Scientific Papers. Series D. Animal Science. Vol. LXVIII, No. 1, 2025
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

TESTING GENETIC ASSOCIATIONS OF THE SNP C.1053C>T POLYMORPHISM FROM DGAT1 GENE WITH MILK QUALITATIVE PARAMETERS IN RIVER BUFFALO (*Bubalus bubalis*)

Viorica COȘIER[1], Daniel INCICĂU[2], Lucas COȘIER[3], Mariana TĂTARU[4], Alexandru RUSU[5], Monica TRIF[6], Călin LAȚIU[7]

[1]University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Faculty of Animal Sciences and Biotechnologies, Department of Genetics, 3-5 Calea Manastur Street, 400372, Cluj-Napoca, Romania
[2]University of Zürich, Department of Molecular Life Sciences, Winterthurerstrasse 190, 8057, Zürich, Switzerland
[3]ETH Zürich, Department of Computer Science, Universitätstrasse 6, 8092, Zürich, Switzerland
[4]University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Faculty of Veterinary Medicine, Department of Preclinical Sciences, 3-5 Calea Manastur Street, 400372, Cluj-Napoca, Romania
[5]Biozoon GmbH, D-27572 Bremerhaven, Germany
[6]Centre for Innovative Process Engineering (Centiv) GmbH, Department of Food Research, 28857, Syke, Germany
[7]University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Faculty of Animal Sciences and Biotechnologies, Department of Fundamental Sciences, 3-5 Calea Manastur Street, 400372, Cluj-Napoca, Romania

Corresponding author email: viorica.cosier@gmail.com

Abstract

Improving milk yield and milk composition are objectives of interest in the selective breeding of animals. Milk fatty acids and proteins are important in the manufacturing of many buffalo dairy products, the best-known being mozzarella cheese. DGAT1 is part of the DGAT gene family (diacylglycerol acyltransferases) that codify key enzymes involved in the final step of triacylglycerol biosynthesis in various tissues and milk. In many cattle breeds the non-synonymous polymorphism K232A, from the 8th exon of the DGAT1 is a genetic marker, with major effects on milk yield and composition. In river buffaloes, the presence of the fixed K allele strongly indicates an increase in milk fat content as a result of selection. To date, only a few polymorphisms from the buffalo DGAT1 gene have been associated with milk composition. This study aims to test the associations between the synonymous polymorphism c.1053C>T from the 13th exon of the buffalo DGAT1 gene with milk qualitative parameters (fat percentage, protein percentage, and lactose content) in the Romanian buffalo breed. The properties of 200 milk samples were analysed using a mixed-effect model applied to longitudinal data spanning four seasons. Results indicated that the season had a significant impact ($p < 0.001$) on the fat and protein percentage, as well as lactose content in the milk. Additionally, a noteworthy association ($p < 0.001$) was observed between buffalo age and fat percentage. However, no significant association was found between genotype and milk quality.

Key words*: buffalo, DGAT1 gene, polymorphisms, milk composition, genetic associations.*

INTRODUCTION

Comparative analysis of the composition of buffalo's milk and cattle shows that the superior nutritional values of the milk and the dairy products of buffaloes are beneficial for human health (Contarini and Povolo, 2013; Khan et al., 2017, Marcone et al., 2017; Reddi et al., 2018). Compared to cow's milk, buffalo milk is richer in almost all milk nutrients (fat, lactose, protein, casein, ash and Ca, vitamins C and E) (Zicarelli, 2004; Abd El-Salam and El-Shibiny, 2011; Khan et al., 2019). Apart from being renowned for their mozzarella cheese production, buffaloes possess numerous other superior characteristics compared to cattle: meat composition is considerably lower in total fat content, including cholesterol (Naveena et Kiral, 2014; Guerrero-Legarreta et al., 2020), and the species' total methane production is lower

(Moss et al., 2000; Calabro et al., 2013; Khan et al., 2021). These properties, which match a perspective shift in consumers towards a healthier diet, have led the global buffalo population to increase at a steady rate of 14.7 ± 5.0% per annum, in the last 50 years (1968-2018). Buffalo milk production accounts for only 15.14% of global milk production. Europe contributes 2.8% to this market, but also with the highest added value obtained from Italian mozzarella. (Minervino et al., 2020). Advances in the molecular field have led to more efficient approaches in improving the genetic value of animals, using molecular information in breeding schemes. Many QTLs (Quantitative Trait Loci) with a major effect on milk yield and composition were first identified in the cattle genome, in the centromeric region of BTA14 that includes the DGAT1 gene, encoding the diacylglycerol acyltransferase1 (Dekkers, 2004; Bouwman et al., 2011). The K232A polymorphism inside the DGAT1 gene has been shown to be associated with milk composition and yield in various cattle breeds, like Holstein-Friesian, Fleckvieh, and Jersey (Grisart et al., 2002; Spelman et al., 2002; Winter et al., 2002; Kuhn et al., 2004; Ripoli et al., 2006). In several cattle breeds, the A allele was associated with milk yield, and the K allele with higher fat and protein content (Grisart et al., 2002; Winter et al., 2002; Spelman et al., 2002; Thaller et al., 2003; Weller et al., 2003; Gautier et al., 2007; Argov-Argaman et al., 2013). In buffaloes, the first studies on gene polymorphisms were performed using the cattle reference genome. Following the recent sequencing, re-sequencing, and annotation of the buffalo genome, numerous studies have subsequently concentrated on pinpointing genomic polymorphisms linked to significant economic traits (Yuan et al., 2007; Mishra et al., 2007; Shi et al., 2012; Cardoso et al., 2015; de Freitas et al., 2016; Silva et al., 2016; Khan et al., 2021). Initial investigations in buffalo have focused on the K232A polymorphism observed in cattle, due to the strong correlation of the DGAT1 polymorphism with milk FP in cattle (Conte et al., 2010). In contrast, research indicates that within water buffalo populations, the K allele appears to be consistently prevalent, owing to both natural and artificial selection processes spanning numerous generations (Tantia et al., 2006; Shi et al., 2012). This phenomenon provides an explanation for the elevated fat content observed in buffalo milk. DGAT (diacylglycerol acyltransferases) is a key enzyme group involved in the final steps of triacylglycerol biosynthesis in the adipose tissue, intestines, liver, and milk. Generally, these enzymes are controlled by a gene family DGAT (Yen et al., 2008), which includes several subfamilies, DGAT1 and DGAT2, DGAT3, and WAX-DGAT identified in different organisms (Turchetto-Zolet et al., 2016; Bhatt-Wessel et al., 2018). Comparative analysis of collinearity and gene synteny reveals a high conservation degree between buffalo and cattle genomes (Wang et al., 2021; Liu et al., 2020). Many other candidate genes for lipid metabolism have been identified in the buffalo genome by different authors, including A2M, DGAT1, GHRL, LEP, MC4R, PRL, SCD, SREBF1, STAT1, and TG. Another 4 genes (APOB, CTNND2, FHIT, and ESSRG) were identified as candidates in Genome-Wide Association Studies (GWAS) (Camargo et al., 2015; Du et al., 2019). GWAS is considered the golden standard in genomic selection technology. Currently, few GWAS exist on river buffaloes (*Bubalus bubalis*), and few or no associations of the DGAT1 gene polymorphisms with milk yield and composition were established (Liu et al., 2020). The most important polymorphisms associated with milk yield and composition were identified in the 5' UTR region and the 13th and 17th exons of the DGAT1 gene (Yuan et al., 2007; Cardoso et al., 2015; Li et al., 2018), while other SNPs were found in various buffalo breeds, but no associations were studied (Mishra et al., 2007; Yuan et al., 2007; Raut et al., 2012; Silva et al., 2016; Gu et al., 2019). For this reason, the main goal of this research is to test the associations of the DGAT1 polymorphism with lactogenic parameters. The DGAT1 gene polymorphism, specifically c.1053C>T, was detected through transcript analysis of the DGAT1 gene in Italian Mediterranean and Romanian buffalo breeds (Gu et al., 2019). Thus, the scope of this study is to test the associations between this polymorphism and fat percentage (FP), protein percentage (PP) and milk lactose content, in a cohort of 50 female Romanian buffaloes, from the Research and Development Station for Buffalo Breeding Sercaia (Brasov County).

MATERIALS AND METHODS

Biological material

The formation of the Romanian buffalo breed took place in the early 1960s through the introgression of the European river buffalo *(Bubalus bubalis*) with the Bulgarian Murrah breed. Today over 90% of the buffalo livestock is raised on small farms from Transylvania (Brașov, Cluj, Sibiu, and Mureș counties) (Matiuti et al., 2020). Two different populations, with distinct genetic backgrounds were identified by Noce et al (2021) in genetic diversity studies (90k SNPs): the Mera buffalo population in Cluj County, distributed among small farms, with a genetic background admixture from Italian-Mediterranean, Hungarian, and Bulgarian origins; and the animals from the Research and Development Station for Buffalo Breeding Sercaia (Brasov County), which have a defined genotype, due to intense selection programs and environmental adaptation to the cold climate.

In the present study, we proposed to analyse this population of Sercaia Station, to test the associations between c.1053C>T polymorphism, from the 13th exon of DGAT1 gene, and milk production characteristics. The animals from this facility are in the official production control program.

Blood and milk samples collection

For the gene polymorphisms study and associations procedures with milk qualitative parameters, we used blood samples collected from 50 buffalo females, randomly selected from the Research and Development Station for Buffalo Breeding Sercaia, Brasov County (45o50'01.4" N and 23o27'07.1"). The animals, in different lactation phases, were fed identically during the sampling period. From these selected females, 200 individual morning milk samples (50 ml/sample and 50 sample/season) were collected in four successive seasons (autumn, winter, spring and summer). Vacutainers with K2EDTA were used to collect blood samples, which were immediately refrigerated (4ºC ± 1ºC) and transported to the laboratory for DNA extraction. The same procedure was applied for milk samples, which were collected in 100 ml Falcon tubes (50 ml/sample). Throughout the sampling process, particular attention was paid to animal welfare.

In silico analysis of the c.1053C>T polymorphism of the DGAT1 gene

In this study we aimed to identify the c.1053C>T polymorphism from the 13th exon, in a 483 bp DNA fragment of the buffalo DGAT1 gene. In the buffalo genome, this gene is located on the 15th (BBU15) chromosome, has 17 exons and a total length of ~8,8 kb (NCBI Gene ID 102390126). The sequence of interest corresponds to the genomic positions g.81,363,550 - g.81,364,033 in the NCBI genomic sequence NC_059171.1/NCBI (GCF_019923935.1/NDDB_SH_1 *Bubalus bubalus*, Murrah breed).

The sequence of interest was amplified by PCR with a specific primers pair (forward 5'-ATGGGCGACCGCGGCGG-3' and reverse 5' – GGAGCATGGGCTTGTAGA-3' (Gu et al., 2017) corresponding to the region between the 12th and 15th exons on the GenBank DGAT1 gene sequence DQ886485.1 of water buffalo. The two sequences produced a perfect alignment in the Blast analysis, and the position of the SNP mutation, recognized by *Dde*I restriction enzyme, is highlighted in Figure 1. The C>T mutation, which falls at the genomic position g.81,363,844 corresponds to the mutation position - c.1053C>T in the NCBI sequence DQ120929.1 *Bubalus bubalis* diacylglycerol O-acyltransferase 1 (DGAT1) mRNA, complete cds. The C>T is a synonymous mutation that does not modify the amino acid sequence ($Y^{Ala/Ala}$) specified by the two codons.

DNA extraction

The DNA samples were obtained from 200 µl blood, collected on vacuum tubes with K2EDTA, with Quick DNA Microprep Plus Kit, following the manufacturer's instructions (BioZyme, St Joseph, Missouri, USA). All samples were analysed to determine the DNA concentration and purity on Spectrophotometer NanoDrop ND1000, and range between 1.8-2 purity (optimum), and a quantity between 50 and 180 ng/µl.

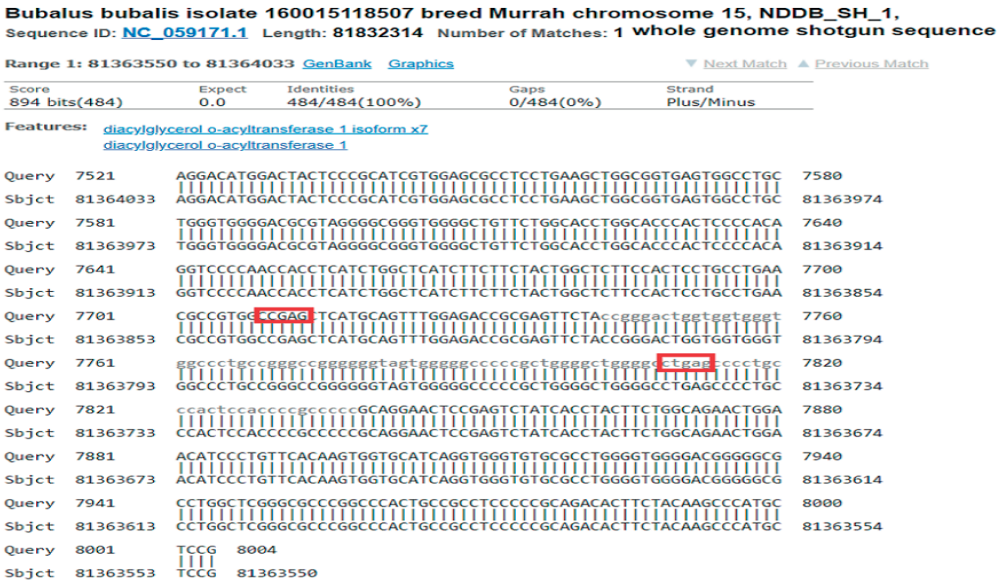

Figure 1. Blast analysis of the genomic sequence NC_059171.1 (g. 81,363,550 - g.81,364,033) and DGAT1 gene from GenBank (DQ886485.1), amplified by PCR (exons: 12-15) and the position of the *Dde*I restriction enzyme sites with potential mutation C>T (DQ886485.1-7710)

Genetic analysis

A final volume of 25 µl PCR mix was set up for each sample with 5x FirePol Master mix (BioZyme) – 5 µl; 1 µl of each primer (forward and reverse) (10 pmol/µl), DNA template – 2 µl (100 ng) and molecular grade water -16 µl. The following conditions were used for the PCR assay: 97ºC for 4 min; followed by 35 cycles at 97ºC (45 s), 63.2ºC (45 s), and 72ºC (45 s); and a final extension step at 72ºC for 8 min, maintaining 4ºC thereafter, in an Eppendorf MasterCycler (Eppendorf, Germany). All amplimers, of 483 pb, were subjected to digestion with the *Dde*I restriction enzyme. The enzymatic digestion mixture includes 10 µl PCR product, 5U of *Dde*I endonuclease (New England, Biolab), and 2.5 µl of the enzyme's buffer. For digestion, an overnight incubation at 37 ºC was performed. The digestion products were analysed in agarose gel (2.5%) electrophoresis, with 1x TBE buffer, and stained with RedSafe (Intron Biotechnology). The 483 DNA fragment amplified by PCR can be cleaved by the *Dde*I restriction enzyme in two positions, that correspond to TT genotypes, with two visible bands on the agarose gel, of 189/194 bp and 100 bp. After digestion, the C allele also presents two fragments but with different lengths of 289 bp and 194 bp. The heterozygous (CT) genotype can be distinguished in the agarose gel through a profile of 289 bp, 189/194 bp, and 100 bp.

Milk quality parameters analysis

In each season (autumn, winter, spring, summer) 50 milk samples (50 ml/sample) collected from buffalo females were analysed on an Ultrasonic milk analyser (Lactoscan MCCW/UASVM of Cluj-Napoca). The working method aimed to determine milk quality parameters (fat % (FP), protein % (PP), and lactose content).

Statistical analysis

Allelic and genotype frequencies were determined by simple counting. Possible deviations from Hardy-Weinberg equilibrium (HWE) were tested by chi-squared test, using the HWE.chisq function call from the genetics package (1.3.8.1.3) in R (Table 1).

The association analysis between the SNP locus and milk traits was performed using a linear mixed-effects model due to the nested structure of the longitudinal data (Bates et al., 2015). Apart from the genotype, two other variables that may also influence milk production were inserted in the model as fixed effects, the season (categorical with four levels), and the age group (categorical with three levels, discretizing the

buffalo age in ranges [4, 7], [7, 10], and [10, 20] years). The milk samples were collected over the course of seasons, with multiple observations per buffalo. Because of that, milk properties for the individual buffaloes are correlated throughout seasons, hence the necessity to introduce nested random effects (intercepts) for each buffalo into the model.
The model formula is the following:

$$y_{hjkl} = \beta_0 + \beta_1 \times GENOTYPE_h + \beta_2 \times SEASON_j + \beta_3 \times AGE_{GROUP_k} + ID_l + \epsilon_{hjkl}$$

Where: Y_{hjkl} represents the modeled phenotype of the milk (FP, PP, lactose) for each buffalo ID_l in season $SEASON_j$ and AGE_{GROUPk}; β_0 is the overall mean of the measured phenotype; β_1, β_2, β_3 are the regression coefficients of the genotype, season and age group classes respectively; ϵ_{hjkl} represents the residual error. $GENOTYPE_h$, $SEASON_j$ and AGE_{GROUPk} are set as the fixed effects of the model and the ID_l of each buffalo as the random effect. The model assumptions for multicollinearity were tested using Variance Inflation Factors (VIFs). For the lactose and protein percentage residuals, normality tests including the Shapiro-Wilk, Anderson-Darling, and Lilliefors (Kolmogorov-Smirnov) indicated no significant departure from normality, with p-values well above the 0.05 threshold. However, for fat percentage residuals, these normality tests failed to meet the assumption of normality. Consequently, a sensitivity analysis was conducted, to evaluate the model's robustness across each 25% segment of quantiles, to ensure the model's reliability despite the non-normality observed in the fat percentage residuals. All analyses were performed using R (R Core Team 2023). The restricted maximum likelihood (REML) estimates of the parameters in the linear mixed-effects models were determined by using lmer function in the lme4 package (1.1.33). Additionally, ggplot2 package (3.4.2) was used for data visualization.

RESULTS AND DISCUSSIONS

From the PCR amplimers digested with *Dde*I enzyme, all possible genotypes, CC, CT, and TT (Figure 2) have been identified in the analysed population. The results of allelic and genotype frequencies and the departure from HWE can be viewed in Table 1.

Table 1. Allelic and genotype frequencies and the departure from Hardy-Weinberg equilibrium

Genotype (SNP c.1053 C>T) frequency			No of animals	Allele frequency		HWE
CC	CT	TT		C	T	χ^2=0.0868
0.26	0.52	0.22	N=50	0.52	0.48	*P*=0.7835

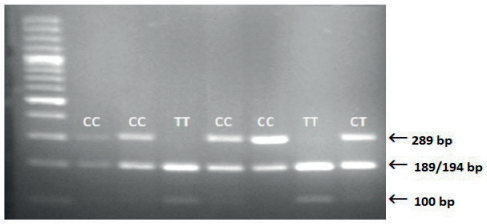

Figure 2. Genotypes of DGAT1 gene (c.1053C>T polymorphism) in Romanian buffalo breed. Line 1:100 bp step ladder (Promega, USA), lines 2-8: CC genotypes with 289 bp and 194 bp bands, TT genotypes with 189/194 bp and 100 bp bands, and CT genotypes with 289, 189/194 and 100 bp bands

The results of the gene polymorphism study in the Romanian buffalo breed, regarding the C>T mutation (c.1053) in exon 13 of the DGAT1 gene, are consistent with those reported by Gu et al. (2019) in studies on differential gene expression. They indicate a higher frequency of the C allele (0.52) and a lower frequency of the T allele (0.48) in the Romanian buffalo compared to the Italian Mediterranean breed, in which the C allele was determined in a lower proportion (C=0.46). To the best of our knowledge, currently, the synonymous mutation c.1053 C>T has only been identified in these two breeds. Querying the relevant literature on this mutation shows the presence of only the T allele at this locus in the Chinese buffalo breed (Yuan et al., 2007), while in various other water buffalo breeds (Bhadawari, Mehsana, Murrah, and Surti buffaloes), including Indian Murrah, studies show the presence of only the C allele (Mishra et al., 2007; Venkatachalapathy et al., 2008). The descriptive statistics of the milk composition parameters are presented in Table 2.

Table 2. Descriptive statistics of the milk composition parameters in Romanian Buffalo breed

Trait	Season	N	Mean ± s_x	SD	Minimum	Maximum	CV%
FP	Autumn	50	8.015 ± 0.027	0.188	7.322	8.501	2.35
	Winter	44	7.974 ± 0.057	0.377	7.111	8.719	4.73
	Spring	49	7.945± 0.045	0.317	7.308	8.487	3.99
	Summer	50	7.735 ± 0.022	0.154	7.466	8.224	1.99
	All	193	7.916 ± 0.021	0.290	7.111	8.719	3.66
PP	Autumn	50	4.028 ± 0.013	0.090	3.912	4.347	2.24
	Winter	44	4.093 ± 0.032	0.213	3.809	4.592	5.21
	Spring	49	3.964 ± 0.027	0.187	3.702	4.391	4.71
	Summer	50	4.353 ± 0.023	0.164	3.999	4.610	3.77
	All	193	4.111 ± 0.016	0.225	3.702	4.610	5.46
Lactose	Autumn	50	4.138 ± 0.022	0.162	3.738	4.453	3.93
	Winter	44	3.896 ± 0.016	0.109	3.716	4.113	2.81
	Spring	49	3.930 ± 0.028	0.197	3.605	4.466	5.01
	Summer	50	3.813 ± 0.023	0.164	3.524	4.230	4.31
	All	193	3.946 ± 0.015	0.202	3.524	4.466	5.12

N = number of samples, s_x = standard error; SD = standard deviation, CV = coefficient of variation, Fat Percentage FP, Protein Percentage PP

Regarding fat percentage and protein percentage, the minimums were observed in winter (7.11% FP) and spring (3.70% PP), while for lactose the lowest value was found in summer (3.52 lactose content). Furthermore, the winter and spring seasons generally show the highest variances, with a maximum standard deviation of 0.37% FP and 0.21% PP in winter, and a variance of ~0.2 in lactose content during spring.

Association Study

Tables 3, 4, and 5 present the estimates of the linear mixed effect models on the association between genotype, season, and age groups. For all mixed effect models, the reference group was chosen among the buffalos between 4 and 7 years old, having the CC genotype, and for the measurements taken in the Autumn season. The CC genotype represents the wild type allele, while the age group and season were the first groups among the observations. All found differences are present between these reference groups and others. Moreover, in the case of the genotype, we are interested in whether any polymorphism is associated with a change in the milk quality.

The overall mean fat percentage in milk samples was estimated to be 7.967% (95% confidence interval (CI): 7.861%, 8.073%) at the reference level (for genotype CC, in Autumn for buffaloes in the age group [4,7] years). In the case of protein percentage, the average was found to be 4.046% (95% CI: 3.975%, 4.117%), while the mean lactose content of the milk was 4.150 (CI: 4.085, 4.216) within the reference group.

To interpret the fixed effects of the model, the estimated coefficients can be used. For instance, for milk samples taken in summer from buffaloes in the same age group, we observe an increase in protein percentage of +0.325% (CI: +0.262%, +0.389%) compared to the reference level.

Table 3. Coefficient estimates β of the linear mixed-effect models for fat percentage of the milk samples

Mixed Effect Model of Fat Percentage (FP)				
Independent Variables		Estimated Coefficients β	Lower 95% CI	Upper 95% CI
INTERCEPT		7.967	7.861	8.073
GENOTYPE	CC	Ref.		
	CT	0.020	-0.070	0.109
	TT	0.071	-0.034	0.176
SEASON	Autumn	Ref.		
	Winter	-0.043***	-0.148	0.061
	Spring	-0.070***	-0.172	0.031
	Summer	-0.280***	-0.381	-0.179
AGE GROUP	[4, 7]	Ref.		
	(7, 10]	-0.053***	-0.143	0.035
	(10, 20]	0.123***	0.035	0.212

95% CI = Confidence Interval; *p < 0.05, **p < 0.01, ***p < 0.001; Ref. stands for reference in the analysis.

Table 4. Coefficient estimates of the linear mixed-effect models for protein percentage of the milk samples

Mixed Effect Model of Protein Percentage (PP)				
Independent Variables		Estimated Coefficients β	Lower 95% CI	Upper 95% CI
INTERCEPT		4.046	3.975	4.117
GENOTYPE	CC	Ref.		
	CT	-0.028	-0.090	0.034
	TT	-0.057	-0.130	0.016
SEASON	Autumn	Ref.		
	Winter	0.068***	0.002	0.134
	Spring	-0.064***	-0.128	0.000
	Summer	0.325***	0.262	0.389
AGE GROUP	[4, 7]	Ref.		
	(7, 10]	0.003	-0.059	0.065
	(10, 20]	0.026	-0.036	0.087

95% CI = Confidence Interval; *p < 0.05, **p < 0.01, ***p < 0.001; Ref. stands for reference in the analysis

The mixed effects model reveals a notable association ($p < 0.001$) between buffalo age groups and seasons concerning FP as seen in Table 3. Likewise, both the PP and lactose models exhibit a significant ($p < 0.001$) association with seasons (Tables 4 and 5).

The combined effect of milk parameters for different genotype groups and seasons can be seen in Figure 3. Notably, the results reveal the absence of influences of the genotypes on milk parameters. A possible source of statistical ambiguity is the limited number of samples available. Specifically, we note that all of the outliers excluded from the analysis are found in Winter and Spring, as these seasons have overall higher variability between individuals. We posit that as a synonymous mutation, further studies on this mutation and testing for linkage disequilibrium between loci, could provide additional information about this polymorphism.

Insights from Research on the Buffalo Genome

The limited number of association studies on gene polymorphisms and productive traits in the water buffalo is primarily explained by the later appearance of the *Bubalus bubalis* genomic reference (Williams, 2017) compared to the *Bos taurus* genome. Thus, this study aims to complement the research on the possible influences of gene mutations on the expression of milk productive parameters such as fat percentage (FP), protein percentage (PP), and lactose.

Table 5. Coefficient estimates of the linear mixed-effect models for the lactose in the milk samples

Mixed Effect Model of Lactose				
Independent Variables		Estimated Coefficients β	Lower 95% CI	Upper 95% CI
INTERCEPT		4.150	4.085	4.216
GENOTYPE	CC	Ref.		
	CT	0.025	-0.031	0.080
	TT	-0.003	-0.068	0.062
SEASON	Autumn	Ref.		
	Winter	-0.241***	-0.306	-0.177
	Spring	-0.207***	-0.270	-0.144
	Summer	-0.325***	-0.387	-0.262
AGE GROUP	[4, 7]	Ref.		
	(7, 10]	-0.036	-0.092	0.019
	(10, 20]	-0.041	-0.096	0.014

95% CI = Confidence Interval; *p < 0.05, **p < 0.01, ***p < 0.001; Ref. stands for reference in the analysis

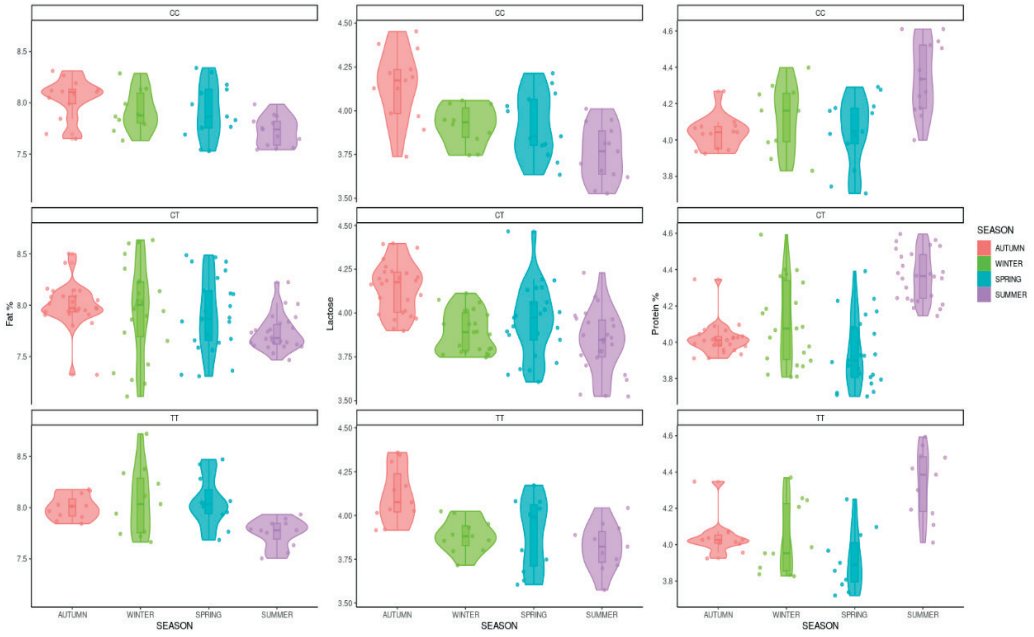

Figure 3. Fat Percentage, Lactose and Protein Percentage for different genotype groups and seasons. The violin plot combines the probability density and box plots, while individual milk sample measurements are visible as dots

A priori hypotheses about the effects of synonymous mutations in genes, which can affect gene expression through various molecular processes such as splicing, mRNA stability, folding, and translation (Wang et al., 2021), have led to the identification of several synonymous mutations associated with productive traits, including the DGAT1 gene (Cardoso et al., 2015). Certain synonymous mutations in genes, although they do not change the specified amino acid in the protein, can be in linkage disequilibrium with other SNPs in the genome, affecting the expression of complex traits (Bouwman et al., 2011).

The role of the DGAT1 gene in the biosynthesis and storage of milk fat has been demonstrated in double-knockout lactation-deficient mice (Smith et al., 2000). The mutations that can have substantial effects on the differentiated expression of genes affect the regulatory regions of the genes (promoters, enhancers, silencers, and insulators). Among the studies identifying mutations in the DGAT1 gene, the VNTR polymorphism in the 5'UTR region of the DGAT1 gene (Cardoso et al., 2015) is notable, accounting for 32% of the additive genetic variance (VA) of milk fat percentage (FP) in the Murrah buffalo breed.

Other studies of SNP associations in buffalo have been tested in specific breeds and need to be confirmed in association studies for each breed individually (Tantia et al., 2006; Mishra et al., 2007; Raut et al., 2012; Venkatachalapathy et al., 2013; Cardoso et al., 2015; de Freitas et al., 2016). Thus, significant associations ($P<0.05$) have been identified between the nonsynonymous polymorphism g.11785 T> C in exon 17, which changes the amino acids Ala484Val and the milk protein percentage (PP) and fat percentage (FP) in the Murrah buffalo breed, and in the Italian buffalo breed (De Freitas et al., 2016; Li et al., 2018). In the river buffalo, an SNP polymorphism (g.8330T>C) in exon 13 of the gene appears to be associated with the protein and fat percentage in milk (Li et al., 2020). Several other studies identified different SNP polymorphisms in intron 1, exons 7-9, and intron 7 of the DGAT1 gene from different buffalo breeds, but many without association studies (Mishra et al., 2007; Yuan et al., 2007; Raut et al., 2012; Silva et al., 2016).

GWAS, despite being a standard in genomic selection, face limitations due to the reliance on the number of allelic variants on microchips. The lead SNP with the tightest association p-value may not always be the causal SNP. This

necessitates considering numerous other SNPs in linkage disequilibrium as potentially causal (Chatterjee and Ahituv, 2017). A comprehensive GWAS, investigating the DGAT1 gene's impact on milk's fatty acid composition was conducted by Cruz et al. in 2019. The authors utilized various statistical methods to elucidate its correlation with milk composition variability. This study established the DGAT1 gene as a key determinant in the variation of milk's fatty acid profile, in conjunction with two other genes, PLBD1 and MGST1, particularly when DGAT1 was included as a fixed variable in the analysis. The exclusion of DGAT1 as a fixed covariate led to the discovery of numerous SNPs. Therefore, the effects of genomic polymorphisms should be cautiously analysed to identify optimal associations with quantitative traits.

Additionally, Liu et al. (2020) linked polymorphisms in the DGAT2, DGAT2L7, and DGAT2Ln genes to the quantity of milk fat in river buffalo through GWAS explaining for the extended role of the genes in the DGAT family. Further genetic studies have pinpointed specific polymorphisms in a haplotype of the DGAT1 gene that are associated with both, milk protein and fat percentages (Liu et al., 2020).

CONCLUSIONS

The composition and fat content of milk are crucial factors in the dairy sector, influencing both the economic success of farms and the consumer preference for high nutritional value dairy products. Notably, buffalo milk is valued for its lower levels of saturated fatty acids and cholesterol, making it an alternative dietary choice and a key point of breeding programs aimed at enhancing milk yield and quality.

In the context of the population examined in this research, we suggest that future studies should aim to sequence the entire exon in a broader sample group. Considering the role of synonymous polymorphism, additional research on this specific mutation, along with analyses of linkage disequilibrium among loci, could yield deeper insights into the nature and implications of this genetic variation.

REFERENCES

Abd El-Salam, M., & El-Shibiny, A (2011). A comprehensive review on the composition and properties of buffalo milk. *Dairy Sci & Technol, 91* (6):663-699. https://doi.org/10.1007/s13594-011-0029-2

Argov-Argaman, N., Mida, K., Cohen, B.C., Visker, M., & Hettinga, K. (2013). Milk fat content and DGAT1 genotype determine lipid composition of the milk fat globule membrane. *PLoS One, 8*(7). https://doi.org/10.1371/journal.pone.0068707

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Soft., 67*(1), 48.

Bhatt-Wessel, B., Jordan, T.W., Miller, J.H., & Peng, L. (2018). Role of DGAT enzymes in triacylglycerol metabolism. Arch. Biochem. *Biophys.*, 655, 1-11. https://doi.org/10.1016/j.abb.2018.08.001

Bouwman, A.C., Bovenhuis, H., Visker, M.H., & Van Arendonk, J.A. (2011). Genome-wide association of milk fatty acids in Dutch dairy cattle. *BMC Genet*., 12, 43. https://doi.org/10.1186/1471-2156-12-43

Calabro, S., Infascelli, F., Tudisco, R., Musco, N., Grossi, M., Monastra, G., & Cutrignelli, M.I. (2013). Estimation of in vitro methane production in buffalo and cow. *Buffalo Bull*., 32, 924–927.

Camargo, G.D., Aspilcuetaborquis, R.R., Fortes, M., Portoneto, R., Cardoso, D.F., Santos, D., Lehnert, S.A., Reverter, A., Moore, S.S., & Tonhati, H. (2015). Prospecting major genes in dairy buffaloes. *BMC Genom.,* 16, 1–14. https://doi.org/10.1186/s12864-015-1986-2

Cardoso, D. F., de Souza, G. F. P., Aspilcueta-Borquis, R. R., Araujo Neto, F. R., de Camargo, G. M. F., Hurtado-Lugo, N. A., Scalez, D. C. B., de Freitas, A. C., Albuquerque, L. G., & Tonhati, H. (2015). Variable number of tandem repeat polymorphisms in DGAT1 gene of buffaloes (*Bubalus bubalis*) is associated with milk constituents. *J. Dairy Sci*., 98, 3492–3495. https://doi.org/10.3168/jds.2014-8729

Chatterjee, S., & Ahituv, N. (2017). Gene Regulatory Elements, Major Drivers of Human Disease. *Ann. Rev. Genomics Hum. Genet.,* 18, 45-63. https://doi.org/10.1146/annurev-genom-091416-035537

Contarini, G., & Povolo, M. (2013) Phospholipids in milk fat: composition, biological and technological significance, and analytical strategies. *Int. J. Mol. Sci., 14*(2), 2808-2831. https://doi.org/10.3390/ijms14022808

Conte, G., Mele, M., Chessa, S, Castiglioni, B, Serra, A, Pagnacco, G, & Secchiari, P. (2010) Diacylglycerol acyltransferase 1, stearoyl-CoA desaturase 1, and sterol regulatory element binding protein 1 gene polymorphisms and milk fatty acid composition in Italian Brown cattle. *J. Dairy Sci., 93*(2), 753-63. https://doi.org/10.3168/jds.2009-2581

Cruz, V.A.R., Oliveira, H.R., Brito, L.F., Fleming, A., Larmer, S., Miglior, F., & Schenkel, F.S. (2019). Genome-Wide Association Study for Milk Fatty Acids in Holstein Cattle Accounting for the DGAT1. *Gene Effect. Animals (Basel), 9*(11): 997 doi: 10.3390/ani9110997.

de Freitas, A.C., de Camargo, G.M., Stafuzza, N.B., Aspilcueta-Borquis, R.R., Venturini, G.C., Dias, M.M., Cardoso, D.F., & Tonhati, H. (2016). Genetic association between SNPs in the DGAT1 gene and milk production traits in Murrah buffaloes. *Trop. Anim. Health Prod., 48*(7), 1421-6 https://doi.org/10.1007/s11250-016-1110-x

Dekkers, J. C. (2004). Commercial application of marker- and gene-assisted selection in livestock: strategies and lessons. *J. of Anim. Sci., 82* (suppl_13), E313-E328. https://doi.org/10.2527/2004.8213_supple313x

Du, C., Deng, T., Zhou, Y., Ye, T., Zhou, Z., Zhang, S., Shao, B., Wei, P., Sun, H., Khan, F.A., et al. (2019). Systematic analyses for candidate genes of milk production traits in water buffalo (*Bubalus bubalis*). *Anim. Genet*., 50, 207–216. https://doi.org/ 10.1111/age.12739

Gautier, M., Capitan, A., Fritz, S., Eggen, A., Boichard D., & Druet T. (2007). Characterization of the DGAT1 K232A and variable number of tandem repeat polymorphisms in French dairy cattle. *J. Dairy Sci*., 90, 2980–2988. https://doi.org/10.3168/jds.2006-707

Grisart, B., Coppieters, W., Farnir, F., Karim, L., Ford, C., Berzi, P., Cambisano, N., Mni M., Reid, S., Simon, P., Spelman, R., Georges, M., & Snell, R. (2002). Positional candidate cloning of a QTL in dairy cattle: Identification of a missense mutation in the bovine DGAT1 gene with major effect on milk yield and composition. *Genome Res*., 12, 222–231. https://doi.org/10.1101/gr.224202

Gu, M., Cosenza, G, Nicolae I., Bota A, Guo, Y., Di Stasio, L., & Pauciullo, A. (2017) Transcript analysis at DGAT1 reveals different mRNA profiles in river buffaloes with extreme phenotypes for milk fat. *J. Dairy Sci., 100*(10), 8265-8276. https://doi.org/10.3168/jds.2017-12771

Guerrero-Legarreta, I., Napolitano, F., Cruz-Monterrosa, R., Mota-Rojas, D., Mora, P., Ramírez-Bribiesca, E., Bertoni, A., Berdugo, J., & Braghieri, A. (2020) A River buffalo meat production and quality: sustainability, productivity, chemical composition and sensory properties. *J Buffalo Sci*., 9, 159-169. https://doi.org/10.6000/1927-520X.2020.09.17

Khan, I.T., Nadeem M., Imran, M., Ayaz M., Ajmal, M., Ellahi, M.Y., & Khalique, A. (2017). Antioxidant capacity and fatty acids characterization of heat treated cow and buffalo milk. *Lipids Health Dis., 16*(1), 163. https://doi.org/10.1186/s12944-017-0553-z

Khan, I.T., Nadeem, M., Imran, M. et al. (2019). Triglyceride, fatty acid profile and antioxidant characteristics of low melting point fractions of Buffalo Milk fat. *Lipids Health Dis*., 18, 59. https://doi.org/10.1186/s12944-019-0995-6

Khan, M.Z., Ma, Y., Ma, J., Xiao, J., Liu, Y., Liu, S., Khan, A., Khan, I.M., & Cao, Z. (2021). Association of DGAT1 With Cattle, Buffalo, Goat, and Sheep Milk and Meat Production Traits. *Front Vet Sci*., 8, 712470. https://doi.org/10.3389/fvets.2021.712470

Kühn, C.G., Thaller A., Winter, O. R. P., Bininda-Emonds, B., Kaupe, G., Erhardt, J., Bennewitz, M., Schwerin, I., & Fries, R. (2004). Evidence for multiple alleles at the DGAT1 locus better explains a quantitative trait locus with major effect on milk fat content in cattle. *Genetics*, 167, 1873–1881. https://doi.org/10.1534/genetics.103.022749

Li, J., Liu, S., Li, Z., Zhang, S., Hua, G., Salzano, A., & Yang, L. (2018). DGAT1 polymorphism in Riverine buffalo, Swamp buffalo and crossbred buffalo. *J. Dairy Res, 85*(4), 412-415. https://doi.org/10.1017/s0022029918000468

Liu, J., Wang, Z., Li, J. et al. (2020). Genome-wide identification of Diacylglycerol Acyltransferases (DGAT) family genes influencing Milk production in Buffalo. *BMC Genet.*, 21, 26. https://doi.org/10.1186/s12863-020-0832-y

Marcone, S., Belton, O., & Fitzgerald, D. J. (2017). Milk-derived bioactive peptides and their health promoting effects: a potential role in atherosclerosis. *Br. J. Clin. Pharmacol, 83*(1), 152-162. https://doi.org/10.1111/bcp.13002

Matiuti, M., Matiuti, C. L., Garleac, C., & Hutu, I. (2020). Particularities of buffalo breeding in Romania. *One Health Int. J.*, 6, 1–5.

Minervino, A.H.H., Zava, M, Vecchio, D, Borghese. (2020). *Bubalus bubalis*: A Short Story. *Front Vet. Sci.,* 7, 570413. https://doi.org/10.3389/fvets.2020.570413

Mishra, B., Tantia, M. S., Kumar, S. T. B., & Vijh, R. K. (2007). Characterization of the DGAT1 gene in the Indian buffalo (*Bubalus bubalis*). *Genet. Mol. Biol.,* 30, 1097–1100. https://doi.org/10.1590/S1415-47572007000600012

Moss, A. R., Jouany, J. P., & Newbold, J. (2000). Methane production by ruminants: its contribution to global warming. *Ann. De Zootech*., 49, 231–253. https://doi.org/10.1051/animres:2000119

Naveena, B.M., & Kiran, M. (2014). Buffalo meat quality, composition, and processing characteristics: Contribution to the global economy and nutritional security, *Anim. Front., 4*(4), 18–24. https://doi.org/10.2527/af.2014-0029

Noce, A., Qanbari, S., González-Prendes, R., Brenmoehl, J., Luigi-Sierra, M. G., Theerkorn, M., Fiege, M. A., Pilz, H., Bota, A., Vidu, L., Horwath, C., Haraszthy, L., Penchev, P., Ilieva Y., Peeva T., Lüpcke W., Krawczynski, R., Wimmers, K., Thiele, M., & Hoeflich, A. (2021). Genetic Diversity of *Bubalus bubalis* in Germany and Global Relations of Its Genetic Background. *Front. Genet*., 11, 610353. https://doi.org/10.3389/fgene.2020.610353

R Core Team (2023). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Raut, A.A., Kumar, A., Kala, S.N., Chhokar, V., Rana, N., Beniwal, V., Jaglan, S., Samuchiwal, SK., Singh, J.K., & Mishra, A. (2012). Identification of novel single nucleotide polymorphisms in the DGAT1 gene of buffaloes by PCR-SSCP https://doi.org/10.1590/s1415-47572012005000043.

Genet. Mol. Biol., 35, 610–613. https://doi.org/10.1590/s1415-47572012005000043

Reddi, S., Shanmugam, V.P., Tanedjeu, K.S., Kapila, S., & Kapila, R. (2018). Effect of buffalo casein-derived novel bioactive peptides on osteoblast differentiation. *Eur J Nutr., 57*(2), 593-605. https://doi.org/10.1007/s00394-016-1346-2

Ripoli, M.V., Corva, P., & Giovambattista, G. (2006). Analysis of a polymorphism in the DGAT1 gene in 14 cattle breeds through PCR-SSCP methods. *Res. Vet. Sci., 80*(3), 287-90. https://doi.org/10.1016/j.rvsc.2005.07.006

Shi, D.S., Wang, J., Yang, Y., Lu, F.H., Li, X.P., Liu, Q.Y. (2012). DGAT1, GH, GHR, PRL and PRLR polymorphism in water buffalo (*Bubalus bubalis*). *Reprod Domest Anim., 47*(2), 328–34. https://doi.org/10.1111/j.1439-0531.2011.01876.x

Silva, C.S., Silva Filho, E., Matos, A.S., Schierholt, A.S., Costa, M.R., Marques, L.C., Costa, J.S., Sales, R.L., Figueiró, M.R., & Marques, J.R. (2016). Polymorphisms in the DGAT1 gene in buffaloes (*Bubalus bubalis*) in the Amazon. *Genet. Mol. Res.,15*(3). https://doi.org/10.4238/gmr.15038720

Smith, S.J, Cases, S., Jensen, D.R., Chen, H.C., Sande, E., Tow, B., Sanan, D.A., Raber, J., Eckel, R.H., & Farese, R.V. Jr. (2000). Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking DGAT. *Nat. Genet., 25*(1), 87-90. https://doi.org/10.1038/75651

Spelman, R. J., Ford, C. A., McElhinney, P., Gregory, G. C., & Snell, R. G. (2002). Characterization of the DGAT1 gene in the New Zealand dairy population. *J. of Dairy Sci., 85*(12), 3514-3517. https://doi.org/10.3168/jds.S0022-0302(02)74440-8

Tantia, M. S., Vijh, R. K., Mishra, B. P., Mishra, B., Kumar, S. B., & Sodhi, M. (2006). DGAT1 and ABCG2 polymorphism in Indian cattle (Bos indicus) and buffalo (*Bubalus bubalis)* breeds. *BMC Vet. Res*., 2, 32. https://doi.org/10.1186/1746-6148-2-32

Thaller, G., Krämer, W., Winter, A., Kaupe, B., Erhardt, G., & Fries, R. (2003). Effects of DGAT1 variants on milk production traits in German cattle breeds. *J. Anim. Sci.,* 81, 1911–1918. https://doi.org/10.2527/2003.8181911x

Turchetto-Zolet, A.C., Christoff, A.P., Kulcheski, F.R., Loss-Morais, G., Margis, R., & Margis-Pinheiro, M. (2016). Diversity and evolution of plant diacylglycerol acyltransferase (DGATs) unveiled by phylogenetic, gene structure and expression analyses. *Genet. Mol. Biol., 39*(4), 524-538. https://doi.org/10.1590/1678-4685-GMB-2016-0024

Venkatachalapathy, R.T., Sharma, A., Sukla, S., & Bhattacharya, T.K. (2008). Cloning and characterization of DGAT1 gene of Riverine buffalo. *DNA Seq., 19*(3), 177-84. https://doi.org/10.1080/10425170701461748

Wang, S.Y., Cheng, Y.Y., Liu, S.C., Xu, Y.X., Gao, Y., Wang, C.L., Wang, Z.G., Feng T.Q., Lu, G.H., Song, J., Xia, P.J., & Hao, L.L. (2021). A synonymous mutation in IGF-1 impacts the transcription and translation process of gene expression. *Mol. Ther. Nucleic Acids,* 26, 1446-1465. https://doi.org/10.1016/j.omtn.2021.08.007

Warnes, G., Gorjanc, W.C.F.G, Leisch, F., & Man, M. (2021). Genetics: Population Genetics. *Rpackageversion*, 1.3.8.1.3, https://doi.org/10.32614/CRAN.package.genetics

Weller, J.I., Golik, M., Seroussi, E., Ezra, E., & Ron, M. (2003). Population-wide analysis of a QTL affecting milk-fat production in the Israeli Holstein population. *J. Dairy Sci*., 86, 2219–2227. https://doi.org/10.3168/jds.S0022-0302(03)73812-0

Williams, J.L., Iamartino, D., Pruitt, K.D., Sonstegard, T., Smith, T.P.L., Low, W.Y., Biagini, T., Bomba, L., Capomaccio, S., Castiglioni, B., Colettam A., Corrado, F, Ferré, F., Iannuzzi, L., Lawley, C., Macciotta, N., McClure, M., Mancini, G., Matassino, D., Mazza, R., Milanesi, M., Moioli, B., Morandi, N., Ramunno, L., Peretti, V., Pilla, F., Ramelli, P., Schroeder, S., Strozzi, F., Thibaud-Nissen, F, Zicarelli, L., Ajmone-Marsan, P., Valentini, A., Chillemi, G., & Zimin, A. (2017). Genome assembly and transcriptome resource for river buffalo, *Bubalus bubalis* (2n = 50). *Gigasci., 6*(10), 1-6. https://doi.org/10.1093/gigascience/gix088

Winter, A., Krämer, W., Werner, F.A., Kollers, S., Kata, S., Durstewitz, G., Buitkamp J., Womack, J.E., Thaller, G., & Fries, R. (2002). Association of a lysine-232/alanine polymorphism in a bovine gene encoding acyl-CoA:diacylglycerol acyltransferase (DGAT1) with variation at a quantitative trait locus for milk fat content. *Proc. Natl. Acad. Sci. USA, 99*(14), 9300-5. https://doi.org/10.1073/pnas.142293799

Yen, C.L., Stone, S.J., Koliwad, S., Harris, C., & Farese, R.V. Jr. (2028). Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *J. Lipid. Res., 49*(11), 2283-301. https://doi.org/10.1194/jlr.r800018-jlr200

Yuan, J., Zhou, J., Deng, X., Hu, X., & Li, N. (2007). Molecular cloning and single nucleotide polymorphism detection of buffalo DGAT1 gene. *Biochem Genet*., 45, 611–21. doi: 10.1007/s10528-007-9100-3https://doi.org/10.1007/s10528-007-9100-3

Zicarelli, L. (2004) Buffalo milk: its properties, dairy yield and mozzarella production. *Vet. Res. Commun*., 28, 127-35. https://doi.org/10.1023/B:VERC.0000045390.81982.4d