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Frequencies Evaluation of β-Casein Gene Polymorphisms in Dairy Cows Reared in Central Italy

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Simple Summary: Bovine milk contains several β-casein variants, with the A1 and A2 variants occurring most frequently. The presence of some variants, such as A1, B, and C, is considered a risk factor for disease in humans who consume milk. These variants are probably involved in intolerance to milk and some human diseases due to the production of a bioactive peptide with opioid activity during digestion, β-casomorphin 7 (BCM-7). In contrast, the A2 variant is not involved in pathogenetic mechanisms; thus, its presence in milk is a desirable feature. The difference between the A1 and A2 variants is a mutation at position 67 of the β-casein gene (*CSN2*), which causes an amino acid to change from histidine (in the A1, B, and C variants) to proline (in the A2 variant). To select dairy cows on the basis of the presence of the β-casein variant A2, allele frequencies of *CSN2* variants were evaluated in Italian dairy cows reared in central Italy. The results of this study may help with the selection of animals with the β-casein gene variant A2 to produce a more digestible milk that only contains the β-casein variant A2.

Abstract: The majority of proteins in cow's milk are caseins, which occur in four groups (α-s1, α-s2, β, and k) encoded by different genes (*CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3*, respectively). In this study, we focused on the β-casein allele variants A1 and A2 due to their influence on milk's technological characteristics and human health. Digestion of the β-casein variant A1 leads to the formation of β-casomorphin 7 (BCM-7), a bioactive peptide that has been suggested to be a possible cause of various human diseases and associated with low milk digestibility. The potential negative role of the β-casein variant A1 in human health has stimulated the planning of cattle breeding programs based on genetic selection to increase the frequency of the A2 variant, which is associated with increased milk digestibility. The aim of this work was to evaluate the frequencies of the different β-casein variants in Italian Holstein Friesian dairy cows from cattle farms located in central Italy to select a population of A2 homozygous animals. β-casein genotypes were identified by evaluating the presence of single nucleotide polymorphisms (SNPs) of the *CSN2* gene using PCR and sequencing analysis. The frequency of the desirable β-casein variant A2 in the studied bovine population was 0.61. The frequency of the undesirable A1 variant in the studied bovine population was 0.30. The frequency of the A2 allele was higher than expected for the breed; therefore, genetic selection for the A2 variant in these animals could be achieved in a fairly short time using A2 homozygous bulls.

Keywords: β-casein; polymorphisms; bovine; milk

1. Introduction

Cow's milk is considered an important source of food for humans and plays a fundamental role in human health due to its valuable protein, fat-soluble vitamins, and mineral salt content, particularly calcium, which counteracts bone fragility and the risk of osteoporosis. Bovine milk is composed of 87% water, 3.68% lipids, 3.51% proteins, 4.98% lactose, and 0.74% microelements [1]. Of the proteins, the most important are caseins, which represent 82% of the total protein content [2]. Caseins are subdivided into four groups, αs1, αs2, β, and k, respectively encoded by the *CSN1S1*, *CSN1S2*, *CSN2*, and *CSN3* genes located on chromosome 6 [3]. The main function of caseins is the transport of calcium phosphate [4]. However, β-casein, which accounts for 36% of the total protein content, is also important for curd formation and determining the surface properties of micelles, which are useful features for cheese production [5,6]. Dairy cattle have 12 β-casein variants (A1, A2, A3, B, C, D, E, F, H1, H2, I, and G); however, only seven of these (A1, A2, A3, B, C, I, and E) have been detected in European cattle breeds (Table 1) [7,8]. The A2 variant is considered the oldest variant, from which the others originated via mutation. The most common variants are A1 and A2; the B variant is less common [9]. The I variant is usually detected at low frequencies [8], the A3 and C variants are rare [9], the E variant is detectable only in the Italian Piedmontese breed [10], and the F variant has been detected in the Emilia Romagna region (Northern Italy) at a very low frequency (0.006) [8]. The difference between the A1 and A2 variants is due to a mutation in position 67, which causes an amino acid to change from histidine (in the A1, B, and C variants) to proline (in the A2, A3, E, and I variants). The allele variants A1, B, and C differ from each other with respect to an amino acid in position 122 (serine in A1 and C, arginine in B) and one in position 37 (glutamic acid in the A1 and B variants and lysine in the C variant). The I and A3 variants were derived from a mutation of the A2 variant; specifically, the I variant has a leucine instead of a methionine in position 93 and the A3 variant has a glutamine instead of a histidine in position 106. To summarize, the A1 and A3 variants originated from the A2 variant; subsequently, the I variant was derived from the A3 variant, and the B and C variants were derived from the A1 variant. Each variant exhibits the same behavior as the variants from which it arose in terms of β-casomorphin 7 (BCM-7) formation [11] (Table 1).

Table 1. The change in the amino acid sequence of β-casein variants (in bold: amino acid variations).

βcasein Variant	Amino Acid Position								
	36	**37**	**67**	**72**	**88**	**93**	**106**	**122**	**138**
A2 *	Glu (E)	Glu (E)	Pro (P)	Gln (Q)	Leu (L)	Met (M)	His (H)	Ser (S)	Pro (P)
A1 *	Glu (E)	Glu (E)	His (H)	Gln (Q)	Leu (L)	Met (M)	His (H)	Ser (S)	Pro (P)
A3 *	Glu (E)	Glu (E)	Pro (P)	Gln (Q)	Leu (L)	Met (M)	Gln (Q)	Ser (S)	Pro (P)
B *	Glu (E)	Glu (E)	His (H)	Gln (Q)	Leu (L)	Met (M)	His (H)	Arg (R)	Pro (P)
C *	Glu (E)	Lys (K)	His (H)	Gln (Q)	Leu (L)	Met (M)	His (H)	Ser (S)	Pro (P)
E *	Lys (K)	Glu (E)	Pro (P)	Gln (Q)	Leu (L)	Met (M)	His (H)	Ser (S)	Pro (P)
I *	Glu (E)	Glu (E)	Pro (P)	Gln (Q)	Leu (L)	Leu (L)	His (H)	Ser (S)	Pro (P)
D	Glu (E)	Glu (E)	Pro (P)	Gln (Q)	Leu (L)	Met (M)	His (H)	Ser (S)	Pro (P)
F	Glu (E)	Glu (E)	His (H)	Gln (Q)	Leu (L)	Met (M)	His (H)	Ser (S)	Leu (L)
G	Glu (E)	Glu (E)	His (H)	Gln (Q)	Leu (L)	Met (M)	His (H)	Leu (L)	Pro (P)
H1	Glu (E)	Glu (E)	Pro (P)	Gln (Q)	Ile (I)	Met (M)	His (H)	Ser (S)	Pro (P)
H2	Glu (E)	Glu (E)	Pro (P)	Glu (E)	Leu (L)	Leu (L)	His (H)	Ser (S)	Glu (E)

Arg: arginine; Gln: glutamine; Glu: glutamic acid; His: histidine; Ile: isoleucine; Leu: leucine; Lys: lysine; Met: methionine; Pro: proline; Ser: serine; * Allele variants detected in European cattle breeds.

We paid particular attention to the allele variants A1 and A2 due to their influence on milk's technological characteristics and implications for human health. The A1 variant improves curd consistency, milk coagulation, and micelle size, but results in lower milk digestibility compared with the A2 variant [12]. The β-casein variants A1 and A2 are differently processed; digestive enzymes are able to perform a proteolytic cleavage of the β-casein chain when a histidine is present at position 67

to form a peptide of seven amino acids named β-casomorphine 7 (BCM-7). Evidence showed that BCM-7 has strong opioid activity with an additional oxidizing effect and can interact with endogenous opioid systems at the gastrointestinal wall in both newborns and adults [13]. According to Deth et al. (14), consumption of milk containing the A2 variant increases the natural production of glutathione (GSH), an antioxidant that is widely recognized for its association with a series of health benefits. The study showed that the consumption of milk containing only the A2 variant increases the GSH level in the blood to approximately twice the level associated with the consumption of milk containing both the β-casein variants A1 and A2 [14]. Therefore, the allele variants that are considered risk factors for human disease are those that contain a histidine in position 67 (A1, B, and C variants), whereas those that contain a proline, i.e., the A3 and I variants, behave like the A2 variant even though they contain other single-nucleotide polymorphisms (SNPs) in position 106 (histidine to glutamine) and position 93 (methionine to leucine), respectively. Some studies reported a correlation between the consumption of milk containing the β-casein variant A1 and heart disease, sudden infant death syndrome, and aggravation of symptoms associated with schizophrenia, autism, type 1 diabetes, and milk intolerance [15–20].

In contrast, a scientific report of the European Food Safety Authority (EFSA) in 2009 concluded that no cause–effect relationship exists between the consumption of milk containing the A1 variant and the etiology of the abovementioned diseases and that further investigations are necessary [13]. More recently, a scientific report highlighted a lack of a correlation between adverse human health effects and consumption of the β-casein variant A1 in comparison to the β-casein variant A2 [21].

Therefore, the discussion on the adverse human health effects of the β-casein variant A1 remains open. However, milk obtained from cows with the A2/A2 genotype seems to be more digestible than milk obtained from cows with the A1 genotype in terms of an increase in gastrointestinal transit [12]. A2 cow's milk, or milk without the A1 variant, is commercially available in a number of countries, including Australia, the United Kingdom, the United States, New Zealand, and the Netherlands, and is widely recommended for people who are milk-intolerant. Formula for newborns that contains the β-casein variant A2 is now sold in China and Australia and is promoted commercially as being more gentle on an infant's digestive system [12]. The potential negative role of the β-casein variant A1 in human health has stimulated the planning of cattle breeding programs based on the selection of β-casein gene polymorphisms.

The purpose of this work was to evaluate the frequency of occurrence of different β-casein variants in dairy cows from cattle farms in central Italy that supply milk to an important drinking-milk-producing plant, with the aim of selecting a population of A2 homozygous animals and commercializing an A2 milk that only contains the β-casein variant A2.

2. Materials and Methods

A total of 1629 whole blood samples were collected from Italian Holstein Friesian cows from 17 farms located in central Italy. The samples were collected in tubes containing Ethylenediaminetetra-acetic acid (EDTA) as an anticoagulant and stored at −20 °C until analysis. The samples were taken in a single withdrawal, at the same time of the mandatory periodic tests required by Italian National Health Programs and during farmer's voluntary health controls. Genomic DNA was extracted using a High Pure PCR Template Preparation Kit (Roche Life Science, Mannheim, Germany) according to the manufacturer's instructions. Specific primers for the exon 6 and 7 portions of the *CSN2* gene were selected from the literature [22] and modified using Primer Express®v3.0.1 software (Applied Biosystems; Thermo Fisher Scientific Inc., Waltham, MA, USA) to increase the efficiency of the assay (Table 2).

Table 2. Primer sequences (For, forward primer; Rev, reverse primer).

Target Gene	Target Sequence	Primer Sequences	Amplification Product (bp)	Reference
CSN2	Exon 6	For CATCAATAAGGTAAAACCCCTCATATT Rev TTGTCAAAGTTTTTATTTCTTGCACTG	274	This study
	Exon 7	For TTTCCAGGATGAACTCCAGGAT Rev CATCAGAAGTTAAACAGGCACAGTTAG	547	

The PCR was set up in a total reaction volume of 50 µL as follows: for exon 6, 1.5 X GoTaq®Flexi Buffer, 1 mM $MgCl_2$, 2.5 U of GoTaq®G2 Flexi DNA Polymerase (Promega Corporation, Madison, WI, USA), 200 µM dNTPs (GE Healthcare, Buckinghamshire, England), 0.3 µM of each primer (Invitrogen; Thermo Fisher Scientific Inc., Waltham, MA, USA) (Table 2), and 4 µL of the extracted DNA template; for exon 7, 1 X GoTaq®Flexi Buffer, 1.5 mM $MgCl_2$, 2.5 U of GoTaq®G2 Flexi DNA Polymerase (Promega Corporation) 200 µM dNTPs (GE Healthcare), 0.2 µM of each primer (Invitrogen; Thermo Fisher Scientific Inc.) (Table 2) and 2 µL of the extracted DNA template.

PCR amplifications for both protocols were conducted on a Mastercycler Ep Gradient S (Eppendorf AG, Hamburg, Germany) with the following thermal cycling profile: an initial denaturation at 95 °C for 10 min, followed by 35 cycles at 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a final elongation step at 72 °C for 7 min. Amplification products were analyzed by electrophoresis on 2% agarose gel containing Midori Green Advanced DNA Stain (Nippon Genetics Europe GmbH, Düren, Germany) and purified with a QIAquick®PCR Purification Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Sequencing reactions were completed for both strands using a BrilliantDye^{TM} Terminator Cycle Sequencing Kit v3.1 (NimaGen BV, Nijmegen, The Netherlands) according to the manufacturer's instructions with the same primers that were used for PCR amplification. Sequencing reactions were analyzed in a 3500 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific Inc.). Sequences were then aligned to the bovine β-casein gene (Accession number X14711.1) with the ClustalW tool of the BioEdit v7.2.5 software [23]. Electropherograms were checked at each investigated mutation point to identify heterozygous peaks indicating the presence of both alleles. The polymorphisms at positions 36 and 37 of exon 6 and positions 67, 72, 88, 93, 106, 122, and 138 of exon 7 were analyzed to discriminate between the different β-casein variants.

Allele and genotype frequencies were directly calculated dividing the number of copies of each allele and genotype by the total alleles and by the total individuals, respectively. The Hardy-Weinberg (HW) equilibrium was verified using Chi-square test ($p < 0.05$) by Genalex 6.5 software [24,25].

3. Results and Discussion

The main goal of our study was to evaluate the frequency of occurrence of the β-casein *CSN2* gene alleles in dairy cattle of the Italian Holstein Friesian breed reared in central Italy. We paid particular attention to the frequencies of the A2 variant and its related genotypes given its association with health benefits. Although the Italian Holstein Friesian breed is not among those breeds characterized by the highest frequency of the A2/A2 genotype, it has a sufficiently high frequency to allow for effective genetic selection of this feature [26]. Therefore, the estimation of allele and genotype frequencies is important for planning an efficacious genetic selection program for these animals with the final goal of producing A2 cow's milk. Our results show the distribution of the allele and genotype frequencies of the different *CSN2* gene variants in the population under study. Considering the polymorphic sites evaluated in this study, all the alleles conformed to the HW equilibrium and no deviation was detected.

The sequencing analysis performed in both directions on the PCR products showed that, of the nine considered polymorphic sites spanning the regions of exons 6 and 7 of the *CSN2* gene, a total of five were polymorphic: E37K, P67H, M93L, H106Q, and S122R (Table 1). They produced six β-casein variants (A1, A2, A3, B, C, and I) in 13 genotypes, 3 of which were homozygous (A1/A1, A2/A2,

and B/B) and 10 of which were heterozygous (A1/A2, A1/A3, A1/B, A1/C, A1/I, A2/A3, A2/B, A2/I, A3/B, and B/I) as shown in Table 3.

Table 3. The allele and genotype frequencies (%) in the examined animals (the data are sorted by decreasing allele and genotype frequency).

Allele	Allele Frequency (%)	Genotype	Genotype Frequency (%)
A2	60.65	A2/A2	36.96
A1	30.39	A1/A2	35.79
B	5.68	A1/A1	9.88
I	3.10	A2/B	7.55
A3	0.15	A2/I	3.93
C	0.03	A1/B	3.07
		A1/I	2.03
		B/I	0.25
		B/B	0.18
		A2/A3	0.12
		A3/B	0.12
		A1/A3	0.06
		A1/C	0.06

The A2 allele was the most commonly found, with a frequency of 60.65%, followed by the A1 allele with a frequency of 30.39%, the B allele at 5.68%, the I allele at 3.10%, the A3 allele at 0.15%, and the C allele with a frequency of 0.03%. These results agree with those reported from a study conducted in cattle of the same breed from farms located in the Emilia Romagna region (Northern Italy) by Massella et al. (8), who found a high frequency of the A2 allele (54.6%), followed by the A1 (37.1%), B (5.0%), I (2.7%), and F (0.6%) alleles.

In our samples, we did not observe the F variant, which is very rare and for which few data are available about its frequency in milk-producing breeds [8]. We did not find the E variant, which, to date, has only been observed in the Piemontese breed [27] or the D, G, H1, and H2 variants, which have never been found in European breeds [7]. These results are also consistent with those reported by Kaminsky et al. (16), which showed that the A1 and A2 variants are the most common in the Holstein Friesian breed.

Regarding the genotype, the homozygous genotype A2/A2 was the most common, with a frequency of 36.96%, followed by the heterozygous genotypes A1/A2 and A1/A1 with frequencies of 35.79% and 9.88%, respectively. The remaining genotypes showed lower frequencies that ranged from 7.55% (A2/B) to 0.06% (A1/A3–A1/C) (Table 3). A total of 2.46% of the examined animals were found to carry the A3 and I variants (A1/I, B/I, A3/B, and A1/A3), while 4% were A2/A3 and A2/I. These individuals could be considered as A2 heterozygous and homozygous, respectively.

A breed is considered suitable for selection of the A2 allele if its frequency is close to 50%. In this case, given the Mendelian inheritance of the trait and presuming that the population is in Hardy-Weinberg equilibrium, in the herd we would expect to find 25% homozygous A1/A1 animals, 50% A1/A2 heterozygotes, and 25% A2/A2 homozygous individuals, and that the produced bulk milk contains 50% β-casein variant A1 and 50% β-casein variant A2 [26]. To change the herd's composition with the aim of producing a milk containing only the β-casein variant A2, A2/A2 bulls must be used for the artificial insemination of A2 carrier cows. The Italian Holstein Friesian breed provides an appropriate genetic background for increasing the frequency of this favorable allele through appropriate breeding.

In the farms analyzed, we found an A2 allele frequency of 60.65%, slightly greater than those reported in the recent literature (8). The presence of other variants similar to A2 (A3 and I), relative to BMC-7 production, accounting for another 3.25%, increased the frequency of favorable variants to 64%. Consequently, we found high frequencies of A2/A2 individuals (37%) and A2 heterozygous carriers (47.4%). As the A3 and I variants behave similarly to the A2 variant, the percentage of A2/A2-like

genotypes (A2/A2, A2/A3, and A2/I) was 41%, increasing the overall percentage of genotypes with at least one A2-like allele to 87%.

4. Conclusions

In this study, we focused on the β-casein variants A1 and A2 due to their influence on milk's technological characteristics and their implications for human health. The β-casein variant A2 is desirable in milk because it increases the digestibility of milk. A2 cow's milk can be immediately produced in a herd if the cows present on farms are screened to identify animals with the A2/A2 genotype and the milk of these individuals is separated from that of the others. The identification of A2 carrier cows then allows the planning of animal couplings with the aim of generating A2 homozygous progenies. In conclusion, genotyping by sequencing is a fast and reliable method for monitoring the allele frequencies of β-casein variants in a population of dairy cows where selection of the β-casein variant A2 is the aim.

Marker-assisted selection (MAS) should be applied to align Italian dairy breeds with those of other countries that have already invested in A2 cow's milk production. In many countries, A2 cow's milk has already been commercialized as a product with beneficial properties with a resulting economic gain.

These data will allow us to manage animal couplings to increase the frequency of the favorable A2 allele, simultaneously decrease the frequency of undesirable alleles, and finally create herds of A2/A2 animals from the population of dairy cow breeds oriented to A2 cow's milk production.

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Marker-assisted selection of dairy cows for β-casein gene A2 variant

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PAPER

Abstract

Many studies highlighted potential associations of β-casein A1 with specific human diseases and a minor digestibility of milk, due to the bioactive peptide β-casomorphin 7 (BCM-7) release during digestion. Conversely, the ancestral β-casein A2 variant seems to be a favorable trait because it is not associated with BMC-7 release. The aim of this work was to evaluate frequencies of β-casein variants in offspring of previously genotyped cows inseminated with A2 homozygous semen. The frequency of the A2/A2 animals has almost doubled from 37 to 69%. These are encouraging results with the perspective of reaching the goal of producing A2 milk.

Keywords: β-casein; bovine; marker-assisted selection (MAS); milk; polymorphisms; variants

Introduction

Marker-Assisted Selection (MAS) is a methodology that allows for the selection of important genetic animal traits in the population of interest by exploiting the genetic information at specific markers. Advancement in genomics made easier the identification of markers which could be ultimately utilized in MAS. In particular, the Genome Wide Association Study (GWAS) approach, initially used in human genetics research to associate genetic variations with particular diseases, is of special interest (Raina *et al*., 2020). The method is based on scanning the genome of many different individuals for genetic markers that can be used to predict the presence of a disease in the population under study. More recently, GWAS has been applied to the field of domestic animal breeding and genetics, and many genetic markers affecting important economical traits have been described (Du *et al*., 2021; Sharma *et al*., 2015).

Cattle breeding programs, consisting of marker-assisted selection (MAS), has been applied to the selection of dairy cows for the presence of β-casein A2 variant, known to confer better digestibility to milk (Duarte-Vazquez *et al*., 2017; Kaminski *et al*., 2007; Park *et al*., 2021).

Milk and dairy products are among the main components of the food tradition of many countries and they play a fundamental role in human health due to their valuable nutritional properties. In spite of various sources of milk being available on the market, bovine milk represents the most consumed variety of milk worldwide (Faye and Konuspayeva, 2012). It contains essential nutrients such as proteins with high biological value, lipids, carbohydrates (mainly lactose), minerals (calcium, phosphorus, zinc, and magnesium), and vitamins (i.e., B2, B12, D, and A) (Jenness *et al*., 1979; Muehlhoff *et al*., 2013). Regarding milk protein fraction, it is composed by soluble

proteins, also known as whey proteins, and by insoluble proteins, that are caseins, which represent about 80% of the total bovine milk proteins. These are subdivided into four groups, αs1, αs2, β, and k, encoded respectively by the *CSN1S1*, *CSN1S2*, *CSN2*, and *CSN*3 genes, all located on chromosome 6 (Rijnkels, 2002). In spite of high milk consumption all over the world, some people experience digestive disorders following the intake of milk and dairy products, because of lactose malabsorption or digestive difficulties due to other dairy components such as β-casein, which represents about 36% of milk protein content (Milan *et al*., 2020). Specifically, some studies have shown a correlation between human health and some β-casein variants (Kay *et al*., 2021; Thiruvengadam *et al*., 2021). Indeed, *Bos taurus CSN2* gene harbors many nucleotide substitutions leading to the formation of 12 protein variants (A1, A2, A3, B, C, D, E, F, G, H1, H2, and I), seven of which (A1, A2, A3, B, C, I, and E) have been identified mainly in European cattle breeds (Barroso *et al*., 1999; Daniloski *et al*., 2021; Hohmann *et al*., 2021; Massella *et al*., 2017) (Table 1).

Among the dairy cattle breeds, A1 and the ancestral A2 variants are the most common while B and I variants are generally less frequent with some variability depending on the breed (Farrel *et al*., 2004). Other variants, such as A3 and C, are rarely found (Farrel *et al*., 2004; Massella *et al*., 2017) and others are related to specific breeds or geographic areas, as it happens for the E and F variants that have been found with a very low frequency, only in the Italian Piedmontese breed and in animals reared in the Emilia-Romagna region (northern Italy), respectively (Massella *et al*., 2017; Voglino *et al*., 1972).

In the last two decades, much attention has been paid to the A1 and A2 β-casein content of milk, due to their suggested role in human health. A1 and A2 variants differ from each other at the gene level for a point mutation, which causes an amino acid change. Particularly, at position 67 of the protein chain, a histidine in the A1 variant is replaced by a proline in the A2 variant. Because of this difference in the protein sequence, the β-casein variants A1 and A2 are differently processed during digestion. Actually, digestive enzymes perform a proteolytic cleavage at position 67 of the β-casein chain only when a histidine is present generating a seven amino acids peptide named β-casomorphin 7 (BCM-7), while cleavage is prevented by the presence of a proline at the same position. Other variants are characterized by a proline at position 67 (A3, E, D, I, and H) and could exhibit the same behavior of the A2 variant as well as other variants, with a histidine at the same position (B, C, F, and G), and could behave as the A1 variant resulting in the formation of the β-casomorphin 7 (BCM-7) (Bodnar *et al*., 2018).

BMC-7 is a bioactive peptide with strong opioid activity and an oxidant effect (EFSA Scientific Report 2009; Kay *et al*., 2021) and its release has been related to the alteration of the physiology of different organ systems. In particular, several studies demonstrated the correlation with the onset of various human pathological conditions, such as heart disease, sudden infant death syndrome, milk intolerance, and also with the aggravation of symptoms associated with schizophrenia, autism, and type 1 diabetes (Caroli *et al*., 2009; Cieslinska *et al*., 2015; Kaminski *et al*., 2007; Kay *et al*., 2021; McLachlan, 2001; Pal *et al*., 2015; Reichelt *et al*., 2012). In more detail, it

Table 1. Differences in the amino acid sequence of β-casein variants.

β-casein Variant	Amino Acid Position								
	36	37	67	72	88	93	106	122	138
A2*	Glu (E)	Glu (E)	Pro (P)	Gln (Q)	Leu (L)	Met (M)	His (H)	Ser (S)	Pro (P)
A1*			**His (H)**						
A3*							**Gln (Q)**		
B*			**His (H)**					**Arg (R)**	
C*		**Lys (K)**	**His (H)**						
E*	**Lys (K)**								
I*						**Leu (L)**			
D									
F			**His (H)**						**Leu (L)**
G			**His (H)**					**Leu (L)**	
H1					**Ile (I)**				
H2				**Glu (E)**		**Leu (L)**			**Glu (E)**

In bold: amino acid variations with respect to the A2 ancestral variant.
Arg: arginine; Gln: glutamine; Glu: glutamic acid; His: histidine; Ile: isoleucine; Leu: leucine; Lys: lysine; Met: methionine; Pro: proline; Ser: serine;
*Allele variants detected in European cattle breeds.

has been reported in the literature that BMC-7, binding to μ receptor in the gastrointestinal (GI) tract, may alter gut microbiota, can increase inflammatory response, and may induce mucin production, thus triggering lactose intolerance-like symptoms. Moreover, BMC-7 may promote oxidative stress, may deregulate insulin metabolism, and can modulate DNA methylation reactions affecting neurodevelopment (Kay *et al*., 2021). However, the European Food Safety Authority (EFSA) in 2009 carried out a meta-analysis of data present in the literature, releasing a scientific report that supports the absence of a cause–effect relationship between the consumption of milk containing the A1 variant and the etiology of the aforementioned diseases, so further studies are necessary in this field (EFSA Scientific Report, 2009). Therefore, the discussion on the adverse human health effects of the β-casein variant A1 remains still open also because other studies demonstrated that milk obtained from A2 homozygous cows seems to be more digestible than milk containing β-casein A1 (Deth *et al*., 2016; He *et al*., 2017; Ramakrishnan *et al*., 2020). This effect could be traced back to the increase in the rate of gastrointestinal transit of A2 milk and to the lack of the pro-inflammatory effect instead associated with A1 milk consumption (Brooke-Taylor *et al*., 2017; Kay *et al*., 2021). In addition, A2 milk consumption increases the natural production of glutathione (GSH), which is reported to be a key antioxidant, widely recognized for its association with many health benefits. The consumption of A2 milk induces a twofold increase of blood GSH levels compared to the levels derived from conventional milk intake (Deth *et al*., 2016). Interestingly, human breast milk, which is recommended by the World Health Organization (WHO) as the exclusive food for newborn feeding, is characterized by the presence of a β-casein protein carrying a proline residue in position 67 which is therefore very similar to the bovine A2 variant (Kay *et al*., 2021).

As a consequence, in many countries, including Australia, the United Kingdom, the United States, New Zealand, the Netherlands, China and more recently also Italy, A2 cow's milk has been made commercially available, and it is widely recommended for people who suffer from milk-intolerance and for newborns who need formulas more soft to their digestive system (Brooke-Taylor *et al*., 2017).

Considering this scenario, this study focused on the planning and execution of a breeding program based on the MAS selection of the β-casein A2 variant, in farms located in central Italy providing milk for an important drinking milk producing plant. A2 heterozygous and homozygous cows, previously genotyped (Sebastiani *et al*., 2020), were artificially inseminated with semen from bulls homozygous for the A2 variant, and their offspring have been likewise analyzed in order to identify A2/A2 animals for A2 milk production.

Materials and methods

Sampling

A total of 1452 Italian Holstein Friesian cows, reared in farms located in central Italy and previously genotyped (Sebastiani *et al*., 2020), were subjected to artificial insemination with semen from A2/A2 selected bulls (Co.S.A.P.A.M. Soc. Coop., Lodi, Italy; ABS Italia Srl, Cremona, Italy; INSEME Spa, Modena, Italy). Among these, 640 were A2 homozygous and 812 were A2 carriers (A1/A2, A2/B, A2/I) animals.

From the pregnant cows, 534 heifers were born. From these animals, whole blood samples were collected in tubes containing ethylenediaminetetra-acetic acid (EDTA) as an anticoagulant and stored at –20 °C until genetic analysis. Samples were taken in a single withdrawal, simultaneously with the mandatory periodic tests required by Italian National Health Programs and during breeders' voluntary health controls.

DNA extraction and sequencing

Genomic DNA was extracted using High Pure PCR Template Preparation Kit (Roche Life Science, Mannheim, Germany) according to the manufacturer's instructions. PCR reactions of both exons 6 and 7 were performed as previously described (Sebastiani *et al*., 2020). Amplicons were analyzed through 2% agarose gel electrophoresis containing Midori Green Advanced DNA Stain (Nippon Genetics Europe GmbH, Düren, Germany). PCR products were purified with QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced in both directions using BrilliantDye™ Terminator Cycle Sequencing Kit v3.1 (NimaGen BV, Nijmegen, Netherlands) according to the manufacturer's instructions.

Sequencing reactions were analyzed in a 3500 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific Inc.). The obtained nucleotide sequences were aligned to the bovine β-casein gene (Accession number X14711.1) using the ClustalW tool of the BioEdit v7.2.5 software (Hall, 1999). Electropherograms were analyzed at each investigated mutation point to identify peaks in heterozygosity. In particular, polymorphisms at positions 36 and 37 of exon 6 and at positions 67, 72, 88, 93, 106, 122, and 138 of exon 7 were analyzed to discriminate the different β-casein variants.

Statistical analysis

Allele and genotype frequencies were directly calculated dividing the number of copies of each allele and genotype

by the total alleles and by the total individuals, respectively. Furthermore, the Hardy–Weinberg (HW) equilibrium was verified using Chi-square test ($P < 0.05$) by the R Studio software (R Core Team, 2020).

Results

In this study, 1452 already genotyped dams (Sebastiani *et al*., 2020), at least carrier of the A2 allele (A2 heterozygous and homozygous animals), were artificially inseminated using commercial semen from A2 homozygous bulls in order to increase A2 variant and A2/A2 genotype frequencies in the female progeny.

From inseminated cows that got pregnant, 534 heifers were born. Among these, 238 derived from A2 homozygous parents were analyzed to confirm the A2/A2 genotype as they have to be used for the production of A2 certified milk. The remaining 296 heifers, born from A2 heterozygous dams, were analyzed in order to define their genotype and separate the A2 homozygous ones in the herds with the same purpose.

Here, we report the results of the analysis carried out on this offspring in terms of allele and genotype frequencies of the different CSN2 gene variants. No deviation of HW equilibrium was observed at the considered polymorphic sites.

In the female offspring population obtained by the artificial insemination of A2 heterozygous and homozygous cows, sequencing analysis of the CSN2 gene PCR products highlighted the presence of four β-casein variants (A1, A2, B, and I) and four genotypes, (A2/A2, A1/A2, A2/B, A2/I) as shown in Table 2. After the application of the MAS on the herds participating in the project, the frequencies of A2 and I alleles, both characterized by a proline in position 67, increased from 60.65 to 84.46% and from 3.10 to 3.28%, respectively, in the progeny compared to dams. At the same time, the frequencies of the unfavorable A1 and B alleles decreased from 30.39 to 10.86% and from 5.68 to 1.40%, respectively. A3 and C alleles and their related genotypes, that were present with low frequencies in the population of dams, were not further found in the offspring. Regarding genotypes, the most interesting result concerned the about twofold increase of A2/A2 frequency from 36.96 to 68.91%. A similarly intriguing result, derived from the MAS application, was the consistent reduction of the frequency of A1/A2 animals, from 35.79 to 21.72%. The frequencies of the other A2 heterozygous genotypes found in the progeny, that is A2/I and A2/B, varied from 7.55 to 2.81% and from 3.83 to 6.55% (Table 2, Figure 1). Interestingly, since the I variant should behave in the same manner as A2

Table 2. Allele and genotype frequencies (%) in the examined animals, before (dams) and after (heifers) MAS selection.

Allele	Allele frequency (%)		Genotype	Genotype frequency (%)	
	Dams	Heifers		Dams	Heifers
A2	60.65	84.46	A2/A2	36.96	68.91
A1	30.39	10.86	A1/A2	35.79	21.72
B	5.68	1.40	A1/A1	9.88	/
I	3.10	3.28	A2/B	7.55	2.81
A3	0.15	/	A2/I	3.83	6.55
C	0.03	/	A1/B	3.07	/
			A1/I	2.03	/
			B/I	0.25	/
			B/B	0.18	/
			A2/A3	0.12	/
			A3/B	0.12	/
			A1/A3	0.06	/
			A1/C	0.06	/

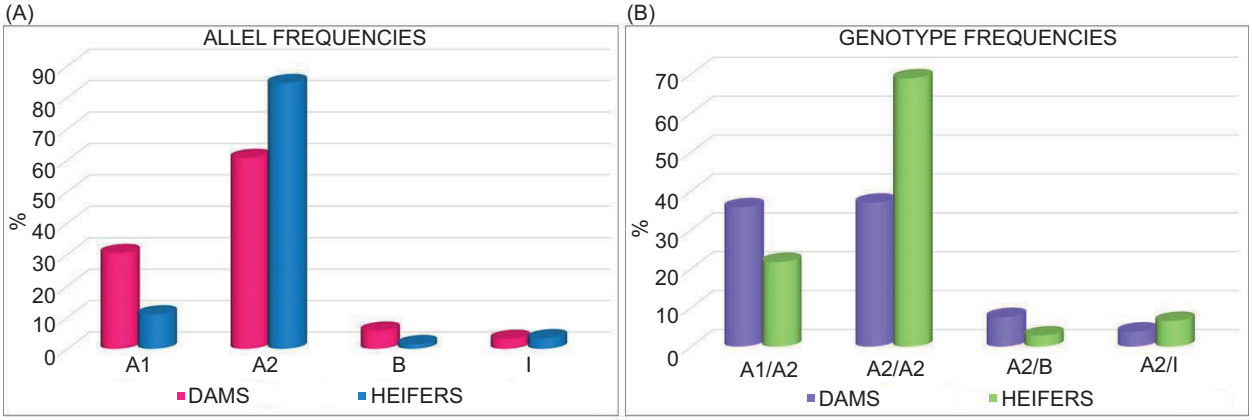

Figure 1. Graphics of the allele (A) and genotype (B) frequencies variation (%) in the examined dams and heifers after MAS selection.

variant in term of BMC-7 formation, A2/I animals could at least be used for the production of a more digestible milk, even though they could not contribute to the marketing of a certified A2 milk.

In conclusion, the first generation from the genotyped dams produced an extra number of 368 A2/A2 cows in addition to the A2 homozygous cows in reproductive age still present in the herds. So, both groups of animals may be used for reproduction and for the related A2 drinking milk production.

Discussion

The data presented here were derived from a larger research project whose purpose was primarily to evaluate the frequencies of the β-casein *CSN2* gene alleles in Italian Holstein Friesian dairy cattle reared in farms supplying milk to an important milk plant in central Italy.

In this study, we wanted to evaluate the increase of the A2 allele and A2/A2 genotype frequencies, in the female progeny of dams, previously genotyped and fertilized with A2/A2 semen in a selection program based on MAS. The final goal was the production of A2 cow's milk, due to its supposed association with health benefits for humans.

In fact, the presence in milk of the A2 isoform of β-casein is increasingly considered a desirable characteristic, because it confers greater digestibility to milk. This could allow milk intake even by people who suffer from lactose intolerance-like symptoms despite not being really lactose intolerant (Park *et al*., 2021).

The Italian Holstein Friesian breed is not among those breeds characterized by the highest frequency of the A2/A2 genotype, but it has a sufficiently high frequency to allow an effective genetic selection of this trait (Canavesi, 2016).

Production of A2 milk can be accomplished following a MAS scheme, which is based on selecting animal carriers of specific polymorphisms that characterize the A2 variant.

During the course of a MAS-based selection plan, aimed at obtaining a consistent number of A2 homozygous cows in the herd, milk coming from A2/A2 animals can be collected separately and directed to A2 milk commercialization. This approach requires organizing, logistical, and management efforts of cow sheds and milking barns, which however is rewarded by an economic gain in the sale of a type of milk with beneficial properties for human health.

In the last years, the commercialization of A2 milk has conquered large market portions in many non-European countries, while in Europe and in Italy it still remains a niche product (Brooke-Taylor *et al*., 2017). Actually, few medium/large Italian dairy industries have taken this route commercializing certified A2 milk, whose distribution is slowly spreading, starting to generate the interest of consumers.

The data reported here are very encouraging because they confirm the expected genetic improvement in the farms analyzed in this survey. The use in the future of certified A2/A2 sexed semen could help to accelerate the increase in the number of female animals to be used for A2 milk production.

Conclusions

In recent decades, advances in assisted reproductive technologies, animal molecular genetics, and statistical analysis applied to animal genetic improvement helped to maximize the genetic gain in livestock breeding worldwide.

In the past, cows produced milk whose β-casein protein was represented only by A2 isoform, considered to be ancestral, but over time changes in the genetic heritage led to the occurrence of other variants (Farrel *et al*., 2004). Cows have thus acquired the ability to produce milk with different β-casein isoforms, in particular A1 and A2.

Nowadays, β-casein A2 milk can be considered "a return to the origins" because it comes from selected cows that produce only the ancestral β-casein A2 protein. It has to be noted that in addition to the indexes evaluated for the selection of the best breeding bulls regarding traits related to morphology, health, and productivity, information about milk quality, such as β-casein genotype, has also been included.

In conclusion, the valorization of this genetic trait and production of A2 milk could be advantageous for the whole drinking milk chain, from producers to consumers.

Author Contributions

S.F., G.C., and M.B. conceptualized the study; C.S. and M.B. formulated the study; C.A. and C.S. did the formal analysis; C.A., M.T., M.C., and C.S. were in charge of investigation; G.C., S.F., and N.D. arranged the resources; C.A. and C.S. prepared the original draft; M.B., C.S., M.T., C.A, and G.C. reviewed and edited the manuscript; M.B. and C.S. supervised the study; M.B., G.C., and S.F.

were in charge of project administration; and S.F. and G.C acquired funding.

Disclosure Statement

All authors report no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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