



Effect of *DGAT1*, *FASN* and *PRL* genes on milk production and milk composition traits in Simmental and crossbred Holstein cattle

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Received: 1 December 2016; Accepted: 6 January 2017

ABSTRACT

The aim of this study was to evaluate the effects of polymorphisms in the acylCoA diacylglycerol acyltransferase (*DGAT1*), fatty acid synthase (*FASN*) and prolactin (*PRL*) genes on milk production traits and the potential influence of the *PRL* gene on SCC of Simmental and crossbred Holstein cattle as well as comparatively among them. A total of eighty-three cows were genotyped by PCR-RFLP method. Crossbred Holsteins with *DGAT1* genotype KA had significantly higher fat content than cows with AA genotype and for *FASN* diplotype TW/AR had significantly higher fat content compared to diplotype AR/AR. In the observed group of animals, there was no association found between *PRL* SNP G8398A and milk production traits and SCC (SCS). For all three genes, cross bred Holstein had significantly higher milk yield, fat and protein content in certain genotypes/diplotypes. The results of combined genotype analysis of the three SNPs showed that GG/TWAR/KK genotype combination had the lowest SCS.

Key words: Crossbred Holstein, *DGAT1*, *FASN*, Milk production, *PRL*, Simmental

The physiological regulation of milk production is a complex process under the control of many genetic and hormonal factors. Molecular genetics attempts to identify genes influencing polygenic economically relevant traits in farm animals. Many studies with diverse breeds of cattle have indicated *DGAT1*, *FASN* and *PRL* as promising candidate genes for milk production and composition (Kadlečová *et al.* 2014, Singh *et al.* 2015, Li *et al.* 2016).

The *DGAT1* gene encodes acyl-CoA diacylglycerol acyltransferase 1 enzyme that catalyzes the final step in triglyceride synthesis (Kadlečová *et al.* 2014). A non-conservative dinucleotide polymorphism (AA>GC) causing lysine to alanine substitution (K232A) is associated with variations in milk production and milk fat content (Kuhn *et al.* 2004, Molee *et al.* 2012). Fatty acid synthase (*FASN*) regulates the *de novo* biosynthesis of long chained fatty acids (FA) and considered to be a potential candidate gene for fat content and FA composition in milk and meat (Ciecierska *et al.* 2013, Li *et al.* 2016). Two non-synonymous SNPs with a potential link to lactation traits were found - 5848bpA/G that causes amino acid

substitution from threonine to alanine (T1950A) and 5863bpT/C from tryptophan to arginine (W1955R) (Matsumoto *et al.* 2012). The same authors found that the genotypes of the T1950A corresponded to those of W1955R, so they suggested that these SNPs were completely linked in Holstein cattle, as was also reported by Abe *et al.* (2009) in Japanese Black cattle. Prolactin is a multifunctional polypeptide hormone that is secreted mainly by the anterior pituitary gland (Othman *et al.* 2011), but also produced by a number of other cells and tissues, including the mammary gland (Mehmannavaz *et al.* 2009). It participates in many biological functions associated with reproduction and lactation (Mehmannavaz *et al.* 2009) as well as in homeostatic biological functions including immune functions (Othman *et al.* 2011, Singh *et al.* 2015). A silent A-G mutation in the exon 3 of the *PRL* gene (position 8398), is often used for genetic characterization of cattle populations by PCR-RFLP method (Othman *et al.* 2011).

The present study aimed to estimate the frequency of the polymorphic alleles of *DGAT1*, *FASN* and *PRL* genes in Simmental and crossbred Holstein cattle, and to evaluate the relationship of these polymorphisms on milk production parameters, as well as the potential influence of the *PRL* gene on SCC. In addition, the interaction of SNPs of investigated genes and their combined influence on economic traits were evaluated.

MATERIALS AND METHODS

A total of 83 milk samples were taken from 49 Simmental and 34 crossbred Holstein (Holstein × Simmental) cows.

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Information about milk traits (daily milk yield, fat and protein content, and somatic cell score - SCS) for the first lactation cows were provided by the Croatian Agricultural Agency, which is responsible for milk recording systems according to the International Committee for Animal Recording rules (ICAR 2011).

Genomic DNA was isolated from milk samples (350 µl) using PathoProof™ DNA Extraction Kit. To amplify the targeted fragments through PCR, specific primers and programs were used according to literature (*DGAT1* K232A - Kuhn *et al.* 2004; *FASN* T1950A/ W1955R - Matsumoto *et al.* 2012; *PRL* G8398A-Othman *et al.* 2011). PCR reaction mix was made up of 50 ng genomic DNA, 200 nM of each primer, 1 × EmeraldAmp MAX HS PCR Master Mix, and

additionally, 5% DMSO in the case of *DGAT1* gene. Amplified PCR products (5 µl) were digested by *Cfr* I (*DGAT1*), *Hha* I (*FASN* T1950A), *Nci* I (*FASN*W1955R) and *Afa* I (*PRL*) enzyme at 37°C in a dry bath incubator for 3–10 h following the supplier's manual. The resulting fragments for *DGAT1* (allele K, one uncut fragment of 411 bp; allele A, two fragments of 208 and 203 bp) (Fig. 1a), *FASN* (T1950A: allele A, one uncut fragment of 336 bp; allele G, two fragments of 262 and 74 bp; W1955R: allele T, one uncut fragment of 336 bp; allele C, two fragments of 247 and 89 bp) (Fig. 1b) and *PRL* (allele G, one uncut fragment of 294 bp; allele A, two fragments of 162 and 132 bp) (Fig. 1c) were separated on a 3% agarose gel. As the genotypes of the T1950A corresponded to those of W1955R, they were later analysed as diplotypes (TW/TW, TW/AR, AR/AR).

Calculation of allele and genotypes frequencies was based on direct counting. Polymorphisms were tested for deviation from the Hardy-Weinberg equilibrium using the χ^2 test. Gene heterozygosity (H_E) and fixation index (F_{IS}) were performed by POPGENE32 software, version 1.32 (Yeh *et al.* 2000). All traits were analyzed by a least square method using GLM (general linear models) procedure in the statistical package SAS (SAS 2009).

$$Y_{ijkl} = \mu + B_i + G_{ij} + C_k + b_1(X_{ijkl} - \bar{x})^2 + b_2(t_{ijkl} - \bar{t}_{ijkl})^2 + e_{ijkl}$$

where μ is the overall mean; breed (B_i), gene (G_j), calving season (C_k) are fixed class effects. Age at first calving (x_{ijklmn}) and days in milk (t_{ijklmn}) were fitted in the model as quadratic regression. Before GLM analysis, SCS was normalized using formula $SCS = \text{LOG}_2(\text{SCC}/100.000) + 3$ (Wiggans and Shook 1987).

RESULTS AND DISCUSSION

Frequencies and Hardy-Weinberg equilibrium: The allele/haplotype and genotype/diplotypes frequencies of all genes are shown in Table 1. In both groups, regarding *DGAT1* gene, the genotype KA prevailed over both homozygous genotypes with allele K as less frequent (Table 1). The results from the current study differed from those reported in a previous study for the Simmental breed, where the frequency for the allele K was 0.055 (Hanusova *et al.* 2014). Results for crossbred Holstein were in accordance with earlier findings (0.36) (Molee *et al.* 2012) and Holstein (0.28) (Kadlecova *et al.* 2014).

In *FASN*, diplotype AR/AR was the most frequent in both Simmental and crossbred Holstein cattle, whereas diplotype TW/TW was not found in the crossbreeds. Consequently, the frequencies of TW haplotype were low in both groups (Table 1). Similar results were reported by Abe *et al.* (2009) in Holstein (0.171), Angus (0.015) and Hereford (0.071) cattle, while the same authors stated a frequency of 0.667 in Japanese Black. The same allele was found in frequencies of 0.37 and 0.29 (Ciecierska *et al.* 2013, Li *et al.* 2016) in Holstein breed. This wide range of frequencies indicated the large genetic variability of the mentioned SNP in different breeds (Ciecierska *et al.* 2013).

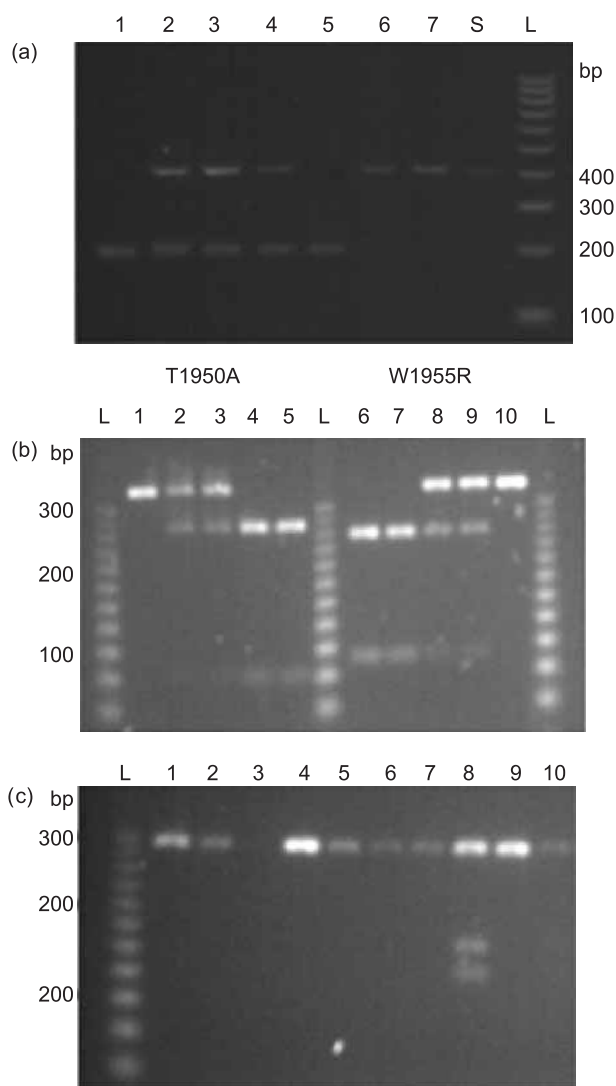


Fig. 1. PCR-RFLP gel electrophoresis. (a) *DGAT1* SNP K232A (AA > GC). Lane 6–8, KK genotype; lane 2–4, KA genotype; lane 1 and 5, AA genotype; L, 100 bp ladder. (b) *FASN* SNPs T1950A (A > G) and W1955R (T > C). T1950A: lane 1, AA genotype; lane 2–3, AG genotype; lane 4–5, GG genotype; L, 25 bp ladder; W1955R: lane 10, TT genotype; lane 8–9, TC genotype; lane 6–7, CC genotype. (c) *PRL* SNP G8398A (A > G). Lane 1–7 and 9–10, GG genotype; lane 8, AG genotype (AA genotype was not found in the studied population); L, 25 bp ladder.

Table 1. Allele/haplotype and genotype/diplotype frequencies of *DGAT1*, *FASN* and *PRL* in Simmental and crossbred Holstein cattle

Gene	Breed	Allele/ haplotype	Allele/ haplotype frequency	Genotype/ diplotype	n	Genotype/ diplotype frequency	χ^2	P-value	Hetero- zygosity	Fixation index (F_{IS})
<i>DGAT1</i>	Simmental	K	0.458	KK	6	0.125	5.64	<0.05	0.667	-0.343
		A	0.542	KA	32	0.667				
				AA	10	0.208				
	Crossbred Holstein	K	0.455	KK	5	0.152	1.63	>0.05	0.606	-0.222
		A	0.545	KA	20	0.606				
				AA	8	0.242				
<i>FASN</i>	Simmental	TW	0.194	TW/TW	2	0.041	0.02	>0.05	0.306	0.022
		AR	0.806	TW/AR	15	0.306				
				AR/AR	32	0.653				
	Crossbred Holstein	TW	0.132	TW/TW	0	0	0.79	>0.05	0.265	-0.156
		AR	0.868	TW/AR	9	0.265				
				AR/AR	25	0.735				
<i>PRL</i>	Simmental	A	0.053	AA	0	0	0.15	>0.05	0.106	-0.056
		G	0.947	AG	5	0.106				
				GG	42	0.894				
	Crossbred Holstein	A	0.094	AA	0	0	0.34	>0.05	0.188	-0.104
		G	0.906	AG	6	0.188				
				GG	26	0.813				

Table 2. Effect of *DGAT1*, *FASN* and *PRL* gene on daily milk yield (DMY) and fat and protein contents, as well as SCS in *PRL*, in Simmental and crossbred Holstein cattle. Values are expressed as means with standard error by least squares estimates.

Gene	Genotype	Simmental				Crossbred Holstein			
		DMY (kg) LSM±SE	Fat (%) LSM±SE	Protein (%) LSM±SE	SCS LSM±SE	DMY (kg) LSM±SE	Fat (%) LSM±SE	Protein (%) LSM±SE	SCS LSM±SE
<i>DGAT1</i>	KK	19.60±1.99	3.87±0.32	3.67±0.16	-	17.32±2.32	3.99±0.47	3.60±0.19	-
	KA	17.71±0.97 ^a	3.92±0.16	3.60±0.08	-	21.33±1.04 ^a	4.36±0.18 ^b	3.81±0.09	-
	AA	18.38±1.35	3.77±0.22	3.57±0.11	-	19.36±1.81	3.62±0.30 ^b	3.79±0.15	-
<i>FASN</i>	TW/TW	21.19±3.00	3.43±0.51	3.49±0.26	-	-	-	-	-
	TW/AR	18.93±1.30	3.93±0.22 ^b	3.61±0.11	-	22.46±1.52	4.68±0.26 ^{b,c}	3.83±0.13	-
	AR/AR	18.45±0.86 ^a	3.99±0.15	3.64±0.07	-	19.83±1.03 ^a	3.98±0.18 ^c	3.79±0.09	-
<i>PRL</i>	AG	18.33±1.85 ^a	4.33±0.33	3.57±0.16	9.98±0.77	25.24±2.11 ^{a,c}	3.94±0.38	3.64±0.07	8.45±0.88
	GG	18.93±0.78	3.94±0.14	3.64±0.07 ^b	8.66±0.32	19.97±0.92 ^c	4.32±0.17	3.88±0.08 ^b	8.41±0.40

^{a,b,c}Means with same superscripts within same row or column of same gene differ significantly at $P < 0.05$.

Most animals included in the study were genotyped as GG homozygous genotype for the *PRL* gene, while genotype AA was not found in the studied population. Accordingly, there was a noticeably higher incidence of allele G in both groups (Table 1) as well as in literature – 0.931 in Holstein (Mehmannavaz *et al.* 2009) and 0.75 in Holstein crossbreed (Singh *et al.* 2015).

The distribution of genotypes was not within the Hardy-Weinberg equilibrium only in Simmental breed in *DGAT1* gene. This could be attributed to the fact that breeding is not random, but planned and done by artificial insemination. A slight excess of heterozygosity, revealed by negative F_{IS} , was found in all SNPs, except in Simmental breed in *FASN* gene (Table 1).

Effects of *DGAT1* K232A polymorphism: In many studies

that analyzed the connection of various genes with milk production traits, *DGAT1* gene stood out as a potential genetic marker (Molee *et al.* 2012). It has been found that it has a pronounced effect on milk production traits, especially allele K on fat production (Kadlecova *et al.* 2014).

The effects of the genotypes on lactation traits showed that in crossbred Holstein the KA genotype had a significantly higher fat content than the AA genotype ($P < 0.05$) (Table 2). These results were in accordance with the findings of other authors (Molee *et al.* 2012, Hanusova *et al.* 2014, Kadlecova *et al.* 2014) who reported that the K allele is responsible for variations in the amount of milk fat.

In the studied population, there was no observed

association between SNP K232A and protein content ($P>0.05$) (Table 2). On the other hand, previous research found a significant positive effect of allele K on milk protein (Molee *et al.* 2012). Molee *et al.* (2012) suggested that these results clearly show that *DGAT1* gene not only affects fat content, but also other aspects of milk composition, especially protein, but that the function of this gene in the synthesis of milk proteins still needs to be clarified.

Most previous studies found a negative effect of allele K on milk yield (Molee *et al.* 2012, Kadlecova *et al.* 2014). In this study, the SNP K232A had no effect on milk yield ($P>0.05$) (Table 2). Furthermore, it was observed that crossbred Holstein cows with genotype KA had significantly higher milk yield compared to cows of the same genotype of the Simmental breed ($P<0.05$) (Table 2).

Effects of *FASN* T1950 and W1955R polymorphisms: Fatty acid synthase (*FASN*) is an enzyme that regulates the *de novo* biosynthesis of long chain FA and plays an essential role in the determination of FA synthesis and release of newly synthesized FAs (Matsumoto *et al.* 2012, Li *et al.* 2016).

Matsumoto *et al.* (2012) reported that T1950 and W1955 R mutation had a significant impact on fat content of Holstein cattle. In their study, diplotype AR/AR had a higher fat content than diplotype TW/AR (diplotype TW/TW was excluded from analysis due to the insufficient number of samples). In contrast, in this study, crossbred Holstein with diplotype TW/AR had significantly higher fat content compared to diplotype AR/AR ($P<0.05$). The findings of this study are consistent with the research by Ciecierska *et al.* (2013). There was no effect found in the Simmental breed ($P>0.05$). From this, it may be concluded that the association of this *FASN* SNP varies depending on breed and population.

Crossbred Holstein cows with TW/AR diplotype had significantly higher fat content ($P<0.05$) compared to the Simmental breed. Also, crossbred cattle of diplotype AR/AR had significantly higher milk yield ($P<0.05$) (Table 2).

Effects of *PRL* G8398A polymorphism: Prolactin is a polypeptide hormone involved in over 300 functions in the body, including water and electrolyte balance, growth and development, endocrinology and metabolism, behaviour, reproduction and immuno-regulation.

Results of previous studies showed variable effects of the *PRL* gene depending on the breed and population studied. In the investigated group of animals, there was no association found between SNP G8398A and milk production traits (Table 2). Only genotype AG of the crossbred Holstein had significantly higher milk yield than genotype GG ($P<0.05$) (Table 2). Similar results were obtained by Mehmannaavaz *et al.* (2009), who reported that allele G in the Holstein breed has a negative impact on milk and protein yield. However, Singh *et al.* (2015) found that genotypes AA and GG had significantly ($P<0.05$) greater milk yield than genotype AG in crossbred Holstein (Indian Frieswal).

Among groups, crossbred Holstein cows with genotype AG had significantly higher milk yield compared to the

same genotype in Simmental cows ($P<0.05$). Moreover, crossbred cows of GG genotype had significantly higher protein content compared to cows with the same genotype in the Simmental breed ($P<0.05$) (Table 2).

Apart from its well-known effect on reproduction and lactation, prolactin also acts as an immunoregulatory hormone on inflammatory properties. In their research, Boutet *et al.* (2007) found that prolactin stimulates pro-inflammatory activity in mammary epithelial cells. They also positively correlated prolactin in chronic mastitis affected quarters to SCC and the number of neutrophils. In conclusion, the authors suggest that prolactin might modulate the inflammatory response in the bovine mammary gland, and play a role in mastitis pathogenesis. In a previous study on crossbred Holstein, it was found that genotype AA had a significantly lower SCC than genotypes AG and GG (Singh *et al.* 2015). In contrary, in this study no statistically significant differences were found among genotypes for SCC (SCS) ($P>0.05$) (Table 2).

Effect of genotype combination analysis: As it is known, the genotype combination effect is a reflection of the interactions of multiple SNPs because the genotype effect of one SNP may be influenced by other SNPs (Zheng *et al.* 2011). Therefore, analysis of genotype combinations is superior to the analysis of one single SNP. In this work, 10 combined genotypes, consisting of three SNPs from three genes (*PRL* G8398A, *FASN* T1950/W1955R, *DGAT1* K232A), were identified and the association of combined genotypes, milk production parameters and SCS were analyzed. A significant association was found for SCS ($P<0.05$) (data not shown). As the combination of genotypes had a significant effect on SCS ($P=0.019$), while the effect of breed was not significant ($P=0.209$), all cows were further analyzed as a single group (Table 3).

Table 3. Effects of combinations of three SNPs from three genes (*PRL* G8398A, *FASN* T1950/W1955R, *DGAT1* K232A) on cattle SCS (n=60)

Combination of genotype (<i>PRL</i> / <i>FASN</i> / <i>DGAT1</i>)	SCS LSM±SE
AG/TWAR/KA	11.12±0.99
AG/ARAR/KA	9.36±0.77
AG/ARAR/KK	6.64±1.52
GG/TWTW/KA	9.35±1.54
GG/TWAR/KK	4.62±2.07
GG/TWAR/KA	8.22±0.47
GG/TWAR/AA	10.35±1.08
GG/ARAR/KK	7.88±0.61
GG/ARAR/KA	9.18±0.32
GG/ARAR/AA	8.06±0.48
	P-values
B*	0.209
GC**	0.019
B × GC***	0.070

*B, effect of breed; **GC, effect of genotype combination; ***B × GC, effect of breed and genotype combination interaction.

The statistical results show that the cows with AG/TWAR/KA genotype combination had the highest SCS, and GG/TWAR/KK genotype combination had the lowest SCS (Table 3). This may indicate that increasing the frequency of GG/TWAR/KK combined genotype in a dairy herd could have a beneficial effect of lowering the average SCS. However, for better comprehension the interaction between these three genes should be studied further.

In this study, milk yield and fat and protein contents were assessed per genotype/diplotype of the three analyzed genes in the Simmental breed and crossbred Holstein cattle, as well as comparatively between them. For all three genes, crossbred Holstein had significantly higher milk yield for certain genotypes/diplotypes (genotype KA of *DGAT1* gene, diplotype AR/AR of *FASN* gene and genotype AG of *PRL* gene). In addition, crossbred Holstein with the TW/AR diplotype of *FASN* gene had significantly higher fat content, and for the *PRL* gene, GG crossbreds had significantly higher protein content. In the combined genotype analysis, the only association was found with SCS. Cows with GG/TWAR/KK genotype combination had the lowest SCS. These results suggest that the three analyzed genes influence milk performance traits, but the interaction between these three genes should be studied further so that they may be implemented in breeding programs to improve milk performance.

ACKNOWLEDGEMENTS

In memoriam to Professor Igor Stokovic who tragically passed away during the trials, but greatly contributed in the study idea, design and problem solving.

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