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Unbalanced expression of beta-casein variants A1 and A2 in Holstein-Friesian cows*

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Variant A1 of bovine beta-casein (*CSN2*) is known for producing beta-casomorphin-7 (BCM7), which is an opioid-like peptide released during gastrointestinal digestion. The aim of the study was to measure how much A1 and A2 protein variants occur in the milk of cows with different *CSN2* genotypes. In a population of 113 A1A2 Holstein-Friesian cows, using the ELISA method, it was found that variant A2 was present at low content in milk (mean 6.31±3.09 ng/mL), but variant A1 reached almost seven times higher concentration (43.40 ng/mL±15.68 ng/mL). This unbalanced expression of the *CSN2* alleles was not associated with the single nucleotide polymorphism within the 5' flanking sequence known as beta-casein enhancer (BCE). Moreover, the origin of allele A1 (whether inherited from a sire or dam) did not explain its overexpression. Furthermore, using qRT-PCR, it was shown that the mRNA levels of the *CSN2* A1 and A2 alleles are at similar levels in the milk somatic cells of 16 A1A2 cows, suggesting that the unbalanced expression of *CSN2* alleles could be determined by post-transcriptional events. Two SNPs were identified within the *CSN2* 3' UTR in 109 cows with A1A2 genotype. The STarMiR software was used to predict microRNA targets and indicated that G/A was located within the canonical seed sites of bta-miR-145, potentially affecting miRNA-mRNA binding and translational repression of the *CSN2* variant.

KEY WORDS: Holstein-Friesian cattle / beta-casein / variant A1 / milk protein

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The casein gene cluster in cattle is organized within 250 kb and consists of four genes: alpha S1-(*CSN1S1*), beta-(*CSN2*), alpha S2-(*CSN1S2*), and kappa-casein (*CSN3*) [Rijnkels 2002]. Casein genes are expressed at high levels in epithelial cells during late pregnancy, lactation, and early involution of the mammary gland [Wickramasinghe *et al.* 2012, Qian and Zhao 2014, Lee *et al*. 2023]. Holstein cattle are the world's most common breed, and as reported by Cieslinska *et al*. [2022], the two most prevalent genetic variants of CSN2 in this breed are A1 and A2, out of the fifteen variants reported. The difference between A1 and A2 variants is caused by single amino acid substitution where Proline (A1) is exchanged by Histidine (A2) due to single nucleotide polymorphism (SNP) at codon 67 in the exon 7 (H67P) of the *CSN2* gene. In recent years, special attention has been drawn to these two variants after the discovery that only variant A1 during gastrointestinal digestion is a source of the opioid-like peptide - β-casomorphin-7 (BCM-7), which impairs gastrointestinal wellbeing and may be a risk factor for different diseases [McLachlan 2001, Thiruvengdam *et al*. 2021, Cieslinska *et al*. 2022].

Since the discovery of the milk protein polymorphism [Aschaffenburg 1961], it was assumed that casein alleles undergo the codominant mode of inheritance when both alleles are expressed in relatively similar quantities. The first report signaling that this assumption should be verified was published by Ehrmann *et al.* [1997], who investigated the association between different milk protein genotypes and the number of different milk proteins measured by densitometry from a polyacrylamide gel. Those authors noted a slight but significant difference between beta-casein A1A1 and A2A2 expressed as a % of total milk protein. A potential unbalanced expression of beta-casein alleles was indirectly observed by highly significant differences between beta-casomorphin-7 content originating from milk A2A2 and A1A1 cows [Cieslinska *et al*. 2012]. In the meantime, monoallelic expression of many human genes was observed; for example, Sanova *et al.* [2016] showed that 10-20 % of human and mouse autosomal genes are the subject of monoallelic expression in multiple cell types. This phenomenon is due to genomic imprinting or by extinguishing or activation of only one allele [Oczkowicz *et al*. 2022]. The unbalanced expression of alleles was also reported by Kravitz *et al*. [2023], who found 2.762 autosomal genes in which one allele tends to be expressed more than the other. They found that such genes are associated with rapidly evolving regions in the human genome, adaptive signaling processes, and genes linked to age-related diseases such as neurodegeneration and cancer.

All these findings encouraged the authors to analyze milk produced by cows with *CSN2* A1A2 genotype by measuring the quantity of each variant to better understand the relationship between these two alleles. This paper presents results showing highly unbalanced expression of beta-casein alleles A1 and A2 in cow's milk and provides new evidence that suggests an explanation for this phenomenon.

Material and methods

Animals

To collect cows with different *CSN2* genotype, 1,511 Holstein-Fresian (Black-and-White variety) cows from 23 herds were included in the study. Tissue was collected from each cow's ear using the Allflex sampling system, followed by genomic DNA isolation using the NucleoSpin Tissue Mini Kit for DNA from Cells and Tissue (Macherey-Nagel).

Milk samples

In the second step, out of 1,511 cows, 113 cows (kept in 12 herds) were chosen to collect 50 mL milk samples. All cows had A1A2 genotype, were in the first lactation (with age ranged between 28-32 months), show very similar milk yield (ranged between 9100-9550 kg), milk total protein percentage (ranged between 3.21 and 3.29%) and low somatic cell count (<250000 cell/mL) (according to national milk recording database SYMLEK). Cows showed no clinical symptoms of potential influence on milk composition. Feeding schemes were very similar in all herds: total mixed ration (TMR) based on maize silage, grass and alfalfa haylage and brewer's spent grain supplemented by minerals and vitamins. Cows in all herds were kept in free-stall system and milked two times a day in parallel parlor. Milk samples were taken in the peak of lactation. Cows were the offspring of 43 different sires.

Measuring the content of CSN2 A1 and A2 variants in milk by ELISA

Milk samples (50 mL) were tested for A1 and A2 variants using the Bovine beta-Casein ELISA Kit from Biosensis. This kit consists of a pre-coated rabbit anti-bovine beta-casein polyclonal capture antibody, a chicken anti-bovine A1 (or A2) beta-casein detection antibody and a horseradish peroxidase (HRP)-conjugated donkey anti-chicken IgY antibody. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a colored reaction product which is directly proportional to the concentration of Bovine A1 (or A2) beta-casein present in samples and protein standards. Five hundred milliliters of raw milk samples were taken from each cow and transferred in a chilled container to the lab within four hours. The samples were then frozen at -20°C. Re-frozen milk samples with the volume of 10 µL were then diluted to 1:40.000 dilution by the essay diluent, and 100 µL of diluted sample was then put into precoated microplate wells. A1 (or A2) beta-casein standard and blank (sample diluent only) were also placed in the wells. After sealing the plate, the samples were incubated using an Orbital Shaker (OHRUS) for 120 min. Solutions inside the wells were then discarded, and five washes with 1x wash buffer 200 µL per well were performed. The incubation, discarding and washing steps were repeated each time after adding 100 µL of the detection antibody, 100 µL of the donkey anti-chicken IgY-HRP conjugate and finally, 100 µL of TMB, but the duration of incubation took 120, 60 and 20 min, respectively. The reaction was stopped by adding 100 µL of stop

solution into each well. The absorbance at 450 nm was read on a Multiscan FC reader (Thermo Scientific). Each milk sample was processed in duplicate. The readings were averaged for each beta-casein standard, blank and sample. A standard curve was plotted on the x-axis, and OD at 450 nm on the y-axis. MyAssays software (www.myassays.com) was used to calculate the true concentration of beta-casein variants.

Identification of single nucleotide polymorphisms within the CSN2 gene

Genotypes of beta-casein alleles A1 and A2 were identified by using the Illumina Bovine MDv2 Chip (Eurogenomics). This polymorphism is depicted as marker CSN2_X14711_8101 and is capable of differentiating between A1 and A2 alleles.

Polymorphism within 5' and 3' flanking regions

Beta-Casein Enhancer (BCE) polymorphism was identified by the Polymerase Chain Reaction Amplification Created Restriction Site (PCR-ACRS) method described earlier [Oleński *et al.* 2012]. Briefly, a pair of primers (forward: 5'-GGAAGGACATGCTTTCTTTTG**A**A-3' and reverse: 5'-ATATGGCCCAGGAGGAAGTC-3') were used to amplify a 183 bp fragment of the BCE sequence. Underlined Adenosine is a base non-complimentary to the template artificially inserted to create restriction site enabling for allele identification. The PCR thermal profile consisted of 35 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 30 s and a final extension at 72°C for 5 min. PCR was performed in a reaction mix containing 40-60 ng of genomic DNA, 0.4 µL (25 pM) of each primer, 3.0 µL PCR Buffer (10x, Biotools, B&M Labs), 1.5 µL dNTPs mix (2.0 mM each), 0.7 unit of Biotools Taq DNA Polymerase and deionized water added to reach a volume of 25 µL. The PCR products were digested with FastDigest *Eco*RI at 37°C (Thermo Scientific). Restriction fragments were electrophoresed in standard 2.5 % agarose gel stained with ethidium bromide and observed by Fluor-S™ MultiImager (Bio-Rad).

In addition, 3' UTR of *CSN2* was screened for polymorphism by sequencing. Briefly, a pair of PCR primers (forward: 5'AGCCGATGTTTGGTCCTAGA3' and reverse: 5'TCCAATTTTAATTTTCCACAGC3') were used to amplify a 600 bp fragment. The PCR thermal profile consisted of 35 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and a final extension at 72°C for 5 min. PCR was performed in a reaction mix containing 40-60 ng of genomic DNA, 1.0 µL (25 pM) of each primer, 1.8 µL Pol PCR Buffer (10x, Eurx), 1.8 µL dNTPs mix (2.0 mM each), 0.7 unit of Eurx Taq DNA Polymerase and deionized water added to reach a volume of 18 µL. PCR products were then sequenced according to the standard protocol in an external laboratory. Sequences were analyzed in BioEdit software (v.7.7.1).

Milk somatic cell isolation

Milk somatic cells were obtained from 16 cows with the A1A2 beta-casein genotype cows (selected from 133 already sampled for genotyping) and kept in one herd. In batches of 2-4 cows, 250 mL morning milk samples were collected,

transported to the laboratory within 30 minutes and processed immediately. For the isolation of somatic cells from milk, the method of Feng *et al*. [2007] was used with minor modifications. Purified cell pellets from five 50 mL Falcon-type conical tubes from each animal were combined in a 2 mL Eppendorf tube and treated with 1.5 mL Fenozol Plus reagent (A&A Biotechnology).

Total RNA extraction and cDNA synthesis

Total RNA from milk somatic cells was extracted using the Total RNA Mini Plus Kit (A&A Biotechnology) according to the manufacturer's protocol. The resulting RNA preparations were digested with DNase, purified and concentrated using the Clean-Up RNA Concentrator Kit (A&A Biotechnology) according to the manufacturer's protocol. RNA concentration, purity and integrity were determined by measurements using a NanoDrop ND1000 spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano Kit (Agilent Technologies). RNA samples were stored at -80°C until cDNA synthesis. 1000 ng of oligo$(dT)_{18}$ primed total RNA was reverse transcribed with Transcriptor reverse transcriptase using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. The final cDNA products were diluted 100-fold prior to use in RT-qPCR, aliquoted and stored at -20°C.

RT-qPCR

RT-qPCR was used to analyze the transcript levels of A1 and A2 beta-casein variants in milk somatic cells of heterozygous A1A2 cows. Identification of the alleles was carried out using an LNA probe-based assay [Giglioti *et al*. 2021]. For amplification of the 73 bp fragment of the casein beta gene, the following primers were used: sense primer: 5′-ACAGTCTCTAGTCTATCCCTTC-3′; antisense primer: 5′-TTGAGTAAGAGGAGGGATGT-3′ and allele-specific probes: Probe A1, 5′ -(FAM) agGCtGttATggat (BHQ1)- 3′; Probe A2, 5′-(HEX) ctGTtAGggatGg (BHQ1)-3′(nucleotides modified by locked nucleic acid in the probes are indicated in capital letters). Real-time PCR reactions were performed in 96-well plate format on a LightCycler® LC 480 II (Roche) using the LightCycler 480 Probes Master Reagent Kit (Roche) according to the manufacturer's instructions. The cycling conditions were as follows: pre-incubation at 95°C for 5 min followed by 45 cycles with each cycle including 95°C for 10 s, annealing temperature 60°C for 10 s and extension at 72°C for 10 s. For RT-qPCR data normalization, a pair of RPLP0 and UCHL5 reference genes were used, as described in Brym *et al*. [2013]. For each primer pair, PCR efficiency (E) and error were calculated using a standard curve derived from a pooled cDNA mixture serially diluted 4-fold over five measurement points. Each assay was performed in duplicate. The quantification cycle (Cq) was automatically determined for each reaction by the LightCycler® 480 SW 1.5 software using default parameters and the second derivative maximum method. The $2^{-\Delta\Delta Ct}$ method of Schmittgen and Livak [2008] was used to calculate the relative expression levels of beta-casein A1 to A2 alleles in milk somatic cells.

miRNA target prediction

MicroRNA target prediction analysis was performed using the STarMir and STarMirDB modules of the Sfold package (Software for Statistical Folding of Nucleic Acids and Studies of Regulatory RNAs), available at http://sfold.wadsworth.org [Rennie *et al*. 2019].

Statistical analysis

Differences between the means of A1 or A2 variant concentrations in milk from cows with different genotypes within the 5'-flanking region, coding and 3'UTR of CSN2 were estimated by Student's t-test or one-way analysis of variance and Duncan's test by Statistica software package, version 13.0 (TIBCO Software Inc., CA, USA; StatSoft Polska, Kraków, Poland). (Statistica v13).

Results and discussion

Genotypes of CSN2 polymorphisms

All 1,511 cows were successfully genotyped, and the frequencies of A1A1, A1A2 and A2A2 genotypes in the Polish Holstein population were 12, 43 and 45%, respectively. The SNP cluster (CSN2_X14711_8101), which discriminates between A1 and A2 alleles, showed very good quality (Fig. 1). Out of 1,511 cows, 113 cows

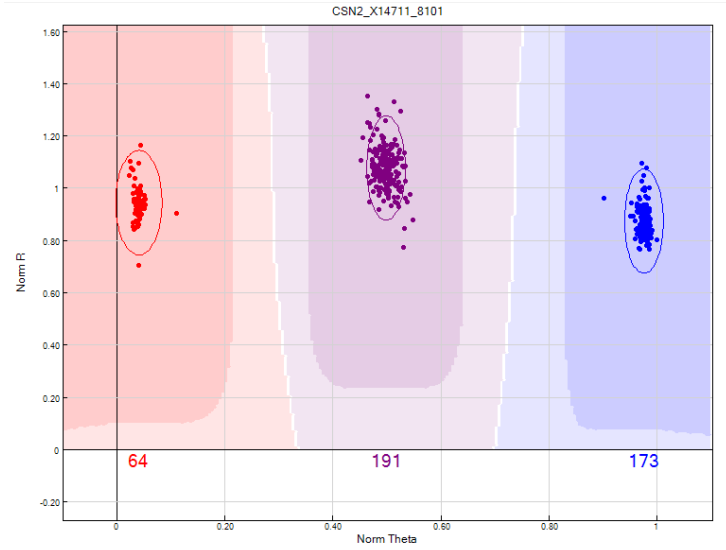

Fig. 1. Example of cluster quality of beta-casein genotyping using Illumina Bovine EuroG_MDv2 Chip. In a group of 428 cows (being a part of 1511 individuals), 64, 191 and 173 were genotyped as A1A1, A1A2 and A2A2, respectively. Each dot represents a single individual.

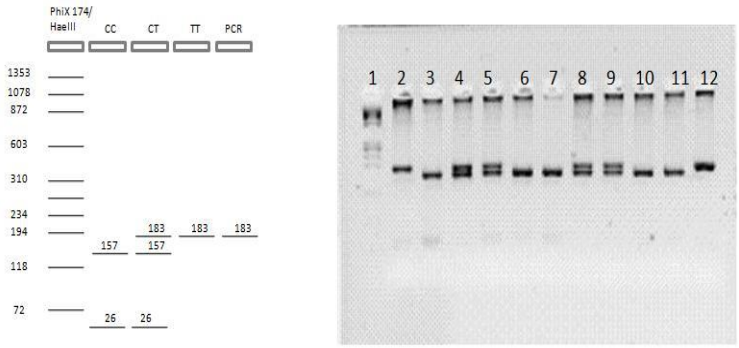

Fig. 2. Scheme (A) and electropherogram (B) showing genotyping of C/T (rs1116662227) polymorphic site at position -1578 within beta-casein enhancer (BCE) sequence by PCR-ACRS method. Line 1 – DNA size marker ΦX174/HaeIII. Line – 2 TT genotype. Lines 3, 6, 7, 10, 11 – CC genotypes. Lines 4, 5, 8, 9 – CT genotypes. Line 12 – undigested PCR product. The restriction fragment of 26 bp is not visible on the gel.

having *CSN2* A1A2 genotypes were selected and genotyped in the 5' flanking region of *CSN2* (Fig. 2). In this group, 61, 39 and 13 cows with CC, CT and TT genotypes were revealed, respectively.

Sequencing of 3' UTR of *CSN2* performed for 109 A1A2 cows (for 4 cows sequencing was unsuccessful) identified two polymorphisms: G>A transition (rs11068836) and T>A transversion (rs109439791) (Fig. 3).

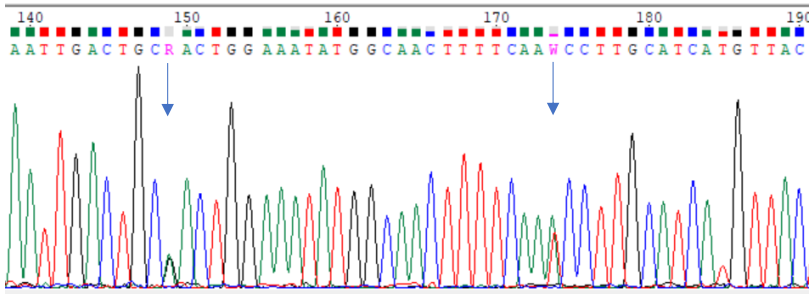

Fig. 3. Representative Sanger sequencing electropherogram of CSN2 A1A2 3'UTR sequence fragment, showing G>A transition (rs11068836) and T>A transversion (rs109439791) indicated by arrows.

CSN2 A1 and A2 variants measured by ELISA

In milk samples from 113 A1A2 cows, the A2 variant was present at a trace level (mean 6.31±3.09 ng/mL), but the A1 variant concentration was almost seven times higher (43.40 ng/mL±15.68 ng/mL). The difference was highly significant at $p<0.01$ (Tab. 1).

Table 1. Concentration of A1 and A2 beta-casein variants in milk samples from 113 A1A2 cows

Item	CSN2 A1 variant content ng/mL	CSN2 A2 variant content ng/mL
Mean	43.40^{A}	6.31^{B}
Standard deviation	15.68	3.09
Minimum value	15.90	3.69
Maximum value	96.31	11.32

^{AB}Means bearing different superscript differ significantly at $p<0.01$.

Table 2. Concentration of A1 and A2 variants in milk samples from 113 cows with A1A2 genotype at the beta-casein locus (rs43703011) and with different genotypes at the beta-casein enhancer (BCE) located in 5'-flanking region (rs1116662227)

CSN2 5'-flanking genotype	N	CSN2 A1 variant content ng/mL		CSN2 A2 variant content ng/mL	
		mean	SD	mean	SD
CC	61	43.39^{A}	15.22	6.15^{A}	2.86
CT	39	42.37^{B}	15.87	6.80^{B}	3.54
TT	13	46.57^{C}	17.98	5.60^{C}	2.64

$^{AA...}$Means for each BCE genotype marked with the same superscript differ significantly at $p<0.01$.
$^{AB...}$Means in columns for A1 and A2 variants marked with different superscript were not significant ($p=0.708$ and $p=0.402$ for A1 and A2 variants, respectively).

Table 3. Concentration of A1 and A2 variants in milk samples from 109 cows with A1A2 genotype at the beta-casein locus (rs43703011) and two SNPs in the 3'UTR region; G>A transition (rs11068836) and T>A transversion (rs109439791)

Diplotype	N	CSN2 A1 variant content (ng/mL)		CSN2 A2 variant (content ng/mL)	
		mean	SD	mean	SD
GGTT	57	44.03	16.42	5.82*	2.47
AGAT	52	42.12	15.22	6.92*	3.66

*Means for each diplotype marked with the asterisk show trend ($p=0.067$).

It was also observed that the unbalanced expression of the A1 allele of the *CSN2* locus was not associated with the single nucleotide polymorphism within the *CSN2* 5' flanking region (Tab. 2). This result eliminates polymorphism in the 5' flanking region as a causal factor responsible for the 7-fold differences between variant A1 and A2 in the milk.

For two polymorphisms within 3'UTR, only two diplotypes occurred in the population of 109 A1A2 cows: GGTT and AGAT. They did not significantly influence the concentration of A1 and A2 variants, although a trend for higher A2 content in cows

Table 4. Concentration of A1 and A2 variants in milk samples from 10 cows that have inherited A1 from the sire and A2 from the dam and vice versa

Sire's genotype	Dam's genotype	A1 variant (ng/mL)	A2 variant (ng/mL)
A1A1	A2A2	39.00	6.76
A1A1	A2A2	53.38	9.00
A1A1	A2A2	78.13	11.32
A1A1	A2A2	48.63	5.57
A1A1	A2A2	50.03	15.51
A1A1	A2A2	48.83	11.94
Mean (SD)		53.00 (13.21)^{A}	10.01 (3.66)^{A}
A2A2	A1A1	34.10	6.75
A2A2	A1A1	47.88	8.90
A2A2	A1A1	43.08	8.98
A2A2	A1A1	38.63	5.45
Mean (SD)		40.92 (4.99)^{B}	7.52 (4.51)^{B}

$^{AB...}$Means in columns for A1 and A2 variants marked with different superscript were not significant (p=0.12 and p=0.24 for A1 and A2 variants, respectively).

with diplotype AGAT was noted (Tab. 3). Sequencing of 4 samples were unsuccessful.

Among 113 A1A2 individuals, it was only possible (by analysis of pedigree data) to find six cows which inherited A1 from a sire and A2 from a dam and four cows which inherited the A2 variant from a sire and A1 variant from a dam. A high content of variant A1 persisted in milk from A1A2 cows regardless of whether the individual inherited the A1 allele from the sire or the dam (Tab. 4).

Analysis of beta-casein allele expression at the transcript level in milk somatic cells of A1A2 cows showed that the A1 allele was transcribed at approximately 1.21-fold more quantity than the A2 allele, but the difference was not statistically significant (p=0.44) (Fig. 4). These observations suggest that post-transcriptional events are responsible for the unbalanced expression of the beta-casein alleles in milk.

In the next step, the STarMiR module of the Sfold package was used to predict microRNA targets within the 600 bp fragment of 3'-UTR of *CSN2*. This analysis showed that SNP c.950G>A (rs11068836) was located within the canonical seed site of bta-miR-145 (Fig. 5), potentially affecting miRNA-mRNA binding and translational repression or decay of *CSN2* variants.

For over twenty years, there has been a discussion about the potential negative influence of the beta-casein A1 variant on human health. According to this thesis, the digestion of cows' milk with A1 releases an opioid peptide - β-casomorphin-7 (BCM-7), which impairs gastrointestinal well-being and is a risk factor for different diseases [McLachlan 2001, Kamiński *et al.* 2007, Thiruvengdam *et al*. 2021, Cieslinska *et al.* 2022]. Attempts to eliminate allele A1 β-casein were initiated in New Zealand, which is a leader in promoting milk with the A2 variant on the dairy market. In 2000, A2 Corporation Ltd. was established in New Zealand to test cows and market milk with

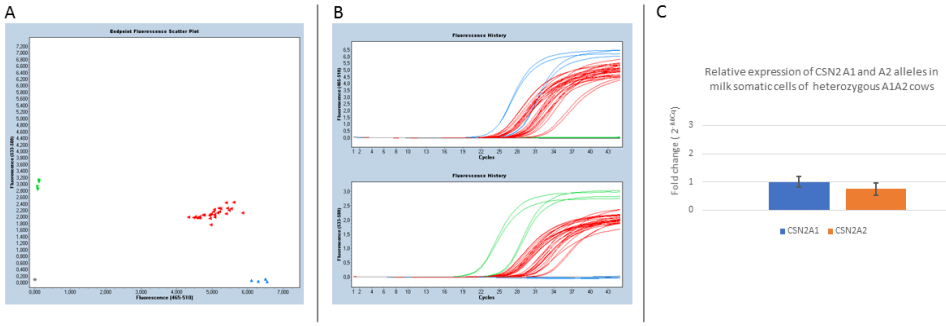

Fig. 4. RT-qPCR analysis of CSN2 A1 and A2 allele expression in milk somatic cells from A1A2 heterozygous cows. A) Scatter plot of CSN2 genotyping assay performed with Taqman probes specific for A1 and A2 CSN2 alleles and cDNA samples from milk somatic cells of 16 A1A2 (red triangles), 2 A1A1 (blue triangles) and 2 A2A2 (green triangles) individuals (each sample performed in duplicate). B) Comparison of RT-qPCR amplification curves for FAM-dye channel (A1-specific probe, upper panel) and HEX-dye channel (A2-specific probe, lower panel). C) Relative expression of CSN2 A1 and A2 alleles in milk somatic cells of heterozygous cows after normalization with RPLP0 and UCHL5 reference genes. The observed difference was not significant (p = 0.441).

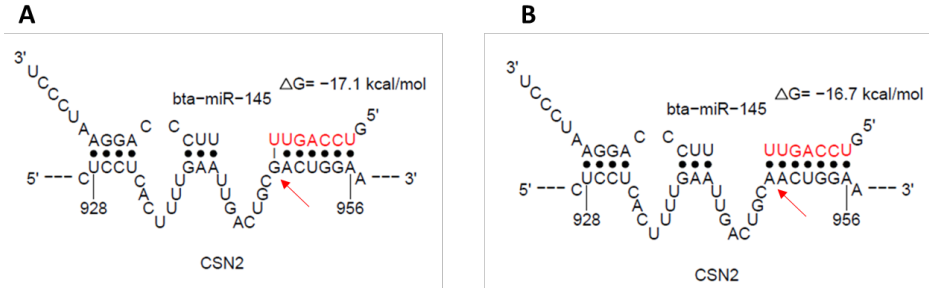

Fig. 5. Schematic representation of the bta-miR-145 binding site on the 3'UTR variants found in the bovine *CSN2* gene (rs11068836/c.950G>A, indicated by arrows). The seed region of the miRNA is highlighted in red.

only the A2 variant of beta-casein. Since 2003, A2 milk has been sold in New Zealand and Australia as a premium brand. The global market for products based on A2 milk is predicted to reach $3.7 billion in 2027 [Allied Market Research, 2024]. Therefore, cows and bulls with the *CSN2* A2 variant are preferred in reproductive schemes by an increasing number of farmers and insemination centers.

The frequency of allele A1 in Holstein cattle (or Holstein-Friesian) is still at a relatively high level – approximately 30% [Sanchez *et a*l. 2020, Sebastiani *et al.* 2020, Kolenda and Sitkowska 2021, Kamiński *et al*. 2022], which is caused by a high proportion of A1A2 heterozygotes, reaching up to approximately 50 % of the cow population. In a rapidly growing number of papers about beta-casein variants and their importance for dairy production [Cieslinska *et al.* 2022], a common assumption was

made that both variants are inherited co-dominantly, which means their content in milk is roughly the same. To verify this assumption, milk samples from cows having A1A2 genotype were collected in the way that minimized the influence of environmental factors on milk yield and content: all cows have very similar milk yield, total protein percentage, somatic cell count, feeding and milking system, lactation number and stage. In these aligned milk samples the content of A1 and A2 variants were measured by ELISA. Surprisingly, a high disproportion was observed between the two variants, with variant A1 occurring in content approximately seven times higher than variant A2.

The quantity of beta-casein variants cannot be compared to work published by Duarte-Vazques *et al*. [2017], in which the mean relative abundance of A1 to A2 variant in raw Holstein cow's milk accounted for 0.574 and 0.426, respectively. Because this milk was collected from 40 Holstein cows of unknown beta-casein genotype, it is suspected that this milk contained both alleles, but the ratio of protein variants might be different (it depends on how many cows were A1A1, A1A2 and A2A2). In the same paper, the A1:A2 ratio was also measured in four infant formulas, but it was not substantially different, although it was more diverse, depending on the individual type of infant formula. The ratio between the two variants also cannot be compared to results obtained by mass spectrometry detection [Fuerer *et al*. 2020] because of the different ionization efficiencies of individual proteoforms; in effect, the MS signals of A1 and A2 protein variants do not represent the actual mass ratio of protein.

Unbalanced or monoallelic expression of a gene can be explained in at least three ways: 1) by the presence of polymorphisms at regulatory binding sites for transcription factors (TFs) leading to changes in the transcription rate for the phased allele, 2) the difference in DNA methylation between maternal and paternal inherited beta-casein gene and 3) the suppression of one allele by miRNA.

In the current study, several analyses were performed to determine the source of the highly unbalanced expression of A1 and A2 variants. Genomic imprinting was excluded because it was shown that a higher content of variant A1 in the milk of daughters occurs regardless of the origin of variant A1 (whether it was inherited from a dam or sire) (Tab. 4). The next possible explanation of the dominance of allele A1 could come from the unbalanced mRNA synthesis of beta-casein alleles. An RT-qPCR analysis, however, showed that both alleles are transcribed in only slightly different efficiency (Fig. 4). Moreover, the possible influence of polymorphism in the 5' flanking region was excluded because milk from cows of different genotypes (CC, CT or TT) did not correlate with differences between A1 and A2 variant quantity (Table 2). A polymorphism within 5'UTR as a causal factor for unbalanced expression of milk proteins was reported by Davis *et al*. [2022] for bovine beta-lactoglobulin. A synonymous SNP G/A at position +78 bp (exon1) resulted in a 60% relative reduction in mRNA expression for variant A, compared to the wild-type G sequence allele. Unfortunately, the actual quantity of beta-lactoglobulin protein A and B in milk was not measured.

Two single nucleotide polymorphisms were identified in the 3'UTR of beta-casein, which is known to be targeted by microRNA, leading to gene silencing [Kilikevicius *et al*. 2022]. To investigate whether this mechanism is involved in the disproportion between A1 and A2 variants, the 600 bp amplicon was screened using the STarMiR module. It was found that one of the polymorphic sites is bound by bta-miR-145 with a different DeltaG value, depending on the 3'UTR allele. DeltaG is a measure of the stability of hybrid miRNA vs. target [Rehmsmeier *et al.* 2004]. Lower DeltaG means that more energy is required for binding miRNA to the target sequence; higher DeltaG means that the hybridization between miRNA and the target sequence is easier, which consequently more likely leads to gene silencing [Naeli *et al*. 2023]. The presence of bta-miR-145 in the epithelial cell of the cow mammary gland was confirmed by other authors [Wenqing *et al*. 2020].

SNPs may create, destroy or modify miRNA target sites and, therefore, might be important effectors of phenotypic variation. The importance of polymorphism within 3'UTR as a target for microRNA was demonstrated by Clop *et al*. [2006]. They showed that the G to A transition in the 3' UTR of *GDF8* in Texel sheep creates a target site for mir1 and mir206, which causes translational inhibition of the myostatin gene, leading to muscular hypertrophy. Different interactions of microRNA with polymorphic 3'UTR targets were also observed in the human *AGTR1* gene that is involved in hypertension [Sethupathy *et al*. 2007].

The current results need more research to confirm that microRNA indeed targets the polymorphic sequence in 3' UTR and has a suppressive effect on the quantity of variant A2.

In conclusion, variant A1 of bovine beta-casein, a source of beta-casomorphin-7, occurs in approximately 7-fold higher concentration than variant A2 in milk produced by Holstein-Friesian cows with the A1A2 genotype. Overexpression of variant A1 takes place after transcription, probably due to microRNA targeting the polymorphic sequence within 3'UTR, leading to the silencing of variant A2. Further experiments are necessary to verify whether microRNA is binding to polymorphic sequences of beta-casein and about microRNA itself, its quantity, sequence variation and methylation status.

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