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What is the impact of amino acid mutations in the primary structure of caseins on the composition and functionality of milk and dairy products?

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ABSTRACT

The impact of amino acid mutations within the peptide structure of bovine milk protein is important to understand as it can effect processability and subsequently effect its physiological properties. Genetic polymorphisms of bovine caseins can influence the chemical, structural, and technological properties, including casein micelle morphology, calcium distribution, network creation upon gelation, and surface activity. The A1 and A2 genetic variants of β-casein have recently acquired growing attention from both academia and industry, prompting new developments in the area. The difference between these two genetic variants is the inclusion of either proline in β-casein A2 or histidine in β-casein A1 at position 67 in the peptide chain. The aim of this review was to examine the extent to which milk and ingredient functionality is influenced by β-casein phenotype. One of the main findings of this review was although β-casein A1 was found to be the dominant variant in milks with superior acid gelation and rennet coagulation properties, milks comprised of β-casein A2 possessed greater emulsion and foam formation capabilities. The difference in the casein micelle assembly, hydrophobicity, and chaperone activity of caseins may explain the contrast in the functionality of milks containing β-casein from either A1 or A2 families. This review provides new insights into the subtle variations in the physicochemical properties of bovine milks, which could potentially support dairy producers in the development of new dairy products with different functional properties.

1. Introduction

Bovine milk is produced in the mammary gland and comprises a large and diverse collection of proteins, fats, carbohydrates and micronutrients, which vary in abundance across stages of lactation, age, and cow's health (McSweeney and Fox, 2013). The protein composition of milk is complex, which influences its physicochemical, functional, and nutritional properties. There are two main categories of milk proteins, namely caseins and whey proteins, present in milk at a ratio of ~80 : 20 (Gazi, Johansen and Huppertz, 2022). Caseins originate from the family of phosphoproteins found in all mammals (O'Mahony and Fox, 2013) and are categorised into four different types: αs_1-, αs_2-, β- and κ-casein, present at the ratio of ~4.0 : 1.0: 3.5 : 1.5 in bovine milk; all caseins contribute to a colloidal structure known as the casein micelle (Horne, 2020). On dry matter basis, the casein micelle consists of ~93% protein and ~7% salts, the latter commonly referred to as micellar calcium phosphate (MCP) (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015; Huppertz et al., 2018; Huppertz and Gazi, 2022).

To form a casein micelle, the four individual caseins interact with each other via predominantly non-covalent, but also some covalent, interactions, as well as via ionic interactions with calcium phosphate nanoclusters (Lucey and Horne, 2018). The nature of the interactions between caseins has been extensively discussed, with some suggesting that hydrophobic interactions are essential (Horne, 2020), while others argue that backbone interactions are the most important (Carver and Holt, 2019). De Kruif et al. (2012) outlined that interactions between caseins involve collective hydrophobic and hydrogen bonding, electrostatic interactions, and van der Waals attractions. Different models for casein micelle assembly and structure have been proposed, e.g., the sub-micelle model (Slattery, 1976; Slattery and Evard, 1973), the

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dual-binding model (Horne, 1998, 2017a, 2020), the nano-cluster model (Holt, 1992, 2004, 2016), the water channel model (Dalgleish, 2011; Dalgleish and Corredig, 2012; Dalgleish et al., 2004), and the network model (Huppertz et al., 2017). Nevertheless, despite its ubiquity and numerous investigations, there is also no general consensus either on the interactions, or on the fine structure of the casein micelle assembly since the structure of the casein micelle cannot be simply visualised (Day et al., 2015; De Kruif et al., 2012; Huppertz and Gazi, 2022).

Milk protein genes are able to produce a substantial number of polymorphisms. The autosomal genes CSN1S1 (αs_1-casein), CSN1S2 (αs_2-casein), CSN2 (β-casein), and CSN3 (κ-casein) encoding for the caseins are organised as a cluster in a DNA stretch of about 200 kb mapped on chromosome 6 (Gai, Uniacke-Lowe, O'Regan, Faulkner and Kelly, 2021). There are at least 39 genetic bovine casein variants reported (Gazi et al., 2022): 8 for αs_1-casein (A, B, C, D, E, F, G, and H), 4 for αs_2-casein (A, B, C, and D), 15 for β-casein (A1, A2, A3, A4, B, C, D, E, F, G, H1, H2, I, J, K), and 12 for κ-casein (A, B, B2, C, E, F1, F2, G1, G2, H, I, J) (Fig. 1). Important differences in occurrence and frequency of the variants arise among species and breeds (Adamov et al., 2020; Gallinat et al., 2013; Gazi et al., 2022a; Huppertz et al., 2018). Genetic variants of milk proteins differ in amino acid sequence, which can affect the isoelectric point and protein charge, and ultimately can impact the physicochemical and the functional properties of milk proteins, milk and dairy products (Day et al., 2015; Gai, Uniacke-Lowe, O'Regan, Faulkner and Kelly, 2021; Lucey and Horne, 2018). Recently, different genotypes of β-casein in bovine milk have gained significant attention within the dairy industry, as they have been related to affecting the functionality of dairy products, and even been linked to having an impact on human health (Daniloski, McCarthy and Vasiljevic, 2021b; Mendes et al., 2019; Milan et al., 2020; NguyenSchwendel et al., 2018; Sebastiani et al., 2022).

Bovine β-casein is considered an intrinsically disordered protein (IDP) and constitutes between 33 and 45% of the total casein in bovine milk. Due to the location of the mutation (single nucleotide polymorphism) on exon VII and the 6th chromosome of CSN2 gene, the transfer from cytosine (CCT) to adenine (CAT) contributes to the substitution of proline (Pro) with histidine (His) at position 67 in the β-casein polypeptide chain (Caroli, Chessa, & Erhardt, 2009; Jann et al., 2002; Kumar et al., 2018; Sebastiani et al., 2022) (Fig. 2). Namely, β-caseins A1 and A2 are the most commonly secreted variants in milk (Aschaffenburg, 1968; Farrell et al., 2004). It is postulated that the A2 variant of β-casein (β-casein A2-5P; considered as a reference proteoform) carries the original amino acid sequence, before a point mutation caused the appearance of the β-casein A1 variant in some European herds (Daniloski et al., 2021a; De Poi et al., 2020; Gallinat et al., 2013; Gazi et al., 2022). The evolution of the β-casein A1 proteoform led to the grouping of the β-casein variants into two families, i.e., the A2 family with the A2, A3, and I variants of β-casein, and A1 family with the A1, B, and F variants of β-caseins (Table 1) (Caroli et al., 2004; Daniloski, McCarthy, Markoska, Auldist and Vasiljevic, 2022a; Ng-Kwai-Hang & Grosclaude, 2003). Bovine milk possessing β-casein A2/A2 carrying Pro is called A2/A2 milk, whereas milks carrying His in the β-casein A1/A1 or A1/A2 polypeptide chains are known as A1/A1 and A1/A2 milks, respectively (Daniloski et al., 2022a; NguyenSolah et al., 2019).

To date many studies have focused on the effects of β-casein genetic polymorphism on animal and human health using *in vitro*, *ex vivo* and *in vivo* trials (Daniloski et al., 2021a, 2021b; Haq et al., 2014; He et al., 2017; Hockey et al., 2021; Ramakrishnan et al., 2022; Ramakrishnan et al., 2020; Yadav et al., 2020), with limited focus on the technical and functional properties of β-casein phenotype on milk products. Therefore, the main purpose of this review is to address the differences between the physicochemical properties of these β-casein phenotypes, but also the other caseins and their genetic variants. This will include the structure of the casein micelle, chaperone activity, hydrophobicity, emulsion, foaming and rheological properties of milk and the process of acid gelation and rennet coagulation.

2. Polymorphism and protein profiling of β-casein genetic variants

Single-nucleotide polymorphisms are the most abundant form of genetic variation and a resource for mapping complex genetic traits through the amino acid modifications within the structure of milk proteins (Chessa et al., 2007; Gazi et al., 2022a; Martin et al., 2013). Whenever these genetic mutations are correlated with milk protein composition or milk production traits, the data can be useful when choosing for breeding cows that produce milk with improved value, such as higher casein levels or an altered concentration of other proteins

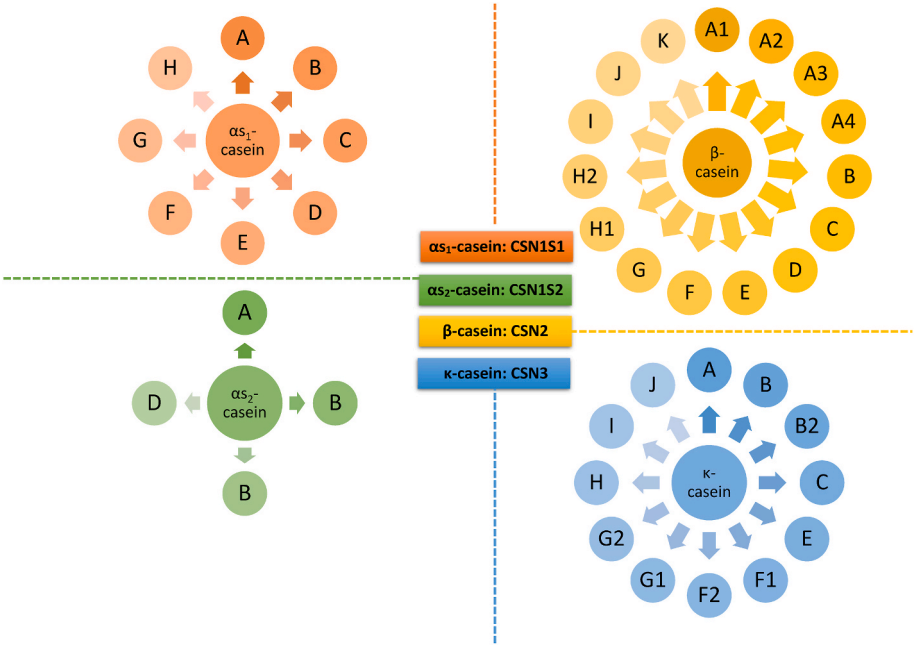

Fig. 1. Different casein forms, their genetic variants and genes.

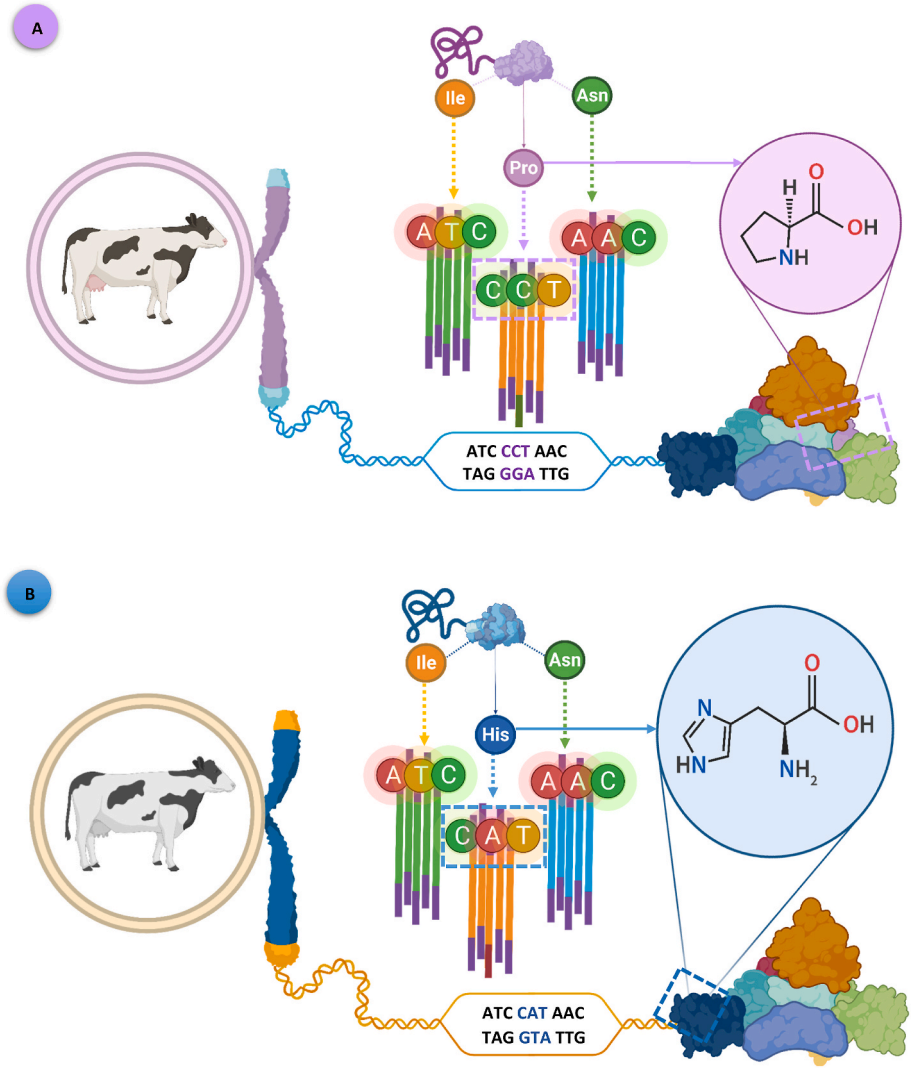

Fig. 2. Bovine genetic polymorphism, variation sequences of A) β-casein A2 genetic variant; and B) β-casein A1 genetic variant. *Iso (Isoleucine), Pro (Proline), His (Histidine), Asn (Asparagine); A (Adenine), T (Thymine), C (Cytosine), G (Guanine).*

Table 1
Differences in the amino acid sequence of the most common genetic variants of β-casein found in the milk of *Bos* genus compared to the reference proteoform β-casein A2-5P.

	β-casein A1 family			β-casein A2 family		
Position	**Proteofroms**					
	β-casein A1-5P	β-casein A2-5P	β-casein A3-5P	β-casein B–5P	β-casein F–5P	β-casein I–5P
	Genetic variants					
	β-casein A1	β-casein B	β-casein F	β-casein A2	β-casein A3	β-casein I
67	His	His	His	Pro	Pro	Pro
93	Met	Met	Met	Met	Met	Leu
106	His	His	His	His	Gln	His
122	Ser	Arg	Ser	Ser	Ser	Ser
152	Pro	Pro	Leu	Pro	Pro	Pro
Average mass (Da)	24022.91	24092.02	24038.96	23982.89	23973.88	23964.85
Isoelectric point (pI)	4.73	4.82	4.73	4.66	4.58	4.66

(Berry et al., 2020). The gene polymorphism in β-casein was first identified in the *Bos* genus, particularly in Jersey and Guernsey cows, by Aschaffenburg in the 20th century and occurred in three genetic variants identified as A, B, and C (Aschaffenburg, 1963; Thompson et al., 1969). Some years later, Peterson and Kopfler (1966) demonstrated, by polyacrylamide gel electrophoresis at acidic pH, that β-casein A variant was not a unique casein but three different variants, denoted as β-caseins A1, A2 and A3. Furthermore, β-casein A4 has also been detected (Chung et al., 1995), however, neither its amino acid sequence or clinical based studies on this genetic sub-type have been reported to date. Despite the significance of the amino acid sequence of these proteins, limited attention was given to the genetic polymorphism of bovine β-casein until the occurrence of the A1/A1, A1/A2, and A2/A2 milk recognition in the 1990s (Elliot et al., 1996).

Over the last two decades, as a result of a greater commercial interest in certain parts of the world, including Australia, Canada, China, New

Zealand and the USA, the selection of cows producing A2/A2 milk has increased (Milan et al., 2020). Therefore, since various β-casein phenotypes can appear in bovine milk and are found to affect the dairy product manufacturing (Section 4), and possibly human health, there is an increased need for reliable analytical methods capable of authenticating a milk labelled as A1/A2 or A2/A2 milk (Daniloski, McCarthy, O'Callaghan and Vasiljevic, 2022c; De Poi et al., 2020). The methods and analytical strategies for the genetic identification and quantification of different β-casein variants in either cows or bovine milks are presented in Table 2. These methods have been primarily based on liquid chromatography, electrophoresis, polymerase chain reaction test, spectroscopy, and microsphere-based immunoassay (Broadbent et al., 2021; Daniloski et al., 2022a; Daniloski et al., 2022c; De Poi et al., 2020; Elferink et al., 2022; Fuerer et al., 2020; Gai et al., 2021; NguyenSolah et al., 2020; Vigolo et al., 2022b).

3. The importance of β-casein phenotypes, other caseins and minerals on the structure and organisation of casein micelle

Bovine β-casein comprises 209 amino acids and is considered the most hydrophobic of the caseins. The hydrophobicity seems to play an important role in the attachment of β-casein within the casein micelle. This protein has a flexible and open conformation, molecular mass of 23.6 kDa (primary structure prior to posttranslational phosphorylation; ~24.0 kDa following phosphorylation), and little tertiary structure (Huppertz et al., 2018). The phosphorylation of all five phosphoserine (SerP) residues (Ser15, Ser17, Ser18, Ser19, and Ser35) in β-casein results in the strong amphipathic nature of this protein (McCarthy, Kelly, O'Mahony and Fenelon, 2013). The first four SerP residues form a centre of phosphorylation, which facilitates the association of β-casein with the calcium phosphate nanoclusters (Gazi et al., 2022a; Horne, 2020; Huppertz et al., 2018). At neutral pH, the C-terminal area of β-casein (residues 41–209) is strongly hydrophobic and shows balanced charges, whereas the N-terminal domain (residues 1–40) is polar and strongly negatively charged (McCarthy et al., 2013). Owing to its amphiphilic nature, β-casein shows a strong propensity to self-assemble into the micelles comprising of 15–60 protein molecules (radius of gyration values ranging between 7.3 and 13.5 nm), where hydrophobic interactions between those molecules are the primary attractive forces (Huppertz, 2013). The formation of β-casein micelles is described by the shell model, also referred to as the consecutive (stepwise) micellization model (De Kruif and Grinberg, 2002), and considers a series of sequential additions of primary particles (dimer forms) to a growing micelle (Atamer et al., 2017). The β-casein micelle has a detergent-like micellar structure with a hydrophobic core and a charged hydrophilic surface (McCarthy et al., 2013). While the presence of a hydrophilic domain in the N-terminus enhances the solubility of β-casein in aqueous media, the hydrophobic domains in the C-terminal section facilitate non-covalent associations with other molecules (Liyanaarachchi and Vasiljevic, 2018).

A large proportion of polyproline II (PPII) conformational motifs, mainly within their Pro and glutamine (P,Q)-rich regions, were observed in all β-casein genetic variants (Sanders et al., 2020), but more in β-casein A2 (Syme et al., 2002) and in A2/A2 milk (Daniloski et al., 2022a). Such PPII regions, known as initiators of the hydrophobic environments of β-casein, were active within the β-casein micelle whilst interacting with other proteins (Thorn et al., 2015). Accordingly, the interactions of β-caseins with other proteins, in particular other caseins, would be enhanced, thus leading to tighter packing within the casein micelle or smaller aggregates when undergoing self-association (especially in the case of β-casein A2) (Raynes et al., 2015). Thorn et al. (2005) revealed that β-casein may manipulate the inherently unstable monomers of native κ-casein by binding to and shielding their hydrophobic surfaces, thus prohibiting interactions with other κ-casein molecules that would otherwise facilitate self-assembly into fibrillar structures. Moreover, due to the presence of one additional Pro in its

Table 2
Identification of β-casein genetic variants and phenotypes in bovine milk or cows by using different methods.

Genetic variant(s) and phenotype(s)	Methodology	Reference
Electrophoresis		
A, B, and C (A varinat does not exist anymore in common nomenclature)	Paper electrophoresis	Aschaffenburg (1963); Thompson et al. (1964)
A1, A2, and A3	Polyacrylamide gel electrophoresis	Peterson and Kopfler (1966)
A4	Urea gel electrophoresis	Chung et al. (1995)
H1	Agarose gel electrophores; Polymerase chain reaction	Han et al. (2000)
A1 and A2	Capillary zone electrophoresis	de Jong et al. (1993)
A1, A2, B, and A1/A2		Noni (2008)
A1 and A2	Capilary electrophoresis	Raynes et al. (2015)
A1 and A2	Urea Polyacrylamide gel electrophoresis	Duarte-Vázquez et al. (2018)
DNA sequencining and polymerase chain reaction		
D	Amino acid composition	Thompson et al. (1969)
A1 and A2	Duplex artificially created restriction site-polymerase chain reaction	Şahin and Boztepe (2022)
A1, A2, A3, B, C, D, E, F, G, H1, H2, I, J, and K	DNA sequencing	Gallinat et al. (2013)
A1, A2, B, I; A2/A2, A1/A2, A2/B, and A2/I	DNA sequencing	Sebastiani et al. (2022)
A1 and A2	Allele Specific Polymerase Chain Reaction	Ristanić et al. (2022)
A1, A2, B, and I	DNA sequencing; Reversed Phase-High Performance Liquid Chromatography	Bonfatti et al. (2008)
A1, A2, A1/A2, and B	Polymerase chain reaction-restriction fragment length polymorphism; Amplification refractory mutation system-polymerase chain; Reversed Phase-High Performance Liquid Chromatography	Vigolo et al. (2022b)
I	Polymerase chain reaction; Reversed Phase-High Performance Liquid Chromatography	Daniloski et al. (2022c); Jann et al. (2002)
High Performance-Liquid Chromatography		
F	Reversed Phase-High Performance Liquid Chromatography	Poulsen et al. (2017); Visser et al. (1995)
G		Visser et al. (1995)
A1/A2, A1/A1, A2/A2, B/A1, B/A2, and I/H2		Bisutti et al. (2022)
A1, A2, and B		Vigolo et al. (2022a)
A1/A1, A1/A2, and A2/A2		Daniloski et al. (2022a); Daniloski et al. (2022b)
A1 and A2	High Performance-Liquid Chromatography Tandem Mass Spectrometry	Guo et al. (2022)
A1, A2, and B	Liquid Chromatography-Mass Spectrometry	De Poi et al. (2020)
A1, A2, A3, B		Miranda et al. (2020)
H2		Senocq et al. (2002)
A1, A2, B, and C	High Performance-Liquid Chromatography-Mass Spectrometry	Givens et al. (2013)
A1/A1, A1/A2, A2/A2, A1/A3, A2/A3, A1/B, A2/B, I/I, A1/I, A2/I, B/I, A1/F, and A2/F	Liquid Chromatography-Electrospray Ionization-Mass Spectrometry	Poulsen et al. (2017)
A1, A2, B, and I	Liquid Chromatography-Electrospray Ionization-Quadrupole-Time of Flight-Mass Spectrometry	Vincent et al. (2016)
A1/A2 and A2	Ultraperformance Liquid Chromatography-High-Resolution Mass Spectrometry	Fuerer et al. (2020)

(continued on next page)

Table 2 (*continued*)

Genetic variant(s) and phenotype(s)	Methodology	Reference
A1/A1, A1/I, A1/A2, A2/A2, and A2/I	Ultra-High-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (Orbitrap™)	NguyenSolah et al. (2020)
A1 and A2	Quantitative Liquid Chromatography-Mass Spectrometry	Broadbent et al. (2021)
A2 and other A named as Am	Top-down high-resolution mass spectrometry-based metabolomics and lipidomics	Jia et al. (2022)
	Spectroscopy	
A1/A1, A1/A2, and A2/A2	Fourier-Transform Mid-Infrared spectroscopy	Cendron et al. (2021)
A1/A1 and A2/A2	Fourier Transform-Infrared spectroscopy	Joshi et al. (2021)
A1, A1/AI, A1/A2, A2, A2/I, and I		Daniloski et al. (2022c)
A1/A1 and A2/A2	Mid-Infrared spectroscopy	Xiao et al. (2022)
A1/A1, A1/A2, and A2/A2	Nuclear Magnetic Resonance spectroscopy	Daniloski et al. (2022a); Daniloski et al. (2022b)
	Immunoassay	
A1 and A2	Microsphere-Based Immunoassay	Elferink et al. (2022)

structure, β-casein A2, had lower hydrophobicity and appeared more frequently as a monomer relative to β-casein A1 (Raynes et al., 2015). Isolated and purified β-casein A1 solutions created larger β-casein micelles, compared to that of β-casein A2. However, various studies found that the milk carrying β-casein A1 possessed smaller casein micelle sizes compared to A2/A2 milks (Daniloski et al., 2022a; Daniloski, McCarthy and Vasiljevic, 2022b; Day et al., 2015).

For casein micelles, it is generally agreed that the κ-casein content was negatively correlated with size of the casein micelles (Bijl, de Vries, van Valenberg, Huppertz and Van Hooijdonk, 2014a; Dalgleish, 1993; Day et al., 2015). A number of studies have associated κ-casein and its genetic variants with the casein micelle size of individual bovine milk samples (Bijl et al., 2014a; Bonfatti et al., 2014; Day et al., 2015; Hallén et al., 2008; Vallas et al., 2012; Walsh et al., 1998). Bijl et al. (2014); Dalgleish et al. (1989); Daniloski et al. (2022a); Day et al. (2015) confirmed that κ-casein possesses the path-terminating function, and thus it can impact the casein micelle size. Both the A and B variants of κ-casein and glycosylation of κ-casein were correlated significantly with average casein micelle size in milks obtained from individual cows; however, κ-casein B was predominately associated with a smaller casein micelle size (Bijl et al., 2014a; Ketto et al., 2019; Ketto et al., 2017; Walsh et al., 1998). Differences in the casein micelle size may also be influenced by factors other than β- and κ-casein concentration, specifically their genetic variants and post-translational modifications (phosphorylation and glycosylation of casein molecules). This would include cow genetics, protein content, farming practices, environmental factors (feed and season), and the mineral content (Bijl et al., 2020; Day et al., 2015; Devold et al., 2000).

Almost a decade ago, Gustavsson et al. (2014) related small casein micelle size with a greater level of total and ionic calcium in milk. A similar trend was observed in the study of Daniloski et al. (2022a) as the authors postulated that A1/A1 and A1/A2 milks possessed greater amounts of total and ionic calcium and smaller casein micelle sizes compared to A2/A2 milk. In the casein protein fraction of milk, calcium is associated both in the form of calcium phosphate nanoclusters and also with amino acid residues via ionic bonds (Huppertz et al., 2021), e.g., with SerP, glutamic acid (Glu) and asparagine (Asp) residues that are not part of the calcium phosphate nanoclusters (Bijl et al., 2019; Lucey and Horne, 2018). The caseins can readily bind calcium ions to their phosphate cluster residues in order of $\alpha s_2 > \alpha s_1 > \beta >$ κ-caseins (O'Mahony and Fox, 2013). Hence, Daniloski et al. (2022b) explained that the higher mineral content in milk carrying β-caseins A1/A1 and A1/A2 was due to the higher presence of αs_2-, αs_1-, and β-caseins in both milks compared to A2/A2 milk.

4. The impact of the casein variants on processing and production of dairy products

Rennetability, heat stability, emulsion and foaming characteristics, renowned as technologically essential properties of milk and dairy products, are influenced in part by the genetic polymorphisms of β-casein (Gai et al., 2021; Goulding, Fox, & O'Mahony, 2020; NguyenSchwendel et al., 2018). Table 3 summarises the influence of specific β-casein genetic variants on the technological properties of milk and dairy products, including heat treatment of milks carrying β-casein A1/A1, A1/A2, or A2/A2, behaviour of either A1/A1, A1/A2, or A2/A2 milks during acid gelation and rennet coagulation, including yoghurt and cheese production, and the colloidal and interfacial properties of β-casein variants (Table 3).

4.1. Heat treatment and stability

Heat treatment is one of the most common methods employed in the dairy industry in order to reduce bacterial and enzymatic activity, thus ensuring safety and prolonging the shelf-life of milk and dairy products (Anema, 2019; McCarthy et al., 2022). Nevertheless, some additional effects might take place during or after heat treatment, including gelling or coagulation during processing, thickening during storage, and constituent fouling, hence the exploration of heat stability of milk is essential for the food industry (Dumpler et al., 2020). It is well established that during the heat treatment of milk, whey proteins, especially β-lactoglobulin, can denature and associate with casein micelles via κ-casein linkages or form κ-casein/β-lactoglobulin complexes in the serum phase depending on pH (Anema, 2021). While extensive research has been carried out on the effects of various genetic variants of κ-casein on heat-treated milk and its stability (Choi & Ng-Kwai-Hang, 2002; McLean et al., 1987; Robitaille, 1995), currently, the knowledge of the influence of thermal treatment on milks carrying β-casein phenotypes A1/A1, A1/A2, and A2/A2 is limited. At elevated temperatures (50–145 °C), β-casein may behave as a molecular chaperone (Zhang et al., 2005), whereby it could interact with partially unfolded whey proteins, via hydrophobic domains, preventing their normal thiol-disulphide interchange with other whey proteins and subsequent aggregation (Liyanaarachchi and Vasiljevic, 2018; Yousefi et al., 2009). For instance, β-casein was shown to reduce heat-induced aggregation of β-lactoglobulin, α-lactalbumin, and bovine serum albumin, suggesting some potential chaperone action (Kehoe and Foegeding, 2011). Daniloski et al. (2022b) recently showed that lower levels of soluble β-casein and non-denatured whey proteins were found in heated A1/A1 and A1/A2 milks compared to in A2/A2 milk. This may suggest that β-casein A2 showed a stronger molecular chaperone activity towards heat-induced aggregation of whey proteins than β-casein A1 (Daniloski et al., 2022b).

The phenotypes of κ-casein have been related to functionality and stability of heat-treated bovine milks and manufactured dairy products. In this regard, upon heat treatment of bovine milk, the B variant of κ-casein possessed some ability to stabilise β-lactoglobulin against heat-induced denaturation (Choi & Ng-Kwai-Hang, 2002), but was less effective stabiliser of the casein micelle compared to κ-casein A (Jensen, Holland, Poulsen and Larsen, 2012a). Compared to κ-caseins A/A and A/B, the greater heat stability of milk was correlated with κ-casein B/B at natural milk pH (6.6–6.8) (Robitaille, 1995). Milk containing κ-casein A/B showed a longer maximum in heat coagulation time compared to κ-casein A/A, and the composite B/B-A/B haplotype of κ-casein--β-lactoglobulin, was associated with more heat-stable milk compared to A/A-A/A of the same haplotype (McLean et al., 1987; Robitaille, 1995).

Table 3
The impact of various β-casein genetic variants on the technological properties of milk.

Sample type	Technological trait	Outcome	Reference
	Milk ingredients (caseinate)		
Milk samples (n = 2) - A1/A2 milk - A2/A2 milk Cow's breed: unknown.	Physicochemical properties of sodium caseinates: Visosity, internal structure and particle size of A1/A2 and A2/A2 sodium caseinates.	The study did not find any noticeable differences between the structural and interfacial properties of sodium caseinate obtained from A1/A2 and A2/A2 milks.	Hemar et al. (2021)
Milk samples (n = 3) - Australian Holstein A1/A1 (n = 1) - Australian Holstein A1/A2 (n = 1) - Australian Holstein A2/A2 (n = 1).	Structure of sodium caseinates was assessed with FTIR and NMR spectroscopies. Physicochemical and interfacial properties were evaluated by analysing adsorbed protein content, hydrophobicity, solubility, and emulsion stability of the samples.	The β-casein A2 in both, A1/A2 and A2/A2 sodium caseinates, appeared to be able to more rapidly reach the oil droplet surface. Sodium caseinates carrying β-casein A2 were more efficient as emulsifying agent, compared to sodium caseinates with β-casein A1.	Daniloski et al. (2022e)
	Milk coagulation and gelation		
Milk samples (n = 892) - Jersey (coagulation): good, n = 27; poor, n = 25; - Holstein-Friesian (coagulation): good, n = 26; poor, n = 18; none, n = 6).	Rennet (chymosin)-induced coagulation	Significantly lower contents of total protein, total casein, minerals (Ca, P, Mg), and κ-casein were identified in A2/A2 as part of poorly coagulating milks.	Jensen et al. (2012b)
Milk samples (n = 892) - Jersey (n = 24); - Holstein-Friesian (n = 24).		The high prevalence of the β-casein B in milk was related to good coagulation ability, whereas poorly coagulating milk was associated with β-casein A2 variant.	Jensen et al. (2012a)
Milk samples (n = 121) - Swedish Red breed (n = 75); - Swedish Holstein breed (n = 46).		The A2/A2 phenotype in milk was associated with poor and the A1/A2 genotype with good coagulating properties and higher firmness.	Hallén et al. (2007)
Milk samples (n = 1299) - Danish Holstein (n = 456); - Danish Jersey (n = 436); Swedish Red (n = 407).		Most pronounced effect was the negative influence of A2 and I β-caseins on milk coagulation compared with β-casein A1, which was essential for curd firming rate and rennet coagulation time.	Poulsen et al. (2013)
Milk samples (n = 888) - Danish Holstein (n = 455); - Danish Jersey (n = 433).		The possible association between β-casein F and noncoagulation milk still remains to be elucidated as it was not directly related to the relative β-casein content.	Poulsen et al. (2017)
Milk samples (n = 299) - Italian Holstein Friesian mixed; milk samples contained different amont of either A1, A2, and B varinats.		The β-casein A1 family, but especially β-casein B varinat showed shorter rennet coaulation time, curd-firming time, and firmer gel compared to β-casein A2.	Vigolo et al. (2022a)
Milk samples (n = 1133; 50 mL from each cow) - Italian Holstein Friesian mixed; milk samples contained different amont of either: 1. A1/A1, A1/A2 or A2/A2 β-caseins; 2. A/A, A/B, or B/B κ-caseins; 3. A/A and B/B β-lactoglobulins.		The β-casein A1/B presented the best performace with a lowest rennet coagulation time and higher curd firmness at 30 min, followed by β-casein A1/A1. The worst cheese-making ability was attributed to β-casein A2/A2.	Bisutti et al. (2022)
Milk samples (n = unknown; 20 L per each milk type) - Brazilian A1/A1 (n = unknown) - Brazilian A2/A2 (n = unknown).	Rennet (chymosin)-induced coagulation; *Petit Suisse* and *Minas Fresca* cheeses manufacturing	A2/A2 compared to A1/A1 cheeses were characterised as a softer and creamier cheesess, but it did not compromised its sensory acceptance.	Mendes et al. (2019)
Milk samples (n = 2; 30 L per each milk type) - Kiwi Cross A1/A1 (n = 1) - Kiwi Cross A2/A2 (n = 1).	Acid-induced gelation Yoghurt manufactoring	Gels produced from A2/A2 milk were more porous, contained thinner protein strands, and had lower gel strength compared to gels from A1/A1 milk.	NguyenSchwendel et al. (2018)
Milk samples (n = 114) - Australian Holstein A1/A1 (n = 5) - Australian Holstein A1/A2 (n = 5) - Australian Holstein A2/A2 (n = 5).	Acid-induced gelation	The associated findings of the more porous A2/A2 milk gel compared to A1/A1 and A1/A2 gels might be related to the increased content of random/PPII structures due to the fact that Pro possesses a tendency to create these conformations.	Daniloski et al. (2022d)
Milk samples (n = 2) - Ultra-high temperature A2 milk - Normal bovine milk (Purchase: JDcom direct-sale store of "Ren Yang Yi Tou Niu" brand)	Acid-induced gelation - Commercial fermentation bacteria (*Lactobacillus delbrueckii* subsp. *bulgaricus* 6047 and *Streptococcus thermophilus* 6038. Mixed with: - *Lactiplantibacillus plantarum* (MWLp-12) and *Limosilactobacillus fermentum* (MWLf-4) isolated from human milk.	Fermented A2 milk possessed smoother microstructure, better texture and rheological properties than the fermented normal milk. Supplementation with MWLp-12 and MWLf-4 would bring in various advantages on firmness, consistency, water holding capacity, and acidity of fermented milk compared with only using commercial fermentation bacteria.	Wang et al. (2022)
	Heat stability		
Milk samples (n = 114) - Australian Holstein A1/A1 (n = 5) - Australian Holstein A1/A2 (n = 5) - Australian Holstein A2/A2 (n = 5).	Heating treatment 1. 72 °C for 15 s; 2. 121 °C for 2.6 min; 3. 140 °C for 3 s	A1/A1 and A1/A2 milks were characterised with greater amounts of calcium and phosphorus, and a higher net negative zeta potential than A2/A2 milk. Histidine present in A1/A1 milk govern the formation of dehydroalanine. Intramolecular β-sheet, β-turn, and random coil were found in A1/A1, A1/A2, and A2/A2	Daniloski et al. (2022b)

(*continued on next page*)

Table 3 (*continued*)

Sample type	Technological trait	Outcome	Reference
		milks; increasing the temperature decreased the intramolecular β-sheets in all three milk types.	
		Emulsion and foam	
Milk samples (n = 2; 30 L per each milk type) - Kiwi Cross A1/A1 (n = 1) - Kiwi Cross A2/A2 (n = 1).	Foam formation and stability	The reconstituted A2/A2 milk showed significantly better foam formation but minimal differences were observed between foam stabilities compared to A1/A1 milk; A2/A2 milk might be a good natural ingredient for dairy products where milk foam is important.	NguyenSchwendel et al. (2018)
Milk samples (n = unknown) Crude casein: protein prepartion β-casein A1 and A2 genetic variants.		The β-casein A1 exhibited the best foaming properties; It would thus appear that the β-casein A1 spread more extensively at the interface and facilitated a faster build up of a coherent interfacial layer. That corresponded in a foam that was both more voluminous and had increased stability compared to the β-casein A2.	Ipsen and Otte (2004)
Milk samples (n = unknown) - Holstein - Jersey - Geman black - German white Whole casein extracted.	Emulsion formation and stability	Both B and A1 variants of β-casein had a higher surface load and higher content of ordered structure in the absorbed state than the β-casein A2, which postulated a correlation with the emulsion-stabilising properties. However, A2 variant of β-casein was able to more rapidly reach the oil droplet surface; consequently more efficient as emulsion forming agent.	Darewicz and Dziuba (2007)
Bovine β-casein containing mainly the genetic variants A1 and A2.		The hydrophobic teflon surface layer favored the transformation of the loop fragments of β-casein and into α-helix. Moreover, dephosphorylation increased the helix-forming propensity. Suggested relationship between surface load and emulsions stabilising properties.	Darewicz et al. (2000)

4.2. Acid gelation and rennet coagulation

The gel strength, curd formation, water holding capacity, and syneresis of milk during acid gelation and rennet coagulation are important functional attributes essential for the end-product functionality for a variety of products, including yogurt and cheese (Lucey, 2020). In the last decade, several studies showed that β-casein genetic variants in milk can be correlated to milk gelation and coagulation properties (Bisutti et al., 2022; Gustavsson et al., 2014; NguyenSchwendel et al., 2018; Poulsen et al., 2013; Poulsen et al., 2017; Vigolo, Franzoi, Penasa and De Marchi, 2022a). During acid-induced milk gelation of Kiwi breeds and rennet-induced milk coagulation of Scandinavian and Italian breeds, bovine β-casein A2/A2 as part of the casein haplotype was found to be the dominant in non-coagulating and poor-coagulating milk samples (Bisutti et al., 2022; NguyenSchwendel et al., 2018; Poulsen et al., 2013).

Very recently, Bisutti et al. (2022) and Vigolo et al. (2022a) found during rennet-induced coagulation that milk containing β-casein A2/A2 showed extended rennet coagulation time and lowered curd firmness compared with the other genetic variants, particularly with respect to the milk containing β-casein A1/A1, which was also observed in other studies (Frederiksen et al., 2011; Jensen et al., 2012a, 2012b; Kumar et al., 2018; Poulsen et al., 2013, 2017). Mendes et al. (2019) reported that a longer time was needed for the rennet coagulation of A2/A2 milk, during the *Petit Suisse* cheese processing. The gel used for manufacturing the cheese was also more porous, contained thinner protein strands and showed low strength. As a result of that, the cheese was softer, creamier, and possessed different sensory characteristics. However, those attributes did not result in A2/A2 cheese samples to be unacceptable by the panellists (Mendes et al., 2019).

Moreover, NguyenSchwendel et al. (2018) showed that the storage modulus was significantly lower for acid-induced gels from A2/A2 milk compared to A1/A1 milk, and gels also had a more porous microstructure, thinner protein strands, and lower gel strength. This suggested that the acid-induced gels and yoghurt from A2/A2 milk were more prone to breakage and deformation by external mechanical powers (NguyenSchwendel et al., 2018). Similarly, observing the rheological and the structural characteristics of acid-induced A1/A1, A1/A2, and A2/A2 gels, Daniloski, McCarthy, Gazi, and Vasiljevic (2022d) determined that the firmer gels obtained from A1/A1 and A1/A2 milks possessed a greater storage modulus. On the contrary, Wang et al. (2022) concluded that acidified and fermented A2/A2 milk had smoother microstructure, better texture, and rheological properties than the fermented A1/A2 milk. Nevertheless, the authors neither stated the amount of proteins (ratio of caseins and whey proteins) in both milks, nor they declared the content of milk's minerals, both of which are crucial for firmness and structure of milk gels (Lucey, 2020).

Upon the gel creation, the system relies on re-arrangement of the bonds among individual caseins creating the original casein micelles (Lucey, 2020). Therefore, an improved gel firmness is rather associated with a higher number of such bonds (Lucey, 2002; Lucey et al., 2000; Van Vliet, Van Dijk, Zoon and Walstra, 1991). Namely, the rearrangements of the casein particles into a more compact structure would increase the number of bonds, which could lead to a gradual formation of more protein-protein bonds at each junction between the casein particles, resulting in firmer gels and decreased total free energy of the system (Daniloski et al., 2022d; Lucey et al., 2022; NguyenSchwendel et al., 2018). The reason behind this phenomena can be related to the difference in κ-casein contents in A1/A1, A1/A2, and A2/A2 gel types (Daniloski et al., 2022d; NguyenSchwendel et al., 2018; Poulsen et al., 2013). On that account, lower amount of κ-casein indeed translates into fewer interactions, at least at the surface of the casein micelles during coagulation (Lucey, 2020); milks comprised of β-casein A1 contained more κ-casein, that theoretically led to a much larger number of particles and higher surface area (Daniloski et al., 2022d; NguyenSchwendel et al., 2018). Therefore, in A2/A2 milk a lower number of interactions were created, compared to A1/A1 and A1/A2 milks and therefore a softer gel (Daniloski et al., 2022d).

The total and ionic calcium contents were found to greatly influence the acid-induced gelation and rennet-induced coagulation of bovine milk (Hallén et al., 2007; Poulsen et al., 2013; Poulsen and Larsen, 2021). In this regard, milk samples carrying β-casein A1 contained higher calcium amount, especially ionic calcium in A1/A2 milk compared to A2/A2 milk and smaller casein micelle sizes (Daniloski et al., 2022d; Day et al., 2015; Jensen et al., 2012a; Poulsen et al., 2013). Almost a decade ago, Gustavsson et al. (2014) revealed that higher calcium content in milk was related to smaller casein micelle size and improved rennet-induced gelation properties.

Compared to other caseins, the genetic variants of κ-casein have been most discussed and related to acid gelation and rennet coagulation of bovine milk (Bijl et al., 2014a; Bonfatti et al., 2010; Poulsen and Larsen, 2021). Different studies have found that the genetic polymorphisms of κ-casein might substantially affect the rennet-induced coagulation characteristics; milks containing κ-casein B variant were represented with a higher amount of κ-casein and smaller casein micelles, decreased curd firming time, and increased whey protein expulsion compared with milks carrying κ-casein A (Bisutti et al., 2022; Gambra et al., 2013). Similarly, Poulsen et al. (2013) and Day et al. (2015) suggested that lower levels of total κ-casein and the presence of κ-casein A, were associated with poor rennet-induced coagulation of milk and greater casein micelle size. In contrast, Ketto et al. (2017) found that the highest levels of micellar κ-casein and a high prevalence of κ-casein A variant were connected with milks possessing good acid-induced coagulation ability, firmer gels, and smaller casein micelles, also identified in the study of Daniloski et al. (2022d).

An important role during and upon milk processing is played by αs_1-casein and its genetic variants and phenotypes. When comparing the genetic variants of this protein, αs_1-casein C possesses a smaller net charge compared to αs_1-casein B. As a consequence, C variant of αs_1-casein has greater association constants and ultimately stronger self-association, which contributes to a firmer curd in cheese making (Fox et al., 2015; Sadler et al., 1968; Schmidt, 1970). Frederiksen et al. (2011) found that an increased content of αs_1-casein B in milk was considered as a main differentiating feature for the occurrence of the non-coagulating milks. Additionally, Poulsen et al. (2013) determined that αs_1-casein C improved milk coagulation. Even though, B and C are generally most discussed αs_1-casein variants, variant A is the most different compared to other variants. Its residues f14 - 26 are deleted, thus it is less hydrophobic, thus the curd formed during cheese making from milk with αs_1-casein A variant was found to be softer (Creamer et al., 1982; Sadler et al., 1968). On the contrary, the genetic variants of αs_2-casein did not show a substantial effect during and upon milk processing (Cipolat-Gotet et al. 2018), simply as a matter that it is hardly to see any genetic variation in this protein. A number of years ago, Ketto et al. (2017) found that the content of αs_2-casein was negatively correlated with properties of acid-induced gels. Furthermore, these authors also observed that due to the increased concentration of αs_2-casein in milk, and its correlation to κ-casein B, was a reason for the poor acid gelation of milk (Ketto et al., 2019).

The composite genotype of αs_1-β-κ-casein was found to have a stronger relationship with acid gelation and rennet coagulation properties than only a single protein phenotype (Gai et al., 2021). In this regard, an improved acid gel firming rate and firmness at 30 and 60 min, and shorter gelation times were correlated to B/B-A2/A2-A/A haplotypes compared to the other proteins' genotypes (Ketto et al., 2017). In contrast, Jensen et al. (2012a) stated that the same composite haplotype was predominant in poorly coagulating milks. The B/B-A2/A2-A/A (αs_1-β-κ-casein) haplotype was positively associated with percentages of fat and protein in Holstein cows, Brown Swiss cows (Boettcher et al., 2004), Finnish Ayrshire cows (Ikonen et al., 2001), and Italian Reggiana cows (Caroli et al., 2004), but negatively associated with milk yield (Boettcher et al., 2004). Interestingly, the composite β-κ-casein genotype, namely A1/A1-A/B, A1/A2-A/B, and A2/A2-A/B were associated with better firmness and shorter coagulation time (Comin et al., 2008). Frederiksen et al. (2011) actually related the composite A/B-A1/A2 (β-κ-casein) haplotype, considered as a sufficient factor for good milk coagulation properties, with a higher content of κ-casein in the gels. Thus, genetic selection of dairy cows for milk with good acid gelation or rennet coagulation abilities, should be highly considered, since they can potentially lead to an improvement in yoghurt and cheese production (Poulsen and Larsen, 2021).

4.3. Interfacial properties

The formation and stability of emulsions and foams are strongly dependent on the interactions between air and liquid (interfacial layer), and the surfactants adsorbed to this surface (Darewicz et al., 2000). The faster adsorption of surfactants on the interfacial layer and their greater capacity to minimise surface tension are crucial for the development of emulsions and foams (NguyenSchwendel et al., 2018). The fact that β-casein is a major constituent of casein micelles and is also commonly used as a foaming or emulsifying agent means that its association behaviour is of importance in the food industry (Chen et al., 2018; Neill and Jingsi, 2021). When comparing emulsion and foam formation and stabilisation, A2/A2 milk showed better foam formation than A1/A1 milk, however both milk types indicated similar foam stability (NguyenSchwendel et al., 2018). In addition, Darewicz and Dziuba (2007) revealed that the superior emulsion properties of β-casein A2 compared to β-casein A1 could be attributed to improved solubility of this protein, its faster migration and adsorption to the interfacial layer, driven mainly by hydrophobic interactions between its C-terminal tail and the surface, and less ordered structure (Darewicz and Dziuba, 2007; Raynes et al., 2015). On the contrary, almost two decades ago, Ipsen and Otte (2004) found that β-casein A1 possessed greater foaming properties (more voluminous foam with an increased stability) than β-casein A2, which appeared in accordance with their results from the measurements of surface pressure and interfacial rheology. Namely, the authors explained that β-casein A1 spread more extensively at the interface and facilitated a faster build up of a coherent interfacial layer (Ipsen and Otte, 2004). Thus, the additional Pro^{67} (found to form a hinge between the polar C-terminal and the primarily hydrophobic N-terminal region) provided for a less extensive part of the hydrophobic domain of β-casein A2 to be adsorbed on the interfacial layer, thus explaining why the β-casein A2 was less space filling compared to β-casein A1 (Ipsen and Otte, 2004).

Hemar et al. (2021) reported no noticeable differences between the physicochemical and interfacial properties of sodium caseinate dispersions obtained from A1/A2 and A2/A2 milks. However, Daniloski, McCarthy, Auldist, and Vasiljevic (2022e) observed that the sodium caseinates carrying β-casein A2 were more efficient as emulsifying agents than the sodium caseinate with β-casein A1. The authors explained that the presence of α-helixes was the main driver for the different protein structure of A1/A1, A1/A2, and A2/A2 sodium caseinates (Daniloski et al., 2022e). The α-helical conformational motifs were found predominately in A1/A1, A1/A2 milks (Daniloski et al., 2022c) and β-casein A1 (Darewicz and Dziuba, 2007). These conformations display a tight structure with no cavities, which may play a role in driving different functionalities. The superior emulsion and foam forming capabilities indicate that A2/A2 milk can potentially be a good natural ingredient for dairy products where milk foam is essential, such as ice cream, whipped cream, mousses and cappuccino's milk with better alternative quality and enhanced sensory attributes.

Although the impact of the genetic variants of all 4 caseins on physicochemical and functional properties of dairy products have been extensively studied and reviewed (Gai et al., 2021; Mendes et al., 2019; NguyenSchwendel et al., 2018; Poulsen and Larsen, 2021), there is no consensus on the structure of casein micelle governed by the major milk proteins and their genetic variants. Therefore, greater scale and extensive data studies that would contain different levels of αs_1-, αs_2-, β-, and κ-caseins including their polymorphic variants, degree of κ-casein glycosylation, composite αs_1-αs_2-β-κ-casein variants are needed to further elaborate on the impact of these genetic variants on both milk and micellar casein. These genetic variants, as stated above, may influence various interactions in the casein micelle and its size, mineral levels, protein conformation, and most importantly, the functionality of milk and dairy products (Bijl et al., 2014a, 2020; Daniloski et al., 2022a; Ketto et al., 2017; Vallas et al., 2012).

5. Conclusion

It was hypothesised in this review that differences in β-casein genetic variants can have important implications on certain milk product characteristics. Based on the studies performed over the last number of years, it is obvious that β-casein A1 and A2 phenotypes are quite different, not only by their composition but based on their functionality and behaviour to environmental factors. Despite the importance of these two β-casein variants, most studies have focused on the association between κ-casein genetic variants and the physio-chemical and functional properties of bovine milk. Milk samples carrying β-casein A2 possess a larger average micelle size than samples carrying β-casein A1, simply as a result of less κ-casein present on the micelle surface. In contrast, limited research has been performed on αs_2-casein protein fractions because it is difficult to identify genetic variation in this protein. As far as technological traits are concerned, milk comprised of β-casein A2 has usually been associated with poorer acid gelation and rennet coagulation properties, but superior emulsion and foam formation capabilities. For instance, milk with β-casein A2 is less suitable for cheese- or yoghurt making, however, the weak gel it produces could potentially be responsible for its proposed easier digestibility (Milan et al., 2020), which might be advantageous for certain applications. On the other hand, isolated and purified β-casein A2 produces smaller β-casein micelles and creates poorer and less stable foams compared to β-casein A1, which explains the complexity of the milk system and the importance to define the factors that influence these variations. Therefore, the mechanism of producing dairy products with the same properties using either β-casein A1 or A2, or the factors, which influence gelation, emulsion and foam stability, are not yet fully understood. Given the significant role that milk composition plays in functional properties, further functionality testing correlated to β-casein phenotype is required to fully identify how a single amino acid substitution can have such a significant impact on milk functionality.

CRediT authorship contribution statement

Davor Daniloski: conceived the study and research question, designed, Writing – original draft, Conceptualization, reviewed, edited the manuscript, designed the tables and the figures, Methodology, Formal analysis, Investigation. **Noel A. McCarthy:** Formal analysis, provided critical feedback and analysis, Funding acquisition, reviewed and edited the manuscript, supervised the study. **Thom Huppertz:** provided critical feedback, reviewed and edited the manuscript, All authors have contributed to the manuscript and reviewed the final version. **Todor Vasiljevic:** Formal analysis, provided critical feedback and analysis, Funding acquisition, reviewed and edited the manuscript, supervised the study.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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