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USING OF INFRARED HIGH-PERFORMANCE SPECTROMETRY DATA FOR GENOME-WIDE ASSOCIATIONS STUDY OF FATTY ACID COMPOSITION AND MILK COMPONENTS IN DAIRY CATTLE (*Bos taurus*)

A.A. SERMYAGIN✉, L.P. IGNATIEVA, I.A. LASHNEVA, A.A. KOSITSIN, O.V. KOSITSINA, A.S. ABDELMANOVA, N.A. ZINOVIEVA

Ernst Federal Research Center for Animal Husbandry, 60, pos. Dubrovitsy, Podolsk District, Moscow Province, 142132 Russia, e-mail alex_sermyagin85@mail.ru (✉ corresponding author), ignatieva-lp@mail.ru, lashnevaira@gmail.com, ksicins@gmail.com, ok.kositsina@mail.ru, preevetic@mail.ru, n_zinovieva@mail.ru
ORCID:
Sermyagin A.A. orcid.org/0000-0002-1799-6014
Ignatieva L.P. orcid.org/0000-0003-2625-6912
Lashneva I.A. orcid.org/0000-0009-4276-8782
Kositsin A.A. orcid.org/0000-0001-8484-4902
Kositsina O.V. orcid.org/0000-0002-3637-4202
Abdelmanova A.S. orcid.org/0000-0003-4752-0727
Zinovieva N.A. orcid.org/0000-0003-4017-6863

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Abstract

Milk fat percentage is highly variabile and depends on environmental conditions which include feeding and farm technology, and on genetic factors such as breed and genotype features. The content of fatty acids (FA) is a biomarker for the physiological state of animals and a parameter of raw milk suitability for processing (yield of cheese, butter and cream). FA profile of mild in terms of C number, the chain length and saturation degree differs between individuals and at the population level. Therefore, the study of genetic and genomic variability of milk production traits to improve the efficiency of animal selection remains relevant. This study aimed at searching for genome-wide associations and polymorphisms in genes involved in milk fatty acid production. In the study, infrared spectrometry was used as an accurate and rapid method to analyze milk composition. Population variability of milk fatty acid profiles was studied using 36982 milk samples from Holsteinized Black-and-White and Holstein cows of 14 breeding herds from the Moscow region in 2017-2018. The heritability (h^2) and correlation (r_g) coefficients for cows' milk components were calculated using REML (residual maximum likelihood) method with BLUPF90 family software. SNPs were detected for a dataset of Holsteinized Black-and-White cows from an experimental herd (the breeding farm Ladozhsky, branch of Ernst Federal Research Center for Animal Husbandry, Krasnodar Territory, 2020-2021). Milk composition was determined using an infrared spectroscopy-based automatic MilkoScan 7 DC analyzer (FOSS, Denmark). A group of 144 cows subjected to phenotyping for fatty acids and milk components were individually genotyped (Bovine GGP 150K biochip, Neogen, USA). Plink 1.9 software was applied to control genotyping quality (110884 SNPs) and to perform GWAS (genome-wide association study) analysis and multidimensional scaling (MDS). Searching genes by identified significant polymorphisms was performed using the bovine genome assembly *Bos taurus* UMD 3.1.1 (https://www.ncbi.nlm.nih.gov/assembly/) and the Ensembl genome browser. QTL annotation was carried out using the Animal QTLdb database. In general, milk fatty acids showed a heritability level that ranged from low to moderate, varying from $h^2 = 0.018$ for polyunsaturated fatty acids to $h^2 = 0.125$ for medium-chain FAs, $h^2 = 0.155$ for long-chain FAs, $h^2 = 0.155$ for myristic acid, $h^2 = 0.176$ for monounsaturated FAs, and $h^2 = 0.196$ for oleic acid. Visualizing experimental cows' population structure by multidimensional scaling showed a moderate range of variability (PC1 = 7.82 %, PC2 = 4.65 %). For myristic and palmitic acids, common QTL clusters are identified on BTA5, BTA10, BTA14, BTA18, and BTA27. For stearic and oleic acids (as members of the long-chain FA family), similar location of QTLs is found on BTA9, BTA10, BTA11, BTA14, BTA17, BTA18, BTA19, BTA20, and BTA29. For short- and medium-chain FAs, there are associations revealed on BTA1, BTA5, BTA10, BTA11, BTA14, BTA18, BTA19, and BTA24. For long-chain FAs, QTLs are detected on BTA6, BTA7, BTA9, BTA10, BTA11, BTA17, BTA18, and BTA29. For short- and medium-chain FAs, saturated FAs, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$, the genes *CACNA1C*, *GCH1*, *ATG14*, *KCNH5*, *PRKCE*, *CTNNA2*, *CYHR1*, *VPS28*, *DGAT1*, *ZC3H3*, *RHPN1*, *TSNARE1* are identified which

form QTLs on BTA10, BTA11 and BTA14. Short- and medium-chain FAs, myristic and palmitic acids and saturated FAs show associations with polymorphisms in the *MED12L*, *EPHB1*, *GRIN2B*, *PRMT8*, *ERC1*, *PELI2*, *ARHGAP39*, *MROH1*, *MAF1*, *GSDMD*, and *LY6D* genes. For long-chain, monounsaturated fatty acids, stearic and oleic acids, there are significant associations with genes *RPS6KA2*, *CPQ*, *CPE*, *FTO*, *FAT3*, and *LUZP2* which may be valuable for genetic improvement of dairy cattle. Continued study of the inheritance of cows' milk fatty acids and other components is necessary to develop a strategy for breeding dairy cattle with a better fatty acid profile and milk composition.

Keywords: cow, fatty acids, milk components, heritability, GWAS, SNP, QTL, genes

Milk fat has the highest energy value and a wide range of biological activity. It is necessary for the absorption of various vitamins, tocopherols, phosphates and other important nutrients. In milk, milk fat is a suspension consisting of small fat globules ranging in size from 0.1 to 20 microns. According to its chemical composition, it is a derivative of the alcohol glycerol and fatty acids (FA), which account for 93-95% of the fat mass. The content of fatty acids in milk can vary significantly depending on the conditions of feeding animals, the season of the year, the stage of lactation and other factors. Fatty acids are divided into two categories, saturated and unsaturated FA. The latter, in turn, are divided into monounsaturated and polyunsaturated. The cow milk is 50.3-73.8% saturated FA and 49.3-6.2% unsaturated FA. Of the unsaturated fatty acids, milk contains the most monounsaturated fatty acids and the least polyunsaturated fatty acids [1].

Fatty acids are organic compounds that differ in the number of carbon atoms and position, as well as the number of double bonds they contain. Cow milk contains on average 3.6 to 4.8% fat by weight. Fatty acids enter milk both free and bound, in the form of glycerides or other lipids. Triacylglycerides, which are composed of glycerol and three fatty acids, account for 96 to 99% of milk fat, while free fatty acids account for only 0.1 to 0.4% [2].

The set of fatty acids differs depending on the breed, the season and the applied zootechnologies. The feeding conditions play an important role. The composition of milk fat changes during lactation. In the early period during lactation, the animal's body mainly uses C_{16} (palmitic) and C_{18} (stearic) fatty acids from the fat depot of tissues. During lactation, the proportion of newly synthesized (de novo) fatty acids ($C_{4:0}$-$C_{14:0}$) increases, while the proportion of fatty acids with 17 or more carbon atoms decreases [3].

Milk fat contains approx. 140 fatty acids, however, only 13 main FAs with an even number of carbon atoms ($C_{4:0}$-$C_{18:3}$) are found in an amount that is more than 1% each. The remaining acids (for example, $C_{10:1}$, $C_{12:1}$), present in amounts less than 1% and in the form of traces, belong to the so-called minor fatty acids [2-4]. In minor fatty acids, the proportion of milk lipids in triglycerides is 2.0-4.2% for butyric acid, 1.5-3.0% for caproic acid, 1.0-2.0% for caprylic acid, and 2.0-3.5%% for capric acid, 0.2-0.4% for decenoic acid, 2.0-4.0% for lauric acid, 0.6-1.5% for myristinoleic acid, 1.5-2.0% for palmitoleic acid, 3.0-5.5% for linoleic acid, up to 1.5% for linolenic acid, up to 0.3% for arachidic acid, and up to 0.1% for behenic acid. In the main fatty acids, this indicator for myristic acid is 8.0-13.0%, for palmitic acid 22.0-33.0%, for stearic acid 9.0-13.0%, for oleic acid 22.0-32.0 % [4-7].

Fatty acids act differently on the human body. Myristic acid has a negative effect on the cardiovascular system, causing diseases, while stearic acid does not have such an effect. The presence of fatty acids in the animal's body is due to a greater extent by their genetically determined synthesis than by intake with feed or mobilization from body fat tissues. The formation of $C_{6:0}$-$C_{16:0}$ fatty acids, according to the literature, is characterized by high heritability (h^2 = 0.41-0.43), and this increases the selection efficiency. Production of fatty acids important for human health ($C_{18:2}$ cis-9, 12) is characterized by relatively low heritability (h^2 = 0.17-0.33), but

since their production negatively correlates with the synthesis of short and medium-length fatty acids, then selection for this trait can also be successful [2].

It is known that compounds with a molecular weight of carbon $C_{18:0}$-$C_{18:1}$ affect fertility at an early stage of lactation, and the amount of $C_{18:1}$ cis-9 indirectly indicates the energy status of the cow and can be used for early prediction of ketosis. $C_{16:0}$ and $C_{17:1}$ cis-9 are a convenient tool for assessing methane production and feed conversion in cows (the lower the methane emission, the better the feed is digested) [8, 9].

To improve the efficiency of animal breeding and the search for informative DNA markers of productivity traits, it is of great interest to analyze the function of each of the components of milk in connection with one or another biological trait and to study the genetic and genomic variability of traits. Thus, research is underway to identify causal nucleotides (point mutations) for quantitative traits (QTL), which, along with many known non-coding polymorphic substitutions (SNPs), can increase the accuracy of detection of the corresponding mutations and the prediction of the breeding value of an animal. Work is underway to optimize the number of SNPs with a high degree of variability in causal variants, which is sufficient to construct a genomic matrix of relatedness, taking into account information on a large number of genotypes and improve the accuracy of estimates [10). So, causal SNPs, strongly associated with economically useful traits in dairy cattle were detected on chromosomes 5, 6, 9, 14, 15 and 20. The polymorphisms located close to or within the *DGAT1* (BTA14), *GHR* (BTA20), *ABCG2* (BTA6) genes had the highest genetic dispersion in turms of milk productivity [11-15].

Increasing the density of SNPs (reducing the distance between SNPs) will increase the likelihood of QTL detection and, to some extent, the accuracy of mapping. Genome-wide associations were used to analyze the composition of fat in the milk of Holstein and Jersey cows of Danish origin [16]. In addition to the standard genotyping procedure with high density chips (777K), this study used the KEGG PATHWAY Database (bioinformatics resource for genome analysis, https://www.genome.jp/kegg/pathway.html). The candidate gene *DGAT1* which very often appears in studies of the milk productivity in cattle was not defined as playing a significant role in the milk fat composition. This once again indicates the complexity of the inheritance of the trait. However, significant associations with the milk fatty acid composition were found for the *SCD* gene involved in the catalyzed conversion of $C_{10:0}$ to $C_{18:0}$ acids and the *ACSS3* gene involved in the activation and intracellular transport of fatty acids.

F. Kawaguchi [17] found 1993 polymorphisms in 23 genes in Japanese black cattle based on allelic differences between groups with high and low content of oleic acid $C_{18:1}$ using a genome-wide association study (GWAS). Among these 23 genes, based on the analysis of their function in the metabolism of fatty acids, three candidate genes were identified, the *CYB5R4*, *MED23*, and *VNN1* that affect the variability of the oleic fatty acid content.

In the Italian population of Simmentals and Holsteins, GWAS for milk fatty acids revealed significant signals on the BTA19 and BTA26 chromosomes. Further analysis identified not only some well-known genes (*FASN*, *SCD*, and *DGAT1*) of quantitative trait loci for milk FA components, but also other significant candidate genes that were associated with functional roles in lipid metabolism pathways. The identified mutations that are associated with the fatty acid profile are found in the *ECI2*, *PCYT2*, *DCXR*, *G6PC3*, *PYCR1*, *ALG12*, *CYP17A1*, *ACO2*, *PI4K2A*, *GOT1*, *GPT*, *NT5C2*, *PDE6G*, *POLR3H*, and *COX15* genes [18].

The discovery of quantitative trait loci and genes associated with milk fat composition can provide an insights into the complex metabolic networks that underlie changes in fatty acid synthesis and point to possible “points of impact” for

improving milk fat composition through breeding. C. Li et al. [19] performed a GWAS analysis for 22 milk fatty acids in 784 Holstein cows from a Chinese population. A total of 83 significant SNPs and 314 putative suggestive SNPs were found for 18 traits associated with milk fatty acid metabolism. Chromosome regions affecting the properties of milk FAs were mainly localized on BTA1, BTA2, BTA5, BTA6, BTA7, BTA9, BTA13, BTA14, BTA18, BTA19, BTA20, BTA21, BTA23, BTA26, and BTA27. Of these, 146 SNPs were associated with more than one trait in milk fatty acid metabolism; most traits were statistically significantly associated with several SNPs, especially $C_{18:0}$ (105 SNPs), C_{18} (93 SNPs), and C_{14} (84 SNPs) FAs. Several SNPs are found near or within the *DGAT1*, *SCD1* and *FASN* genes, which are known to affect milk composition in dairy cattle. In addition, 20 new highly significant candidate polymorphisms for $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{14:1}$, indeces of C_{14}, $C_{18:0}$, $C_{18:1n9c}$, and index C_{18} were identified, including mutations in the *HTR1B*, *CPM*, *PRKG1*, *MINPP1*, *LIPJ*, *LIPK*, *EHHADH*, *MOGAT1*, *ECHS1*, *STAT1*, *SORBS1*, *NFKB2*, *AGPAT3*, *CHUK*, *OSBPL8*, *PRLR*, *IGF1R*, *ACSL3*, *GHR*, and *OXCT1* genes [19].

The fatty acid concentration is particularly relevant for milk chemical analysis, since the milk quality parameters, e.g., yield of cheese, butter and cream, largely depend on lipid metabolism. In studies by P. Gottardo [20] on 2977 Holstein, Brown Swiss and Simmental cows, it was found that the Holstein cows have the best ratio of saturated to unsaturated fatty acids in the total milk fat. Simmental cows are on an intermediate position followed by Brown Swiss cows [20].

In many countries, infrared spectrometry is a widely used method to detect and quantify fatty acids in milk. Metabolism of milk fatty acids is under influence of many factors. Therefore, estimation of variability and heritability indicators for such traits is necessary to choose the most effective breeding strategy. In Russia, FAs are relatively new trait for cow breeding and improving milk quality. In Western European countries, the fatty acid analysis quantifies saturated and unsaturated fatty acids. Besides, FA profile serves as an indicator of animal physiological state and numtrition level. In this regard, information obtained in herds at a population level and in experiments will clarify whether breeding for FA composition is prospective. Validation of genetic polymorphisms associated with the variability of the cow milk fatty acids along with other components, will provide new knowledge on the genomic architecture of milk productivity indicators.

Here, for the first time, we assessed the genetic variability of the milk fatty acid fractions in the Russian populations of Holsteinized Black-and-White and Holstein cows to involve these traits into breeding programs and genetic improvement of animals. In experiments, using infrared spectrometry of milk composition and high-performance genomic scanning, we created a database of cows' individual phenotypes and genotypes. Eventually, the quantitative trait loci and functional mutations that regulate the synthesis of milk lipids were revealed.

Our aim was to search for genome-wide associations and polymorphisms in the genes that determine the fatty acid composition of cow milk. To determine it, infrared spectrometry was used as one of the fastest and most accurate express methods for physicochemical analysis of milk composition.

Materials and methods. The results of interpopulation observations and studies in the experimental population were used to create the databases. At the first stage, in 14 breeding herds of Holsteinized Black-and-White and Holstein cattle from the Moscow region (n = 11529), fatty acid profiles were obtained using IR spectra. The milk samples (n = 36982 in total) were collected during 9 months of 2017-2018 in control milkings. Based on the milk composition, the population genetic parameters and variability of the content of the following fatty acids and components in milk: myristic ($C_{14:0}$), palmitic (C16:0), stearic ($C_{18:0}$), oleic

($C_{18:1}$) acids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), short-chain fatty acids (SCFA), medium-chain fatty acids (MCFA), long-chain fatty acids (LCFA) and trans-isomers FA (TIFA), mass proportions of fat (MPF), protein (MPP), casein (MPC), lactose (MPL), dry matter (DM), DSMR (dry skimmed milk residue), traces of acetone and beta-hydroxybutyrate (BHB), urea concentrations, freezing points and acidity.

To calculate the heritability of the milk fatty acid composition and genetic correlations, the mixed model equation was used:

$$y = \mu + HFMTD_j + Age_k + Lact_l + sire_m + e_{jklm},$$

where y is the studied milk indicator of daughter cows; μ is the mean population constant for a sample of 14 herds; HFMTDj is the effect of the farm, month and date of control milking; Age$_k$ is the age of the 1st calving; Lact$_l$ is the effect of the last completed lactation No.; sire$_m$ is the effect of the father bull; e$_{jklm}$ is random error (unallocated variant). The residual maximum likelihood (REML) method based on maximization of the variance value likelihood through a multiple iteration procedure using the BLUPF90 family programs [21, 22] was applied in calculation for 14 herds.

Primaryly, the milk fatty acid profiles in the experimental herd of Holsteinized black-and-white cows were obtained at the PZ Ladozhsky (branch of the Ernst FRC VIZh, Krasnodar Territory, 2020-2021). The test group consisted of 144 cows previously phenotyped for milk FA spectra and milk components and genotyped using a Bovine GGP 150K biochip (Neogen, USA). DNA isolation from fragments pinched off ears of the cows, SNP genotyping and analysis of milk samples were performed according to standard protocols at the scientific facility Animal Biotechnology (OSIS BioTechZh) of the Ernst FRC VIZh.

In the herds and in the test group, the milk fatty acids and other milk componens indicated hereinabove were determined as recommended (an automatic MilkoScan™ 7 DC analyzer based on Fourier Transform InfraRed analysis, FOSS, Denmark). Animal productivity was assessed individually in 5 to 12 control milkings, on average 8.9 per animal. Milk samples were collected into 50 ml cups with the preservative (Microtabs, USA) during milking in the morning, afternoon and evening.

Genotyping quality control (110884 SNPs), analysis of genome-wide association studies (GWAS), and multidimensional scaling (MDS) were performed (Plink 1.9 software) [23]. As a result, genotypes representing 143 genomic SNP profiles with a genotyping level from 99.3 to 99.7% were selecte among the test cows.

The search for genes by identified significant polymorphisms based on the GWAS data was carried out using the assembly of the bovine genome version UMD 3.1 (Ensembl browser, https://www.ensembl.org/index.html). To determin quantitative trait loci on animal chromosomes, annotation of genes was performed using the international database Animal QTLdb [24].

The Data Analysis package in the MS Excel 2013 environment was used to calculate the mean values (M), standard errors of means (±SEM), and standard deviations (SD). The degree of trait variability was assessed by the coefficient of phenotypic variation (C_{vp}). To calculate heritability (h^2), we used the ratio of genetic variance to the sum of genetic and residual variance.

Results. Fatty acids are highly variable components of milk. In cows, milk FA quantitative profiles are influenced by both environmental conditions (eg feeding, housing) and genetic factors (breed, ancestors, and genotype). The ratio of fatty acids depending on the number of carbon atoms, the length of the chain and the degree of saturation differs both between individuals and at the population level. Understanding the mechanisms of fatty acid synthesis is important to determine

associated quantitative trait loci (QTL). We assessed the genetic variability of the milk fatty acid content in populations of the Holsteinized Black-and-White and Holstein cows on the example of several herds of the Moscow region in order to clarify the prospects of these traits for selection (Table 1).

The heritability of the milk fatty acid quantitative composition varied from low values for polyunsaturated fatty acids ($h^2 = 0.018$) to moderate values for medium ($h^2 = 0.125$), long-chain ($h^2 = 0.155$) and myristic acid ($h^2 = 0.155$), monounsaturated fatty acids ($h^2 = 0.176$) and oleic acid ($h^2 = 0.196$).

1. Phenotypic and genetic parameters of milk fatty acid composition in cows (*Bos taurus*) from 14 breeding herds of Holsteinized Black-and-White and Holstein cows (control milkings, Moscow Province, 2017-2018)

Fatty acid, fatty acid group	g/100 g milk		C_{vp}, %	h^2
	M±SEM	SD		
Myristic	0.680±0.001	0.148	21.8	0.155
Palmitic	1.845±0.003	0.633	34.3	0.071
Stearic	0.585±0.001	0.195	33.4	0.125
Oleic	0.875±0.001	0.281	32.2	0.196
Saturated	3.600±0.004	0.861	23.9	0.083
Monounsaturated	1.002±0.001	0.213	23.0	0.176
Polyunsaturated	0.005±0.000	0.011	238.8	0.018
Short chain	0.538±0.001	0.113	21.1	0.114
Medium chain	2.206±0.003	0.652	29.6	0.125
Long chain	1.486±0.002	0.463	31.2	0.155

N o t e. The total number of milk samples $n = 36982$. C_{vp} is the coefficient of phenotypic variation, h^2 is the coefficient of heritability.

Genetic correlations between milk yield, fat mass fraction (MFA) and fatty acid composition of milk are submitted in Table 2 (if $r_g > 0.050$, the obtained coefficients are significant at $p < 0.001$). It was found that there was practically no genetic relationship between daily milk yield and MFF ($r_g = –0.032$), while a closer negative correlation occurred with the content of trans-FA isomers ($r_g = –0.129$), myristic acid ($r_g = –0.110$) and MCFA ($r_g = –0.106$). The relationship between MFF and the content of various fatty acids ranged from 0.393 for oleic to 0.955 for SFA, for TIFA ($r_g = –0.286$) and more desirable PUFA ($r_g = –0.465$) the values were negative. The content of myristic and palmitic saturated fatty acids negatively correlated with the amount of unsaturated oleic (r_g from –0.160 to –0.427), MUFA (r_g from –0.072 to –0.337), PUFA (r_g from –0.554 to –0.584) and with the content of acids from more complex high molecular weight groups LCFA (r_g from –0.030 to –0.325). The relationship between the amounts of fatty acids $C_{14:0}$, $C_{16:0}$, on the one hand, and TIFA, on the other hand, showed that with an increase in the content of saturated fatty acids in milk, trace amounts of isomers blocking the synthesis of milk fat decreased (r_g from –0.469 to –0.637, respectively).

2. Genetic correlations (r_g) between daily milk yield, milk fat mass fraction and quantitative fatty acid composition in 14 breeding herds of Holsteinized Black-and-White and Holstein cows (*Bos taurus*) (control milkings, $n = 11529$, Moscow Province, 2017-2018)

Trait	1	2	3	4	5	6	7	8	9	10	11	12
MFF	–0.032											
$C_{14:0}$	–0.110	0.634										
$C_{16:0}$	–0.101	0.801	0.874									
$C_{18:0}$	–0.023	0.615	–0.122	0.300								
$C_{18:1}$	0.053	0.393	–0.427	–0.160	0.764							
SFA	–0.031	0.955	0.781	0.905	0.485	0.150						
MUFA	–0.008	0.463	–0.337	–0.072	0.767	0.983	0.211					
PUFA	0.075	–0.465	–0.554	–0.584	–0.037	0.113	–0.569	0.097				
SCFA	0.045	0.815	0.551	0.608	0.482	0.264	0.852	0.255	–0.455			
MCFA	–0.106	0.684	0.980	0.920	–0.033	–0.384	0.818	–0.293	–0.596	0.554		
LCFA	0.053	0.499	–0.325	–0.030	0.831	0.979	0.276	0.970	0.038	0.350	–0.276	

Continued Table 2

TIFA	–0.129	–0.286	–0.637	–0.469	0.282	0.384	–0.383	0.355	0.469	–0.300	–0.643	0.396

N o t e. The total number of milk samples n = 36982; 1 — daily milk yield, 2 — mass fraction of milk fat (MFF), 3 — $C_{14:0}$, 4 $C_{16:0}$, 5 — $C_{18:0}$, 6 — $C_{18:1}$, 7 — saturated fatty acids (SFA), 8 — monounsaturated fatty acids (MUFA), 9 — polyunsaturated fatty acids (PUFA), 10 — short chain fatty acids (SCFA), 11 — medium chain fatty acids (MCFA), 12 — long chain fatty acids (LCFA). TIFA — trans isomer of fatty acids. If $r_g > 0{,}050$, correlations are statistically significant at $p < 0.001$.

The amount of PUFA was negatively correlated with the amount of SCFA (r_g = –0.455) and SCFA (r_g = –0.596), but the relationship between the production of PUFA and TIFA was the highest and most positive (r_g = 0.469), which, in combination with the heritability coefficient, indicates a complex selection the process of increasing these indicators in the milk of cows. However, it should be noted that the problem of improving the ratio of saturated and unsaturated fatty acids in milk in favor of the latter remains unresolved. An increase in the proportion of oleic acid, MUFA, PUFA, LCFA in milk during the selection of animals simultaneously leads to a change in the fatty acid composition of trans-isomers, fatty acids with a short and medium length of carbon chain.

Summarizing the obtained data (see Tables 1, 2), we can conclude that the revealed correlations and indicators of genetic variability of fatty acids in cow's milk are promising for further GWAS analysis in order to adjust cattle breeding programs.

The results of the analysis of milk samples obtained in the control milkings of cows from the experimental group are shown in Table 3. It was found that 50.5% of the daily milking was in the morning; the rest was distributed approximately equally between daytime and evening milkings, 23.5 and 26.0 %, respectively. There was a clear inverse linear relationship between the amount of daily milk yield and the component composition of milk. With a smaller volume of milk for lunch milking of 6.1 kg, the percentage of fat fraction (up to 4.15%, including fatty acids) and dry matter (13.33%) increased.

For milk protein and casein, lactose, DSMR, BHB and urea, no significant quantitative differences were found when sampling at different times. The values of the molar mass of acetone detected in trace amounts were higher in the morning and evening milk samples (0.047 and 0.040 mmol/l, respectively), while the freezing point was lower ($–536.5\times10^{-3}$ °C and $–537.2\times10^{-3}$ °C, respectively). The coefficient of phenotypic variation (C_{vp}) regardless of the time (morning, lunch, evening) of milk sampling (20.0-24.2%) was higher for the mass fraction of fat than for other selectively significant traits of milk quality. Based on phenotypic variability, it can be assumed that the potential efficiency of selection for fatty acids will be higher for palmitic (C_{vp} = 22.0-25.0%), stearic (C_{vp} = 24.6-32.1%) acids, long-chain fatty acids (C_{vp} = 20.2-27.8%), short-chain fatty acids (C_{vp} = 23.0-27.5%), as well as the sum of saturated fatty acids (C_{vp} = 21.5-25.8%).

The repeatability (r) between adjacent control milkings (morning and evening) according to the studied indicators of milk composition was quite high, the exception was traces of metabolites — acetone, BHB and urea (r = 0.565-0.630) and the freezing point of milk (r = 0.505). Relatively moderate values were obtained for oleic acid (r = 0.625) and polyunsaturated fatty acids (r = 0.590). In general, it can be concluded that it is quite advisable to control the component composition of cows' milk either by an average sample or separately in the morning and evening, while accounting for the volume of milk produced from a cow should be equal to the number of milkings per day. We believe that when analyzing genome-wide associations, there will be no significant shift in the identified QTLs when using data obtained with a 2-fold control of productivity per day, that is, such control is sufficient.

3. An extended analysis of the cows' milk composition depending on the time of sampling (n = 144, Holsteinized Black-and-White experimental herd, PZ Ladozhsky, Krasnodar Territory, 2020-2021)

Trait	Control milking												repetability		
	morning				afternoon				evening						
	M	±SEM	SD	C_{vp}, %	*M*	±SEM	SD	C_{vp}, %	*M*	±SEM	SD	C_{vp}, %	m/a	a/e	m/e
Milk yield,kg	13.0	0.1	3.6	27.3	6.1	0.1	1.8	30.1	6.70	0.10	1.90	27.8	0.750	0.724	0.762
MFF, %	3.22	0.03	0.78	24.2	4.15	0.03	0.80	19.3	3.81	0.03	0.76	20.0	0.596	0.740	0.652
MFP (actual), %	3.24	0.02	0.43	13.3	3.25	0.02	0.42	12.8	3.26	0.02	0.44	13.6	0.869	0.915	0.867
MFP (raw), %	3.44	0.02	0.44	12.7	3.46	0.02	0.42	12.2	3.46	0.02	0.45	12.9	0.868	0.914	0.866
MFC, %	2.71	0.01	0.36	13.4	2.76	0.01	0.35	12.8	2.75	0.01	0.38	13.7	0.868	0.914	0.862
MFL, %	4.78	0.01	0.20	4.2	4.80	0.01	0.20	4.1	4.79	0.01	0.20	4.1	0.765	0.855	0.751
DM, %	12.33	0.04	1.11	9.0	13.33	0.04	1.09	8.1	12.97	0.04	1.12	8.7	0.734	0.835	0.764
DSMR, %	9.06	0.02	0.49	5.4	9.11	0.02	0.49	5.4	9.10	0.02	0.52	5.8	0.835	0.886	0.826
Acetone, mmol/l	0.047	0.002	0.049	105.1	0.036	0.002	0.040	111.5	0.040	0.002	0.056	139.0	0.608	0.681	0.569
Acetone, logarithm	–1.758	0.028	0.794	45.2	–1.930	0.030	0.817	42.3	–1.827	0.027	0.775	42.4	0.581	0.623	0.630
BHB, mmol/l	0.017	0.001	0.024	137.7	0.017	0.001	0.024	136.7	0.017	0.001	0.027	156.5	0.631	0.717	0.570
BHB, logarithm	–2.279	0.026	0.739	32.4	–2.282	0.027	0.738	32.3	–2.291	0.026	0.733	32.0	0.644	0.685	0.600
Urea, mg · 100 ml^{-1}	41.3	0.2	4.9	12.0	42.1	0.2	4.8	11.5	39.9	0.2	4.8	12.1	0.617	0.746	0.565
Freezing point, ×10^{-3} °C	–536.5	0.3	8.1	1.5	–538.5	0.4	9.5	1.8	–537.2	0.3	9.6	1.8	0.561	0.669	0.505
Acidity, pH	6.57	0.00	0.06	0.9	6.56	0.00	0.06	0.9	6.56	0.00	0.06	0.9	0.647	0.758	0.658
$C_{14:0}$, g/100 g	0.307	0.003	0.074	24.1	0.374	0.003	0.082	21.8	0.357	0.003	0.084	23.5	0.685	0.797	0.711
$C_{16:0}$, g/100g	0.794	0.007	0.198	25.0	0.969	0.008	0.225	23.2	0.923	0.008	0.220	23.8	0.684	0.806	0.718
$C_{18:0}$, g/100 g	0.295	0.003	0.095	32.1	0.383	0.003	0.094	24.6	0.352	0.003	0.091	25.8	0.658	0.775	0.709
$C_{18:1}$, g/100 g	1.016	0.009	0.247	24.3	1.327	0.009	0.247	18.6	1.194	0.008	0.231	19.3	0.577	0.692	0.625
LCFA, rg/100 g	1.243	0.012	0.345	27.8	1.664	0.012	0.336	20.2	1.494	0.011	0.315	21.1	0.590	0.705	0.633
MCAF, g/100 g	1.241	0.011	0.316	25.4	1.489	0.013	0.339	22.8	1.432	0.012	0.345	24.1	0.712	0.825	0.735
SCFA, g/100 g	0.437	0.004	0.120	27.5	0.576	0.005	0.133	23.0	0.524	0.004	0.125	23.8	0.609	0.758	0.637
MUFA, g/100 g	0.931	0.008	0.229	24.6	1.223	0.009	0.232	19.0	1.103	0.008	0.215	19.5	0.577	0.687	0.633
PUFA, g/100 g	0.124	0.001	0.025	20.3	0.150	0.001	0.026	17.3	0.135	0.001	0.024	18.0	0.545	0.656	0.590
SFA, g/100 g	2.139	0.019	0.551	25.8	2.730	0.022	0.587	21.5	2.542	0.020	0.571	22.5	0.636	0.775	0.676

N o t e. The total number of milk samples n = 2340. MFF — mass fraction of milk fat, MFP — mass fraction of protein, MFC — mass fraction of caseine, MFL — mass fraction of lactose, DM — dry matter, DSMR — dry skimmed milk residue, BHB — beta-hydroxybutyrat, LCFA — long chain fatty acids, MCFA — medium chain fatty acids, SCFA — short chain fatty acids, MUFA — monounsaturated fatty acids, PUFA — polyunsaturated fatty acids, SFA — saturated fatty acids; m/a — orning/afternoon repeatability, a/e — afternoon/evening repeatability, m/e — moning/evening repeatability.

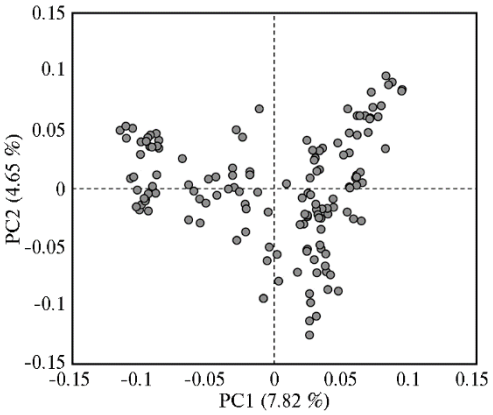

Fig. 1. Analysis of the genetic structure of a group of cows using the multidimensional scaling method (n = 144, an experimental herd of Holsteinized Black-and-White cows, PZ Ladozhsky, Krasnodar Territory, 2020-2021).

We also determined the genetic structure of the experimental animal group using the multivariate scaling (MDS) method (Fig. 1). Since the herd is represented by Holsteinized black-and-white dairy cattle, a moderate range of variability was observed for the components of variability (PC1 = 7.82%, PC2 = 4.65%). Currently, the experimental herd is consolidated due to individual selection of parental pairs (cows and Holstein bulls) in order to obtain as genetically homogeneous individuals as possible to study the inheritance of quantitative traits, including the component composition of milk. In our opinion, this will make it possible to more accurately assess the genotype of cows based on the phenotyping of economically useful qualities of each animal. We used the results of the MDS analysis of the sample as covariants along the PC1/PC2 axes to correct the effect of genetic variability on the population structure of the experimental herd and reduce the likelihood of obtaining false positive GWAS values of associations with direct phenotypic data on a number of milk components and the content of fatty acids in it.

Previously, in one of the studies, we detected 32 ($p < 0.001$-0.00001) causal SNP mutations associated with the evaluation of the breeding value of bulls by the content of fatty acids in the milk of daughter cows (the most significant were on the chromosomes BTA1, BTA5, BTA6, BTA10, BTA11, BTA14 , BTA19, BTA22 and BTA26) [25]. The genes *CHST11*, *ACO2*, *PPARGC1A*, *NRXN1*, *LPIN1*, *ASIC2*, *PCDH15*, *PRKG1* were directly associated with the synthesis of C_{14}, C_{16}, C_{18} fatty acids, conjugated linoleic acid, with an index of saturated and unsaturated fatty acids. In addition, genes located in QTL were found that are associated with animal fertility indicators, linear measurements of the udder and limbs (*NCAM2*, *FGD4*, *KCNIP4*, *SFXN1*, *NBAS*, *PGR*, *MON1B*, *GPLD1*, *PRKG1*). An analysis of the international database NCBI (https://www.ncbi.nlm.nih.gov/) on identified polymorphisms showed that the identified genes often exhibit a pleiotropic effect. This once again confirms the complex nature of the heritability of the quantitative composition of fatty acids and at the same time indicates the possibility of using the content of fatty acids in milk to control the health and fertility of dairy cows [25].

To further search for genome-wide associations with the quantitative composition of milk FAs, we correlated the data of GWAS analysis with the results of direct phenotyping of cows of the experimental herd for this trait and identified SNPs that are associated with the own productivity of daughters of sires assessed on populations from the Moscow region (14 herds). For indicators of the breeding value of these bulls-fathers by FA, the first results of the search for associations were previously obtained.

It has been established that all the studied traits of milk productivity of cows were characterized by the polygenic nature of inheritance and the multiple action of genes involved in the control of indicators of the quantitative composition of milk fatty acids with different selection significance. Thus, for the daily milk yield, we found quantitative trait loci (QTL) on the BTA1, BTA4, BTA7, BTA9, BTA14, BTA15, BTA18, and BTA25 chromosomes (Fig. 2). The variability in the mass

fraction of fat (MFF) in the GWAS analysis served as an indicator of the reliability of phenotyping of the other studied traits: the spectra of essential fatty acids and MFA were determined in the same samples, so the identified associations can be considered significant. On the BTA14 chromosome, we found clusters associated with the percentage of milk fat, which contain the milk fat candidate gene *DGAT1* (SNP mutation ARS-BFGL-NGS-4939) and a number of other genes linked to it. QTLs were also detected by the mass fraction of fat on chromosomes BTA5, BTA10, BTA11, and BTA19.

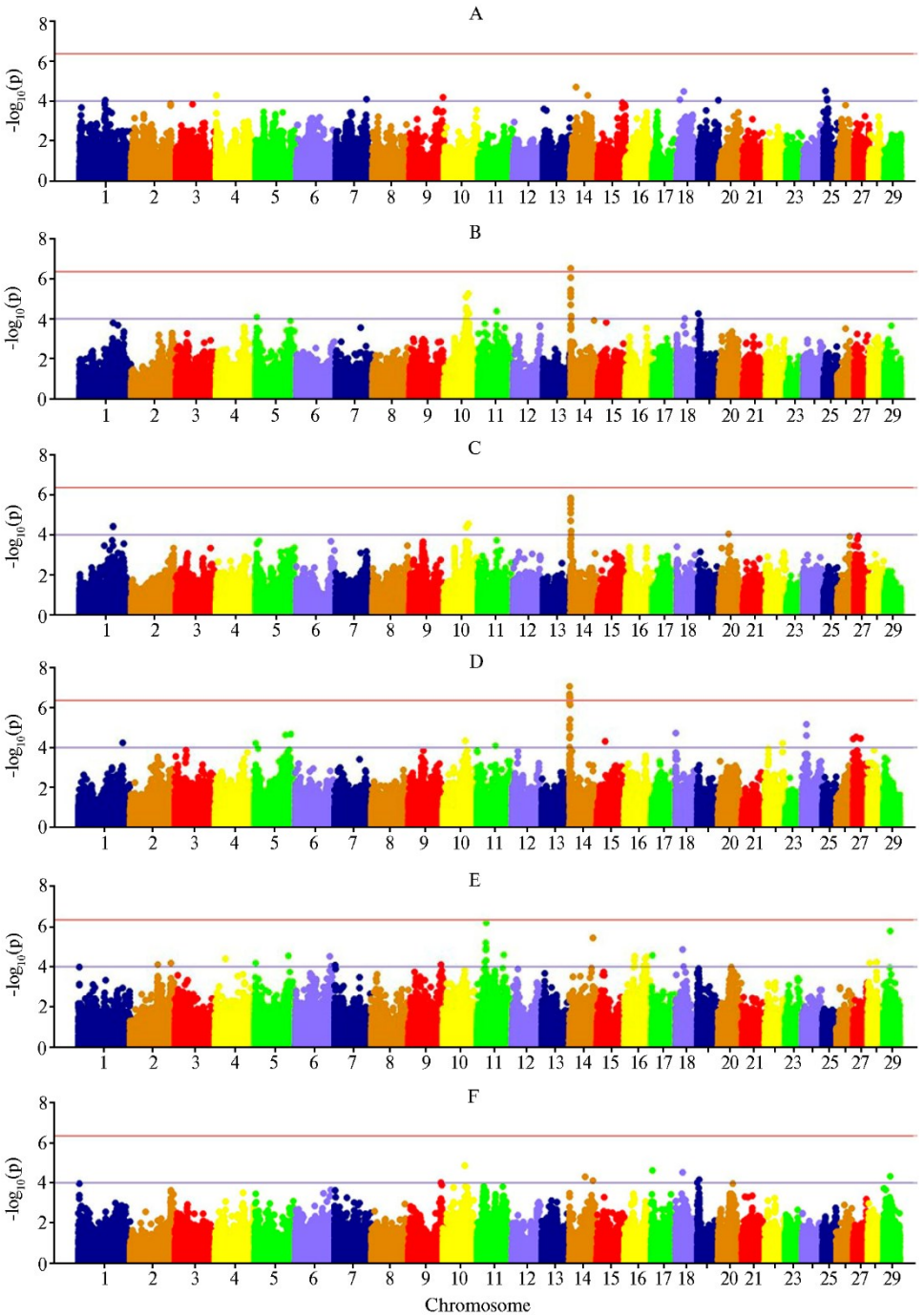

Fig. 2. GWAS analysis for daily milk yield (A)**, mass fraction of milk fat** (B) **and essential fatty acids,**

myristic (C)**, palmitic** (D)**, stearic** (E) **and oleic** (F) **based on direct phenotypic indicators** (n = 144, an experimental herd of Holsteinized black-and-white cows, Ladozhsky PZ, Krasnodar Territory, 2020-2021).

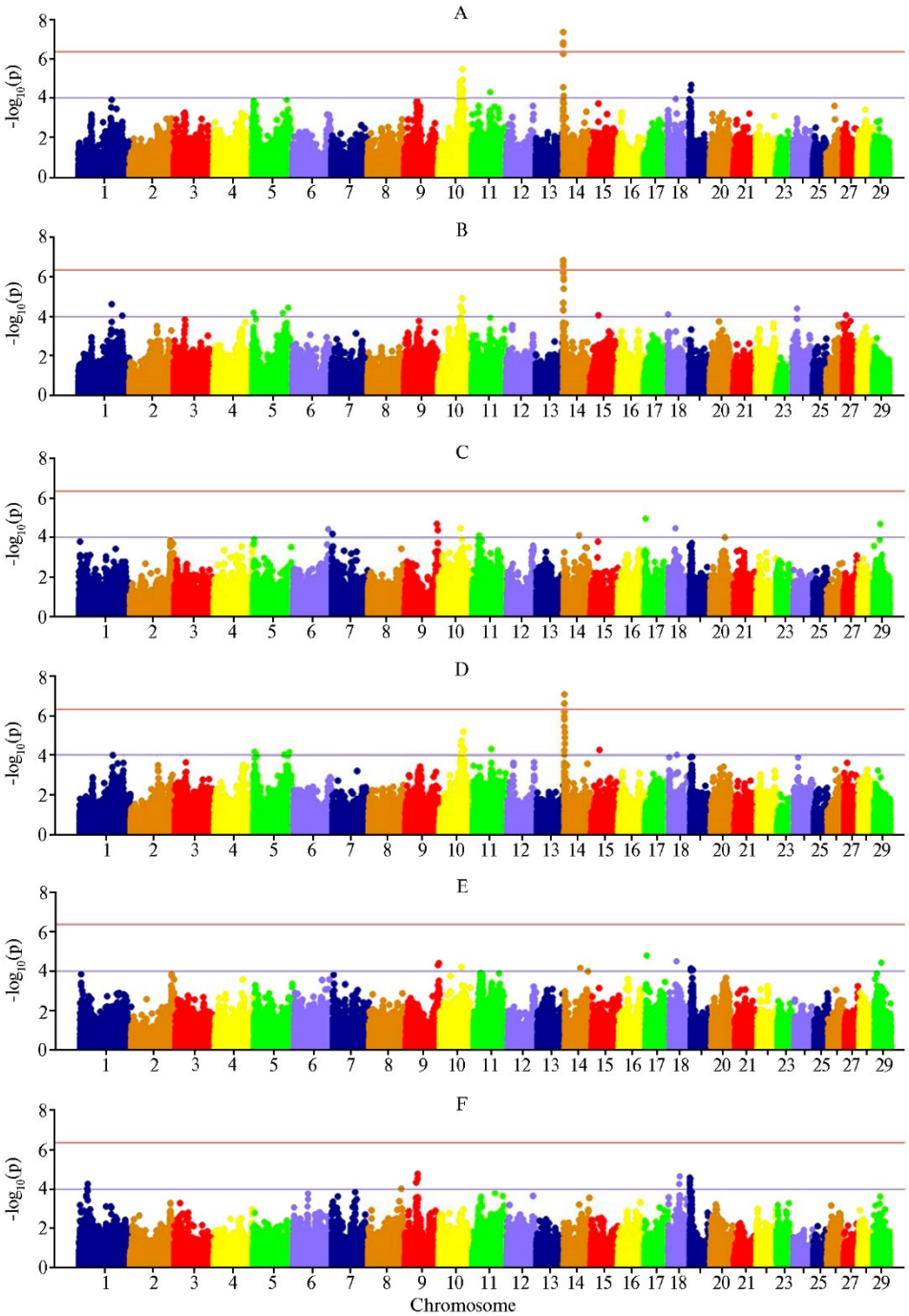

Fig. 3. GWAS analysis for short chain fatty acids (A)**, medium chain fatty acids** (B)**, long chain fatty acids** (C)**, saturated fatty acids** (D)**, monounsaturated fatty acids** (E) **and polyunsaturated fatty acids** (E) **based on direct phenotypic indicators** (n = 144, an experimental herd of Holsteinized black-and-white cows, Ladozhsky PZ, Krasnodar Territory, 2020-2021).

For myristic and palmitic FAs, we identified common clusters for BTA5, BTA10, BTA14, BTA18, and BTA27, which was largely consistent with the association profile for MFF. Stearic and oleic FAs, as long-chain FAs, showed similar

localization on the BTA9, BTA10, BTA11, BTA14, BTA17, BTA18, BTA19, BTA20, and BTA29 chromosomes (see Fig. 2). For short- and medium-chain FAs, associations were detected on the BTA1, BTA5, BTA10, BTA11, BTA14, BTA18, BTA19, and BTA24 chromosomes. In this regard, SCFA and MCFA turned out to be more similar to myristic and palmitic acids, the content of which in milk showed close genetic correlations: for SCFA, r_g = 0.551 and r_g = 0.608, respectively, for MCFA, r_g = 0.920 and r_g = 0.980. For long-chain fatty acids, QTLs were detected for BTA6, BTA7, BTA9, BTA10, BTA11, BTA17, BTA18, and BTA29 (see Fig. 2), which generally agreed with the data for stearic and oleic acids, which have a similar association profile, and with the identified genetic relationship between these traits (r_g = 0.831 for stearic acid, r_g = 0.979 for oleic acid).

The group of saturated and unsaturated fatty acids showed different patterns of QTL localization according to the identified associations, which, in our opinion, is mainly associated with the features of their synthesis and metabolic pathways (Fig. 3). Mono- and polyunsaturated fatty acids (as the most significant in terms of the nutritional value of milk) showed total QTLs for BTA1, BTA2, BTA9, BTA11, BTA18 and BTA19 (see Fig. 3). The presence of different loci that control the formation and secretion of milk fatty acids and their location on different chromosomes can also be associated with the pressure of artificial selection.

4. Annotations of identified significant polymorphisms ($p < 0.0001$) **on bovine chromosomes** (BTA) (n = 144, an experimental herd of Holsteinized Black-and-White cows, PZ Ladozhsky, Krasnodar Territory, 2020-2021)

Gene	Traits	BTA	Position, bp	
			start	end
MED12L	MCFA, $C_{14:0}$	1	117,548,538	117,917,463
EPHB1	MCFA	1	135,191,077	135,518,801
GRIN2B	MCFA, SFA, $C_{16:0}$	5	96,408,804	96,761,516
PRMT8	MCFA, SFA, $C_{16:0}$	5	106,812,249	106,812,249
ERC1	MCFA, $C_{16:0}$	5	108,308,618	108,549,124
CACNA1C	SFA, $C_{16:0}$	5	109,152,548	109,417,890
ARFGEF3	TIFA	9	77,035,587	77,158,234
RPS6KA2	LCFA, MUFA	9	102,918,982	103,074,109
GCH1	MFF, LCFA, MCFA, SCFA, SFA, $C_{14:0}$, $C_{18:1}$	10	67,576,390	67,631,089
ATG14	MFF, MCFA, SCFA, SFA	10	680,734,07	68,110,299
PELI2	SCFA	10	68,778,347	68,974,093
KCNH5	MFF, LCFA, MCFA, SCFA, SFA, $C_{14:0}$, $C_{18:1}$	10	75,235,434	75,637,242
PRKCE	MFF, $C_{18:0}$	11	27,935,104	28,472,632
CTNNA2	MFF	11	54,723,190	55,906,462
ARHGAP39	SCFA	14	1,563,866	1,600,378
CYHR1	MFF, MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$	14	1,663,923	1,677,519
VPS28	MFF, MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$, $C_{18:1}$	14	1,693,641	1,698,490
DGAT1	MFF, MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$	14	1,795,351	1,804,562
MROH1	MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$	14	1,844,664	1,894,424
MAF1	MCFA, SCFA, SFA	14	1,921,784	1,924,818
GSDMD	MCFA, $C_{16:0}$	14	2,341,290	2,346,302
ZC3H3	MFF, MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$	14	2,354,390	2,418,557
RHPN1	$C_{14:0}$	14	2,462,544	2,471,434
LY6D	MCFA, $C_{16:0}$	14	2,801,383	2,803,020
TSNARE1	MFF, MCFA, SCFA, SFA, $C_{14:0}$, $C_{16:0}$	14	3,054,763	3,171,546
CPQ	$C_{18:0}$, $C_{18:1}$	14	69,287,302	69,893,052
CPE	LCFA, MUFA	17	546,398	697,915
CDH13	Daily milk yield	18	9,512,739	10,162,782
AKTIP	Daily milk yield	18	21,926,577	21,937,955
FTO	MUFA, $C_{18:0}$, $C_{18:1}$	18	22,118,201	22,541,532
ABCC1	Daily milk yield	25	14,469,282	14,570,639
TNKS	TIFA, $C_{16:0}$	27	24,632,930	24,789,416
FAT3	LCFA, MUFA, $C_{18:0}$, $C_{18:1}$	29	1,965,869	2,605,125
LUZP2	LCFA, MUFA, $C_{18:0}$, $C_{18:1}$	29	20,259,769	20,557,376

Note. MFF — mass fraction of milk fat, LCFA — long chain fatty acids, MCFA — medium chain fatty acids, SCFA — short chain fatty acids, MUFA — monounsaturated fatty acids, PUFA — polyunsaturated fatty acids, SFA — saturated fatty acids, TIFA — trans isomer of fatty acids.

No significant QTLs were detected for trans-isomers FA, except for those detected for BTA1, BTA6, BTA18, BTA22, and BTA27, which is probably due to

the small dispersion in this parameter (data not shown in the figures).

We annotated the identified polymorphisms in the genes associated with the daily milk yield, fat mass fraction and fatty acid composition of milk in cows from the experimental group (Table 4). The comparison was carried out using the international database Animal QTLdb (https://animalgenome.org/cgi-bin/QTLdb/BT/index).

When mapping loci of quantitative traits for daily milk yield, we revealed the presence of three highly significant associations with polymorphisms in the *CDH13* and *AKTIP* (BTA18) and *ABCC1* (BTA25) genes (see Table 4). They are also associated with milk cholesterol content, animal fertility, long-term use, and somatic cell count in milk [24]. The *CACNA1C*, *GCH1*, *ATG14*, *KCNH5*, *PRKCE*, *CTNNA2*, *CYHR1*, *VPS28*, *DGAT1*, *ZC3H3*, *RHPN1*, *TSNARE1* genes which form QTLs on chromosomes BTA10, BTA11, and BTA14 have been identified for MFF, short-, medium-chain, saturated fatty acids, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$. It should be noted that all of the listed genes had a pleiotropic effect on a number of fatty acids. Annotation revealed genes associated with energy metabolism, which determines resistance to ketosis [26], content of conjugated linoleic acid in milk, percentage and yield of milk fat and protein, cholesterol content in milk, content of palmitic and palmitoleic fatty acids, milk yield per lactation, reproductive qualities of animals. Using GWAS analysis, we identified the diacylglycerol-O-acyltransferase 1 gene, which can serve as a marker of polymorphism in the study of milk fat indicators and allows us to indirectly assess the accuracy of the results obtained [14, 27]. We identified 70 different QTLs, predominantly associated with the fatty acid profile in cow's milk, casein content, animal energy status, calcium, potassium and phosphorus content in milk [24, 28].

Short- and medium-chain fatty acids, myristic and palmitic acids, saturated fatty acids had an association with polymorphisms in the *MED12L*, *EPHB1*, *GRIN2B*, *PRMT8*, *ERC1*, *PELI2*, *ARHGAP39*, *MROH1*, *MAF1*, *GSDMD*, *LY6D* genes (see Table 4), which were are associated with the number of successful inseminations, ease of calving, pregnancy rate of bull daughters, percentage of fat and protein in milk, palmitic FA, attachment and depth of the udder of cows, mastitis and the number of somatic cells in milk [24].

For long-chain, monounsaturated fatty acids, stearic and oleic acids, the annotation revealed the following selectable genes: *RPS6KA2*, *CPQ*, *CPE*, *FTO*, *FAT3*, *LUZP2* (see Table 4). Their polymorphisms are also associated with variability in the predisposition of cows to mastitis, linear measurements of the animal's exterior (limbs and udder), fertility, milk fat yield, and the number of somatic cells in milk (29). Trans FAs, despite their low variability compared to other fractions of milk fatty acids, in our study showed an association with polymorphisms in the *ARFGEF3* and *TNKS* genes, which are known to be associated with cow milk yield (30).

Thus, based on the study of the genetic and phenotypic variability of milk composition at the population level in 14 herds of dairy cattle (Moscow Province) and in the experimental herd of the Holsteinized Black-and-White cows (Krasnodar Territory), selection constants and a number of significant associations have been established between the identified gene polymorphisms and formation of milk fatty acids. Using a population genetic analysis based on the ratio of intergroup and general group variance, the highest heritability for oleic acid ($h^2 = 0.196$), monounsaturated fatty acids ($h^2 = 0.176$), long- and medium-chain fatty acids ($h^2 = 0.125$-0.55), stearic acid ($h^2 = 0.125$) was shown. These features can be recommended when evaluating sires for the quality of offspring. Studies conducted on a group of cows genotyped and phenotyped for the expanded component composition of milk have provided new data on the localization of QTL fatty acid composition in the genomes of animals of Russian origin. As a result of annotation in the genes

CACNA1C, *ARFGEF3*, *RPS6KA2*, *GCH1*, *ATG14*, *PELI2*, *KCNH5*, *PRKCE*, *CTNNA2*, *ARHGAP39*, *CYHR1*, *VPS28*, *DGAT1*, *MROH1*, *MAF1*, *GSDMD*, *ZC3H3*, *RHPN1*, *LY6D*, *TSNARE1*, *CPQ*, *CPE*, *FTO*, *TNKS*, *FAT3*, and *LUZP2* polymorphisms were found to be significantly associated with the variability of fatty acid content in milk. Saturated fatty acids compared to unsaturated fatty acids showed more variability in GWAS, probably due to stronger selection pressure. Further study of the genetic mechanisms of inheritance of the fatty acid composition of milk will make it possible to develop the basis for a selection strategy for this trait.

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