$R_{f\ (final)}$	Total fouling coefficient
$R_{f\ irrev}$	Irreversible fouling coefficient
$R_{f\ rev}$	Reversible fouling coefficient
R_m	Membrane resistance
RP-HPLC	Reverse-phase High Performance Liquid Chromatography
s	Seconds
SCC	Somatic cell count
SD	Standard deviation

SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SGF	Simulated gastric fluid
SMP	Skim milk powder
TCA	Trichloroacetic acid
T_d	Denaturation temperature
TMP	Trans-membrane pressure
TN	Total nitrogen
TP	True protein
TS	Total solids
U	Units
UF	Ultrafiltration
UHT	Ultra high temperature
VCF	Volume concentration factor
w/w	Weight/weight
w/v	Weight/volume
WPI	Whey protein isolates

List of publications and conference contributions

Peer-reviewed publications:

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Kelly, L. M., O'Mahony, J. A., & Tobin, J. T. Factors influencing the macro composition and protein fraction of milk among individual cows. *Food Chemistry & Technology Departmental Seminar,* Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork. August 29 nd, 2022

Kelly, L. M., McCarthy, W. P., O'Mahony, J. A., & Tobin, J. T. The impact of genetic variation on the functional properties of beta-lactoglobulin. *Vistamilk Stakeholders and Industry Partners Conference,* Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork. March 31st, 2023.

Poster presentation:

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Kelly, L. M., McCarthy, W. P., O'Mahony, J. A., & Tobin, J. T. The impact of genetic variation on the functional properties of beta-lactoglobulin. *Internation Dairy Federation, World Dairy Summit,* McCormick Place, Martin Luther King St, Chicago, Illinois. October 15th – 19th, 2023.

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Best poster presentation - *Internation Dairy Federation, World Dairy Summit,* McCormick Place, Martin Luther King St, Chicago, Illinois. October 15th – 19th, 2023.

Statement of research objectives

The overall objective of the research reported in this thesis was to determine the impact of genetic variation on the functional properties of β-lactoglobulin, and to determine how these differences affect process efficiency, protein functionality and digestion. In addition, some of these differences are as a result of the amino acid substitution at position 64, in which the A variant contains an aspartic acid and the B variant contains a glycine. This causes a difference in the net negative charge between the variants due to the functional group of the aspartic acid containing an additional negative charge. Below are the specific objectives for the experimental chapters contained within this thesis:

- To conduct a review of the scientific literature regarding the genetic variation of the six major milk proteins, to gain knowledge in the area as well as to identify gaps in the literature that could inform specific topics to be addressed.
- To determine the distribution of protein variants among 48 high genetic merit dairy cows at the dairy research herd in Teagasc Moorepark. This will provide an insight into how the breeding program is defining protein variants that may become dominant in the national herd. Many factors can influence the composition of milk, such as diet, and breed/genetics. It has been shown from previous studies that the AA and BB genotypes of β-lg can impact the expression levels of β-lg, with the A variant of β-lg typically being expressed more than the B variant. This research will also highlight implications for macro level protein distributions, such as total protein and casein to whey ratios as influenced by protein variants.
- To determine the impact of β-lg variants on the permeation of whey proteins and the accumulation of foulants, both of which govern overall filtration efficiency

during separation of whey proteins and caseins in skim milk using microfiltration. The impact of β-lg genetic variants on the efficiency of membrane processes is relatively unknown. Processes such as microfiltration are used to enrich/isolate whey proteins directly from milk and it is also extensively used in the production of whey protein concentrates and isolates from cheese whey.

- To determine the effects of genetic variation on the functional properties of β-lg (especially denaturation and aggregation) independently of confounding effects of other components of milk. Studying the functionality of β-lg in milk can be somewhat misleading as the complexity of milk makes it difficult to correlate minor changes in amino acid composition and structure within individual proteins, with the overall behaviour of the milk system. By removing these other components, the true impact of the genetic variation on the functional properties of β-lg can be understood.
- To study the possible effects of genetic variants of β-lg on the digestive properties of infant formula. A lot of the interest in the genetic variation of milk proteins arose from a study which suggested that milk containing the A1A1 genotype of β-casein caused adverse health effects when consumed. While the variants of β-casein have been extensively studied, the nutritional effects of other milk protein genetic variants still remains unreported.

Chapter 1

Genetic variation in bovine milk proteins: Implications for functional and nutritional properties

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Declaration

This chapter was written by author LMK and reviewed by his co-authors.

Abstract

The six major bovine milk proteins are encoded by highly polymorphic chromosomes. Among the caseins (α_{s1}-, α_{s2}-, β-, κ-) and whey proteins, (α-lactalbumin, β-lactoglobulin), 53 variants have been identified, with the earliest being discovered in 1946. The effect of genetic variation of milk proteins on the nutritional properties of milk and milk products has become an area of much interest, and controversy, in recent years. In particular, the purported adverse health implications of milk with the A1 variant of β-casein have received much attention. However, outside of this more controversial topic, other proteins, and variants thereof, can also affect nutrition and health outcomes. The functional implications of genetic variants have been well characterised, specifically heat stability and coagulation (i.e., rennet, acid) properties of milk. The research surrounding the impact of genetic variants on heat stability has suggested that variants of β-lactoglobulin and κ-casein are the most influential in altering the heat stability of milk. It has been proposed that milk with the B variants of both β-lactoglobulin and κ-casein result in better heat stability than milk with the A variant. Variants of four of the major milk proteins are reported to influence the coagulation properties of milk. The genotypes of BC, BB, BB, and BB were conducive with improved coagulation properties for milk proteins α_{s1}-casein, β-casein, κ-casein, and β-lactoglobulin, respectively. Genetic variants of milk proteins provide considerable potential to create new dairy products and provide opportunities for farmers, milk processors and retailers to add value to milk by tailoring physical and nutritional properties to best suit consumer preferences and needs.

1.1. Introduction

Protein is one of the most important components in milk as it is the primary macromolecule responsible for the production of many dairy products, such as cheese and yoghurt (Bobe et al., 1999; Gustavsson et al., 2014). The protein content of milk can be influenced by various animal factors such as age, health, diet, stage of lactation, and genetics (Bobe et al., 1999; Fox, Uniacke-Lowe, et al., 2015a; Heck et al., 2008). However, of these factors, genetics is the only one to have a major impact on protein composition (Heck, Schennink, et al., 2009a). Milk is comprised of two major families of protein, casein and whey. In milk, 80% of the total protein is casein, which exists in the form of large suspended colloidal structures that precipitate at pH 4.6, i.e., casein micelles (Fox & Brodkorb, 2008; Goulding et al., 2020b). Whey proteins make up the remaining 20% of the total milk protein content and are solubilised in the serum fraction of milk (Formaggioni et al., 1999; Fox et al., 1998; Goulding et al., 2020b; Wong, 2012). The proportions of casein to whey proteins can vary between different bovine breeds, as well as among individual cows of the same breed (Litwińczuk et al., 2011; Mackle et al., 1999). There are six major proteins in milk, four of which are micellar caseins (α_{s1}-, α_{s2}-, β-, and κ- casein) and the remaining two are whey proteins, β-lactoglobulin (β-lg) and α-lactalbumin (α-lac), which are present in the serum phase of milk (Farrell Jr et al., 2004; Kroeker et al., 1985; O'Mahony & Fox, 2014a). Within the whey fraction of milk, there is a wide variety of other minor milk proteins such as blood serum albumin, immunoglobulins and lactoferrin, as well as proteose peptones, each of which has a very specific biological function (Fox, Uniacke-Lowe, et al., 2015a).

Genetic polymorphism has been observed in all six major milk proteins, with 53 variants identified (Gai et al., 2021). The region coding for the expression of the four casein proteins is located in a tight 250 kb cluster, commonly referred to as a casein locus, and is situated within bovine chromosome 6 (Bevilacqua et al., 2006; Hewa Nadugala et al., 2022). The two major

whey proteins, β-lg and α-lac, are coded from chromosomes 5 and 11, respectively (Boland & Singh, 2019; Martin et al., 2002). Mutations to the coding region of one of the caseins can lead to secondary effects on the other non-mutated caseins. This can manifest as an increased or decreased synthesis of one of the other caseins due to the close proximity of the coding regions within the casein locus (Heck, Schennink, et al., 2009a).

1.2. History of milk protein variants

1.2.1. β-lactoglobulin

Before 1946, milk proteins were considered homogeneous. It wasn't until the work of Li (1946) that the first protein genetic variant was observed. A timeline in which the variants of the six major milk proteins were discovered is presented in Fig 1.1. By employing more sensitive electrophoretic techniques than in previous studies, Li (1946) could identify two forms of crystalline β-lg. Following this work, Polis et al. (1950) were the first to assign names to the two forms of β-lg. The names assigned were β_1-lg and β_2-lg for the two separate bands of β-lg observed. The first suggestion that the occurrence of β_1-lg and β_2-lg was genetically determined was by Aschaffenburg, and Drewry (1957), who then bestowed the nomenclature of A and B for the two β-lg variants. The pool of genetic variants of β-lg expanded in 1962 when Bell (1962) found a new variant, C, by starch gel electrophoresis, in the milk of Jersey cows. A D variant subsequently emerged from the work of Grosclancle et al. (1966), which was confirmed by Larsen, and Thymann (1966). Three new genetic variants of β-lg would emerge in 1981 from Southeast Asian Banteng cattle and were given the names E, F and G in the order of their discovery (Bell, McKenzie, et al., 1981). Variant H of β-lg was discovered in Italian Friesians in 1988 by Conti et al. (1988). The three most recently discovered β-lg variants are I, J, and W

(Godovac-Zimmermann et al., 1996; Godovac Zimmermann et al., 1990). The first of these three variants to be discovered is W, and is named out of alphabetical order because it is a silent mutation in which isoleucine (Ile) 56 is substituted for leucine (Leu) 56, with no change in charge (Farrell Jr et al., 2004; Godovac Zimmermann et al., 1990). The I and J variants were discovered as part of the same study, using ion-exchange chromatography, in 1996 and are currently the most recently discovered variants of β-lg (Gai et al., 2021; Godovac-Zimmermann et al., 1996).

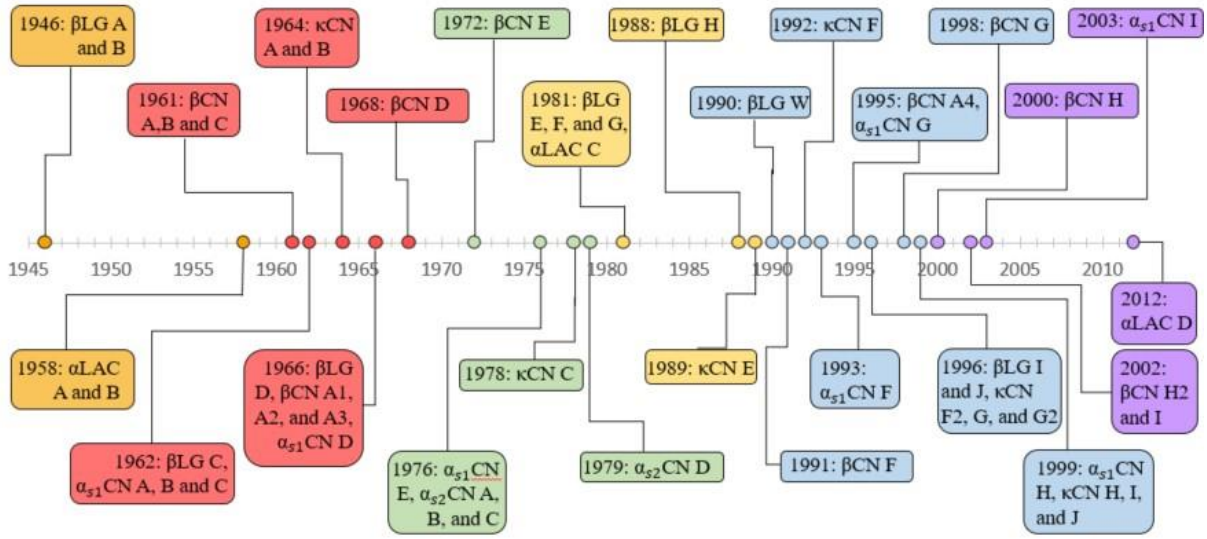

Figure 1.1 Timeline of the discovery of milk protein genetic variants. • < 1960, • 1960 – 1969, • 1970 – 1979, • 1980 – 1989, • 1990 – 1999, • > 2000. The notations for the milk proteins are as follows; β-lactoglobulin (βLG), α-lactalbumin (αLAC), β-casein (βCN), α_{s1}-casein (α_{s1}CN), κ-casein (κCN) and α_{s2}-casein (α_{s2}CN). The letter proceeding the milk protein indicates the genetic variant.

1.2.2. α-lactalbumin

The first identification of polymorphism in α-lac was in 1958, in the milk of African Zebu cattle (Blumberg & Tombs, 1958). Two forms, A and B, were identified based on their electrophoretic mobility at pH 8.6. Milk with the A variant is said to occur mainly in Asian breeds and the AB haplotype may also be found; however, the homozygous B genotype is more

commonly found in western breeds (Ebner & Brodbeck, 1968). The C variant of α-lac was first discovered by starch gel- and filter paper- electrophoresis in Banteng cattle in 1981 (Bell et al., 1981). The D variant of α-lac was identified by capillary zone electrophoresis in 2012 by Visker et al. (2012).

1.2.3. β-casein

The first variants of β-casein were reported by Aschaffenburg (1961) using electrophoretic approaches. In these findings, three variants were found and denoted A, B and C, based on decreasing mobility. The importance of breed, and thus genetics, on the frequency of specific variants was also highlighted even at this early stage of understanding of milk protein polymorphism (Aschaffenburg, 1961; Thompson et al., 1964). In 1966, the A variant was subdivided into three groups that were given the names A^1, A^2, and A^3 due to the substitution of histidine for proline at positions 67 for A^2 and A^3 (Caroli et al., 2009; Peterson & Kopfler, 1966). All amino acid substitutions for β-casein are presented in Fig 1.2, with reference to the A1 variant, providing a chronological sequence of the β-casein variants. The amino acid substitution between the A1A1 and BB genotypes replaces a serine for arginine at position 122, which could also impact the degree of phosphorylation between the two variants. The first observation of the D variant of β-casein was found in Indian Deshi and East African Boran cows, which was highlighted as a rare variant due to its lower frequency of observation in specific regions (Aschaffenburg et al., 1968). The β-casein variant E was discovered in 1972 by Voglino (1972). After the discovery of β-casein variant E, the first suggestion of a new variant occurred in 1991, when Visser et al. (1991) discovered an unknown β-casein component and denoted it as βX. Four years later, βX was confirmed as a new β-casein variant and named F (Visser et al., 1995). Not long before the discovery of β-casein F, a new variant, A^4, was discovered in Korean cattle by Chung et al. (1995). Dong and Ng-Kwai-Hang (1998) identified

the G variant of β-casein by reverse-phase high-performance liquid chromatography (RP-HPLC). The first reported discovery of the H variant of β-casein was by Han et al. (2000). However, Senocq et al. (2002) also discovered a new variant and labelled it as the H variant of β-casein. It wasn't until 2004 that the two variants were given their appropriate nomenclatures of H^1 and H^2 by Han et al. (2000) and Senocq et al. (2002), respectively. The most recently discovered β-casein variant is the I variant, which was discovered in 2002 by Jann et al. (2002) (Fig 1.2).

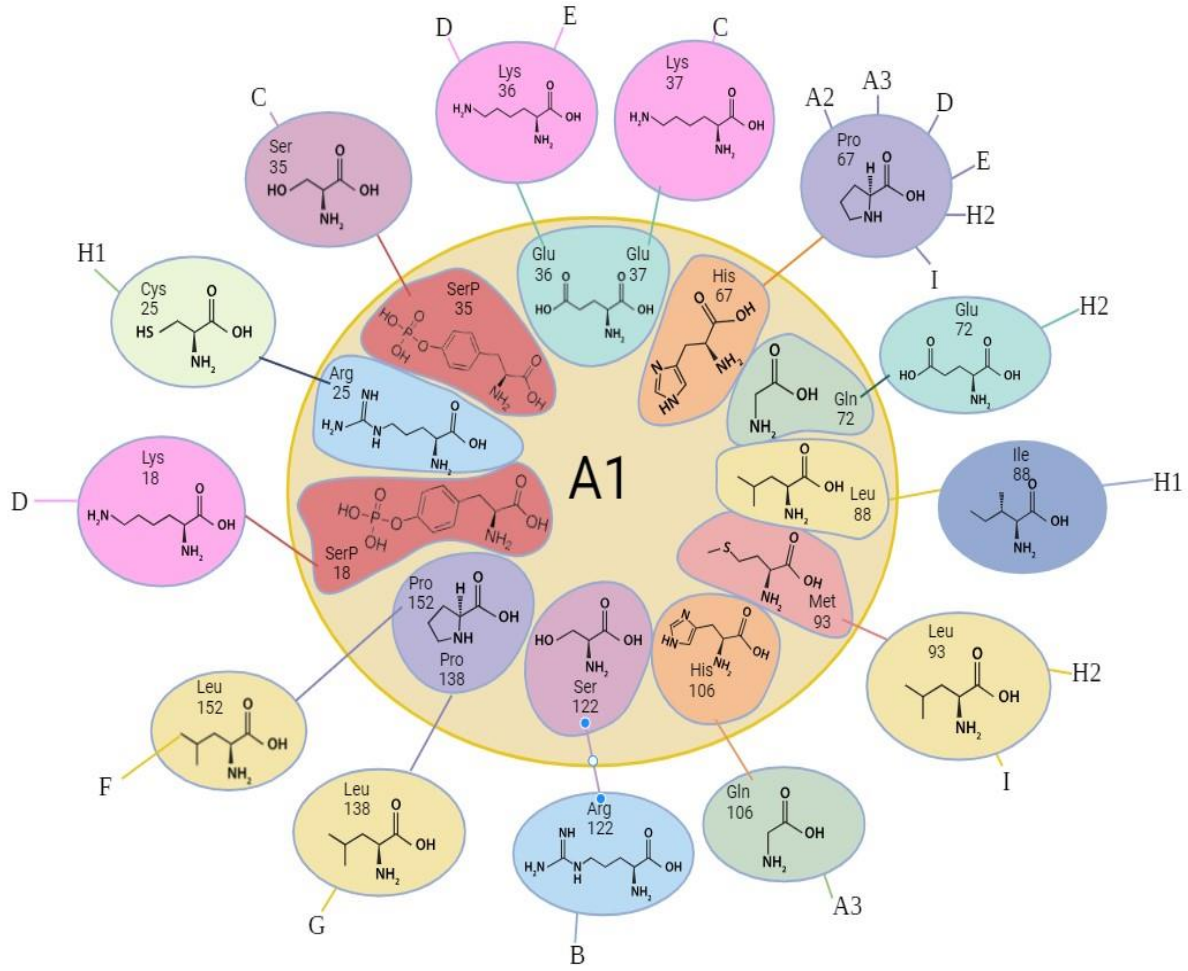

Figure 1.2 The amino acid substitution of β-casein with respect to the A1 variant. Created using Biorender.com.

1.2.4. α-casein

Variants of α-casein were first discovered by Thompson et al. (1962), who found three genetic variants A, B and C. The A variant differs from the B variant by the deletion of the amino acid sequence from position 14-26 and differs from the C variant by a substitution of glutamic acid to glycine at position 192 (Caroli et al., 2009). However, the separation of the α-casein fraction of milk into α_{s1}- and α_{s2}- casein was not properly elucidated until the work of Waugh et al. (1962). However, Thompson et al. (1965) confirmed that the variants they discovered in 1962 were those of α_{s1}-casein, with no variants of α_{s2}-casein yet discovered at the time of their publication. Several years after the last α_{s1}-casein variants were found, Grosclancle (1966) discovered a new variant D in French Flamande cattle in 1966. The first variants of α_{s2}-casein were found in 1976 in Nepalese cattle, with the discovery of variants A, B, and C, as well as the first report of the E variant of α_{s1}-casein (Brunner, 1981; Eigel et al., 1984; Grosclaude et al., 1976). The A variant of α_{s2}-casein differs from the B variant by a substitution of serine for phenylalanine at position 8 and differs from the C variant by three substitutions at positions 33, 47 and 130. At these positions, glutamic acid is replaced with glycine, alanine is replaced with threonine, and threonine is replaced with isoleucine, respectively (Farrell Jr et al., 2004). Three years after discovering the first α_{s2}-casein variants, another variant, D, was found in European cattle (Grosclaude et al., 1979). Throughout the 1990s, three new variants, F, G and H of α_{s1}-casein were discovered in 1993, 1995 and 1999, respectively, with F and G being found in European cattle breeds and the H variant found in African cattle (Erhardt, 1993; Farrell Jr et al., 2004; Mahé et al., 1999; Mariani et al., 1995). The earliest reports of the I variant of α_{s1}-casein were in 2003, in which an intermediate between the B and C variants was observed during isoelectrofocusing of milk from Cameroon cattle (Caroli et al., 2008).

1.2.5. κ-casein

Variants of κ-casein were first identified by Neelin (1964), although it remained unconfirmed until the work of Woychik (1965). Schmidt et al. (1966) found that the differences among variants were due to the substitution of aspartic acid (κ-casein A) with alanine (κ-casein B). This substitution causes the B variant to be more extensively glycosylated than A. The earliest report of the C variant of κ-casein was in 1978, by Di Stasio, and Merlin (1978). Using isoelectric focusing, Seibert et al. (1987) discovered a new variant, which they named D, in German Simmental cows. However, this variant would later be discarded as it was revealed that it was the same as the C variant (Farrell Jr et al., 2004). The E variant of κ-casein was discovered by Erhardt (1989) in German cattle using polyacrylamide gel electrophoresis (PAGE) (Formaggioni et al., 1999). The F variant was discovered by Sulimova et al. (1992), and later expanded upon by Farrell Jr et al. (2004), who suggested the updated nomenclature for the F variants due to the discovery by Prinzenberg et al. (1996) of a new variant which they also called F. The new names suggested were F^1, and F^2 for the variants identified by Sulimova et al. (1992) and Prinzenberg et al. (1996), respectively. Prinzenberg et al. (1996) were the first to discover the G variant in 1996; however, not long after, Sulimova et al. (1996) also reported a G variant. Farrell Jr et al. (2004), once again, termed the variants with respect to their discovery as G^1 and G^2. Genetic variants H and I were found in 1999 by Prinzenberg et al. (1999). Lastly, variant J was discovered by Mahé et al. (1999) in the Ivory Coast using starch and polyacrylamide-gel electrophoresis.

1.3. Protein modification due to genetic variation

1.3.1. DNA mutation

Genetic variants of milk proteins originate as a result of a mutation to the DNA sequence that codes for a particular protein. There are a number of ways in which DNA can mutate, including environmental influences, exposure to certain chemicals, or errors during DNA replication (Chatterjee & Walker, 2017; Clancy, 2008). The most relevant form of mutation for the synthesis of milk protein variants is single nucleotide polymorphism (SNP) or nucleotide insertion or deletion (Fig 1.3) (Caroli et al., 2009; Venturini et al., 2014). A SNP refers to a point mutation within the DNA where a nucleotide has been replaced with a different base pair (Børsting & Morling, 2013; Venturini et al., 2014). During protein synthesis, a single-stranded copy of the DNA called messenger RNA (mRNA), is transported to a ribosome where amino acids are ordered, and peptide bonds are formed (Bierema, 2021; Emery, 2013). Amino acids within a protein are ordered based on a three-nucleotide sequence or codon within the mRNA (O'Connor et al., 2010; Parvathy et al., 2022). When a genetic mutation causes the replacement of a nucleotide it can result in a different amino acid being assigned within the protein, and thus a genetic variant is produced (Clancy, 2008; O'Connor et al., 2010; Venturini et al., 2014).

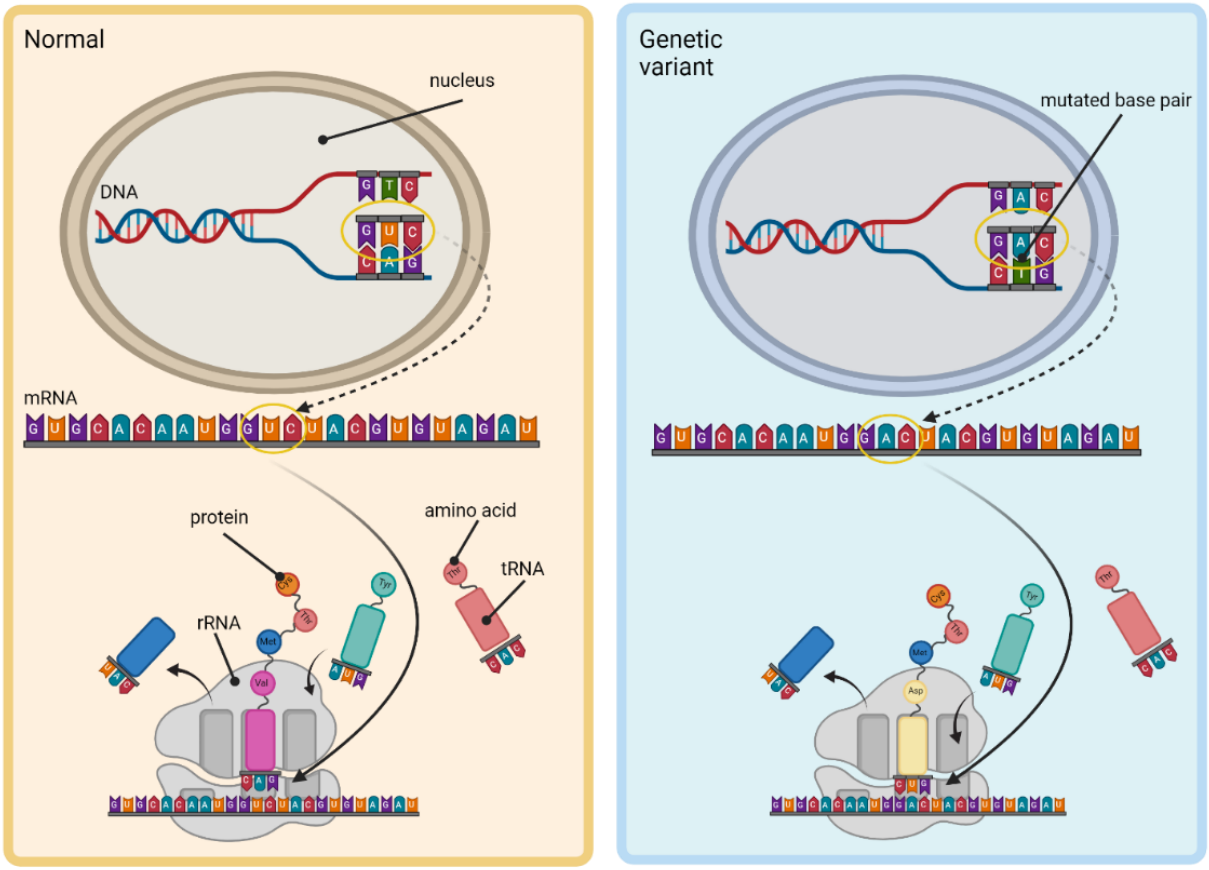

Figure 1.3 Genetic mutation within the coding region of DNA, resulting in the amino acid substitution within the protein's primary structure. Abbreviations are as follows; transfer RNA (tRNA), ribosomal RNA (rRNA). Created using Biorender.com.

1.3.2. Phosphorylation

Post-translational modification takes place mostly in the form of phosphorylation, which exclusively affects casein due to its strong calcium-binding properties and the resulting phosphate groups attach to casein, which cause further variation between proteins (Fang et al., 2016; Heck et al., 2008). The amino acids serine, threonine, and tyrosine are the only amino acids that are susceptible to phosphorylation in milk; therefore, substitutions which add or remove one of these amino acids to the primary structure could result in a change in the degree of phosphorylation which may further alter the structure and functional properties of the

proteins (Nilsson et al., 2020). Phosphorylation of milk proteins occurs in the Golgi apparatus where the phosphorylation-inducing enzyme, casein kinase, is located (Fang et al., 2016; Marks, 2008). In short, the phosphorylation mechanism is initiated by binding ATP to kinase, after which the active site of kinase binds to the substrate protein. Once attached, a phosphate from ATP is transferred to either serine, threonine or tyrosine, which converts ATP to ADP, and the now phosphorylated protein is released. The final stage in phosphorylation is the release of ADP from kinase (Ardito et al., 2017; Ubersax & Ferrell Jr, 2007), and a graphical representation of the phosphorylation process is presented in Fig 1.4.

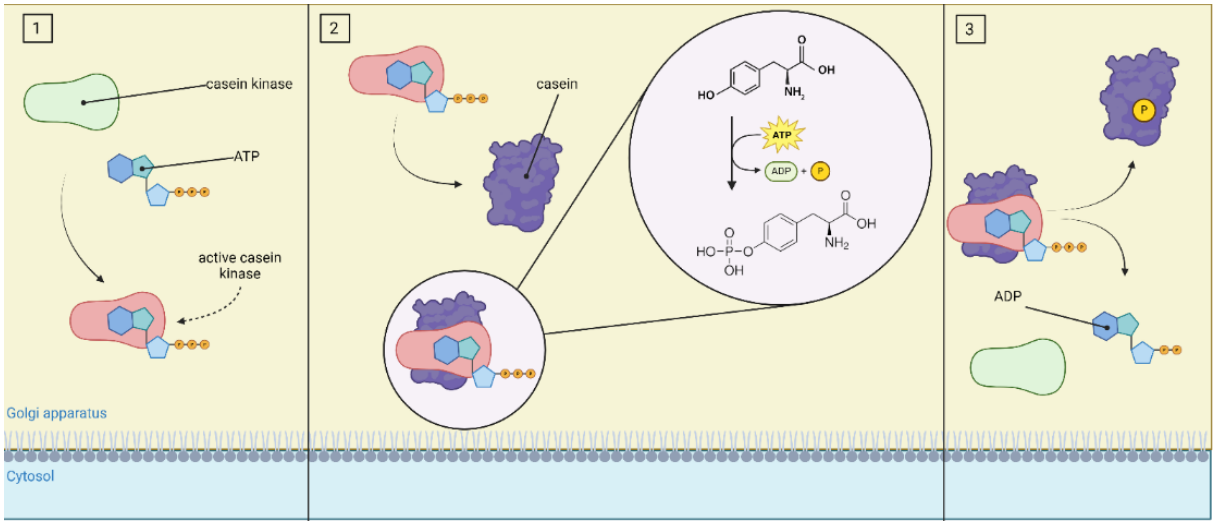

Figure 1.4 Phosphorylation of casein. In panel 1, an adenosine triphosphate (ATP) molecule binds with casein kinase, activating the active site of casein kinase. In panel 2, the activated casein kinase and the bound ATP attach to either serine, threonine or tyrosine of casein. At this point, the ATP binds one of its phosphate groups to the attached amino acid completing phosphorylation. In panel 3, the phosphate has been removed from the ATP and broken down into adenosine diphosphate (ADP). The ADP disconnects from casein kinase, and the casein kinase is disconnected from casein and released into the lumen of the Golgi apparatus. Created using Biorender.com.

1.3.3. Glycosylation

Among the casein proteins, κ-casein is the only one that is glycosylated. Within κ-casein, a region referred to as the casein-glycomacropeptide is heavily glycosylated by O-linked glycan's (Thesbjerg et al., 2024). O-linked glycosylation is first formed due to the addition of a precursor sugar called N-acetlgalactosamine (GalNAc) to the hydroxyl groups of either serine or threonine within a protein sequence (Steen et al., 1998). Once the GalNAc has been attached to the serine or threonine, specific glycosyltransferases add other sugars, such as galactose or glucose, to GalNAc, which then forms a chain of sugars (Hounsell et al., 1996; Thesbjerg et al., 2024). Due to the linking of GalNAc to only serine or threonine at the beginning of glycosylation, it is likely that genetic variation which causes the substitution of these amino acids, could impact the degree of glycosylation, resulting in alterations to the functional properties of the parent protein. The degree of glycosylation of κ-casein could be altered when the BB genotype is present, due to the substitution of threonine for isoleucine at position 136, and also for the EE genotype, due to the substitution of serine for glycine at position 155 (Farrell et al., 2004).

1.4. Genotype frequencies

Different protein variants have been strongly associated with individual cow breeds. Depending on the breed, and even the herd, the variance of specific genotypes can be high, low, or relatively balanced (Bonfatti et al., 2010). In this section of the review, the frequency of protein genotypes among various breeds is discussed.

1.4.1. Holstein Friesian

Holstein Friesian cows are the most common dairy cow breed in the world. Among Holstein Friesian cows, differences between the prevalence of genotypes were observed in the studies by Çardak (2005) and Sanchez et al. (2020). Both of these authors determined that the most frequent genotypes for α_{s1}-casein was BB, followed by the heterozygous BC allele, and finally, the least frequent, homozygous CC variant. However, only Sanchez et al. (2020) reported the DD genotype, which was the α_{s1}-casein variant with the lowest frequency. In a separate study that did not report any BB or CC genotypes of α_{s1}-casein; however it has been reported that the genotype AG was the most common variant of α_{s1}-casein in Holstein Friesian cows, followed by the AA genotype (Asmarasari et al., 2020). A great variety of β-casein genotypes have been observed, with as many as 12 variants identified (Gai et al., 2021; Marko et al., 2020). The most common genotype of β-casein is that of the heterozygous A1A2 variant, followed by the A2A2 and then the A1A1 genotype (Marko et al., 2020). Other alleles of A3, B, C, F, G and I for β-casein have been found in Holstein Friesians, but with very low frequencies (Çardak, 2005; Massella et al., 2017). Very little information on the frequency of α_{s2}-casein variants is found in the literature. Kolenda and Sitkowska (2021) reported that the DD homozygous genotype of α_{s2}-casein was the most common for Holstein Friesian cows, followed by the only other reported AD haplotype. For κ-casein, in Holstein Friesian cows, two alleles dominate, A and B. The most frequent genotype of κ-casein is the heterozygous form, AB; thereafter, the most common genotype is AA, then BB (Adamov et al., 2020; Çardak, 2005); however, some studies have reported the most common genotype of κ-casein to be AA (Čítek et al., 2019). The κ-casein allele E has been reported in Holstein Friesian cows but at very low frequencies (Adamov et al., 2020; Čítek et al., 2019). The most common alleles of β-lg are A and B. The most frequent genotype is AB, followed by AA and then BB (Lukač et al., 2013). The C allele of β-lg has been detected in Holstein Friesian cows but at low frequencies (Burr, 1996;

Makamu & Tyasi, 2024). The A and B alleles of α-lac are the most common alleles of this protein. The most common genotype of α-lac reported was that of AA (Kaura et al., 2021). It should be noted that in many studies, variants that had a frequency of less than 1% were not reported in their results (Sumaiya et al., 2020).

1.4.2. Jersey

Jersey cows are a popular breed of dairy cow due to their high levels of protein and fat in milk. Breeding practices have led to the development of some new variants which are uncommon among other cow breeds. The most common β-casein genotype for Jersey cows is A2A2 (Sanchez et al., 2020; Zepeda-Batista et al., 2015). Genotypes containing a C and A3 allele for β-casein are not found in Jersey cows (Sanchez et al., 2020). The uncommon BB genotype of β-casein is also found in this breed at higher frequencies than Danish Holstein or Swedish Red. However, the occurrence of β-casein genotypes containing a C allele is still low (Gustavsson et al., 2014). The BB genotype of κ-casein is the most frequent genotype among Jersey cows; however, a relatively high frequency of the AA genotypes is also present among the Jersey breed (Sanchez et al., 2020). Genotypes containing the B and C alleles of α_{s1}-casein are commonly found in Jersey cows. Information regarding which of the two alleles is more common is conflicting; however, more studies have suggested that the homozygous CC genotype is less common, than either the homozygous BB or heterozygous BC genotypes of α_{s1}-casein (Jensen, Poulsen, et al., 2012; Poulsen et al., 2013; Sanchez et al., 2020). The most common β-lg genotype in Jersey milk is the AA genotype (Wolanciuk, 2015). While still uncommon, genotypes containing a C allele of β-lg has been found at higher frequencies in Jersey cattle than most other breeds (Caroli et al., 2009; Casas & Kehrli Jr, 2016). However, other studies have found the AB haplotype to be more common among Jersey cows (Jensen,

Poulsen, et al., 2012; Sanchez et al., 2020). The only reported genotype of α_{s2}-casein found in literature is the AA genotype (Jensen, Poulsen, et al., 2012).

1.4.3. Guernsey

The most commonly found β-casein genotype in Guernsey cows is the A2A2 genotype; in fact, this genotype is more commonly found in Guernsey milk than any other cow breed (Bell et al., 2006; Jiménez-Montenegro et al., 2022; Thompson et al., 1964). The α_{s1}-casein BB genotype is the most common among Guernsey cows (Caroli et al., 2009). The κ-casein homozygous genotypes are limited to AA and BB, with the AA genotype being more common than the BB genotype (Chen et al., 2017). Guernsey cows more commonly produce milk with the B variant of β-lg than the A variant; however, the AA genotype is still present at relatively high frequencies (Caroli et al., 2009; Zikakis et al., 1974). No literature has reported on the frequency of α_{s2}-casein variants in Guernsey cattle.

1.4.4. Rare variants

There are around fifty-three variants among the six major milk proteins. While a lot of genotypes can be found within the milk of many breeds, it is important to note that some of the less common genotypes are specific to breeds and are often exclusive to geographical regions due to differences in breeding practices (Caroli et al., 2009; Lisson et al., 2013). The less common alleles of α_{s1}-casein are F and H, found in Ayrshire and Kuri breeds, respectively (Caroli et al., 2009). While not particularly rare, the least common of the α_{s2}-casein alleles is C, found primarily in Mongolian cattle (Formaggioni et al., 1999). The milk protein with the most variants is β-casein, and in conjunction with this, it also has the greatest proportion of uncommon genotypes. The following is a list of the uncommon genotypes of β-casein with their respective breed; B2B2 = Russian cattle, DD = Indian Deshi and East African Boran, EE

= Piemontese, FF = Meuse-Rhine-Yssel, GG = Holstein, H1H1 = Korean cattle, H2H2 = Norwegian cattle (Caroli et al., 2009; Formaggioni et al., 1999). A number of rare κ-casein genotypes have also been identified and linked to specific breeds; DD = Italian Brown, F1F1 = Yakut, F2F2 = Finnish Ayrshire, G1G1 = Pinzgauer, II = Asturian Mountain and Menorquina, JJ = Ivory Coast and Burkina Faso (Caroli et al., 2009; Jann et al., 2004). The rare C allele of α-lac is found exclusively among Australian breeds, specifically Banteng cattle (Bell, Hopper, et al., 1981). Finally, the rare protein genotypes of β-lg are as follows; HH = Italian Friesian, HH = Polish Red, JJ = Hungarian Grey, WW = Murnau-Werdenfelser, Jersey, Red Holstein and Simmental (Caroli et al., 2009; Cieślińska et al., 2019; Formaggioni et al., 1999).

1.5. Impact of milk protein variants on nutritional properties

Milk has a high nutritional value, due to a host of essential nutrients that are vital for human health and development. Milk consumption has been linked with numerous health benefits, such as reducing the risk of osteoporosis, colon cancer, high blood pressure, cardiovascular disease, and type two diabetes (Bell et al., 2006; Clare & Swaisgood, 2000; Daniloski et al., 2021; Korhonen, 2009). Interest in bioactive peptides found in milk has increased in the last number of years due to their association with some of these health benefits. These bioactive peptides are found within the parent protein, where their physiological effects cannot be expressed (Mohanty et al., 2016). When milk is consumed, gastric enzymes break down the milk proteins, releasing peptides into the digestive system, where they can diffuse into the blood and be transported to their corresponding receptors elsewhere in the body where they can induce a physiological effect (Nielsen et al., 2017). The physiological effect of these bioactive peptides can be altered due to genetic variation within the parent protein, but is also based on chain length, which can vary from 2 to 20 amino acid residues (Korhonen, 2009;

Mohanty et al., 2016). Amino acid substitution by genetic variation at the cleavage site of a bioactive peptide can alter the rate at which the peptide is released and the extent of the physiological effect (Nair & Jayachandran, 2019). Bioactive peptides can be present in milk before ingestion due to the action of indigenous enzymes, such as plasmin, elastase and cathepsin D, during storage (Deeth, 2021; Kelly & McSweeney, 2003; McSweeney, 2004). The main mechanism by which a peptide can be liberated from its parent protein is through enzymatic action by either gastrointestinal digestion of milk, fermentation with proteolytic starter cultures, or hydrolysis by proteolytic enzymes (Korhonen & Pihlanto, 2006; Mohanty et al., 2016).

1.5.1. A1 and A2 β-casein

The influence of milk protein variants on human health and nutrition is caused by changes to amino acid sequences which can change a proteins cleavage site and ultimately lead to the loss of peptides or the release of new peptides that may positively or adversely affect human health (Albenzio et al., 2017; Martin et al., 2013). None of the milk protein variants have gained more attention for their associated health implications than the A1 and A2 variants of β-casein. A controversial hypothesis was proposed by Elliott (1992) linking the consumption of A1 milk with type 1 diabetes, coronary heart disease, schizophrenia and autism. The A1 and A2 variants of β-casein differ by a single amino acid substitution at position 67. At this position, histidine is found in A1, and proline is found in A2 milk (Fig 1.2). The implication of this substitution is the alteration to the elastase cleavage site, resulting in the release of an opioid-like bioactive peptide β-casomorphin-7 (BCM7). Opioids are a class of substances that interact with the opioid receptors within the body and induce a morphine-like effect (Che & Roth, 2023; Sebastiani et al., 2020). To liberate BCM7 from the parent protein, two other gastrointestinal proteases are required (pepsin and leucine aminopeptidase), after which the bioactive opioid

peptide of BCM7 is released (Asledottir et al., 2017; Jinsmaa & Yoshikawa, 1999). BCM7 was first identified as a bioactive compound in 1979; however, it wasn't until the 1990s that a negative association with health was suggested in the literature (Bell et al., 2006; Elliott, 1992; Truswell, 2005). One of the main concerns surrounding the consumption of A1 milk and the subsequent release of BCM7 is its potential to pass through the gastrointestinal tract of infants. Due to their underdeveloped nervous system, BCM7 could localise in the central nervous system, affecting the respiratory system by inducing inflammation (Daniloski et al., 2021; Sun et al., 2003; Umbach et al., 1985). In adults, the supposed harmful effects of BCM7 are not as prevalent due to decreased permeability of BCM7 through the gastrointestinal tract, preventing it from transiting to the nervous system where it can express its physiological effects such as heart disease, diabetes or respiratory issues (Bell et al., 2006; Hohmann et al., 2020).

However, a 2009 report from the European Food Safety Authority suggested no sufficient link between the consumption of A1 milk and the onset of physiological and psychological diseases (Authority, 2009). However, recent studies have linked the potential benefit of A2 consumption with increased production of the antioxidant glutathione which can help neutralise free radicals which have the potential to damage cell in the body (Deth et al., 2015; Noctor et al., 2011). Deth (2015) compared the levels of glutathione in Chinese men and women who consumed either A1 or A2 milk, with the results showing a significant increase in glutathione levels in those who drank A2 milk compared to those who drank A1 milk. The hypothesis for the difference in glutathione levels was claimed to be linked to the negative effect of BCM7 on cellular uptake of cysteine, a rate-limiting precursor for glutathione production (Lu, 2009). A recent study by Daniloski et al. (2024), who investigated the effects of A1 and A2 β-casein on the digestive characteristics of reconstituted skim milk, highlighted a difference in the rate of protein breakdown, with the A1 variant being considerably faster. In their study, they indicated

that this difference in protein breakdown could significantly affect human nutrition; however, a health implication/outcome was not proposed.

Although BCM7 has largely been associated with A1 milk (Bell et al., 2006; Daniloski et al., 2021; Swinburn, 2004; Truswell, 2005; Woodford, 2011), it is still a product of A2 milk digestion (Gai et al., 2021). While the release of BCM7 from A2 milk is significantly lower than that of A1 milk, often being reported as approximately 75% lower than A1 milk, it is still noteworthy to acknowledge its release during digestion (Asledottir et al., 2018; Priyadarshini et al., 2018). Aside from the A1 and A2 variants of β-casein, BCM7 has also been observed as a digestive product of variants F, I and B of β-casein (Asledottir et al., 2018; Cieślińska et al., 2007; Noni, 2008).

1.5.2. Allergic response to milk protein genetic variants

Allergic reaction to milk affects around 15% of children, and is largely due to the presence of β-lg and α_{s1}-casein (Lisson et al., 2014; Monaci et al., 2006). The implications of the effect of β-lg variants on its allergenic properties are ill-defined, and indeed contradictory, in the literature. In mice, the development of an allergic reaction to β-lg was found to be stronger in milk containing the B variant of β-lg than in the A variant (Oliveira, Valente-Mesquita, et al., 2001). However, Malik et al. (1988) found that milk with the A variant of β-lg induced a greater allergic response than milk with the B variant and attributed this to the amino acids on the surface of the protein, which are only found in the A variant of β-lg. It has been proposed that the presence of either A or B variants of β-lg can affect the health status of the cow's udder (Chaneton et al., 2011). These authors found that milk with the A variant of β-lg had greater antibacterial activity against *Strep. uberis* and *Staph. aureus* than milk with the B variant. They attributed this activity, at least in part, to the A variant β-lg being more acidic than the B variant and while the exact mechanism of this activity was not addressed in their study, it was

suggested that anionic domains of β-lg could be the cause of the antimicrobial activity. The allergenicity of genetic variants of α_{s1}-casein was investigated by determining the IgE-binding properties of peptides after *in vitro* gastrointestinal digestion (Lisson et al., 2014). These authors found that the B variant of α_{s1}-casein had a more intense immune response than any of the other variants, as indicated by an increase in IgE binding of milk with the B variant (Lisson et al., 2014).

1.6. Functional properties of milk protein genetic variants

The assessment of indicators of milk functionality is an important means of determining potential processing performance and finished product quality. Such functional indicators include heat stability, rennet and acid coagulation properties and emulsification properties (Gai et al., 2021). Changes in the structure of a protein caused by genetic variation can have a significant impact on its functional performance.

1.6.1. Heat stability

Thermal processes such as pasteurisation, evaporation, spray drying, sterilisation, and ultra-high temperature (UHT) heat treatment are used to produce long shelf-life milk products (Fox, Uniacke-Lowe, McSweeney and O'Mahony, 2015; Singh, 2004). Heat-induced coagulation of milk proteins during thermal processing is a major ongoing focus area for dairy processors. During the heat-treatment of milk, coagulants which cause fouling can result in blockages and poor heat transfer between the equipment and the milk and can greatly affect cleaning efficiency and discharge of milk solids to effluent (Fuquay et al., 2011; Huppertz, 2016b).

The milk proteins that influence heat stability most strongly are β-lg and κ-casein and thus, these proteins, and their respective variants, will be the focus of this section (Huppertz, 2016b;

Singh & Fox, 1987; Singh, 2004). The most common genotypes, in western herds, for both β-lg and κ-casein are the AA and BB genotypes, respectively (de Wit, 2009; Molina et al., 2006). Changes to the primary structure of β-lg and κ-casein by a genetic mutation can have marked effects on the heat coagulation time (HCT) of milk(Choi & Ng-Kwai-Hang, 2003; Imafidon et al., 1991b). During heat stability testing, the interactions between β-lg and κ-casein are responsible for the type A HCT-pH profile (Gai et al., 2021). A study by McLean et al. (1987) found that the maximum heat stability of skim milk samples was significantly affected by protein variants of β-lg and κ-casein, with the milk containing the B variant of both proteins displaying higher maximum HCT and the A-type displaying the lowest maximum HCT. This could be a result of milk with the β-lg A variant, often being associated with higher total protein and whey protein content, or possibly due to the difference in the reactivity of thiol groups between the β-lg molecules which may cause a greater extent of cysteine bonds forming between protein molecules (de Wit, 2009; Heck, Schennink, et al., 2009a; Sawyer, 1968). A higher protein content typically reduces the heat stability of milk due to the closer proximity of proteins, and higher whey protein content can cause a faster association of β-lg with κ-casein, thereby reducing the heat stability. Milk with the κ-casein AA genotype is associated with lower κ-casein concentration, which can reduce the electrostatic repulsion between casein micelles (Heck, Schennink, et al., 2009a; Molina et al., 2006). A similar study by Robitaille (1995) agreed with the finding of McLean et al. (1987), but added that β-lg only influenced heat stability when the genotype of κ-casein containing an A allele was present. In contrast, Bikker et al. (2000) found that milk with the B variant of β-lg was the most heat-labile, followed by the A variant, whereas milk with the C variant of β-lg was the most heat-stable (Bikker et al., 2000; Brunner, 1981). Daniloski, McCarthy, and Vasiljevic (2022) highlighted an indirect effect that some variants of β-casein have on κ-casein. In their study, it was found that milk with the A2A2 genotype of β-casein had lower levels of κ-casein than the A1A1 and A1A2

genotype; these lower levels of κ-casein could lead to alterations in the thermal stability of milk by reducing the electrostatic repulsion between casein micelles.

The variation in heat stability between the different genotypes of β-lg is probably due to structural changes and self-association due to differences in primary structure, particularly that of the substitution of aspartic acid for glycine at position 64 which causes the A variant to have a greater net negative charge than the B variant (Bikker et al., 2000; Olsen et al., 2022). The negative charge of β-lg could impact heat stability by hindering the binding of β-lg with κ-casein due to the charge repulsion between both proteins (Fox, Uniacke-Lowe, et al., 2015; Fox et al., 1998).The results of Bikker et al. (2000) found that milk with the C variant of β-lg is the most heat-stable than milk with either the A or B variant. However, in contrast to Bikker et al. (2000), Keppler et al. (2014) found milk with the B variant of β-lg to be more heat stable than the A variant. Research conducted in 1968 obtained different results than those of Keppler et al. (2014), with milk containing the A variant of β-lg being the most stable, followed by B and finally the C variant; however, they suggested that the difference in heat stability among the variants of β-lg was due to variation in the reactivity of the thiol/disulphide groups (de Wit, 2009; Sawyer, 1968). Upon heating of milk at temperatures in excess of 80˚C, β-lg beings to unfold, exposing charged sites and thiol groups, which ultimately bind to κ-casein, destabilising the casein micelle (de Wit, 2009). Among three variants of β-lg, the negative net charge of the proteins is in the order of A>B>C (Keppler et al., 2014; Manderson et al., 1998a). The variation in heat stability that occurs between variants A and B for κ-casein is due to the substitution at amino acid positions 136 and 148. At position 136, threonine is replaced with isoleucine and at position 148, aspartic acid is replaced with alanine for the A and B variants, respectively (Farrell Jr et al., 2004; Gai et al., 2021). The influence of genetic variation of κ-casein on heat stability could be dueto the location at which the amino acid substitution occurs (Azevedo et al., 2008; Bonfatti et al., 2014). These substitutions take place close to

glycosylation sites and may impact the glycosylation pattern, which in itself, influences heat stability due to alterations to the steric stability or net negative charge of κ-casein (Azevedo et al., 2008; Dziuba & Minkiewicz, 1996; Fan et al., 2019).

1.6.2. Milk coagulation

The micellar destabilisation of the casein proteins in milk is an important process in producing various dairy products, such as cheese and yoghurt (Fox et al., 2017; Jensen, Holland, et al., 2012). There are two main actions by which caseins are destabilised. The first is rennet-induced coagulation caused by the enzymatic cleavage of κ-casein at or near phenylalanine$_{105}$-methionine$_{106}$, and the second is acid-induced coagulation upon reaching the isoelectric point of pH 4.6 (Fox et al., 1998; Li et al., 2020).

Rennet coagulation is a two-stage process where the first stage is the removal of the negatively charged casein macropeptide (amino acid residues 106-169) from κ-casein by rennet-like enzymes, such as chymosin, and the second stage is the aggregation of caseins by calcium phosphate bonds (Gai et al., 2021; Horne & Lucey, 2017). When approximately 85% of κ-casein has been enzymatically removed from the micelle by rennet-like enzymes, or in the case of acid gelation a pH of 4.6 is achieved, aggregation of the casein micelles occurs, resulting in the formation of a gel matrix (Britten & Giroux, 2022; Huppertz & Chia, 2021). The coagulation ability of milk is not exclusively determined by κ-casein; it has also been found that the other caseins, particularly β-casein influence coagulation through a chaperone-like activity, in which β-casein binds with unfolded proteins preventing them from joining together to form a gel matrix (Akbari et al., 2018; Zhang et al., 2005).

Milk protein polymorphism can influence coagulation properties in several ways, such as increasing or decreasing protein content, affecting the size of the casein micelles, and

alterations to other important components that influence coagulation, such as fat, lactose and salts (Bijl et al., 2014; Heck, Schennink, et al., 2009a). Jensen (2012) investigated the impact of protein variants on rennet coagulation, in which they found that milk with the B variant of β-casein had better gel-forming properties such as higher gel strength and lower rennet coagulation time (RCT) when compared to milk with the A1 or A2 variants of β-casein. A study on the influence of protein genetic polymorphism in Scandinavian milk stated that the beneficial properties of milk with the B variant of β-casein were observed in Danish Holsteins but not in Danish Jersey cows (Poulsen et al., 2013). Milk containing the A1A2 haplotype of β-casein is reported to have better coagulation properties than milk with either the A1A1 or A2A2 genotype of β-casein (Hallén et al., 2007). Research conducted by St-Gelais, and Haché (2005) showed that changes in the protein composition of milk could have marked effects on the gel structure and strength. Depending on the genetic variation of a milk protein the composition of milk can be altered which can effect the gelation properties of milk (Heck et al., 2007). Additionally, a protein that is synthesised from a region of DNA close to where another protein is synthesised can cause the expression of both proteins to change (Heck, Schennink, et al., 2009a). One such interlinked effect is that of α-casein and β-casein; whereby, up-regulation in terms of expression leads to changes in the ratios of individual caseins or whey proteins, effectively altering the ratio of α-casein to β-casein (St-Gelais & Haché, 2005). Milk with the A2 variant of β-casein was conducive to weaker gels, possibly due to an increase in micelle size compared to other variants; however, the influence of β-casein variants on casein micelle size is poorly understood (Daniloski, McCarthy and Vasiljevic, 2022; Day et al., 2015). Large micelles can also contribute to weaker gels due to formation of a more porous structure compared to smaller micelles (Glantz et al., 2010; Mahé et al., 1999). Milk with the I variant of β-casein was found by Poulsen et al. (2013) to produce gels with a longer RCT and a weak gel structure. The comparison of acid gelation properties of milk with different genetic variants

of β-casein found that milk with the A2 variant produced weaker gels and had poorer water holding capacity compared to the A1 variant or the A1A2 haplotype, which produced gels of similar strength (Daniloski, McCarthy, Gazi, et al., 2022).

Comin et al. (2008), suggested that κ-casein genetic variants had a greater impact on milk coagulation properties than any of the β-casein variants analysed in their study. They also reported that, when both κ-casein and β-casein contained at least one B allele, this gave the best coagulation properties indicated by shorter rennet coagulation time and stronger gels. However, other studies have shown no significant interactions between the genetic variants of β-casein and κ-casein (Hallén et al., 2007). The tendency for milk with the AA or BB genotypes or the AB haplotype of κ-casein to coagulate and form a strong gel are in the order of BB>AB>AA (Davoli et al., 1990; Kübarsepp et al., 2005; Petrovska et al., 2017). A study by Wedholm et al. (2006) found that milk with a higher concentration of κ-casein forms stronger gels possibly due to better crosslink formation between casein micelles. The concentration of κ-casein is influenced by its genetic variant, with milk containing the B variant producing more κ-casein followed by the AB haplotype, and finally the A variant (Heck, Schennink, et al., 2009a). It is therefore possible that the results of Davoli et al. (1990) and Petrovska et al. (2017), which indicate that milk with the B variant of κ-casein has better coagulation properties than the A variant or AB haplotype is, at least in part, due to the differences in κ-casein concentrations.

κ-casein and its genetic variants, have been shown to have the greatest impact on micelle size out of all the proteins in milk (Glantz et al., 2010). Milk with the B variant of κ-casein is associated with a smaller micelle size than the AB haplotype or the A variant, which was attributed to the differences in the degree of glycosylation among the variants, in which the more glycosylated κ-casein was linked to a smaller micelle (Bijl et al., 2014). Bonfatti (2014) stated that, with an increase in glycosylation of κ-casein, a considerably shorter RCT is

achieved, possibly due to the increase in intermolecular interactions available to proteins that are more glycosylated. κ-casein is the only glycosylated casein protein and, of the κ-casein variants, the B variant is the most glycosylated, followed by the A variant (Bijl et al., 2014; Bonfatti et al., 2014; Gregersen & Lucey, 2016). It is worth noting; however, that the A variant of κ-casein has more glycosylation sites than the B variant but, because of the structural conformation of the A variant, not all of these sites are accessible, which results in fewer overall glycosylated regions (Bonfatti et al., 2014).

Milk containing the haplotype of BC for α_{s1}-casein was conducive to improved coagulation properties, including faster curd firming rate and higher gel strength, which was likely due to a smaller micelle size that is typical of the BC haplotype (Jõudu et al., 2007; Ketto et al., 2017). Conversely, Poulsen et al. (2013) found that milk containing the CC genotype of α_{s1}-casein had a shorter RCT and higher curd firming rate than milk with the B variant. It was also reported by Andrén (2007) that milk with the BC haplotype was linked to increased milk total protein and casein content, which is known to improve milk coagulation properties. Higher levels of α_{s2}-casein were correlated with poor or non-coagulating milk (Jõudu et al., 2008). It could, therefore, be assumed that milk with variants of α_{s2}-casein that produce lower levels of α_{s2}-casein could be more favourable. There have been few studies on the impact of α_{s1}-casein variants, and even less on α_{s2}-casein variants, on milk coagulation properties; an explanation for this could be due to the small number of alleles and the minor effects of α_{s1}- and α_{s2}-casein on milk coagulation properties (Bittante et al., 2012).

The two most common variants of β-lg are the A and B variants. Jersey cows are more likely to contain the AA genotype, while Holstein cows have an almost equal frequency of the AA and BB genotypes (Wolanciuk, 2015). The effects of β-lg variants on milk coagulation properties are relatively small in comparison to the effect of casein variants, attributable to the fact that whey proteins are not directly involved in rennet coagulation (Bonfatti et al., 2010).

However, some studies have reported variation in coagulation properties, potentially due to variations in protein concentration associated with certain genetic variants of β-lg (Bonfatti et al., 2010; Gai et al., 2021; Meza-Nieto et al., 2007). Some reports have suggested that milk with the B variant is associated with higher gel strength than milk with the A variant (Jensen, Holland, et al., 2012; Meza-Nieto et al., 2007; Meza-Nieto et al., 2013), possibly due to increased protein, casein and total solids linked with the B variant of β-lg (Meza-Nieto et al., 2013). It is possible that the association of the B variant of β-lg with the casein micelle before rennet addition, which the A variant was not shown to do, contributed to improved rennet coagulation properties of milk (Meza-Nieto et al., 2007). Other studies have reported conflicting results, with milk containing the A variant of β-lg having a shorter gelation time and a higher gel strength than milk with the B variant (Glantz et al., 2011; Marziali & Ng-Kwai-Hang, 1986a; Matějíček et al., 2008). Jensen, Holland, et al. (2012) reported that, regardless of their paired allele, either A or B, milk with the allele of C showed poor rennet coagulation properties; however, no explanation was given for this. The literature regarding the effect of milk protein polymorphism on coagulation properties is often conflicting and inconsistent. This could potentially be due to the difficulty in assessing a single milk component without the confounding influence of other components within milk, such as fat and other proteins. This challenge could be alleviated by purifying the milk component of interest, eliminating the other conflicting component; however, this tends to be technically very difficult to achieve.

1.6.3. Emulsifying properties

The foaming and emulsifying properties of proteins are some of the most important functional characteristics within the food industry (Zayas, 1997). Proteins form foams and emulsions by decreasing the interfacial tension between the immiscible solution, which is achieved by the

organisation of the protein's hydrophobic and hydrophilic regions at these interfaces (Lam & Nickerson, 2013). The ability to form good, stable foams or emulsions is dependent on several important factors, such as the type of protein, genetic variant, concentration, temperature, pH and the viscosity of the dispersing liquid (Euston et al., 1999; Pearce & Kinsella, 1978; Zayas, 1997).

The emulsification properties of three variants of β-casein (A1, A2 and B) were studied by Darewicz, and Dziuba (2007), with results showing that milk with the B variant had the best emulsification properties, followed by milk with the A1 variant and then milk with the A2 variant. The emulsification properties of milk containing the B variant were characterised by a larger, but more stable, droplet, which was attributed to the B variant's larger surface coverage compared to the A1 or A2 variants, which was possibly due to the differences in isoelectric point among the three variants tested (Darewicz & Dziuba, 2007). Between the A1 and A2 variants, milk with the A1 variant was shown to have good foaming properties, while milk with the A2 variant has poor foaming properties. This was shown to be due to a more extensive spread of the A1 variant at the interface, which facilitates a faster build-up of an interfacial layer (Ipsen & Otte, 2004).

The emulsification properties of β-lg variants A, B and C were assessed by Euston et al. (1999). The variant that produced the smallest droplet size was in the order of A<B<C. Overall, it was stated that the β-lg variant with the best emulsifying ability was the A variant, with B being better than C. The differences between the variants were due to the change in net charge and protein conformation induced by substituting amino acids within its primary structure; however, no specific values for net charge were presented. Between variants A and B of β-lg, the A variant showed a greater capacity to adsorb at the air-water interface. This was attributed to the greater flexibility of the A variant, due in part to its lower thermal stability, allowing it to unfold to a greater extent at the air-water interface (Ipsen & Otte, 2004).

1.7. Conclusion

Genetic variants of milk have been identified since 1946, with the most recent variants being discovered in 2003, comprising a total of 53 variants among the six primary milk proteins. Genetic variants of milk proteins are, and continue to be, an area of growing interest among dairy scientists and milk processors. Genetic variants of milk proteins have been a topic of interest and controversy for many decades, especially with regard to the A1 and A2 variants of β-casein. Some studies have aimed to link the A1 variant of β-casein with heart disease, type 2 diabetes and autism; however, no definitive link has been established. This controversy has attracted researchers to this area of study, increased the volume of literature, and expanded the understanding of the importance of genetic variants to the functional and nutritional properties of milk. Some studies have investigated the impact of genetic variants on the allergenic properties of milk; however, at the time of this review, no consistent or conclusive results have been published. The importance of genetic variation on the functional properties of milk is highlighted in terms of its effect on heat stability, milk coagulation properties and emulsification properties. Genetic variation of the six major milk proteins can influence heat stability, with the genetic variants of β-lg and κ-casein being the most influential. Genetic variation of the casein proteins, particularly κ- and β-casein, can affect rennet and acid coagulation properties of milk, impacting coagulation time and gel strength. The effect of genetic variation of β-casein on the emulsification properties of milk found that milk with the B variant formed the most stable droplets. Among the A, B and C variants of β-lg, it was found that milk with the A variant had the best emulsification properties. Depending on the breed, some genetic variants can be more prevalent than others, and with the continual development of breeding practices, new variants will likely be discovered. Future breeding practices might be tailored for the selection of specific variants of milk proteins, which could be achieved at the herd, regional and even national levels through the bull and artificial insemination selection

processes. The selection of specific variants could be used to aid the production of dairy products like yoghurt and cheese or create milk with an added nutritional benefit. The literature on milk protein variants is growing, but more research is needed on the genetic variants of minor serum milk proteins, such as immunoglobulins, blood serum albumin and lactoferrin. More research in this area could showcase the emergence of more milk variants which has the potential to significantly impact milk composition, functionality and nutritional properties.

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Declaration of competing interest

Authors declare no conflicts of interest.

1.8. References

Adamov, N., Atanasov, B., Ilievska, K., Nikolovski, M., Dovenska, M., Petkov, V., & Dovenski, T. (2020). Allele and genotype frequencies of the κappa-casein (CSN3) locus in Macedonian Holstein-Friesian cattle. *Macedonian Veterinary Review, 43*, 45-54.

Akbari, A., Bamdad, F., & Wu, J. (2018). Chaperone-like food components: from basic concepts to food applications. *Food & Function, 9*, 3597-3609.

Albenzio, M., Santillo, A., Caroprese, M., Della Malva, A., & Marino, R. (2017). Bioactive peptides in animal food products. *Foods, 6*, 35.

Andrén, A. (2007). Influence of genetic milk protein variants on milk quality. *Journal of Animal and Feed Sciences, 16*, 143-150.

Ardito, F., Giuliani, M., Perrone, D., Troiano, G., & Lo Muzio, L. (2017). The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy. *International Journal of Molecular Medicine, 40*, 271-280.

Aschaffenburg, R., & Drewry, J. (1957). Genetics of the β-Lactoglobulins of Cow's Milk. *Nature, 180*, 376-378.

Aschaffenburg, R. (1961). Inherited casein variants in cow's milk. *Nature, 192*, 431-432.

Aschaffenburg, R., Sen, A., & Thompson, M. P. (1968). Genetic variants of casein in Indian and African Zebu cattle. *Comparative Biochemistry and Physiology, 25*, 177-184.

Asledottir, T., Le, T. T., Petrat-Melin, B., Devold, T. G., Larsen, L. B., & Vegarud, G. E. (2017). Identification of bioactive peptides and quantification of β-casomorphin-7 from bovine β-casein A1, A2 and I after ex vivo gastrointestinal digestion. *International Dairy Journal, 71*, 98-106.

Asledottir, T., Le, T. T., Poulsen, N. A., Devold, T. G., Larsen, L. B., & Vegarud, G. E. (2018). Release of β-casomorphin-7 from bovine milk of different β-casein variants after ex vivo gastrointestinal digestion. *International Dairy Journal, 81*, 8-11.

Asmarasari, S. A., Sumantri, C., Gunawan, A., Taufik, E., & Anggraeni, A. (2020). Genetic Variants of Milk Protein Genes and Their Association with Milk Components in Holstein Friesian Cattle. *Jurnal Ilmu Ternak dan Veteriner, 25*, 99-111.

Authority, E. F. S. (2009). Review of the potential health impact of β-casomorphins and related peptides. *European Food Safety Authority Journal, 7*, 231.

Azevedo, A., Nascimento, C., Steinberg, R., Carvalho, M., Peixoto, M., Teodoro, R., Verneque, R., Guimarães, S., & Machado, M. (2008). Genetic polymorphism of the kappa-casein gene in Brazilian cattle. *Genetics and Molecular Research, 7*, 623-630.

Bell, K. (1962). One-dimensional starch-gel electrophoresis of bovine skim-milk. *Nature, 195*, 705-706.

Bell, K., Hopper, K., & McKenzie, H. (1981). Bovine a-Lactalbumin C and aS1-, ß-and?-Caseins of Bali (Banteng) Cattle, Bos (Bihos) Javanicus. *Australian Journal of Biological Sciences, 34*, 149-160.

Bell, K., McKenzie, H., & Shaw, D. (1981). Bovine ß-Lactoglobulin E, F and G of Bali (Banteng) Cattle, Bos (Bihos) Javanicus. *Australian Journal of Biological Sciences, 34*, 133-148.

Bell, S. J., Grochoski, G. T., & Clarke, A. J. (2006). Health implications of milk containing β-casein with the A2 genetic variant. *Critical Reviews in Food Science and Nutrition, 46*, 93-100.

Bevilacqua, C., Helbling, J. C., Miranda, G., & Martin, P. (2006). Translational efficiency of casein transcripts in the mammary tissue of lactating ruminants. *Reproduction Nutrition Development, 46*, 567-578.

Bierema, A. (2021). Protein Synthesis Overview. *An Interactive Introduction to Organismal and Molecular Biology* (Second Edition): Michigan State University.

Bijl, E., de Vries, R., van Valenberg, H., Huppertz, T., & Van Hooijdonk, T. (2014). Factors influencing casein micelle size in milk of individual cows: Genetic variants and glycosylation of κ-casein. *International Dairy Journal, 34*, 135-141.

Bikker, J., Anema, S., Li, Y., & Hill, J. (2000). Thermal denaturation of β-lactoglobulin A, B and C in heated skim milk. *Milchwissenschaft, 55*, 609-613.

Bittante, G., Penasa, M., & Cecchinato, A. (2012). Invited review: Genetics and modeling of milk coagulation properties. *Journal of Dairy Science, 95*, 6843-6870.

Blumberg, B. S., & Tombs, M. (1958). Possible polymorphism of bovine α-lactalbumin. *Nature, 181*, 683-684.

Bobe, G., Beitz, D., Freeman, A., & Lindberg, G. (1999). Effect of milk protein genotypes on milk protein composition and its genetic parameter estimates. *Journal of Dairy Science, 82*, 2797-2804.

Boland, M., & Singh, H. (2019). *Milk proteins: from expression to food* (Third Edition): Academic Press.

Bonfatti, V., Di Martino, G., Cecchinato, A., Degano, L., & Carnier, P. (2010). Effects of β-κ-casein (CSN2-CSN3) haplotypes, β-lactoglobulin (BLG) genotypes, and detailed protein composition on coagulation properties of individual milk of Simmental cows. *Journal of Dairy Science, 93*, 3809-3817.

Bonfatti, V., Chiarot, G., & Carnier, P. (2014). Glycosylation of κ-casein: Genetic and nongenetic variation and effects on rennet coagulation properties of milk. *Journal of Dairy Science, 97*, 1961-1969.

Børsting, C., & Morling, N. (2013). Single-Nucleotide Polymorphisms. In *Encyclopedia of Forensic Sciences* (Second Edition) (pp. 233-238): Academic Press.

Breitling, J., & Aebi, M. (2013). N-linked protein glycosylation in the endoplasmic reticulum. *Cold Spring Harbor Perspectives in Biology, 5*, a013359.

Britten, M., & Giroux, H. J. (2022). Rennet coagulation of heated milk: A review. *International Dairy Journal, 124*, 105179.

Brunner, J. R. (1981). Cow Milk Proteins: Twenty-Five Years of Progress1. *Journal of Dairy Science, 64*, 1038-1054.

Burr, R. G. (1996). Frequency of occurrence of novel milk protein variants in a New Zealand dairy cattle study population: a thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Biochemistry, *Massey University*. Massey University.

Çardak, A. (2005). Effects of genetic variants in milk protein on yield and composition of milk from Holstein-Friesian and Simmentaler cows. *South African Journal of Animal Science, 35*, 41-47.

Caroli, A., Chessa, S., Chiatti, F., Rignanese, D., Meléndez, B., Rizzi, R., & Ceriotti, G. (2008). Short Communication: Carora Cattle Show High Variability in αs1-Casein. *Journal of Dairy Science, 91*, 354-359.

Caroli, A. M., Chessa, S., & Erhardt, G. J. (2009). Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *Journal of Dairy Science, 92*, 5335-5352.

Casas, E., & Kehrli Jr, M. E. (2016). A review of selected genes with known effects on performance and health of cattle. *Frontiers in Veterinary Science, 3*, 113.

Chaneton, L., Pérez Sáez, J. M., & Bussmann, L. E. (2011). Antimicrobial activity of bovine β-lactoglobulin against mastitis-causing bacteria. *Journal of Dairy Science, 94*, 138-145.

Chatterjee, N., & Walker, G. C. (2017). Mechanisms of DNA damage, repair, and mutagenesis. *Environmental and Molecular Mutagenesis, 58*, 235-263.

Che, T., & Roth, B. L. (2023). Molecular basis of opioid receptor signaling. *Cell, 186*, 5203-5219.

Chen, B., Grandison, A. S., & Lewis, M. J. (2017). Best use for milk-a review I-Effect of breed variations on the physicochemical properties of bovine milk. *International Journal of Dairy Technology, 70*, 3-15.

Choi, J., & Ng-Kwai-Hang, K. (2003). Effects of Genetic Variants of κ-casein and β-lactoglobulin and Heat Treatment on Coagulating Properties of Milk. *Asian-Australasian Journal of Animal Sciences, 16*, 1212-1217.

Chung, E., Han, S., & Rhim, T. (1995). Milk protein polymorphisms as genetic marker in Korean native cattle. *Asian-Australasian Journal of Animal Sciences, 8*, 187-194.

Cieślińska, A., Kamiński, S., Kostyra, E., & Sienkiewicz-Szłapka, E. (2007). Beta-casomorphin 7 in raw and hydrolyzed milk derived from cows of alternative β-casein genotypes. *Milchwissenschaft, 62*, 125-127.

Cieślińska, A., Fiedorowicz, E., Zwierzchowski, G., Kordulewska, N., Jarmołowska, B., & Kostyra, E. (2019). Genetic polymorphism of β-Casein gene in Polish Red Cattle—Preliminary study of A1 and A2 frequency in genetic conservation herd. *Animals, 9*, 377.

Čítek, J., Hanusová, L., Lískovcová, L., Samková, E., Hanuš, O., Hasoňová, L., Křížová, Z., & Večerek, L. (2019). Polymorphisms in CSN3, CSN2 and LGB genes and their relation to milk production in dairy cattle in the Czech Republic. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 67*, 19-24.

Clancy, S. (2008). Genetic mutation. *Nature Education, 1*, 187.

Clare, D. A., & Swaisgood, H. E. (2000). Bioactive Milk Peptides: A Prospectus1. *Journal of Dairy Science, 83*, 1187-1195.

Comin, A., Cassandro, M., Chessa, S., Ojala, M., Dal Zotto, R., De Marchi, M., Carnier, P., Gallo, L., Pagnacco, G., & Bittante, G. (2008). Effects of Composite β- and κ-Casein Genotypes on Milk Coagulation, Quality, and Yield Traits in Italian Holstein Cows. *Journal of Dairy Science, 91*, 4022-4027.

Conti, A., Napolitano, L., Cantisani, A. M., Davoli, R., & Dall'Olio, S. (1988). Bovine beta-lactoglobulin H: isolation by preparative isoelectric focusing in immobilized pH gradients and preliminary characterization. *Journal of Biochemical and Biophysical Methods, 16*, 205-214.

Corfield, A. (2017). Eukaryotic protein glycosylation: a primer for histochemists and cell biologists. *Histochemistry and Cell Biology, 147*, 119-147.

Daniloski, D., Cunha, N. M., McCarthy, N. A., O'Callaghan, T. F., McParland, S., & Vasiljevic, T. (2021). Health-related outcomes of genetic polymorphism of bovine β-casein variants: A systematic review of randomised controlled trials. *Trends in Food Science & Technology*.

Daniloski, D., McCarthy, N. A., Gazi, I., & Vasiljevic, T. (2022). Rheological and structural properties of acid-induced milk gels as a function of β-casein phenotype. *Food Hydrocolloids, 131*, 107846.

Daniloski, D., McCarthy, N. A., & Vasiljevic, T. (2022). Impact of heating on the properties of A1/A1, A1/A2, and A2/A2 β-casein milk phenotypes. *Food Hydrocolloids, 128*, 107604.

Daniloski, D., Hailu, Y., Brodkorb, A., Vasiljevic, T., & McCarthy, N. A. (2024). Impact of β-casein phenotype on the physical properties of skim milk powders and their subsequent digestion characteristics. *Food Hydrocolloids, 152*, 109918.

Darewicz, M., & Dziuba, J. (2007). Formation and stabilization of emulsion with A1, A2 and B β-casein genetic variants. *European Food Research and Technology, 226*, 147-152.

Davoli, R., Dall'Olio, S., & Russo, V. (1990). Effect of κ-casein genotype on the coagulation properties of milk. *Journal of Animal Breeding and Genetics, 107*, 458-464.

Day, L., Williams, R. P. W., Otter, D., & Augustin, M. A. (2015). Casein polymorphism heterogeneity influences casein micelle size in milk of individual cows. *Journal of Dairy Science, 98*, 3633-3644.

de Wit, J. N. (2009). Thermal behaviour of bovine β-lactoglobulin at temperatures up to 150°C. a review. *Trends in Food Science & Technology, 20*, 27-34.

Deeth, H. C. (2021). Heat-induced inactivation of enzymes in milk and dairy products. A review. *International Dairy Journal, 121*, 105104.

Deth, R., Clarke, A., Ni, J., & Trivedi, M. (2015). Clinical evaluation of glutathione concentrations after consumption of milk containing different subtypes of β-casein: results from a randomized, cross-over clinical trial. *Nutrition Journal, 15,* 1-6.

Di Stasio, L., & Merlin, P. (1978). A new k-casein variant in cattle. *International Society for Animal Blood Group Research, 97,* 273-278.

Dong, C., & Ng-Kwai-Hang, K. F. (1998). Characterization of a Non-electrophoretic Genetic Variant of β-Casein by Peptide Mapping and Mass Spectrometric Analysis. *International Dairy Journal, 8,* 967-972.

Dziuba, J., & Minkiewicz, P. (1996). Influence of glycosylation on micelle-stabilizing ability and biological properties of C-terminal fragments of cow's κ-casein. *International Dairy Journal, 6,* 1017-1044.

Ebner, K. E., & Brodbeck, U. (1968). Biological Role of α-Lactalbumin: A Review. *Journal of Dairy Science, 51,* 317-322.

Eigel, W. N., Butler, J. E., Ernstrom, C. A., Farrell, H. M., Harwalkar, V. R., Jenness, R., & Whitney, R. M. (1984). Nomenclature of Proteins of Cow's Milk: Fifth Revision. *Journal of Dairy Science, 67,* 1599-1631.

Elliott, R. B. (1992). Epidemiology of diabetes in polynesia and New Zeland: child health research unit. *Pediatric and Adolescent Endocrinology, 21,* 66-71.

Emery, P. W. (2013). Amino acids: Metabolism. In B. Caballero (Ed.), *Encyclopedia of Human Nutrition* (Third Edition): Academic Press.

Erhardt, G. (1989). K-Kaseine in Rindermilch—Nachweis eines weiteren Allels (k-CnE) in verschiedenen Rassen. *Journal of Animal Breeding and Genetics, 106,* 225-231.

Erhardt, G. (1993). A new αS1-casein allele in bovine milk and its occurrence in different breeds. *Animal Genetics, 24,* 65-66.

Euston, S., L. Hirst, R., & P. Hill, J. (1999). The emulsifying properties of β-lactoglobulin genetic variants A, B and C. *Colloids and Surfaces B: Biointerfaces, 12,* 193-202.

Fan, X., Zhang, Z., Qiu, L., Zhang, Y., & Miao, Y. (2019). Polymorphisms of the kappa casein (CSN 3) gene and inference of its variants in water buffalo (Bubalus bubalis). *Archives Animal Breeding, 62,* 585-596.

Fang, Z.-H., Visker, M., Miranda, G., Delacroix-Buchet, A., Bovenhuis, H., & Martin, P. (2016). The relationships among bovine αS-casein phosphorylation isoforms suggest different phosphorylation pathways. *Journal of Dairy Science, 99,* 8168-8177.

Farrell Jr, H., Jimenez-Flores, R., Bleck, G., Brown, E., Butler, J., Creamer, L., Hicks, C., Hollar, C., Ng-Kwai-Hang, K., & Swaisgood, H. (2004). Nomenclature of the proteins of cows' milk: Sixth revision. *Journal of Dairy Science, 87,* 1641-1674.

Formaggioni, P., Summer, A., Malacarne, M., & Mariani, P. (1999). Milk protein polymorphism: Detection and diffusion of the genetic variants in Bos genus. *Annali della Facolta di Medicina Veterinaria, Univerista di Parma, 19*, 127-165.

Fox, P. F., McSweeney, P. L., & Paul, L. (1998). *Dairy chemistry and biochemistry* (First Edition): Springer

Fox, P. F., & Brodkorb, A. (2008). The casein micelle: Historical aspects, current concepts and significance. *International Dairy Journal, 18*, 677-684.

Fox, P. F., Uniacke-Lowe, T., McSweeney, P. L. H., & O'Mahony, J. A. (2015). *Dairy Chemistry and Biochemistry* (Second Edition): Springer.

Fox, P. F., Guinee, T. P., Cogan, T. M., & McSweeney, P. L. (2017). *Fundamentals of cheese science* (Second Edition): Springer

Fuquay, J. W., McSweeney, P. L., & Fox, P. F. (2011). *Encyclopedia of Dairy Sciences* (Second Edition): Academic Press.

Gai, N., Uniacke-Lowe, T., O'Regan, J., Faulkner, H., & Kelly, A. L. (2021). Effect of Protein Genotypes on Physicochemical Properties and Protein Functionality of Bovine Milk: A Review. *Foods, 10*, 2409.

Glantz, M., Devold, T., Vegarud, G., Månsson, H. L., Stålhammar, H., & Paulsson, M. (2010). Importance of casein micelle size and milk composition for milk gelation. *Journal of Dairy Science, 93*, 1444-1451.

Glantz, M., Lindmark Månsson, H., Stålhammar, H., & Paulsson, M. (2011). Effect of polymorphisms in the leptin, leptin receptor, and acyl-coenzyme A:diacylglycerol acyltransferase 1 (DGAT1) genes and genetic polymorphism of milk proteins on cheese characteristics. *Journal of Dairy Science, 94*, 3295-3304.

Godovac-Zimmermann, J., Krause, I., Baranyi, M., Fischer-Frühholz, S., Juszczak, J., Erhardt, G., Buchberger, J., & Klostermeyer, H. (1996). Isolation and rapid sequence characterization of two novel bovine β-lactoglobulins I and J. *Journal of Protein Chemistry, 15*, 743-750.

Godovac Zimmermann, J., Krause, I., Buchberger, J., Weiss, G., & Klostermeyer, H. (1990). Genetic Variants of Bovine β-Lactoglobulin. A Novel Wild-type β-Lactoglobulin W and its Primary Sequence. *Biological Chemistry Hoppe-Seyler, 371*, 255-260.

Goulding, D. A., Fox, P. F., & O'Mahony, J. A. (2020). Chapter 2 - Milk proteins: An overview. In *Milk Proteins* (Third Edition): Academic Press.

Gregersen, V. R., & Lucey, J. A. (2016). Cheese: Rennet-Induced Coagulation of Milk. In *Reference Module in Food Science* (First Edition) (pp. 1-8): Elsevier.

Grosclancle, F., Pujolle, J., Garnier, J., & Ribadeau-Dumas, B. (1966). Evidence for Two Additional Variants in Proteins of Cow's Milk: α S1-Casein D and β-Lactoglobulin, D. *Analytical Biochemistry, 6*, 215.

Grosclaude, F., Marie-Françoise, M., Mercier, J., Bonnemaire, J., & Teissier, J. (1976). polymorphisme des lactoprotéines de bovinés népalais i.-mise en evidence, chez le yak, et caractérisation biochimique de deux nouveaux variants: βlactoglobuline dyak et caséine αs1e. In *Annales de génétique et de sélection animale* (First Edition) (pp. 461-479): EDP Sciences.

Grosclaude, F., Joudrier, P., & Mahé, M.-F. (1979). A genetic and biochemical analysis of a polymorphism of bovine αs2-casein. *Journal of Dairy Research, 46*, 211-213.

Gustavsson, F., Buitenhuis, A., Johansson, M., Bertelsen, H., Glantz, M., Poulsen, N., Månsson, H. L., Stålhammar, H., Larsen, L., & Bendixen, C. (2014). Effects of breed and casein genetic variants on protein profile in milk from Swedish Red, Danish Holstein, and Danish Jersey cows. *Journal of Dairy Science, 97*, 3866-3877.

Hallén, E., Allmere, T., Näslund, J., Andrén, A., & Lundén, A. (2007). Effect of genetic polymorphism of milk proteins on rheology of chymosin-induced milk gels. *International Dairy Journal, 17*, 791-799.

Han, S. K., Shin, Y. C., & Byun, H. D. (2000). Biochemical, molecular and physiological characterization of a new β-casein variant detected in Korean Cattle. *Animal Genetics, 31*, 49-51.

Heck, J., Olieman, C., Schennink, A., Van Valenberg, H., Visker, M., Meuldijk, R., & Van Hooijdonk, A. (2008). Estimation of variation in concentration, phosphorylation and genetic polymorphism of milk proteins using capillary zone electrophoresis. *International Dairy Journal, 18*, 548-555.

Heck, J., Schennink, A., Van Valenberg, H., Bovenhuis, H., Visker, M., Van Arendonk, J., & Van Hooijdonk, A. (2009). Effects of milk protein variants on the protein composition of bovine milk. *Journal of Dairy Science, 92*, 1192-1202.

Hewa Nadugala, B., Pagel, C. N., Raynes, J. K., Ranadheera, C. S., & Logan, A. (2022). The effect of casein genetic variants, glycosylation and phosphorylation on bovine milk protein structure, technological properties, nutrition and product manufacture. *International Dairy Journal, 133*, 105440.

Hohmann, L. G., Yin, T., Schweizer, H., Giambra, I. J., König, S., & Scholz, A. M. (2020). Comparative effects of milk containing A1 versus A2 β-casein on health, growth and β-casomorphin-7 level in plasma of neonatal dairy calves. *Animals, 11*, 55.

Horne, D. S., & Lucey, J. A. (2017). Rennet-induced coagulation of milk. *Cheese*, 115-143.

Hounsell, E. F., Davies, M. J., & Renouf, D. V. (1996). O-linked protein glycosylation structure and function. *Glycoconjugate journal, 13*, 19-26.

Huppertz, T. (2016). Heat stability of milk. In *Advanced Dairy Chemistry* (Fourth Edition) (pp. 179-196): Springer.

Huppertz, T., & Chia, L. W. (2021). Milk protein coagulation under gastric conditions: A review. *International Dairy Journal, 113*, 104882.

Imafidon, G. I., Ng-Kwai-Hang, K. F., Harwalkar, V. R., & Ma, C. Y. (1991). Effect of Genetic Polymorphism on the Thermal Stability of β-Lactoglobulin and κ-Casein Mixture. *Journal of Dairy Science, 74*, 1791-1802.

Ipsen, R., & Otte, J. (2004). The relation between protein structure, interfacial rheology and foam formation for various milk proteins. *Annual Transactions of the Nordic Rheology Society, 21*, 143-178.

Jann, O., Ceriotti, G., Caroli, A., & Erhardt, G. (2002). A new variant in exon VII of bovine β-casein gene (CSN2) and its distribution among European cattle breeds. *Journal of Animal Breeding and Genetics, 119*, 65-68.

Jann, O. C., Ibeagha-Awemu, E. M., Özbeyaz, C., Zaragoza, P., Williams, J. L., Ajmone-Marsan, P., Lenstra, J. A., Moazami-Goudarzi, K., & Erhardt, G. (2004). Geographic distribution of haplotype diversity at the bovine casein locus. *Genetics Selection Evolution, 36*, 1-15.

Jensen, H. B., Holland, J. W., Poulsen, N. A., & Larsen, L. B. (2012). Milk protein genetic variants and isoforms identified in bovine milk representing extremes in coagulation properties. *Journal of Dairy Science, 95*, 2891-2903.

Jensen, H. B., Poulsen, N. A., Andersen, K. K., Hammershøj, M., Poulsen, H. D., & Larsen, L. B. (2012). Distinct composition of bovine milk from Jersey and Holstein-Friesian cows with good, poor, or noncoagulation properties as reflected in protein genetic variants and isoforms. *Journal of Dairy Science, 95*, 6905-6917.

Jiménez-Montenegro, L., Alfonso, L., Mendizabal, J. A., & Urrutia, O. (2022). Worldwide research trends on milk containing only A2 β-casein: A bibliometric study. *Animals, 12*, 1909.

Jinsmaa, Y., & Yoshikawa, M. (1999). Enzymatic release of neocasomorphin and β-casomorphin from bovine β-casein. *Peptides, 20*, 957-962.

Jõudu, I., Henno, M., & Värv, S. (2007). Milk protein genotypes and milk coagulation properties of Estonian native cattle. *Agricultural and Food Science, 16*, 222-231.

Jõudu, I., Henno, M., Kaart, T., Püssa, T., & Kärt, O. (2008). The effect of milk protein contents on the rennet coagulation properties of milk from individual dairy cows. *International Dairy Journal, 18*, 964-967.

Kaura, R., Kaur, S., Arora, J., & Kashyap, N. (2021). Screening and Association of Polymorphism in Alpha-Lactalbumin Gene in Holstein Friesian Crossbred Cattle. *Journal of Animal Research, 11*, 351-355.

Kelly, A. L., & McSweeney, P. L. H. (2003). Indigenous Proteinases in Milk. In *Advanced Dairy Chemistry* (Third Edition) (pp. 495-521): Springer US.

Keppler, J. K., Sönnichsen, F. D., Lorenzen, P.-C., & Schwarz, K. (2014). Differences in heat stability and ligand binding among β-lactoglobulin genetic variants A, B and C using 1H NMR and fluorescence quenching. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1844*, 1083-1093.

Ketto, I. A., Knutsen, T. M., Øyaas, J., Heringstad, B., Ådnøy, T., Devold, T. G., & Skeie, S. B. (2017). Effects of milk protein polymorphism and composition, casein micelle size and salt distribution on the milk coagulation properties in Norwegian Red cattle. *International Dairy Journal, 70*, 55-64.

Ketto, I. A., Abdelghani, A., Johansen, A.-G., Øyaas, J., & Skeie, S. B. (2019). Effect of milk protein genetic polymorphisms on rennet and acid coagulation properties after standardisation of protein content. *International Dairy Journal, 88*, 18-24.

Kolenda, M., & Sitkowska, B. (2021). The Polymorphism in Various Milk Protein Genes in Polish Holstein-Friesian Dairy Cattle. *Animals, 11*, 389.

Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal, 16*, 945-960.

Korhonen, H. (2009). Milk-derived bioactive peptides: From science to applications. *Journal of Functional Foods, 1*, 177-187.

Kroeker, E. M., Ng-Kwai-Hang, K. F., Hayes, J. F., & Moxley, J. E. (1985). Effects of Environmental Factors and Milk Protein Polymorphism on Composition of Casein Fraction in Bovine Milk. *Journal of Dairy Science, 68*, 1752-1757.

Kübarsepp, I., Henno, M., Kärt, O., & Tupasela, T. (2005). A comparison of the methods for determination of the rennet coagulation properties of milk. *Acta Agriculturae Scand Section A, 55*, 145-148.

Lam, R. S. H., & Nickerson, M. T. (2013). Food proteins: A review on their emulsifying properties using a structure–function approach. *Food Chemistry, 141*, 975-984.

Larsen, B., & Thymann, M. (1966). Studies on Milk Protein Polymorphism in Danish Cattle and the Interaction of the Controlling Genes. *Acta Veterinaria Scandinavica, 7*, 189-205.

Li, C. H. (1946). Electrophoretic Inhomogeneity of Crystalline Beta-lactoglobulin. *Journal of the American Chemical Society, 68*, 2746-2747.

Li, S., Ye, A., & Singh, H. (2020). Effect of seasonal variations on the acid gelation of milk. *Journal of Dairy Science, 103*, 4965-4974.

Lisson, M., Lochnit, G., & Erhardt, G. (2013). Genetic variants of bovine β- and κ-casein result in different immunoglobulin E-binding epitopes after in vitro gastrointestinal digestion. *Journal of Dairy Science, 96*, 5532-5543.

Lisson, M., Novak, N., & Erhardt, G. (2014). Immunoglobulin E epitope mapping by microarray immunoassay reveals differences in immune response to genetic variants of caseins from different ruminant species. *Journal of Dairy Science, 97*, 1939-1954.

Litwińczuk, Z., Król, J., Brodziak, A., & Barłowska, J. (2011). Changes of protein content and its fractions in bovine milk from different breeds subject to somatic cell count. *Journal of Dairy Science, 94*, 684-691.

Lu, S. C. (2009). Regulation of glutathione synthesis. *Molecular Aspects of Medicine, 30*, 42-59.

Lukač, D., Vidović, V., Nemeš, Ž., Stupar, M., & Popović-Vranješ, A. (2013). Genotypic frequencies of the β-lactoglobulin, κ-casein and transferrin in Serbian Holstein-Friesian dairy cattle. *Mljekarstvo: časopis za unaprjeđenje proizvodnje i prerade mlijeka, 63*, 203-210.

Mackle, T., Bryant, A., Petch, S., Hooper, R., & Auldist, M. (1999). Variation in the composition of milk protein from pasture-fed dairy cows in late lactation and the effect of grain and silage supplementation. *New Zealand Journal of Agricultural Research, 42*, 147-154.

Mahé, M.-F., Miranda, G., Queval, R., Bado, A., Zafindrajaona, P. S., & Grosclaude, F. (1999). Genetic polymorphism of milk proteins in African Bos taurus and Bos indicus populations. Characterization of variants αs1-Cn H and κ-Cn J. *Genetics Selection Evolution, 31*, 239-253.

Makamu, T. C., & Tyasi, T. L. (2024). Systematic Review of Single Nucleotide Polymorphisms of Cattle Beta-Lactoglobulin Gene and their Association with Milk Production Traits. *American Journal of Animal anf Veterinary Sciences.*

Malik, Z., Bottomley, R., & Austen, B. (1988). Allergenic Properties of the Genetic Variants A and B of Bovine Beta-Lactoglobulin. *International Archives of Allergy and Immunology, 86*, 245-248.

Manderson, G., Hardman, M., & Creamer, L. (1998). Effect of heat treatment on the conformation and aggregation of β-lactoglobulin A, B, and C. *Journal of Agricultural and Food Chemistry, 46*, 5052-5061.

Mariani, P., Summer, A., Anghinetti, A., Senese, C., Di Gregorio, P., Rando, P., & Serventi, P. (1995). Effects of the αsl-CN G allele on the percentage distribution of caseins αs1-, αS2-, β-, and κ-in Italian Brown cows. *Latte, 31*, 3-13.

Marko, R., Uros, G., Branislav, V., Milan, M., Danijela, K., Vlado, T., & Zoran, S. (2020). Beta-casein gene polymorphism in Serbian Holstein-Friesian cows and its relationship with milk production traits. *Acta Veterinaria, 70*, 497-510.

Marks, F. (2008). *Protein Phosphorylation* (First Edition): John Wiley & Sons.

Martin, P., Szymanowska, M., Zwierzchowski, L., & Leroux, C. (2002). The impact of genetic polymorphisms on the protein composition of ruminant milks. *Reproduction Nutrition Development, 42*, 433-459.

Martin, P., Bianchi, L., Cebo, C., & Miranda, G. (2013). Genetic Polymorphism of Milk Proteins. In *Advanced Dairy Chemistry* (Fourth Edition) (pp. 463-514): Springer US.

Marziali, A., & Ng-Kwai-Hang, K. (1986). Effects of milk composition and genetic polymorphism on coagulation properties of milk. *Journal of Dairy Science, 69*, 1793-1798.

Massella, E., Piva, S., Giacometti, F., Liuzzo, G., Zambrini, A. V., & Serraino, A. (2017). Evaluation of bovine beta casein polymorphism in two dairy farms located in northern Italy. *Italian Journal of Food Safety, 6*, 6904-6904.

Matějíček, A., Matějíčková, J., Štípková, M., Hanuš, O., Genčurová, V., Kyseľová, J., Němcová, E., Kott, T., Šefrová, J., & Krejčová, M. (2008). Joint effects of CSN3 and LGB genes on milk quality and coagulation properties in Czech Fleckvieh. *Czech Journal of Animal Science, 53*, 246-252.

McLean, D. M., Graham, E. R. B., Ponzoni, R. W., & McKenzie, H. A. (1987). Effects of milk protein genetic variants and composition on heat stability of milk. *Journal of Dairy Research, 54*, 219-235.

McSweeney, P. L. (2004). Biochemistry of cheese ripening. *International Journal of Dairy Technology, 57*, 127-144.

Meza-Nieto, M. A., Vallejo-Cordoba, B., González-Córdova, A. F., Félix, L., & Goycoolea, F. M. (2007). Effect of β-Lactoglobulin A and B Whey Protein Variants on the Rennet-Induced Gelation of Skim Milk Gels in a Model Reconstituted Skim Milk System. *Journal of Dairy Science, 90*, 582-593.

Meza-Nieto, M. A., González-Córdova, A. F., Piloni-Martini, J., & Vallejo-Cordoba, B. (2013). Effect of β-lactoglobulin A and B whey protein variants on cheese yield potential of a model milk system. *Journal of Dairy Science, 96*, 6777-6781.

Mohanty, D. P., Mohapatra, S., Misra, S., & Sahu, P. S. (2016). Milk derived bioactive peptides and their impact on human health – A review. *Saudi Journal of Biological Sciences, 23*, 577-583.

Molina, L., Kramm, J., Brito, C., Carrillo, B., Pinto, M., & Ferrando, A. (2006). Protein composition of milk from Holstein–Friesian dairy cows and its relationship with the genetic variants A and B of κ-casein and β-lactoglobulin (Part I). *International Journal of Dairy Technology, 59*, 183-187.

Monaci, L., Tregoat, V., van Hengel, A. J., & Anklam, E. (2006). Milk allergens, their characteristics and their detection in food: A review. *European Food Research and Technology, 223*, 149-179.

Moremen, K. W., Tiemeyer, M., & Nairn, A. V. (2012). Vertebrate protein glycosylation: diversity, synthesis and function. *Nature reviews Molecular cell biology, 13*, 448-462.

Nair, I. C., & Jayachandran, K. (2019). Aspartic Proteases in Food Industry. In *Green Bio-processes: Enzymes in Industrial Food Processing* (First Edition) (pp. 15-30): Springer Singapore.

Neelin, J. M. (1964). Variants of κ-Casein Revealed by Improved Starch Gel Electrophoresis1. *Journal of Dairy Science, 47*, 506-509.

Nilsson, K., Buhelt Johansen, L., de Koning, D. J., Duchemin, S. I., Stenholdt Hansen, M., Stålhammar, H., Lindmark-Månsson, H., Paulsson, M., Fikse, W. F., & Glantz, M. (2020). Effects of milk proteins and posttranslational modifications on noncoagulating milk from Swedish Red dairy cattle. *Journal of Dairy Science, 103*, 6858-6868.

Noctor, G., Queval, G., Mhamdi, A., Chaouch, S., & Foyer, C. H. (2011). Glutathione. *The Arabidopsis Book American Society of Plant Biologists, 9*.

Noni, I. D. (2008). Release of β-casomorphins 5 and 7 during simulated gastro-intestinal digestion of bovine β-casein variants and milk-based infant formulas. *Food Chemistry, 110*, 897-903.

O'Riordan, N., Kane, M., Joshi, L., & Hickey, R. M. (2014). Structural and functional characteristics of bovine milk protein glycosylation. *Glycobiology, 24*, 220-236.

O'Connor, C. M., Adams, J. U., & Fairman, J. (2010). Essentials of cell biology. *Cambridge, MA: NPG Education, 1*, 54.

O'Mahony, J., & Fox, P. (2014). Milk: an overview. In *Milk Proteins* (Second Edition) (pp. 19-73): Academic Press.

Oliveira, K. M. G., Valente-Mesquita, V. L., Botelho, M. M., Sawyer, L., Ferreira, S. T., & Polikarpov, I. (2001). Crystal structures of bovine β-lactoglobulin in the orthorhombic space group C2221. *European Journal of Biochemistry, 268*, 477-484.

Olsen, K., Orlien, V., & Skibsted, L. H. (2022). Pressure denaturation of β-lactoglobulin: Volume changes for genetic A and B variants. *International Dairy Journal, 133*, 105416.

Parvathy, S. T., Udayasuriyan, V., & Bhadana, V. (2022). Codon usage bias. *Molecular Biology Reports, 49*, 539-565.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry, 26*, 716-723.

Peterson, R. F., & Kopfler, F. C. (1966). Detection of new types of β-casein by polyacrylamide gel electrophoresis at acid pH: A proposed nomenclature. *Biochemical and Biophysical Research Communications, 22*, 388-392.

Petrovska, S., Jonkus, D., Zagorska, J., & Ciprovica, I. (2017). The influence of k-casein genotype on the coagulation properties of milk collected from the local Latvian cow breeds. *Agronomy Research, 15*, 1411-1419.

Polis, B. D., Schmukler, H. W., Custer, J. H., & McMeekin, T. L. (1950). Isolation of an Electrophoretically Homogeneous Crystalline Component of β-Lactoglobulin2. *Journal of the American Chemical Society, 72*, 4965-4968.

Poulsen, N. A., Bertelsen, H. P., Jensen, H. B., Gustavsson, F., Glantz, M., Lindmark Månsson, H., Andrén, A., Paulsson, M., Bendixen, C., Buitenhuis, A. J., & Larsen, L. B. (2013). The occurrence of noncoagulating milk and the association of bovine milk coagulation properties with genetic variants of the caseins in 3 Scandinavian dairy breeds. *Journal of Dairy Science, 96*, 4830-4842.

Prinzenberg, E. M., Hiendleder, S., Ikonen, T., & Erhardt, G. (1996). Molecular genetic characterization of new bovine kappa-casein alleles CSN3F and CSN3G and genotyping by PCR-RFLP. *Animal Genetics, 27*, 347-349.

Prinzenberg, E. M., Krause, I., & Erhardt, G. (1999). SSCP analysis at the bovine CSN3 locus discriminates six alleles corresponding to known protein variants (A, B, C, E, F, G) and three new DNA polymorphisms (H, I, A1). *Animal Biotechnology, 10*, 49-62.

Priyadarshini, P., Mishra, C., Mishra, B., Swain, K., Rout, M., & Mishra, S. P. (2018). Impact of milk protein on human health: A1 verses A2. *International Journal of Chemical Studies, 6*, 531-535.

Robitaille, G. (1995). Influence of κ-casein and β-lactoglobulin genetic variants on the heat stability of milk. *Journal of Dairy Research, 62*, 593-600.

Sanchez, M. P., Fritz, S., Patry, C., Delacroix-Buchet, A., & Boichard, D. (2020). Frequencies of milk protein variants and haplotypes estimated from genotypes of more than 1 million bulls and cows of 12 French cattle breeds. *Journal of Dairy Science, 103*, 9124-9141.

Sawyer, W. H. (1968). Heat Denaturation of Bovine β-Lactoglobulins and Relevance of Disulfide Aggregation. *Journal of Dairy Science, 51*, 323-329.

Schmidt, D. G., Both, P., & de Koning, P. J. (1966). Fractionation and Some Properties of κ-Casein Variants. *Journal of Dairy Science, 49*, 776-782.

Schwarz, F., & Aebi, M. (2011). Mechanisms and principles of N-linked protein glycosylation. *Current Opinion in Structural Biology, 21*, 576-582.

Sebastiani, C., Arcangeli, C., Ciullo, M., Torricelli, M., Cinti, G., Fisichella, S., & Biagetti, M. (2020). Frequencies Evaluation of β-Casein Gene Polymorphisms in Dairy Cows Reared in Central Italy. *Animals, 10*, 252.

Seibert, B., Erhardt, G., & Senft, B. (1987). Detection of a new K-casein variant in cow's milk. *Animal Genetics, 18*, 269-272.

Senocq, D., Mollé, D., Pochet, S., Leonil, J., Dupont, D., & Levieux, D. (2002). A new bovine beta-casein genetic variant characterized by a Met93 --> Leu93 substitution in the sequence A2. *Le Lait, 82*, 171-180.

Singh, H., & Fox, P. F. (1987). Heat stability of milk: role of β-lactoglobulin in the pH-dependent dissociation of micellar κ-casein. *Journal of Dairy Research, 54*, 509-521.

Singh, H. (2004). Heat stability of milk. *International Journal of Dairy Technology, 57*, 111-119.

Steen, P. V. d., Rudd, P. M., Dwek, R. A., & Opdenakker, G. (1998). Concepts and principles of O-linked glycosylation. *Critical reviews in biochemistry and molecular biology, 33*, 151-208.

St-Gelais, D., & Haché, S. (2005). Effect of β-casein concentration in cheese milk on rennet coagulation properties, cheese composition and cheese ripening. *Food Research International, 38*, 523-531.

Sulimova, G., Sokolova, S., Semikozova, O., Nguet, L., & Berberov, E. (1992). Analysis of DNA polymorphism of cluster genes in cattle: casein genes and major histocompatibility complex (MHC) genes. *Tsitologiia i Genetika, 26*, 18-26.

Sulimova, G. E., Badagueva Iu, N., & Udina, I. G. (1996). Polymorphism of the kappa-casein gene in populations of the subfamily Bovinae. *Genetika, 32*, 1576-1582.

Sumaiya, R., Hossain, M., Hossain, M. M., Alim, M., Nusrat, J., Islam, M., Md, S., Ara, B. A., & Jahangir, A. (2020). Genotyping of cattle based on kappa-casein and alpha-lactalbumin genes. *Indian Journal of Biotechnology, 19*, 211-218.

Sun, Z., Zhang, Z., Wang, X., Cade, R., Elmir, Z., & Fregly, M. (2003). Relation of β-casomorphin to apnea in sudden infant death syndrome. *Peptides, 24*, 937-943.

Swinburn, B. (2004). Beta casein A1 and A2 in milk and human health. *Report to New Zealand Food Safety Authority*, 1-43.

Thesbjerg, M. N., Poulsen, K. O., Astono, J., Poulsen, N. A., Larsen, L. B., Nielsen, S. D.-H., Stensballe, A., & Sundekilde, U. K. (2024). O-linked glycosylations in human milk casein and major whey proteins during lactation. *International Journal of Biological Macromolecules, 267*, 131613

Thompson, M., Kiddy, C., Pepper, L., & Zittle, C. (1962). Variations in the αs-casein fraction of individual cow's milk. *Nature, 195*, 1001-1002.

Thompson, M. P., Kiddy, C. A., Johnston, J. O., & Weinberg, R. M. (1964). Genetic Polymorphism in Caseins of Cows' Milk. II. Confirmation of the Genetic Control of β-Casein Variation. *Journal of Dairy Science, 47*, 378-381.

Thompson, M. P., Tarassuk, N. P., Jenness, R., Lillevik, H. A., Ashworth, U. S., & Rose, D. (1965). Nomenclature of the Proteins of Cow's Milk—Second Revision: Report of the

Committee on Milk Protein Nomenclature, Classification, and Methodology of the Manufacturing Section of ADSA for 1963-64. *Journal of Dairy Science, 48*, 159-169.

Truswell, A. (2005). The A2 milk case: a critical review. *European Journal of Clinical Nutrition, 59*, 623-631.

Ubersax, J. A., & Ferrell Jr, J. E. (2007). Mechanisms of specificity in protein phosphorylation. *Nature reviews Molecular Cell Biology, 8*, 530-541.

Umbach, M., Teschemacher, H., Praetorius, K., Hirschhäuser, R., & Bostedt, H. (1985). Demonstration of a β-casomorphin immunoreactive material in the plasma of newborn calves after milk intake. *Regulatory Peptides, 12*, 223-230.

Venturini, G., Cardoso, D., Baldi, F., Freitas, A., Aspilcueta-Borquis, R., Santos, D., Camargo, G., Stafuzza, N., Albuquerque, L., & Tonhati, H. (2014). Association between single-nucleotide polymorphisms and milk production traits in buffalo. *Genetics and Molecular Research, 13*, 10256-10268.

Visker, M. H. P. W., Heck, J. M. L., van Valenberg, H. J. F., van Arendonk, J. A. M., & Bovenhuis, H. (2012). Short communication: A new bovine milk-protein variant: α-Lactalbumin variant D. *Journal of Dairy Science, 95*, 2165-2169.

Visser, S., Slangen, C. J., & Rollema, H. S. (1991). Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chromatography. *Journal of Chromatography A, 548*, 361-370.

Visser, S., Slangen, C. J., Lagerwerf, F. M., Van Dongen, W. D., & Haverkamp, J. (1995). Identification of a new genetic variant of bovine ß-casein using reversed-phase high-performance liquid chromatography and mass spectrometric analysis. *Journal of Chromatography A, 711*, 141-150.

Voglino, G. (1972). A new β-casein variant in Piedmont cattle. *Animal Blood Groups and Biochemical Genetics, 3*, 61-62.

Waugh, D. F., Ludwig, M. L., Gillespie, J. M., Melton, B., Foley, M., & Kleiner, E. S. (1962). The αs-Caseins of Bovine Milk. *Journal of the American Chemical Society, 84*, 4929-4938.

Wedholm, A., Larsen, L. B., Lindmark-Månsson, H., Karlsson, A. H., & Andrén, A. (2006). Effect of Protein Composition on the Cheese-Making Properties of Milk from Individual Dairy Cows. *Journal of Dairy Science, 89*, 3296-3305.

Wolanciuk, A. (2015). The association of genetic variants of β-lactoglobulin and κ-casein with yield and chemical composition of milk obtained from four breeds of cow. *Scientific Annals of Polish Society of Animal Production, 1*, 21-32.

Wong, N. P. (2012). *Fundamentals of dairy chemistry*: Springer Science & Business Media.

Woodford, K. (2011). Milk proteins and human health: A1 versus A2 Beta-casein. *General Practitioners Conference, 1*, 1-6.

Woychik, J. H. (1965). Phenotyping of k-caseins. *Journal of Dairy Science, 48*, 496-497.

Zayas, J. F. (1997). Emulsifying properties of proteins. In *Functionality of proteins in food* (First Edition) (pp. 134-227): Springer.

Zepeda-Batista, J. L., Alarcón-Zúñiga, B., Ruíz-Flores, A., Núñez-Domínguez, R., & Ramírez-Valverde, R. (2015). Polymorphism of three milk protein genes in Mexican Jersey cattle. *Electronic Journal of Biotechnology, 18*, 1-4.

Zhang, X., Fu, X., Zhang, H., Liu, C., Jiao, W., & Chang, Z. (2005). Chaperone-like activity of β-casein. *The International Journal of Biochemistry & Cell Biology, 37*, 1232-1240.

Zikakis, J. P., Haenlein, G. F. W., Hines, H. C., Mather, R. E., & Tung, S. (1974). Gene Frequencies of Electrophoretically Determined Polymorphisms in Guernsey Blood and Milk1, 2. *Journal of Dairy Science, 57*, 405-410.

Chapter 2

Factors influencing the variation in macro chemical composition and protein profile in milk among individual cows

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Declaration

This chapter was written by author LMK and reviewed by his co-authors. JBM assisted in milk collection. BH assisted in organising the cows for milk collection.

Abstract

Many factors, such as health, breed, and diet can influence the macro-composition of milk. Among 48 individual cows, of which 24 were Holstein-Friesian and 24 were Jersey-Friesian cross breeds, the macro-composition and protein fractions of their milk was analysed. Almost all cows exhibited a linear relationship between milk yield and milk dry matter content. Cows which did not follow this trend, likely experienced poor udder health in which somatic cell count exceeded 400,000 cells/mL. The HF breed produced more kgs of protein per day than the JFX breed. Among the 48 cows analysed, 17 genetic variants were identified among the milk proteins β-casein, κ-casein and β-lactoglobulin. Milk containing the A2I variant of β-casein was associated with the highest total protein production among the β-casein variants. No significant ($P > 0.05$) difference in total protein production was observed relative to the κ-casein or β-lactoglobulin variants. However, milk with the A variant of β-lactoglobulin had significantly ($P < 0.05$) more total whey proteins than any other β-lactoglobulin variant. The findings in this study highlight the variability in protein profile even in a group of cows representing a relatively small genetic pool. Selecting for specific protein genetic variants, which have been associated with higher protein yields or other unique functional or nutritional qualities, could increase the profitability of milk for Irish dairy producers.

2.1 Introduction

Milk is produced by mammals postpartum and contains the macronutrients required for the growth and development of their offspring (Boro et al., 2018). Milk is synthesised in the mammary epithelial cells by absorbing nutrients from the blood, which are then used to produce many of the components in milk (Kim & Wu, 2009) such as protein, fat and lactose which are present at concentrations of approximately 3.5, 3.7, and 4.8%, respectively (Fox, Uniacke-Lowe, McSweeney and O'Mahony, 2015). The composition of milk can be altered depending on a number of intrinsic and extrinsic factors affecting the cow (Phelan et al., 1982). Such factors include health, age, parity, breed and diet (Alothman et al., 2019; Heck, Van Valenberg, et al., 2009; Mele et al., 2016). However, individual genetic variation between cows often has a greater impact on milk composition than variation due to the other factors, particularly in terms of the protein profile. Therefore, it is of interest to determine the protein profile at a macro level in terms of the nitrogen fractions therein, but also as a function of the genetic variants of the proteins themselves to assess their distribution among individual cows.

The Holstein-Friesian (HF) breed has dominated dairy herds in Ireland since the 1990s due to their greater capacity for milk yield relative to other breeds and suitability to facilitate increased milk production in a market that, at the time, rewarded higher milk volume over milk solids (Horan et al., 2005). However, since 2007, the payment model for milk has transitioned to have a primary focus on milk solids, particularly protein and fat (Sneddon et al., 2013). The implementation of the economic breeding index, in which breeding choices increase profits for dairy farmers, has helped increase milk solids among HF herds throughout Ireland (O'Sullivan et al., 2019). There is also a growing interest in crossbreeding HF cows with Jersey cows, to also achieve higher milk solids (Prendiville et al., 2009). Additionally, these Jersey × Holstein-Friesian (JFX) cows are well suited to a seasonal pasture-based feeding system due to their high intake capacity at pasture (Shortall et al., 2018). However, even with the progress that has

been made as a result of implementation of economic breeding indices which increase milk solids, in both HF and JFX cows, the diversity of the components therein among individual cows, especially their protein profile, is an area that has garnered little interest at farm level. Despite this genetic variants provide opportunities for diversification of dairy products, with the evolution of A2 milk products being one such example extensively reported in both media and academic research articles (Daniloski et al., 2024; EFSA, 2009; Woodford, 2009).

Genetic variation is an important factor affecting the composition and functionality of milk. Of the six major milk proteins, which are; α_{s1}-casein, α_{s2}-casein, β-casein , κ-casein , α-lactalbumin and β-lactoglobulin (β-lg), 53 genetic variants have been identified (Bittante et al., 2012; Gai et al., 2021; Kelly et al., 2024). Genetic variation primarily affects proteins, driven by mutations in the region of DNA that codes for a specific protein (Heck, Schennink, et al., 2009a). This mutation manifests as a substitution of an amino acid within the primary structure of the protein (Heck et al., 2008). These small changes in the amino acid sequence of a protein can significantly affect the compositional and functional properties of milk, with some variants also increasing or decreasing the concentration of milk proteins (Bobe et al., 1999). Equally, some variants can alter the functional behaviour of milk, causing changes to important techno-functional properties, such as heat stability and rennet/acid coagulation properties (Daniloski, McCarthy, Gazi, et al., 2022; Marziali & Ng-Kwai-Hang, 1986b).

Due to extensive breeding programmes over the last number of decades, the genetic pool in relation to milk proteins has become diverse among Irish herds. However, few studies have reported on the extent of this genetic diversification of milk proteins; therefore, the aim of this study was to quantify the frequency of the genotypes of β-casein, κ-casein and β-lg among individual cows within an Irish herd, and highlight some of the dominating genotypes. In addition, the impact that these genotypes may have on the protein fraction of milk was also analysed, and may be of benefit to Irish dairy farmers who want to improve milk protein yield.

2.2 Materials and methods

2.2.1 Sample collection

Morning and evening milk from 48 individual pasture-based cows was collected during four periods at five-week intervals across a lactation cycle at the Teagasc Curtins research farm, Moorepark, Fermoy, Co. Cork, Ireland between April and August 2021. Cows were randomised based on age, breed, and parity and allocated into either Holstein Friesian (HF) or Jersey Friesian crossbred (JFX) groups in which each breed had a population of 24 cows. Both breeds were further divided in feeding groups assigned to grazed diets of perennial ryegrass (PRG), multi-species swards (MSS) or perennial ryegrass with white clover (PRGWC). This resulted in each test group comprising of 8 cows. Morning and evening milks were combined in equal quantities to create a milk sample, representative of a full day. The combined morning and evening milk was then stored at 4°C prior to compositional analysis.

2.2.2 Compositional analysis

The macro chemical composition of the combined morning and evening milk samples were analysed in duplicate, using a MilkoScan 7 (FOSS Analytical A/S, 69 Slangerupgade, DK-3400 Hillerod, Denmark). The somatic cell count of each milk sample was determined using a Fossomatic FC (FOSS Analytical A/S, 69 Slangerupgade, DK-3400 Hillerod, Denmark).

2.2.3 Nitrogen fractions

The methods described by the International Dairy Federation were used to determine crude protein (CP) (IDF, 2014), non-casein nitrogen (NCN) (IDF, 2004), and non-protein nitrogen (NPN) (IDF, 2016) contents. A nitrogen to protein conversion factor of 6.38 was used for all

nitrogen fractions. The NCN was determined by diluting the raw milk samples in deionised water and acidifying them to a pH of 4.6 using 1M acetic acid and 1M sodium acetate, after which the samples were then filtered through Whatman No. 1 filter paper to remove any precipitated material. Total nitrogen was measured using the Kjeldahl standard reference method on the sample filtrate, and using the nitrogen conversion factor, the NCN was determined. NPN was determined by the precipitation of the protein component of each sample using trichloroacetic acid (15% w/v). After acid addition, the mixture was filtered through Whatman No. 1 to remove precipitated material, and nitrogen was measured using the Kjeldahl method on the filtrate to determine the NPN. The CP, TP, casein, and whey protein contents were determined using the approaches and formulas described by (O'Callaghan et al., 2016), presented as follows: CP= TN × 6.38, TP = (TN – NPN) × 6.38, casein = (TN – NCN) × 6.38, whey protein = (NCN – NPN) × 6.38.

2.2.4 Protein profile analysis

The genotypes of κ-casein and β-lg were determined using the high performance liquid chromatography (HPLC) method outlined by Gaspard, and Brodkorb (2019). In brief, the raw milk samples were diluted in a buffer containing 7 M urea, 20 mM bis-tris propane, 0.19 mM dithiothreitol. The samples were filtered using a PES 0.22 μm syringe filter, before being injected onto a Poroshell 300SB-C18 (Size: 2.1×7.5 mm, 5 μm; Agilent Technologies, Ireland). Peak analysis and integration were carried out using Agilent Chemstation Software. While genotypes of β-casein could not be verified by HPLC, they were instead identified using a genomic database provided by the Irish Cattle Breeding Federation (ICBF). Genotypes of κ-casein and β-lg were verified using the same database.

2.2.5 Statistical analysis

Statistical significance between individual milk sample was determined using IBM SPSS Statistics (IBM Statistics Inc. Armonk, NY) using paired-samples t-test, with results at a *P*-value <0.05 deemed significant.

2.3 Results and Discussion

The values of each milk component displayed in Tables 2.1 and 2.2 are the averages from the four milk collection points described in Section 2.2.1. These averages are presented as kg of component (i.e., protein) per day. The compositions that were determined by either the mid infrared spectroscopy as described in Section 2.2.2, or by nitrogen fractions determination described in Section 2.2.3, were initially expressed on a percentage in milk basis, with the kg of a given component produced per day calculated based on individual cow yield.

2.3.1. Macro Chemical Composition

The daily milk yields of the 48 individual cows ranged from 13.23-27.05 kg/day (Table 2.1). The lowest value of 13.23 kg/day was recorded from a cow within the PRGWC JFX group, while the highest yield of 27.05 kg/day was recorded from a cow within the MSS HF group. In general, the HF cows produced, on average, 1.6 kg/day more milk than the JFX cows. The higher milk yield of HF compared to JFX is well reported in the literature (Prendiville et al., 2009; Vance et al., 2013). The PRG diet was associated with the highest average milk yield among individual HF cows, whereas the PRGWC diet had the lowest average yield. The MSS diet for the JFX breed was associated with the highest average milk yield, while similar to the HF breed, the PRGWC diet had the lowest average milk yield. In general, among the cows in this study, there was a linear relationship between milk yield and dry matter (milk solids as a

function of yield on a dry basis), meaning that as the milk yield increased, so too did the dry matter content (Fig 2.1). In comparing between the two breeds, the JFX cows had a more linear relationship than the HF cows, with R^2 values of 0.91 and 0.87, respectively. Of the diets, the MSS and PRGWC had the same R^2 value of 0.92 and the PRG diet was the least linear, with a R^2 value of 0.70. The highest value recorded for total dry matter produced was from a cow in the PRGWC JFX group, with a value of 3.87 kg/day and a milk yield of 25.28 kg/day. The milk from a cow within the PRGWC HF group recorded the lowest total dry matter, with a value of 2.06 kg/day and a milk yield of 14.28 kg/day. The HF cows on a MSS diet had the highest average dry matter content at 3.23 kg/day, although the PRG diet had, on average, a greater milk yield; however, the differences in dry matter content and milk yield between PRG HF and MSS HF groups were not statistically significant ($P>0.05$), with differences of 0.18 and 0.01 kg/day, respectively. Among the JFX breed, the MSS diet had both the highest average dry matter and highest milk yield.

The protein yield of the milk from the 48 individual cows ranged from 0.56-1.00 kg/day. The lower value of 0.56 kg was recorded from the milk of a cow within the PRGWC HF group, whereas the higher value of 1.00 kg/day was recorded from the milk of a cow within the PRGWC JFX group. In general, the HF breed produced more kgs of protein per day than the JFX breed. Typically, the JFX breed produces milk with higher concentrations of protein than the HF breed (Yoo et al., 2019); however, due to the larger volume of milk produced from the HF breed, the kg of protein per day was higher. Within all groups, there was a significant correlation ($P<0.001$) between milk yield and kg of protein per day. From the cows analysed, most followed a trend in which higher milk yield correlated positively with kg of protein; however, some individual cows did not follow this general trend. For example, within the PRGWC JFX group, cow 26 had a milk yield of 19.0 kg and produced 0.72 kg of protein per

day; while, cow 396 yielded 17.4 kg of milk per day and produced 0.73 kg of protein per day. It is possible that udder health is one of the reasons why some cows produce more protein per day. For example, comparison of cows 26 and 396, showed a significant ($P < 0.05$) difference in somatic cell count (SCC), with cow 26 having a SCC of 25,000 cells/mL, whereas cow 396 had an average SCC of 149,000 cells/mL. The SCC of cow 396 increased to 489,000 cells/mL during the latter collection points, as evident by the high standard deviation associated with this cow. It has been reported that a SCC greater than 400,000 cells/mL is an indication of poor udder health and can lead to reductions in milk yield (Sumon et al., 2020). Additionally, it has been reported that the increase in SCC, and the subsequent infections can cause a significant increase in the protein content of milk due to increased permeability of the milk-blood barrier, which allows more protein from the blood to enter the milk (Gonçalves et al., 2020). While this is likely the case for the difference between cows 26 and 396, there are other incidences of cows within the same group having higher kg of protein per day, despite having lower milk yields, possibly linked to variation in economic breeding index among the individual cows (O'Sullivan et al., 2019).

Among the individual cows, the kg of fat per day ranged from 0.75 to 1.44 kg/day with the lowest and highest recorded for PRG HF and PRGWC JFX groups, respectively. On average, the MSS HF group had the highest average kg of fat per day (1.18 kg/day). It is well reported that on a milk percentage basis JFX cows have a higher fat contents than HF cows (Kostovska et al., 2024; Prendiville et al., 2009; Vance et al., 2012). While both breeds on the MSS diet recorded higher average kg of fat per day than the other diets, the greater milk yield of the MSS HF group is the likely cause for the higher kg of fat per day than the MSS JFX group. The lowest average kg of fat per day was recorded for the PRGWC HF group, with an average value of 1.01 kg/day.

The lowest kg of lactose per day was recorded from cow 410 within the PRGWC JFX group, with a value of 0.65 kg/day, and is likely linked to low milk yield, with no incidences of high SCC recorded. The highest kg of lactose per day was also recorded from a cow within the PRGWC JFX group, with a value of 1.26 kg/day. The highest average kg of lactose per day was associated with the PRG HF group, whereas the lowest average was associated with PRG JFX.

Table 2.1 Macro chemical component yield of the milk from 48 individual cows, averaged across four time points.

Breed and Diet	Cow Number	Milk Yield (kg/day)	Protein (kg/day)	Fat (kg/day)	Lactose (kg/day)	Dry matter (kg/day)	SCC/mL (×1000)
	158	23.05 ± 4.8	0.85 ± 0.17	1.23 ± 0.20	1.13 ± 0.24	3.39 ± 0.63	35.5 ± 23.3
	162	25.53 ± 4.1	0.90 ± 0.15	1.25 ± 0.29	1.19 ± 0.19	3.56 ± 0.63	24.8 ± 14.8
	390	21.60 ± 1.4	0.78 ± 0.04	0.86 ± 0.13	1.02 ± 0.09	2.85 ± 0.14	32.5 ± 14.5
	397	21.08 ± 5.4	0.70 ± 0.15	0.97 ± 0.32	1.02 ± 0.25	2.86 ± 0.76	44.3 ± 26
PRG HF	553	19.38 ± 3.2	0.66 ± 0.11	0.83 ± 0.07	0.94 ± 0.17	2.57 ± 0.37	89.3 ± 125
	559	18.93 ± 2.7	0.68 ± 0.11	0.75 ± 0.18	0.89 ± 0.14	2.48 ± 0.45	93.3 ± 80.4
	7956	23.28 ± 7.8	0.79 ± 0.29	0.96 ± 0.28	1.11 ± 0.40	3.04 ± 1.02	391 ± 432
	8247	26.23 ± 5.8	0.92 ± 0.23	1.29 ± 0.38	1.22 ± 0.27	3.66 ± 0.90	412 ± 439
Mean		**22.38 ± 2.7**	**0.79 ± 0.10**	**1.01 ± 0.21**	**1.07 ± 0.12**	**3.05 ± 0.44**	**140 ± 163**
	16	18.35 ± 5.7	0.73 ± 0.25	1.23 ± 0.13	0.87 ± 0.28	2.95 ± 0.62	24.8 ± 14.9
	151	22.25 ± 4.2	0.85 ± 0.16	1.15 ± 0.14	1.02 ± 0.20	3.21 ± 0.50	27.0 ± 19.1
	195	19.60 ±2.6	0.84 ± 0.09	1.28 ± 0.21	0.92 ± 0.11	3.21 ± 0.38	24.8 ± 15.1
	405	20.60 ± 6.3	0.75 ± 0.21	1.04 ± 0.27	1.01 ± 0.29	2.96 ± 0.81	62.8 ± 58.2
PRG JFX	547	18.24 ± 5.8	0.71 ± 0.25	0.86 ± 0.31	0.90 ± 0.28	2.62 ± 0.88	42.3 ± 22.4
	569	17.78 ± 1.6	0.66 ± 0.09	1.02 ± 0.13	0.87 ± 0.07	2.69 ± 0.22	323 ± 126
	8233	21.38 ± 5.7	0.83 ± 0.21	1.23 ± 0.29	1.03 ± 0.28	3.25 ± 0.75	378 ± 429
	8424	22.98 ± 2.8	0.91 ± 0.12	1.28 ± 0.12	1.12 ± 0.14	3.47 ± 0.33	26.5 ± 15.1
Mean		**20.15 ± 2.0**	**0.79 ± 0.08**	**1.14 ± 0.15**	**0.96 ± 0.09**	**3.05 ± 0.29**	**114 ± 147**
MSS HF	18	26.90 ± 4.5	0.93 ± 0.16	1.36 ± 0.34	1.23 ± 0.20	3.77 ± 0.71	95.8 ± 125

	27	24.28 ± 4.8	0.92 ± 0.18	1.38 ± 0.27	1.10 ± 0.24	3.62 ± 0.71	353 ± 431
	153	20.48 ± 5.2	0.85 ± 0.22	1.28 ± 0.38	0.98 ± 0.27	3.28 ± 0.87	26.0 ± 20.4
	176	19.28 ± 3.7	0.70 ± 0.14	1.01 ± 0.23	0.93 ± 0.19	2.80 ± 0.57	77.5 ± 112
	430	18.95 ± 4.4	0.65 ± 0.14	0.94 ± 0.23	0.88 ± 0.23	2.64 ± 0.62	23.3 ± 18.1
	562	21.80 ± 4.0	0.75 ± 0.10	1.07 ± 0.20	1.04 ± 0.21	3.05 ± 0.51	165 ± 130
	7098	27.05 ± 5.8	0.94 ± 0.19	1.35± 0.35	1.23 ± 0.29	3.76 ± 0.87	635 ± 553
	7852	19.88 ± 3.9	0.75 ± 0.14	1.01 ± 0.17	0.94 ± 0.21	2.92 ± 0.59	32.3 ± 25.4
Mean		**22.33 ± 3.3**	**0.81 ± 0.11**	**1.18 ± 0.19**	**1.04 ± 0.14**	**3.23 ± 0.45**	**176 ± 215**
	157	21.65 ± 5.0	0.86 ± 0.20	1.21 ± 0.26	1.08 ± 0.27	3.30 ± 0.74	33.3 ± 24.4
	192	23.10 ± 3.8	0.92 ± 0.16	1.28 ± 0.10	1.07 ± 0.20	3.49 ± 0.48	35.0 ± 28.0
	411	17.40 ± 3.6	0.73 ± 0.14	1.02 ± 0.16	0.84 ± 0.17	2.73 ± 0.50	26.3 ± 9.00
	421	24.99 ± 8.9	0.98 ± 0.35	1.36 ± 0.41	1.22 ± 0.43	3.77 ± 0.39	29.0 ± 9.10
MSS JFX	546	19.40 ± 3.4	0.69 ± 0.11	0.96 ± 0.09	0.93 ± 0.18	2.74 ± 0.40	23.5 ± 14.6
	577	15.85 ± 3.8	0.61 ± 0.14	0.90 ± 0.27	0.75 ± 0.19	2.39 ± 0.62	376 ± 390
	6810	20.53 ± 4.4	0.80 ± 0.20	1.14 ± 0.18	0.96 ± 0.21	3.07 ± 0.61	322 ± 252
	8219	23.95 ± 6.2	0.83 ± 0.21	1.23 ± 0.28	1.13 ± 0.31	3.40 ± 0.80	279 ± 237
Mean		**20.86 ± 3.2**	**0.80 ± 0.12**	**1.13 ± 0.16**	**0.99 ± 0.17**	**3.11 ± 0.43**	**140 ± 155**
	391	25.15 ± 5.2	0.92 ± 0.18	1.33 ± 0.36	1.22 ± 0.29	3.66 ± 0.84	32.5 ± 25.4
	394	21.03 ± 4.1	0.82 ± 0.17	1.20 ± 0.23	1.02 ± 0.20	3.21 ± 0.63	19.3 ± 9.70
PRGWC HF	420	22.78 ± 3.9	0.87 ± 0.15	0.96 ± 0.14	1.10 ± 0.22	3.13 ± 0.52	16.0 ± 8.3
	550	18.85 ± 3.7	0.72 ± 0.15	1.12 ± 0.29	0.87 ± 0.17	2.89 ± 0.65	420 ± 198

	556	19.43 ± 3.6	0.70 ± 0.14	1.08 ± 0.29	0.91 ± 0.19	2.86 ± 0.62	25.8 ± 7.00
	8017	14.28 ± 3.5	0.56 ± 0.14	0.68 ± 0.20	0.70 ± 0.18	2.06 ± 0.53	44.3 ± 13.3
	8421	22.95 ± 4.0	0.89 ± 0.16	1.32 ± 0.25	1.04 ± 0.20	3.45 ± 0.65	21.0 ± 8.30
	8438	23.08 ± 4.2	0.79 ± 0.16	1.02 ± 0.23	1.10 ± 0.24	3.10 ± 0.65	14.8 ± 7.90
Mean		**20.94 ± 3.4**	**0.78 ± 0.12**	**1.09 ± 0.21**	**0.99 ± 0.16**	**3.05 ± 0.48**	**74 ± 140**
	26	18.98 ± 2.9	0.72 ± 0.11	0.93 ± 0.09	0.90 ± 0.17	2.71 ± 0.28	25.0 ± 11.0
	152	16.83 ± 1.9	0.66 ± 0.07	0.88 ± 0.10	0.81 ± 0.09	2.49 ± 0.27	50.8 ± 22.8
	396	17.40 ± 2.0	0.73 ± 0.08	1.12 ± 0.10	0.82 ± 0.10	2.82 ± 0.28	149 ± 227
	410	13.23 ± 3.5	0.59 ± 0.17	0.82 ± 0.22	0.63 ± 0.17	2.15 ± 0.58	73.5± 48.6
PRGWC JFX	557	17.53 ± 2.1	0.62 ± 0.07	0.92 ± 0.18	0.85 ± 0.09	2.53 ± 0.36	29.8 ± 5.10
	567	25.28 ± 5.3	1.00 ± 0.23	1.41 ± 0.26	1.22 ± 0.25	3.87 ± 0.69	171 ± 94.2
	7756	26.15 ± 5.1	0.90 ± 0.19	1.12 ± 0.15	1.26 ± 0.27	3.50 ± 0.62	351 ± 171
	7965	23.75 ± 4.2	0.89 ± 0.15	1.44 ± 0.29	1.15 ± 0.22	3.67 ± 0.69	48.3 ± 28.0
Mean		**19.91 ± 4.6**	**0.76 ± 0.15**	**1.08 ± 0.24**	**0.96 ± 0.23**	**2.97 ± 0.63**	**112 ± 110**

Macro chemical composition determined by Milkoscan. Abbreviations for the above table are as follows; perennial ryegrass (PRG), multi species swards (MSS), perennial ryegrass white clover (PRGWC), Holstein Friesian (HF), Jersey Friesian (JFX), somatic cell count (SCC).

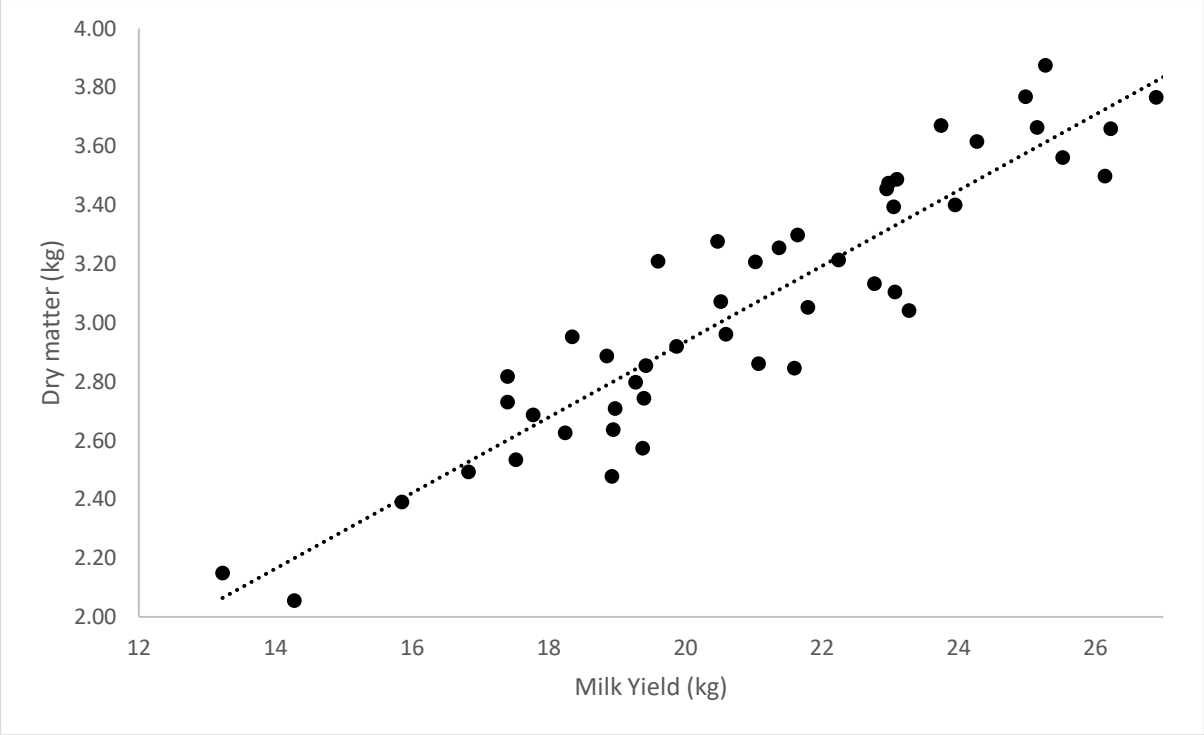

Figure 2.1 Relationship between milk yield (x-axis) and milk dry matter content (y-axis) for all cows in this study

2.3.2 Protein composition

Due to the similarities in the protein contents determined spectroscopically to the crude protein (CP) values determined by Kjeldahl, the values for true protein, whey protein as determined by NCN and NPN will be discussed instead of CP. The yield and distribution of nitrogen fractions (CP, TP, NCN and NPN) expressed on a kg per day basis is presented in Table 2.2. The highest concentration of true protein (TP) was present in the milk from cow 567, at 0.97 kg TP per day. The lowest concentration of TP was recorded from cow 8017, at 0.54 kg TP per day. The highest and lowest kg of TP per day among individual cows were recorded in the PRGWC JFX and PRGWC HF groups respectively, with these values likely being a reflection of the milk yield of these cows. The MSS HF group had the highest average kg of TP per day at 0.78, with

the lowest observed for the PRGWC HF group at 0.73 kg of TP per day, despite having the highest value for an individual cow.

Analysis of the milk from individual cows, using the Kjeldahl method, determined that the non-protein nitrogen (NPN) ranged between 0.02-0.05 kg/day. Two cows had the lower value of 0.02 kg/day, and were from the PRGWC HF and PRGWC JFX groups, while three cows had the higher value of 0.05 kg/day and were from the MSS HF, MSS JFX and PRGWC JFX groups. Diets consisting of white clover are reported to have a much higher nitrogen content than those in which white clover is absent (O'Callaghan et al., 2016; Winters et al., 2004). With this in mind, it is surprising that the kg of NPN per day among the groups that were fed PRGWC and MSS did not significantly ($P > 0.05$) differ from the groups fed PRG. Additionally, the average kg of NPN per day of the PRG HF group was actually slightly higher at 0.04, compared to the PRGWC diet at 0.03 kg of NPN per day; however, this was not significantly ($P > 0.05$) different.

The whey protein content of the milk among the 48 cows analysed was equally the lowest for three cows (cow numbers; 553, 8017 and 557), at a value of 0.08 kg/day. Interestingly, these values don't appear to be as closely linked to the milk yield as the CP and TP. The milk yield ranged from 14.3-19.4 kg/day among the three cows. Considering that each of these cows were from different groups, it would indicate that some factor other than breed and diet, such as individual animal genetics, influenced the whey protein content among these cows. The highest whey protein content was observed in cow 7098, with a value of 0.21 kg/day; this cow produced more milk than any other cow analysed, and is likely a major contributing factor to why the whey protein content was so high. In addition, another contributing factor is the SCC, in which this cow also recorded a high average value of 635,000 cells/mL, with a standard deviation of +/- 553,000 cells/mL. This SCC is not acceptable for milk, which usually has an upper limit of around 400,000 cells/mL by European standards (Ruegg & Pantoja, 2013). The high somatic

cell count indicates an infection, which can lead to a decrease in casein, but also an increase in whey proteins, due to more α-lactalbumin and immunoglobulins entering the mammary glands to help fight infection (Bisutti et al., 2022; Hamed et al., 2012).

The casein content was the highest at 0.80 kg/day for cow 421, within the MSS JFX group. The lowest value was recorded for cow 8017 at a value of 0.46 kg/day. Even though cow 7098 had the highest milk yield due to the high SCC, the casein content was lower that what might have been expected, at a value of 0.67 kg/day. The MSS JFX group had the highest average casein content, at 0.65 kg/day. Additionally, the influence of genetics as a contributor to the differences among the groups is possible as it has been reported in previous studies (Heck et al., 2009) that certain casein genotypes can influence the casein content.

The highest casein to whey protein ratio of 87:13 was recorded for two cows, one from PRG HF and the other from PRGWC HF. The lowest casein:whey protein was recorded from cow 7098 from group MSS HF, with a ratio of 75:25. This low ratio is possibly linked to clinical or sub-clinical mastitic infection, as indicated by the high SCC. While there were a number of cows with high SCC, indicative of poor udder health, cow 7098 was somewhat an outlier in terms of the casein to whey protein ratio. It is possible that cow 7098 was infected with clinical mastitis, whereas the other cows with high SCC may have been in the subclinical stages of mastitis in which the protein composition was not altered. While the results of each individual milk collection are not presented, the casein:whey protein ratio decreased proportional with increasing SCC for cow 7098.

Table 2.2 Protein component yield of the milk from 48 individual cows, averaged over four collection time points.

Breed and Diet	Cow Number	CP (kg/ day)	TP (kg/ day)	NPN (kg/ day)	NCN (kg/ day)	Whey (kg /day)	Casein (kg/ day)	C: W
PRG HF	158	0.85 ± 0.16	0.81 ± 0.16	0.04 ± 0.01	0.17 ± 0.03	0.13 ± 0.03	0.68 ± 0.13	84:16
	162	0.90 ± 0.16	0.86 ± 0.15	0.04 ± 0.01	0.19 ± 0.04	0.15 ± 0.03	0.71 ± 0.12	82:18
	390	0.79 ± 0.04	0.75 ± 0.03	0.04 ± 0.01	0.17 ± 0.01	0.14 ± 0.00	0.61 ± 0.03	82:18
	397	0.75 ± 0.23	0.71 ± 0.22	0.04 ± 0.01	0.17 ± 0.04	0.13 ± 0.03	0.58 ± 0.20	81:19
	553	0.67 ± 0.11	0.63 ± 0.11	0.04 ± 0.01	0.12 ± 0.01	0.08 ± 0.01	0.55 ± 0.10	87:13
	559	0.68 ± 0.11	0.64 ± 0.10	0.04 ± 0.01	0.13 ±0.03	0.09 ± 0.02	0.55 ± 0.09	86:14
	7956	0.80 ± 0.29	0.75 ± 0.28	0.04 ± 0.02	0.19 ± 0.06	0.15 ±0.04	0.60 ± 0.24	80:20
	8247	0.91 ± 0.23	0.87 ± 0.22	0.04 ± 0.01	0.20 ±0.04	0.16 ± 0.03	0.71 ± 0.19	81:19
Mean		**0.79 ± 0.09**	**0.75 ± 0.09**	**0.04 ± 0.01**	**0.17 ± 0.03**	**0.13 ± 0.03**	**0.62 ± 0.07**	**83:17**
PRG JFX	16	0.73 ± 0.24	0.69 ± 0.23	0.03 ± 0.01	0.16 ± 0.05	0.12 ± 0.04	0.57 ± 0.19	82:18
	151	0.85 ± 0.14	0.81 ± 0.14	0.04 ± 0.01	0.16 ± 0.02	0.12 ± 0.02	0.69 ± 0.12	85:15
	195	0.84 ± 0.08	0.81 ± 0.08	0.03 ± 0.01	0.17 ± 0.02	0.14 ± 0.01	0.67 ± 0.07	83:17
	405	0.75 ± 0.21	0.72 ± 0.19	0.04 ± 0.01	0.17 ± 0.06	0.13 ± 0.05	0.58 ± 0.15	82:18
	547	0.71 ± 0.24	0.68 ± 0.23	0.03 ± 0.01	0.15 ± 0.05	0.12 ± 0.05	0.56 ± 0.18	83:17
	569	0.66 ± 0.07	0.63 ± 0.07	0.03 ± 0.01	0.14 ± 0.01	0.10 ± 0.01	0.52 ± 0.06	83:17
	8233	0.83 ± 0.21	0.80 ± 0.21	0.04 ± 0.01	0.20 ± 0.04	0.16 ± 0.03	0.64 ± 0.18	80:20
	8424	0.91 ± 0.11	0.88 ± 0.10	0.04 ± 0.01	0.19 ± 0.02	0.15 ± 0.02	0.73 ± 0.09	83:17
Mean		**0.79 ± 0.09**	**0.75 ± 0.08**	**0.03 ± 0.01**	**0.17 ± 0.02**	**0.13 ± 0.02**	**0.62 ± 0.07**	**82:18**

	18	0.94 ± 0.16	0.90 ± 0.15	0.05 ± 0.01	0.17 ± 0.02	0.12 ± 0.02	0.77 ± 0.14	86:14
	27	0.91 ± 0.18	0.88 ± 0.17	0.04 ± 0.01	0.19 ± 0.03	0.15 ± 0.02	0.72 ± 0.16	83:17
	153	0.85 ± 0.21	0.82 ± 0.21	0.03 ± 0.01	0.17 ± 0.04	0.14 ± 0.03	0.68 ± 0.17	83:17
	176	0.71 ± 0.13	0.68 ± 0.13	0.03 ± 0.01	0.13 ± 0.02	0.10 ±0.01	0.58 ± 0.12	86:14
MSS HF	430	0.66 ± 0.14	0.62 ± 0.13	0.03 ± 0.01	0.15 ± 0.04	0.11 ± 0.03	0.51 ± 0.12	81:19
	562	0.76 ± 0.11	0.72 ± 0.09	0.04 ± 0.01	0.15 ± 0.02	0.12 ± 0.01	0.60 ± 0.08	84:16
	7098	0.93 ± 0.18	0.88 ± 0.17	0.04 ± 0.01	0.26 ± 0.05	0.21 ± 0.04	0.67± 0.18	75:25
	7852	0.75 ± 0.14	0.72 ± 0.13	0.03 ± 0.01	0.17 ± 0.02	0.14 ± 0.01	0.58 ± 0.13	80:20
Mean		**0.81 ± 0.11**	**0.78 ± 0.11**	**0.04 ± 0.01**	**0.17 ± 0.04**	**0.14 ± 0.04**	**0.64 ± 0.09**	**82:18**
	157	0.86 ± 0.19	0.82 ± 0.19	0.04 ± 0.01	0.15 ± 0.03	0.11 ± 0.02	0.71 ± 0.17	86:14
	192	0.92 ± 0.14	0.88 ± 0.14	0.05 ± 0.01	0.20 ± 0.03	0.16 ± 0.02	0.72 ± 0.12	82:18
	411	0.73 ± 0.14	0.70 ± 0.13	0.03 ± 0.01	0.13 ± 0.03	0.10 ± 0.02	0.60 ± 0.11	86:14
	421	0.99 ± 0.11	0.95 ± 0.34	0.04 ± 0.01	0.19 ± 0.07	0.15 ± 0.06	0.80 ± 0.28	85:15
MSS JFX	546	0.69 ± 0.11	0.66 ± 0.10	0.04 ± 0.01	0.15 ± 0.02	0.11 ± 0.02	0.55 ± 0.09	83:17
	577	0.61 ± 0.14	0.58 ± 0.14	0.03 ± 0.01	0.13 ± 0.04	0.10 ± 0.03	0.48 ±0.11	82:18
	6810	0.80 ± 0.19	0.76 ± 0.18	0.04 ± 0.01	0.16 ± 0.04	0.12 ± 0.02	0.63 ± 0.17	83:17
	8219	0.85 ± 0.19	0.81 ± 0.19	0.04 ± 0.01	0.15 ± 0.02	0.11 ± 0.01	0.69 ± 0.18	86:14
Mean		**0.81 ± 0.12**	**0.77 ± 0.12**	**0.04 ± 0.01**	**0.16 ± 0.03**	**0.12 ± 0.02**	**0.65 ± 0.10**	**84:16**
	391	0.91 ± 0.15	0.87 ± 0.14	0.04 ± 0.01	0.18 ± 0.04	0.14 ± 0.03	0.72 ± 0.12	84:16
PRGWC HF	394	0.82 ± 0.17	0.79 ± 0.17	0.03 ± 0.01	0.16 ± 0.03	0.13 ± 0.03	0.66 ± 0.14	83:17
	420	0.88 ± 0.15	0.84 ± 0.14	0.04 ± 0.01	0.18 ± 0.05	0.14 ± 0.04	0.71 ± 0.11	84:16

	550	0.73 ± 0.15	0.70 ± 0.15	0.03 ± 0.01	0.12 ± 0.03	0.09 ± 0.02	0.61 ± 0.13	87:13
	556	0.69 ± 0.15	0.66 ± 0.14	0.03 ± 0.01	0.13 ± 0.04	0.09 ± 0.03	0.56 ± 0.11	86:14
	8017	0.56 ± 0.15	0.54 ± 0.14	0.02 ± 0.01	0.11 ± 0.03	0.08 ± 0.02	0.46 ± 0.12	84:16
	8421	0.89 ± 0.17	0.85 ± 0.16	0.04 ± 0.01	0.17 ± 0.02	0.13 ± 0.01	0.72 ± 0.15	84:16
	8438	0.79 ± 0.16	0.76 ± 0.15	0.03 ± 0.01	0.17 ± 0.03	0.14 ± 0.02	0.62 ± 0.13	82:18
Mean		**0.78 ± 0.12**	**0.75 ± 0.11**	**0.03 ± 0.01**	**0.15 ± 0.03**	**0.12 ± 0.02**	**0.63 ± 0.09**	**84:16**
	26	0.71 ± 0.10	0.68 ± 0.09	0.03 ± 0.01	0.17 ± 0.04	0.13 ± 0.03	0.54 ± 0.07	80:20
	152	0.67 ± 0.08	0.64 ± 0.07	0.03 ± 0.01	0.14 ±0.02	0.11 ± 0.01	0.53 ± 0.06	83:17
	396	0.73 ± 0.08	0.70 ± 0.07	0.03 ± 0.01	0.16 ± 0.02	0.13 ± 0.02	0.57 ± 0.06	82:18
	410	0.59 ± 0.17	0.57 ± 0.16	0.02 ± 0.01	0.12 ± 0.04	0.10 ± 0.03	0.47 ± 0.13	83:17
PRGWC JFX	557	0.63 ± 0.08	0.60 ± 0.07	0.03 ± 0.01	0.11 ± 0.02	0.08 ± 0.01	0.51 ± 0.06	86:14
	567	1.01 ± 0.24	0.97 ± 0.22	0.05 ± 0.01	0.22 ± 0.06	0.17 ± 0.04	0.79 ± 0.18	82:18
	7756	0.90 ± 0.17	0.85 ± 0.16	0.04 ± 0.02	0.19 ± 0.03	0.14 ± 0.02	0.71 ± 0.14	83:17
	7965	0.90 ± 0.15	0.86 ± 0.15	0.04 ± 0.01	0.20 ± 0.03	0.16 ± 0.02	0.70 ± 0.12	82:18
Mean		**0.77 ± 0.15**	**0.73 ± 0.14**	**0.03 ± 0.01**	**0.16 ± 0.04**	**0.13 ± 0.03**	**0.60 ± 0.12**	**83:17**

Protein composition determined by the Kjeldahl method. Abbreviations for the above table are as follows; crude protein (CP), true protein (TP), non-protein nitrogen (NPN) and non-casein nitrogen (NCN), perennial ryegrass (PRG), multispecies sward (MSS), perennial ryegrass with white clover (PRGWC), Holstein Friesians (HF), Jersey Friesians Cross (JFX), casein to whey protein ratio (C:W).

2.3.3 Protein profile analysis

Based on previous studies it has been reported that β-casein, κ-casein and β-lg, are the most polymorphic milk proteins (Caroli et al., 2009; Farrell et al., 2004a). With this in mind, they will be the main focus of the investigation and discussion on the milk protein variants among individual cows.

Protein profile analysis of milk from individual cows using RP-HPLC analysis showed that each cow had a unique milk protein profile. Distinct regions and peaks within each profile allowed for the identification of the six major milk proteins of β-casein, κ-casein, α_{s1}-casein, α_{s2}-casein, β-lg, and α-lactalbumin. The protein profiles, coupled with genetic predictions from the Irish Cattle Breeding Federation (ICBF (2022), showed the potential to identify genetic variants of certain proteins based on differences in retention time and morphology of peak areas. This analysis showed that the most distinguishable genotypes from the protein profiles related to β-lg (Fig 2.2). In a haplotype AB β-lg milk sample, the two alleles that produce β-lg are co-dominant, meaning that both the A and the B variants of the β-lactoglobulin proteins are produced. The haplotype AB β-lg samples are identifiable by their protein profiles due to the presence of two peaks with retention time of 16-18 min. The homozygous forms of β-lg can also be identified from the milk protein profile due to the presence of only one β-lg peak, with the AA and BB genotypes of β-lg identified based on differences in retention time. The AA genotype typically elutes at approximately 17.5 min and the BB genotype at 16 min. Two other variants of β-lg were also identified through the genomic database, haplotypes AC and BC. The C variant of β-lg had the same retention time as the BB genotype. Therefore, it was co-eluted with the BB genotype or, in the case of the AC haplotype, could be mistaken for BB genotype. The C genotype; however, was only found in three cows, all of which were JFX. Of the β-lg variants identified, the AA genotype was the most common among HF cows, followed

by the AB haplotype and then BB genotype, with relative frequencies of 42, 32 and 26%, respectively (Fig 2.5). The BB genotype was the most common β-lg variant present in the milk of JFX cows, with a relative frequency of 33%. The AB haplotype and AA genotype were the next most common β-lg variants at 29 and 27%, respectively; however, unlike the HF cows, the JFX cows had two additional genotypes of β-lg, that of AC and BC, which were both present at a relative frequency of 6%.

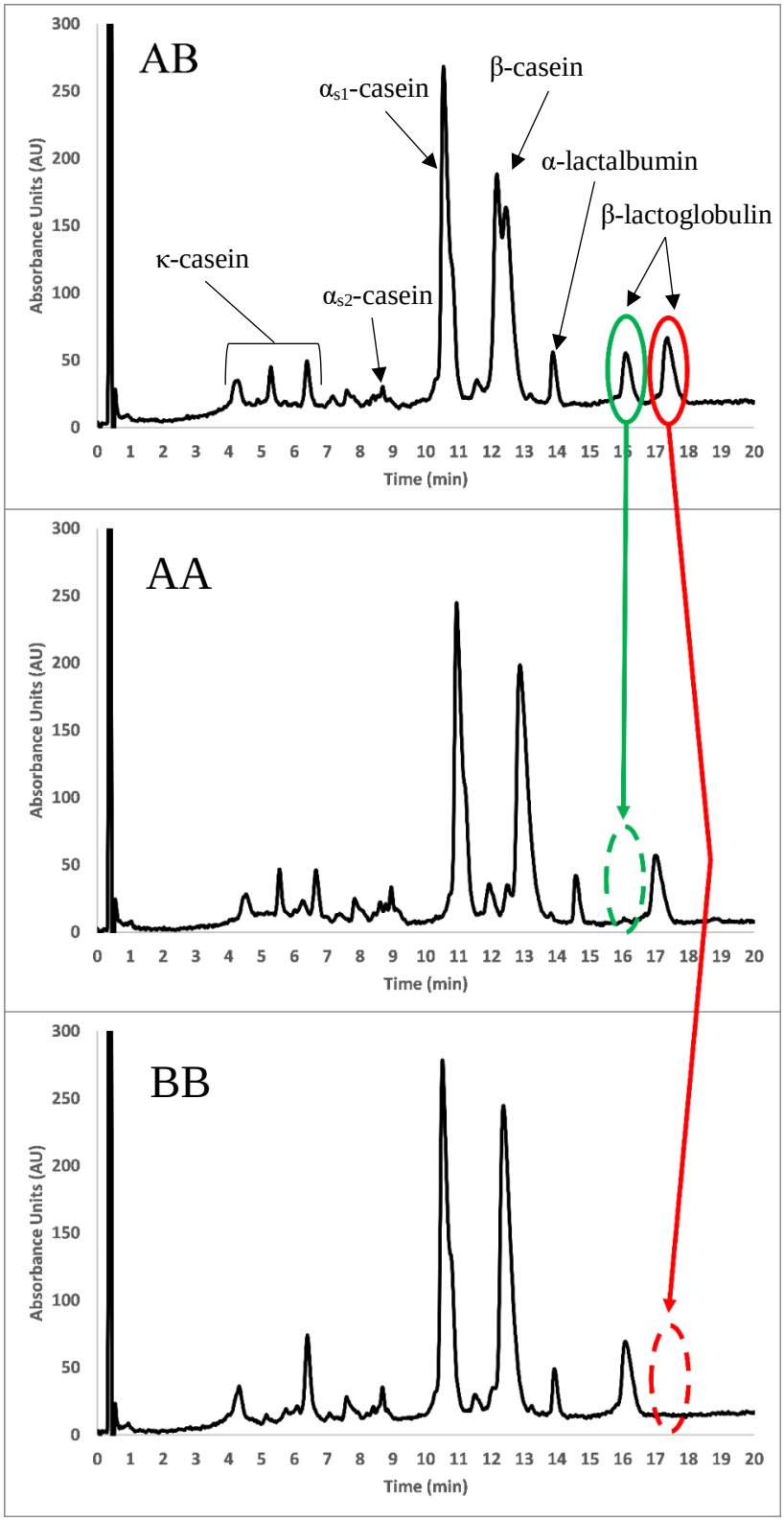

Figure 2.2 Milk protein profile by high performance liquid chromatography, identifying the AB, AA and BB genotypes of β-lg.

With the aid of the genomic database from (ICBF, 2022), it was observed that the genotypes of κ-casein could also be identified from the protein profiles (Fig 2.3). In the protein profile the κ-casein retention time spans between 4 and 7 min. The genetic variants of κ-casein are distinguishable by small changes to the peak area within the κ-casein region. The AA genotype is identifiable by a main peak at 5.5 min, the BB genotype by a main peak at 6.5 min, and the AB haplotype by two peaks at 5.5 and 6.5 min. The AB haplotype of κ-casein was the most common among both breeds, with a relative frequency of 41 and 63%, for HF and JFX, respectively; (Fig 2.5). The AA genotype is the second most common genotype, at 36 and 21%, for HF and JFX respectively, and finally, BB is the least common genotype among the HF and JFX breeds, with a relative frequency of 23 and 17%, respectively.

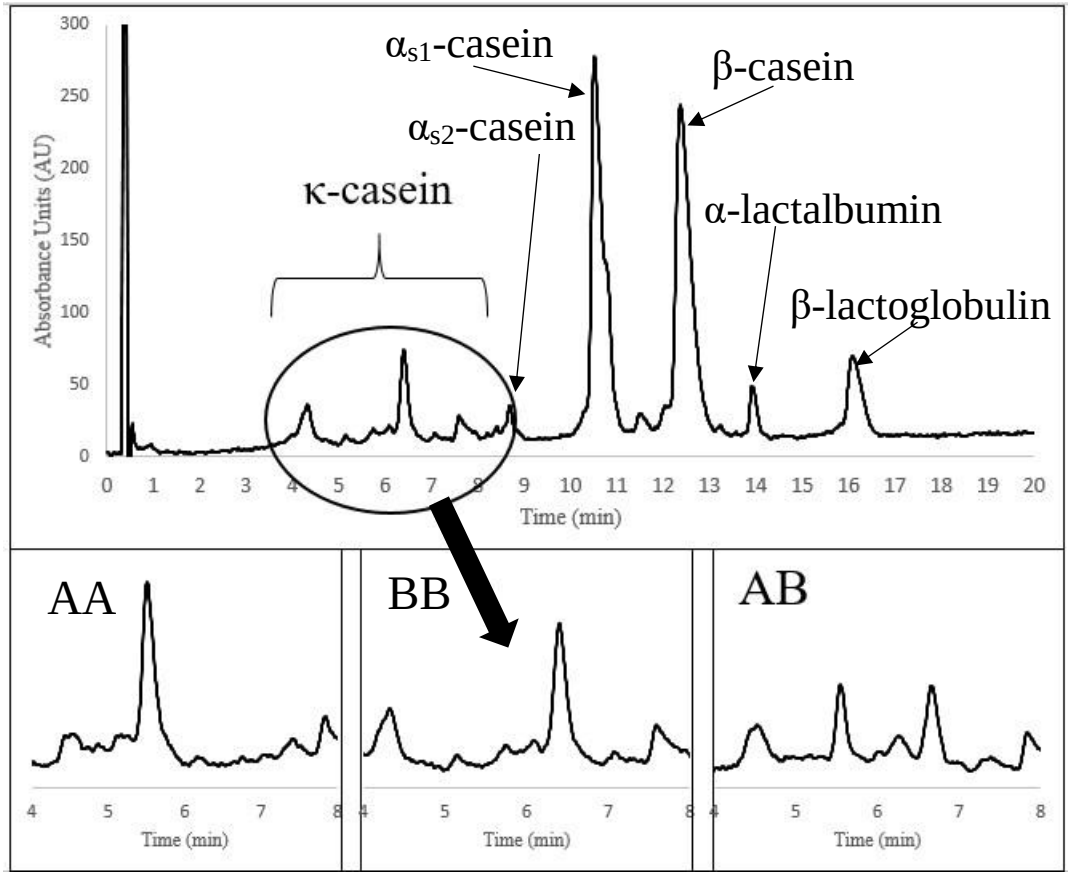

Figure 2.3 Milk protein profile by high performance liquid chromatography, in which the AA, BB and AB genotypes of κ-casein can be identified within a retention time between 4and 7 min.

The genomic database provided by the ICBF (2022) highlighted β-casein protein variants not easily identifiable from the HPLC profiles. While some variants of β-casein produce two peaks within the β-casein retention time region, it was not possible to distinguish specific genetic variants due to similarities in retention times (Fig 2.4). In particular, β-casein was found to be highly polymorphic, with nine variants identified from the database. A possible reason why the HPLC could not identify the different variants of β-casein is due to the larger size (209 amino acids) of the protein, which might mask the effect of different amino acid substitutions as a function of protein retention time compared to β-lg (162 amino acids) and κ-casein (169 amino acids) (Slattery, 1976). The β-casein variants found among the 48 cows are presented in Fig

2.5. Of the nine β-casein genotype identified, the relative frequencies for those found among the HF cows are 5, 14, 52, 10, 5, and 14% for the A1A1, A1A2, A2A2, A1I, A2F and A2I, respectively. The β-casein genotype found among the JFX were present in the relative frequencies of 4, 33, 38, 4, 8, 4, 4 and 4% for the genotypes A1A1, A1A2, A2A2, A2F, A2I, IB, A1B and A2B, respectively.

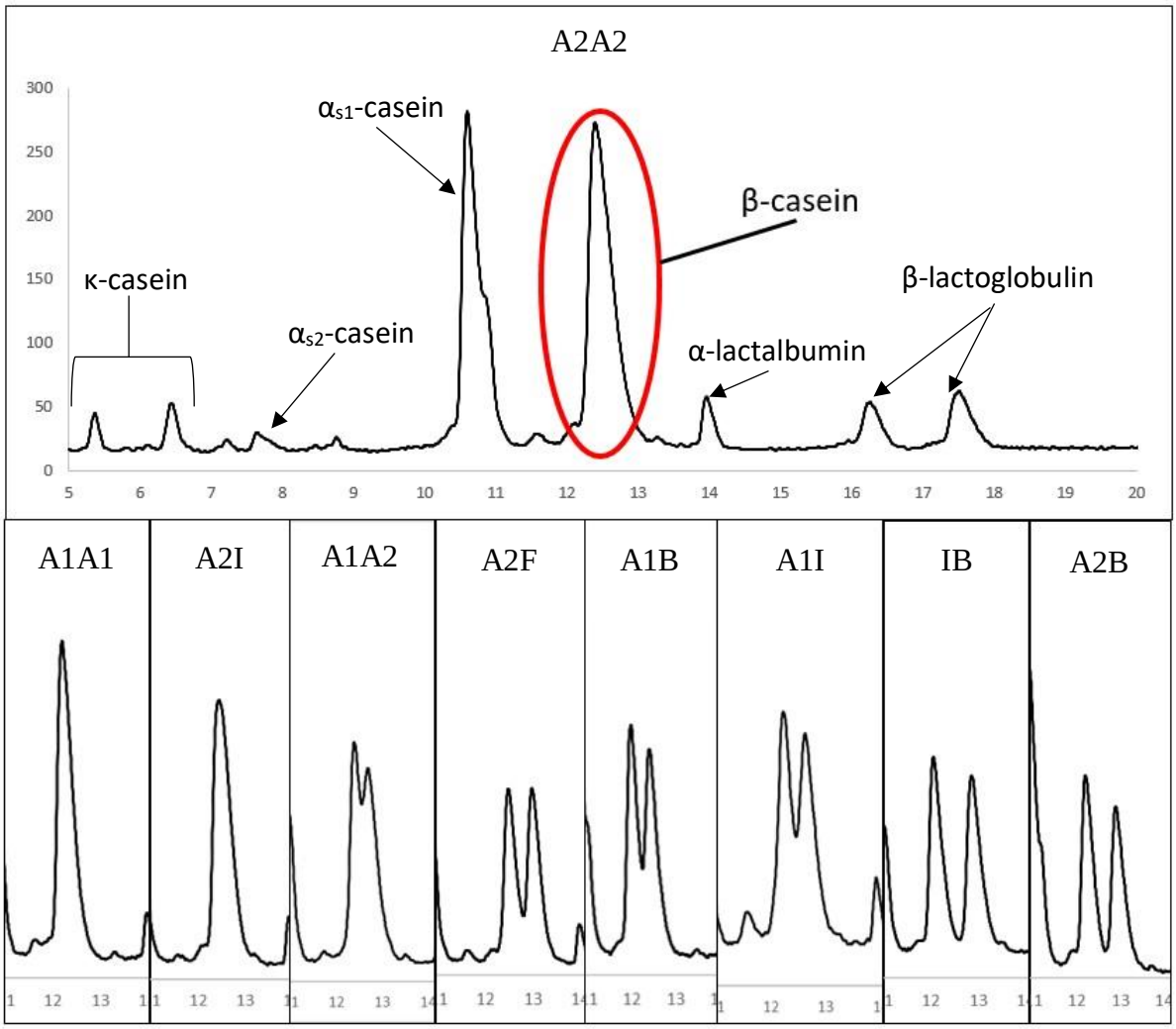

Figure 2.4 Milk protein profiles by high performance liquid chromatography profiles of the β-casein genotypes A1A1, A1A2, A2A2, A2F, A1I, A2I, IB, A1B and A2B.

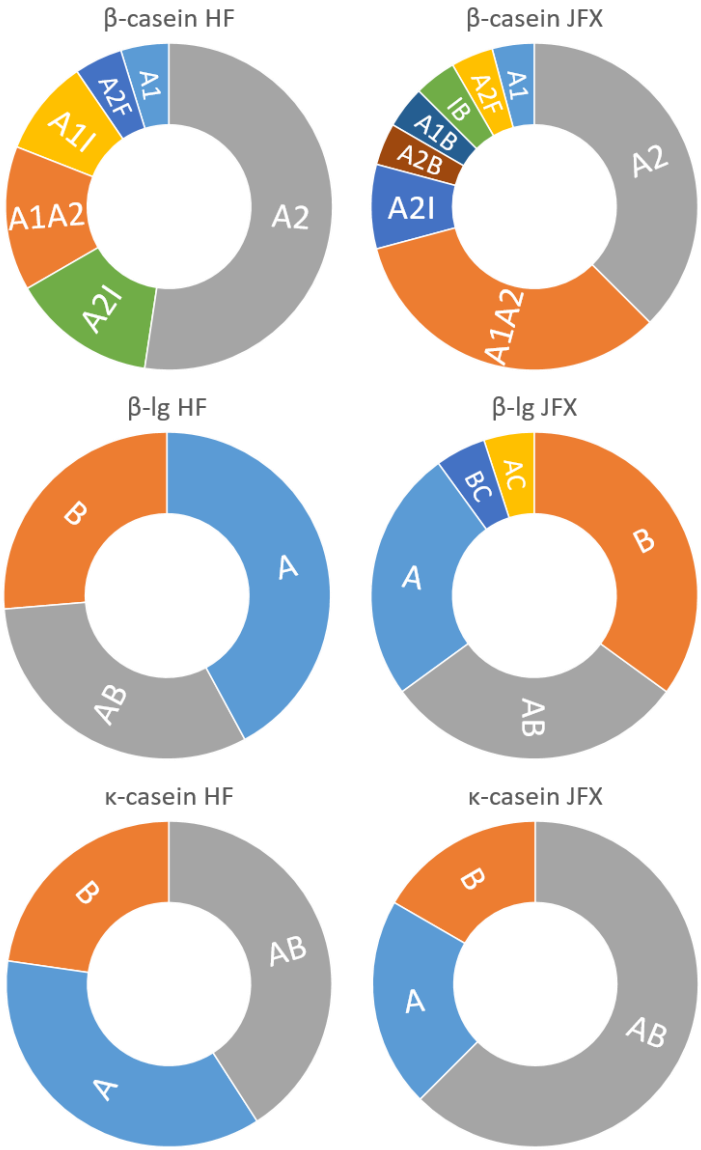

Figure 2.5 Genotype distribution among individual cows from two breeds; Holstein Friesian (HF) and Jersey Friesian Cross (JFX), for three milk proteins; β-casein, β-lactoglobulin (β-lg) and κ-casein. Letters contained within the donut chart indicates the genetic variant.

2.3.4 Impact of β-casein variants on protein content

Among the β-casein genotypes, the A1I and A2B haplotypes were associated with the highest average total nitrogen (TN) at 0.92 kg per day and true protein (TP) at 0.88 kg per day, compared to any of the other genotype, with a significant ($P < 0.05$) difference between the A1I variant and all other variants not containing B in their genotype (Table 2.3). The genotypes

associated with the lowest TP in milk was the A2I and IB haplotype, with an average of 0.66 kg per day, in which the A2I haplotype was significantly *(P* < 0.05) different from all other genotypes, with the exception of IB. The A1I haplotype also had the highest average casein content of all genotypes, and was significantly (*P* < 0.05) different to all other genotypes, except the haplotypes A1B and A2B. In contrast to the present study, in which no significant (*P* > 0.05) difference was found between the protein fractions of milk containing the A1A1, A2A2 genotypes or the A1A2 haplotypes, Ng-Kwai-Hang et al. (1986b) found that milk comprising the A1A1 genotype had the highest casein content among the A1A1, A2A2, and A1A2 genotypes, followed by the A1A2 haplotype and then the A2A2 genotype; however, their results were recorded as percentages and were not calculated on a kg per day basis where milk yield is considered. Studies by Bech, and Kristiansen (1990), Heck, Schennink, et al. (2009a) and Marko et al. (2020) found that the milk with the A2A2 genotype was correlated with higher protein concentration. Relative to the HF group of cows, applying a breeding program which prioritises higher protein contents, results in a greater frequency of the A2A2 genotype. While this observation is specific to the cows examined in this study, this trend would likely also apply to the broader Irish herd. Although observations reported within the literature suggest that genetic variants of β-casein may not be a true contributor to the protein concentration in milk (Gai et al., 2021; Heck, Schennink, et al., 2009b). The effect of β-casein variants on the whey protein content of milk found that no variant significantly (*P*> 0.05) altered the whey protein content of milk.

Table 2.3. Protein fraction composition of nine genotypes of β-casein identified among 48 individual cows.

	β-casein Genotypes								
	A2A2	A1A2	A1A1	A2I	A2F	A1I	A1B	A2B	IB
NPN (kg/ day)	0.04 (± 0.01)^{a}	0.04 (± 0.01)^{a}	0.04 (± 0.01)^{a}	0.03 (± 0.01)^{a}	0.04 (± 0.01)^{a}	0.05 (± 0.00)^{a}	0.04 (± 0.01)^{a}	0.04 (± 0.01)^{a}	0.03 (± 0.01)^{a}
NCN (kg/ day)	0.17 (± 0.04)^{a}	0.16 (± 0.03)^{a}	0.15 (± 0.03)^{a}	0.14 (± 0.03)^{a}	0.15 (± 0.01)^{a}	0.19 (± 0.02)^{a}	0.18 (± 0.05)^{a}	0.19 (± 0.02)^{a}	0.13 (±0.04)^{a}
TN (kg/ day)	0.80 (± 0.09)^{a}	0.79 (± 0.11)^{a}	0.80 (± 0.12)^{a}	0.69 (± 0.12)^{b}	0.75 (± 0.14)^{a}	0.92 (± 0.02)^{c}	0.88 (± 0.13)^{abc}	0.92 (± 0.10)^{abc}	0.69 (± 0.14)^{ab}
TP (kg/ day)	0.76 (± 0.09)^{a}	0.75 (± 0.11)^{a}	0.76 (± 0.12)^{a}	0.66 (± 0.12)^{b}	0.71 (± 0.13)^{a}	0.88 (± 0.02)^{c}	0.84 (± 0.14)^{abc}	0.88 (± 0.10)^{abc}	0.66 (± 0.14)^{ab}
Casein (kg/ day)	0.63 (± 0.07)^{a}	0.63 (± 0.10)^{a}	0.65 (± 0.10)^{a}	0.56 (± 0.10)^{a}	0.60 (± 0.13)^{a}	0.74 (± 0.04)^{b}	0.71 (± 0.11)^{ab}	0.73 (± 0.09)^{ab}	0.56 (± 0.11)^{a}
Whey Protein (kg/ day)	0.13 (± 0.03)^{a}	0.12 (± 0.02)^{a}	0.11 (± 0.02)^{a}	0.10 (± 0.02)^{a}	0.11 (± 0.01)^{a}	0.14 (± 0.03)^{a}	0.14 (± 0.04)^{a}	0.15 (± 0.02)^{a}	0.09 (± 0.03)^{a}
C:W	82:18	83:17	85:15	84:16	84:16	83:17	84:16	83:17	84:16

Values within a row not sharing a common superscript differed significantly ($P < 0.05$). Abbreviations are as follows; non-protein nitrogen (NPN), non-casein nitrogen (NCN), casein to whey protein ratio (C:W). Genotype populations; A2A2 (n = 23), A1A2 (n = 11), A1A1 (n = 2), A2I (n = 5), A2F (n = 2), A1I (n = 2), A1B (n = 1), A2B (n = 1), IB (n = 1).

2.3.5 κ-casein

The genetic variants of κ-casein had no significant (P> 0.05) effect on any of the protein fractions of milk (Table 2.4). This disagrees with the results reported by Ng-Kwai-Hang et al. (1986b) and Botaro et al. (2009), who found that milk with the BB genotype of κ-casein had significantly higher quantities of casein than milk with the AA genotype or the AB haplotype. However, the discrepancy between the current study and those which have reported significant differences between protein fractions among genetic variants of κ-casein, is likely due to the fact that the results in this study were calculated on a kg/day basis where milk yield is considered, whereas most other studies report them on a percentage in milk basis.

Table 2.4. Protein fraction composition of milk samples comprising three genotypes of κ-casein

	κ-casein Genotype		
	AA	BB	AB
NPN (kg/ day)	0.04 (± 0.00)[a]	0.04 (± 0.01)[a]	0.04 (± 0.01)[a]
NCN (kg/ day)	0.17 (± 0.02)[a]	0.15 (± 0.04)[a]	0.17 (± 0.03)[a]
TP (kg/ day)	0.77 (± 0.08)[a]	0.75 (± 0.16)[a]	0.76 (± 0.10)[a]
TN (kg/ day)	0.81 (± 0.08)[a]	0.79 (± 0.15)[a]	0.80 (± 0.11)[a]
Casein (kg/ day)	0.64 (± 0.07)[a]	0.63 (± 0.13)[a]	0.63 (± 0.08)[a]
Whey Protein (kg/ day)	0.13 (± 0.01)[a]	0.11 (± 0.02)[a]	0.13 (± 0.02)[a]
C:W	82:18	85:15	83:17

Values within a row not sharing a common superscript differed significantly (P < 0.05). Abbreviations are as follows; non-protein nitrogen (NPN), non-casein nitrogen (NCN), casein to whey protein ratio (C:W). Genotype population; AA (*n* = 13), BB (*n* = 9), AB (*n* = 26)

2.3.6 β-lactoglobulin

The genotypes of β-lg had a significant ($P< 0.05$) effect on the whey protein content as determined by NCN measurement, but had no significant effect on the NPN content. Milk with the AA genotype had the highest NCN, followed by AB, AC, BB, and then finally the BC haplotype, with significant ($P< 0.05$) differences found between the AA and BB/BC variants of β-lg (Table 2.5). There was no significant ($P> 0.05$) difference in total nitrogen, true protein, NPN or casein between each genotype. The different genotypes of β-lg had the greatest impact on the whey protein content of milk. These results are in agreement with Berry et al. (2010), who reported that milk from JFX cows with the AA genotype of β-lg produced milk with 30% more β-lg than those with the BB genotype. This difference in whey protein content between the variants could be due to the mutations that cause the genetic variation to occur being located near the promoter region of the DNA, leading to the increased synthesis of β-lg (Heck, Schennink, et al., 2009a).

Table 2.5. Protein composition of milk samples containing five different variants of β-lg; A, B, AB, AC and BC.

	β-lg Genotype				
	AA	BB	AB	AC	BC
TN (kg/ day)	0.77 (± 0.13)[a]	0.79 (± 0.12)[a]	0.83 (± 0.11)[a]	0.69 (± 0.15)[a]	0.73 (± 0.13)[a]
TP (kg/ day)	0.73 (± 0.13)[a]	0.75 (± 0.11)[a]	0.79 (± 0.09)[a]	0.66 (± 0.16)[a]	0.70 (± 0.13)[a]
NPN (kg/ day)	0.04 (± 0.01)[a]	0.04 (± 0.01)[a]	0.04 (± 0.01)[a]	0.03 (± 0.01)[a]	0.03 (± 0.01)[a]
NCN (kg/ day)	0.17 (± 0.04)[a]	0.15 (± 0.02)[b]	0.16 (± 0.02)[ab]	0.15 (± 0.05)[ab]	0.13 (± 0.03)[b]
Casein (kg/ day)	0.60 (± 0.09)[a]	0.64 (± 0.09)[a]	0.65 (± 0.09)[a]	0.55 (± 0.12)[a]	0.60 (± 0.11)[a]
Whey Protein (kg/ day)	0.14 (± 0.03)[a]	0.11 (± 0.02)[b]	0.13 (± 0.02)[ab]	0.11 (± 0.03)[b]	0.10 (± 0.02)[b]
Casein: Whey	81:19	86:14	83:17	83:17	86:14

Values within a row not sharing a common superscript differed significantly ($P < 0.05$). Abbreviations are as follows; total nitrogen (TN), true protein (TP), non-protein nitrogen (NPN), non-casein nitrogen (NCN), casein to whey protein ratio (C:W). Genotype population; AA (n = 12), BB (n = 14), AB (n = 20), AC (n = 1), BC (n = 1).

2.4 Conclusion

Of the 48 HF and JFX cows examined in this study it was found that typically the JFX breed produces milk with higher concentrations of protein than the HF breed. However, due to the fact that the HF breed has a higher yield producing ~ 1.6 kg more milk/day, the kg of protein produced per day was also higher for this group. The diversity in casein to whey protein ratio among individual cows provided interesting observations ranging from 87:13 to 75:25, with the latter ratio linked to high SCC and likely mastitic infection. Of the cows analysed, 17

genetic variants were identified among β-casein, κ-casein and β-lg. This highlights the diversity of milk protein variants in a relatively small herd. Milk containing the A1I and A2B haplotypes of β-casein had higher TP and casein content than milk with the other genetic variants. This indicates that selecting for cows with these genotypes could be of benefit to farmers, who are typically paid, in part on the protein content of milk. Milk with the AA genotype of β-lg had the highest whey protein content and was present at the highest frequency among HF cows, although total protein content was not affected. While cow breeding programs will always strive to maximise the monetary return to both farmers and milk processors in terms of milk price, it is important to monitor the evolution of the protein profile, relative to genetic polymorphism, in the dairy herd to ensure that functional and nutritional characteristics of the milk are well understood

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Declaration of competing interest

Authors declare no conflicts of interest.

2.5 References

Alothman, M., Hogan, S. A., Hennessy, D., Dillon, P., Kilcawley, K. N., O'Donovan, M., Tobin, J., Fenelon, M. A., & O'Callaghan, T. F. (2019). The "grass-fed" milk story:

understanding the impact of pasture feeding on the composition and quality of bovine milk. *Foods, 8*, 350.

Bech, A.-M., & Kristiansen, K. R. (1990). Milk protein polymorphism in Danish dairy cattle and the influence of genetic variants on milk yield. *Journal of Dairy Research, 57*, 53-62.

Berry, S., Lopez-Villalobos, N., Beattie, E., Davis, S., Adams, L., Thomas, N., Ankersmit-Udy, A., Stanfield, A., Lehnert, K., & Ward, H. (2010). Mapping a quantitative trait locus for the concentration of β-lactoglobulin in milk, and the effect of β-lactoglobulin genetic variants on the composition of milk from Holstein-Friesian x Jersey crossbred cows. *New Zealand Veterinary Journal, 58*, 1-5.

Bisutti, V., Vanzin, A., Toscano, A., Pegolo, S., Giannuzzi, D., Tagliapietra, F., Schiavon, S., Gallo, L., Trevisi, E., Negrini, R., & Cecchinato, A. (2022). Impact of somatic cell count combined with differential somatic cell count on milk protein fractions in Holstein cattle. *Journal of Dairy Science, 105*, 6447-6459.

Bittante, G., Penasa, M., & Cecchinato, A. (2012). Invited review: Genetics and modeling of milk coagulation properties. *Journal of Dairy Science, 95*, 6843-6870.

Bobe, G., Beitz, D., Freeman, A., & Lindberg, G. (1999). Effect of milk protein genotypes on milk protein composition and its genetic parameter estimates. *Journal of Dairy Science, 82*, 2797-2804.

Boro, P., Debnath, J., Das, T. K., Naha, B. C., Debbarma, N., Debbarma, P., Debbarma, C., & Bala, L. S. (2018). Milk composition and factors affecting it in dairy Buffaloes: A review. *Journal of Entomology and Zoology Studies, 340*, 340-343.

Botaro, B. G., Lima, Y. V. R. d., Cortinhas, C. S., Silva, L. F. P., Rennó, F. P., & Santos, M. V. d. (2009). Effect of the kappa-casein gene polymorphism, breed and seasonality on physicochemical characteristics, composition and stability of bovine milk. *Revista Brasileira de Zootecnia, 38*, 2447-2454.

Caroli, A. M., Chessa, S., & Erhardt, G. J. (2009). Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *Journal of Dairy Science, 92*, 5335-5352.

Daniloski, D., McCarthy, N. A., Gazi, I., & Vasiljevic, T. (2022). Rheological and structural properties of acid-induced milk gels as a function of β-casein phenotype. *Food Hydrocolloids, 131*, 107846.

Daniloski, D., Hailu, Y., Brodkorb, A., Vasiljevic, T., & McCarthy, N. A. (2024). Impact of β-casein phenotype on the physical properties of skim milk powders and their subsequent digestion characteristics. *Food Hydrocolloids, 152*, 109918.

EFSA. (2009). Review of the potential health impact of β-casomorphins and related peptides. *European Food Safety Authority Journal, 7*, 231.

Farrell, H., Jimenez-Flores, R., Bleck, G., Brown, E., Butler, J., Creamer, L., Hicks, C., Hollar, C., Ng-Kwai-Hang, K., & Swaisgood, H. (2004). Nomenclature of the proteins of cows' milk: Sixth revision. *Journal of Dairy Science, 87,* 1641-1674.

Fox, P. F., Uniacke-Lowe, T., McSweeney, P. L. H., & O'Mahony, J. A. (2015). *Dairy Chemistry and Biochemistry* (Second Edition): Springer.

Gai, N., Uniacke-Lowe, T., O'Regan, J., Faulkner, H., & Kelly, A. L. (2021). Effect of Protein Genotypes on Physicochemical Properties and Protein Functionality of Bovine Milk: A Review. *Foods, 10*, 2409.

Gaspard, S. J., & Brodkorb, A. (2019). The use of high performance liquid chromatography for the characterization of the unfolding and aggregation of dairy proteins. In *Protein Self-Assembly* (First Edition) (pp. 103-115): Springer.

Gonçalves, J. L., Kamphuis, C., Vernooij, H., Araújo Jr, J., Grenfell, R. C., Juliano, L., Anderson, K., Hogeveen, H., & Dos Santos, M. (2020). Pathogen effects on milk yield and composition in chronic subclinical mastitis in dairy cows. *The Veterinary Journal, 262*, 105473.

Hamed, H., Trujillo, A.-J., Juan, B., Guamis, B., ElFeki, A., & Gargouri, A. (2012). Interrelationships between somatic cell counts, lactation stage and lactation number and their influence on plasmin activity and protein fraction distribution in dromedary (Camelus dromedaries) and cow milks. *Small Ruminant Research, 105*, 300-307.

Heck, J., Olieman, C., Schennink, A., Van Valenberg, H., Visker, M., Meuldijk, R., & Van Hooijdonk, A. (2008). Estimation of variation in concentration, phosphorylation and genetic polymorphism of milk proteins using capillary zone electrophoresis. *International Dairy Journal, 18*, 548-555.

Heck, J., Schennink, A., Van Valenberg, H., Bovenhuis, H., Visker, M., Van Arendonk, J., & Van Hooijdonk, A. (2009a). Effects of milk protein variants on the protein composition of bovine milk. *Journal of Dairy Science, 92*, 1192-1202.

Heck, J., Van Valenberg, H., Dijkstra, J., & Van Hooijdonk, A. (2009). Seasonal variation in the Dutch bovine raw milk composition. *Journal of Dairy Science, 92*, 4745-4755.

Heck, J. M. L., Schennink, A., van Valenberg, H. J. F., Bovenhuis, H., Visker, M. H. P. W., van Arendonk, J. A. M., & van Hooijdonk, A. C. M. (2009b). Effects of milk protein variants on the protein composition of bovine milk. *Journal of Dairy Science, 92*, 1192-1202.

Horan, B., Dillon, P., Faverdin, P., Delaby, L., Buckley, F., & Rath, M. (2005). The Interaction of Strain of Holstein-Friesian Cows and Pasture-Based Feed Systems on Milk Yield, Body Weight, and Body Condition Score. *Journal of Dairy Science, 88*, 1231-1243.

ICBF (2023). *National Genotyping Programme.* https://www.icbf.com/national-genotyping-programme/

IDF. (2004). Milk - Determination of the casein-nitrogen content - Part 1: Indirect method (Reference method). In *International Dairy Federation* (Vol. IDF 29-1 :2004). Brussels, Belgium: International Dairy Federation.

IDF. (2014). Milk and milk products - Determination of nitrogen content - Part 1: Kjeldahl prinicple and crude protein calculation. In *International Dairy Federation* (Vol. IDF 20-1 :2014). Brussels, Belgium: International Dairy Federation.

IDF. (2016). Milk and milk products - determination of nitrogen content - Part 4: Determination of protein and non-protein nitrogen content calculation (Reference Method). In *International Dairy Federation* (Vol. IDF 20-4 :2016). Brussels, Belgium: International Dairy Federation.

Kelly, L. M., O'Mahony, J. A., & Tobin, J. T. (2024). Genetic variation in bovine milk proteins: Implications for functional and nutritional properties. *International Journal of Dairy Technology*.

Kim, S. W., & Wu, G. (2009). Regulatory role for amino acids in mammary gland growth and milk synthesis. *Amino Acids, 37*, 89-95.

Kostovska, R., Horan, B., Drouin, G., Tobin, J. T., O'Callaghan, T. F., Kelly, A. L., & Gómez-Mascaraque, L. G. (2024). Multispecies pasture diet and cow breed impacts on milk composition and quality in a seasonal spring-calving dairy production system. *Journal of Dairy Science*.

Marko, R., Uros, G., Branislav, V., Milan, M., Danijela, K., Vlado, T., & Zoran, S. (2020). Beta-casein gene polymorphism in Serbian Holstein-Friesian cows and its relationship with milk production traits. *Acta Veterinaria, 70*, 497-510.

Marziali, A. S., & Ng-Kwai-Hang, K. F. (1986). Effects of Milk Composition and Genetic Polymorphism on Coagulation Properties of Milk. *Journal of Dairy Science, 69*, 1793-1798.

Mele, M., Macciotta, N. P. P., Cecchinato, A., Conte, G., Schiavon, S., & Bittante, G. (2016). Multivariate factor analysis of detailed milk fatty acid profile: Effects of dairy system, feeding, herd, parity, and stage of lactation. *Journal of Dairy Science, 99*, 9820-9833.

Ng-Kwai-Hang, K. F., Hayes, J. F., Moxley, J. E., & Monardes, H. G. (1986). Relationships Between Milk Protein Polymorphisms and Major Milk Constituents in Holstein-Friesian Cows. *Journal of Dairy Science, 69*, 22-26.

O'Sullivan, M., Horan, B., Pierce, K. M., McParland, S., O'Sullivan, K., & Buckley, F. (2019). Milk production of Holstein-Friesian cows of divergent Economic Breeding Index evaluated under seasonal pasture-based management. *Journal of Dairy Science, 102*, 2560-2577.

O'Callaghan, T. F., Hennessy, D., McAuliffe, S., Kilcawley, K. N., O'Donovan, M., Dillon, P., Ross, R. P., & Stanton, C. (2016). Effect of pasture versus indoor feeding systems on raw milk composition and quality over an entire lactation. *Journal of Dairy Science, 99*, 9424-9440.

Phelan, J., O'Keeffe, A., Keogh, M., & Kelly, P. (1982). Studies of milk composition and its relationship to some processing criteria: 1. Seasonal changes in the composition of Irish milk. *Irish Journal of Food Science and Technology*, 1-11.

Prendiville, R., Pierce, K. M., & Buckley, F. (2009). An evaluation of production efficiencies among lactating Holstein-Friesian, Jersey, and Jersey×Holstein-Friesian cows at pasture. *Journal of Dairy Science, 92*, 6176-6185.

Ruegg, P., & Pantoja, J. (2013). Understanding and using somatic cell counts to improve milk quality. *Irish Journal of Agricultural and Food Research*, 101-117.

Shortall, J., Foley, C., Sleator, R. D., & O'Brien, B. (2018). The effect of dairy cow breed on milk production, cow traffic and milking characteristics in a pasture-based automatic milking system. *Livestock Science, 209*, 1-7.

Slattery, C. W. (1976). Review: Casein Micelle Structure; An Examination of Models. *Journal of Dairy Science, 59*, 1547-1556.

Sneddon, N., Lopez-Villalobos, N., Hickson, R., & Shalloo, L. (2013). Review of milk payment systems to identify the component value of lactose. In *Proceedings of the New Zealand Society of Animal Production* (First Edition) (pp. 33-36): New Zealand Society of Animal Production.

Sumon, S. M. R., Parvin, M. S., Ehsan, M. A., & Islam, M. T. (2020). Relationship between somatic cell counts and subclinical mastitis in lactating dairy cows. *Veterinary World, 13*, 1709.

Vance, E. R., Ferris, C. P., Elliott, C. T., McGettrick, S. A., & Kilpatrick, D. J. (2012). Food intake, milk production, and tissue changes of Holstein-Friesian and Jersey × Holstein-Friesian dairy cows within a medium-input grazing system and a high-input total confinement system. *Journal of Dairy Science, 95*, 1527-1544.

Vance, E. R., Ferris, C. P., Elliott, C. T., Hartley, H. M., & Kilpatrick, D. J. (2013). Comparison of the performance of Holstein-Friesian and Jersey×Holstein-Friesian crossbred dairy cows within three contrasting grassland-based systems of milk production. *Livestock Science, 151*, 66-79.

Winters, A. L., Minchin, F. R., Davies, Z., Kingston-Smith, A. H., Theodorou, M. K., Griffith, G. W., & Merry, R. J. (2004). Effects of manipulating the protein content of white clover on silage quality. *Animal Feed Science and Technology, 116*, 319-331.

Woodford, K. B. (2009). *Devil in the Milk: Illness, health and politics of A1 and A2 milk* (First Edition): Chelsea Green Publishing.

Yoo, J., Song, M., Park, W., Oh, S., Ham, J.-s., Jeong, S.-g., & Kim, Y. (2019). A comparison of quality characteristics in dairy products made from Jersey and Holstein milk. *Food Science of Animal Resources, 39*, 255.

Chapter 3

Impact of genetic variants on the separation efficiency of β-lactoglobulin during microfiltration of skim milk

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Declaration

This chapter was written by author LMK. WPM and SS both assisted in the operation of the microfiltration unit. BH assisted in organising the cows for milk collection

Abstract

Genetic variants of milk proteins are often overlooked by dairy processors as a variable with the potential to influence process efficiency. This study assessed the impact of the A and B genetic variants of β-lactoglobulin on the isolation of whey proteins from skim milk by microfiltration. The A and B genetic variants of β-lactoglobulin differ by two amino acids at positions 64 (A = aspartic acid, B = glycine) and 118 (A = valine, B = alanine), with the A variant thus having a higher negative charge. Mass balance calculations indicated that 46.7% more whey proteins permeated during microfiltration for the A compared to the B variant, with the A variant also having significantly ($P < 0.05$) more accumulation of total and reversible fouling material than the B variant. These findings could be of importance to the dairy industry to improve microfiltration efficiency and increase protein yield during manufacture of native whey protein ingredients.

3.1 Introduction

Milk proteins can be categorised into two major groups, casein and whey proteins. Caseins comprise approximately 80% of total milk protein and are colloidally suspended in milk, largely in the form of micelles. Whey proteins comprise the remaining ~20%, solubilized within the serum phase of milk (Fox, Uniacke-Lowe, McSweeney and O'Mahony, 2015). β-lactoglobulin (β-lg) is the most abundant of the whey proteins, comprising approximately 62% of total whey protein (Boland, 2011). β-lg is a 162 amino acid protein with a molecular weight of approximately 18 kDa (Aich et al., 2015; Sawyer, 2012). While, the function of β-lg is still largely unknown, some suggest that it belongs to the lipocalin family and is a transporter of hydrophobic molecules, with others speculating that it aids in the transport and absorption of vitamin A (Oliveira, Valente-Mesquita, et al., 2001).

Genetic variants are proteins that differ from one another, typically by the substitution of an amino acid in its primary structure (Caroli et al., 2009), and form in response to a mutation within the coding region of DNA for that protein (Bhattacharya et al., 2017). The first genetic variant of a milk protein was discovered for β-lg in 1946 (Li, 1946). These genetic variants, later called A and B, differ by only two amino acids, where aspartic acid and valine in the A variant are substituted for glycine and alanine in the B variant, at position 64 and 118, respectively (Aschaffenburg & Drewry, 1957; Farrell Jr et al., 2004). These alterations to the amino acid sequence can profoundly affect the secondary and tertiary structure of the protein, leading to changes in functionality, which in some instances could be due to the differences in charges between variants (Bhattacharya et al., 2017; Wang et al., 2016).

Membrane filtration is a technology commonly used for separating milk components based on their molecular size. The four main membrane processes used in the dairy industry are microfiltration (MF), ultrafiltration, nanofiltration and reverse osmosis (Carter et al., 2021).

The primary use of MF in the dairy industry is for separating casein and whey proteins in milk, as well as removing bacteria and spores from milk and other liquid dairy streams (Nelson & Barbano, 2005; Zulewska, Newbold, & Barbano, 2009). The pore size of MF membranes can range between 0.1-2.0 μm; however, the average size of the casein micelle is 0.2 μm, therefore a pore size of ~0.1 μm is typically used for the efficient separation of casein and whey protein (Carter et al., 2021; Glantz et al., 2010). Whey proteins are much smaller (3.6×10^{-3} μm) than casein micelles and can pass through a MF membrane into the permeate stream, selectively removing them from the casein fraction of milk (Zulewska et al., 2009). The efficiency of removing whey proteins is largely determined by the type of membrane used, any pre-treatments applied to the feed and the operational conditions applied during filtration (Carter et al., 2021). Zulewska et al. (2009) reported that ceramic MF, operating under uniform transmembrane pressure, removed 25.78% more whey proteins than polymeric spiral-wound membranes. With diafiltration, ceramic and polymeric MF could remove 95% of the whey proteins from skim milk; however, the ceramic MF required three stages, and the polymeric MF required seven stages of diafiltration to achieve an equivalent extent of whey protein removal (Beckman et al., 2010; Hurt et al., 2010).

The impact of various processing conditions, such as temperature, concentration factor and membrane configuration on the separation efficiency of skim milk, has been well reported in the literature (Granger-Delacroix et al., 2023; Neocleous et al., 2002; Subhir et al., 2022; Subhir et al., 2023). However, the influence of genetic variation in the primary whey protein β-lg on the removal efficiency of whey proteins during MF has not been reported before. The aim of this paper was to assess the impact of genetic variants A and B of β-lg, on their partitioning behaviour during MF of skim milk, focusing on efficiency of protein removal from a mass balance perspective and accumulation of foulants. This research could highlight genetic

variants of β-lg that potentially offer more efficient membrane processing for the production of whey protein products.

3.2 Materials and methods

3.2.1 Feed material

Raw milk samples were obtained from 29 cows, which were categorised into two groups, cows that contained the AA genotype of β-lg (n=15) and those that contained the BB genotype of β-lg (n=14).Due to low milk volumes that would have been produced, casein genotypes were not screened as part of this study. Prior to milk collection, the genotypes of the individual cows within the herd were identified using the national genotyping program, provided by the Irish Cattle Breeding Federation (ICBF (2022), and validated by high performance liquid chromatography (HPLC) analysis using the method outlined by Gaspard and Brodkorb (2019). Milk was collected from the Teagasc, Curtins Research Farm, Moorepark, Fermoy, Co. Cork, Ireland, with each cow's milk individually collected and segregated depending on β-lg variant in separate bulk tanks across four consecutive milkings and stored at 4°C prior to collection for a maximum of 24 h. Individual batches of milk, segregated based on β-lg variant, were skimmed using a centrifugal cream separator (GEA Westfalia Oelde, Germany) to a final fat concentration of 0.025%, *w/w*. Pasteurisation of the milk was carried out using an APV plate heat exchanger (SPX Flow Technology, Crawley, UK), at 73°C for 15 s, then cooled to 4°C. Following pasteurisation, each milk sample was transferred to a jacketed tank and held at 4°C prior to further processing.

3.3.2 Membrane processing

All filtration experiments were performed using a pilot-scale membrane filtration unit (GEA Process Technologies, Dublin, Ireland) equipped with three 0.1 µm ceramic graded permeability (GP) MF membranes (Membralox® model, EP 3730, 0.1 µm alumina, Pall Corp, California, CA, USA) with a total surface area of 1.05 m². For each trial, ~370 kg of pasteurised skim milk were subjected to MF under continuous operating conditions, using a volume concentration factor (VCF) of 3 at 50°C. During processing, both the retentate and the permeate streams, and any associated plant flushes, were collected in separate tanks for mass balance purposes, and composite samples were collected for compositional analysis.

The specific operational conditions utilised for the filtration trials are provided in Table 3.1. Constant recirculation pressures (310 kPa retentate pressure in and 100 kPa retentate pressure out) were maintained throughout the filtration process to ensure a consistent trans-membrane pressure (TMP). A permeate back pressure of 80 kPa was also applied during MF. Under these operating pressure conditions, the membrane experienced an overall pressure drop (ΔP) and TMP of 210 and 125 kPa, respectively. Prior to and following filtration, the membrane plant was subjected to standard cleaning-in-place procedures as described previously by McCarthy et al. (2022). All filtration experiments were conducted in duplicate, and a discrete batch of pasteurised skim milk was used for each trial.

Table 3.1 Operational parameters during ceramic microfiltration of skim milk containing either the A or B genetic variant of β-lactoglobulin (β-lg).

Parameters	Skim milk (β-lg A)	Skim milk (β-lg B)
Recirculation flow rate (L h^{-1})	13200 ± 100.4	13400 ± 44.1
Feed flow rate (L h^{-1})	97.5 ± 14.1	97.1 ± 6.9
Retentate flow rate (L h^{-1})	34.8 ± 6.0	33.3 ± 2.7
Permeate flux (L m^{-2} h^{-1})	62.0 ± 7.7	62.7 ± 4.4
Clean water flux (L m^{-2} h^{-1})	361 ± 1.4	360 ± 0.0
Fouled water flux (L m^{-2} h^{-1})	182 ± 2.2	176 ± 4.1
Retentate pressure in (kPa)	310 ± 0.0	310 ± 0.0
Retentate pressure out (kPa)	100 ± 0.0	100 ± 0.0
TMP (kPa)	125 ± 0.1	125 ± 0.0
Pressure drop (kPa)	210 ± 0.0	210 ± 0.0
R_{f_final} (m^{-1})	$3.46 \times 10^5 \pm 4.30 \times 10^4$	$2.94 \times 10^5 \pm 3.07 \times 10^4$
R_{f_irrev} (m^{-1})	$1.75 \times 10^5 \pm 1.99 \times 10^4$	$2.00 \times 10^5 \pm 5.26 \times 10^3$
R_{f_rev} (m^{-1})	$1.71 \times 10^5 \pm 2.31 \times 10^4$	$9.35 \times 10^4 \pm 3.60 \times 10^4$

TMP = Trans-membrane pressure, R_{f_final} = Total fouling accumulated, R_{f_irrev} = Foulant that can only be removed by chemical cleaning. R_{f_rev} = Foulant that can be removed by flushing with water.

3.2.3 Sample preparation and compositional analysis

The skim milk was sampled before MF, while composite retentates and permeates were sampled after MF. The retentate flush was collected and sampled to facilitate mass balance calculations. Samples for dry matter and nitrogen fraction analysis were analysed fresh, while samples for other analyses were stored at 4°C with the addition of a preservative (Broad Spectrum Microtabs® II, D & F Control Systems, Inc., CA, USA).

3.2.4 Dry matter content

The dry matter (DM) content of each sample was determined using a microwave moisture analyser (Smart trac, 5 turbo, CEM Corporation NC, USA).

3.2.5 Analysis of nitrogen fractions

The distribution of nitrogen fractions in skim milk, MF retentate and permeate samples was determined using International Dairy Federation (IDF) standard methods: IDF 20-1 (2014), IDF 20-4 (2016) and IDF 29-1 (2004) to determine crude protein, non-protein nitrogen (NPN) and casein nitrogen content (NCN), respectively. The NPN content was determined by initially adding 15% trichloroacetic acid (Sigma-Aldrich, Wicklow, Ireland) to each sample to precipitate all of the protein within the samples. The NCN was determined by adjusting the pH of each sample to the isoelectric point of casein (pH 4.6), at which point casein precipitates from the solution. The precipitate from the NPN and NCN solutions was removed by filtering through Whatman No.1 filter paper. After filtering, the nitrogen content of each solution was determined by the Kjeldahl method, and the protein content of each sample was determined using a nitrogen-to-protein conversion factor of 6.38.

3.2.6 Protein profile analysis

3.2.6.1 SDS-PAGE

The protein profiles in skim milk, and MF retentates and permeates were determined using pre-cast sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) from Novex Technologies (Thermo Fisher Scientific, Dublin, Ireland), following the method described by Buggy et al. (2017). In brief, all samples were diluted in a sodium dodecyl sulphate (SDS) buffer under reducing conditions at pH 8.4 and a volume of 10 µL of each sample was loaded into the wells of a 12% Bis-Tris Gel. Electrophoresis was carried out using an X-Cell Surelock electrophoresis unit from Novex Technologies, with a MOPS SDS running buffer (Thermo Fisher Scientific, Dublin, Ireland). The samples were standardised based on their protein content, to achieve 10 µg of protein per well, and loading volumes (10 µL) remained constant for all samples. Following electrophoresis, the gels were stained with instant blue stain from Expedeon (Cambridge, UK). Subsequently, the gels were destained in MilliQ® water until a clear background was obtained.

3.2.6.2 High performance liquid chromatography for protein profiling

The method used for this analysis is the same as that described in detail by Gaspard, and Brodkorb (2019). Each sample was dispersed in a 7 M urea (Fisher Scientific, Dublin, Ireland), 20 mM bis tris propane (Sigma-Aldrich, Wicklow, Ireland), and 0.19 mM dithiothreitol (Thermo Fisher Scientific, Dublin, Ireland) buffer, adjusted to pH 7.5. After incubation at room temperature for 1 h, each sample was filtered prior to injection. A sample volume of 5 µL was injected onto a Poroshell 300SB-C18 (Size: 2.1×7.5 mm, 5 µm; Agilent Technologies, Ireland) column with a flow rate of 0.5 mL min^{-1}. The column operated at a constant temperature of 35°C throughout the analysis. Two solvents (denoted A and B) were used as the mobile phases.

The components of the A solvent were water, acetonitrile and trifluoroacetic acid, present in the ratio 900:100:1, respectively, and the components of the B solvent were water, acetonitrile and trifluoroacetic acid in the ratio 100:900:1, respectively. A linear gradient was used in which the non-polar B solvent concentration increased from 26 to 100% over a 36.5 min timespan. A UV-vis detector (61365D MWD Agilent Technologies 1200 series) at an absorbance of 214 nm was used for peak detection throughout sample analysis. Peak integration and system operation was performed using Agilent Chemstation software.

3.2.7 Calculations

The mass balance and the recovery of the total solids and protein fractions were determined as described by Subhir et al. (2022). In brief, the total masses of all streams from MF were recorded and using the corresponding total solids, or nitrogen fraction, the recovery of each component was calculated using equation 1.

$$Recovery = \frac{(component\ \% \times kg\ of\ permeate) + (component\ \% \times kg\ of\ retentate)}{component\ \% \times kg\ of\ feed} \quad (1)$$

The calculations for the quantification of reversible and irreversible fouling, followed the approach described by Subhir et al. (2023). The fouling resistance was calculated by initially measuring the membrane resistance (R_m) using TMP, clean water flux (J_{CWF}) and reverse osmosis water viscosity (η_{water}) according to equation 2.

$$R_m\ (m^{-1}) = \frac{TMP\ (kPa)}{J_{CWF\ (\mathrm{L\ m{-}2\ h{-}1})} \cdot \eta_{water(\mathrm{Pa \cdot s})}} \quad (2)$$

The total fouling resistance (R_f) was calculated using equation 3. The values of TMP, permeate flux ($J_{permeate}$) and permeate viscosity ($\eta_{permeate}$) were used to calculate the total fouling resistance.

$$R_f\ (m^{-1}) = \frac{TMP\ (kPa)}{J_{permeate\ (\mathrm{L\ m{-}2\ h{-}1})} \cdot \eta_{permeate(\mathrm{Pa \cdot s})}} - R_m\ (m^{-1}) \quad (3)$$

The irreversible fouling, that is the foulants that can only be removed by chemical cleaning, was calculated according to equation 4. The irreversible fouling was calculated using TMP, fouled water flux (J_{FWF}) and the water viscosity (η_{water}).

$$R_{f_irrev}(m^{-1}) = \frac{TMP\ (kPa)}{J_{FWF\ (\mathrm{L\ m{-}2\ h{-}1})} \cdot \eta_{water(\mathrm{Pa \cdot s})}} - R_m(m^{-1}) \quad (4)$$

The reversible fouling, or the foulants that can be removed by flushing the membrane system, was calculated according to equation 5 where the R_{f_final} is the fouling resistance calculated before flushing the membrane system.

$$R_{f_rev}(m^{-1}) = R_{f_final}(m^{-1}) - R_{f_irrev}\ (m^{-1}) \quad (5)$$

3.2.8 Statistical data analysis

Statistical analysis was performed using IBM SPSS Statistics (IBM Statistics Inc. Armonk, NY). Statistical significance was determined by one-way ANOVA and paired-samples t-test. Results were considered significant at a *P*-value <0.05.

3.3 Results and discussion

In this study, milk samples with two discrete variants of β-lg were investigated, namely β-lg A and β-lg B. For clarity and consistency, when describing the skim used and the retentates and permeates generated in this study, they will be referred to as the A or B variant respectively, denoting the β-lg variant contained therein. The permeate flux was recorded at one min intervals throughout the process and is shown in Fig 3.1. The average flux for the filtration processes was 61.97 and 62.72 L m^{-2} h^{-1} for the skim milk containing the A and B variant of β-

lg, respectively, with no significant difference ($P > 0.05$) between the two variants. These values are higher than the permeate flux of 45.5 L m^{-2} h^{-1} reported by Subhir et al. (2022) who used similar processing parameters, with the exception of a lower TMP (105 kPa). The MF flux reported by Zulewska, and Barbano (2014), of 71.8 L m^{-2} h^{-1}, is closer to that reported in this study, whereby these authors performed continuous MF using similar process conditions such as temperature, feed volume, and membrane type as applied here. On initial transition from water to skim milk, the flux of the A and B variants was 327 and 249 L m^{-2} h^{-1}, which decreased to 92.3 and 97.7 L m^{-2} h^{-1} respectively, within the first 20 min of processing. After this, the flux for both A and B variant skim milk batches declined gradually until it reached an average steady state of 56.6 and 59.9 L m^{-2} h^{-1} for the skim milk with the A and B variants of β-lg, respectively, for the remainder of the filtration process (Fig 3.1). A study by Tan et al. (2014) investigated the cause of the decline in flux by sampling a piece of the ceramic membrane after MF of skim milk, with their study demonstrating that a dense gel-like fouling layer formed on the surface of the membrane, as well as within the pores, likely due in part to the high TMP (159 kPa) typically used for ceramic filtration. This layer caused a distinct obstruction within the membrane and was highlighted as the main cause of the initial decrease in permeate flux. It has been reported that the composition of membrane fouling material during MF, is mostly made up of whey proteins, with β-lg being the most relevant in terms of foulant material (Mourouzidis-Mourouzis & Karabelas, 2006; Tong et al., 1988). Hence, differences in MF performance as a function of fouling accumulation with respect to the A and B variants of β-lg are of particular interest to dairy processors.

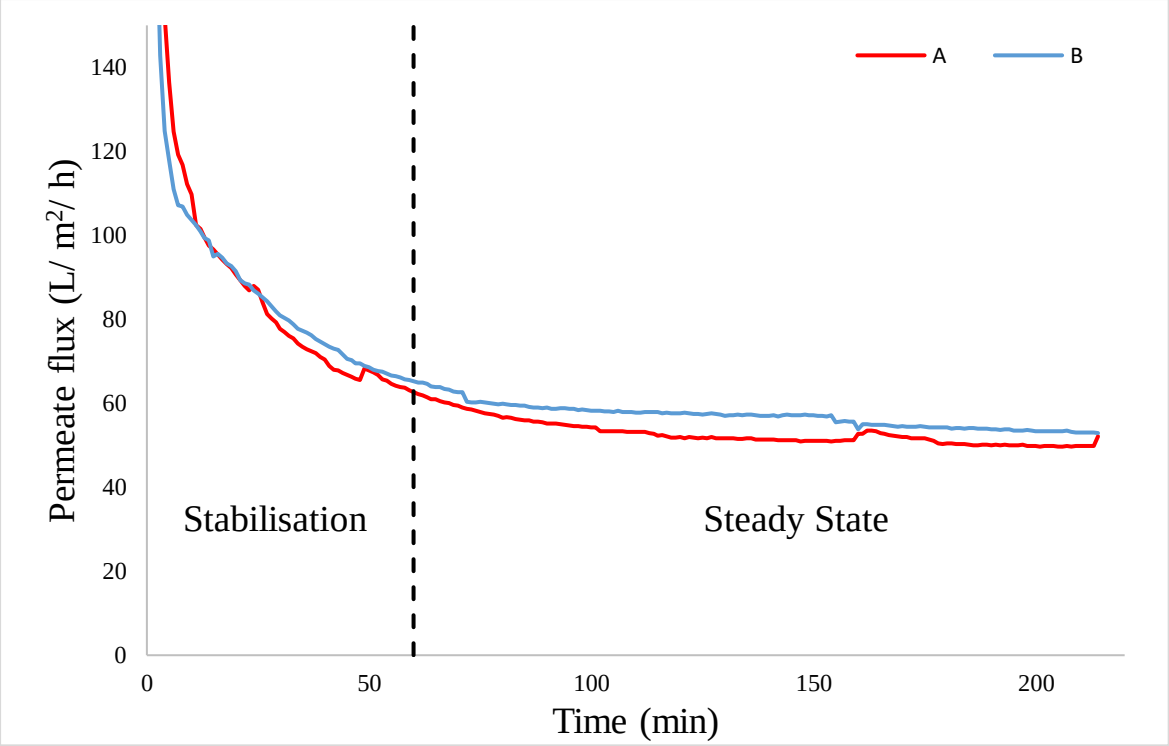

Figure 3.1 Permeate flux as a function of time during ceramic microfiltration. The skim milk containing the A variant of β-lg is indicated by a red line (-) and the skim milk containing the B variant of β-lg is indicated by a blue line (-).

3.3.1 Fouling

While the caseins within skim milk, and the genetic variants thereof are likely to contribute to the accumulation of fouling material, based on the studies by Tong et al. (1988), Mourouzidis-Mourouzis, and Karabelas (2006) and Tan et al. (2014), whey proteins dominate in terms on interaction with the membrane and subsequent fouling phenomena. Therefore, the casein fraction and the genetic variants contain therein will not be discussed in regards to fouling. The fouling resistance as a function of time during filtration is presented in Fig 3.2. The fouling resistance of the MF process increased rapidly in the first few minutes of filtration, before increasing more gradually as a function of processing time/cumulative permeate volume. This trend was common among both variants; however, the degrees to which the discrete skim milk

batches fouled the membrane differed after the initial increase in fouling resistance. Imafidon et al. (1991a) reported structural differences between the A and B variants of β-lg, which could be an important contributing factor to the differences in fouling between the two variants. These differences in molecular structure of the protein are due to the amino acid substitutions, in which an aspartic acid of the A variant is substituted with glycine in the B variant at position 64, and a valine of the A variant is substituted with alanine in the B variant, at position 118. In their study, Imafidon et al. (1991a) found that the B variant had a greater proportion of β-sheets than the A variant. This may have led to the A structure being less stable during processing, which can lead to greater aggregation of β-lg, resulting in higher deposition rates at the membrane surface and increased fouling resistance. Previous studies have highlighted the differences in the stability of milk based on their β-lg genotype. Keppler et al. (2014) reported that the A variant of β-lg was less heat-stable than the B variant. This could be a contributing factor to the difference in fouling between the two variants, with more denatured A variant of β-lg contributing to a greater accumulation of foulant at the membrane surface.

To better understand the type of fouling accumulation, clean and fouled water fluxes were recorded before and after the MF of skim milk at 50°C. The clean water flux was 360 and 362 L m^{-2} h^{-1} for the skim milk containing the A and B variants, respectively. The fouled water flux was 174 and 189 L m^{-2} h^{-1} for A and B variant trials, respectively, indicating that the fouled water flux was 48.3 and 52.2% of the initial clean water flux for the skim milk containing the A and B variants, respectively. These values are in broad agreement with those reported by Zulewska et al. (2009), who processed skim milk using similar membrane and process conditions as this study, and reported that the fouled water flux was ~55% of the initial clean water flux in their study.

Based on the resistance in series model proposed by Kühnl et al. (2010), the skim milk containing the A variant had a final fouling resistance (R_{f_final}) of 3.46×10^5 m^{-1} and the B

variant had a R_{f_final} of 2.95×10^5 m^{-1}, which was significantly ($P = 0.043$) lower than that of the A variant. The resistance due to foulants which can be removed by flushing with water, R_{f_rev} (reversible), and foulants that require removal through chemical cleaning, R_{f_irrev} (irreversible) differed between the variants. The R_{f_rev} for the skim milk containing the A variant was 1.71×10^5 m^{-1}, which was significantly ($P = 0.039$) greater than that for the B variant, at 9.35×10^4 m^{-1}. The skim milk containing the A variant had an R_{f_irrev} of 1.75×10^5 m^{-1}, in comparison with 2.00×10^5 m^{-1} for the B variant, which was not significantly ($P = 0.19$) different. This indicates that the two variants contributed to irreversible fouling to the same extent, and would be expected to require a similar cleaning procedure to restore the membrane.

Comparison of reversible and irreversible fouling indicates that more reversible foulants accumulated at the membrane surface for the skim milk containing the A variant, possibly as a result of the higher mass transfer of whey proteins to the permeate, which will be discussed later. Additionally, the alumina material from which the ceramic membrane is made has an isoelectric point (pI) at pH ~9, and has a positive charge at pH values less than the pI (Franks & Meagher, 2003). As there is a difference in charge between the A and B variants of β-lg, this may contribute to the discrete fouling behaviour. The difference in charge between A and B variants is due to the amino acid substitution at position 118, in which the A variant has an aspartic acid and the B variant has a glycine. The aspartic acid substitution has a carboxylic acid side chain, which carries a negative charge, while glycine contains only a single carbon side chain (Keppler et al., 2014; Wu, 2021). The amino acid substitution of valine with alanine, which occurs at position 64 for the A and B variant respectively, is unlikely to contribute to the net charge of the proteins as these amino acids contain no charged sidechains (Wu, 2021). With this in mind, it is possible that the higher net negative charge of the A variant, coupled with the positive charge of the membrane, leads to greater concentration polarisation at the membrane surface and hence increased reversible fouling rates compared to the B variant. In addition, the

difference in the concentration of the whey proteins in the skim milk, in which the skim milk with the A variant of β-lg had significantly ($P< 0.05$) higher whey protein content than the skim milk containing the B variant of β-lg could also be a contributing factor to the differences observed in the accumulation of foulant on the membrane surface. The fouling values reported in the present study are much higher than those reported by Subhir et al. (2023), who microfiltered 300 kg of skimmed milk at 50 °C; however, the difference in results between their study and the current study is likely due to the larger surface area and lower TMP applied using polymeric MF membranes.

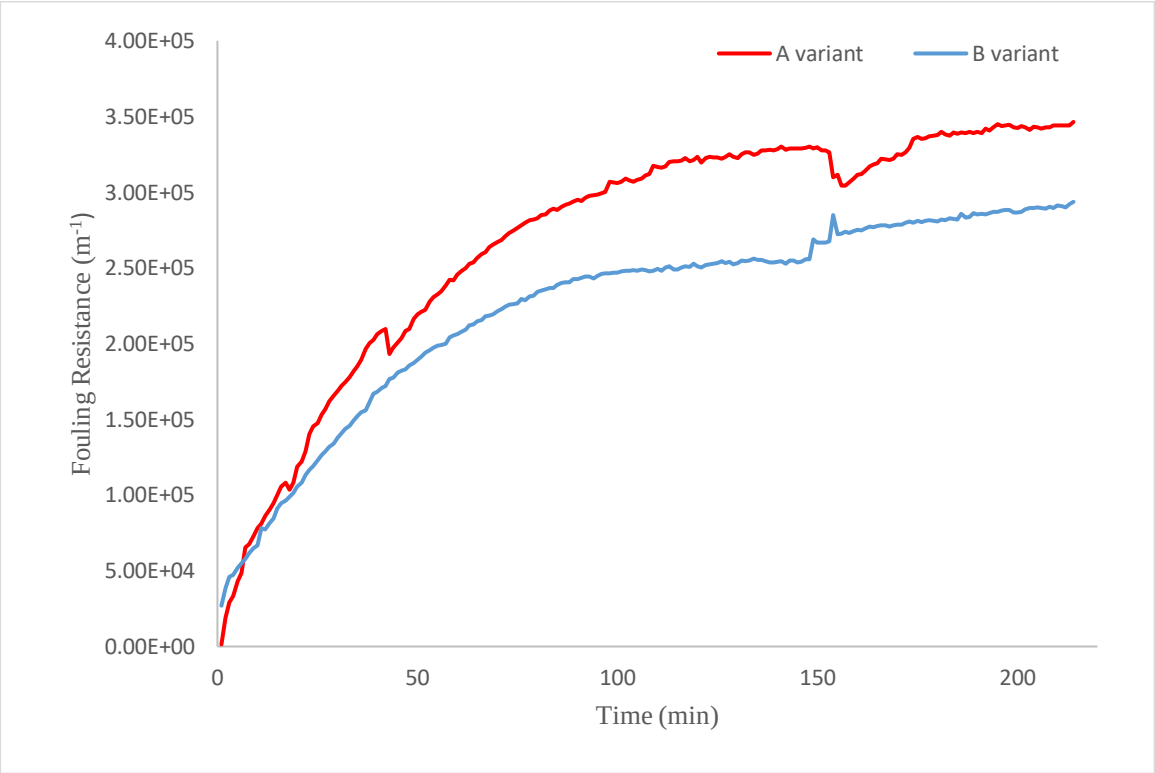

Figure 3.2 Accumulation of fouling resistance as a function of time during MF of skim milk with β-lg variants A (red) and B (blue)

3.3.2 Mass balance and whey protein partitioning

The mass balance and compositional analysis of the retentate, permeate, and feed streams are presented in Table 3.2. The average recoveries of the duplicated trials for dry matter, crude

protein, and true protein for the samples containing A variant were 101, 95.2, and 95.5%, respectively, in comparison with 102, 100, and 99.3% for the samples with the B variant. Challenges in quantifying the recovery of whey proteins using the standard NCN method (IDF 29-1:2004) have already been reported (Nelson & Barbano, 2005; Subhir et al., 2022). This issue manifests as an under or over estimation of whey protein in processed streams, including permeates and retentates generated during MF of skim milk, linked to both serum caseins and partially unfolded whey proteins that are quantified incorrectly according to the test. In the present study, the NCN analysis provided a mass balance for whey protein recovery of 112.7 and 97.2% for the samples containing the A and B variants, respectively. While an overestimation of whey protein recovery was reported for the samples containing the A variant of β-lg, it is not as great as that reported by Subhir et al. (2022), for MF of commercially sourced skim milk (134.5%). Low quantities of serum casein detected by SDS-PAGE in the permeate stream indicates good separation of micellar casein between the permeate and retentate streams. While there were faint casein bands present in the permeate samples, their presence in the SDS-PAGE gels is negligible, and unlikely to contribute meaningfully to the inaccuracies in the determination of whey protein recoveries (Fig 3.3). Additionally, these faint bands could also be minor whey proteins that migrate with casein. Results from the Kjeldahl analysis indicate the presence of casein within the permeate streams; however, this is not supported by the protein profile generated by HPLC (Table 3.2, Fig 3.4). These HPLC results are in agreement with those of Jørgensen et al. (2016) and Song et al. (2022), who reported very little or no casein in the permeate stream while operating at the same temperature and using a similar MF membrane to that used in the present study. The factors contributing to the overestimation of whey proteins and underestimation of casein recoveries include, overestimation of casein in the pasteurised skim milk, due to isoelectric precipitation of denatured whey proteins, quantified as casein according to the NCN test, and underestimation

of casein in the retentate due to the presence of unprecipitated serum casein in NCN filtrates (Subhir et al., 2022).

It is interesting to note the disparity in the recoveries of the whey proteins between the A (112.7%) and B (97.2%) variants of β-lg. This difference could further indicate a variation in the stability of the A and B variants to thermal and mechanical damage during processing; however, the exact reason for the differences in recoveries between the milk samples is not known.

Table 3.2 Mass balance and nitrogen fraction analysis of milk, retentate and permeate streams.

Sample	Mass collected (kg)	Dry Matter (% w/w)	Crude Protein (% w/w)	True Protein (% w/w)	Non-protein nitrogen (% w/w)	Whey proteins (% w/w)	Casein (% w/w)	Whey protein :Casein
A Raw	442.3 ± 10.1	14.00 ± 0.35[a]	3.51 ± 0.02[a]	3.35 ± 0.04[a]	0.14 ± 0.01[a]	0.53 ± 0.04[a]	2.65 ± 0.04[a]	16.7:83.3
B Raw	438.4 ± 8.3	13.54 ± 0.28[b]	3.38 ± 0.11[b]	3.21 ± 0.11[b]	0.15 ± 0.00[a]	0.47 ± 0.03[b]	2.58 ± 0.07[b]	15.4:84.6
A Skim	370.8 ± 6.01	9.65 ± 0.10[a]	3.58 ± 0.02[a]	3.40 ± 0.02[a]	0.16 ± 0.01[a]	0.60 ± 0.01[a]	2.80 ± 0.02[a]	17.6:82.4
B Skim	371.8 ± 1.06	9.38 ± 0.23[b]	3.46 ± 0.11[b]	3.28 ± 0.11[b]	0.18 ± 0.00[a]	0.53 ± 0.01[b]	2.75 ± 0.03[b]	16.2:83.8
A Retentate	157.5 ± 3.54	12.83 ± 0.04[a]	7.01 ± 0.04[a]	6.86 ± 0.04[a]	0.15 ± 0.00[a]	0.87 ± 0.02[a]	5.99 ± 0.04[a]	12.7:87.3
B Retentate	158.5 ± 4.95	13.07 ± 0.22[b]	7.29 ± 0.03[b]	7.05 ± 0.06[b]	0.17 ± 0.01[a]	0.72 ± 0.06[b]	6.13 ± 0.02[b]	10.5:89.5
A Permeate	258.3 ± 2.47	5.77 ± 0.10[a]	0.62 ± 0.05[a]	0.48 ± 0.03[a]	0.14 ± 0.00[a]	0.44 ± 0.01[a]	0.04 ± 0.01[a]	91.7:8.3
B Permeate	258.3 ± 3.89	5.69 ± 0.04[b]	0.51 ± 0.02[b]	0.36 ± 0.01[b]	0.15 ± 0.00[a]	0.30 ± 0.01[b]	0.06 ± 0.01[a]	83.3:16.7
% A Recovery		101.0	95.2	95.5	100.8	112.7	91.9	
% B Recovery		101.5	100.1	99.3	98.2	97.2	96.5	

Samples with an A or B refer to the genetic variant of β-lg. Results of the same sample type and that are within the same column, are denoted as significantly ($P < 0.05$) different if they contain a different superscript letter. These values are the results of duplicate microfiltration experiments.

The efficiency of removing whey proteins from skim milk was measured by calculating the mass of whey proteins in the skim milk and the permeate stream. Even though the levels of whey proteins and whey protein:casein are higher in skim milk containing the A variant compared to the B variant, the removal efficiency of the whey proteins from the skim milk with the A variant was higher than the B variant. The percentage of whey proteins that permeated, relative to the concentration in the initial skim milk during MF (without diafiltration), was 48.5 and 37.6% for the A and B variants, respectively. It is also noteworthy that, even with higher levels of reversible fouling, more of the whey proteins within the skim milk containing the A variant permeated through the membrane than the skim milk with the B variant. This would also suggest that the reversible fouling layer is not a rate-limiting factor when it comes to the partitioning of β-lg. Skim milk containing the A variant had significantly higher total protein (P=0.027), casein (P=0.009) and whey proteins (P=0.033) than the B variant. While there was no genotype screening for caseins, it is possible that genetic variation among the caseins may have contributed to the partitioning of the whey proteins; however, that was out of the scope for this study. This difference in protein content in milk linked to β-lg genetic variation has been reported before (Heck, Schennink, et al., 2009a). In addition, while there was 13.1% more whey protein in the skim milk containing the A variant than the B variant, 46.7% more whey protein was permeated from the skim milk containing the A variant compared to the B variant during MF. This indicates that there was a much higher rejection of whey proteins from skim milk containing the B variant of β-lg.

The protein profiles by SDS-PAGE of the various MF streams, and the feed, are shown in Fig 3.3. Distinct bands for the six major milk proteins of α_{s1}-, α_{s2}-, β- and κ-caseins, β-lg and α-lactalbumin, as well as some minor whey proteins, were observed in the raw milk and skim milk lanes. Although SDS-PAGE reflects a more qualitative analysis, more intense bands of the A variant of β-lg were observed in the skim milk and retentate lanes (2 and 4) compared to

those of the B variant. This is likely correlated with the higher whey protein content in the skim milk containing the A variant of β-lg. The MF permeate lanes for both genetic variants have faint bands, indicating the presence of some caseins within the samples. The presence of serum casein in the permeate generated using ceramic MF has been reported in previous studies (Subhir et al., 2022); the authors demonstrated that, during ceramic microfiltration of skim milk, non-micellar serum caseins permeated through the membrane. It is therefore possible that the casein bands observed in the permeate stream of the present study are non-micellar serum caseins. The very faint bands, or negligible levels of serum casein identified using SDS-PAGE for the MF permeates also calls into question the relatively high casein contents reported in Table 3.2 according to the NCN test. This indicates that the reported casein was likely predominantly composed of whey proteins denatured during processing (which would have lost their solubility at pH 4.6), and were thus incorrectly quantified as casein according to the test. Other studies that have found casein in the permeate streams of microfiltration have suggested that it could be due to the transition of β-casein from the colloidal to the serum phase, in which state it is more susceptible to permeation through the membrane (Subhir et al., 2023). However, as transition of β-casein from the colloidal to serum phase is driven by temperatures much lower than those used in this study (50°C), it is unlikely to be present in the permeate. Thus the faint protein bands observed within the molecular weight range typical of individual caseins, may actually reflect the presence of minor whey proteins.

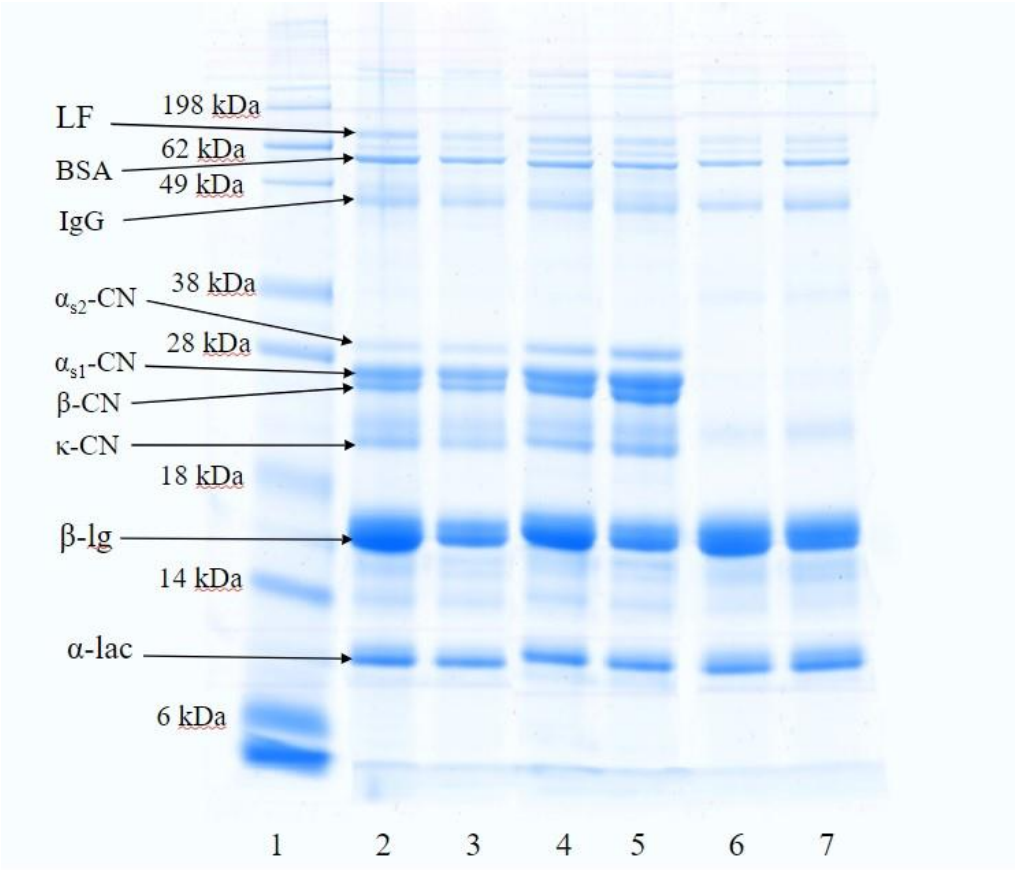

Figure 3.3 SDS PAGE of the protein profiles of feed, retentate, and permeate streams of micro-filtration. (1) Molecular mass ladder, (2) skim milk A, (3) skim milk B, (4) MF retentate A, (5) MF retentate B, (6) MF permeate A, (7) MF permeate B. Abbreviations are as follows: lactoferrin (LF), bovine serum albumin (BSA), heavy-chain immune globulin (IgG), casein (CN), β-lactoglobulin (β-lg) and α-lactalbumin (α-lac).

The protein profiles generated by HPLC complement the SDS-PAGE results, and are presented in Fig 3.4. The HPLC analysis confirmed that all the MF streams contained either the A or B variant of β-lg and that no cross-contamination between the variants occurred. The six main protein fractions of milk elute from the column in the following order: κ-casein, α_{s2}-casein, α_{s1}-casein, β-casein, α-lactalbumin, and lastly β-lg. The A and B variants of β-lg elute at retention times differing by around two min, with the B variant eluting first, at 15 min 30 s, and the A variant at 17 min 30 s. The HPLC data also indicated the effective retention of the casein fraction by the MF membrane, which further supports the inaccuracies attributed to the NCN

method when quantifying whey proteins in MF streams. As the HPLC analysis did not detect the presence of caseins within the permeate streams, this may further support the possibility that the faint bands observed by SDS-PAGE are not casein and may instead be minor whey proteins that have permeated during MF.

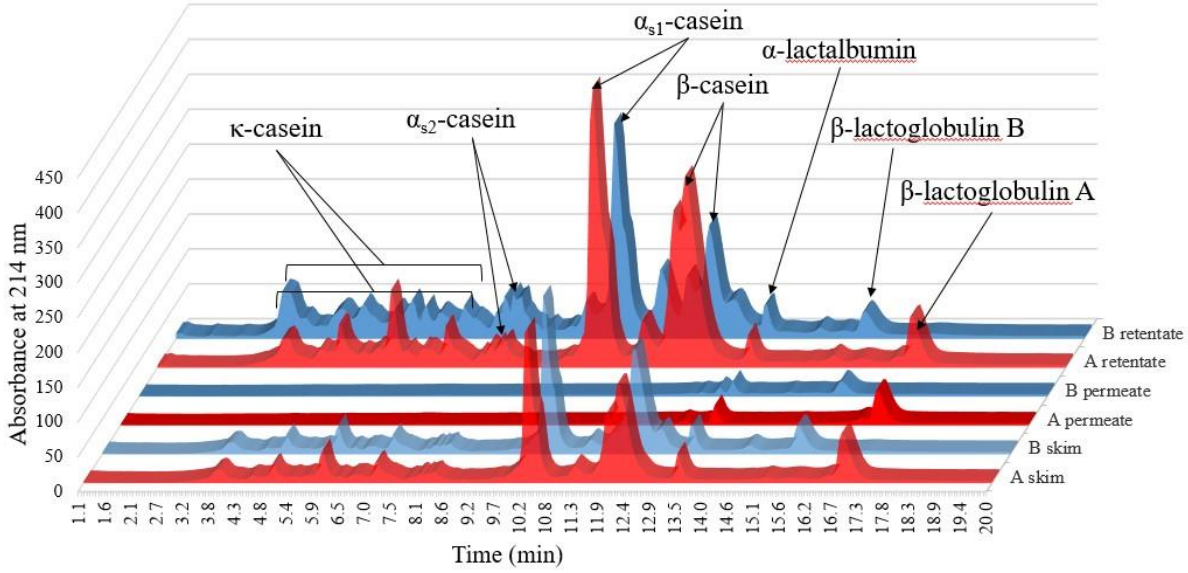

Figure 3.4 High performance liquid chromatography protein profiles of the skim milk, microfiltration permeate and retentate streams for A and B variants.

3.4 Conclusion

The objective of this study was to investigate the effects of β-lg genetic variants A and B on the separation efficiency of whey proteins during MF, which to the best of our knowledge, has not been reported previously. Transmission of whey proteins during MF was higher for skim milk containing the A variant of β-lg compared to the B variant, independent of the initial whey protein content of the skim milk. Separately, the skim milk containing the A variant had higher total and reversible fouling accumulation than the B variant, although it was deemed not rate-

limiting from a whey protein transmission perspective. While the observations reported here pertain to separation of whey proteins directly from skim milk using ceramic membranes, it is more common to use polymeric membranes within the dairy industry. Thus, future studies could assess the effect of β-lg variants on the performance of polymeric MF systems as applied to skim milk or cheese whey during manufacture of whey protein ingredients, which may be of interest to whey processors.

Declaration of competing interest

The authors declare that they have no known competing financial or personal interests that could have appeared to influence the work reported in this paper.

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3.5 References

Aich, R., Batabyal, S., & Joardar, S. N. (2015). Isolation and purification of beta-lactoglobulin from cow milk. *Vet World, 8*, 621-624.

Aschaffenburg, R., & Drewry, J. (1957). Genetics of the β-Lactoglobulins of Cow's Milk. *Nature, 180*, 376-378.

Beckman, S. L., Zulewska, J., Newbold, M., & Barbano, D. M. (2010). Production efficiency of micellar casein concentrate using polymeric spiral-wound microfiltration membranes1. *Journal of Dairy Science, 93*, 4506-4517.

Bhattacharya, R., Rose, P. W., Burley, S. K., & Prlić, A. (2017). Impact of genetic variation on three dimensional structure and function of proteins. *Public Library of Science One, 12*, e0171355.

Boland, M. (2011). Whey proteins. In *Handbook of Food Proteins* (First Edition) (pp. 30-55): Woodhead Publishing.

Buggy, A. K., McManus, J. J., Brodkorb, A., Carthy, N. M., & Fenelon, M. A. (2017). Stabilising effect of α-lactalbumin on concentrated infant milk formula emulsions heat treated pre-or post-homogenisation. *Dairy Science & Technology, 96*, 845-859.

Caroli, A. M., Chessa, S., & Erhardt, G. J. (2009). Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *Journal of Dairy Science, 92*, 5335-5352.

Carter, B. G., Cheng, N., Kapoor, R., Meletharayil, G. H., & Drake, M. A. (2021). Invited review: Microfiltration-derived casein and whey proteins from milk. *Journal of Dairy Science, 104*, 2465-2479.

Farrell Jr, H., Jimenez-Flores, R., Bleck, G., Brown, E., Butler, J., Creamer, L., Hicks, C., Hollar, C., Ng-Kwai-Hang, K., & Swaisgood, H. (2004). Nomenclature of the proteins of cows' milk—Sixth revision. *Journal of Dairy Science, 87*, 1641-1674.

Fox, P. F., Uniacke-Lowe, T., McSweeney, P. L. H., & O'Mahony, J. A. (2015). *Dairy Chemistry and Biochemistry* (Second Edition): Springer.

Franks, G. V., & Meagher, L. (2003). The isoelectric points of sapphire crystals and alpha-alumina powder. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 214*, 99-110.

Gaspard, S. J., & Brodkorb, A. (2019). The use of high performance liquid chromatography for the characterization of the unfolding and aggregation of dairy proteins. In *Protein Self-Assembly* (First Edition) (pp. 103-115): Springer.

Glantz, M., Devold, T., Vegarud, G., Månsson, H. L., Stålhammar, H., & Paulsson, M. (2010). Importance of casein micelle size and milk composition for milk gelation. *Journal of Dairy Science, 93*, 1444-1451.

Heck, J., Schennink, A., Van Valenberg, H., Bovenhuis, H., Visker, M., Van Arendonk, J., & Van Hooijdonk, A. (2009). Effects of milk protein variants on the protein composition of bovine milk. *Journal of Dairy Science, 92*, 1192-1202.

Hurt, E., Zulewska, J., Newbold, M., & Barbano, D. M. (2010). Micellar casein concentrate production with a 3X, 3-stage, uniform transmembrane pressure ceramic membrane process at 50°C1. *Journal of Dairy Science, 93*, 5588-5600.

ICBF (2023). *National Genotyping Programme.* https://www.icbf.com/national-genotyping-programme/

Imafidon, G. I., Ng-Kwai-Hang, K. F., Harwalkar, V. R., & Ma, C. Y. (1991). Differential Scanning Calorimetric Study of Different Genetic Variants of β-Lactoglobulin. *Journal of Dairy Science, 74*, 2416-2422.

Jørgensen, C. E., Abrahamsen, R. K., Rukke, E.-O., Johansen, A.-G., Schüller, R. B., & Skeie, S. B. (2016). Optimization of protein fractionation by skim milk microfiltration: Choice of ceramic membrane pore size and filtration temperature. *Journal of Dairy Science, 99*, 6164-6179.

Keppler, J. K., Sönnichsen, F. D., Lorenzen, P.-C., & Schwarz, K. (2014). Differences in heat stability and ligand binding among β-lactoglobulin genetic variants A, B and C using 1H NMR and fluorescence quenching. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1844*, 1083-1093.

Kühnl, W., Piry, A., Kaufmann, V., Grein, T., Ripperger, S., & Kulozik, U. (2010). Impact of colloidal interactions on the flux in cross-flow microfiltration of milk at different pH values: A surface energy approach. *Journal of Membrane Science, 352*, 107-115.

Li, C. H. (1946). Electrophoretic Inhomogeneity of Crystalline Beta-lactoglobulin. *Journal of the American Chemical Society, 68*, 2746-2747.

McCarthy, W. P., Blais, H. N., O'Callaghan, T. F., Hossain, M., Moloney, M., Danaher, M., O'Connor, C., & Tobin, J. T. (2022). Application of nanofiltration for the removal of chlorate from skim milk. *International Dairy Journal, 128*, 105321.

Mourouzidis-Mourouzis, S. A., & Karabelas, A. J. (2006). Whey protein fouling of microfiltration ceramic membranes. *Journal of Membrane Science, 282*, 124-132.

Nelson, B. K., & Barbano, D. M. (2005). A Microfiltration Process to Maximize Removal of Serum Proteins from Skim Milk Before Cheese Making. *Journal of Dairy Science, 88*, 1891-1900.

Oliveira, K. M., Valente-Mesquita, V. L., Botelho, M. M., Sawyer, L., Ferreira, S. T., & Polikarpov, I. (2001). Crystal structures of bovine β-lactoglobulin in the orthorhombic space group C2221: Structural differences between genetic variants A and B and features of the Tanford transition. *European Journal of Biochemistry, 268*, 477-484.

Sawyer, L. (2012). β-Lactoglobulin. In *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects*, (Fourth Edition) (pp. 211-259): Springer.

Song, B., Zhang, S., Zhang, Y., Zhang, W., Pang, X., & Lv, J. (2022). Ceramic membrane filtration of skim milk for the production of a casein-enriched permeate. *International Dairy Journal, 135*, 105478.

Subhir, S., McSweeney, P. L. H., Fenelon, M. A., Magan, J. B., & Tobin, J. T. (2022). Suitability of nitrogen fractions determination to assess serum protein separation efficiency from a mass balance perspective during microfiltration of skim milk. *International Dairy Journal, 128*, 105319.

Subhir, S., McSweeney, P. L. H., Murphy, E. G., & Tobin, J. T. (2023). The impact of temperature on flux, protein transmission and energy usage during microfiltration of skim milk using polymeric membranes. *International Dairy Journal*, 105729.

Tan, T. J., Wang, D., & Moraru, C. I. (2014). A physicochemical investigation of membrane fouling in cold microfiltration of skim milk. *Journal of Dairy Science, 97*, 4759-4771.

Tong, P. S., Barbano, D. M., & Rudan, M. A. (1988). Characterization of Proteinaceous Membrane Foulants and Flux Decline During the Early Stages of Whole Milk Ultrafiltration. *Journal of Dairy Science, 71*, 604-612.

Wang, Q., Hulzebosch, A., & Bovenhuis, H. (2016). Genetic and environmental variation in bovine milk infrared spectra. *Journal of Dairy Science, 99*, 6793-6803.

Wu, G. (2021). *Amino acids: biochemistry and nutrition* (Second Edition): CRC Press.

Zulewska, J., & Barbano, D. M. (2014). The effect of linear velocity and flux on performance of ceramic graded permeability membranes when processing skim milk at 50°Cs, *Journal of Dairy Science, 97*, 2619-2632.

Chapter 4

The impact of genetic variation on the techno-functional properties of β-lactoglobulin

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Declaration

This chapter was written by author LMK and reviewed by his co-authors. WPM and SS assisted in the production of whey protein isolate. BH assisted in organising the cows for milk collection. AJ assisted in milk collection.

Abstract

β-lactoglobulin (β-lg) is the most abundant whey protein in milk. There are two main genetic variants of β-lg, namely the A and B variant, which differ in the specific amino acids at positions 64 and 118, whereby aspartic acid is replaced with glycine and alanine is replaced with valine, respectively. In this study, whey protein isolate (WPI) powders were produced containing exclusively the A or B variant of β-lg. Analysis of the secondary structure of the A and B variants by fourier transform infrared (FTIR) spectroscopy indicated that the quantity of beta sheets was higher in the B than the A variant. Differential scanning calorimetry (DSC) analysis indicated that the A and B variants had thermal denaturation onset temperatures ($T_{d\ onset}$) of 72.01 and 75.58°C, and peak denaturation temperatures (T_d) of 74.50 and 78.39°C, respectively. The difference in $T_{d\ onset}$ and T_d are possibly associated with the differences in the proportion of beta sheets between the genetic variants. Analysis of the heat-induced gelation properties demonstrated that, in the pH range of 6.6-7.2, the B variant of β-lg produced stronger gels than the A variant, as evidenced by higher storage modulus values. This was possibly due to increased electrostatic repulsion between the protein molecules, caused by the additional charged amino acid in the A variant of β-lg. These findings could be of importance to processors who manufacture whey products, as the differences in thermal behaviour between the A and B variant could impact fouling during processing and subsequent functionality in finished products.

4.1 Introduction

The whey protein fraction accounts for around 20% of the total protein in milk (Gai et al., 2021). The proteins β-lactoglobulin (β-lg), α-lactalbumin, blood serum albumin, and immunoglobulin, are individual soluble proteins within whey, with β-lg found in the highest concentration (Farrell et al., 2004a; Manderson et al., 1998a). β-lg is an 18 kDa globular protein comprised of 162 amino acids, the biological function of which has been suggested to involve the transport of hydrophobic retinol and fatty acids through the stomach to receptors in the intestine, in addition to enzyme regulation, while also contributing to the passive immunity of the calf (Fogolari et al., 1998; Kontopidis et al., 2004). The secondary structure of β-lg is comprised largely of random coils (47%) and β-sheets (45%), with a small number of α-helices (8%), which gives rise to a conical β-barrel structure (Deeth & Bansal, 2019; Kontopidis et al., 2004). A study by Boye et al. (1997) found that the proportions of α-helices and β-sheets can vary, depending on the genetic variant of β-lg, and these structural changes may lead to differences in functional properties.

In 1946, the first genetic variants of milk proteins were discovered in β-lg (Li, 1946). Since then, 11 genotypes of β-lg have been identified in bovine milk, with genotypes AA and BB being the most frequently occurring homozygous genotypes (Farrell et al., 2004a; Gai et al., 2021). These common genotypes can be expressed in one of three ways, two homogenous genotypes, AA or BB, and a heterogeneous haplotype AB (Farrell et al., 2004a; Gai et al., 2021). The heterogeneous alleles of β-lg are co-dominant, meaning, in the case of an AB genotype, both A and B variants of β-lg are expressed in the cow's milk (Sawyer & Kontopidis, 2000). The changes in the primary structure between genetic variants are often small, differing by only one or two amino acids (Farrell et al., 2004a). These small changes can significantly impact the structure of the protein and, ultimately, its functional properties (Heck, Schennink, et al., 2009a). The changes in amino acid sequence between β-lg A and B occur at position 64,

where the A variant has an aspartic acid and the B variant has a glycine. A second substitution occurs at position 118, where the A variant has alanine, and the B variant has a valine (Caroli et al., 2009). Previous studies have shown that the genetic variants of β-lg can impact the milk protein composition, in which it has been reported that milk with the A variant typically has a higher whey protein content than the B variant, due to a greater expression of β-lg (Heck, Schennink, et al., 2009a).

The literature regarding the impact of genetic variants of β-lg on protein functionality is somewhat contradictory and mostly pertains to β-lg in milk. For example, milk containing the B variant of β-lg has been the most consistently identified variant associated with improved rennet gelation properties, including shorter gelation onset time, faster gel firming rate and stronger gels (Jensen, Poulsen, et al., 2012; Meza-Nieto et al., 2007). An increase in protein, casein number and total solids, linked to milk containing the B variant of β-lg, and a concomitant improvement in rennet gelation properties, compared to milk with the A and AB variant, has been reported (Meza-Nieto et al., 2013). In contrast, Glantz et al. (2011) reported that milk with the A variant of β-lg had better rennet gelation properties than milk with the B variant; however, no explanation was provided to support this observation. Instead, the authors highlighted the contradictory reports within the literature, pertaining to the impact of genetic variation of β-lg on the rennet gelation properties of milk, and suggested that more studies are needed before these variants can be used as a genetic marker for future breeding programmes. The contradictory results found within the literature could potentially be explained by certain differences within the composition of the samples and the methodology between the different studies. For instance, the pH and calcium to negative charge ratio of β-lg has been reported to influence the heat inducted aggregation of the different variants of β-lg (Khaldi et al., 2018; O'Kennedy et al., 2006).

The heat stability of milk is also influenced by genetic variants of β-lg (McLean et al., 1987; Michalcová & Krupová, 2007); for example, milk with the A variant of β-lg has been demonstrated to have a longer heat coagulation time (HCT) (Bikker et al., 2000; Michalcová & Krupová, 2007). Early research on the impact of β-lg on heat stability of milk suggested that one of the reasons for the more stable nature of β-lg A, is the greater net negative charge of the unfolded protein compared to other variants of β-lg (B and C), which increases the electrostatic repulsion between β-lg molecules, reducing aggregation (de Wit, 2009; Manderson et al., 1998a). However; in contrast with the results of Bikker et al. (2000) and Michalcová, and Krupová (2007), Keppler et al. (2014) suggested that the B variant was more heat stable than the A variant, whereby purified samples of β-lg were assessed for heat stability using nuclear magnetic resonance spectroscopy. The authors reported that the difference in heat stability between the two variants was due to a shift in the α-helix secondary structural element of the A variant, which exposed a cysteine group, and also due to the A variant having a less stable methyl group than the B variant which help with hydrophobic interactions.

To further elucidate differences in functional properties of β-lg between the two most common variants in milk, the present study produced whey protein isolate (WPI) powders with the β-lg genetic variants A or B using a combination of microfiltration (MF), ultrafiltration (UF), and spray drying. The objective of this study was to analyse the functional implications of genetic variants of β-lg in a protein isolate form, whereby possible confounding effects of the other components of milk are removed. This study will provide deeper insights into the impact of genetic variation of β-lg on thermal stability and gel forming properties. These results could help identify variants of β-lg that would be of importance to whey protein concentrate/isolate manufacturers, as the differences in their functional properties might provide options for improving process efficiency and also highlight variants that can benefit product formulation.

4.2 Materials and methods

4.2.1 Assembly and processing of milk

Within a herd of 127 cows, raw milk samples were obtained from 45 cows, which were categorised into two groups based on their β-lg genotype, β-lg AA (n=19) and β-lg BB (n=26), based on their gene expression. Prior to milk collection, the genotypes of the individual cows within the herd were identified using the national genotyping program, provided by the Irish Cattle Breeding Federation (ICBF, 2022), and validated by high performance liquid chromatography (HPLC) analysis. Milk was collected from the Teagasc research farm Curtins, Moorepark, Fermoy, Co. Cork, Ireland. Each cow's milk was individually collected and segregated depending on β-lg variant in separate bulk tanks across four consecutive milkings and stored at 4°C prior to collection. Milk collection was carried out in duplicate, across a two week period.

Individual batches of milk were skimmed using a centrifugal cream separator (GEA Westfalia Oelde, Germany) to a final fat concentration of 0.025% w/w. Pasteurisation of the milk was performed using a semi commercial scale heat exchanger (Moorepark Technology Ltd, Fermoy, Co. Cork, Ireland), at 73°C for 15 s, then cooled to 4°C. Following pasteurisation, each milk sample was transferred to a jacketed tank and held at 4°C prior to membrane processing.

4.2.2 Membrane processing

On the day of processing, the skim milk was heated to 50°C before filtration. A pilot-scale membrane filtration plant (GEA Process Technologies, Dublin, Ireland) was used, operating in continuous mode, with separate collection tanks for retentate and permeate streams. The

membrane plant was equipped with three 0.1 µm ceramic graded permeability (GP) microfiltration (MF) membranes (Membralox® model, EP 3730, 0.1 µm alumina, Pall Corp, California, CA, USA) with a total surface area of 1.05 m², and was operated in continuous mode. Filtration was carried out in duplicate, and a discrete batch of pasteurised skim milk was used for each trial. Prior to and following filtration, the membrane plant and membranes were subjected to standard cleaning-in-place procedures (McCarthy et al., 2022). On the day of processing, approximately 500 kg of pasteurised skim milk was subjected to MF, to a volume concentration factor (VCF) of 3 at 50°C. The MF permeate was concentrated and diafiltered, reaching a final dry matter of ~15%, using a combination of pilot- and lab-scale UF equipment, fitted with 10 kDa spiral-wound polymeric UF membranes, operating at a temperature of 15°C (Model ST, Synder Filtration, Vacaville, CA 95688, United States).

4.2.3 Spray drying

Four whey protein isolate (WPI) powders were produced by drying the UF retentate using a single-stage spray dryer (Anhydro F1 Lab Dryer; Copenhagen, Denmark). The spray dryer was equipped with a two-fluid nozzle atomisation system (Type 1/8 JAC 316ss), operating under counter-flow drying conditions. The atomisation pressure was set at approximately 2-3 bar, while the air inlet and outlet temperatures were maintained at 185 and 85°C, respectively. The powders were then packaged and sealed in aluminium bags before storage in a moisture controlled room at 16°C.

4.2.4 Sample preparation

Prior to functional analysis, solutions of either 3 or 10% protein (w/v) were prepared from the four WPI powders produced, which contained either the A and B variants of β-lg (duplicate powders for each variant). Powders were rehydrated with Milli-Q water, and agitated for 2 h

using a magnetic stirrer at 500 rpm before being maintained at 4°C overnight to enable complete rehydration. Composite solutions of each variant were prepared by mixing equal volumes of the 3 or 10% protein (w/v) solution prepared from the duplicate A and B powders, respectively. The reconstituted samples are denoted as A and B, with respect to the genetic variant contained therein, throughout this study. To mimic the heterogenous AB haplotype of β-lg, equal aliquots of the A and B solutions were mixed together, and this mixture is denoted as AB throughout this study. It should be noted that, in milk, the A variant of β-lg has a higher expression than the B variant; however, for the purposes of the analysis in this study, a direct comparison of the impact of genetic variation of β-lg on it functional properties, without the influence of natural difference in concentration, was conducted.

4.2.5 Powder composition

The nitrogen fractions of the A and B WPI powders were analysed using the Kjeldahl method. The total nitrogen content of the powders was determined as described by IDF (2014). The non-protein nitrogen (NPN) and the non-casein nitrogen (NCN) contents were analysed using reconstituted 10% protein (w/v), samples, using the methods described in IDF (2016) and IDF (2004), respectively.

For mineral analysis, an aliquot of 1 g of each sample was pre-digested at room temperature using 10 mL HNO_3, before being further digested using a Mars 6 Microwave Digestion System (CEM Corporation, NC, USA). The digested samples were then diluted until a final volume of 50 mL was achieved. The mineral composition of the diluted samples was then determined using optical emission spectroscopy, specifically an Agilent 5110 ICP-OES instrument (Agilent Technologies, Santa Clara, CA, United States), using a method adopted from the AES standard method (ISO:15151, 2018).

The moisture content was determined by weighing 3 g of each powder into an aluminium pan, and placing it into an oven at 83°C for 6 h, after which the powders were removed from the oven, and the weight recorded. The moisture content was calculated by the change in weight before and after oven drying.

4.2.6 Protein profile analysis by liquid chromatography

The protein profiles of the individual cow milk samples and WPIs (10% protein solutions) were determined using the method outlined by McCarthy et al. (2017), in which samples were dispersed in a buffer containing 7 M urea (Fisher Scientific, Dublin, Ireland), 20 mM bis-tris propane (Sigma-Aldrich, Wicklow, Ireland) and 0.19 mM dithiothreitol (Thermo Fisher Scientific, Dublin, Ireland), adjusted to pH 7.5 using 4 M HCl (Fisher Scientific, Dublin, Ireland). The samples were injected onto a Poroshell 300SB-C18 (Size: 2.1×7.5 mm, 5 µm; Agilent Technologies, Ireland). The HPLC was equipped with a UV–vis detector (61365D MWD Agilent Technologies 1200 series), and analysis of the chromatograms was performed using Agilent Chemstation software (Agilent Technologies, Ireland). It should be noted that the chromatograms were used solely for qualitative analysis to indicate the presence or absence of proteins in the skim milk and WPIs.

4.2.7 Protein secondary structure analysis by Fourier Transform Infrared Spectroscopy

Each sample was analysed using a Bruker Invenio S FTIR (Bruker Optik, Ettlingen, Germany) with a BioATRcell II attachment. A mercury cadmium telluride detector was cooled with liquid nitrogen, and the sample compartment was purged to remove moisture and carbon dioxide. The attenuated total reflectance (ATR) crystal was heated to 25°C, and each sample was analysed in duplicate, with each spectrum consisting of an average of 64 scans. The background measure

was taken using a blank ATR crystal. Water spectra were measured and subsequently subtracted from the sample spectra. Spectra were atmospheric corrected for water vapour and carbon dioxide and subsequently normalised. The amide 1 region (1600-1700 cm^{-1}) was analysed to observe any secondary structural changes in protein.

4.2.8 Heat-induced gelation

Heat-induced gelation properties were analysed using an Anton Paar Physica MCR 501 (Anton Paar Ltd, Dublin, Ireland) with a concentric cylinder geometry. The viscoelastic behaviour of the WPI solutions/gels was analysed by heating the samples from 25 to 90°C at a rate of 2°C/min, at which point it was held for 5 min before cooling to 50°C at a rate of 5 °C/min. Low shear oscillatory conditions of 0.5% strain at 1 Hz were used during analysis. An aliquot of 19 mL of the 10% (w/v) protein solution of variants A, B and the mixture AB was transferred into the rheometer cup and sealed using paraffin oil to prevent evaporation of the sample during testing. The storage (G′) and loss (G″) moduli were measured during each test, and the G'_{max} was taken as the highest gel strength recorded during testing. The gel onset temperature was determined as the temperature at which the G′ was greater than G″ by 1 Pa. The influence of pH on gelation was determined by measuring the rheological properties of a series of samples with pH in the range 6.2-7.2, at 0.2 pH unit intervals. The pH of the samples were adjusted at room temperature (20°C) using either 0.1 M HCl or NaOH.

4.2.9 Heat stability

The heat stability of variants A, B and AB (3%, w/v, protein solution) of β-lg were analysed using an Elbanton oil bath (Hettich Benelux Laboratory Equipment, Geldermalsen, the Netherlands). An aliquot (3.4 g) of each variant was transferred into a glass tube, sealed and submerged into the oil bath at 140°C, operating at 8 oscillations per min. The heat coagulation

time (HCT) was taken as the time required for visible coagulation to occur. Samples were pH adjusted to 6.2-7.2, in 0.2 pH unit intervals, and subjected to heat stability testing to generate a heat coagulation time-pH profile. Samples were pH adjusted at 20°C approximately 2 h prior to analysis, using 0.1 M HCl or NaOH, with the pH of all samples checked, and adjusted if necessary, immediately before heat stability testing.

4.2.10 Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was performed on 10% w/v protein solutions using a Q2000 DSC (TA Instruments, New Castle, DE, USA). Murphy et al. (2014) described the method used for this analysis, in which 20 mg of sample was heated from 40 to 100°C at a rate of 5°C min^{-1}, in a nitrogen-purged environment. The enthalpy, denaturation temperature (T_d) and onset temperature ($T_{d\ onset}$) of each sample was determined by linear integration using Universal Analysis 2000 software (TA Instruments).

4.2.11 Statistical Analysis

All statistical analysis was performed using IBM SPSS Statistics v27 (IBM Statistics Inc. Armonk, NY). Results were deemed significant at a P value <0.05. All graphs were created using GraphPad Prism 9.1.0 (GraphPad Software Inc. San Diego, CA).

4.3 Results and discussion

4.3.1 Compositional analysis

The protein fraction of the milk from individual cows containing either the A or B variant of β-lg was not significantly (P> 0.05) different in terms of total protein concentration, casein fraction or non-protein nitrogen (NPN) (Table 4.1). This agrees with the findings of Ojala et al. (1997), who also reported no statistically significant (P>0.05) differences in protein concentration for milks with different genetic variants of β-lg in a sample size of 1032 cows. Milk with the A variant had a significantly (P <0.001) higher whey protein content than the B variant, which is in agreement with other studies (Bobe et al., 1999; Heck, Schennink, et al., 2009a; Ng-Kwai-Hang & Kim, 1996). In addition, the ratio of casein:whey proteins was significantly (P<0.05*)* different between milk containing either the A or B variants, with the A variant having a higher proportion of whey protein than the B variant. This difference in ratio could be of particular interest to cheese manufacturers that process sweet whey for the production of whey protein-based products. In the study of Ng-Kwai-Hang, and Kim (1996), the authors found that milk with the A variant of β-lg had a higher concentration of β-lg than milk with the B variant; however, these results were breed specific. A possible explanation for the higher β-lg content in milk with the A variant is due to the mutations which cause the genetic variation occurring in the promoter region of DNA which code for β-lg, leading to an increase in the synthesis of β-lg A (Bobe et al., 1999; Heck, Schennink, et al., 2009a).

The composition of the powders produced in this study is presented in Table 4.2. Analysis of the nitrogen fractions of the WPI powders made from milk with β-lg A and B variants, highlighted potential inaccuracies associated with the determination of whey protein contents

using the standard IDF method for non-casein nitrogen (NCN) in processed dairy product such as WPI, as described in Subhir et al. (2022). The NCN analysis suggests that the powders contain large quantities of casein, approximately 30% of true protein, and a lower than expected whey protein content, of approximately 56% of true protein. The results are likely linked to the formation of soluble aggregates of whey proteins, produced as a result of heat-induced denaturation during pasteurisation, microfiltration, ultrafiltration and/or spray drying. These soluble aggregates may be susceptible to precipitation during acidification to pH 4.6, which are then quantified as casein according to the NCN test (Ryan et al., 2013). While the Kjeldahl results highlight inconsistencies in quantification of the NCN content in the WPI produced, it does however, provide insights into the stability of the different β-lg variants during processing. The higher NCN value for the WPI containing the A variant of β-lg indicates that more of the proteins in this sample remained native after processing compared to the B variant WPI. The chromatograms presented in Fig 4.1 also confirmed the presence/absence of the A or B variant in both the initial milk and the final WPI powders produced. From these chromatograms, it was evident that casein was extensively removed during MF. This further questions the reliability of the quantification of the nitrogen fractions in processed whey streams using standard methods designed for raw milk.

No significant (P>0.05) differences were found in calcium, magnesium or potassium contents between the WPIs containing the A and B variant of β-lg (Table 4.2). However, significant differences (P<0.005) for iron, sodium and phosphorous were determined between the WPI containing the A and B variant of β-lg. The WPI containing the A variant of β-lg had 392 ppm more sodium, whereas the WPI containing the B variant had 4.59 and 488 ppm more iron and phosphorous, respectively - an observation that does not appear to have been reported in the literature previously. Considering how extensively the MF permeate was diafiltered, it is unlikely that these minerals are free (i.e., not bound to protein molecules), indicating that the

A and B variants of β-lg may have different abilities to bind minerals, possibly linked to different protein structuring; however, this hypothesis was not investigated further in this study but is discussed in the suggestions for future research in Chapter 6. The higher iron content associated with the B variant is interesting as it could help support immune function, as iron that is bound to β-lg can be transported to immune cells to help combat pathogens (Roth-Walter et al., 2021). It could, therefore, be advantageous to select dairy ingredients with the B variant of β-lg that binds more iron, for use in infant nutrition products, where iron-binding proteins (e.g., lactoferrin) are sometimes added.

Table 4.1 Nitrogen fraction analysis of milk from cows containing either the A or B variant of β-lg

g/kg milk	Milk (β-lg A) n = 19	Milk (β-lg B) n = 26
True Protein	36.98 ± 3.52[a]	35.30 ± 2.12[a]
Whey Protein	6.56 ± 0.59[a]	4.92 ± 0.55[b]
Casein	30.41 ± 3.20[a]	30.38 ± 1.92[a]
NPN	1.75 ± 0.37[a]	1.59 ± 0.21[a]
NCN	8.31 ± 0.66[a]	6.51 ± 0.55[b]
Casein:Whey Protein	83:17[a]	86:14[b]

Samples in the same row which do not share a common superscript letter are significantly ($P<0.05$) different. Non-protein nitrogen (NPN), Non-casein nitrogen (NCN).

Table 4.2 Compositional analysis of WPI samples containing the A or B variant of β-lg

	WPI (β-lg A) n = 2	**WPI (β-lg B) n = 2**
Total Nitrogen (g/100 g)	90.0 ± 0.6 [a]	87.6 ± 0.3 [b]
NPN (g/100 g)	1.48 ± 0.10 [a]	1.92 ± 0.28 [a]
NCN (g/100 g)	65.7 ± 16.2 [a]	51.4 ± 15.4 [a]
True Protein (g/100 g)	88.5 ± 0.7 [a]	85.6 ± 0.4 [b]
Casein (g/100 g)	24.3 ± 16.3 [a]	36.1 ± 15.5 [a]
Whey Protein (g/100 g)	64.2 ± 16.3 [a]	49.5 ± 15.2 [a]
Moisture (g/100 g)	5.67 ± 0.06 [a]	6.89 ± 0.35 [b]
Calcium (ppm)	2850 ± 79 [a]	2930 ± 62 [a]
Iron (ppm)	8.74 ± 1.27 [a]	13.33 ± 2.54 [b]
Magnesium (ppm)	598 ± 21[a]	568 ± 11 [a]
Phosphorous (ppm)	1250 ± 16 [a]	1740 ± 153 [b]
Potassium (ppm)	4080 ± 142 [a]	4390 ± 160 [a]
Sodium (ppm)	1710 ± 65 [a]	1320 ± 84 [b]

Components that do not share a common a superscript letter were significantly ($P < 0.05$) different. Abbreviations are as follows; non-protein nitrogen (NPN), non-casein nitrogen (NCN).

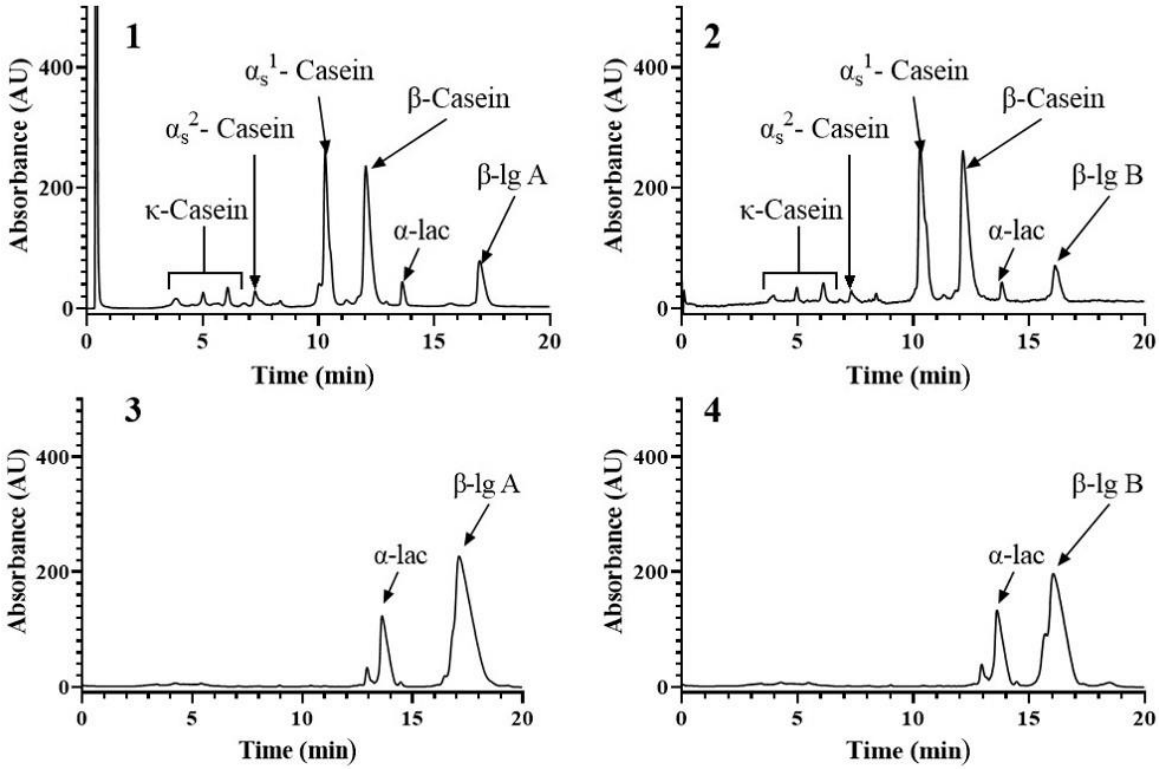

Figure 4.1 High performance liquid chromatography (HPLC) chromatographs of raw milk (1 & 2) and the whey protein isolate (3 & 4) produced thereof containing either the A (1 & 3) or B (2 & 4) variant of β-lg.

4.3.2 Protein structure

The FTIR spectra of the amide one second derivative of the three samples containing either the A and B variant of β-lg or the AB mixture, are presented in Fig 4.2. Among the variants of β-lg, B had a greater proportion of β-sheets, as indicated by a higher absorbance between the wavelengths of 1620-1635 cm^{-1}. On the other hand, the A variant had a higher absorbance than either the AB mixture or B variants at wavelengths between 1635-1650 cm^{-1}, representing regions of random coil and unordered structures. These differences indicate that the B variant of β-lg has a more ordered secondary structure than the AB mixture or the A variants. These results are in agreement with those of Imafidon et al. (1991a) who also reported upon the

secondary structure of purified protein variants A and B of β-lg by FTIR. The use of FTIR for the comparative analysis of the secondary structure between milk protein genetic variants has been reported previously. Daniloski, McCarthy, O'Callaghan, et al. (2022) reported the structural differences of β-casein genetic variants by FTIR and found that the A2 genetic variant had a greater proportion of β-sheets and random coils than the A1 variant.

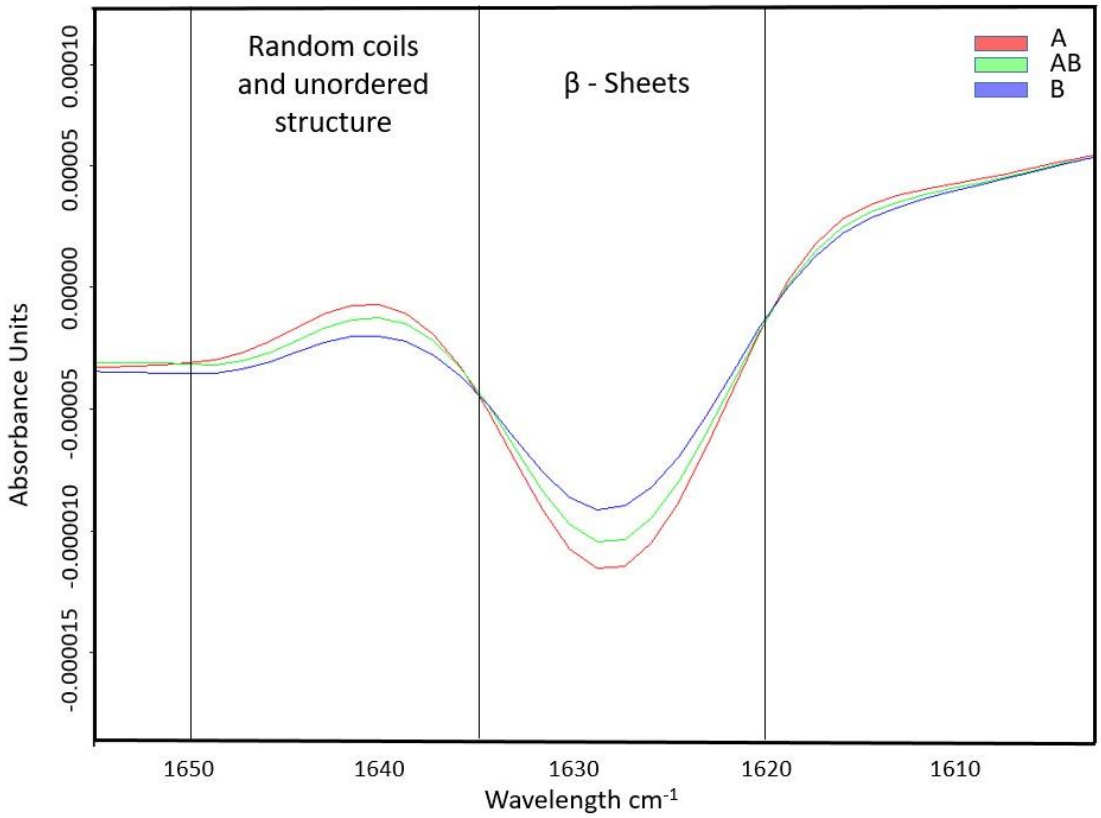

Figure 4.2 Fourier transform infra-red spectra of variants A and B of β-lg and the AB mixture in the amide 1 region. Wavelengths between 1620-1635 cm^{-1} indicates β-sheets, and between wavelengths 1635-1650 cm^{-1} indicates random coils and unordered structures. For clarity the AB sample is an equal mixture of a 10% protein solution of the whey protein isolate (WPI) containing the A variant and a 10% protein solution of the WPI containing the B variant of β-lg.

4.3.3 Differential scanning calorimetry

Differential scanning calorimetry (DSC) was used to determine the denaturation onset temperature ($T_{d\ onset}$) and peak denaturation temperature (T_d) of 10%, w/v, β-lg solutions, as influenced by genetic variants. Fig 4.3 shows the peak denaturation temperatures of the protein solutions containing either the A or B variant of β-lg or the AB mixture at pH 6.2. The enthalpy of the denaturation reaction was 0.28, 0.27 and 0.25 J g^{-1}, for the A, B and AB samples, respectively, which was not significantly ($P>0.05$) different. A significant ($P<0.05$) difference was observed in both the $T_{d\ onset}$ and T_d between each variant, with the lowest temperatures recorded for the protein solution containing the A variant of β-lg, followed by the AB mixture, and then the B variant. The average $T_{d\ onset}$ was 72.01, 75.58 and 73.51°C for variants A, B and AB of β-lg, respectively. The average T_d of each protein solution was 74.68, 78.68, and 76.26°C for A, B and AB samples, respectively. Imafidon et al. (1991a) determined the T_d of purified variants of β-lg in different media, reporting similar results to this study, with the A variant having a considerably lower T_d than the B variant in most media. However, in contrast to this study, the authors reported that the mixture of A and B variants (i.e., AB) had a lower T_d than the A or B genotypes in most media.

While the exact reason for the differences in T_d among the variants of β-lg in the present study is not fully understood, it is likely due to the conformational differences between the variants resulting from amino acid substitutions, leading to some β-lg structures being more stable than others. A possible reason for the difference in the T_d between the variants A and B of β-lg, is due to the differences in their secondary structures. As indicated in Section 3.2, the B variant has a greater proportion of β-sheets than the A variant or the AB mixture. The β-sheet secondary structure is made of β-strands which are stabilised by inter-strand hydrogen bonds, and it is these additional bonds which provide stability to the protein structure against thermal

denaturation (Branden & Tooze, 2012). As a result, it could be suggested that the B variant of β-lg has a more robust structure to withstand heat-induced denaturation than the A variant or the AB mixture. Boye et al. (1997) proposed that the increased polarity of the A variant, due to its additional aspartic acid residue at position 64, could cause variation in T_d between A and B. While the results are not shown here, altering the pH of the samples did not affect the T_d , regardless of the variant. While Paulsson, and Dejmek (1990) found no difference in T_d of β-lg as a function of pH, other studies have reported an effect of pH on the T_d of β-lg, possibly linked to differences in the ionic composition of the solvent phase (Arakawa & Timasheff, 1987; Dannenberg & Kessler, 1988).

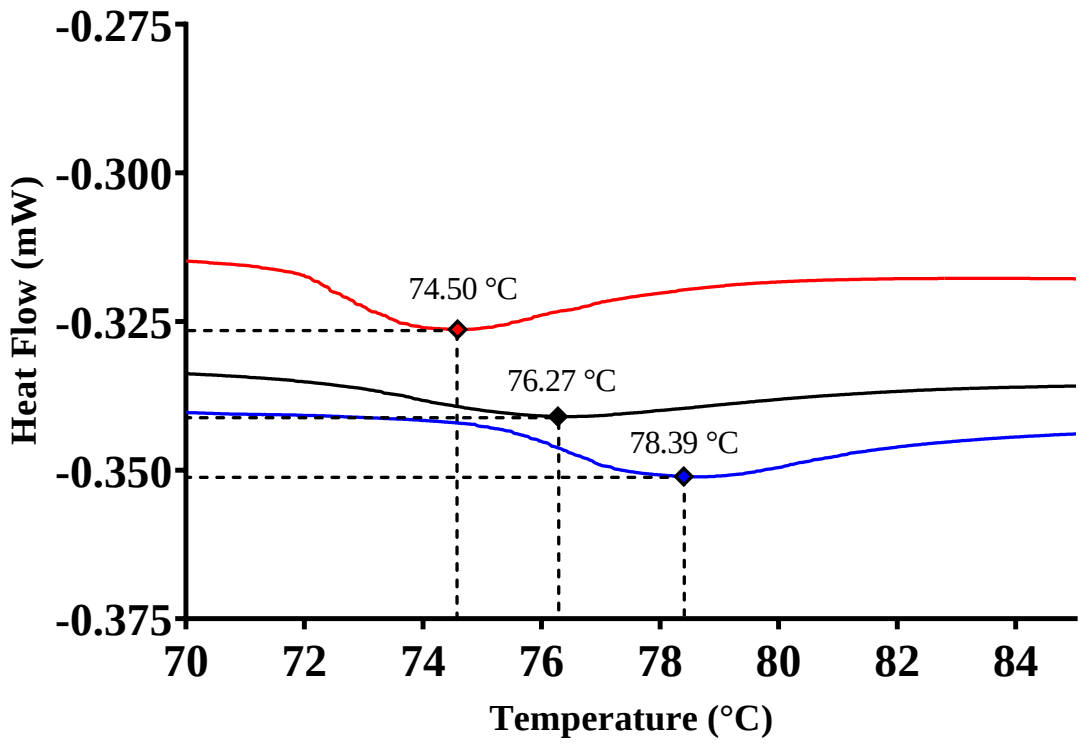

Figure 4.3 Differential scanning calorimetry (DSC) of 10% w/w protein solutions containing the β-lg variants A (♦) or B (♦) variants and the AB (♦) mixture. The peak minimum (♦) is highlighted as the denaturation temperature for each variant. For clarity the AB sample is an equal mixture of a 10% protein solution of the whey protein isolate (WPI) containing the A variant and a 10% protein solution of the WPI containing the B variant of β-lg.

4.3.4 Gelation properties

Heat-induced gelation profiles were different between the variants of β-lg. The gel onset temperatures at pH 6.2 for the protein solutions containing the A or B variant of β-lg or the AB mixture were 76.55, 78.86 and 77.49°C, respectively, with a graphical representation of same presented in Fig 4.4. Considering the purity of the samples, it is likely that the main cause of the difference in the gel onset temperatures at this pH, are linked to differences in the T_d. However, the mineral distribution could also play a role in contributing to the differences observed, in which the higher sodium content of the A variant may be a contributing factor to the lower T_d and gel onset temperature observed. Huang et al. (1994) also investigated the gelation properties of the A and B variants of β-lg and obtained similar results to those found in this study. In the study of Huang et al (1994), the authors used milk samples which had the casein fraction removed by acid precipitation, leaving only the serum proteins remaining. The acid whey was adjusted to pH 2, after which it was concentrated by UF, coupled with diafiltration, until a pH in the range of 6-7 was reached. Their study also reported that the A variant had a lower gel onset temperature of 72.3°C than the B variant at 80°C. The greater difference in gel onset temperature of their study compared to the current study, could be due to a combination of a lower protein concentration (7%) and the addition of 50 mM NaCl during solution preparation.

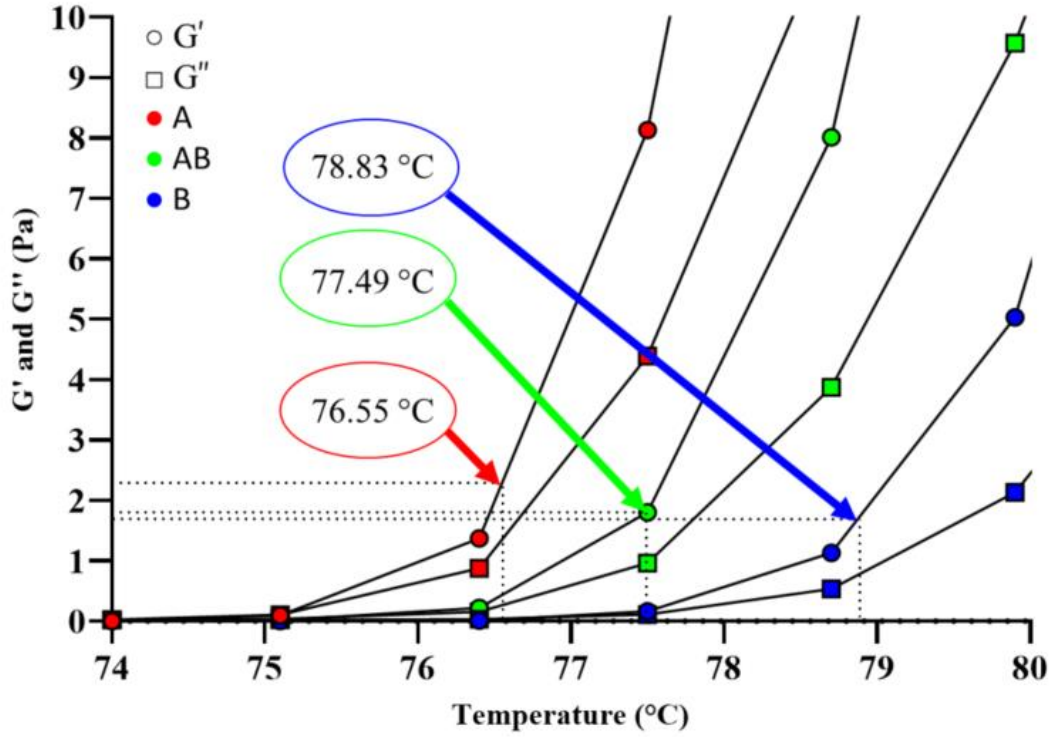

Figure 4.4 Gelation onset temperature of β-lg variants A (●), B (●) and the AB (●) mixture at pH 6.2, in which the onset temperature was determined as the point in which the G′(○) is 1 Pa higher than the G″ (□). For clarity the AB sample is an equal mixture of a 10% protein solution of the whey protein isolate (WPI) containing the A variant and a 10% protein solution of the WPI containing the B variant of β-lg.

The effect of pH on the maximum strength of gels prepared from the protein solutions containing either the A or B variant of β-lg or the AB mixture are displayed in Fig 4.5. The results indicate that, at pH 6.2 and 6.4, all protein solutions, regardless of β-lg variant, produced strong gels, with the protein solutions containing the A variant producing slightly stronger gels than either the B variant or the AB mixture. However, as the pH increased to >6.6, the gel-forming properties of the genetic variants showed considerable differences. The protein solutions containing the β-lg A variant or the AB mixture formed weak gels, or did not gel between pH 6.6 and 7.2. In contrast, the protein solutions containing the B variant of β-lg formed gels between pH 6.6 and 7.2, which decreased in strength as the pH increased. A representative graph of the full gelation profiles of the protein solutions containing the A or B

variants of β-lg or the AB mixutre, at pH 6.2 and 6.8, is displayed in Fig 4.6. An important characteristic of the heat-induced gelation of β-lg is the two-step process by which the gel forms. The first stage of this process is unfolding of the protein, by breaking the hydrogen bonds that help stabilise the protein structure, exposing the more hydrophobic core of the protein. The second stage is the aggregation of unfolded proteins by hydrogen bonding and hydrophobic interactions, eventually creating a gel matrix (Bischof & He, 2006; Boye et al., 1997). Based on the results from DSC analysis, it is unlikely that the pH influenced the first stage of gelation of β-lg, that is, the unfolding of the protein. However, the pH could have affected the second stage of β-lg gelation, which involves protein-protein aggregation and matrix formation.

The differences in the gelation properties between the protein solutions containing the A and B variants of β-lg are likely due to differences in charge of the unfolded proteins. The A variant has been reported to have a higher net negative charge than B, due to the A variant having an aspartic acid at position 64, whereas the B variant has a glycine (Farrell et al., 2004a). This substitution could cause higher repulsive forces between the unfolded proteins, preventing them from interacting and ultimately forming a gel structure (Euston et al., 1999; Keppler et al., 2014). Typically, as the pH of protein solutions moves further away from their isoelectric point, the charge increases (Feng et al., 2020), which may also help explain the weaker gel forming properties of β-lg (which has an isoelectric point of 5.2), at higher pH. Studies on the effects of minerals on the gelling properties of WPI have found that higher levels of ionic Na increase gel strength (Kuhn et al., 2010). However, even though the WPI containing the A variant of β-lg has higher levels of Na, the B variant had a higher gel strength. This could possibly indicate that the Na is already bound to the protein and that the difference in charge

and secondary structure between the A and B variants plays a more important role in their gelation properties than the observed differences in mineral distribution (Table 4.2).

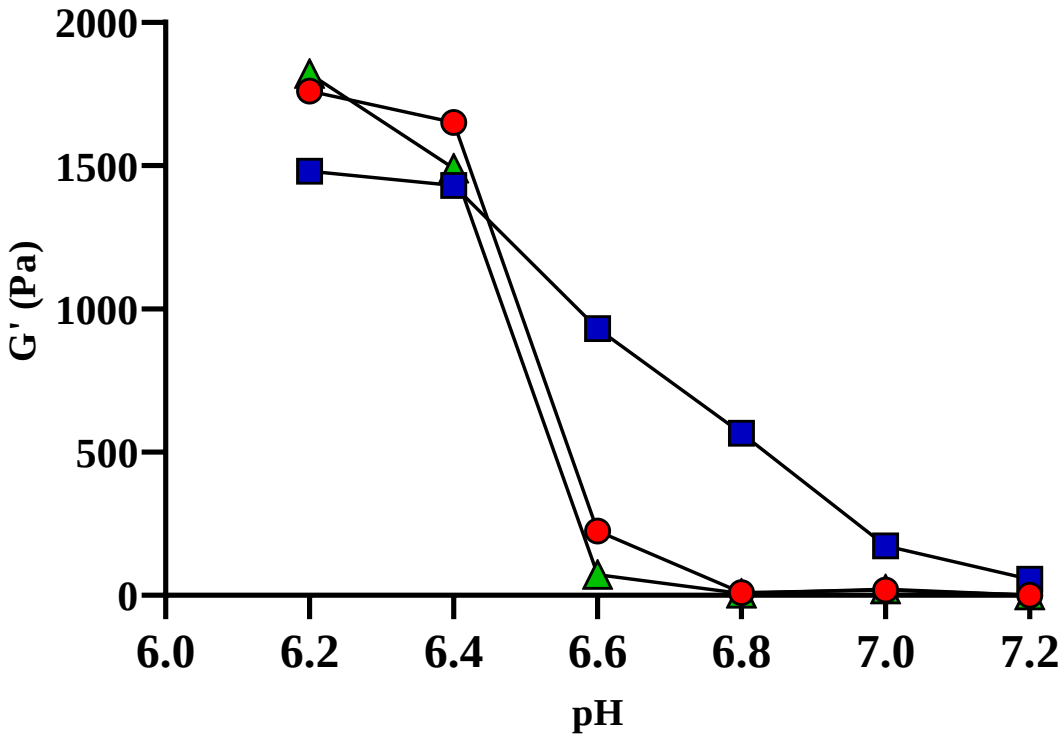

Figure 4.5 Effect of pH on the maximum gel strength (G'_{max}) of variants A (●) or B (■) variant of β-lg or the AB (▲) mixture between pH 6.2 and 7.2 at intervals of 0.2 in a 10 % protein solution. For clarity the AB sample is an equal mixture of a 10% protein solution of the whey protein isolate (WPI) containing the A variant and a 10% protein solution of the WPI containing the B variant of β-lg.

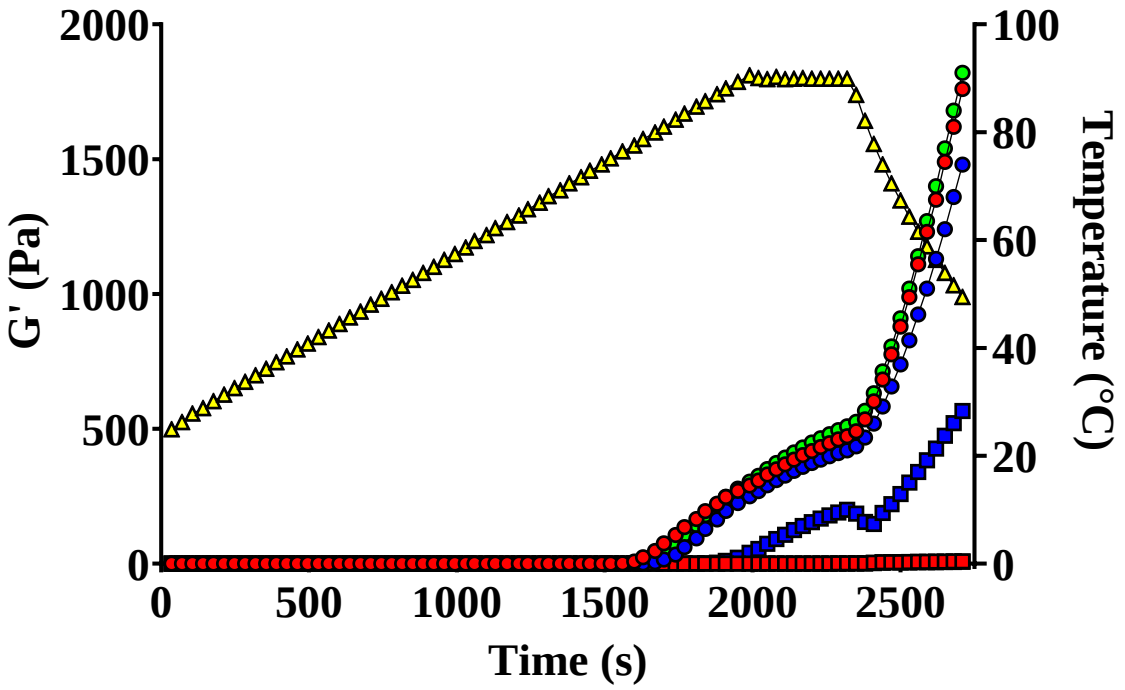

Figure 4.6 Heat-induced gelation profiles of 10% protein solution made from whey protein isolates containing either the A (●) or B (●) variant of β-lg or the AB (●) mixture at pH 6.2 (●) and 6.8 (■). The temperature profile is indicated by the secondary y-axis (▲). For clarity the AB sample is an equal mixture of a 10% protein solution of the whey protein isolate (WPI) containing the A variant and a 10% protein solution of the WPI containing the B variant of β-lg.

4.3.5 Heat stability

No significant (P>0.05) difference was found in the heat stability of the A and B variants (3%, w/v, protein solutions), at their natural pH of ~6.8. The heat stability of the β-lg variants at their natural pH was 14 min 42 s, 14 min 17 s, and 14 min 10 s for variants A, B and the mixture of AB, respectively. It should be noted that most of the published literature on the effects of variants of β-lg on heat stability has been conducted using milk as opposed to the WPI, used in this study. McLean et al. (1987), assessed the influence of β-lg variants on the heat stability of milk at 140°C, and found that the variants of β-lg did not affect the heat stability of milk at its natural pH of 6.7. In contrast, Sawyer (1968) purified variants of β-lg by fractionation using ammonium sulphate, and heated the purified samples in test tubes at 97.5°C for 150 min. In

their study they reported that the A variant had lower heat stability, indicating that it may be due to the A variant having a higher reactivity to disulphide bonding during aggregation, than the B variant of β-lg. In addition, the lower heat stability of milk with the A variant of β-lg could be due to the higher whey protein content therein. A greater amount of whey protein has been shown to effect heat stability due to protein-protein interactions between whey proteins and the casein micelle, which reduces heat stability by neutralising the electrostatic charge present on the surface of the micelle (Dumpler et al., 2020b).

The only pH value that showed a significant (P <0.05) difference between the variants was 6.4 (Fig 4.7). At this pH, the protein solutions containing the B variant and the AB mixture had lower heat stability than A by >10 min. It is possible that, at this pH, the higher electrostatic repulsion of the A variant due to an additional charged amino acid at position 64, conferred the higher heat stability. The results from Section 3.4 indicated that the A variant could potentially be more heat stable than the B variant, due to its resistance to forming a gel at certain pH's. While the A variant did form a gel at pH 6.4, the differences between results in this section, and those in Section 4.4, could be due to the lower protein concentration used for testing heat stability (3%) compared to that used for the analysis of gelation properties (10%). Nielsen et al. (1996) investigated the effect of heating time and protein concentration on isolated variants of β-lg which were obtained from acid whey by a combination of salt fractionation and freeze drying. Their study reported that, at protein concentrations <5%, w/v, the B variant was more heat labile than the A variant, but at concentrations >5%, w/v, the A variant was more heat labile. Although the authors suggested some reasons for the concentration dependence of thermal aggregation, including the A variant being more sensitive to disulphide bonding and non-covalent interactions at higher concentrations, no definitive conclusions were established. The results from the present study indicate that at pH 6.4, the A variant has better heat stability than the B variant or the AB mixture. This pH is of particular interest as it is the natural pH of

many milk concentrates and could be of significance during thermal processing of such products, as it has the potential to reduce fouling, with associated benefits for overall efficiency of thermal processes (Corredig et al., 2019; Tamime, 2009).

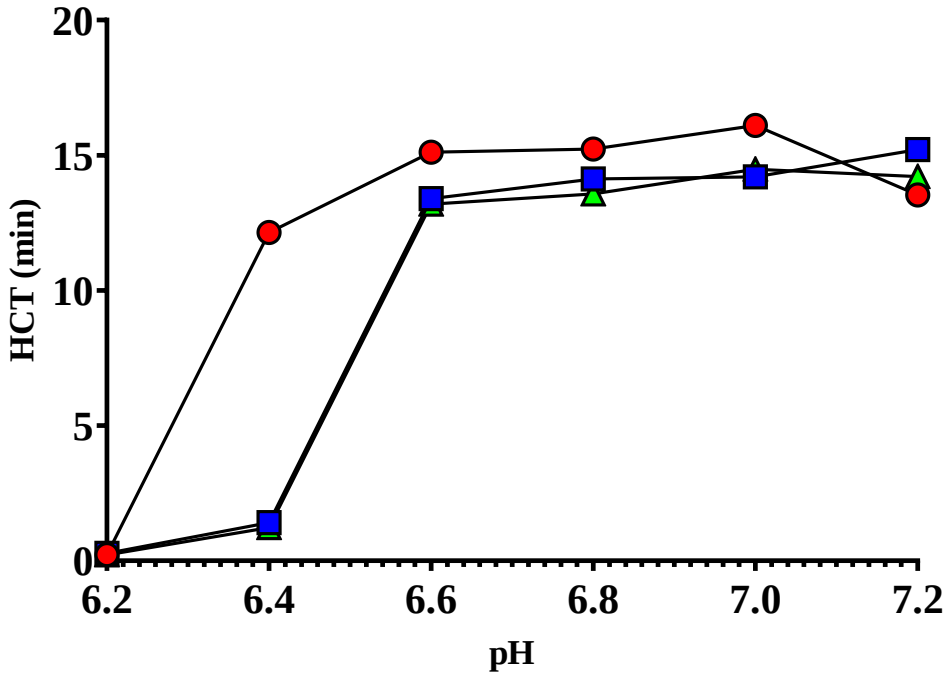

Figure 4.7 The heat stability of A (●), B (■), and AB (▲) variants of β-lg, over a pH range of 6.2-7.2, at 0.2 pH unit intervals. For clarity the AB sample is an equal mixture of a 3% protein solution of the whey protein isolate (WPI) containing the A variant and a 3% protein solution of the WPI containing the B variant of β-lg.

4.4. Conclusion

Few studies have assessed the functional properties of variants of β-lg with protein purity as high as that achieved in this study. The thermal denaturation temperatures of each genotype increased in the order of AA<AB< BB. The differences in denaturation temperature are likely due to the variation in β-sheets, which was in the order of A<AB<B. The gelation onset temperature followed the same pattern as the thermal denaturation temperature. Still, despite the A variant having a lower denaturation temperature, it had better stability against heat-

induced gelation at pH in the range 6.6-7.2, than the B variant, possibly due to the additional charge on the A variant. Mineral analysis of the WPI's found a significant (P<0.05) difference in the levels of sodium, phosphorous and iron, between the A and B variants, thereby suggesting that the A and B variants of β-lg have different mineral binding properties. The differences in thermal stability of the variants of β-lg could be of particular interest to milk processors who use ultra-high temperature heat treatment as the more heat stable variants could reduce the propensity for fouling and thereby support effective heat transfer. In addition, the better gelation properties of the B variant of β-lg at pH 6.6-7.0 could be of benefit to manufacturers that formulate gel based products, as the stronger gels at these pH's could help improve product stability.

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Declaration of competing interest

Authors declare no conflicts of interest.

4.5 References

Arakawa, T., & Timasheff, S. N. (1987). Abnormal solubility behavior of .beta.-lactoglobulin: salting-in by glycine and sodium chloride. *Biochemistry*, *26*, 5147-5153.

Bikker, J., Anema, S., Li, Y., & Hill, J. (2000). Thermal denaturation of β-lactoglobulin A, B and C in heated skim milk. *Milchwissenschaft*, *55*, 609-613.

Bischof, J. C., & He, X. (2006). Thermal stability of proteins. *Annals of the New York Academy of Sciences, 1066*, 12-33.

Bobe, G., Beitz, D., Freeman, A., & Lindberg, G. (1999). Effect of milk protein genotypes on milk protein composition and its genetic parameter estimates. *Journal of Dairy Science, 82*, 2797-2804.

Boye, J. I., Ma, C. Y., Ismail, A., Harwalkar, V. R., & Kalab, M. (1997). Molecular and Microstructural Studies of Thermal Denaturation and Gelation of β-Lactoglobulins A and B. *Journal of Agricultural and Food Chemistry, 45*, 1608-1618.

Branden, C. I., & Tooze, J. (2012). *Introduction to Protein Structure,* (Second Edition): Garland Science.

Caroli, A. M., Chessa, S., & Erhardt, G. J. (2009). Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *Journal of Dairy Science, 92*, 5335-5352.

Daniloski, D., McCarthy, N. A., O'Callaghan, T. F., & Vasiljevic, T. (2022). Authentication of β-casein milk phenotypes using FTIR spectroscopy. *International Dairy Journal, 129*, 105350.

Dannenberg, F., & Kessler, H.-G. (1988). Reaction Kinetics of the Denaturation of Whey Proteins in Milk. *Journal of Food Science, 53*, 258-263.

de Wit, J. N. (2009). Thermal behaviour of bovine β-lactoglobulin at temperatures up to 150°C. a review. *Trends in Food Science & Technology, 20*, 27-34.

Deeth, H., & Bansal, N. (2019). Whey Proteins: An Overview. In *Whey Proteins* (First Edition) (pp. 1-50): Academic Press.

Dumpler, J., Huppertz, T., & Kulozik, U. (2020). Invited review: Heat stability of milk and concentrated milk: Past, present, and future research objectives. *Journal of Dairy Science, 103*, 10986-11007.

Euston, S., L. Hirst, R., & P. Hill, J. (1999). The emulsifying properties of β-lactoglobulin genetic variants A, B and C. *Colloids and Surfaces B: Biointerfaces, 12*, 193-202.

Farrell, H., Jimenez-Flores, R., Bleck, G., Brown, E., Butler, J., Creamer, L., Hicks, C., Hollar, C., Ng-Kwai-Hang, K., & Swaisgood, H. (2004). Nomenclature of the proteins of cows' milk: Sixth revision. *Journal of Dairy Science, 87*, 1641-1674.

Feng, Y., Kilker, S. R., & Lee, Y. (2020). Surface charge (zeta-potential) of nanoencapsulated food ingredients. In *Characterization of Nanoencapsulated Food Ingredients* (First Edition) (pp. 213-241): Academic Press.

Fogolari, F., Ragona, L., Zetta, L., Romagnoli, S., De Kruif, K. G., & Molinari, H. (1998). Monomeric bovine β-lactoglobulin adopts a β-barrel fold at pH 2. *Federation of European Biochemistry Societies Letters, 436*, 149-154.

Gai, N., Uniacke-Lowe, T., O'Regan, J., Faulkner, H., & Kelly, A. L. (2021). Effect of Protein Genotypes on Physicochemical Properties and Protein Functionality of Bovine Milk: A Review. *Foods, 10*, 2409.

Glantz, M., Lindmark Månsson, H., Stålhammar, H., & Paulsson, M. (2011). Effect of polymorphisms in the leptin, leptin receptor, and acyl-coenzyme A:diacylglycerol acyltransferase 1 (DGAT1) genes and genetic polymorphism of milk proteins on cheese characteristics. *Journal of Dairy Science, 94*, 3295-3304.

Heck, J., Schennink, A., Van Valenberg, H., Bovenhuis, H., Visker, M., Van Arendonk, J., & Van Hooijdonk, A. (2009). Effects of milk protein variants on the protein composition of bovine milk. *Journal of Dairy Science, 92*, 1192-1202.

Huang, X. L., Catignani, G. L., Foegeding, E. A., & Swaisgood, H. E. (1994). Comparison of the Gelation Properties of .beta.-Lactoglobulin Genetic Variants A and B. *Journal of Agricultural and Food Chemistry, 42*, 1064-1067.

ICBF (2023). *National Genotyping Programme.* https://www.icbf.com/national-genotyping-programme/

IDF. (2004). Milk - Determination of the casein-nitrogen content - Part 1: Indirect method (Reference method). In *International Dairy Federation* (Vol. IDF 29-1 :2004). Brussels, Belgium: International Dairy Federation.

IDF. (2014). Milk and milk products - Determination of nitrogen content - Part 1: Kjeldahl prinicple and crude protein calculation. In *International Dairy Federation* (Vol. IDF 20-1 :2014). Brussels, Belgium: International Dairy Federation.

IDF. (2016). Milk and milk products - determination of nitrogen content - Part 4: Determination of protein and non-protein nitrogen content calculation (Reference Method). In *International Dairy Federation* (Vol. IDF 20-4 :2016). Brussels, Belgium: International Dairy Federation.

Imafidon, G. I., Ng-Kwai-Hang, K. F., Harwalkar, V. R., & Ma, C. Y. (1991). Differential Scanning Calorimetric Study of Different Genetic Variants of β-Lactoglobulin. *Journal of Dairy Science, 74*, 2416-2422.

ISO:15151. (2018). Milk, milk products, infant formula and adult nutritionals - Determination of minerals and trace elements - Inductively coupled plasma atomic emission spectrometry (ICP-AES) method: International Standard Organisation.

Jensen, H. B., Poulsen, N. A., Andersen, K. K., Hammershøj, M., Poulsen, H. D., & Larsen, L. B. (2012). Distinct composition of bovine milk from Jersey and Holstein-Friesian cows with good, poor, or noncoagulation properties as reflected in protein genetic variants and isoforms. *Journal of Dairy Science, 95*, 6905-6917.

Keppler, J. K., Sönnichsen, F. D., Lorenzen, P.-C., & Schwarz, K. (2014). Differences in heat stability and ligand binding among β-lactoglobulin genetic variants A, B and C using

1H NMR and fluorescence quenching. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1844*, 1083-1093.

Khaldi, M., Croguennec, T., André, C., Ronse, G., Jimenez, M., Bellayer, S., Blanpain-Avet, P., Bouvier, L., Six, T., & Bornaz, S. (2018). Effect of the calcium/protein molar ratio on β-lactoglobulin denaturation kinetics and fouling phenomena. *International Dairy Journal, 78*, 1-10.

Kontopidis, G., Holt, C., & Sawyer, L. (2004). Invited Review: β-Lactoglobulin: Binding Properties, Structure, and Function. *Journal of Dairy Science, 87*, 785-796.

Kuhn, K. R., Cavallieri, Â. L. F., & Da Cunha, R. L. (2010). Cold-set whey protein gels induced by calcium or sodium salt addition. *International Journal of Food Science & Technology, 45*, 348-357.

Li, C. H. (1946). Electrophoretic Inhomogeneity of Crystalline Beta-lactoglobulin. *Journal of the American Chemical Society, 68*, 2746-2747.

Manderson, G., Hardman, M., & Creamer, L. (1998). Effect of heat treatment on the conformation and aggregation of β-lactoglobulin A, B, and C. *Journal of Agricultural and Food Chemistry, 46*, 5052-5061.

McCarthy, N. A., Wijayanti, H. B., Crowley, S. V., O'Mahony, J. A., & Fenelon, M. A. (2017). Pilot-scale ceramic membrane filtration of skim milk for the production of a protein base ingredient for use in infant milk formula. *International Dairy Journal, 73*, 57-62.

McCarthy, W. P., Blais, H. N., O'Callaghan, T. F., Hossain, M., Moloney, M., Danaher, M., O'Connor, C., & Tobin, J. T. (2022). Application of nanofiltration for the removal of chlorate from skim milk. *International Dairy Journal, 128*, 105321.

McLean, D. M., Graham, E. R. B., Ponzoni, R. W., & McKenzie, H. A. (1987). Effects of milk protein genetic variants and composition on heat stability of milk. *Journal of Dairy Research, 54*, 219-235.

Meza-Nieto, M. A., Vallejo-Cordoba, B., González-Córdova, A. F., Félix, L., & Goycoolea, F. M. (2007). Effect of β-Lactoglobulin A and B Whey Protein Variants on the Rennet-Induced Gelation of Skim Milk Gels in a Model Reconstituted Skim Milk System. *Journal of Dairy Science, 90*, 582-593.

Meza-Nieto, M. A., González-Córdova, A. F., Piloni-Martini, J., & Vallejo-Cordoba, B. (2013). Effect of β-lactoglobulin A and B whey protein variants on cheese yield potential of a model milk system. *Journal of Dairy Science, 96*, 6777-6781.

Michalcová, A., & Krupová, Z. (2007). Influence of composite kappa-casein and beta-lactoglobulin genotypes on composition, rennetability and heat stability of milk of cows of Slovak Pied breed. *Czech Journal of Animal Science, 52*, 292.

Murphy, E. G., Fenelon, M. A., Roos, Y. H., & Hogan, S. A. (2014). Decoupling macronutrient interactions during heating of model infant milk formulas. *Journal of Agricultural and Food Chemistry, 62*, 10585-10593.

Ng-Kwai-Hang, K. F., & Kim, S. (1996). Different amounts of β-lactoglobulin A and B in milk from heterozygous AB cows. *International Dairy Journal, 6*, 689-695.

Nielsen, B. T., Singh, H., & Latham, J. M. (1996). Aggregation of bovine β-lactoglobulins A and B on heating at 75 °C. *International Dairy Journal, 6*, 519-527.

O'Kennedy, B., Mounsey, J., Murphy, F., Pesquera, L., & Mehra, R. (2006). Preferential heat-induced denaturation of bovine β-lactoglobulin variants as influenced by pH. *Milchwissenschaft, 4*, 366-369.

Ojala, M., Famula, T. R., & Medrano, J. F. (1997). Effects of Milk Protein Genotypes on the Variation for Milk Production Traits of Holstein and Jersey Cows in California. *Journal of Dairy Science, 80*, 1776-1785.

Paulsson, M., & Dejmek, P. (1990). Thermal Denaturation of Whey Proteins in Mixtures with Caseins Studied by Differential Scanning Calorimetry. *Journal of Dairy Science, 73*, 590-600.

Roth-Walter, F., Afify, S. M., Pacios, L. F., Blokhuis, B. R., Redegeld, F., Regner, A., Petje, L.-M., Fiocchi, A., Untersmayr, E., Dvorak, Z., Hufnagl, K., Pali-Schöll, I., & Jensen-Jarolim, E. (2021). Cow's milk protein β-lactoglobulin confers resilience against allergy by targeting complexed iron into immune cells. *Journal of Allergy and Clinical Immunology, 147*, 321-334.e324.

Ryan, K. N., Zhong, Q., & Foegeding, E. A. (2013). Use of Whey Protein Soluble Aggregates for Thermal Stability—A Hypothesis Paper. *Journal of Food Science, 78*, R1105-R1115.

Sawyer, L., & Kontopidis, G. (2000). The core lipocalin, bovine β-lactoglobulin. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1482*, 136-148.

Sawyer, W. H. (1968). Heat Denaturation of Bovine β-Lactoglobulins and Relevance of Disulfide Aggregation. *Journal of Dairy Science, 51*, 323-329.

Subhir, S., McSweeney, P. L. H., Fenelon, M. A., Magan, J. B., & Tobin, J. T. (2022). Suitability of nitrogen fractions determination to assess serum protein separation efficiency from a mass balance perspective during microfiltration of skim milk. *International Dairy Journal, 128*, 105319.

Chapter 5

Influence of heat treatment and genetic variation of β-lactoglobulin on the *in vitro* gastric digestion characteristics of a model infant formula

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Declaration

This chapter was written by author LMK and reviewed by his co-authors. CJF assisted in the development of an *in vitro* infant digestion model. EH performed the FTIR analysis. WPM performed SDS-PAGE of the digested IMF samples. AB assisted in reviewing the manuscript.

Abstract

The most abundant protein in bovine milk-based infant milk formula (IMF) is β-lactoglobulin (β-lg). The most common genetic variants of β-lg are the homozygous A and B variants, and the heterozygous AB variant. Two types of IMF were manufactured using either a low heat treatment (72°C × 15 s), or a high heat treatment (135°C × 2 s). Each IMF produced using the low and high heat treatments contained either exclusively A, B or the AB variant of β-lg. In lieu of a global consensus for an infant digestion model, an adjusted version of the INFOGEST adult model was used. Analysis using SDS-PAGE indicated that the high heat treated IMF samples had extensive breakdown of β-lg, whereas protein bands remained visible among the low heat treatment IMFs at the end of gastric digestion. The model IMF prepared using the high heat treated AB variant had significantly ($P < 0.05$) more free amines released during digestion than any other sample, possibly due to the production of smaller (whey protein dimer-based) aggregates with this variant on heat treatment. Overall, this study demonstrated that the severity of thermal processing applied during IMF manufacture was more impactful than genetic variation in the primary whey protein, on the digestion of model IMF.

5.1 Introduction

It is recommended by the World Health Organisation that infants are breast fed for at least the first six months of life (Gallier et al., 2015; Martin et al., 2016). However, breastfeeding is not always possible, and in some situations a substitute in the form of infant formula is required (Alles et al., 2004). In the United States, around 67% of three-month-old infants rely on infant milk formula (IMF) as the sole source of nutrition for their growth and development (Martin et al., 2016). Cow's milk is the most widely used milk for the production of IMF, but to best mimic the composition of breast milk, the concentration of certain components need to be adjusted (Masum et al., 2020). The protein content of human milk differs to that of cow's milk, with human milk containing approximately 0.9% protein, in contrast with 3.5% protein in cow's milk. Additionally, the proportion of casein to whey protein in human milk is 40:60, whereas in cow milk, that proportion is 80:20; therefore, to mimic human milk effectively, the proportion of the whey protein fraction in cow milk needs to be increased. This increase in the whey protein fraction is often achieved by adding whey-protein-rich ingredients, such as whey protein concentrates and demineralised whey to the IMF formulation (Masum et al., 2020). The addition of whey protein rich ingredients during formulation, results in β-lactoglobulin (β-lg) being the most abundant protein component of IMF, despite the fact that human milk does not actually contain β-lg (Murphy et al., 2015).

β-lg is an 18 kDa whey protein, comprising 162 amino acids (Sawyer, 2012). Genetic polymorphisms are common among milk proteins, with 11 genetic variants reported to date in β-lg alone (Caroli et al., 2009; Gai et al., 2021). The most common homozygous genotypes of β-lg are AA and BB, which differ by two amino acids, where an aspartic acid is replaced by a glycine, and a valine is replaced by an alanine at positions 64 and 118, respectively (Ganai et al., 2009). These substitutions can result in conformational changes to the protein, which have

been shown to affect the protein's hydrophobicity and heat stability, but may also have the ability to impact proteolysis during digestion (Keppler et al., 2014; Svenning et al., 2000).

Applying heat treatment during the production of IMF primarily meets food safety requirements in terms of microbiological quality, prior to stabilization in the powdered form (Francisquini et al., 2020). However, intense heat treatment can result in undesirable alterations to certain compositional components in IMF, which may affect its technological, sensorial and digestive properties, including changes in protein structure and loss of biological function, especially for heat labile proteins such as immunoglobulins, in addition to the formation of undesirable Maillard compounds (Fracisquini et al., 2017). Within IMF, proteins are some of the components most vulnerable to heat-induced changes (Francisquini et al., 2020; Tunick et al., 2016). The casein fraction of milk proteins is generally described as heat stable due to the lack of any complex tertiary protein structure, whereas the whey proteins are more heat labile (Li et al., 2021). Whey proteins are the dominant protein contributors to heat-induced changes within IMF (Crowley et al., 2016). Different heat treatments have been shown to alter the extent of heat-induced changes, such as protein denaturation and protein-protein interactions, which alters protein digestibility (Rudloff & Lönnerdal, 1992).

The impact of genetic variants on the digestion of milk proteins has been reported previously (Asledottir et al., 2018; Sheng et al., 2021). Sheng et al. (2021) studied the influence of genetic variants of κ-casein on the digestion of skim milk using a static *in vitro* model. The authors found that the A variant of κ-casein had a faster rate of digestion than the B or AB variants, which they attributed to differences in micelle size and glycosylation associated with the genetic variation. Barnett et al. (2014) investigated the effects of genetic variants of β-casein on digestion in rats fed a milk-based diet; the gastric transit time for the milk containing the A1 variant was significantly longer than that for the A2 variant. It was suggested that the delay in transit time of the milk containing the A1 variant was due to the increased levels of β-

casomorphin-7 released during the digestion of the A1 variant of β-casein, which binds to μ-opioid receptors, inhibiting gastrointestinal functions (Asledottir et al., 2018; Barnett et al., 2014). In addition, a recent study by Daniloski et al. (2024) reported that the A1 variant of β-casein was more susceptible to enzymatic breakdown than the A2 variant; however, the health implications of this were not reported.

As the A variant of β-lg has a lower denaturation temperature, the breakdown of β-lg during *in vitro* gastric digestion could be altered as a result of potential differences in unfolding of the protein as a result of heat treatment during IMF manufacture. Due to the short residence time of milk in the infants mouth, the gastric phase acts as the first stage of digestion for an infant, and is subsequently the first stage of proteolysis, hence for this study, only the gastric phase of digestion was conducted. In addition, other studies have found differences in gastric digestion due to genetic variation of milk proteins, which, considering that β-lg is the most abundant protein found in IMF, could highlight important differences in digestive characteristics which may be of significance to infant health.

5.2 Materials and methods

The raw materials used for the formulation of model infant formulas were either manufactured in-house or purchased. The skim milk powder (SMP) was produced from fresh whole milk using a centrifugal cream separator (GEA Westfalia Oelde, Germany), a APV plate heat exchanger (SPX Flow Technology, Crawley, UK) for pasteurisation, a Niro 3-effect falling film evaporator (GEA Niro A/S, Soeborg, Denamrk), and a single stage spray dryer (Anhydro F1 Lab Dryer; Copenhagen, Denmark). The composition of the raw materials used for the production of the IMF is displayed in Table 5.1. The SMP containing only the A variant of β-lg had a protein content of 38.0 % and a lactose content of 55.6 %. The SMP containing only

the B variant had a protein content of 36.7 % and a lactose content of 56.6 %. The WPI was manufactured in-house using a combination of microfiltration and ultrafiltration using a pilot-scale membrane plant (GEA Process Technologies, Dublin, Ireland), described in detail by Kelly et al. (2025), followed by drying using a single-stage-spray dryer (Anhydro F1 Lab Dryer; Copenhagen, Denmark). Based on the work carried out in Chapters 3 and 4, it was assumed that the protein content of the WPI was comprised entirely of whey proteins. The WPI containing only the A variant of β-lg had a protein content of 90.6 %, and the WPI containing only the B variant of β-lg had a protein content of 92.9 %. The lactose powder was purchased from Kerry Group (Tralee, Co. Kerry, Ireland), and had a purity of 99.9 %. The fat source for the IMF was sunflower oil purchased from a local retail outlet.

Table 5.1. Composition of the raw materials used for the production of a model infant formula

Raw material	True Protein (%)	Fat (%)	Lactose (%)	Moisture (%)	Casein (%)	Whey Protein (%)
SMP (β-lg A)	36.9	0.0	59.4	2.6	30.9	6.0
SMP (β-lg B)	35.0	0.0	60.1	3.2	30.8	4.2
WPI (β-lg A)	89.7	0.0	0.0	4.1	0.0	89.7
WPI (β-lg B)	92.0	0.0	0.0	5.0	0.0	92.0
Vegetable oil	0.0	100	0.0	0.0	0.0	0.0
Lactose powder	0.0	0.0	99.9	0.0	0.0	0.0

Abbreviations are as follows; SMP = skim milk powder, WPI = whey protein isolate.

5.1.1 Infant milk formula production

On the day of processing, a 40 % w/w dry matter content solution containing protein, fat and lactose ratios of 11: 30: 59, respectively, was produced. The protein composition was formulated to produce an IMF with a whey protein:casein ratio of 60:40. To prepare this solution, 2.6 L of deionised water was heated using a water bath to 60°C. Once the water was adequately heated, the respective quantities of the individual ingredients outlined in Table 5.2 were added to the water in the order of lactose, WPI, SMP and finally sunflower oil. After the addition of each component, the solution was high shear mixed to fully disperse the ingredients while also creating a coarse emulsion. The solution was then heat-treated at 72 °C for 15 s (HTST), or 135 °C for 3 s (UHT), using a Microthermics tubular heat-exchanger (Microthermics Inc., Raleigh, North Carolina, USA) and subsequently homogenised using a two-stage valve-type homogeniser (Niro Soavi Model NS2006H, Parma, Italy) with a first and second stage pressure of 15000 kPa and 5000 kPa, respectively. The heat-treated and homogenised material was then dried using a single-stage spray drier (Anhydro F1 Lab Dryer, Copenhagen, Denmark), with air inlet and outlet temperatures of 185 and 85 °C, respectively. Six IMF powders were produced; three from the HTST heat treatment, and three from the UHT heat treatment. Each powder, within each heat treatment, contained a different genetic variant of β-lg, either A, B or the AB haplotype. The IMF powders from the HTST heat treatment are denoted as A_{low}, B_{low} and AB_{low}, and the powders from the UHT heat-treatment are denoted as A_{high}, B_{high} and AB_{high}. A graphical representation of the processing steps used for the production of the model infant formula is presented in Fig 5.1.

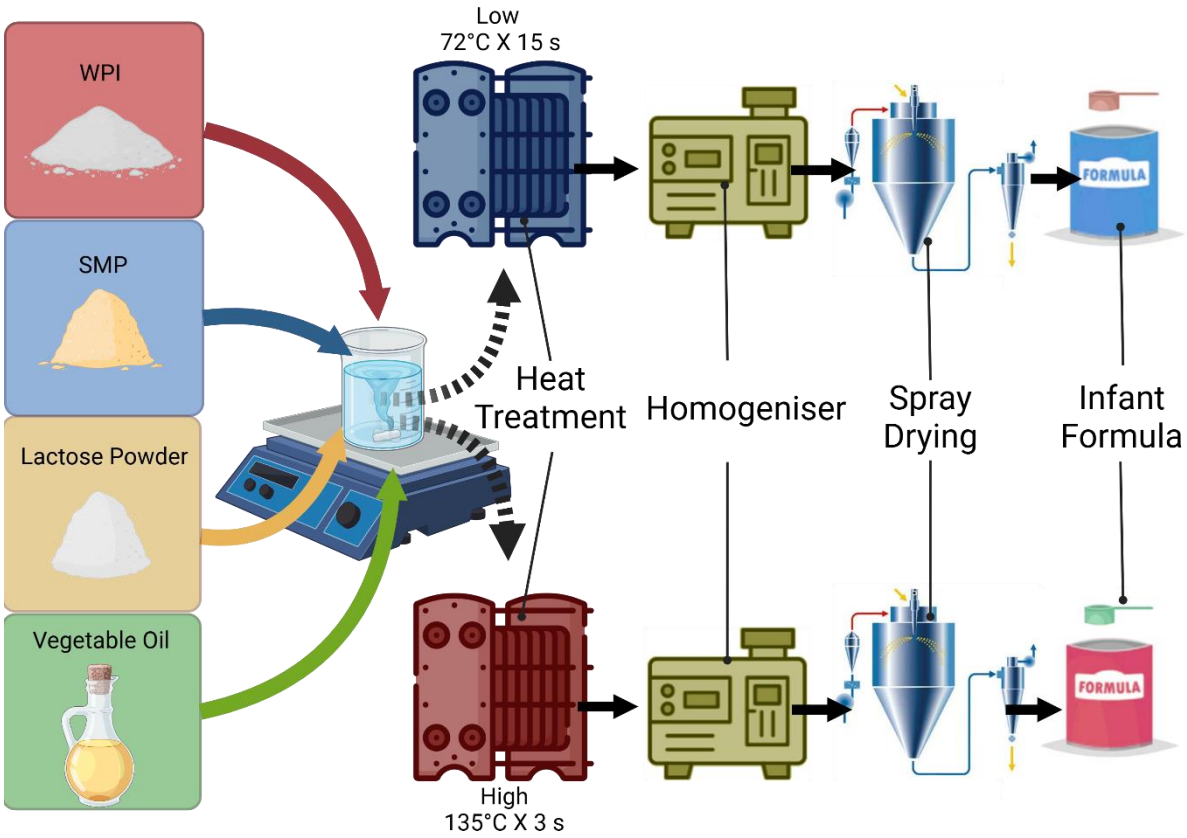


Figure 5.1 Processing steps used for the production of a model infant formula by two different heat treatments in which "high" indicates a heat treatment of 135°C for 3 s and "low" indicates a heat treatment of 72°C for 15 s. Abbreviations are as follows; WPI = whey protein isolate, SMP = skim milk powder.

Table 5.2 Mass of each component used during the formulation of a model infant formula

Raw material	Amount used (g)		
	IMF (βlg-A)	IMF (βlg-B)	IMF (βlg-AB)
SMP (β-lg A)	207	0	104
SMP (β-lg B)	0	215	108
WPI (β-lg A)	84	0	42
WPI (β-lg B)	0	80	40
Vegetable oil	427	427	427
Lactose powder	740	734	737
Water	2600	2600	2600

Materials containing β-lg A or B signifies the β-lg variant contained therein.
Abbreviations are as follows; SMP = skim milk powder, WPI = whey protein isolate, IMF = infant milk formula

5.2.2 Compositional analysis

The protein content of each IMF was determined by analysing the total nitrogen and the non-protein nitrogen contents using the Kjeldahl method, as outlined by the International Dairy Federation (IDF) methods IDF (2014) and IDF (2016), respectively. The true protein content was then determined using Equation 1, in which a nitrogen-to-protein conversion factor of 6.38 was applied. The fat content of each IMF was determined using the Rose Gottlieb method outlined in IDF (2008). The ash content of each IMF was measured using a Leco TGA701 thermogravimetric analyser (Leco Instruments, Stockport, UK). The lactose content was calculated as the remaining percentile, after protein, fat and ash was determined and the composition of the IMFs is presented in Table 5.3.

$$True\ protein = (total\ nitrogen - non\ protein\ nitrogen) \times 6.38 \quad \text{Eq.1}$$

Table 5.3 Macro-composition of the six model infant milk formulas (IMF)

IMF	True Protein (%, w/w)	Fat (%, w/w)	Lactose (%, w/w)	Ash (%, w/w)
A_{low}	10.89 ± 0.03	30.45 ± 0.58	56.16 ± 0.59	2.16 ± 0.04
B_{low}	10.65 ± 0.06	29.55 ± 0.19	57.09 ± 0.19	2.41 ± 0.03
AB_{low}	9.94 ± 0.08	29.98 ± 0.19	56.95 ± 0.19	2.70 ± 0.01
A_{high}	10.17 ± 0.12	30.95 ± 0.02	55.93 ± 0.02	2.53 ± 0.00
B_{high}	10.49 ± 0.01	32.50 ± 0.21	54.26 ± 0.21	2.36 ± 0.01
AB_{high}	10.50 ± 0.16	31.28 ± 0.03	55.83 ± 0.03	2.08 ± 0.00

Infant milk formula (IMF) sample names containing either an A or B indicates the β-lactoglobulin (β-lg) genetic variant contained therein. Sample names containing AB indicates the AB haplotype of β-lg. IMF sample names which contain a subscript "low" indicates a HTST heat treatment and those which contain a subscript "high" indicates a UHT heat treatment was used during IMF production.

5.2.3 In vitro semi-dynamic digestion

The parameters used for the *in vitro* semi-dynamic digestion of IMF mimic gastric digestion by a six month old infant. The gastric conditions were adjusted from the adult semi-dynamic protocol (Mulet-Cabero et al., 2020) based on parameters reported by other studies that have performed infant digestion using static or semi-dynamic models based on the INFOGEST semi-dynamic *in vitro* digestion system (Chen et al., 2022; de Oliveira et al., 2016; Ménard, Bourlieu, et al., 2018). The conditions used for the infant *in vitro* semi-dynamic digestion are listed in Table 5.4. The digestion of each IMF was carried out in duplicate. Among the IMFs heat-treated by HTST, the average protein content at the start of digestion was 1.38, 1.40 and 1.29% for samples A_{low}, B_{low} and $AB_{low,}$ respectively. For the IMFs heat-treated by UHT, the

average protein content at the beginning of digestion was 1.43, 1.44 and 1.22% for samples A_{high}, B_{high} and AB_{high}, respectively.

Table 5.4 Parameters for the semi-dynamic in vitro gastric digestion of a model infant formula (IMF)

Gastric conditions at 37°C			Units
IMF ingested		30.0	mL
Fasted Volume		1.76	mL
Digestion Time		49	min
Gastric Emptying (GE)	Points	4	
	Volume at emptying	9.0	mL
Gastric Secretion	SGF	11.9	mL
	Flow rate	0.24	mL min^{-1}
Meal : SGF		63:37	
Enzyme Requirement	Pepsin activity	400	U mL^{-1}
	Lipase activity	7	U mL^{-1}
Enzyme Secretion	Enzyme solution	1.14	mL
	Flow rate	0.02	mL min^{-1}

Abbreviations are as follows; simulated gastric fluid (SGF)

Based on the study of Ménard, Bourlieu, et al. (2018), a ratio of meal to simulated gastric fluid (SGF) of 63:37 was used, which differs from the adult *in vitro* digestion model, which uses a

ratio of 50:50. Typically, for adult *in vitro* digestions, an initial oral phase marks the beginning of semi-dynamic digestion; however, due to the short residence time of IMF in the mouth during infant digestion, the oral phase can be removed for beverages (Mulet-Cabero et al., 2020). Due to the lack of an international consensus on an infant digestion method, the digestion time half-time ($t_{½}$) was determined using the same method used for adult digestion, in which the $t_{½}$ is based on the caloric content of the food material (Mulet-Cabero et al., 2020). The energy content of 30 mL of IMF was 19.81 kCal, which was determined by calculating the caloric content of the protein, fat and lactose present in the IMF as per the calculations outlined in the INFOGEST adult model. By using an energy emptying rate of 0.4 kCal min^{-1} and a volume emptying rate of 0.7 mL min^{-1}, $t_{½}$ values of 24 min and 45 s were determined. The pH acidification curve followed a linear regression of $pH = –0.059\ t + 7.1$ from the natural pH of the IMF (pH 7.1) to the fasted pH of an infant's stomach at pH 4.19, whereas the adult model aims to reach a fasted pH of 2.1 (Chen et al., 2022). SGF was prepared using the method outlined by Mulet-Cabero et al. (2020). Prior to digestion, a 1:10 dilution of 0.5M HCl with SGF was prepared to obtain a 0.05M HCl and SGF mixture. This mixture was added to the IMF at a rate of 0.24 mL min^{-1} to reach the target pH of 4.19. The rabbit gastric extract (RGE) enzyme activity was determined using the method outlined by Brodkorb et al. (2019). The RGE had a lipase activity of 7 U mg^{-1} and a pepsin activity of 400 U mg^{-1}. An enzyme solution was prepared by dissolving 160 mg of RGE in 3.2 mL of deionised water, resulting in a lipase activity of 19 U mL^{-1} and a pepsin activity of 965 U mL^{-1}. These activities are consistent with the enzyme activity of a six month old infant, weighing approximately 7.5 kg, and are based on the research of Hamosh (1996) and Cook-Sather et al. (1997). The enzyme solution was subsequently added at a rate of 0.0231 mL min^{-1}.

5.2.4 Gastric phase

The parameters of the gastric phase of digestion used are outlined by Chen et al. (2022). An aliquot of 30 mL of IMF was added into a temperature-controlled glass vessel at 37 °C. The fasted volume of SGF (1.76 mL) was then added to the IMF to initiate digestion. The SGF was titrated into the digestion vessel using a pH-stat dosing device (800 Dosino, Switzerland) and a syringe pump (New Era Pump Systems Inc., USA). The total volume of SGF titrated into the digestion vessel was 11.9 mL at a rate of 0.24 mL min^{-1}, and the total volume of enzyme solution pumped into the digestion vessel was 1.14 ml, at a rate of 0.0231 mL min^{-1}. A volume of 9 mL of sample was collected at four gastric emptying (GE) points, which were taken at 12 min and 23 s (GE1), 24 min and 45 s (GE2), 37 min and 8 s (GE3), and finally 49 min and 30 s (GE4), additionally, samples indicated by GE0 refers to the undigested IMF samples. Once taken, the samples were placed into an 85°C water bath for 10 min to stop digestion, after which the samples were placed in a freezer for storage. At each respective GE, the pH was as follows; GE0 ≈ 7.1, GE1 ≈ 6.4, GE2 ≈ 5.6, GE3 ≈ 4.9, GE4 ≈ 4.2.

5.2.5 Protein content in digested samples

The protein content of the digested samples was determined using the Dumas method using a LECO FP628 nitrogen analyser (LECO Corporation, St Joseph, MI, USA), using a nitrogen to protein conversion factor of 6.38. The results from this analysis, while not presented, were used for the dilution of the digested samples prior to the analysis that was conducted as described in Sections 5.2.7 and 5.2.8.

5.2.6 Confocal laser scanning microscopy

A Leica TSC SPS confocal laser scanning microscope (Leica Microsystems, Baden-Wurttemberg, Germany) was used to assess the microstructures of the IMF samples collected at each emptying point during semi-dynamic digestion. A dye comprising of 0.1% Fast Green and 0.02% Nile red mixed at a ratio of 1:1 was added to each sample prior to viewing. To visualise protein and fat respectively. Images were captured using a 20× oil-immersion objective, equipped with a He-Ne laser.

5.2.7 Electrophoretic analysis of protein profile

The method used for the protein profiling by sodium dodecyl sulphate-polyacrylamine gel electrophoresis (SDS-PAGE) of the digested samples was based on the method of Laemmli (1970) and modified as reported by Buggy et al. (2017). The protein concentration of each sample was standardised to achieve a concentration of 20 μg of protein within each well. Samples were dissolved in SDS under reducing conditions and aliquots of 10 μL of each sample were loaded into each well of a 12% NuPAGE Bis-Tris gels (Thermofiser, Waltham, MA, US). Electrophoresis was conducted using an X-Cell Surelock electrophoresis system with 3-morpholinopropanesulfonic acid (MOPS) running buffer. Gels were subsequently stained using instant blue stain (Expedeon, Cambridge, UK) and rinsed using MilliQ water.

5.2.8 Primary amine analysis

The concentration of primary amines from the IMF and the digested samples thereof was determined using the *o*-phthaldialdehyde (OPA) method. The OPA working reagent was prepared using the method outlined by Lorieau et al. (2018). The digested samples were prepared for primary amine analysis by adding 5% trichloroacetic acid to remove any insoluble

protein and then centrifuged at 10000 g for 30 min at 4°C, the supernatant was removed and analysed thereafter (Mulet-Cabero et al., 2019). A calibration curve was generated using a glutamic acid standard at concentrations 0 to 8 mM. An aliquot of 8 µl of sample and 232 µl of OPA working reagent were added to a 96 well microplate and stored in the dark for 10 min, after which the absorbance was recorded at 340 nm. Analysis was carried out in triplicate.

5.2.9 Size exclusion chromatography

The method used for the quantification of peptides within digested samples is that used previously by Chen et al. (2022). All samples were diluted to a protein concentration of 0.2 %. An isocratic gradient with a flow rate of 0.5 mL min^{-1} was applied during analysis using a mobile phase consisting of 30% acetonitrile and 0.1% trifluoroacetic acid. Prior to analysis, the samples were filtered using a 0.22 µm polyethersulfone (PES) filter. A Waters C2695 HPLC unit (Milford, MA, USA) equipped with a TSK 2000 SW_{XL} column (Tosh Biosciences GmbH, Germany) and a UV/vis detector (Waters UV2489) at a wavelength of 214 nm was used. Using Waters Empower software (Milford, MA, USA, peak analysis and integration was achieved. Analysis was carried out in duplicate.

5.2.10 Statistical data analysis

Statistical analysis was performed using IBM SPSS Statistics (IBM Statistics Inc. Armonk, NY). Statistical significance was determined by paired-samples t-test. Results were considered significant at a *P*-value <0.05.

5.3 Results and discussion

5.3.1 Structural changes during in vitro digestion

The initial microstructure of all samples at GE0 prior to digestion showed a uniform distribution of components (Fig 5.2). For all samples, the first indication of digestion was an increase in particle size followed by a subsequent breakdown of these aggregates. These changes are in agreement with other studies (Halabi et al., 2022; Huppertz & Lambers, 2020) and are likely due to enzymatic breakdown of proteins by pepsin. The samples that were subjected to HTST during their production began to form large coagulated particles at GE1, whereas the samples that were subjected to UHT treatment began to coagulate at GE2, indicating a difference in the onset of coagulation between the HTST samples and the UHT samples.

The aggregation of proteins within the samples is likely caused by a combination of the reduction of pH, in which the pH at GE1 and GE2 was 6.4 and 5.6, respectively, and the enzymatic hydrolysis of κ-casein by pepsin, followed by the aggregation of *para* casein micelles; however, this action can be delayed by the heat-induced association of β-lg to the casein micelle (Halabi et al., 2022). This could indicate that the higher heat treatment of the UHT samples during their production caused a greater association of β-lg with κ-casein and, due to the tendency of β-lg to resist proteolysis by pepsin in the initial stages of digestion, results in the delay in aggregation observed between the UHT and HTST samples.

After GE1, the large particles within the digesta of the B_{low} and AB_{low} samples began to break down, and continue to dissociate until the end of digestion. The A_{low} sample differed from those of the B_{low} and AB_{low} samples at GE2. While these samples began to breakdown at GE2 the A_{low} sample continued to coagulate and form larger particles. However, after GE2 the A_{low}

sample began to breakdown and eventually adopted a similar microstructure to those of the B_{low} and AB_{low} samples at GE4. All of the UHT samples had large coagulated particles at GE2. However, the AB_{high} sample had considerably smaller particles at GE3 and GE4 than the A_{high} or B_{high} samples. This difference in aggregate size between β-lg variants has been reported previously by Keppler et al. (2015), who identified that the heterodimer formation of the AB haplotype of β-lg produced smaller aggregates; however, this was in a purified β-lg solution and the exact reason why the AB haplotype induced smaller aggregates is unknown. Additionally, between GE2 and GE3 for the B_{high} samples, the particles that are present at GE2 appear to coalesce into larger particles at GE3, which then breakdown at GE4. At the latter stages of digestion (GE3 and GE4) the UHT samples exhibited much larger coagulated material compared to the HTST samples. Chen et al. (2022) also found that a heat treated IMF, compared to an IMF that was prepared by membrane filtration, had larger sized coagulated material at the end of digestion. The formation of larger aggregates observed from the IMF heat treated by UHT could be due to the greater association of β-lg to the fat globules within the IMF which are stabilised by a protein layer, and has been shown to promote aggregation (Lee & Sherbon, 2002; Wiking et al., 2022). In addition, the more extensive protein denaturation that would have occurred during high heat treatment, could also lead to larger protein aggregates through hydrophobic interactions and disulphide bonding, particularly among whey proteins (Li et al., 2021).

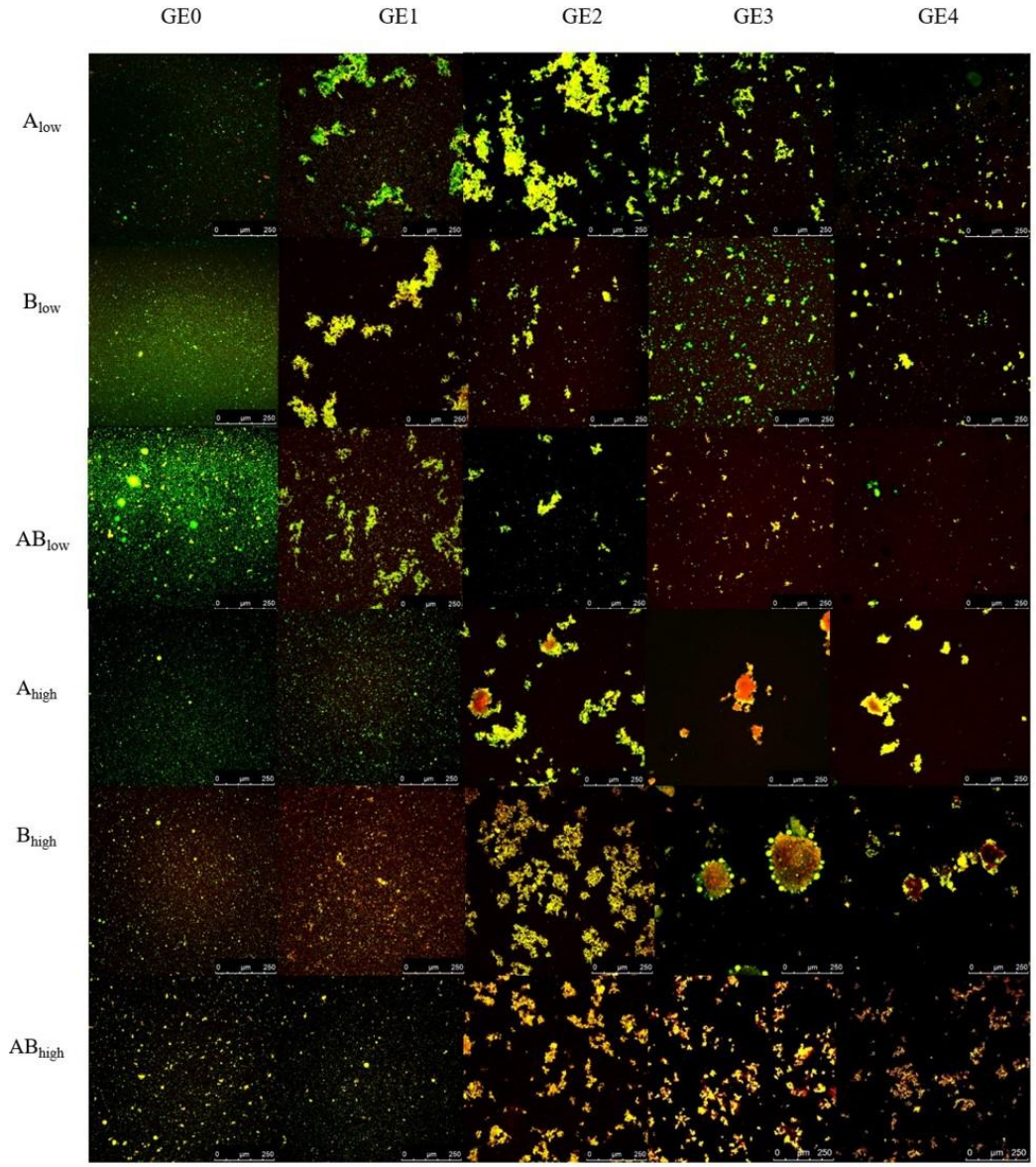


Figure 5.2 Confocal laser scanning microscopy images of the microstructures of six different model infant formulas at five gastric emptying points (GE0-GE4) during *in vitro* digestion. Sample names containing either an A or B indicates the β-lactoglobulin (β-lg) genetic variant contained therein; additionally names containing AB indicates the AB haplotype of β-lg. Sample names containing a subscript low indicates a HTST heat treatment and those with a subscript high indicate a UHT heat treatment.

5.3.2 Identification of proteins in infant milk formula using SDS-PAGE

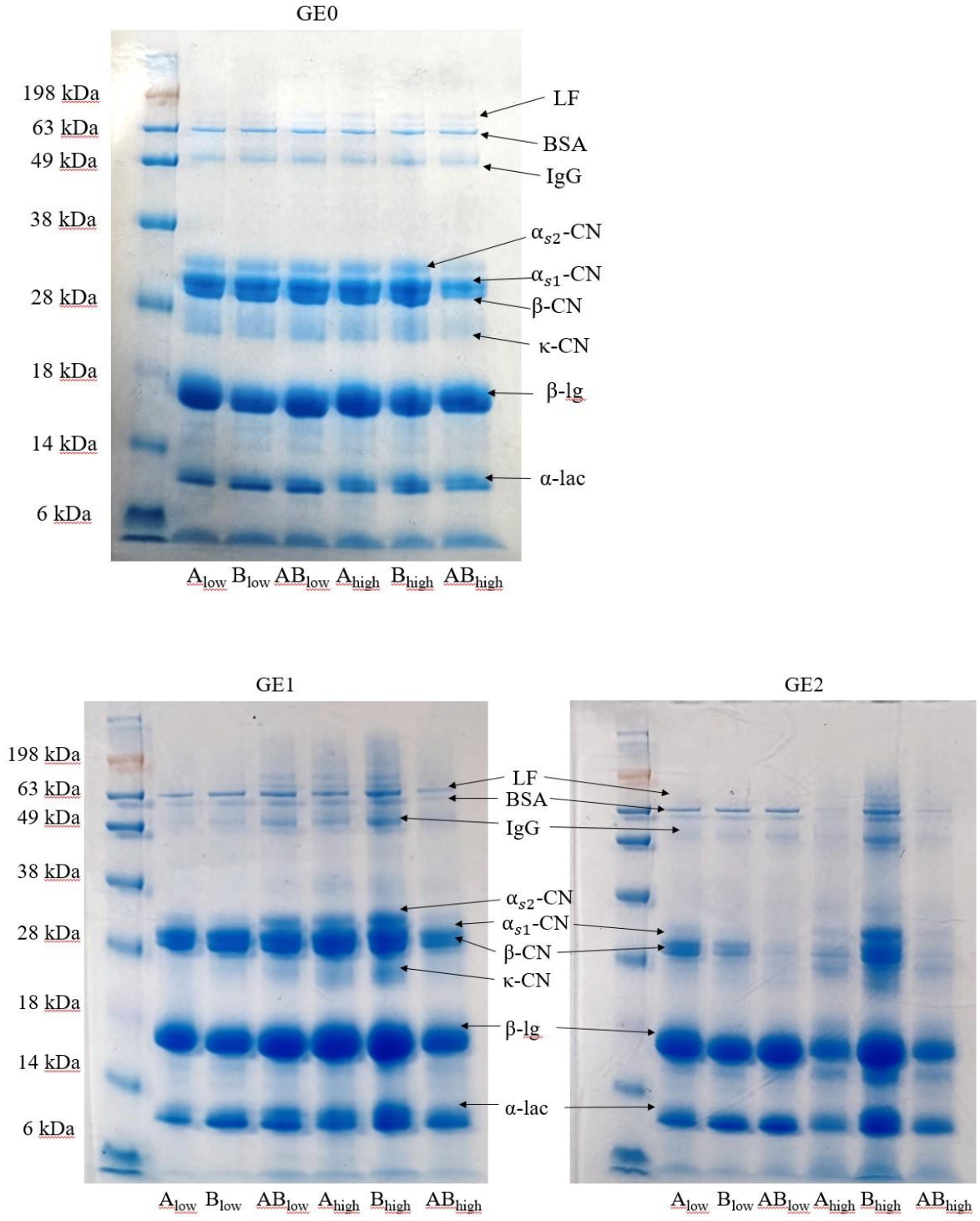
The SDS-PAGE protein profiles highlight the major milk proteins and their progressive digestion at each gastric emptying point (Fig 5.3). At GE0, the intact caseins (α_{s1}-, α_{s2}-, β-, and

κ-casein) can be observed at approximately 30-35 kDa, β-lg at 18 kDa and α-lactalbumin at 14 kDa. Between GE1 and GE2, the intensity of the casein bands decreased for all samples, with the exception of B_{high}. The decrease in band intensity is due to the breakdown of the proteins by pepsin, and dilution of the samples by addition of SGF and removal of the gastric contents at each GE point (Chen et al., 2022). It is unclear why the B_{high} sample resisted proteolysis at GE2, but it could be due to the greater quantity of β-sheets associated with the B variant of β-lg which may alter the proteolytic action of pepsin by reducing the number of available cleavage sites (Imafidon et al., 1991a).

For all samples, the change in the protein profiles between GE2 and GE3 represented a total breakdown of the caseins with bands no longer present in the 30 – 35 kDa region. The samples that were treated under HTST conditions resisted proteolysis of β-lg and α-lactalbumin, with no difference found among the variants. The samples that were treated with UHT had a reduction in the intensity of the β-lg and α-lactalbumin bands between GE2 and GE3. At GE4, the HTST samples retained a distinct β-lg band; however the α-lactalbumin bands for all samples were no longer present. These results agree with those of He et al. (2022) who also found that during static digestion of IMF, β-lg retained a visible band throughout digestion. These results are also in agreement with those of Chen et al. (2022), who reported the absence of β-lg bands at the end of digestion for a heat-treated IMF.

The UHT samples appear to have had a total breakdown of all major proteins, with no bands remaining in the gel at GE4. From the profiles generated from the SDS-PAGE analysis, it is clear that a higher heat treatment, regardless of genetic variant, caused greater break down of β-lg. The poor digestibility of β-lg has been reported previously (Chatterton et al., 2006; Kitabatake & Kinekawa, 1998; van Lieshout et al., 2020). This resistance to gastric digestion is thought to be due to the tightly folded globular structure of β-lg, which restricts access of pepsin to the peptide bonds of the protein backbone (van Lieshout et al., 2020). However, the

high temperatures applied during UHT induces a virtually complete denaturation and aggregation of β-lg. These structural changes render β-lg more susceptible to proteolysis at the gastric phase, which may explain the absence of β-lg bands among the UHT samples at GE3 and GE4.



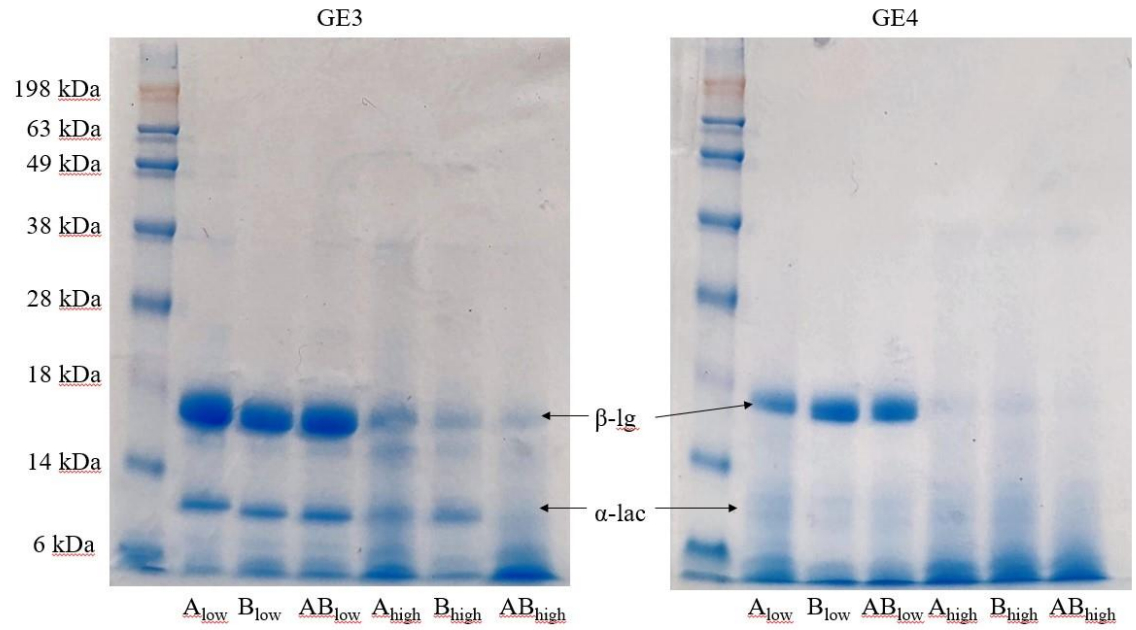


Figure 5.3 SDS PAGE protein profiles of digested samples at five gastric emptying points (GE0-GE4) during *in vitro* digestion. IMF sample names containing either an A or B indicates the β-lactoglobulin (β-lg) genetic variant contained therein; additionally names containing AB indicates the AB haplotype of β-lg. Samples containing a subscript "low" indicates a HTST heat treatment and those with a subscript "high" indicate a UHT heat treatment. The abbreviations of the protein bands are as follows; lactoferrin (LF), bovine serum albumin (BSA), immunoglobulin G (IgG), casein (CN), β-lactoglobulin (β-lg) and α-lactalbumin (α-lac).

5.3.3 Protein hydrolysis

Before digestion, little to no free amines were present within any of the samples (Table 5.4) as measured using the OPA assay. To clarify, the OPA analysis is used to determine the concentration of primary amines, which are the amines present at the N-terminus of a protein or peptide. A higher concentration of primary amines is indicative of the breakdown of peptide bonds. As digestion begins, primary amines were liberated and their concentration continued to increase until the end of digestion at GE4. This is due to the cleavage of peptide bonds by pepsin during gastric digestion. A greater amount of amines were released from the samples subjected to UHT, compared to HTST heat treatment, indicating a more complete hydrolysis of the proteins. This is likely due to conformational changes in the proteins as a result of the

high temperatures applied during their manufacture, allowing for a greater accessibility for cleavage by pepsin. The IMFs containing the AB haplotype of β-lg, within their respective heat treatment groups, had the highest quantity of free amines at the end of digestion (GE4), with the AB_{high} sample having a significantly (P <0.05) higher free amine concentration than any other sample. While it is unclear as to why the AB haplotype was conducive to greater release of primary amines, a possible reason could be due to the formation of smaller aggregates as observed in Fig 5.2. The smaller aggregate size of the AB_{low} and AB_{high} samples, could allow for more enzymatic cleavage of β-lg by pepsin, resulting in greater liberation of primary amines due to an increase in aggregate surface area.

The A_{low} sample had the lowest release of amines at the end of digestion (GE4), and was significantly lower (P <0.05) than the other samples that were subjected to HTST heat treatment at GE1. This could possibly be due to greater electrostatic repulsion between the A variant and pepsin where both molecules have a net negative charge, while the B variant of β-lg is also negatively charged, the A variants has an additional charged amino acid at position 64 (Boeris et al., 2011; Keppler et al., 2014). Of the samples that were UHT treated, the B_{high} IMF had the lowest release of amines, which was significantly (P <0.05) lower than the A_{high} and AB_{high} samples at GE3 and GE4. A possible reason for the contrast in amine liberation between the UHT and the HTST heat treatments of the IMF's containing the A and B variant of β-lg, may be due to differences in the interaction between β-lg and the casein micelle. It was also reported by Halabi et al. (2020), that the hydrolysis of caseins increased when denatured whey proteins, associated with the casein micelle. The A variant of β-lg has been reported to have better solubility than the B variant, which can increase the tendency of the A variant of β-lg to interact with the micelle, resulting in an increase in casein hydrolysis (Fei et al., 2020).

Table 5.4 Concentration of the free amines released from infant formulas during semi-dynamic *in vitro* digestion.

IMF samples	mmol free amines/g total protein				
	GE0	GE1	GE2	GE3	GE4
A_{low}	0.00 ± 0.05^{a}	1.77 ± 0.14^{a}	2.89 ± 0.31^{a}	5.24 ± 0.48^{a}	9.09 ± 0.45^{a}
B_{low}	0.00 ± 0.03^{a}	2.17 ± 0.22^{bd}	3.44 ± 0.11^{a}	6.16 ± 0.30^{a}	9.75 ± 0.40^{ad}
AB_{low}	0.00 ± 0.03^{a}	2.61 ± 0.12^{b}	4.87 ± 0.12^{b}	7.80 ± 0.24^{c}	9.79 ± 0.25^{ad}
A_{high}	0.00 ± 0.01^{a}	1.55 ± 0.25^{acd}	4.28 ± 0.20^{b}	8.32 ± 0.29^{bc}	11.79 ± 0.27^{b}
B_{high}	0.01 ± 0.01^{a}	1.18 ± 0.06^{c}	3.36 ± 0.13^{a}	6.32 ± 0.20^{a}	10.23 ± 0.23^{ad}
AB_{high}	0.00 ± 0.01^{a}	1.50 ± 0.14^{ac}	4.67 ± 0.83^{ab}	9.93 ± 0.92^{c}	12.84 ± 0.42^{c}

IMF sample names containing either an A or B indicates the β-lactoglobulin (β-lg) genetic variant contained therein; additionally names containing AB indicates the AB haplotype of β-lg. Samples containing a subscript "low" indicates a HTST heat treatment and those with a subscript "high" indicate a UHT heat treatment. Samples within the same column, not sharing a common superscript letter were deemed statistically significantly different ($P < 0.05$). These results are the averages from duplicated digestions, with the primary amine analysis performed in triplicate.

5.3.4 Peptide distribution

The size distribution of peptides, as analysed using SEC HPLC, is shown in Fig.5.4. All IMF samples showed a high proportion of proteins with a molecular weight > 9 kDa prior to digestion (GE0), which is indicative of intact/undigested or aggregated proteins. As digestion progressed, the proportion of intact proteins decreases and peptides within the ranges of 1-9 and <1 kDa increase. This is in agreement with the results from Chen et al. (2022), who also performed semi-dynamic *in vitro* digestion of IMF under similar conditions as those in the present study. In their study, they observed an increase in the concentration of < 10 kDa peptides as digestion progressed. The range 1-9 kDa represents small and medium-sized soluble digested peptides. Peptides < 1 kDa are generally recognised as readily absorbed into

the bloodstream during intestinal digestion, or they may possibly breakdown further from the action of brush-border enzymes (Le Roux et al., 2020).

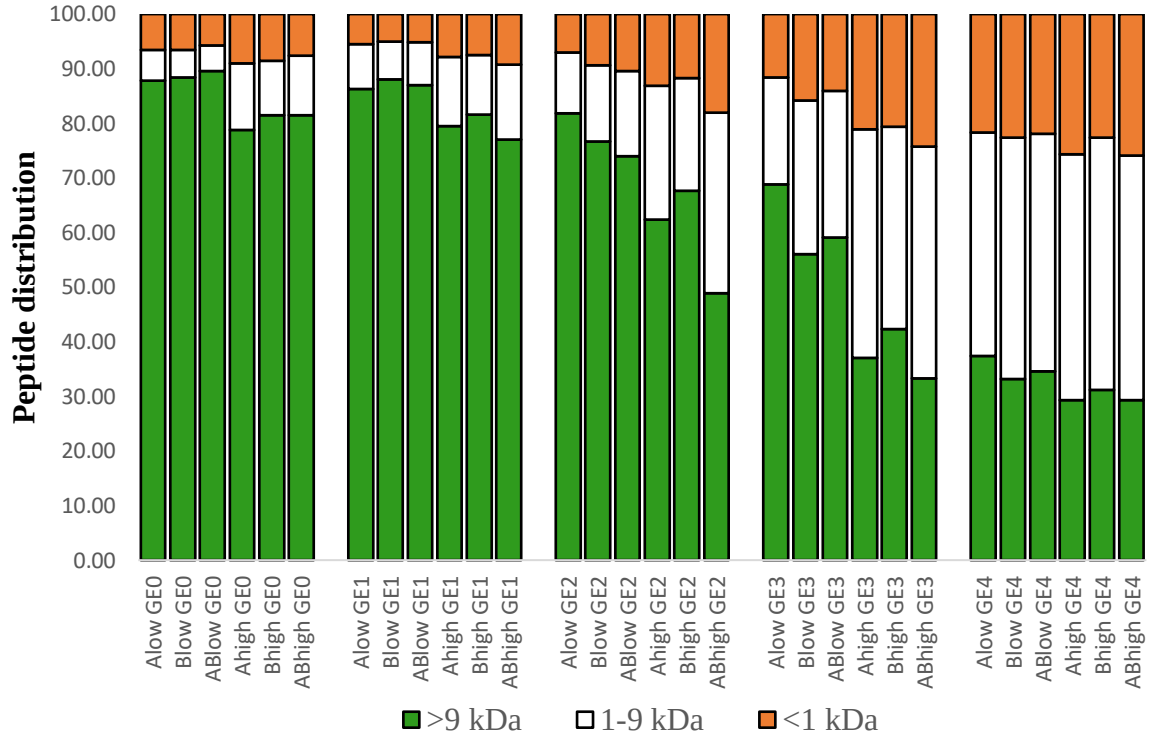


Figure 5.4 Peptide distribution of six model infant formulas at five gastric emptying points (GE0-GE4) during semi dynamic *in vitro* digestion. IMF sample names containing either an A or B indicates the β-lactoglobulin (β-lg) genetic variant contained therein; additionally names containing AB indicates the AB haplotype of β-lg. Sample names containing low indicates a HTST heat treatment and those with high indicate a UHT heat treatment. Peptide distribution is as follows; >9 kDa (■), 1-9 kDa (□) and <1 kDa (■). These results are the averages of duplicated digestions, with the peptide distribution analysis performed in duplicate.

At GE0, the samples subjected to HTST heat treatment had a larger proportion of peptides at >9 kDa than the samples that to UHT heat treatment, which had a larger proportion of 1-9 and < 1 kDa peptides. This is probably due to more proteins being damaged during UHT heat treatment, as a result of the combination of high temperatures and mechanical stress. A study by Ye et al. (2017) in which the effect of the heat treatment of milk on protein digestion during the gastric phase was investigated, also indicated that higher temperature heat treatment of milk yields an increased rate of protein breakdown, linked to denaturation and aggregation of whey

proteins. However, during the gastric phase, the IMF that was subjected to UHT heat treatment was digested faster than the IMF that was subjected to HTST heat treatment. This is evident from the higher concentration of 9-1 and < 1 kDa peptides in the A_{high}, B_{high} and AB_{high} samples between GE2 and GE3 than those of A_{low}, B_{low} and AB_{low} samples. There was a distinct difference between UHT and HTST treated samples throughout digestion until GE4, at which point the distribution of peptides was similar among all samples, indicating that, at the end of the gastric phase, all the samples had a similar molecular weight distribution of proteins entering the small intestine. These results are in agreement with those of Tunick et al. (2016) who reported that whole milk samples that were UHT heat-treated had a considerably lower protein particle size than HTST heat treated whole milk until the end of gastric digestion at which point the size distributions were equivalent.

5.4 Conclusion

This study determined that different heat treatments during production of IMF can have a significant ($P < 0.05$) effect on *in vitro* digestion behaviour. The IMF samples that were subjected to UHT heat treatment, showed extensive breakdown of β-lg during the gastric phase, whereas it remained relatively intact in the IMF samples subjected to the lower temperature HTST treatment. The AB haplotype for both UHT and HTST had the most free amines liberated at the end of digestion, which is indicative of more extensive proteolysis having taken place, possibly due to the smaller aggregate size of the AB haplotype compared to the samples containing the A and B variants. Overall, there was little difference between variants of β-lg in terms of the digestion of IMF, while the use of different heat treatments did influence infant digestion.

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Declaration of competing interest

Authors declare no conflicts of interest.

5.5 References

Alles, M. S., Scholtens, P. A. M. J., & Bindels, J. G. (2004). Current trends in the composition of infant milk formulas. *Current Paediatrics, 14*, 51-63.

Asledottir, T., Le, T. T., Poulsen, N. A., Devold, T. G., Larsen, L. B., & Vegarud, G. E. (2018). Release of β-casomorphin-7 from bovine milk of different β-casein variants after ex vivo gastrointestinal digestion. *International Dairy Journal, 81*, 8-11.

Barnett, M. P. G., McNabb, W. C., Roy, N. C., Woodford, K. B., & Clarke, A. J. (2014). Dietary A1 β-casein affects gastrointestinal transit time, dipeptidyl peptidase-4 activity, and inflammatory status relative to A2 β-casein in Wistar rats. *International Journal of Food Sciences and Nutrition, 65*, 720-727.

Boeris, V., Micheletto, Y., Lionzo, M., Silveira, N. P. d., & Picó, G. (2011). Interaction behavior between chitosan and pepsin. *Carbohydrate Polymers, 84*, 459-464.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., & Carrière, F. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*, 991-1014.

Buggy, A. K., McManus, J. J., Brodkorb, A., Carthy, N. M., & Fenelon, M. A. (2017). Stabilising effect of α-lactalbumin on concentrated infant milk formula emulsions heat treated pre-or post-homogenisation. *Dairy Science & Technology, 96*, 845-859.

Caroli, A. M., Chessa, S., & Erhardt, G. J. (2009). Invited review: Milk protein polymorphisms in cattle: Effect on animal breeding and human nutrition. *Journal of Dairy Science, 92*, 5335-5352.

Chatterton, D. E. W., Smithers, G., Roupas, P., & Brodkorb, A. (2006). Bioactivity of β-lactoglobulin and α-lactalbumin: Technological implications for processing. *International Dairy Journal, 16*, 1229-1240.

Chen, Y., Callanan, M., Giblin, L., Tobin, J., & Brodkorb, A. (2022). Comparison of conventional heat-treated and membrane filtered infant formulas using an in vitro semi-dynamic digestion method. *Food & Function, 13*, 8158-8167.

Cook-Sather, S. D., Tulloch, H. V., Liacouras, C. A., & Schreiner, M. S. (1997). Gastric fluid volume in infants for pyloromyotomy. *Canadian Journal of Anaesthesia, 44*, 278-283.

Crowley, S. V., Dowling, A. P., Caldeo, V., Kelly, A. L., & O'Mahony, J. A. (2016). Impact of α-lactalbumin:β-lactoglobulin ratio on the heat stability of model infant milk formula protein systems. *Food Chemistry, 194*, 184-190.

Daniloski, D., Hailu, Y., Brodkorb, A., Vasiljevic, T., & McCarthy, N. A. (2024). Impact of β-casein phenotype on the physical properties of skim milk powders and their subsequent digestion characteristics. *Food Hydrocolloids, 152*, 109918.

de Oliveira, S. C., Deglaire, A., Ménard, O., Bellanger, A., Rousseau, F., Henry, G., Dirson, E., Carrière, F., Dupont, D., & Bourlieu, C. (2016). Holder pasteurization impacts the proteolysis, lipolysis and disintegration of human milk under in vitro dynamic term newborn digestion. *Food Research International, 88*, 263-275.

Fei, S., Zou, L., Xie, X., Yang, F., Chen, H., & Li, X. (2020). Purification and Characterization of Bovine β-Lactoglobulin Variants A and B (Characterization of Bovine β-Lactoglobulin Variants). *Food Science and Technology Research, 26*, 399-409.

Fracisquini, J. d. A., Nunes, L., Martins, E., Stephani, R., Perrone, Í., & de Carvalho, A. F. (2017). Effect of heat treatment on infant formulas: a review. *Revista do Instituto de Laticínios Cândido Tostes, 72*, 236-244.

Francisquini, J. d. A., Nunes, L., Martins, E., Stephani, R., Perrone, Í. T., & Carvalho, A. F. d. (2020). How the heat treatment affects the constituents of infant formulas: a review. *Brazilian Journal of Food Technology, 23*, e2019272.

Gai, N., Uniacke-Lowe, T., O'Regan, J., Faulkner, H., & Kelly, A. L. (2021). Effect of Protein Genotypes on Physicochemical Properties and Protein Functionality of Bovine Milk: A Review. *Foods, 10*, 2409.

Gallier, S., Vocking, K., Post, J. A., Van De Heijning, B., Acton, D., Van Der Beek, E. M., & Van Baalen, T. (2015). A novel infant milk formula concept: Mimicking the human milk fat globule structure. *Colloids and Surfaces B: Biointerfaces, 136*, 329-339.

Ganai, N., Bovenhuis, H., Van Arendonk, J., & Visker, M. (2009). Novel polymorphisms in the bovine β-lactoglobulin gene and their effects on β-lactoglobulin protein concentration in milk. *Animal Genetics, 40*, 127-133.

Halabi, A., Croguennec, T., Bouhallab, S., Dupont, D., & Deglaire, A. (2020). Modification of protein structures by altering the whey protein profile and heat treatment affects in vitro static digestion of model infant milk formulas. *Food & Function, 11*, 6933-6945.

Halabi, A., Croguennec, T., Ménard, O., Briard-Bion, V., Jardin, J., Le Gouar, Y., Hennetier, M., Bouhallab, S., Dupont, D., & Deglaire, A. (2022). Protein structure in model infant

milk formulas impacts their kinetics of hydrolysis under in vitro dynamic digestion. *Food Hydrocolloids, 126*, 107368.

Hamosh, M. (1996). Digestion in the newborn. *Clinics in perinatology, 23*, 191-209.

He, T., Rombouts, W., Einerhand, A. W. C., Hotrum, N., & van de Velde, F. (2022). Gastric protein digestion of goat and cow milk infant formula and human milk under simulated infant conditions. *International Journal of Food Sciences and Nutrition, 73*, 28-38.

Huppertz, T., & Lambers, T. T. (2020). Influence of micellar calcium phosphate on in vitro gastric coagulation and digestion of milk proteins in infant formula model systems. *International Dairy Journal, 107*, 104717.

IDF. (2008). Dried Milk and Dried Milk Products - Determination of Fat Contant. In *International Dairy Federation* (Vol. IDF 9:2008). Brussels, Belgium: International Dairy Federation

IDF. (2014). Milk and milk products - Determination of nitrogen content - Part 1: Kjeldahl prinicple and crude protein calculation. In *International Dairy Federation* (Vol. IDF 20-1 :2014). Brussels, Belgium: International Dairy Federation.

IDF. (2016). Milk and milk products - determination of nitrogen content - Part 4: Determination of protein and non-protein nitrogen content calculation (Reference Method). In *International Dairy Federation* (Vol. IDF 20-4 :2016). Brussels, Belgium: International Dairy Federation.

Imafidon, G. I., Ng-Kwai-Hang, K. F., Harwalkar, V. R., & Ma, C. Y. (1991). Differential Scanning Calorimetric Study of Different Genetic Variants of β-Lactoglobulin. *Journal of Dairy Science, 74*, 2416-2422.

Kelly, L. M., McCarthy, W. P., Subhir, S., Horan, B., O'Mahony, J. A., & Tobin, J. T. (2025). Impact of genetic variants on the separation efficiency of β-lactoglobulin during microfiltration of skim milk. *International Dairy Journal, 161*, 106115.

Keppler, J. K., Sönnichsen, F. D., Lorenzen, P.-C., & Schwarz, K. (2014). Differences in heat stability and ligand binding among β-lactoglobulin genetic variants A, B and C using 1H NMR and fluorescence quenching. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1844*, 1083-1093.

Keppler, J. K., Martin, D., Garamus, V. M., & Schwarz, K. (2015). Differences in binding behavior of (−)-epigallocatechin gallate to β-lactoglobulin heterodimers (AB) compared to homodimers (A) and (B). *Journal of Molecular Recognition, 28*, 656-666.

Kitabatake, N., & Kinekawa, Y.-I. (1998). Digestibility of Bovine Milk Whey Protein and β-Lactoglobulin in Vitro and in Vivo. *Journal of Agricultural and Food Chemistry, 46*, 4917-4923.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*, 680-685.

Le Roux, L., Ménard, O., Chacon, R., Dupont, D., Jeantet, R., Deglaire, A., & Nau, F. (2020). Are Faba Bean and Pea Proteins Potential Whey Protein Substitutes in Infant Formulas? An In Vitro Dynamic Digestion Approach. *Foods, 9*, 362.

Lee, S. J., & Sherbon, J. W. (2002). Chemical changes in bovine milk fat globule membrane caused by heat treatment and homogenization of whole milk. *Journal of Dairy Research, 69*, 555-567.

Li, H., Zhao, T., Li, H., & Yu, J. (2021). Effect of Heat Treatment on the Property, Structure, and Aggregation of Skim Milk Proteins. *Frontiers in Nutrition, 8*, 714869.

Lorieau, L., Halabi, A., Ligneul, A., Hazart, E., Dupont, D., & Floury, J. (2018). Impact of the dairy product structure and protein nature on the proteolysis and amino acid bioaccessiblity during in vitro digestion. *Food Hydrocolloids, 82*, 399-411.

Martin, C. R., Ling, P.-R., & Blackburn, G. L. (2016). Review of Infant Feeding: Key Features of Breast Milk and Infant Formula. *Nutrients, 8*, 279.

Masum, A. K. M., Huppertz, T., Chandrapala, J., Adhikari, B., & Zisu, B. (2020). Physicochemical properties of spray-dried model infant milk formula powders: Influence of whey protein-to-casein ratio. *International Dairy Journal, 100*, 104565.

Ménard, O., Bourlieu, C., De Oliveira, S. C., Dellarosa, N., Laghi, L., Carrière, F., Capozzi, F., Dupont, D., & Deglaire, A. (2018). A first step towards a consensus static in vitro model for simulating full-term infant digestion. *Food Chemistry, 240*, 338-345.

Mulet-Cabero, A.-I., Mackie, A. R., Wilde, P. J., Fenelon, M. A., & Brodkorb, A. (2019). Structural mechanism and kinetics of in vitro gastric digestion are affected by process-induced changes in bovine milk. *Food Hydrocolloids, 86*, 172-183.

Mulet-Cabero, A.-I., Egger, L., Portmann, R., Ménard, O., Marze, S., Minekus, M., Le Feunteun, S., Sarkar, A., Grundy, M. M.-L., & Carrière, F. (2020). A standardised semi-dynamic in vitro digestion method suitable for food–an international consensus. *Food & Function, 11*, 1702-1720.

Mulet-Cabero, A.-I., Egger, L., Portmann, R., Ménard, O., Marze, S., Minekus, M., Le Feunteun, S., Sarkar, A., Grundy, M. M. L., Carrière, F., Golding, M., Dupont, D., Recio, I., Brodkorb, A., & Mackie, A. (2020). A standardised semi-dynamic in vitro digestion method suitable for food – an international consensus. *Food & Function, 11*, 1702-1720.

Murphy, E. G., Roos, Y. H., Hogan, S. A., Maher, P. G., Flynn, C. G., & Fenelon, M. A. (2015). Physical stability of infant milk formula made with selectively hydrolysed whey proteins. *International Dairy Journal, 40*, 39-46.

Rudloff, S., & Lönnerdal, B. (1992). Solubility and digestibility of milk proteins in infant formulas exposed to different heat treatments. *Journal of Pediatric Gastroenterology and Nutrition, 15*, 25-33.

Sawyer, L. (2012). β-Lactoglobulin. In *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects,* (Fourth Edition) (pp. 211-259): Springer.

Sheng, B., Nielsen, S. D., Poulsen, N. A., & Larsen, L. B. (2021). Differential in vitro digestion rates in gastric phase of bovine milk with different κ-casein phenotypes. *Journal of Dairy Science, 104,* 10462-10472.

Svenning, C., Brynhildsvold, J., Molland, T., Langsrud, T., & Vegarud, G. E. (2000). Antigenic response of whey proteins and genetic variants of β-lactoglobulin—the effect of proteolysis and processing. *International Dairy Journal, 10,* 699-711.

Tunick, M. H., Ren, D. X., Van Hekken, D. L., Bonnaillie, L., Paul, M., Kwoczak, R., & Tomasula, P. M. (2016). Effect of heat and homogenization on in vitro digestion of milk. *Journal of Dairy Science, 99,* 4124-4139.

van Lieshout, G. A. A., Lambers, T. T., Bragt, M. C. E., & Hettinga, K. A. (2020). How processing may affect milk protein digestion and overall physiological outcomes: A systematic review. *Critical Reviews in Food Science and Nutrition, 60,* 2422-2445.

Wiking, L., Gregersen, S. B., Hansen, S. F., & Hammershøj, M. (2022). Heat-induced changes in milk fat and milk fat globules and its derived effects on acid dairy gelation – A review. *International Dairy Journal, 127,* 105213.

Ye, A., Cui, J., Dalgleish, D., & Singh, H. (2017). Effect of homogenization and heat treatment on the behavior of protein and fat globules during gastric digestion of milk. *Journal of Dairy Science, 100,* 36-47.

Chapter 6

General Discussion and Suggestions for Future Work

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Declaration

This chapter was written by author LMK.

6.1 Summary of key findings

The results generated throughout this thesis have helped expand the knowledge base and literature on the impact of genetic variation on the technological, functional and digestive properties of β-lg. By studying the literature regarding the genetic variation of milk proteins in Chapter 1, essential, up to date, background knowledge was gained, which defined the research topics included in this thesis. The extensive pool of genetic variants present within cow's milk underpinned the concept for Chapter 2, which sought to identify the genetic variants of some of the most polymorphic milk proteins (i.e., β-casein, κ-casein and β-lg) within a subset of the high genetic merit research herd at Teagasc Moorepark.

To understand the distribution of β-lg and other protein variants among Irish dairy cows, the author was fortunate to be able access the national dairy research herds in Teagasc, Moorepark, which not only allowed access to milk, but also provided the opportunity to segregate, in relatively large volumes, the milk from cows with a diverse range of variants among the milk proteins. The results from Chapter 2 highlight the difference in the protein fraction among individual cows within a sub-group of 48 cows from the Moorepark herd, capturing the typical Holstein Friesian and Jersey Holstein cross breed cows that represent the majority of Irish milk production systems. The genotypes of β-casein, κ-casein and β-lg were characterised using high performance liquid chromatography coupled with the national genotyping programme facilitated by the Irish Cattle Breeding Federation an organisation that offers breeding information services to farms across Ireland. Of the 53 known variants described in the literature, 17 were present among the 48 individual cows; consists of 9, 3 and 5 for β-casein, κ-casein and β-lg, respectively. These different genotypes were linked to significant ($P<0.05$) differences in protein composition, both at the macro

level and among the nitrogen fractions. The milk containing the A1I genotype of β-casein had the highest total protein and casein contents among all the variants studied, while no significant (P >0.05) differences were found between the variants of κ-casein. Among the β-lg variants, milk with the AA genotype of β-lg had the highest whey protein content, compared with the other β-lg variants. While it is not known why these variants impact milk composition, it has been suggested that point mutations within sections of DNA that cause genetic variation are located in the promoter region of the DNA, which leads to either a promotion or suppression of protein synthesis.

Based on the results from Chapter 2, cows with the homozygous AA or BB genotype of β-lg were identified by HPLC, and their milk was segregated during successive milking's for the purpose of purifying the whey protein fraction, and the β-lg variants therein, by filtration to obtain highly pure whey protein ingredients that could then be subjected to functional characterisation. Chapter 3 highlighted the differences in the microfiltration performance of skim milk containing the A or B variants of β-lg, with the A variant resulting in significantly more reversible fouling than the B variant. However, despite having more reversible fouling, the permeate from the milk with the A variant of β-lg had a higher quantity of serum proteins than the B variant, indicating that the reversible fouling layer is not a rate-limiting factor during the partitioning of β-lg. The tendency for skim milk containing the A variant of β-lg to induce more reversible fouling is likely due to the greater negative charge of this variant, compared to the B variant, which causes a stronger attractive force towards the positively-charged microfiltration membrane. This attraction towards the membrane is also the likely reason for the skim milk with the A variant of β-lg having more serum protein in the permeate stream.

In Chapter 3, WPI powders containing exclusively either the β-lg genetic variants A or B, were produced. FTIR analysis of these powders showed that the secondary structures of the A and B variants are different, with the B variant containing more beta-sheets than the A variant, which had more random coils and unordered structures. Additionally, differential scanning calorimetry analysis demonstrated that the thermal denaturation temperatures (T_d) of the variants were significantly different (P <0.05), with the A variant having a T_d of 74.68°C, and 78.68°C for the B variant. These results are likely due to the difference in the secondary structures, in which the additional β-sheets of the B variant help protect the protein against thermal denaturation (Branden & Tooze, 2012). Rheological analysis indicated that the heat-set gelation properties of whey protein solutions prepared with the variants differ between pH 6.4 and 7. In this range, the B variant produced significantly stronger gels than the A variant. It was also found that the heat stability of A was higher than the B variant at pH 6.4. These results were attributed to the difference in net charge between the A and B variants, which has been reported in previous studies (Keppler et al., 2014).

The differences in the stability of the A and B variants yielded some thought-provoking results, which led to the conception of the studies described in Chapters 4 and 5 of this thesis. These chapters aimed to further investigate the effects of differences in the stability of these genetic variants on industrial processing performance and gastric digestion fate.

Chapter 5 investigated the effect of genetic variation of β-lg on the digestion of infant formula containing β-lg. In addition to this, the effects of different heat treatments, 73°C × 15 s (HTST) and 135°C × 2 s (UHT) during infant formula production were also investigated. Regardless of genetic variant, SDS-PAGE electrophoretic analysis

demonstrated that the samples exposed to the higher heat treatment during UHT had an almost complete breakdown of β-lg at the end of the gastric emptying (GE) phase, whereas intense bands could still be observed for the samples that were produced using the HTST heat treatment. The B variant of the UHT treated infant formula resisted proteolysis until GE3, whereas the other variants of the same heat treatment began to break down at GE2, as observed from SDS-PAGE, and supported by analysis of free amino groups using OPA analysis which showed that this sample also produced lower amounts of free amides than the other variants at the same heat treatment. These results are possibly due to the higher thermal stability of the B variant, which could delay the onset of gastric proteolysis by pepsin. The A variant, coupled with the HTST heat treatment, had the lowest release of amines of all the samples. This was attributed to the greater electrostatic repulsion between the A variant and pepsin, possibly linked to the higher negative charge of the A variant. The IMF containing the AB haplotype of β-lg, regardless of heat treatment, had the highest quantity of free amines at GE4. This is possibly due to the formulation of smaller heat-induced aggregates, which was also observed by confocal microscopy.

6.2 Scientific and industrial impact of the research

The industrial impact of the research conducted in Chapter 2 would be of benefit to primary producers and dairy processors. In Ireland, the payment to farmers for milk is based on protein, fat and volume. This research identified a number of genotypes of β-casein, κ-casein and β-lg that were linked to an increase in the level of protein in milk. By selecting bulls in which the protein genetic variants have been predicted, the widespread development of herds across Ireland with specific protein genetic variants

could be achieved. Additionally, dairy processors would also benefit from different genetic variants. Milk containing the A1I genotype of β-casein was associated with significantly (P <0.05) higher levels of casein. These variants would be of benefit to processors that produce cheese and yogurt products in particular, as the higher amounts of casein would help increase the product yield. The A variant of β-lg was associated with milk that had higher levels of whey proteins, with this variant therefore being of interest to producers of whey protein concentrates and isolates, and infant formula. Milk containing the A variant would increase product yield due to the higher levels of whey proteins. This would also benefit infant formula producers, as the majority of the protein in infant formula comes from whey protein, which is often delivered in infant formula in the form of whey protein concentrates and isolates.

The scientific impact of the research conducted in Chapter 3 has considerably expanded our knowledge of the functionality of β-lg. By purifying β-lg through a combination of micro- and ultra-filtration, the majority of potentially confounding milk components, such as casein and fat, were removed. With this, the results obtained from Chapter 3 are potentially more accurate representations of the functional properties of β-lg than those which have been conducted previously in milk systems (Ng-Kwai-Hang, 1998; Paterson et al., 1999). The differences in heat-set gelation of the A and B variants may also indicate potential benefits for processors. Processors that subject milk to a heat treatment, such as pasteurisation or ultra-high temperature, may prefer the A variant of β-lg, due to the higher thermal stability and lower tendency to aggregate than the B variant. This may help reduce the build-up of foulant material during heat treatment, such as pasteurisation and UHT, which has been known to cause issues such as reduction in heat transfer, interrupted flow and blockages (Huppertz & Nieuwenhuijse, 2022).

Microfiltration of milk containing either the A or B variant of β-lg using ceramic membranes, indicated the beneficial membrane filtration performance of the A variant. One of the main components of the fouling layer during the microfiltration of milk is β-lg (Jeurnink et al., 1996; Rabiller-Baudry et al., 2009). While the B variant had significantly (P <0.05) less reversible fouling than the A variant, there was a greater rejection of the B variant. The B variant also had more irreversible fouling than the A variant. The significance of these findings for processors, particularly those that manufacture whey protein concentrates and isolates, is the higher product yield that would be generated from variant A due to the higher rejection rate of B from the membrane during filtration. Additionally, the B variant had a greater extent of irreversible fouling, meaning that more time, water and potentially more chemicals, would be needed during cleaning, which would increase the cost of production.

The *in vitro* digestion of the infant formulas containing the AB variant of β-lg highlighted potential health benefits for infants upon consumption. The OPA analysis suggests that the infant formulas containing the AB haplotype had the most extensive breakdown of protein, higher than that for either the A or B variants. The more extensive breakdown of food can help prevent digestive discomfort by reducing the amount of substrate material that can be fermented by bacteria in the intestines, which can cause bloating and fatigue. The SDS-PAGE analysis also indicated that the infant formulas subjected to a higher heat treatment had considerably greater breakdown of β-lg than the lower heat treated infant formulas. This difference in the breakdown of β-lg could contribute to differences in the extent of digestive discomfort due to potentially reduced fermentation of β-lg in the intestines.

6.3 Suggestions for future research

The work presented in this thesis highlights the significant impact that the genetic variants of β-lg can have on a variety of different aspects of milk and milk products, particularly its functional, technological and digestive properties. However, there are still gaps in this current field of research, and it merits further studies to help expand the knowledge in this area. The following are some suggestions for topics that are poorly characterised in the literature and could be the basis for future studies.

1. One of the interesting findings during the study of the functional properties of β-lg was that the mineral composition between the A and B variants of β-lg were significantly (P <0.05) different. The WPI containing the A variant had a higher sodium level, and the B variant had higher levels of phosphorous and iron. While it was not possible to expand upon this within the context of this thesis, additional research could highlight the extent to which ions such as sodium and iron bind with the A and B variants of β-lg, as well as the specific binding mechanism of the different variants, helping elucidate the cause of the different mineral contents among the two variants. This could be done by investigating the proteins spatial structuring by x-ray crystallography, NMR spectroscopy or computational modelling.

2. The interfacial properties of different genetic variants of β-lg have not been studied in over twenty years (Ipsen & Otte, 2004). Whey proteins are a popular choice as emulsifiers throughout the food industry, due to their functional properties such as emulsification and foam stability, which allows them to work synergistically with other foods (De Wit, 1998; Królczyk et al., 2016). An integral part of a protein forming a good emulsion is its ability to unfold and arrange its hydrophobic and

hydrophilic regions at the liquid interface (McClements, 2004). The differences in amino acid sequence and secondary structure between the A and B variants would be expected to change how the proteins organise at the surface/interface, which would confer different emulsification, foaming and lipid stabilisation properties of the different variants.

3. An area of milk protein genomics that has largely gone unreported is that of the minor proteins, such as bovine serum albumin, lactoferrin, and immunoglobulin. The functions of bovine serum albumin, lactoferrin and immunoglobulin are transportation of biological molecules, regulation of iron levels and defense against infection, respectively (Deeth & Bansal, 2019). Genetic variation of these minor proteins would be expected to affect their functional properties. Considering how polymorphic the six main milk proteins are, with over 50 variants discovered, it is not unreasonable to consider that this polymorphic behaviour could extend deeper into the serum proteins in milk. Some minor whey proteins, such as immunoglobulin and lactoferrin, are involved in supporting the immune system. Anti-bacterial components, such as macrophages, are also susceptible to genetic variation, which can affect its immune response to potential infections (Berrington & Hawn, 2007)

4. The digestion of milk proteins and the subsequent release of bioactive peptides has been a source of controversy within the dairy industry for the last few decades. The main focus of this controversy has been due to the release of the bioactive peptide BCM7 from the A1 variant of β-casein and purported negative health implications such as heart disease, schizophrenia and diabetes (Daniloski et al., 2021). However, few studies have reported on the bioactive peptides of β-lg, and

even fewer compare the differences in the release of bioactive peptides from the different variants of β-lg. Some bioactive peptides of β-lg, such as Ala-Leu-Pro-Met-His-Ile-Arg and Ile-Pro-Ala-Val-Phe-Lys, which have been reported to help treat Covid-19, could have a stunted release if the amino acids substitutions, caused by genetic variation, are situated at the cleavage sites of these peptides (Çakır et al., 2021). These potential differences in the release of bioactive peptides from β-lg could further add to the controversy around milk peptides, like the A1 variant of β-casein, or highlight potential health benefits from peptides released during the digestion of β-lg.

5. One of the leading causes of milk allergenicity is the immune response to β-lg. study conducted recently, and yet to be published, within Teagasc, Moorepark investigated the effects of the genetic variants of β-lg on the inflammatory response and weight gain in mice on a β-lg rich diet. The results of this study indicated that the AB variant of β-lg resulted in significantly ($P<0.001$) less weight gain than the A and B variants, and no difference was found in the inflammatory response. This study could be expanded upon by developing a human intervention study in which healthy athletes are supplemented with whey protein products such as WPC/WPI containing different β-lg variants, followed by assessment of the impact on weight gain and lean muscle growth.

6. An area of research that remains largely unexplored regarding genetic variants is their influence on the structural properties of ice cream. Ice cream is a dairy product enjoyed by many all over the world with a market value that is expected to reach €126 billion by 2026 (Moreno-Quispe et al., 2023). The structure of ice

cream is complex due to the number of different phases found therein. Whey proteins are sometimes added to ice cream to help improve stability by adsorption at the air-water and fat-water interfaces and forming a protective layer (Barbiroli et al., 2022). By stabilising the air-water interface, it can improve the overrun and quality of ice cream (Patel et al., 2006). The different charges, secondary structures and thermal stabilities between milk protein genetic variants could influence stability of ice cream by altering how the proteins adsorb at the surfaces/interfaces. The effect of the different variants of β-lg, or indeed even that of the other major milk proteins, on the stability of ice cream, has not been investigated. Research in this area could highlight the potential importance of genetic variants on the structure, stability and quality of ice cream.

6.4 References

Barbiroli, A., Iametti, S., & Bonomi, F. (2022). Beta-lactoglobulin as a model food protein: How to promote, prevent, and exploit its unfolding processes. *Molecules, 27*, 1131.

Berrington, W. R., & Hawn, T. R. (2007). Mycobacterium tuberculosis, macrophages, and the innate immune response: does common variation matter? *Immunological Reviews, 219*, 167-186.

Branden, C. I., & Tooze, J. (2012). *Introduction to Protein Structure* (Second Edition): Garland Science.

Çakır, B., Okuyan, B., Şener, G., & Tunali-Akbay, T. (2021). Investigation of beta-lactoglobulin derived bioactive peptides against SARS-CoV-2 (COVID-19): In silico analysis. *European Journal of Pharmacology, 891*, 173781.

Daniloski, D., Cunha, N. M., McCarthy, N. A., O'Callaghan, T. F., McParland, S., & Vasiljevic, T. (2021). Health-related outcomes of genetic polymorphism of bovine β-casein variants: A systematic review of randomised controlled trials. *Trends in Food Science & Technology, 111,* 233-248.

De Wit, J. (1998). Nutritional and functional characteristics of whey proteins in food products. *Journal of Dairy Science, 81*, 597-608.

Deeth, H., & Bansal, N. (2019). Whey Proteins: An Overview. In *Whey Proteins* (First Edition) (pp. 1-50): Academic Press.

Huppertz, T., & Nieuwenhuijse, H. (2022). Constituent fouling during heat treatment of milk: A review. *International Dairy Journal, 126*, 105236.

Ipsen, R., & Otte, J. (2004). The relation between protein structure, interfacial rheology and foam formation for various milk proteins. *Annual Tranactions of the Nordic Rheology Society, 21*, 143-178.

Jeurnink, T. J., Walstra, P., & De Kruif, C. (1996). Mechanisms of fouling in dairy processing. *Netherlands Milk and Dairy Journal, 50*, 407-426.

Keppler, J. K., Sönnichsen, F. D., Lorenzen, P.-C., & Schwarz, K. (2014). Differences in heat stability and ligand binding among β-lactoglobulin genetic variants A, B and C using 1H NMR and fluorescence quenching. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 1844*, 1083-1093.

Królczyk, J. B., Dawidziuk, T., Janiszewska-Turak, E., & Sołowiej, B. (2016). Use of whey and whey preparations in the food industry–A review. *Polish Journal of Food and Nutrition Sciences, 66*, 157-165.

McClements, D. J. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science, 9*, 305-313.

Moreno-Quispe, L. A., Espinoza-Espinoza, L. A., Mauricio-Sandoval, E. A., Palacios, M. S. A., & Portalatino, E. J. V. (2023). Ice cream consumption preferences in Sullana, Peru. *Current Research in Nutrition and Food Science, 11*, 267.

Ng-Kwai-Hang, K. (1998). Genetic polymorphism of milk proteins: relationships with production traits, milk composition and technological properties. *Canadian Journal of Animal Science, 78*, 131-147.

Patel, M. R., Baer, R., & Acharya, M. (2006). Increasing the protein content of ice cream. *Journal of Dairy Science, 89*, 1400-1406.

Paterson, G. R., MacGibbon, A. K. H., & Hill, J. P. (1999). Influence of κ-casein and β-lactoglobulin phenotype on the heat stability of milk. *International Dairy Journal, 9*, 375-376.

Rabiller-Baudry, M., Bouzid, H., Chaufer, B., Paugam, L., Delaunay, D., Mekmene, O., Ahmad, S., & Gaucheron, F. (2009). On the origin of flux dependence in pH-modified skim milk filtration. *Dairy Science and Technology, 89*, 363-385.

Appendix (Part 1)

Additional Preliminary Experimental Studies

**not for examination*

Impact of β-lactoglobulin genetic polymorphism on the gastric digestion of yoghurt using semi dynamic *in-vitro* digestion

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Abstract

This study aimed to determine the impact of the genetic variants of bovine β-lactoglobulin on the (micro) structural properties and gastric digestion dynamics of AA, AB and BB yoghurts. The viscoelastic properties of these yoghurts were analysed using oscillatory rheology. Following this, the gastric digestion behaviour of each yoghurt was examined using the INFOGEST semi-dynamic *in vitro* gastric digestion system. The digestion profiles characterisation was carried out using Size Exclusion - High Performance Liquid Chromatography, Fourier-Transform Infrared Spectroscopy, bicinchoninic acid assay, and o-phthaldialdehyde assay to evaluate extent of protein hydrolysis. Confocal microscopy was employed to assess the microstructure of yoghurt and digesta. Our results showed that AA yoghurt exhibited a less viscous and looser gel, which led to a more rapid protein hydrolysis during gastric digestion, compared to BB yoghurt, which formed a dense protein network and tight curd, resulting in a yoghurt that was more resistant to gastric digestion. This study highlights the differences in the yoghurt matrix structure and protein breakdown following gastric digestion of various β-lactoglobulin phenotypes and outlines how these results correlate with the initial yoghurt structure.

1. Introduction

The properties of bovine milk and its dairy products are affected more by the proteins they contain than by any other milk component (Goulding et al., 2020a). Two major protein groups are found in bovine milk; caseins, known as αs-, β- and κ-caseins, predominantly present in the form of casein micelles and whey proteins, including α-lactalbumin, β-lactoglobulin and other minor proteins, found in the milk serum (Fox & Mulvihill, 1982). These two categories of dairy proteins are crucial for the technological properties of milk, significantly affecting gel formation in the production of cultured dairy products, with yoghurt being the most popular (Lucey, 2004).

During the acid gelation, as the pH drops from around 6.70 to 4.60, the κ-casein layer on the casein micelles collapses, leading to a reduction in electrostatic repulsion between the casein molecules (Fox & Mulvihill, 1982). However, prior to the fermentation process essential for yoghurt production, the milk is normally heat-treated at 80 - 90 °C for 10 min in order to improve the viscoelastic properties of the product (Lucey, 2004). Applied heat treatment causes the whey proteins, but particularly β-lactoglobulin, to denature and associate with κ-casein on the surface of the casein micelles (Lucey & Singh, 1997). Namely, these complexes driven by the protein cross-linking via intermolecular disulphide bonds and hydrophobic interactions of caseins and whey proteins leads to formation of protein aggregates and subsequently the yoghurt network (Lucey et al., 2022). The possibility of improving the yoghurt properties, such as viscosity, texture, curd firmness, water holding capacity and gelation time by selection for certain milk protein genes, has long been a subject of interest (Hallén et al., 2009; Ketto et al., 2018; Ng-Kwai-Hang et al., 1986a).

Phenotypes of κ-casein A/A and β-casein A1/A2, have been shown to have significant effect on production of firmer acid and yoghurt gels (Daniloski et al., 2022a; Hallén et al., 2009). A similar effect may be noticeable as a result of the β-lactoglobulin phenotype due to correlations between its content in the starting milk with the acid gelation properties (Heck, Schennink, et al., 2009b; Ng-Kwai-Hang et al., 1986a).

Among all milk proteins, β-lactoglobulin accounts for approximately 50 % of the total whey protein in bovine milk (Deeth & Bansal, 2019). The full amino acid sequence of β-lactoglobulin was first elucidated by Braunitzer et al. (1972) stating that this protein contains 162 amino acid residues and has a molecular weight of 18,300 Da. This protein contains two intramolecular disulphide bonds, the first between the f66 - 160 in its polypeptide chain and the second, a urea-mediated disulphide interchange, between amino acids 106 and 119. As well as this, a free cysteine exists at position 121 of its structure (O'Mahony & Fox, 2014b). Until present, 11 different genetic variants of bovine β-lactoglobulin have been found in the literature (Farrell et al., 2004b), nevertheless, the genetic variability within the β-lactoglobulin gene gives rise to distinct phenotypes, denoted as AA, AB, and BB, which have been associated with diverse effects on milk and dairy product characteristics (Deeth & Bansal, 2019). Although nearly identical proteins, two amino acid substitutions exist between β-lactoglobulin A and B, in position 64 and 118 within their polypeptide chains, with aspartic^{64} to glycine^{64} and valine^{118} to alanine^{118} for β-lactoglobulin A and β-lactoglobulin B, respectively (Olguin-Arredondo & Vallejo-Córdoba, 1999). Several studies examined the rheological properties of acid gels made from milk containing different β-lactoglobulin phenotypes, finding a consensus that β-lactoglobulin BB is related to a positive effect on the acid gels firmness, when compared to both acid gels with β-lactoglobulin A (Allmere et al., 1998; Hallén et al., 2009). In contrast, very

recently, Şahin Semerci et al. (2024) found out that the stirred yoghurt made from milk with β-lactoglobulin AA showed lower syneresis values and higher values for firmness, apparent viscosity, and consistency compared to the AB yoghurt.

Firmer yoghurts have been related to higher curd firmness and protein connectivity during *in vivo* gastric digestion, but also slower rate of gastric emptying (Le Feunteun et al., 2014). Additionally, this has been repeated in pigs with two iso-caloric yoghurts with differing viscosity, showing that high viscosity yoghurt was emptied more slowly from the gastric phase (Dupont et al., 2018). Since genetic variation in β-lactoglobulin affects the physical properties of yoghurt and the yoghurt matrix, differences may exist in the rate and extent of protein breakdown during gastric digestion, which may affect the rate of release of proteins into the small intestine for further breakdown and absorption. Therefore, the present study aims to investigate the relationship between β-lactoglobulin genetic polymorphisms (AA, AB, and BB) and the gastric digestion kinetics of yoghurt, focusing on the hydrolysis of protein fractions and the alteration of protein structure during gastric digestion.

2. Materials and Methods

2.1. Materials

Yoghurt starter culture (YoFlex Express 1.0: *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) was obtained from CHR Hansen (CHR Hansen, Hørsholm, Denmark). All chemicals used were analytical grade. Porcine pepsin (P-6887) was obtained from Sigma Aldrich (Wicklow, Ireland). Pierce BCA Protein Assay kit was purchased from Thermo Fisher Scientific (Cork, Ireland).

2.2. Skim milk powder

The milk was obtained from 127 Irish Holstein Friesian cows from Curtin's Farm (Teagasc, Moorepark, Fermoy, Co. Cork, Ireland). Genotypic analysis identified 15 cows that were homozygous for β-lactoglobulin A and 14 cows were homozygous for β-lactoglobulin B. Milk from these cows was collected into two separate 5000 L stainless steel tanks, one for each polymorphism. The collected milks in both individual tanks separated based on the genetic variants of β-lactoglobulin, were analysed using Reversed Phase HPLC to detect the β-lactoglobulin polymorphisms present (McCarthy et al., 2017). Milk was processed using the facilities at Moorepark Technology Limited (Teagasc, Moorepark, Fermoy, Co. Cork). Milk was skimmed using a Skimmer pro centrifuge disk separator (GEA, Naas, Ireland) to a fat content of 0.06 and 0.04 % for the A and B polymorphism batched, respectively. The skim milk was then immediately pasteurised by heating the milk to 72 °C for 15 seconds using an APV Fisher stainless steel pasteuriser (SPX Flow Technology Denmark A/S, Soeborg, Denmark). Prior to spray drying, 50 kg of skim milk from each batch was evaporated to a solids content of 40 % using a Scheffers single-effect recirculating evaporator (Scheffers, Schiedam, The Netherlands). The concentrated skim samples were then dried using a using a single-stage spray dryer (Anhydro F1 Lab Dryer; Copenhagen, Denmark) with an inlet temperature of 185 °C and an outlet temperature of 85 °C (Daniloski et al., 2024a). Milk from homozygous A and B cows was mixed in the ratio of 1:1 to create AB milk.

2.3. Yoghurt production

Skimmed milk powder was rehydrated to 15 % total solid and 5 ± 0.3 % (w/w) protein in Milli-Q water (Millipore Corp. Bedford, MA, USA). Protein content was checked using mid-infrared spectrometry (MilkoScan FT6000, Foss Analytical A/S). Skim milk was mixed using a magnetic stirrer and heated to 50 °C for 30 minutes on a hot plate. Milk was left overnight in the fridge at 4 °C so that powder was fully dissolved. Prior to inoculation, milk was heated to 85°C for 10 min and then cooled to 43°C in an icebox and subsequently 0.03 g of thermophilic starter culture was weighed out and added in the rehydrated milk samples. Following this, four individual yoghurts for each group (45 g each), were fermented in beakers at 43 °C until pH 4.60 was reached. The pH measurement (CyberScan pH meter 510, Eutech Instruments, Singapore) was carried out periodically on a 'trial' yoghurt for each condition, preventing distortion of the gel in yoghurts used in further analysis. When the required pH was reached, the beakers were removed from the water bath and were rapidly cooled in an icebox to stop the starter culture activity. Beakers were placed at refrigerated temperature overnight for further analysis.

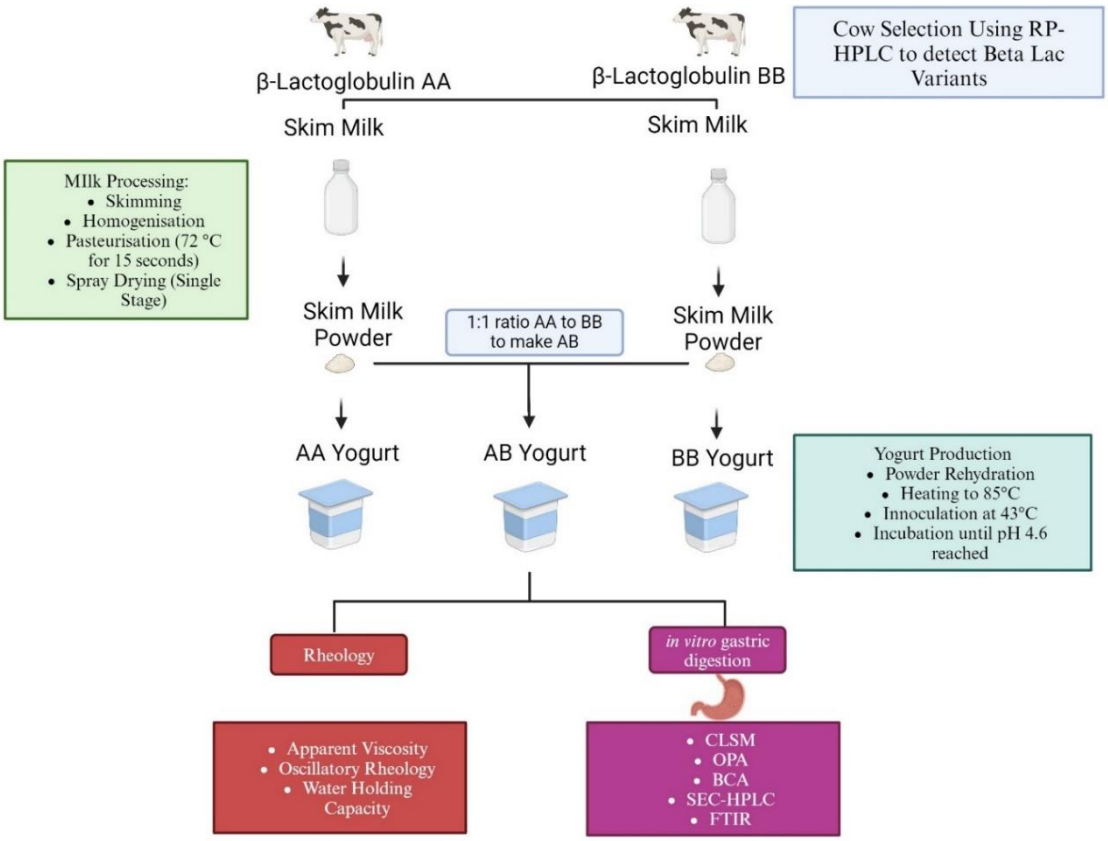

Figure 1 Schematic overview of the experimental design

2.4. Rheological properties of yoghurts

2.4.1. Measurement of Viscoelastic Properties of Yoghurt

Dynamic oscillatory rheology was conducted using an AR G2 Rheometer (TA Instruments, Crawley, UK) with a 60 mm diameter aluminium parallel plate geometry and a 1000 µm plate gap. Approximately 5 mL of yoghurt was loaded onto the lower plate, equilibrated at 20 °C for 2 minutes, and tested without pre-shear or normal force control. Amplitude sweeps were performed to identify the linear viscoelastic region (LVR) by progressively increasing strain and monitoring the storage modulus (G') and loss modulus (G"). A strain of 0.5%, within the LVR, was used for frequency sweeps.

Frequency sweeps (0.628–628 rad/s) were conducted at 20 ℃ with 100 logarithmically spaced measurement points, maintaining a constant strain of 0.5%. The viscoelastic properties, including storage modulus (G'), loss modulus (G''), and loss tangent (tan δ = G''/G'), were recorded. All measurements were performed in triplicate following Baldursdottir et al. (2010).

2.4.2. Apparent Viscosity

Rheological measurements were performed using a controlled stress rheometer (AR G2 Rheometer, TA Instruments, Crawley, UK) equipped with an aluminium plate geometry. 5 mL of yogurt was loaded onto the rheometer plate and allowed to equilibrate at 20°C for 2 minutes before beginning a shear rate sweep from 0.01 to 300 s^{-1}, with 10 points collected per decade. A total of 50 sample points were recorded. The temperature was maintained at 25°C throughout the experiment. The test was set with a sample period of 10 seconds per point, a percentage tolerance of 5%, and a maximum point acquisition time of 1 minute. The experiment required three consecutive data points to fall within the tolerance range to proceed to the next shear rate. Prior to measurement, the sample was subjected to a constant rate shear of 20 s^{-1} for 30 seconds to ensure homogeneous sample distribution and to minimize sample history effects.

Dynamic oscillatory rheological measurements were performed using a 60 mm diameter aluminium parallel plate geometry. The gap between the plates was set to 1000 µm, and approximately 5 mL of yogurt sample was loaded onto the lower plate for testing. Prior to measurements, the sample temperature was set to 20°C, and the system was allowed to equilibrate for 2 minutes. Amplitude sweeps were conducted before dynamic testing to determine the linear viscoelastic region (LVR). A strain

value of 0.5%, comfortably within the LVR, was selected for all frequency sweep experiments. Frequency sweeps were performed from 626 to 0.628 Rad/s, in logarithmic mode, with 100 measurement points distributed across this range. The following rheological parameters were recorded: storage modulus, G' (elastic or solid-like behaviour, G'), loss modulus, G'' (viscous or liquid-like behaviour) and loss tangent (tan δ = G''/G'), indicating the ratio of viscous to elastic behaviour. All measurements were performed in triplicate.

2.4.3. Yoghurt water retention

The water holding capacity (WHC) of yoghurts was measured based on the method described by Daniloski et al. (2024b). Briefly, 5 g batches of yoghurts were made (in duplicate) in 50 mL falcon tubes using the method described in section 2.3. Tubes were then centrifuged at 10,000 x g for 15 minutes at 4 ℃ using a Heraeus Multifuge X1R (Thermo Scientific) centrifuge. The supernatant was then decanted and weighed. The WHC was expressed as a percentage of the mass of the pellet relative to the total mass of the sample.

2.5. In vitro semi-dynamic gastric digestion

Yoghurts were stored in fridge at 4 ℃ for a maximum of 48 hours before semi dynamic *in vitro* digestion. Yoghurts were digested using *in vitro* semi dynamic digestion as described in Mulet-Cabero, Egger, Portmann, Ménard, Marze, Minekus, Le Feunteun, Sarkar, Grundy, Carrière, et al. (2020). Yoghurts contained 5.55 % protein, equalling to 2.5 g protein in 45 mg of yoghurt, as determined using the Dumas method with a nitrogen to protein conversion factor of 6.38.

Oral phase: 45 mL of yoghurt was added to oral phase. This was carried out in the gastric digestion vessel. In the yoghurt, 5.4 mL of eSSF, 33.8 µL $CaCl_2$, and 1.316 mL

water was added. Following this, 5 mL of the mixture was removed for analysis. This 5 mL was added to a 50 mL falcon tube where1 mL was immediately taken for confocal analysis; 1 mL was immediately taken for FTIR analysis.

Gastric phase: The initial volume of gastric phase was 43.00 mL. To begin gastric phase of digestion, the basal phase was added to the digestion vessel as follows, eSGF (0.13M HCL) 3.5 mL, $CaCl_2$ 2.2 µL, sater 7.3 mL, pepsin 0.22 mL. Prior to digestion, porcine pepsin was assayed according to Minekus et al. (2014). Following this, eSGF and pepsin were continuously added at 1.73 mL/min and 0.197 mL/min respectively. The $t_{1/2}$ for gastric digestion was 11 minutes. yoghurt. Four gastric emptying steps were carried out, one every 5.5 minutes and 20 mL was emptied at each emptying point. Gastric emptying points were named as GE 1 - 4 in the text.

2.5.1. Gastric emptying samples

20 mL of gastric digesta was added to a 50 mL falcon tube at each emptying point (every 5.5 minutes for 22 minutes). Samples for confocal and FTIR analysis were removed, and analysis was performed immediately. The remaining gastric emptying sample was heated at 90 °C for 10 minutes to denature enzyme and stop digestion. Samples were snap frozen using liquid nitrogen. Samples were then stored in freezer at - 20°C. When required for OPA and BCA analysis, samples were thawed in fridge at 4 °C.

2.6. Sample analysis

2.6.1. Protein molecular mass distribution

Size exclusion chromatography - high performance liquid chromatography (SEC-HPLC) was used to estimate the molecular mass of proteins in the digesta (O'Loughlin et al., 2013). SEC-HPLC was carried out as described by Freitas et al. (2022). Briefly, Polyether sulfone (PES) membrane with 0.45 µm pore size (Captiva Premium Syringe Filters reference 5190-5095, Agilent Technologies, UK) were used to filter sample supernatants. Briefly, a TSK $G2000_{SWXL}$ column (600 × 7.5 mm; Tosoh Bioscience GmbH, Germany) with a guard column was used, in connection with a Waters 2695 HPLC with UV/Visible detector controlled by the software EMPOWER®. An injection volume of 10 µL was eluted at 0.5 mL/min with buffer (30% v/v Acetonitrile, 0.1% v/v TFA, prepared in HPLC grade water) and monitored over 60 min at 210 nm. Estimations were made using a calibration curve based on the retention times of the following standards bovine serum albumin, carbonic anhydrase, β-lactoglobulin, aprotinin, bacitracin, histidine-leucine, phenylalanine and glycine, with respective molecular weights of 67,000, 29,000, 18,400, 6,500, 1,400, 268, 165 and 75 Da.

2.6.2. Confocal Laser Scanning Microscopy (CLSM)

Confocal Laser Scanning Microscopy (CLSM) (Leica TCAS SP5) (Leica Microsystems CMS GmbH, Wetzlar, Germany) was used to examine the microstructure of the three yoghurts. CLSM was carried out on yoghurt prior to digestion, in the oral phase of digestion, and at each gastric emptying point. CLSM was carried out in parallel to *in vitro* gastric digestion. Aliquots of 500 µl were removed from gastric emptying samples and added to an Eppendorf containing 50 µl 0.1 % a green dye (w/v, Fast Green, FCF; $C_{37}H_{34}N_2Na_2O_{10}S_3$, Cayman Chemical, Ann Arbor, MI, USA). A dropper was used to transfer sample from Eppendorf to glass

plate. Samples were covered with a cover slip and mounted on the CLSM. Images were taken using a 63 x 1.4 oil immersion objective. Fast green dye was excited at 633 nm using a HE/Ne laser, with the corresponding emission filter set at 680 - 720 nm (Gómez-Mascaraque & Pinho, 2021).

2.6.3. OPA assay

Proteolysis was analysed by quantifying the free amine groups using the OPA assay method (Nielsen et al., 2001). Briefly, samples were centrifuged at 10,000 x g for 30 minutes using an Eppendorf Centrifuge 5417R (Mason Technology Ltd.). Supernatant was then removed from the pellet. A standard curve was prepared using L-Leucine in the range of 10 - 0.2 mM. Samples were diluted in PBS so that they were in the linear range of standard curve. 200 µl of OPA reagent was added to 10 µL sample in a 96 well plate that wasmixed using plate shaker for 1 minute. The plate was covered with aluminium foil and incubated at 24 °C for 15 minutes. Absorbance was read using plate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany) at 340 nm.

2.6.4. BCA Assay

Protein released into the supernatant was analysed using the Pierce^{TM} BCA Protein Assay Kit according to the manufacturers protocol. Samples were centrifuged at 3,000 x g for 10 minutes using an Eppendorf Centrifuge 5417R (Mason Technology Ltd.). The soluble fraction was removed and diluted 10:1 in PBS buffer so that they would be in the linear range of the standard curve. A standard curve was prepared using bovine serum albumin in the range of 2000 - 0 µg/mL. Following this, Samples and

working reagent were added to 96 well plate and incubated at 37 °C for 30 minutes. Plates were then cooled to room temperature and subsequently read at 562 nm on a plate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany). The standard curve was used to calculate the protein content of each sample. Dilution factors during assay and digestion were considered during calculations.

2.6.5. Fourier Transformed Infrared Spectroscopy

Fourier Transformed Infrared Spectroscopy (FTIR) of yoghurt digests were analysed using an FTIR spectrometer (Bruker, INVENIO 100453, Billerica, MA, USA) with BioATR attachment controlled by a Haake K20/DC30 external water bath (Thermo Haake, Karlsruhe, Germany) at 37 °C following the method of Daniloski et al. (2024a). Liquid nitrogen was used to cool the MCT detector, and the sample compartment was purged to remove moisture and carbon dioxide. Bruker OPUS 5.5 software was used to obtain the spectra in absorbance mode. All samples were atmospheric corrected to account for peaks caused by atmospheric interferences such as moisture and carbon dioxide. Water subtraction was carried out to remove the water band, which overlaps the protein region. Vector normalization was carried out on all samples between 900 and 1800 cm^{-1} to remove baseline effects. Both band and peak assignment was performed following previously published methods of Grewal et al. (2018) and Daniloski et al. (2022b).

2.7. Data and statistical analysis

Three independent batches of yoghurts were produced for each β-lactoglobulin group (AA, AB, and BB) to be used in *in vitro* digestions. Three further independent batches of yoghurts were produced for rheological measurements. Spectral data analysis was carried out using MATLAB (MATLAB Release 2020b, The MathWorks, Inc., Natick, Massachusetts, USA). Two-way ANOVA and PCA plots were produced using Unscrambler software (Version 10.2, CAMO AS, Trondenheim, Norway). FTIR spectra graphs were produced using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Means for continuous variables (diverse β-lactoglobulins) were compared using Tukey's Studentised Range post-hoc (HDS) test and one-way analysis of variance (ANOVA), thus establishing effects of the main factor (β-lactoglobulins phenotype). Statistical significance was set at 95 % level.

3. Results

3.1. Milk powder composition

Milk powders were standardised based on total nitrogen content. The composition of the skim milk powders is given in the Supplementary Table 1 S. An increased whey content was observed in A (5.95 %) polymorphism compared to B polymorphism (4.97 %). As well as this, the calcium content in BB was significantly higher than in BB (1445.58 mg /100 mL and 1495.28 mg /100 mL respectively, $p = 0.02$), while iron contents were higher in both skim milk powders with β-lactoglobulin A than in the sample with β-lactoglobulin B (0.27 mg/100 mL and 0.52 mg/100 mL, respectively).

3.2. Physicochemical properties of skim milk yoghurts

3.2.1. Viscoelastic properties

Figure 2 shows the viscoelastic properties of skim yoghurts. The storage modulus (*G'*), which is a measure of the elastic behaviour of the yoghurt was higher for all yoghurts than their corresponding viscous modulus (*G''*), which is a measure of the viscous behaviour of the yoghurt. This indicates that the yoghurts had elastic properties. However, a large difference was observed between yoghurts due to the β-lactoglobulin polymorphism present, with BB yoghurt showing the highest *G'* and *G''* values, followed by AB and AA respectively ($p < 0.05$, Figure 2). A clear difference in crossover times among the three yoghurts could be seen, with the transition which occurred earlier for AA, followed by AB, with no crossover occurring in BB yoghurt. This indicates that BB yoghurt had a firmer gel, followed by AB and lastly AA sample.

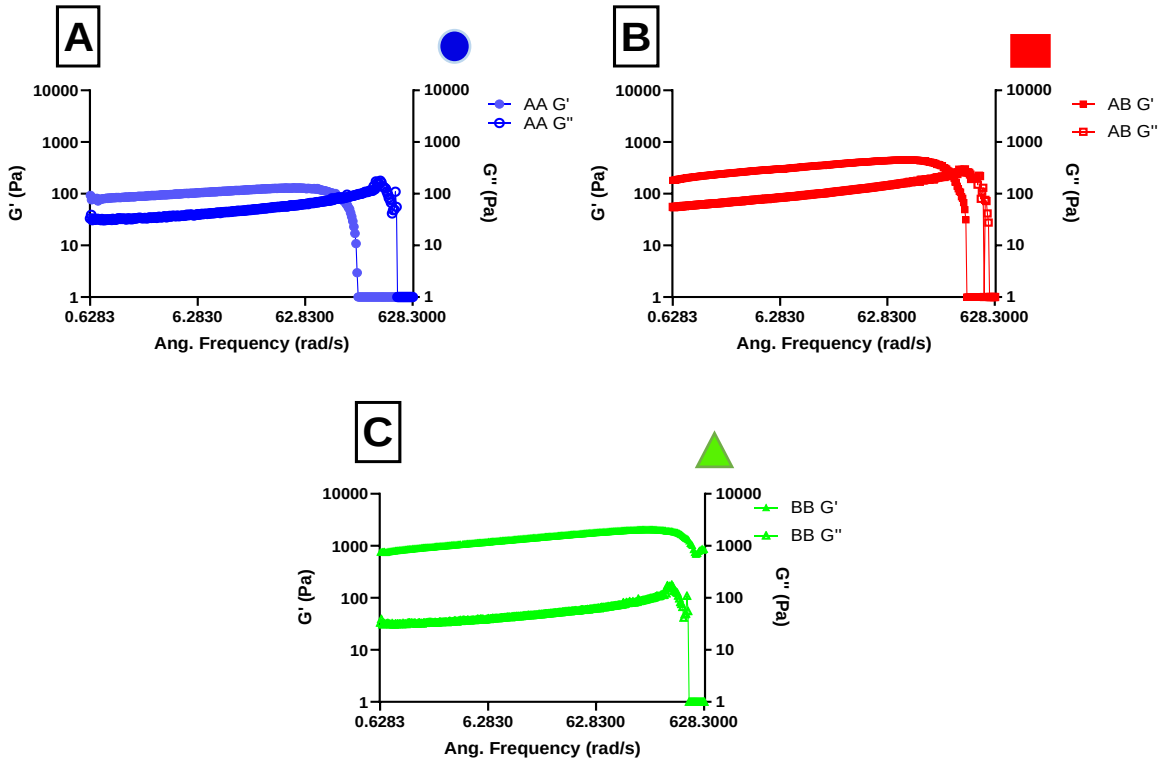

Figure 2. A, B and C: The effect of beta lactoglobulin variants on elastic modulus (G') and loss modulus (G") on skim yoghurts. Graph is representative of yoghurts viscoelastic behaviour. Figure 2 (A) shows the viscoelastic behaviour of AA yoghurt, (B) shows viscoelastic behaviour of AB yoghurt and (C) shows the viscoelastic behaviour of BB yoghurt

3.2.2. Apparent viscosity

The apparent viscosity (η) of the yoghurt samples decreased with increasing shear rate, which indicates non-Newtonian behaviour (Figure 3). Dissimilar flow curves were observed for the yoghurts made of either AA, AB, or BB rehydrated milks. Initially, the η of the yoghurts differed greatly. The highest η was assigned to BB yoghurt (10.04 Pa · s), followed by AB yoghurt (2.55 Pa · s), and AA yoghurt (0.06Pa · s). Accordingly, AA yoghurt displayed lower η across the shear rate ramp, compared to the other yoghurts made from milk samples containing β-lactoglobulin B in their structure. Flow curve of the yoghurt made from AB milk (Figure 3) was between the curve for AA milk and the curve for the yoghurts made from BB milk.-Additionally, the yoghurt produced from milk homozygous for β-lactoglobulin B required a higher shear rate to break these interactions when compared to AA or AB yoghurts, indicating that BB was the most viscous, followed by AB and finally AA yoghurts (Figure 3).

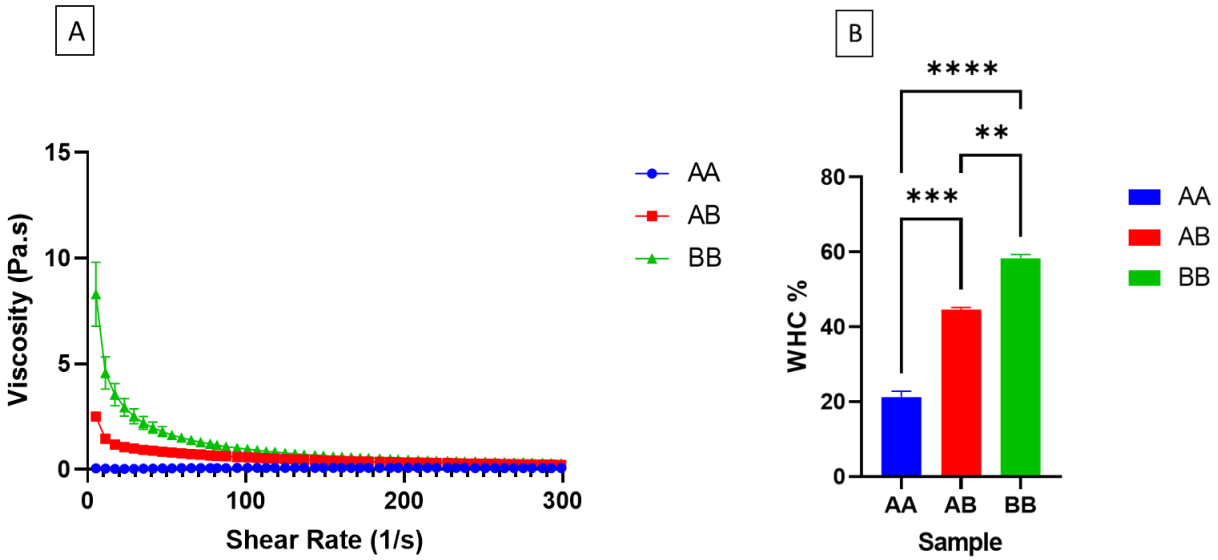

Figure 3 A) Apparent viscosity as a function of shear rate for AA (blue circle), AB (red square), and BB (green triangle) yoghurts. Values are the means of data from triplicate analyses. B) Water Holding Capacity of AA (blue circle), AB (red square), and BB (green triangle) yoghurts.

3.2.3. Water holding capacity

The ability of the yoghurts to retain water was affected by the β-lactoglobulin phenotype ($p < 0.05$) as shown in Figure 3 B. Namely, after maintaining the samples at low temperature overnight, the WHC of the AA yoghurt significantly decreased. Water holding capacity of AB and BB yoghurts was approximately 60 %, indicating greater structural stability and water retention ($p < 0.05$). Moreover, a relationship between WHC and the final G' of the yoghurts can be observed, wherein increased final G' and η positively correlated with increased WHC of the yoghurts prepared from milk samples containing the homozygous β-lactoglobulin BB, when compared to those yoghurts containing the β-lactoglobulin A ($p < 0.05$).

3.3. Semi Dynamic in vitro digestion

Although similar rapid decrease of the pH was observed during the gastric digestion for all yoghurts ($p > 0.05$), the pH of the homozygous AA yoghurt was no altered for the first 5 min of gastric digestion; than all three pH curves decreased gradually, reaching a pH of around 2 after 21 min (Figure 4 A). The starting pH for all yoghurts was approximately 4.60, and continuously decreased to pH 2.00 by the end of the gastric phase of digestion. This was achieved by the gradual automated addition of acidic simulated gastric fluid at a constant flow rate (1.75 mL/min). Additionally, no significant difference in buffering capacity was found between samples, with all samples reaching pH 2 ± 0.12 by the end of the gastric digestion. This is an important factor due to the sensitivity of pepsin to pH (Pletschke et al., 1995).

3.3.1. Visualisation and conformational changes of the gastric digesta

The visual observations showed that AA yoghurt had a looser protein network than AB and BB yoghurts, with BB yoghurt appearing to have the firmest structure of the three, similarly to the viscoelastic properties and the highest *G'* of this yoghurt type ($p < 0.05$). By the end of gastric digestion (21 minutes), AA yoghurt appeared to have lost its white colour compared to the other two yoghurts. This could be due to an accelerated emptying of caseins from this yoghurt compared to AB and BB yoghurts. To confirm the depicted visual differences in the yoghurt digests, CLSM was used to study the microstructure of yoghurts (Figure 4 B). During the oral phase of digestion (0 min), no enzymes had been added; therefore, no breakdown of protein had occurred compared to the raw sample. Despite this, AA yoghurt appeared to have a less dense protein network with larger pores compared to both AB and BB samples (also observed in sections 3.2.1. and 3.2.2.). After 16.5 minutes of gastric digestion, BB yoghurt retains its dense network, while both AA and AB appear to have broken up.

By the end of gastric digestion, all samples had undergone protein hydrolysis as a looser network could be observed for all yoghurts.

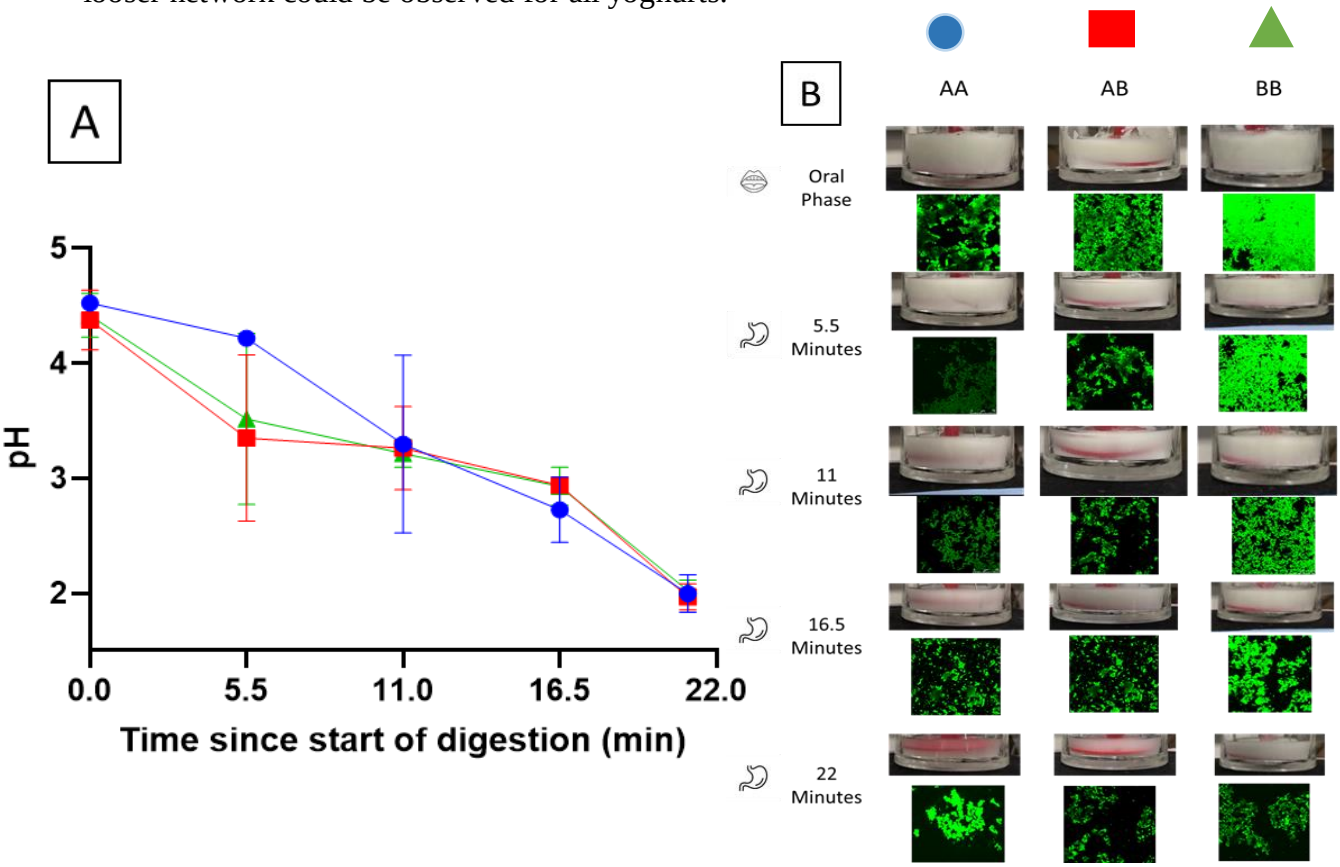

Figure 4. pH curve during semi-dynamic gastric digestion. B) Visualisation and structural properties of gastric digesta observed with CLSM. Scale bars represent 25 µm. Note: observations with the naked eye, but also proven by the viscoelastic properties, suggested that initial yoghurt had a less condensed gel structure in AA yoghurt vs AB or BB at the start of digestion ($p < 0.05$).

3.3.2 Release of protein into supernatant

The BCA assay was used to measure the cumulative release of protein into the supernatant (Figure 5 A) while the OPA assay was used to measure the cumulative release of free amines into the supernatant (Figure 5 B). A gradual increase in protein released into the supernatant was observed for all yoghurts, however AB sample released a signfiicantly higher quantity of protein into the supernatat than BB sample after 16,5 and 22 minutes ($p < 0.05$, Figure 6A). Similarily, a gradual increase in free amines released into the supernatant was observed for all yoghurts, while AB showed a significantly greater release of free amines into the supernatant than BB sample after

22 minutes of gastric digestion *($p < 0.05$, Figure 5 B)*. Figure 5 C shows the release of free amines divided by the release of protein into the supernatant, with no significant differences being found. It can be seen that free amine release is in proportion to the protein released, resulting in a steady value throughout digestion.

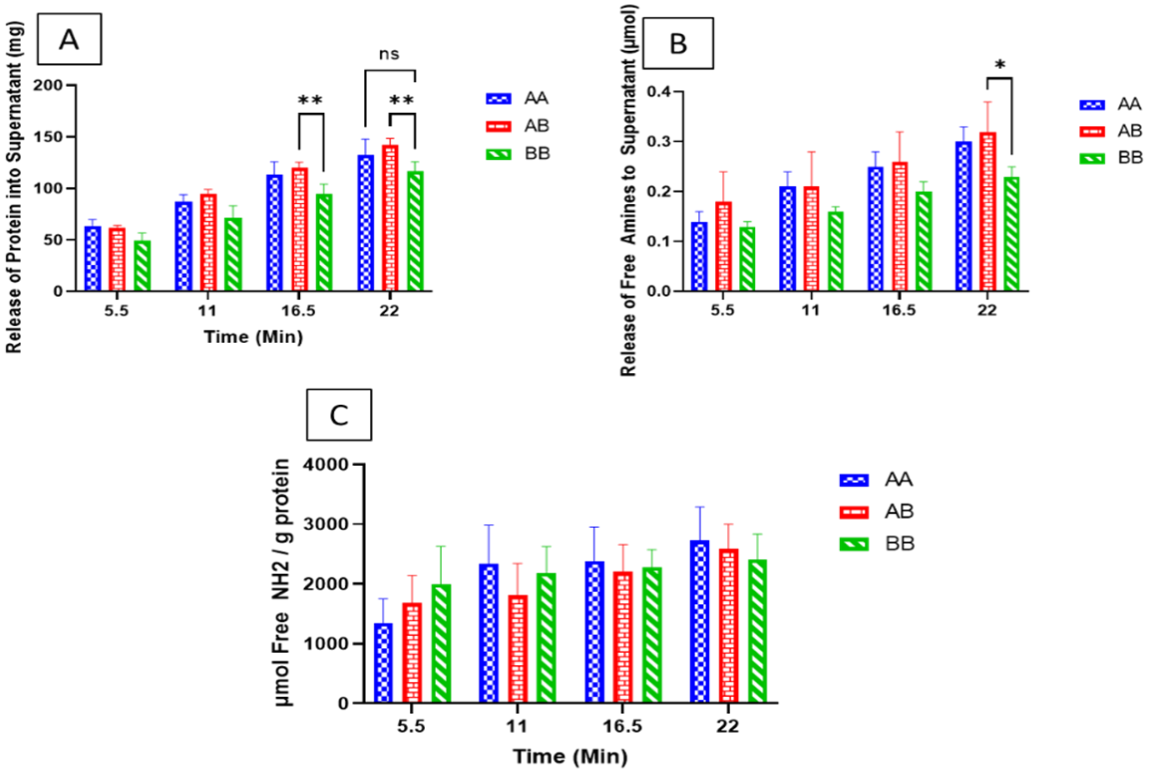

Figure 5. Concentration of Protein and Free Amines released into supernatant as measured by OPA (Free amines) and BCA (Protein) assays of AA (blue circle), AB (red square), and BB (green triangle) yoghurts. All data is cumulative release into supernatant. (A) Release of protein into the supernatant (B) Release of Free Amines into the supernatant (C) cumulative free amines released into supernatant divided by cumulative protein released into supernatant. * indicates significant difference ($p < 0.05$), ** indicates significant difference ($p < 0.01$).

3.3.4. Size exclusion chromatography

SEC-HPLC was used to estimate the molecular weight distributions of proteins in samples taken during the *in vitro* gastric digestion at all four GE points (Figure 6). In

AA yoghurt, two well-defined peaks can be observed in the range of 21 to 11 kDA in the GE 1. In the same range at GE2, a peak shift appears from 19 kDA to 16 kDa, as well as a disappearance of the peak at 13 kDA. Interestingly, a more intense peak was observed at 16 kDA at GE4 compared to GE3, potentially due to sampling difficulties during digestion. For AB yoghurt, 2 two peaks can also be observed at in the range of 21 to 11 kDA in the GE 1, however, they are less well defined compared to AA. These peaks disappear as digestion progresses, however, GE4 showed the highest absorbance, followed by GE 2 and finally GE 3. Similarly, to AA yoghurt, a well-defined peak is observed at 5 kDA at GE3, which is not observed at any other point. Interestingly, well-defined peaks are observed at 2 kDA for all GE points; however, GE1 shows the highest absorbance at this point.

For BB yoghurt, a progressive breakdown can be observed from between GE1, GE2, GE3 and GE4. Similarly, to both AA and AB yoghurts, a well-defined peak can be observed at 5 kDA. This peak can also be observed in GE4, with a slight peak shift to the right and a decrease in intensity.

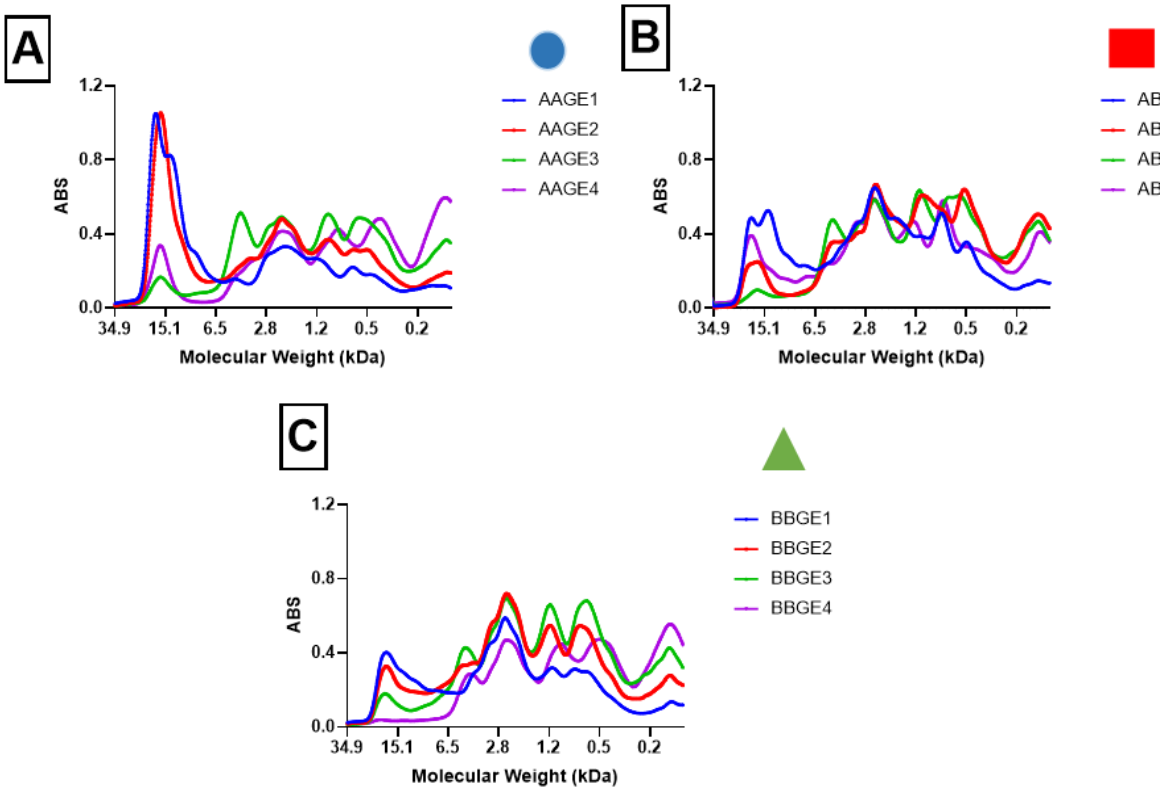

Figure 6. SEC-HPLC graphs depicting the molecular mass distributions of proteins during in vitro semi-dynamic digestion of AA (blue circle), AB (red square), and BB (green triangle) yoghurts.

3.3.5. Conformational features of digesta

The main areas of differentiation between yoghurts containing different β-lactoglobulin polymorphisms (AA, AB, BB) at each stage of digestion can be found in the FTIR spectra between the ranges of 1751 cm^{-1} and 1500 cm^{-1} *(*Figure 7A*)*. Furthermore, PCA plots clearly separated yoghurts containing different β-

lactoglobulin polymorphisms from each other (Figure 7B*)*. During the GE 1 and GE 2,(up to 11.5 minutes), yoghurt containing AA is grouped separately to AB and BB samples along PC 1, while the variation between AB and BB samples is explained by PC 2. According to the loading plot, the main variance related to PC 1 can be attributed to wavenumbers between 1580 - 1550 cm^{-1} and 1660 - 1640 cm^{-1}. Loadings between 1580 - 1570 cm^{-1} have been associated with differences in the amount of β-turns, while loadings between 1560-1550 cm^{-1} have been attributed to differences in the amount of α-helices (Grewal et al., 2017). In the amide I region, loadings in the region of 1660 - 1645 cm^{-1} have been attributed to differences in random coil structures. This may suggest a difference in 'the participation of caseins in the formation of aggregates' (Grewal et al., 2018). Since a peak shift is observed, it is likely that a conformational change exists between yoghurts with β-lactoglobulin AA vs AB and BB. PC2 can be explained by high loadings in the 1585 - 1550 cm^{-1} region. This region has been associated with β-turns and α-helices. Grewal et al. (2018) associated high loadings in this region to differences in $–COO^-$ asymmetric stretching vibrations of glutamic acid (Glu) residues.

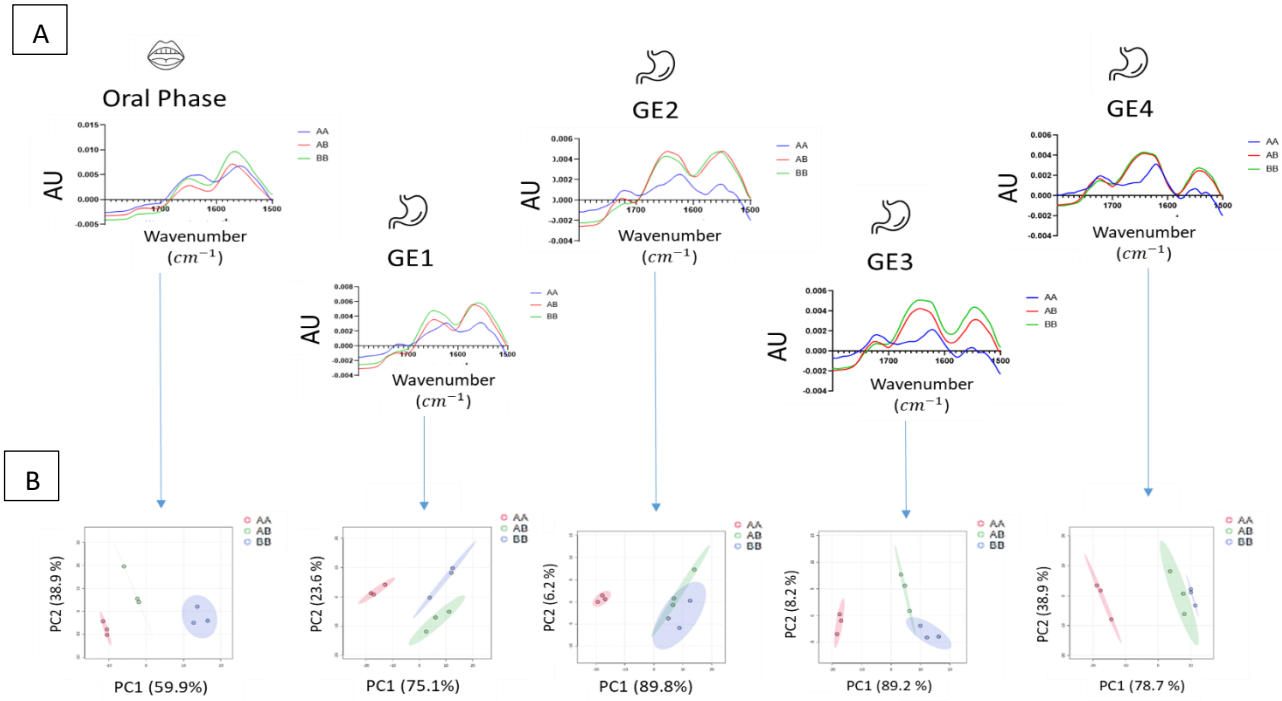

Figure 7. FTIR analysis of digests after 0, 5, 10, 15 and 20 minutes of gastric digestion. PCA plots indicate a clear separation between AA, AB and BB yoghurt digesta at all stages of digestion.

Similarly to the first two emptying points, the final two stages of gastric digestion (GE3 and GE4) of yoghurt containing AA is grouped separately to AB and BB samples along PC 1, while the variation between AB and BB samples is explained by PC 2. As in the first 2 stages of gastric emptying, the region between 1655 to 1645 cm^{-1} had high loading values, which, by observing the spectra graphs can be associated with both a difference in absorbance and a peak shift between yoghurts AA vs AB and BB. However, 1550 to 1530 cm^{-1} region also shows high loading scores. This region is associated with differences in β-sheets (Grewal et al., 2017).

4. Discussion

This study examines the impact of β-lactoglobulin polymorphisms (AA, AB, BB) on the rheological and microstructural properties of yogurt, as well as on its gastric digestibility.

Oscillatory rheology, viscosity analysis, WHC and CLSM analysis of the yoghurt provided information about the differences among AA, AB, and BB yoghurts in terms of techno-functional properties and microstructure. Notably, AA yoghurt was related to a less viscous gel with weaker viscoelastic properties, lower WHC, a visibly looser protein network structure compared to BB yoghurts (Figures 2 - 4, $p < 0.05$). This observation could be attributed to the differing conformational properties of the β-lactoglobulin polymorphisms, influencing protein interactions and network formation, potentially due to the destabilisation of β-lactoglobulin B compared to A of approximately 5 kJ/mol (Pace & Alexander, 1971).

Before milk fermentation, the samples were heat treated, thus the free Cys^{121} in β-lactoglobulin variants was exposed and played an important aggregation role in the formation of yoghurt (Donato & Guyomarc'h, 2009). The decreased stability of β-lactoglobulin B compared to A may result in β-lactoglobulin B exposing its free Cys more readily than β-lactoglobulin A, thereby leading to more favourable yoghurt production characteristics (Manderson et al., 1998b). The fine gels formed due to whey protein coating of casein micelles are less likely to undergo syneresis. In the present study, AA yoghurt expelled significantly higher volumes of whey than BB yoghurt, indicating that a fine gel had not been sufficiently formed in the prior compared to the latter sample.

As well as this, the increased calcium content associated with β-lactoglobulin B may lead to the formation of stronger gels due to the role of Ca^{2+} in enhancing the

association between caseins and β-lactoglobulin (Mohammad-Beigi et al., 2023). It is interesting to note that firmer yoghurts appear to have been produced from BB compared AA milks, despite higher whey protein contents in the reconstituted AA skim milk powder. Based on previous studies, it would be assumed that an increase in whey protein content would lead to a firmer yoghurt (Wilkinson et al., 2000). During the production of the skim milk powders that were used to produce yoghurt, protein content was standardised to total nitrogen content. Because of this, contents of the skim milk powders used to produce the yoghurts varied in terms of whey protein content. This finding is in agreement with other studies that show an increase in whey protein content associated with the β-lactoglobulin A *vs* B (Graml et al., 1989; Ng-Kwai-Hang et al., 1986a). This has also been observed in other studies (McLean et al., 1984).

Moreover, β-lactoglobulin A has a lower isoelectric point due to the presence of an extra negative charge compared to the β-lactoglobulin B, which can be visualised by different elution times from HPLC column (Yamamoto & Ishihara, 1999). The valine to alanine substitution at position 118 is thought to lead to the creation of a cavity at strand H, which may be the reason for β-lactoglobulin A showing more stability than the β-lactoglobulin B (Alexander and Pace 1990). This may explain why several studies have found a significant difference between β-lactoglobulin A and β-lactoglobulin B in the amount of native β-lactoglobulin remaining in milk after heating, with β-lactoglobulin B showing a significantly greater loss than β-lactoglobulin A (Allmere et al., 1997; Allmere et al., 1998; Hillier & Lyster, 1979). Other differences also exist between polymorphisms, β-lactoglobulin B has been shown to be five times more soluble than β-lactoglobulin A in distilled water (Treece et al., 1964). Very recently, Anema (2024) explained that only small variations in

calcium ion binding were observed between the heat-treated β-lactoglobulin A and B genetic variants. Namely, it was found that the A type could potentially bind 13.5 calcium ions per monomer and the B genetic variants could bind 13 calcium ions per monomer (Anema, 2024), which might result in the different outcomes presented in the current study in both yoghurt processing and gastric digestion. Nevertheless, it has to be noted that the milk is a system comprised of numerous micro and macro components which could affected these behaviours.

4.2. Does yoghurt structure affect in vitro gastric digestion?

In vivo, yoghurt proteins exhibit a longer gastric retention time when compared to liquid milk (Gaudichon et al., 1995; Le Feunteun et al., 2014). This suggests that gel matrix has an impact on gastric emptying. The results of the present study indicate that during *in vitro* digestion, AA yoghurt demonstrated distinct emptying behaviour compared to the other polymorphisms. During digestion, differences in protein matrix could be observed between digesta of BB yoghurts vs AA and AB, with BB retaining its structure and both AA and AB samples being broken down faster. Visual observations at the final gastric emptying point confirmed this, showing AA yoghurt losing its colour, indicating increased casein emptying. In contrast, AB and BB yoghurts retained their white appearance, suggesting a slower casein emptying rate and therefore a slower digestion rate. This is in agreement with an *in vivo* study on pigs, which showed that more viscous yoghurts were emptied slower than less viscous yoghurts from the gastric phase (Mpassi et al., 2001).

Interestingly, quantification of protein release into the supernatant using the BCA assay did not show significant difference between AA and BB samples at any stage of digestion. However, AB digesta released significantly higher protein into the

supernatant compared to BB digesta after 16.5 and 22 minutes of gastric digestion (GE 3 and GE 4). This does not agree with *in vivo* findings, where low viscosity yoghurts demonstrated a higher percentage of gastric retention after 120 minutes in mini pigs, therefore we would expect a difference between AA and BB samples (Ménard, Famelart, et al., 2018). Differences between and the present study exist however, such as examining drinking yoghurt, compared to the present study, which examined set-style yoghurt.

Using the OPA assay to determine the release of free amines into the supernatant, results showed that after 22 minutes of gastric digestion, AB yoghurt released a significantly higher amount of free amine groups compared to BB sample. This finding indicated that AB digesta was broken down more easily when compared to BB sample. As well as this, despite not finding a significant difference, both AA and AB digests tended to release a higher amount of free amines than BB digesta at every stage of gastric digestion. Ménard, Famelart, et al. (2018) showed low viscosity yoghurts to be more resistant to gastric proteolysis compared to high viscosity yoghurt in a dynamic *in vitro* system. Our results contrast with these findings, where the highest viscosity yoghurt showed the lowest release of free amines during gastric digestion.

Using FTIR, an increase in absorbance was observed in the region of 1580 - 1550 cm^{-1} that may be related to β-turns motifs which are normally present due to κ-casein/β-lactoglobulin interactions. The increased absorbance in this region could explain the denser microstructure in AB and BB *vs* AA yoghurts observed in the oral phase using CLSM. The formation of gel structure in yoghurt making is dependent upon this κ-casein/β-lactoglobulin interactions. When milk is heated, commonly to 85 °C for 30 minutes, β-lactoglobulin is denatured, allowing complex formation between β-lactoglobulin and κ-CN (Anema, 2020). FTIR allowed the analysis of structural

changes to proteins during digestion. The observed differences in the Amide II and Amide I regions of the FTIR spectra between the yoghurt polymorphisms suggest alterations in secondary structure elements such as β-turns, α-helices, and β-sheets. These structural changes likely contribute to the differential digestion patterns observed among the yoghurt polymorphisms shown using both SEC and the OPA assay.

However, the yoghurts vary in terms of viscosity, with AA showing the least viscous gel, followed by AB and finally BB. Increasing viscosity has been linked to reduced disintegration in the gastric phase *in vitro (Rioux & Turgeon, 2012).* Viscosity has also been shown to influence the feeling of satiation in several *in vivo* studies (Morell et al., 2015). For example, it has been shown that when fed *ad libitum*, healthy adults consumed a higher quantity of liquid milk-based products when compared to semi solid milk-based products (Zijlstra et al., 2008). This may be due to a longer retention of nutrients in the stomach related to increased viscosity (Zijlstra et al., 2008). Although no differences were observed in the present study in terms of protein release, visual observations highlighted a more rapid loss of white colour in AA yoghurt compared to AB or BB samples, indicating a more rapid loss of casein proteins.

β-lactoglobulin structure is also altered at different pH (Yan et al., 2013). In its native form, β-lactoglobulin found in the compact N-form, and transitions to Q-form in acidic conditions (Casal et al., 1988). At neutral pH, β-lactoglobulin occurs as a dimer, however, as pH becomes more acidic, dissociation occurs, which has also been shown to be polymorphism dependent, with β-lactoglobulin A dissociating more readily than the β-lactoglobulin B (Gilbert & Gilbert, 1961). β-lactoglobulin A has been shown to form octamers at pH 4.5, which may be due to the Gly -> Asp substitution at position 64 (Sawyer & James, 1982).Variations in experimental conditions, such as altering the

ratio of β-Lactoglobulin A and B in yogurt, could help to understand the extent of the effect of polymorphism on yogurt production and subsequent digestion.

5. Conclusion

This study investigated the distinct differences in yoghurt production due to β-lactoglobulin polymorphism and the subsequent impact on *in vitro* gastric digestion. Firstly, the results of this study confirm that β-lactoglobulin B is associated with the formation of a firmer gel when compared to reconstituted skim milk powders that carried β-lactoglobulin , which forms weaker gels with lower viscoelasticity and greater syneresis. This was confirmed using CLSM, showing clear differences between AA and AB and BB yogurts. Secondly, this study shows that this difference led to distinct gastric digestion characteristics, with BB yoghurt appearing to be more resistant to digestion compared to AA yoghurt. Moreover, it is possible to clearly separate AA from BB digests at all stages of digestion using Principal Component Analysis of FTIR spectra. Further investigations could investigate the mechanisms causing these differences these differences and their implications for human health and nutrition, such as examining differences in bioactive peptide release. As well as this, it would be of interest to adjust the ratios of β-lactoglobulin A to B, to find an optimum yoghurt, with favourable rheological characteristics paired with ease of digestion. The findings of this study may be of interest to dairy producers, due to β-lactoglobulin A being more frequently observed in Holstein-Friesian cows compared to β-lactoglobulin B, although further study is required. Overall, this study shows the importance of understand the genetics of bovine dairy herds for both production qualities and digestion, particularly when moving towards precision based dairying.

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References

Allmere, T., Andrén, A., & Björck, L. (1997). Interactions between different genetic variants of β-lactoglobulin and κ-casein A during heating of skim milk. *Journal of Agricultural and Food Chemistry*, *45*(5), 1564-1569.

Allmere, T., Andrén, A., Lindersson, M., & Björck, L. (1998). Studies on rheological properties of stirred milk gels made from milk with defined genetic variants of κ-casein and β-lactoglobulin. *International Dairy Journal*, *8*(10), 899-905.

Anema, S. G. (2020). The whey proteins in milk: Thermal denaturation, physical interactions, and effects on the functional properties of milk. *Milk proteins*, 325-384.

Anema, S. G. (2024). Calcium ion binding to native and heat treated β-lactoglobulin genetic variants. *International Dairy Journal*, *159*, 106057.

Braunitzer, G., Chen, R., Schrank, B., & Stangl, A. (1972). Automatische Sequenzanalyse eines Proteins (ß-lactoglobulin AB). *Biol. Chem*, *238*, 622-627.

Casal, H. L., Köhler, U., & Mantsch, H. H. (1988). Structural and conformational changes of β-lactoglobulin B: an infrared spectroscopic study of the effect of pH and temperature. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *957*(1), 11-20.

Daniloski, D., Hailu, Y., Brodkorb, A., Vasiljevic, T., & McCarthy, N. A. (2024a). Impact of β-casein phenotype on the physical properties of skim milk powders and their subsequent digestion characteristics. *Food Hydrocolloids*, *152*, 109918.

Daniloski, D., McCarthy, N. A., Gazi, I., & Vasiljevic, T. (2022a). Rheological and structural properties of acid-induced milk gels as a function of β-casein phenotype. *Food Hydrocolloids*, *131*, 107846.

Daniloski, D., McCarthy, N. A., O'Callaghan, T. F., & Vasiljevic, T. (2022b). Authentication of β-casein milk phenotypes using FTIR spectroscopy. *International Dairy Journal*, *129*, 105350.

Daniloski, D., Vasiljevic, T., Freitas, D., Comunian, T. A., Brodkorb, A., & McCarthy, N. A. (2024b). Physicochemical and simulated gastric digestion properties of A1/A1, A1/A2 and A2/A2 yoghurts. *Food Hydrocolloids*, *157*, 110430.

Deeth, H., & Bansal, N. (2019). Chapter 1 - Whey Proteins: An Overview. In H. C. Deeth & N. Bansal (Eds.), *Whey Proteins* (pp. 1-50). Academic Press.

Donato, L., & Guyomarc'h, F. (2009). Formation and properties of the whey protein/$\kappa $-casein complexes in heated skim milk–A review. *Dairy Science and Technology*, *89*(1), 3-29.

Dupont, D., Le Feunteun, S., Marze, S., & Souchon, I. (2018). Structuring food to control its disintegration in the gastrointestinal tract and optimize nutrient bioavailability.

Innovative Food Science & Emerging Technologies, *46*, 83-90. https://doi.org/https://doi.org/10.1016/j.ifset.2017.10.005

Farrell, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., Hicks, C. L., Hollar, C. M., Ng-Kwai-Hang, K. F., & Swaisgood, H. E. (2004). Nomenclature of the Proteins of Cows' Milk—Sixth Revision. *Journal of Dairy Science*, *87*(6), 1641-1674.

Fox, P. F., & Mulvihill, D. M. (1982). Milk proteins: molecular, colloidal and functional properties. *Journal of Dairy Research*, *49*(4), 679-693.

Freitas, D., Gómez-Mascaraque, L. G., & Brodkorb, A. (2022). Digestion of protein and toxic gluten peptides in wheat bread, pasta and cereal and the effect of a supplemental enzyme mix. *Frontiers in Nutrition*, *9*, 986272.

Gaudichon, C., Mahé, S., Roos, N., Benamouzig, R., Luengo, C., Huneau, J.-F., Sick, H., Bouley, C., Rautureau, J., & Tome, D. (1995). Exogenous and endogenous nitrogen flow rates and level of protein hydrolysis in the human jejunum after [15N]milk and [15N]yoghurt ingestion. *British Journal of Nutrition*, *74*(2), 251-260. https://doi.org/10.1079/BJN19950128

Gilbert, L. M., & Gilbert, G. A. (1961). Sedimentation of β-Lactoglobulin (A, B) under dissociating conditions in acid solution. *Nature*, *192*(4808), 1181-1181.

Gómez-Mascaraque, L. G., & Pinho, S. C. (2021). Microstructural analysis of whey/soy protein isolate mixed gels using confocal Raman microscopy. *Foods*, *10*(9), 2179.

Goulding, D., Fox, P., & O'Mahony, J. (2020). Milk proteins: An overview. *Milk proteins*, 21-98.

Graml, R., Weiss, G., Buchberger, J., & Pirchner, F. (1989). Different rates of synthesis of whey protein and casein by alleles of the β-lactoglobulin and αs1-casein locus in cattle. *Genetics Selection Evolution*, *21*(4), 547-554.

Grewal, M. K., Chandrapala, J., Donkor, O., Apostolopoulos, V., Stojanovska, L., & Vasiljevic, T. (2017). Fourier transform infrared spectroscopy analysis of physicochemical changes in UHT milk during accelerated storage. *International Dairy Journal*, *66*, 99-107.

Grewal, M. K., Huppertz, T., & Vasiljevic, T. (2018). FTIR fingerprinting of structural changes of milk proteins induced by heat treatment, deamidation and dephosphorylation. *Food Hydrocolloids*, *80*, 160-167.

Hallén, E., Allmere, T., Lundén, A., & Andrén, A. (2009). Effect of genetic polymorphism of milk proteins on rheology of acid-induced milk gels. *International Dairy Journal*, *19*(6), 399-404.

Heck, J. M. L., Schennink, A., van Valenberg, H. J. F., Bovenhuis, H., Visker, M. H. P. W., van Arendonk, J. A. M., & van Hooijdonk, A. C. M. (2009). Effects of milk protein

variants on the protein composition of bovine milk. *Journal of Dairy Science*, *92*(3), 1192-1202.

Hillier, R. M., & Lyster, R. L. (1979). Whey protein denaturation in heated milk and cheese whey. *Journal of Dairy Research*, *46*(1), 95-102.

Ketto, I. A., Øyaas, J., Ådnøy, T., Johansen, A.-G., Schüller, R. B., Narvhus, J., & Skeie, S. B. (2018). The influence of milk protein genetic polymorphism on the physical properties of cultured milk. *International Dairy Journal*, *78*, 130-137.

Le Feunteun, S., Barbé, F., Rémond, D., Ménard, O., Le Gouar, Y., Dupont, D., & Laroche, B. (2014). Impact of the Dairy Matrix Structure on Milk Protein Digestion Kinetics: Mechanistic Modelling Based on Mini-pig In Vivo Data. *Food and Bioprocess Technology*, *7*(4), 1099-1113. https://doi.org/10.1007/s11947-013-1116-6

Lucey, J., & Singh, H. (1997). Formation and physical properties of acid milk gels: A review. *Food research international*, *30*(7), 529-542.

Lucey, J. A. (2004). Cultured dairy products: An overview of their gelation and texture properties. *International Journal of Dairy Technology*, *57*(2-3), 77-84.

Lucey, J. A., Wilbanks, D. J., & Horne, D. S. (2022). Impact of heat treatment of milk on acid gelation. *International Dairy Journal*, *125*, 105222.

Manderson, G. A., Hardman, M. J., & Creamer, L. K. (1998). Effect of Heat Treatment on the Conformation and Aggregation of β-Lactoglobulin A, B, and C. *Journal of Agricultural and Food Chemistry*, *46*(12), 5052-5061.

McCarthy, N. A., Wijayanti, H. B., Crowley, S. V., O'Mahony, J. A., & Fenelon, M. A. (2017). Pilot-scale ceramic membrane filtration of skim milk for the production of a protein base ingredient for use in infant milk formula. *International Dairy Journal*, *73*, 57-62.

McLean, D. M., Graham, E. B., Ponzoni, R. W., & McKenzie, H. A. (1984). Effects of milk protein genetic variants on milk yield and composition. *Journal of Dairy Research*, *51*(4), 531-546.

Ménard, O., Famelart, M. H., Deglaire, A., Le Gouar, Y., Guérin, S., Malbert, C. H., & Dupont, D. (2018). Gastric Emptying and Dynamic In Vitro Digestion of Drinkable Yogurts: Effect of Viscosity and Composition. *Nutrients*, *10*(9). https://doi.org/10.3390/nu10091308

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., & Dupont, D. (2014). A standardised static in vitro digestion method suitable for food–an international consensus. *Food & Function*, *5*(6), 1113-1124.

Mohammad-Beigi, H., Wijaya, W., Madsen, M., Hayashi, Y., Li, R., Maria Rovers, T. A., Jæger, T. C., Buell, A. K., Hougaard, A. B., Kirkensgaard, J. J. K., Westh, P., Ipsen, R., & Svensson, B. (2023). Association of caseins with β-lactoglobulin influenced by

temperature and calcium ions: A multi-parameter analysis. *Food Hydrocolloids*, *137*, 108373.

Morell, P., Hernando, I., Llorca, E., & Fiszman, S. (2015). Yogurts with an increased protein content and physically modified starch: rheological, structural, oral digestion and sensory properties related to enhanced satiating capacity. *Food Research International*, *70*, 64-73. https://doi.org/https://doi.org/10.1016/j.foodres.2015.01.024

Mpassi, D., Rychen, G., Mertes, M., & Laurent, F. (2001). Portal absorption of 45Ca from labelled milk, yoghurt or heat treated yoghurt in the growing pig. *International Dairy Journal*, *11*(10), 809-815. https://doi.org/https://doi.org/10.1016/S0958-6946(01)00107-8

Mulet-Cabero, A.-I., Egger, L., Portmann, R., Ménard, O., Marze, S., Minekus, M., Le Feunteun, S., Sarkar, A., Grundy, M. M. L., Carrière, F., Golding, M., Dupont, D., Recio, I., Brodkorb, A., & Mackie, A. (2020). A standardised semi-dynamic in vitro digestion method suitable for food – an international consensus [10.1039/C9FO01293A]. *Food & Function*, *11*(2), 1702-1720. https://doi.org/10.1039/C9FO01293A

Ng-Kwai-Hang, K., Hayes, J., Moxley, J., & Monardes, H. (1986). Relationships between milk protein polymorphisms and major milk constituents in Holstein-Friesian cows. *Journal of Dairy Science*, *69*(1), 22-26.

Nielsen, P., Petersen, D., & Dambmann, C. (2001). Improved method for determining food protein degree of hydrolysis. *Journal of Food Science*, *66*(5), 642-646.

O'Loughlin, I. B., Murray, B. A., Brodkorb, A., FitzGerald, R. J., Robinson, A. A., Holton, T. A., & Kelly, P. M. (2013). Whey protein isolate polydispersity affects enzymatic hydrolysis outcomes. *Food Chemistry 141*(3), 2334-2342.

O'Mahony, J. A., & Fox, P. F. (2014). Chapter 2 - Milk: An Overview. In H. Singh, M. Boland, & A. Thompson (Eds.), *Milk Proteins (Second Edition)* (pp. 19-73). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-405171-3.00002-7

Olguin-Arredondo, H., & Vallejo-Córdoba, B. (1999). Separation and determination of beta-lactoglobulin variants A and B in cow's milk by capillary free zone electrophoresis. *Journal of Capillary Electrophoresis and Microchip Technology*, *6*(5-6), 145-149.

Pace, C., & Alexander, S. S. (1971). Comparison of the denaturation of bovine β-lactoglobulins A and B and goat β-lactoglobulin. *Biochemistry*, *10*(14), 2738-2743.

Pletschke, B. I., Naudé, R. J., & Oelofsen, W. (1995). Ostrich pepsins I and II: a kinetic and thermodynamic investigation. *The International Journal of Biochemistry & Cell Biology*, *27*(12), 1293-1302.

Rioux, L.-E., & Turgeon, S. L. (2012). The Ratio of Casein to Whey Protein Impacts Yogurt Digestion In Vitro. *Food digestion*, *3*(1), 25-35. https://doi.org/10.1007/s13228-012-0023-z

Şahin Semerci, E., Ergin, F., Demir, E., Küçükçetin, A., & Balcıoğlu, M. S. (2024). Influence of different genotype combinations of β-lactoglobulin and β-casein in cow milk on physicochemical and sensory properties of stirred yoghurt. *Available at SSRN 4860048*.

Sawyer, L., & James, M. N. G. (1982). Carboxyl–carboxylate interactions in proteins. *Nature*, *295*(5844), 79-80.

Treece, J. M., Sheinson, R. S., & McMeekin, T. L. (1964). The solubilities of β-lactoglobulins A, B, and AB. *Archives of Biochemistry and Biophysics*, *108*(1), 99-108.

Wilkinson, M., Guinee, T. P., & Fenelon, M. (2000). *Effect of milk composition on the quality of fresh fermented dairy products* (1841701211).

Yamamoto, S., & Ishihara, T. (1999). Ion-exchange chromatography of proteins near the isoelectric points. *Journal of Chromatography A*, *852*(1), 31-36.

Yan, Y., Seeman, D., Zheng, B., Kizilay, E., Xu, Y., & Dubin, P. L. (2013). pH-Dependent aggregation and disaggregation of native β-lactoglobulin in low salt. *Langmuir*, *29*(14), 4584-4593.

Zijlstra, N., Mars, M., de Wijk, R. A., Westerterp-Plantenga, M. S., & de Graaf, C. (2008). The effect of viscosity on ad libitum food intake. *International Journal of Obesity*, *32*(4), 676-683.

Appendix (Part 2)

Peer reviewed publications

**not for examination*

Kelly, L.M., O'Mahony, J.A., Tobin, J.T. (2024). Genetic variation in bovine milk proteins: Implications for functional and nutritional properties. *International Journal of Dairy Technology*. DOI: https://doi.org/10.1111/1471-0307.13152

Kelly, L.M., McCarthy, W.P., Subhir, S., Horan, B., O'Mahony, J.A., Tobin, J.T. (2025). Impact of genetic variants on the separation efficiency of β-lactoglobulin during microfiltration of skim milk. *International Dairy Journal, 161,* 106115

*Hayes, E., Kelly, L.M., Aydogdu. T., O'Shea, N., O'Donnell, C., Green, D., Fenelon, M.A. (2024). Real-time measurement of heat stability of skim milk using attenuated total reflectance (ATR)-FTIR spectroscopy. *International Journal of Dairy Technology*. DOI: https://doi.org/10.1111/1471-0307.13156