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# FADS2-23 POLYMORPHISM: A POTENTIAL MARKER FOR ECONOMIC TRAITS IN HOLSTEIN CATTLE SELECTION

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## ABSTRACT

Milk composition is nutritionally important to consumers and economically important to producers and processors. Fatty acid desaturase-2 (FADS2) is a component of the lipid metabolic pathway and converts essential fatty acids into long-chain polyunsaturated fatty acids. The work aimed to identify the impact of FADS2-23 marker polymorphism on milk production traits in Holstein cows. We established the genetic structure of the analysed population and the possible relationships between the genetic variants of the FADS2-23 marker and the average breeding values for the traits of milk production. The artificially created restriction site-PCR was used to identify genetic polymorphism for the FADS2-23 marker. We detected all genotypes: AA (8.10%), AG (41.90%), and GG (50%). The allele frequencies were 29.05% for the A allele and 70.95% for the G allele. The Holstein cattle kept in the Slovak Republic exhibit a slight increase in homozygosity (0.5878), polymorphism information content (0.3272), the effective number of alleles (1.7013) and a slight decrease in the level of possible variability realisation (41.42%). We observed a positive effect of the FADS2-23 marker polymorphism on the percentage fat content in the milk of Holstein cows. Genotype AA demonstrably increased the average breeding value for fat content in percent by 0.06% compared to genotype GG. Therefore, the rs109772589 polymorphism in the bovine FADS2 gene may be proposed as a potential genetic marker for fat percentage content in Holstein cattle.

**Keywords:** Holstein cattle, milk production, genetic structure, FADS2-23

## INTRODUCTION

Milk is a very complex nutrient system developed by mammalian evolution to support post-natal growth, and it is the first and only food for the offspring of mammals (**Melnik *et al.*, 2013; Urashima *et al.*, 2022**). Good nutrition and access to an adequate healthy diet are essential for growth and development, body maintenance and protection from diseases and as a result, in recent years there has been an increase in the interest in ensuring healthy nutrition (**Cena & Calder, 2020; Carey *et al.*, 2023**). The nutritional quality of milk depends on several factors such as genetics/breed (**Marchitelli *et al.*, 2013**), nutrition (**Šimko *et al.*, 2014**) and animal health (**Šťastná & Šťastný, 2015**). **Bauman *et al.*** (**2006**) and **Li *et al.*** (**2019**) state that milk fat is the essential component of milk, which has a significant impact on nutrition and is one of the most important economic properties of dairy cows' milk.

The milk fat is considered one of the most complex fats and is characterized by a high content of saturated fatty acids (SFA) and a low content of polyunsaturated fatty acids (PUFA). Changing the fatty acid profile of milk can improve the nutritional quality of milk and thus lead to improvements in dairy products that have a positive effect on human health. The PUFA concentrations in blood and tissue lipids are known to be closely related to several positive health consequences for cardiovascular disease morbidity and mortality, reducing excess fat, autoimmune disease, early visual, cognitive and motor development, mental health and psychiatric disorders, as well as early growth and development during pregnancy and early childhood (**Glaser *et al.*, 2010; Ibeagha-Awemu, 2014; Bykova *et al.*, 2023**). In addition, polyunsaturated fatty acids are involved in anti-cancer activity (**Yang *et al.*, 2013**) by eliminating or retarding the neoplastic process of tumours of various cancer types (**Bykova *et al.*, 2023**) and have a beneficial role in the prevention and treatment of various inflammatory immune disorders such as allergies (**Park *et al.*, 2013**). Fatty acid desaturase-2 (FADS2) is one of the rate-limiting enzymes in PUFAs biosynthesis and converts essential fatty acid into long-chain polyunsaturated fatty acids (LC-PUFA) by the introduction of a double bond between carbon atoms at positions Δ6 and Δ7 of fatty acid. The Δ-6-desaturase is a membrane-bound enzyme involved in the conversion of linoleic acid (18:2n-6) into γ-linolenic acid (18:3:n-6) and that of α-linolenic acid (18:3n-3) into stearidonic acid (18:4n-3) (**Proskura et al., 2019; Li et al., 2019, Zhang et al., 2021**). The FADS2 gene, located on BTA29, consists of 12 exons and encodes a protein 444 amino acids long. Ibeagha-Awemu et al. (2014) studied polymorphisms in the FADS2 gene to describe their associations with fatty acid content in milk and identified single nucleotide polymorphism (SNP) rs109772589 with G to A substitution located in the 3´UTR region with a significant effect on fatty acid content and labelled the marker as FADS2-23. **Beak *et al.*** (**2019**) and **Zhang *et al.*** (**2021**) suggested that the FADS2 enzyme is the initial and rate-limiting enzyme of the omega fatty acid metabolic pathway, and thus rs109772589 may still have potential as a genetic marker for omega fatty acid balance. However, regardless of the data collected , the relationship between the marker FADS2-23 and economic traits, such as milk, protein and fat yield expressed in kilograms, and the percentage of fat and protein in milk remains to be explained. Therefore, this study aimed to characterize the genetic polymorphism in marker FADS2-23 and to determine a possible connection with the economic traits of Slovak Holstein cows.

## MATERIAL AND METHODS

### Animals

In this study, were collected samples of hair from 1050 Holstein cows. The cows were from two farms in western Slovakia. The representative group of Holstein cows was formed based on approximately the same level of breeding, breeding technology and nutrition, and high genetic quality of herds based on the Slovak national system of genetic evaluation of Holstein cows with the same scheme of sampling and recording according to ICAR standards (**Interbull, 2025**). Based on the age at calving, the cows included in this study were classified (according to the mean, median and standard deviation) at each lactation into the three groups: (the first lactation: <23 months, 23–25 months and >25 months; the second lactation: <36 months, 36–39 months and >39 months; and the third lactation: <49 months, 49–53 months and >53 months). The basic traits used to describe the genotypic association with milk production were the Slovak Production Index (SPI) (**Interbull, 2025**), the breeding values of three traits expressed in kilograms: breeding values of milk, breeding value of fat and breeding value of protein and two traits expressed in percentage: breeding value of fat and protein percentage. Data on daily milk production, breeding values of milk traits and the Slovak Production Index were provided by the Slovak Breeding Services and the Holstein Cattle Breed Book of the Slovak Republic. Based on the index value and standard deviation we selected three qualitative groups of animals at each holding animal farm (high-quality animals, average and below-average animals). The highest genetic quality is represented by animals with production index values higher than the mean plus one standard deviation, average genetic quality are animals with production index value between plus and minus one standard deviation from the mean, and the lowest genetic quality is represented by animals with production index value lower than the mean minus one standard deviation. We used a general empirical rule (for normal distribution): one standard deviation (+-) from the mean represents approximately 68% of the data (**Moore *et al.*, 2012**). Genomic DNA was extracted using the commercial column kit QIAamp® DNA Mini Kit (Qiagen).

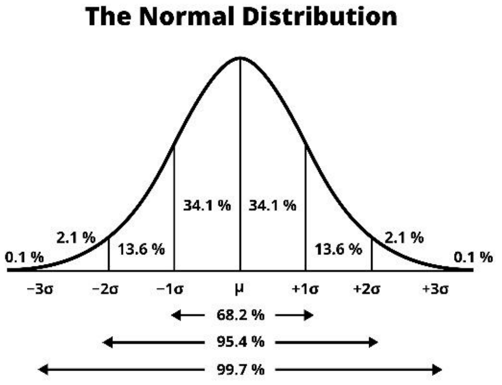

**Figure 1** Classification of Slovak Production Index (SPI) based on Mean and Standard Deviation.

The Slovak Production Index (SPI) values were classified into three performance categories (high, average, and low) using a statistical approach based on the normal data distribution. **High Production:** SPI values that are higher than the mean plus one standard deviation ($SPI > \mu + 1\sigma$). **Average Production:** SPI values falling within the range of the mean minus one standard deviation to the mean plus one standard deviation ($\mu - 1\sigma \leq SPI \leq \mu + 1\sigma$). **Low Production:** SPI values that are lower than the mean minus one standard deviation ($SPI < \mu - 1\sigma$).

### ACRS-PCR analysis and genotyping of FADS2-23

For genotyping of the SNP *rs109772589* (position 40,378,345 on BTA29 in the ARS-UCD2.0 reference genome), which ischaracterised by a G/A substitution in the 3 ´UTR region of the *FADS2-23* gene (Gene ID:521822) described by **Ibeagha-Awemu *et al.* (2014)**, we used the ACRS-PCR (artificially created restriction site-PCR) method. DNA primers used for PCR amplification (forward primer 5´-ACCCGTAGATAGCTCCAGGAGAGGCC-3´ and reverse primer 5´-GTGCTCCCATCGCAAAGCAG-3´) were designed using the program BatchPrimer3 v1.0 (**You *et al.*, 2008**). Each PCR reaction was carried out in a final volume of 20 µl and contained 2 µl template DNA, 1 U MyTaq HS DNA polymerase (**Bioline, London, UK**), 1X MyTaq Reaction buffer (5 mmol × dm$^{-3}$dNTPs, 15 mmol × dm$^{-3}$$MgCl_2$, stabilizers and enhancers) and 0.4 pmol × dm$^{-3}$of each primer. PCR cycling condition with gradient thermocycler C1000 Touch TM (**Biorad, Hercules, CA, USA**) included 94°C for 3 minutes followed by 35 cycles of 94°C for 5 seconds, 60°C for 20 seconds and 72°C for 30 seconds. The reaction was completed by the final elongation step of 72°C for 2 minutes. After PCR, amplified products of 372 bp were digested with 1 µl restriction endonuclease FastDigest *Msp*I (**Thermo Scientific BioScience, Waltham, MA, USA**). The proper restriction enzyme was searched by using the NEBcutter V2.0 program (**Vincze *et al.*, 2003**). The products of restriction digestion were separated on 2.5 % agarose gel (**Serva, Odessa, TX, USA**) with intercalacting dye GelRed$^{TM}$ (**Biotium, Fremont, CA, USA**) in 1×SB buffer (**Brody & Kern, 2004**) at 180 V for 15 minutes. The visualization and recording of restriction fragment results describing the presence of specific alleles was performed using UV light and the Olympus C-7070 documentary system.

### Statistical analysis

The genotypic structure of the population studied for polymorphism rs109772589 in the FADS2-23 marker was tested and the allelic frequencies were estimated using molecular genetics analyses. The Hardy-Weinberg equilibrium was tested by comparing expected and observed genotype frequencies through the Chi-square statistic. The effectiveness of allele occurrence was evaluated using the following parameters: observed heterozygosity ($He_{obs}$), expected heterozygosity ($He_{exp}$) (**Nie, 1973**), expected homozygosity (E) (**Crow & Kimura, 1970**), effective number of alleles (ENA) (**Crow & Kimura, 1970**), polymorphism information content (PIC) (**Boltstein *et al.*, 1980**), and level of possible variability realisation (V%) (**Crow & Kimura, 1970**).

For the association study, we used breeding values of the cows from official holstein national genetic evaluation (milk, protein and fat yield expressed in kilograms, and milk's protein and fat percentage) as the phenotype values. A single trait random regression test-day model for the estimation of dairy cattle production breeding values in the Slovak national genetic evaluation was used. The model included: fixed herd-test-day effect, fixed breed groups effect, fixed age, season and lactation groups effects. The permanent effect and additive genetic regression effect of cows were solved as random effects. The genetic lactation curves as the base of breeding values were modelled using the five regression parameters for all animals. The association between genotypes of the FADS2-23 marker and the breeding value of production traits was evaluated using a linear model with genotype and herd as fixed effect:

$$y=\mu+G_i+H_j+e_{ij},$$

where $y$ is the breeding value (kg milk, kg protein, kg fat, % protein, % fat), $\mu$ is the overall mean, $G_i$ is the fixed effect of genotype (i=1, 2, 3) $H_j$ is the fixed effect of herd (j=1, 2), and $e_{ij}$ is the residual effect.

The statistical analysis using the GLM (General Linear Model) procedures and the LSM (Least Squares Means) adjustment for multiple comparisons was performed using SAS Enterprise Guide version 9.3 (**SAS Inc., 2011**). A probability of less than 0.05 was considered significant (P value <0.05).

## RESULTS AND DISCUSSION

### SNP identification and genotyping

Polymorphism in the *FADS2-23* marker of Slovak Holstein cows was detected using ACRS-PCR. The digestion of 372 bp PCR product with *Msp*I differentiated allele A (372 bp) and G (347 bp, 25 bp). After electrophoresis in 2.5% agarose gel were observed all three genotypes (Fig. 2). The genotype and allele frequencies of Holstein cattle for *FADS2-23* are presented in Table 1.

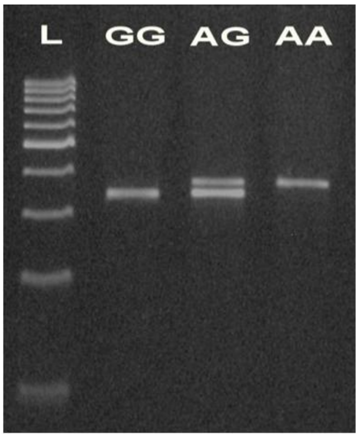

**Figure 2** Illustration of SNP polymorphism rs109772589 in *FADS2-23* genotypes on 2.5% agarose gel. L – GeneRuler 100 -1000 bp ladder (Thermo Scientific BioScience, Waltham, MA, USA); genotype GG (347 bp, 25 bp); genotype AG (372 bp, 347 bp, 25 bp); genotype AA (372 bp)

**Table 1** Genotype and allele frequencies of Holstein cattle for *FADS2-23*

| LOCUS | GENOTYPE FREQUENCIES | | | ALLELIC FREQUENCIES | | $\chi^2$ | P |
|---|---|---|---|---|---|---|---|
| | AA | AG | GG | A | G | | |
| ***FADS2-23*** | 0.0810 | 0.4190 | 0.50 | 0.2905 | 0.7095 | 0.058 | 0.9713 |

$\chi^2$—chi-square test, P —p-value (statistical significance)

Based on the χ2-test, we found that the difference between the expected and observed frequencies of bovine genotypes was not statistically significant, which means that an equilibrium state in the observed population was found by Hardy-Weinberg's law. The effectiveness of alleles *FADS2-23* in the tested population is shown in Table 2.

**Table 2** Effectiveness of *FADS2-23* alleles in Holstein cattle population

| LOCUS | ALLELES | He$_{obs}$ | He$_{exp}$ | PIC | E | ENA | V % |
|---|---|---|---|---|---|---|---|
| ***FADS2-23*** | *A; G* | 0.4190 | 0.4122 | 0.3272 | 0.5878 | 1.7013 | 41.42 |

He$_{obs}$—observed heterozygosity, He$_{exp}$—expected heterozygosity, PIC—polymorphism information content, E—expected homozygosity, ENA—effective number of alleles, V%—level of possible variability realization.

We found that the homozygous form of GG had the highest proportion in the cattle population, which we monitored with a frequency of 50%, the heterozygous genotype AG occurred with the lower frequency (41.90%) and the homozygous genotype AA had the lowest frequency, namely 8.10%, which means that the G allele was more numerous than the A allele and was 70.95% in the population. Our results contradict the findings of **Ibeagha-Awemu *et al.* (2014)** who, in the Canadian population of Holstein cattle observed the superiority of subjects with homozygous genotype AA with the frequency of up to 64%, less frequently occurring animals with the heterozygous genotype AG (33.3%) and the minimum is represented by homozygous genotype GG, representing only 2.6%. **Beak *et al.* (2019)** observed only heterozygous genotype AG in a population of Hanwoo cattle. In our study, we found a slight increase in homozygosity (0.5878) for the FADS2-23 marker in the Holstein cattle population, which also caused a slight decrease in the level of possible variability realization (41.42%). The effective number of alleles (1.7013) also corresponds to these observations.

### Effect of FADS2-23 genotype on milk production traits

Statistical analysis of the effect of genotypes *FADS2-23* marker on milk yield confirmed significant differences between genotypes for percentage fat content only. For the other monitored traits, we did not observe a statistically significant influence of different genotypes on the variability of these traits. The average breeding values for milk production traits to the analysed genotypes of the FADS2-23 marker are shown in Table 3.

**Table 3** Average breeding values for milk production traits of Holstein cows with different *FADS2-23* genotypes

| Breeding values | Genotype (LS mean±SE) | | | p |
|---|---|---|---|---|
| | *AA*<br>(n = 85) | *AG*<br>(n = 440) | *GG*<br>(n = 525) | |
| **BVM** | 909.1±541 | 791.52±541 | 806.80±572 | n.s |
| **BVF** | 19.51±10.96 | 14.59±16.81 | 11.15±14.73 | n.s |
| **BVF%** | -0.20$^{a}$±0.21 | -0.21$^{ab}$±0.21 | -0.26±0.21$^{b}$ | * |
| **BVP** | 23.53±13.18 | 19.89±14.47 | 19.64±15.08 | n.s |
| **BVP%** | -0.07±0.12 | -0.07±0.12 | -0.08±0.14 | n.s. |

BVM – breeding values for the yield of milk (kg); BVF – breeding values for the yield of fat (kg); BVF% – breeding values for the content of fat (%); BVP – breeding values for the yield of protein (kg); BVP% – breeding values for the content of protein (%); SE - standard error; p-values are for the significance of the genotype, we used Two-way ANOVA (genotype, herd); a, b – different superscripts within rows indicate statistically significant differences (*p≤0.05), we used Duncan Multiple Range Test; n.s. – non-significant.

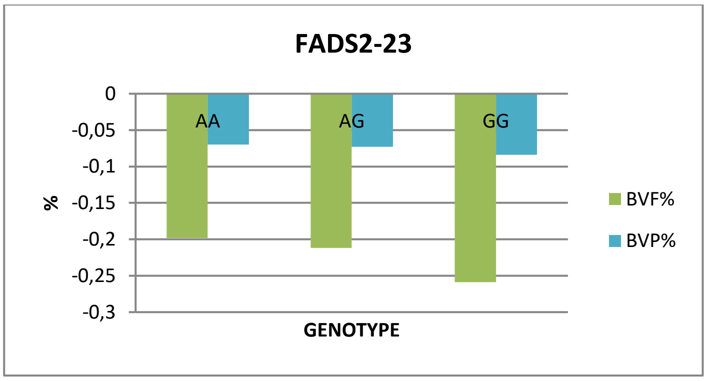

**Figure 3** Effect of different *FADS-23* genotypes on the average breeding values for content of protein and fat in percentages

BVF% – breeding values for the content of fat (%); BVP% – breeding values for the content of protein (%);

A higher percentage of milk fat was observed in cows with the AA genotype of the *FADS-23* marker. Genotype AA significantly increased the average breeding value of the fat content in percent by +0.06% compared to genotype GG.

Delta-6 desaturase is a key enzyme desaturating linoleic acid (LA) or α-linolenic acid (ALA) in the biosynthesis of polyunsaturated fatty acids (PUFAs) (**Shi *et al.*, 2016**). In connection with the FADS2 gene, recent studies have focused mainly on study fatty acid composition of bovine milk. **Ibeagha-Awemu *et al.* (2014)** argued that the marker FADS2-23 (rs109772589) is significantly associated with the content of omega-6 fatty acids dihomo-gamma linolenic and arachidonic, the GG genotype, which was predominant in our population, shows higher values of these acids, while allele A significantly reduces the content of dihomo-gamma linolenic acid by 0.008 g.100g$^{-1}$ of total fat in milk and arachidonic acid by 0.0071 g.100g$^{-1}$ of total fat in milk**. Proskura *et al.* (2019**) studied the effect of rs209202414 polymorphism in the FADS2 on the fatty acid composition of bovine milk. They observed statistically significant associations between the rs209202414 genotypes and the lauric, behenic, lignoceric, oleic, eicosatrienoic, and docosadienoic acids in Jersey cows. In Polish Holstein-Friesian cows they found significant associations between docosahexaenoic and erucic acids. The use of molecular genetic methods to identify animals with a variant of the FADS gene that increases desaturation activity and the subsequent involvement of these animals in breeding programs can lead to an increase in the content of unsaturated fatty acids in milk. The studies of **Ibeagha-Awemu *et al.*** (**2014**) and **Proskura *et al.*** (**2019**) suggest the rs109772589 and rs209202414 in the bovine FADS2 gene as a potential genetic marker for fatty acid composition in cattle milk.

In this study, the FADS2-23 gene was studied in Holstein cattle for economic traits. We performed association studies of the FADS2-23 marker and it has been confirmed that AA genotype determines a higher percentage of milk fat.

## CONCLUSION

The results of this study demonstrate a significant positive association between the polymorphism of the FADS2-23 marker and the milk fat percentage in Holstein cows. In particular, individuals carrying the AA genotype showed a higher proportion of milk fat. These findings suggest that the rs109772589 polymorphism within the bovine FADS2 gene may represent a promising genetic marker associated with this economically important production trait.

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