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Beta-Casein Genotyping in Dairy Cow Herds in Győr-Moson-Sopron County

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The main objective of this study was to determine the beta-casein A1/A2 polymorphism status of animals in two Holstein Friesian dairy cow herds in Győr-Moson-Sopron County, Hungary. The A1/A2 status of cattle is determined by the beta-casein gene on the sixth chromosome. The analysed single nucleotide polymorphism is non-synonymous; A1 and A2 variants of bovine beta-casein differ at position 67 of the amino acid chain: A1 variant codes for histidine and A2 codes for proline, which may affect the milk protein degradation process. The analysed polymorphism leads to key conformational changes in the secondary protein structure of beta-casein. Beta-casomorphin (known as BCM7) is released only from A1-type milk and cannot be completely degraded by enzymes during digestion. DNA isolation was performed from whole blood, and a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method with agarose gel electrophoresis was applied in order to determine individual genotypes. The results from the two dairy farms demonstrate that a high proportion of cows (86.08 and 90.74 %) carry the A2 gene variant without targeted selection. At farm „A", beta-casein polymorphisms were determined in 599 cows and 148 heifers. The genotype distribution of the cows was 47.25 % heterozygous, 38.83 % homozygous A2, whereas 14.02 % of the cows carried the A1A1 genotype. In heifers, A2A2 was already present in a remarkably high frequency (91.89 %), whereas the prevalence of heterozygotes was 7.43 %, and A1A1 animals made up only 0.67 % of the analysed heifer population. In Hungary, a growing number of dairy farms are using verified A2 homozygous breeding bull semen. The introduction of homozygous A2 sperm on the farm „A" remarkably increased A2 frequency in the heifer population. In total, 324 cows were genotyped on farm „B", where the A2A2 genotype was observed in 30.55 % of the animals. The second most common genotype was A1A2, with a genotype frequency of 60.19 %, whereas A1A1 homozygotes occurred with a 9.26 % frequency. The growing popularity of A2 milk due to potential health benefits is driving Hungarian stakeholders towards the targeted selection of dairy populations; animal genotyping is an evident approach to facilitate this transition.

1. Introduction

Research on the relationship between the A1/A2 genotype and milk is ongoing. The scientific community continues to investigate the differences between A1 and A2 milk, including the potential health benefits. However, it is important to note that the current scientific evidence is inconclusive and further research is needed to confirm or refute the claimed benefits. Further research into the A1/A2 genotype and the results are expected to lead to further developments and innovations in the food industry and health advice on this topic. This indicates that the researchers wanted to investigate and categorise the genetic variation in beta-casein proteins among the cows in these herds, specifically differentiating between the A1 and A2 variants of beta-casein. The Holstein-Friesian breed has been bred in the USA and Canada over the last hundred years from the European Blackthorn Shoveler. Because of its particularly high milk yield, the Holstein-Friesian is a globally recognised and desirable breed. It is found all over the world. The most productive herds are in Israel and the USA. In addition, Germany, Italy, Hungary, South Africa and Japan have also made their mark on the breed composition of their respective countries.

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The cattle sector has always played an important role in Hungary's livestock production, but nowadays, there is an increase in the number of people who have allergic reactions to some milk components. As a result, consumers are more interested in products that can replace milk and dairy products. In the dairy industry, A2 beta-casein milk has become increasingly popular with consumers in recent years. However, the scientific evidence supporting these claims is not entirely conclusive, and more research is needed in this area. Determining the A1/A2 polymorphism status of dairy cows is essential for various purposes, including selective breeding to produce milk with specific beta-casein compositions or for research on milk protein genetics and its potential health implications.

The aim of the present study is to determine the distribution of A1/A2 beta-casein genotypes in two Holstein-Friesian breeding herds in the county of Győr-Moson-Sopron. Milk protein plays an important role in providing our animal protein needs. Milk protein is composed of two main types of protein: generally, 80 % casein and 20 % whey protein. Casein can be further subdivided into alpha (αs1-CN, αs2-CN), beta and kappa casein, and gamma casein, which is essentially formed by the degradation of beta-casein (Kamiński et al., 2010). 95 % of the proteins found in ruminant milk are encoded by six structural genes. The four casein genes are located on chromosome 6. The genes (CSN1S1, CSN2, CSN1S2, and CSN3) encode the αs1- CN, β-CN, αs2-CN, and κ-CN proteins. The two main whey proteins, α-LA and β-LG, are encoded by the LAA and LGB genes on chromosome 5 and 3 (Caroli et al., 2009).

Beta-casein is a fraction of milk protein that can make up to 45 % of the total amount of casein. As described in the literature, 12 beta-casein variants have been identified so far: A1, A2, A3, B, C, D, E, F, G, H1, H2 and I (Farrell et al., 2004). Previous research has shown that historically, all primitive cattle breeds carried the A2 variant, and the A1 variant may have evolved as a result of a point mutation a few thousand years ago.

Beta-casein is 209 amino acids long. The mutation caused the proline at position 67 to be replaced by histidine, resulting in beta-casein A1.

This difference allows the formation of betacasomorphin 7 (BCM-7) during digestion (Barłowska et al.,2012). BCM-7 is a heptapeptide with opioid properties (Pal et al., 2015) that has a strong affinity for mu-opioid receptors (Givens et al., 2013). Results from Cieślińska et al. (2012) suggest that most BCM7 is released from hydrolysed and processed milk in association with the A1 allele of beta-casein, regardless of the lactation period.

The genotype distributions presented in Table 1 are shown in (Çardak, 2005): A, (Ristanic et al., 2020): B; (Hanusová et al., 2010): C, and (Kamiński et al., 2006): D, and it can be concluded that based on the data presented, that the A1A2 heterozygous genotype is typically the most prevalent in the Holstein-Friesian breed. In one of the four studies (study A), the proportion of the A2A2 genotype was very low.

In the other three studies (studies B, C, and D), the proportion of A2A2 genotype ranged from 30 % to 40 %.

A1A1 homozygotes were found in relatively low numbers in all four breeding lines in these studies. This suggests that in the Holstein-Friesian breed, A1A2 heterozygotes and A2A2 genotypes are more common compared to A1A1 homozygotes. The variation in A2A2 genotype prevalence between the studies may be due to factors such as the specific populations of cows analysed, geographical location, or breeding practices in the respective regions. The data of the processed results were taken as a guide due to their geographical location, as it allows us to compare the Hungarian population genetically.

Table 1: Beta-casein polymorphism in Holstein-Friesian farms

Genotype	**A % (n=237)**	**B % (n=106)**	**C % (n=92)**	**D % (n=143)**
A1A1	5.9	12.3	13.0	11.2
A1A2	46.0	54.7	83.0	58.0
A2A2	40.9	33.0	4.0	30.8

Human milk, goat's milk, sheep's milk, and milk from many other species are typically A2, meaning that in cattle, proline is found in the polymorphic amino acid position in these species. Milk with more than 99 % beta-casein in the A2 variety is known as "A2 milk" and is commercially available in many countries (Cattel and Nelson, 2010).

In relation to the successful introduction of the "A2 milk" category in the domestic market, Bodnár et al. (2018) conducted a SWOT analysis, highlighting that one of the key issues is the marketing strategy for its introduction and a thorough assessment of consumer needs. Secondly, more information is needed about A2 milk and dairy products made from it. Local consumption of A2 milk should be increased by disseminating adequate knowledge about the specific effects of these products on human health.

The A1 genotype of beta-casein has been associated with the occurrence of several human diseases, such as ischaemic heart disease, diabetes, sudden infant death syndrome (SIDS), autism and schizophrenia (Truswell, 2005). According to Kaskous (2020), homozygous A2 milk and even heterozygous A1A2 milk may be healthier

than homozygous A1 milk. A study by Clarke and Trivedi (2014) showed that not drinking A1 milk improved the health of patients with several diseases.

2. Materials and methods

2.1 Samples

The study was carried out on samples of two dairy cattle herds in Győr-Moson-Sopron County. A total of 824 animals (674 cows and 150 heifers – the total population of the two analysed farms) were genotyped. This information gives context to the scope of the study and the number of animals included in the genetic analysis, which can be crucial for understanding the representativeness of the findings and the potential implications for breeding and management practices in the region. Blood was collected using 9 mL Monovette (Sarstedt, Nümbrech, Germany) blood collection tubes with EDTA-K3 anticoagulant. Samples for DNA isolation were taken from the tail vein of cows and stored at – 20 ºC until use. DNA isolation and further analyses were performed in the genetic laboratory of the Department of Animal Sciences, Faculty of Albert Casimir Mosonmagyaróvár, Széchenyi István University.

2.2 DNA isolation

The isolation protocol steps were performed according to Tempfli (2014). Frozen Monovette blood collection tubes were thawed at room temperature, and then 300 µL of the blood sample was transferred to a 1.5 mL Eppendorf tube. 900 µL of homemade Cell Lysis solution was added to the blood sample and mixed by vortexing. Tubes were centrifuged for 1 min (15.000 × g). After centrifugation, the supernatant was removed, leaving the pellet containing white blood cells at the bottom of the tube. White blood cells were vortexed for 10-20 s, and then 300 µL of Extraction buffer solution was added to the sample. Cells and solution were mixed by pipetting and vortexing. The mixture was incubated in a water bath at 37 °C for at least one hour, and then 100 µL of Sodium Acetate solution was added to the mixture. Samples were then vortexed for about 20 s, followed by centrifugation for 4 min at room temperature (15,000 × g). After centrifugation, the translucent supernatant (300-400 µL) was pipetted into a new Eppendorf tube containing 300 µL isopropanol. DNA precipitated by the gentle shaking of the tube and was observed in thread-like forms. DNA was compressed into pellets by centrifugation (15,000 × g, 1 min). The DNA pellet was washed by adding 300 µL of 70 % ethanol. Ethanol washing was done by repeated inversions. DNA was collected again by centrifugation (15.000 × g for 1 min) to form pellets. The supernatant was removed by pipetting. The pellet was dried at room temperature for 15-20 min and then resuspended in 50 µL nuclease-free water. Rehydrated DNA was stored at – 20 ºC until further processing.

2.3 PCR-FRLP

After isolation, the DNA concentration of the samples was determined using a NanoDrop 2000 spectrophotometer and a uniform DNA concentration of 100 ng/µL was set. For the identification of the beta-casein polymorphism, the primers reported and used by Sodhi et al. (2018) – on sequence characterisation and Lien et al. (1992) on detection of Multiple β-Casein Alleles, were used to amplify the 251 bp long region of the locus under study: forward primer 5’ GAG TCG ACT GCA GAT TTT CAA ATC AGT GAG AGT CAG 3’ and reverse primer 5’ CCT GCA GAA TTC TAG TCT ATC CCT TCC CTG GGC CCA TCG 3’. The amplification of the test sequence was performed by polymerase chain reaction (PCR). PCRs were run in a SensoQuest Labcycler with the following settings: initial denaturation for 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min, elongation at 72 °C for 1 min. Reactions were pooled in 25 µl using PCR MasterMix, nuclease-free water (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and primers (Integrated DNA Technologies, Newark, New Jersey, USA). Amplified products were digested by restriction fragment length polymorphism (RFLP) for at least five hours using the TaqI enzyme. The enzyme cleaves in the presence of the A1 allele, resulting in a 251 bp long PCR product that is cleaved into 213 bp and 38 bp fragments. The digested DNA fragments were separated on 2 % agarose gel electrophoresis.
Agarose gels run for 20-30 min were illuminated under UV light, which allowed the cleavage patterns characteristic of different genotypes to be clearly distinguished. Since there are relatively few base pair differences between the two alleles, the alleles on the heterozygotes are visible close to each other on the gel. An allele with 251 base pairs indicates the presence of the A2 gene variant in the individual, and an allele with 213 base pairs indicates the presence of the A1 gene variant in the individual.

2.4 Statistics

The defined genotypes were recorded in Microsoft Excel 2015, and allele and genotype frequencies were calculated per file. Allele frequencies determined at sites A and B were compared using a chi-square test using

SPSS v.16 (SPSS Inc., Chicago, USA) statistical software. The chi-square test is widely accepted for the comparison of allele and genotype frequencies between populations.

3. Results

The tested beta-casein polymorphism was detected in the herds of both selected dairy farms. Figure 1 shows the different cleavage patterns underlying the genotyping for all three possible genotypes.

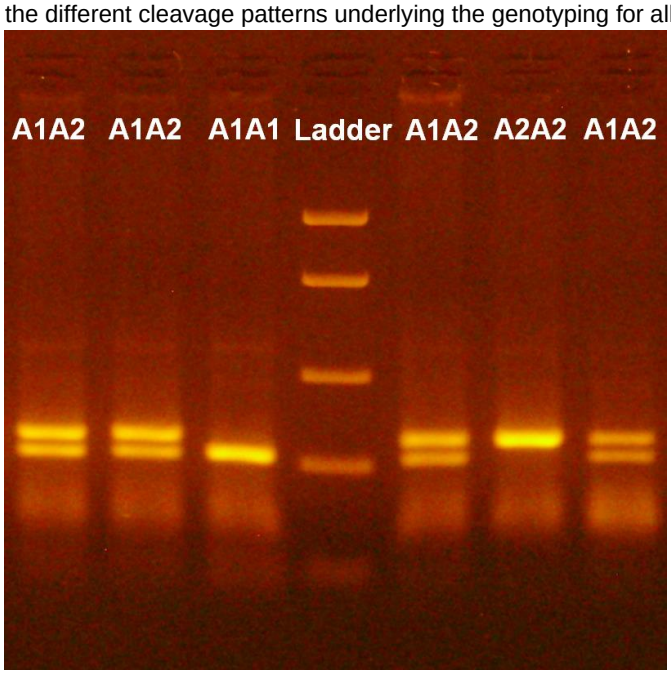

Figure 1: Cleavage pattern of genotypes isolated in agarose gel

3.1 Cows' allele and genotype frequencies of beta-casein A1/A2

Table 2 shows that the overall difference in allele frequency between the two colonies is only about 2 %. The chi-square test showed that there is no significant difference (chi-sqare=3.581; p=0.167) in the beta-casein allele frequencies between the cow herds of the two farms.

Table 2: Prevalence of beta-casein A1/A2 alleles and genotypes in cows from herd A (n=599) and herd B (n=324)

	"A" herd (n=599)	"B" herd (n=324)
A1 allele frequency	37.66 %	39.35 %
A2 allele frequency	62.47 %	60.65 %
A1A1 genotype frequency (n)	14.02 % (84)	9.26 % (30)
A1A2 genotype frequency (n)	47.28 % (283)	60.19 % (195)
A2A2 genotype frequency (n)	38.83 % (232)	30.55 % (99)

These data provide a detailed picture of the genetic composition of both the "A" and "B" herds in terms of beta-casein A1/A2 polymorphism. It's evident that the A1A2 genotype is the most prevalent in both herds, but the percentages and numbers of A2A2 and A1A1 genotypes vary between the two herds. The allele frequencies also differ slightly between the herds. This information can be valuable for breeding and management decisions in these dairy cattle herds. The A1A2 genotype is present in 60.65 % of the cows tested in the "B" herd. The second most common genotype is A2A2 on both farms. In terms of genotype frequencies, it can be observed that compared to the 38.83 % A2A2 genotype prevalence of the A site, the B site lags behind by almost 10 %. The lowest proportion of cows with genotype A1A1 was observed, 14.02 % in site A and 9.26 % in site B. These results are similar to those reported by Ristanic et al. (2020) for Serbian and Kamiński et al. (2006) for Polish conditions, who found a similar genotype distribution in the herds they studied. The A2 allele is present in more than 60 % of individuals. A similar finding was also reported by Ristanic et al. (2020) and OleńskI et al. (2010), while Antonopoulos et al. (2021) reported an allele frequency of 74 %.

3.2 Heifers' allele and genotype frequencies of beta-casein A1/A2

Table 3: Allele and genotype frequencies of beta-casein A1/A2 in the heifer population of farm A (n=150)

Allele and genotype frequencies	Heifer population
A1 allele frequency	4.33 %
A2 allele frequency	95.67 %
A1A1 genotype frequency (n)	0.66 % (1)
A1A2 genotype frequency (n)	7.33 % (11)
A2A2 genotype frequency (n)	92.00 % (138)

Table 3 shows the results of a genotyped heifer population of 150 heifers from colony A. We observed homozygous genotype A2 in 138 individuals, 92 % of the total number of heifers. The A1 homozygous genotype was observed in only one heifer. The heterozygous genotype frequency was 7.33 %. The A2 allele frequency was 95.67 %, which is the result of forward selection. When the genotype frequencies of the two cow groups were compared to the heifer group, the chi-square test determined a significant difference (chi-square=91.612; $p<0.001$), which indicated a considerable increase in the prevalence of the preferable A2 allele in the heifer group. The aim of this work was to evaluate the frequency of beta-casein variants in the offspring of cows inseminated with A2 homozygous semen. These are encouraging results for the achievement of the A2 milk production target. These genotype and allele frequencies in the heifer population provide insights into the genetic composition of this group of animals, which may have implications for future breeding decisions and milk production characteristics in the herd.

4. Conclusion

The beta-casein A1/A2 polymorphism has implications for human health. The proportion of lactose and milk protein-intolerant people in the population is steadily increasing, and we can offer them a healthier option to consume dairy products as opposed to plant-based drinks, which have a lower nutritional value compared to dairy products. As the current purchase price of raw milk is low and, compared to this, A2 milk from Austria is purchased at a significantly higher price, it makes sense to keep A2 milk-producing animals in production through targeted selection and to use insemination material from guaranteed homozygous A2 breeding bulls for inseminations.

In Hungary, more and more dairy farms are using A2 homozygous breeding bull semen. The finding that a high proportion of the animals (60.65 % and 62.47 %) in the breeding herds are A2, even without targeted selection, is an interesting and potentially significant observation. It is possible that the herds in question naturally have a higher prevalence of A2 alleles due to the genetic diversity within their populations. Some cattle breeds, or even specific lines within breeds, may naturally have higher frequencies of A2 alleles. Regardless of the underlying reasons, the observation that a significant proportion of animals in these herds are A2 carriers has practical implications. It may suggest that these herds are well-positioned to meet the growing demand for A2 milk. However, further research and monitoring of allele frequencies may be necessary to ensure the stability of A2 prevalence over time and to meet specific market demands. The observation that in the heifer herd produced by selection or deliberate mating, 92 % of the heifers will already produce A2A2 genotypes, with an A2 allele frequency of 95.67 %, is a significant finding. This suggests that targeted breeding practices and selection for A2 alleles have been highly successful in increasing the prevalence of A2A2 genotypes in the heifer population. Thus, our results suggest that targeted mating based on genetic information significantly increases the proportion of homozygous individuals after only one generation. It demonstrates the potential for dairy farms to adapt to changing market demands and consumer preferences by incorporating genetic management practices that enhance the production of A2 milk. The domestic commercialisation of A2 cow's milk could be of particular importance nowadays, as dairy farms need to take advantage of every opportunity to generate additional revenue in an environment of extremely high energy and feed prices. Genotyping of dairy herds can ensure that farms selling A2 milk on a targeted basis produce only A2 milk and that consumers' rights are not compromised if they choose to buy A2 milk at a higher price to maintain their health.

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