Effects of candidate gene polymorphisms on the detailed fatty acids profile determined by gas chromatography in bovine milk

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ABSTRACT

Association analyses between candidate genes and bovine milk fatty acids can improve our understanding of genetic variation in milk fatty acid profiles and reveal potential opportunities to tailor milk fat composition through selection strategies. In this work, we investigated the association of 51 single nucleotide polymorphisms (SNP) selected from 37 candidate genes using a functional and positional approach, with 47 fatty acids, 9 fatty acid groups, and 5 Δ^9-desaturation indices in milk samples from Brown Swiss cows. Individual milk samples were collected from 1,158 Italian Brown Swiss cows, and gas chromatography was used to obtain detailed milk fatty acid compositions. A GoldenGate assay system (Illumina, San Diego, CA) was used to perform genotype 96 selected SNP located in 54 genes across 22 chromosomes. In total, 51 polymorphic SNP in 37 candidate genes were retained for the association analysis. A Bayesian linear animal model was used to estimate the contribution of each SNP. A total of 129 tests indicated relevant additive effects between a given SNP and a single fatty acid trait; 38 SNP belonging to 30 genes were relevant for a total of 57 fatty acid traits. Most of the studied fatty acid traits (~81%) were relevantly associated with multiple SNP. Relevantly associated SNP were mainly found in genes related to fat metabolism, linked to or contained in previously identified quantitative trait loci for fat yield or content, or associated with genes previously identified in association analyses with milk fatty acid profiles in other cow breeds. The most representative candidate genes were *LEP*, *PRL*, *STAT5A*, *CCL3*, *ACACA*, *GHR*, *ADRB2*, *LPIN1*, *STAT1*, *FABP4*, and *CSN2*. In particular, relevant associations with SNP located on bovine chromosome 19 (BTA19) were found.

Two candidate genes on BTA19 (*CCL3* and *ACACA)* were relevantly associated with de novo short- and medium-chain fatty acids, likely explaining the high heritability values found for these fatty acids (with the exception of C6:0). Two additional genes on BTA19 (*CCL2* and *GH1*) showed associations with saturated and branched-chain fatty acids. Our findings provide basic information on genes and SNP affecting the milk fatty acid composition of dairy cows. These results may support the possibility of using genetic selection to modify milk fatty acid profiles to promote beneficial health-related effects.

Key words: association analyses, candidate genes, milk fatty acids, single nucleotide polymorphism

INTRODUCTION

Dairy products contain a wide range of saturated and unsaturated fatty acids with chain lengths ranging from 4 to >20 carbon atoms. Regulation of the fatty acid composition of milk has received attention because certain fatty acids are believed to have various beneficial or potentially harmful effects on human health. However, this issue is still very controversial, with recent studies seeming to contradict the traditional accepted role of specific fatty acids in health and disease. For instance, recent human meta-analyses found no association between SFA intake and the risk of cardiovascular disease or stroke (Mente et al., 2009; Siri-Tarino et al., 2010). Some epidemiological studies have suggested that coronary heart disease risk may be positively associated with industrial *trans* fatty acids but not those originating from biohydrogenation in ruminants, including vaccenic acid (**VA**) and the naturally occurring isomer of CLA or C18:2 *cis*-9,*trans*-11 (rumenic acid, **RA**; Gebauer et al., 2011). The biologically beneficial effects of CLA have been widely documented (Dilzer and Park, 2012), and both n-6 and n-3 PUFA are shown to reduce cardiovascular risk (Michas et al., 2014). However, the benefits of n-3 fatty acids [e.g., eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**)] seem to be not as pronounced as previously believed, warranting

caution when recommending dietary supplementation of PUFA to the general population without considering the individual intake of total energy and fats.

Because of this increasing awareness of the relationships between diet and health, several feeding and genetic strategies have been evaluated for their ability to engineer milk fat toward a healthier composition (Mele, 2009; Shingfield et al., 2013). In a few European countries (e.g., France and the Netherlands), the dairy industry recently introduced supplementary premiums for farmers based on milk fatty acid compositions (e.g., SFA, n-3 fatty acids, and total C18:1 fatty acids), and contents of α-linolenic acid (C18:3 *cis*-9,*cis*-12,*cis*-15), oleic acid (C18:1 *cis*-9), and palmitic acid (C16:0; Borreani et al., 2013). Feeding trials have clearly demonstrated that altering the cow's nutrition is a very effective way to quickly change the milk fat composition; however, these effects are completely reversible when feeding conditions change and the necessary changes can significantly increase the feeding cost (Shingfield et al., 2013).

In contrast, genetic improvement can gradually induce smaller but permanent trait modifications. Genetic analyses have shown heritable variations in the fatty acid profile of bovine milk (e.g., Bastin et al., 2013; Krag et al., 2013; Pegolo et al., 2016). Moreover, candidate gene studies have shown that SNP in the genes encoding stearoyl-CoA desaturase (**SCD**), diacylglycerol o-acyltransferase 1 (**DGAT1**), and sterol regulatory element binding protein-1 (**SREBP1**) clearly influence the milk fat composition and explain part of the genetic variation of milk fat unsaturation indices seen in Holsteins (Schennink et al., 2007, 2008; Rincon et al., 2012) and Brown Swiss cows (Mele et al., 2007; Conte et al., 2010). Single nucleotide polymorphisms located in other genes involved in lipid biosynthesis have also been shown to affect the milk fatty acid compositions of different bovine breeds (Matsumoto et al., 2012; Marchitelli et al., 2013; Nafikov et al., 2014). Genome-wide studies in Holstein cows have allowed researchers to detect QTL harboring candidate polymorphic genes that are involved in fat synthesis or known to affect fat yield or content, and significantly associated with fatty acids (~20 fatty acid traits were considered) (e.g., Stoop et al., 2009; Bouwman et al., 2011). Recently, a selective DNA-pooling approach was used to identify QTL for milk content of RA, VA, and SCD (Strillacci et al., 2014).

These results collectively suggest that loci found to affect fatty acid profiles could be used in marker-assisted selection programs aimed at modifying the nutritional qualities of milk. However, relatively few fatty acids have been examined to date in this context, and several interesting fatty acids are not yet well known, especially those present at relatively low amounts in milk (and thus hard to detect) but that could have a role in human health.

This work is part of a broader project including the evaluation of the effects of polymorphisms in candidate genes on production traits, milk technological properties, and milk fatty acid profile in Brown Swiss cows. We previously analyzed 96 SNP in 54 candidate genes, and 51 polymorphic SNP in 37 candidate genes were retained for association analyses with productivity and technological traits (Cecchinato et al., 2014, 2015). Here, we extend our association analysis to a very detailed milk fatty acid profile, including 47 individual fatty acids, 9 fatty acid groups, and 5 Δ^9-desaturation indices, determined by GC in the same population. Our aim is to develop genetic markers that will allow breeders to select for animals that produce milk with a healthier fatty acid composition.

MATERIALS AND METHODS

Animals and Milk Sampling

The milk sampling procedure is detailed in Cipolat-Gotet et al. (2013). Briefly, milk samples were collected once per cow during the evening milking from 1,158 Brown Swiss cows from 85 herds (a maximum of 15 cows per herd) located in the Alpine province of Trento (Italy). Each farm was sampled once. The milk samples (without the addition of preservative) were immediately refrigerated at 4°C and transferred to the Cheese-Making Laboratory of the Department of Agronomy, Food, Natural Resources, Animals and Environment of the University of Padova (Legnaro, Padova, Italy). Data regarding the cows and herds were provided by the Superbrown Consortium of Bolzano and Trento (Italy), and pedigree information was obtained from the Italian Brown Swiss Cattle Breeders Association (ANARB, Verona, Italy). Each sampled cow had known ancestors for at least 4 generations and the pedigree file included 8,845 animals; there were 1,326 sires, 264 of which had progeny (between 2 and 80 daughters) with records in the data set.

GC Analysis

Fatty acid methyl esters were prepared by the direct extraction and alkali-catalyzed *trans*-methylation procedure previously described by Feng et al. (2004) and modified as in Pegolo et al. (2016). The fatty acid composition was determined using a ThermoQuest gas chromatograph (ThermoElectron Corp., Waltham, MA) equipped with a flame-ionization detector and a high polar fused-silica capillary column (Chrompack

CP-Sil88 Varian, Middelburg, the Netherlands; 100 m, 0.25 mm i.d.; film thickness 0.20 μm). Individual FAME were identified by comparison with standard mixtures, pure standards, and published GC profiles. In the case of C16:1, C18:1, and C18:2 isomers, risk of peak coelution was assessed by using an Ag-ion solid-phase extraction fractionation, according to Kramer et al. (2008). A reference standard butter (BCR 164; Commission of the European Communities, Community Bureau of Reference, Brussels, Belgium) was used to estimate correction factors for the short-chain fatty acids (**SCFA**), as previously described by Mele et al. (2008). Inter- and intraassay coefficients of variation were calculated using the reference standard butter, and the detection limit of the analysis was 0.001% above that of the total fatty acids amount. The milk fatty acid composition was expressed as grams per 100 g of total fatty acids.

Blood Sampling, DNA Extraction, SNP Selection, and Genotyping

Blood sampling, DNA extraction, and SNP selection and genotyping were performed as previously described (Cecchinato et al., 2014). Briefly, DNA extraction was performed with a DNeasy 96 Blood and Tissue Kit (Qiagen, Hilden, Germany) from 100 μL of individual whole blood. The quantity and quality of the obtained DNA were assessed using the QBit system (Invitrogen, Carlsbad, CA) and 1% agarose gel electrophoresis. Candidate gene selection was carried out using both a functional candidate gene approach (genes involved in the synthesis of proteins, fatty acids, and components of the immune system) and a positional candidate gene approach (location in chromosomal regions associated with milk quality and technological properties). From a total of 113 SNP initially selected, the 89 SNP with the best Illumina designability rank scores, and 7 SNP with scores between 0.5 and 0.6 were chosen. The GoldenGate system (Illumina, San Diego, CA) was used to genotype the 96 selected SNP, which were located in 54 genes on 22 chromosomes. Fifty-one polymorphic SNP [minor allele frequency (**MAF**) >0.05] in 37 candidate genes were retained for the association analysis. Details on all of the genotyped SNP (e.g., chromosomal location, genomic position, type of mutation, MAF) are reported in the Supplemental Data file (http://dx.doi.org/10.3168/jds.2015-10420).

Statistical Analysis

Association studies for all investigated genes were carried out using Bayesian methodology (Sorensen and Gianola, 2002). The following mixed linear animal model was used:

$$y_{ijkl} = \mu + DIM_i + parity_j + h_k + a_l + x_{lm}\beta_m + \varepsilon_{ijkl},$$

where y_{ijkl} is the phenotypic record for the analyzed trait; DIM_i is the effect of the ith class of days in milk ($i = 1$ to 6; class 1, <60 d; class 2, 60 to 120 d; class 3, 121 to 180 d; class 4, 181 to 240 d; class 5, 241 to 300 d; and class 6, >300 d); $parity_j$ is the effect of the jth parity of the cow ($j = 1$ to 5 or more); h_k is the effect of the kth herd ($k = 1$ to 85); a_l is the polygenic effect of individual l; x_{lm} (0, 1, 2) reflects the number of copies of the minor allele at the mth SNP of subject l; β_m is the additive effect of the mth SNP; and ε_{ijkl} is the random residual term.

Bounded uniform priors were used for all environmental variables, and **a** and **h** were assumed a priori to be independent and normally distributed:

$$\mathbf{a} \mid \sigma_a^2 \sim N\left(\mathbf{0}, \mathbf{A}\sigma_a^2\right),$$

$$\mathbf{h} \mid \sigma_h^2 \sim N\left(\mathbf{0}, \mathbf{I}\sigma_h^2\right),$$

where **A** is the known additive genetic relationship matrix, **I** is the identity matrix, and σ_h^2 and σ_a^2 were herd and additive genetic variances, respectively. The pedigree file included information on 8,845 animals; there were 1,326 sires, 264 of which had progeny (between 2 and 80 daughters) with records in the data set.

The marginal posterior distributions of all parameters were obtained using a Gibbs sampler running with a single chain of 1,000,000 points; the first 50,000 were discarded as burn-in, as previously tested by Raftery and Lewis (1996). Samples were saved every 100 iterations. Due to autocorrelations between successive samples, convergence was tested using Geweke's Z-criterion (Geweke, 1992). Monte Carlo sampling errors and the effective sample size were computed using the time-series procedures described by Geyer (1992). The parameters of concern were the dispersion parameters and the additive effects of SNP, as defined by Falconer and Mackay (1996). The posterior mean was used as a point estimate for the parameter of concern. The lower and upper bounds of the 95% highest posterior probability density regions (**HPD95**) for each additive effect were estimated from the Gibbs samples.

For all traits, the model was fitted to separately estimate the contribution of each SNP (i.e., the model was run 51 times per trait for a total of 3,111 times). A SNP was considered as having a relevant effect on the

Table 1. Descriptive statistics for milk production traits and individual SFA (g/100 g of fatty acids)

Milk production trait	Mean	SD	P5[1]	P95[1]	Heritability[2]
Milk yield, kg/d	24.43	7.95	12.11	37.90	0.086
Fat, %	4.23	0.73	3.15	5.45	0.259
Individual fatty acids					
C4:0	3.46	0.91	2.09	4.99	0.033
C6:0	2.15	0.39	1.50	2.79	0.039
C8:0	1.35	0.23	0.97	1.70	0.124
C10:0	3.17	0.63	2.15	4.17	0.201
C11:0	0.06	0.04	0.02	0.13	0.134
C12:0	3.72	0.75	2.47	4.86	0.243
C13:0	0.11	0.04	0.06	0.18	0.078
C13:0 *iso*	0.06	0.04	0.02	0.14	0.034
C14:0	12.08	1.56	9.36	14.15	0.097
C14:0 *iso*	0.17	0.05	0.08	0.26	0.125
C15:0	1.19	0.24	0.82	1.59	0.102
C15:0 *iso*	0.28	0.08	0.17	0.42	0.092
C15:0 *ante*	0.53	0.12	0.35	0.73	0.115
C16:0	30.54	3.72	24.67	36.67	0.255
C16:0 *iso*	0.32	0.09	0.18	0.47	0.077
C17:0	0.54	0.12	0.39	0.75	0.092
C17:0 *iso*	0.32	0.08	0.18	0.45	0.050
C17:0 *ante*	0.42	0.09	0.29	0.56	0.102
C18:0	8.95	1.87	6.19	12.50	0.218
C20:0	0.13	0.04	0.07	0.20	0.097
C22:0	0.06	0.03	0.02	0.12	0.031
C24:0	0.04	0.02	0.01	0.08	0.031

[1]P5 = 5th percentile; P95 = 95th percentile.
[2]From Pegolo et al. (2016).

trait when the posterior means of the additive effect did not include zero in the HPD95 interval. Moreover, as suggested by Ramírez et al. (2014), we computed the posterior probability of the estimated effect of being >0 for positive effects or <0 for negative effects (**PPN0**). Only relevant SNP are presented in the tables. The genetic variance explained by a given SNP (**V_a**) was calculated from the estimated genotypic effects and the observed genotypic frequencies.

Descriptive statistics and estimates of genetic variance components are given in Tables 1, 2, and 3 and were extensively reported and discussed in Pegolo et al. (2016). The present results are expressed as the percentage of the total additive genetic variance obtained using the same data set.

RESULTS AND DISCUSSION

Associations of Candidate Genes with Milk Fatty Acid Profile

Several prior studies have taken the approach of performing association analyses between candidate genes and the milk fatty acid profile, but these studies considered relatively few genes and fatty acid traits (e.g., Rincon et al., 2012; Marchitelli et al., 2013; Nafikov et al., 2014). Moreover, to our knowledge, only one previ-

ous study reported association analyses with the milk fatty acid profile of the Brown Swiss breed (Strillacci et al., 2014). In that study, the authors performed genotyping using the Illumina BovineSNP50 BeadChip, but they considered only VA and RA as fatty acid traits.

In the present work, 38 different SNP belonging to 30 genes were relevant for 57 fatty acid traits (Tables 4, 5, and 6). The most representative and relevantly associated candidate genes are outlined in Table 7.

Leptin

The leptin (*LEP*) gene, which is located on BTA4, was associated with 9 fatty acids and 2 desaturation indices, giving it the greatest number of associations among the genes considered in the present study (Table 7). The *LEP* gene encodes a hormone that plays a key role in the metabolic adaptation of nutrient partitioning during the energy-consuming processes of pregnancy and lactation. At the onset of lactation, LEP in the presence of prolactin (**PRL**) enhances fat synthesis and elevates the expression levels of the genes encoding α-CN and β-LG (Feuermann et al., 2004).

We identified relevant associations between fatty acids and 2 SNP of *LEP*: rs110559656 and rs29004170. The *A* allele of rs110559656 was negatively associated with several short- and medium-chain SFA (from C6:0

Table 2. Descriptive statistics for individual UFA (g/100 g of fatty acids)

Individual fatty acids	Mean	SD	P5[1]	P95[1]	Heritability[2]
MUFA					
C10:1 *cis*-9	0.33	0.09	0.18	0.47	0.124
C14:1 *cis*-9	1.08	0.32	0.54	1.61	0.360
C16:1 *cis*-9	1.21	0.32	0.78	1.80	0.243
C16:1 *trans*-9	0.06	0.03	0.02	0.12	0.033
C17:1 *cis*-9	0.20	0.08	0.11	0.34	0.070
C18:1 *trans*-4	0.03	0.02	0.01	0.08	0.023
C18:1 *trans*-6–8	0.21	0.07	0.11	0.34	0.059
C18:1 *trans*-9	0.18	0.06	0.11	0.27	0.082
C18:1 *trans*-10	0.29	0.10	0.17	0.45	0.056
C18:1 *trans*-11 (vaccenic acid)	1.20	0.38	0.61	1.85	0.074
C18:1 *cis*-9	18.33	3.21	14.03	23.43	0.089
C18:1 *cis*-12	0.24	0.10	0.11	0.43	0.083
C18:1 *trans*-16	0.24	0.08	0.14	0.40	0.058
C20:1 *cis*-9	0.11	0.04	0.05	0.17	0.064
PUFA					
C18:2 *cis*-9,*trans*-11 (rumenic acid)	0.65	0.22	0.32	1.03	0.072
C18:2 *trans*-11,*cis*-15	0.10	0.08	0.04	0.18	0.032
C18:2 *trans*-9,*trans*-12	0.58	0.44	0.35	0.88	0.030
C18:2 *cis*-9,*cis*-12	2.04	0.60	1.23	3.12	0.060
C18:3 *cis*-9,*cis*-12,*cis*-15	0.56	0.17	0.30	0.86	0.093
C18:3 *cis*-9,*trans*-11,*cis*-15	0.04	0.03	0.02	0.10	0.031
C20:3 *cis*-8,*cis*-11,*cis*-14	0.10	0.06	0.05	0.21	0.109
C20:4 *cis*-5,*cis*-8,*cis*-11,*cis*-14	0.13	0.05	0.07	0.22	0.081
C20:5 *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17	0.05	0.02	0.02	0.10	0.045
C22:4 *cis*-7,*cis*-10,*cis*-13,*cis*-16	0.03	0.02	0.01	0.08	0.048
C22:5 *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19	0.08	0.03	0.04	0.14	0.039

[1]P5 = 5th percentile; P95 = 95th percentile.
[2]From Pegolo et al. (2016).

to C12:0; explaining from 2.4 to 8.7% of the genetic variance) and positively associated with C18:1 *cis*-9 (explaining 9% of the genetic variance). Interestingly, high heritability values have been previously reported for most of the de novo synthesized fatty acids (e.g., C8:0 to C12:0; Pegolo et al., 2016). Substitution of the *A* allele for the *G* allele of rs110559656 reduces SFA and increases MUFA (Table 6). Furthermore, the *G* allele

Table 3. Descriptive statistics of groups of fatty acids (g/100 g of fatty acids) and unsaturation indices

Item[1]	Mean	SD	P5[2]	P95[2]	Heritability[3]
Group of fatty acids					
SFA	69.63	4.11	61.98	75.50	0.147
MUFA	24.24	3.46	19.52	29.88	0.132
PUFA	3.78	0.79	2.66	5.13	0.107
Short-chain fatty acids	10.52	1.72	7.68	13.25	0.054
Medium-chain fatty acids	52.81	5.26	43.11	60.79	0.146
Long-chain fatty acids	34.38	5.14	26.94	43.45	0.126
Branched-chain fatty acids	2.08	0.41	1.47	2.78	0.083
Trans fatty acids	2.22	0.53	1.42	3.07	0.107
Trans fatty acids C18:1	2.16	0.52	1.37	3.00	0.111
Unsaturation index, %					
C10:1/(C10:0 + C10:1)	9.54	2.00	0.06	0.13	0.227
C14:1/(C14:0 + C14:1)	8.16	2.04	0.05	0.12	0.438
C16:1/(C16:0 + C16:1)	3.83	0.90	0.03	0.05	0.258
C18:1/(C18:0 + C18:1)	67.22	4.32	0.60	0.73	0.218
RA/(RA + VA)	34.98	5.53	0.26	0.44	0.098

[1]Short-chain fatty acids included the C4:0, C6:0, C8:0, and C10:0 fatty acids; medium-chain fatty acids included all linear fatty acids from C11:0 to C16:1; long-chain fatty acids included all linear fatty acids from C17:0 to C24:0; *trans* fatty acids included all *trans* fatty acids; *trans* fatty acids C18:1 included all *trans* isomers of C18:1. RA = rumenic acid; VA = vaccenic acid.
[2]P5 = 5th percentile; P95 = 95th percentile.
[3]From Pegolo et al. (2016).

Table 4. Estimated marginal posterior densities of additive effect for the relevant SNP on individual SFA

Trait	Gene[1]	rs no.[2]	Allele	Effect[3]	HPD95[4]	PPN0[5]	V_a,[6] %
C4:0	*LXRα*	rs134390757	T vs. C	0.068	0.011; 0.125	0.990	8.8
	PPARGC1A	rs44857081	A vs. C	0.210	0.035; 0.374	0.992	16.1
C6:0	*LEP*	rs29004170	C vs. G	0.030	0.003; 0.056	0.985	7.4
	CCL3	rs109686238	T vs. C	0.039	0.008; 0.069	0.993	11.8
	FABP4	rs110757796	A vs. G	0.056	0.002; 0.110	0.979	14.0
	LEP	rs110559656	A vs. G	−0.035	−0.068; −0.003	0.981	8.7
C8:0	*CCL3*	rs109686238	T vs. C	0.041	0.020; 0.062	0.999	13.1
	ACACA	rs110562092	A vs. G	−0.025	−0.044; −0.006	0.996	5.2
	LEP	rs110559656	A vs. G	−0.025	−0.047; −0.002	0.982	4.5
C10:0	*CCL3*	rs109686238	T vs. C	0.097	0.042; 0.155	0.999	5.8
	ACACA	rs110562092	A vs. G	−0.061	−0.113; −0.011	0.991	2.4
	LEP	rs110559656	A vs. G	−0.067	−0.128; −0.007	0.984	2.5
C11:0	*CCR2*	rs41257559	T vs. C	−0.004	−0.007; −0.001	0.989	0.7
	STAT5A	rs137182814	C vs. G	−0.031	−0.061; −0.001	0.978	4.8
C12:0	*CCL3*	rs109686238	T vs. C	0.112	0.044; 0.179	0.999	4.6
	AGPAT6	rs110445169	T vs. C	0.064	0.001; 0.127	0.975	1.5
	ACACA	rs110562092	A vs. G	−0.077	−0.138; −0.018	0.995	2.3
	LEP	rs110559656	A vs. G	−0.085	−0.157; −0.013	0.988	2.4
C13:0	*CCL2*	rs41255713	T vs. C	0.008	0.002; 0.013	0.921	2.3
	CCR2	rs41257559	T vs. C	−0.005	−0.009; −0.001	0.990	1.1
C13:0 *iso*	*XDH*	rs42890834	A vs. G	−0.003	−0.006; −0.001	0.977	0.4
C14:0	*CCL3*	rs109686238	T vs. C	0.154	0.019; 0.288	0.987	5.2
	CSN2	rs43703011	A vs. C	−0.216	−0.426; −0.005	0.979	7.8
C14:0 *iso*	*LEP*	rs29004170	C vs. G	−0.004	−0.008; −0.001	0.993	0.8
	PRL	rs110684599	A vs. C	0.010	0.004; 0.017	0.999	3.8
	CARD15	rs43710288	T vs. A	−0.004	−0.008; −0.001	0.978	0.8
C15:0	*GHR*	rs109136815	T vs. C	0.021	0.001; 0.042	0.977	3.1
	LPIN1	rs136905033	T vs. C	0.064	0.007; 0.120	0.986	12.3
	ADRB2	rs132839139	A vs. G	−0.100	−0.188; −0.009	0.986	15.8
C15:0 *iso*	*GHR*	rs109136815	T vs. C	0.006	0.001; 0.012	0.975	1.5
	PRL	rs110684599	A vs. C	0.015	0.008; 0.023	0.999	8.4
	XDH	rs42890834	A vs. G	−0.005	−0.011; −0.001	0.978	1.2
C15:0 *ante*	*CCL2*	rs41255714	A vs. G	−0.010	−0.019; −0.002	0.991	0.2
C16:0	*ORL1*	rs135588030	A vs. G	−0.486	−0.874; −0.091	0.994	2.4
C17:0	*CCR2*	rs41257559	T vs. C	−0.009	−0.019; −0.001	0.978	3.5
	GHR	rs109136815	T vs. C	0.010	0.001; 0.019	0.985	4.2
	ADRB2	rs132839139	A vs. G	−0.041	−0.078; −0.002	0.980	16.0
C17:0 *iso*	*CSN1S1*	rs43703010	A vs. G	−0.017	−0.034; −0.001	0.936	5.2
	STAT5A	rs137182814	C vs. G	0.016	0.005; 0.124	0.984	12.8
C17:0 *ante*	*PRL*	rs211032652	T vs. C	0.012	0.005; 0.019	0.999	6.7
	ORL1	rs135588030	A vs. G	0.011	0.001; 0.021	0.986	4.2
	STAT5A	rs109578101	T vs. C	−0.031	−0.049; −0.012	1.000	18.8
C18:0	*PRL*	rs211032652	T vs. C	0.202	0.045; 0.363	0.994	2.8
	TLR2	rs43706433	A vs. G	−0.177	−0.340; −0.016	0.984	2.1
C20:0	*PRL*	rs110684599	A vs. C	0.006	0.001; 0.012	0.978	1.4
	CSN3	rs43703015	T vs. C	0.007	0.001; 0.013	0.980	1.7
	ADRB2	rs132839139	A vs. G	0.022	0.003; 0.040	0.989	4.6
C22:0	*SCD1*	rs41255693	T vs. C	0.007	0.000; 0.014	0.974	1.3
	CSN1S1	rs43703010	A vs. G	−0.012	−0.020; −0.004	0.999	2.6
	ADRB2	rs132839139	A vs. G	0.014	0.001; 0.027	0.983	1.9

[1]All gene symbols were defined in Cecchinato et al. (2014).

[2] http://www.ncbi.nlm.nih.gov/snp.

[3]SNP were considered having a relevant effect on the trait when the posterior mean of the additive effect did not include zero in the HPD interval.

[4]HPD95 = lower and upper bound of the 95% highest posterior density region.

[5]PPN0 = the probability of the additive effect to be over or below zero.

[6]V_a = proportion (%) of genetic variance explained by each SNP.

of rs29004170 increases some PUFA, such as RA (V_a = 6.5%) and C18:2 *trans*-11,*cis*-15 (V_a = 3.1%). In the case of the 2 desaturase indices, the SNP had small but relevant effects (V_a < 0.1%; Tables 6 and 7).

Several polymorphisms in *LEP* have been studied for their associations with milk production traits in dairy cattle (e.g., Buchanan et al., 2003; Clempson et al., 2011; Zetouni et al., 2013). However, contradictory re-

sults have been found, probably reflecting the presence of population-specific haplotypes or the major role of other genes and QTL in milk production. In previous studies (Cecchinato et al., 2014, 2015), our group did not find any relevant association of SNP on *LEP* with milk technological or production traits. In their study on genetic variation of milk fatty acids, Marchitelli and colleagues (2013) found no relevant association between

Table 5. Estimated marginal posterior densities of additive effect for the relevant SNP on individual UFA

Trait	Gene[1]	rs no.[2]	Allele	Effect[3]	HPD95[4]	PPN0[5]	V_a,[6] %
MUFA							
C10:1 *cis*-9	*CCL3*	rs109686238	T vs. C	0.009	0.001; 0.017	0.992	3.8
	ACACA	rs110562092	A vs. G	−0.009	−0.015; −0.002	0.994	2.4
C14:1 *cis*-9	*LPIN1*	rs136905033	T vs. C	0.078	0.001; 0.157	0.974	3.5
C16:1 *cis*-9	*PI*	rs41257077	A vs. G	−0.041	−0.078; −0.003	0.983	2.5
	LPIN1	rs136905033	T vs. C	0.094	0.010; 0.181	0.984	6.6
C16:1 *trans*-9	*FABP4*	rs110757796	A vs. G	0.006	0.001; 0.011	0.989	1.0
C17:1 *cis*-9	*CCL3*	rs109686238	T vs. C	−0.013	−0.020; −0.006	1.000	0.3
	ACACA	rs110562092	A vs. G	0.006	0.001; 0.012	0.977	0.1
	LEP	rs110559656	A vs. G	0.009	0.002; 0.017	0.994	0.1
	ADRB2	rs132839139	A vs. G	−0.042	−0.075; −0.010	0.994	0.7
C18:1 *trans*-4	*CCL2*	rs41255713	T vs. C	0.003	0.001; 0.006	0.986	0.3
	FABP4	rs110757796	A vs. G	0.004	0.001; 0.007	0.988	0.4
	STAT5A	rs109578101	T vs. C	−0.005	−0.010; −0.001	0.984	0.5
C18:1 *trans*-6–8	*STAT1*	rs43705173	T vs. C	−0.006	−0.012; −0.001	0.988	1.7
	FGF2	rs110937773	A vs. G	0.005	0.001; 0.009	0.975	1.2
C18:1 *trans*-9	*AGPAT6*	rs110445169	T vs. C	−0.005	−0.010; −0.001	0.980	1.2
C18:1 *trans*-10	*CSN2*	rs43703011	A vs. C	0.014	0.001; 0.027	0.984	6.9
	GHR	rs109136815	T vs. C	0.011	0.002; 0.019	0.993	5.1
C18:1 *cis*-9	*LEP*	rs110559656	A vs. G	0.414	0.091; 0.739	0.995	9.1
C18:1 *cis*-12	*STAT1*	rs43705173	T vs. C	−0.009	−0.016; −0.002	0.995	3.8
	STAT1	rs43706906	C vs. G	−0.007	−0.013; −0.001	0.981	2.4
	ABCG2	rs41577868	T vs. G	−0.006	−0.013; −0.001	0.978	1.8
C18:1 *trans*-16	*STAT1*	rs43705173	T vs. C	−0.008	−0.014; −0.001	0.990	3.0
	STAT1	rs43706906	C vs. G	−0.006	−0.012; −0.001	0.981	1.8
	CARD15	rs43710288	T vs. A	−0.006	−0.013; −0.001	0.979	1.7
C20:1 *cis*-9	*GHR*	rs109136815	T vs. C	−0.004	−0.008; −0.001	0.989	0.7
	SCD1	rs41255693	T vs. C	0.009	0.001; 0.018	0.982	2.1
PUFA							
C18:2 *cis*-9,*trans*-11 (rumenic acid)	*LEP*	rs29004170	C vs. G	−0.020	−0.033; −0.006	0.998	6.5
	CSN2	rs43703013	G vs. C	−0.032	−0.056; −0.009	0.996	9.2
	PRL	rs110684599	A vs. C	0.036	0.014; 0.057	0.999	16.2
C18:2 *trans*-11,*cis*-15	*LEP*	rs29004170	C vs. G	−0.008	−0.016; −0.001	0.976	3.1
C18:2 *trans*-9,*trans*-12	*PRL*	rs211032652	T vs. C	0.014	0.009; 0.101	0.988	1.5
C18:2 *cis*-9,*cis*-12	*FABP4*	rs110757796	A vs. G	0.074	0.003; 0.145	0.980	6.7
	LPIN1	rs137457402	T vs. G	−0.043	−0.077; −0.007	0.992	4.1
C18:3 *cis*-9,*cis*-12,*cis*-15	*LPIN1*	rs136905033	T vs. C	−0.037	−0.072; −0.003	0.982	8.2
C18:3 *cis*-9,*trans*-11,*cis*-15	*PLCE1*	rs41624917	T vs. C	0.003	0.001; 0.006	0.997	0.0
	STAT5A	rs137182814	C vs. G	−0.043	−0.078; −0.010	0.993	4.2
C20:3 *cis*-8,*cis*-11,*cis*-14	*STAT5A*	rs137182814	C vs. G	−0.047	−0.094; −0.001	0.975	14.5
	STAT1	rs43705173	T vs. C	−0.005	−0.009; −0.001	0.990	1.2
	STAT1	rs43706906	C vs. G	−0.005	−0.009; −0.001	0.990	1.2
C20:4 *cis*-5,*cis*-8,*cis*-11,*cis*-14	*STAT1*	rs43705173	T vs. C	−0.005	−0.009; −0.001	0.990	1.1
	STAT1	rs43706906	C vs. G	−0.005	−0.009; −0.001	0.990	1.2
C20:5 *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17	*LEPR*	rs43349286	T vs. C	−0.003	−0.006; −0.001	0.981	0.3
	ACACA	rs110562092	A vs. G	−0.002	−0.004; −0.001	0.988	0.2
C22:4 *cis*-7,*cis*-10,*cis*-13,*cis*-16	*PLCE1*	rs41624917	T vs. C	−0.002	−0.004; −0.001	0.985	0.2
	PI	rs41257077	A vs. G	0.002	0.000; 0.004	0.980	0.1
C22:5 *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19	*FGF2*	rs110937773	A vs. G	−0.003	−0.006; −0.001	0.998	0.4
	DGKG	rs41608610	T vs. C	−0.006	−0.011; −0.002	0.997	1.0
	STAT5A	rs109578101	T vs. C	−0.008	−0.015; −0.001	0.982	1.3

[1]All gene symbols were defined in Cecchinato et al. (2014).

[2]http://www.ncbi.nlm.nih.gov/snp.

[3]SNP were considered to have a relevant effect on the trait when the posterior mean of the additive effect did not include zero in the HPD interval.

[4]HPD95 = lower and upper bound of the 95% highest posterior density region.

[5]PPN0 = the probability of the additive effect to be over or below zero.

[6]V_a = proportion (%) of genetic variance explained by each SNP.

Table 6. Estimated marginal posterior densities of additive and dominance effects for the relevant SNP on groups of fatty acids and unsaturation indices.

Trait[1]	Gene[2]	rs no.[3]	Allele	Effect[4]	HPD95[5]	PPN0[6]	V_a,[7] %
Group of fatty acids							
SFA	PLCE1	rs41624917	T vs. C	0.395	0.025; 0.775	0.979	2.4
	ABCG2	rs41577868	T vs. G	0.360	0.051; 0.664	0.989	2.9
	AGPAT6	rs110445169	T vs. C	0.330	0.006; 0.667	0.974	2.3
	LEP	rs110559656	A vs. G	−0.573	−0.947; −0.196	0.998	6.2
	GH1	rs41923484	C vs. G	0.395	0.009; 0.797	0.974	2.5
MUFA	PLCE1	rs41624917	T vs. C	−0.342	−0.676; −0.003	0.977	3.3
	LEP	rs110559656	A vs. G	0.499	0.160; 0.837	0.998	7.7
PUFA	LEP	rs29004170	C vs. G	−0.049	−0.097; 0.000	0.976	1.7
Short-chain fatty acids	LEP	rs29004170	C vs. G	0.129	0.010; 0.251	0.983	5.4
	CCL3	rs109686238	T vs. C	0.189	0.054; 0.326	0.996	11.8
	ACACA	rs110562092	A vs. G	−0.128	−0.248; −0.006	0.980	5.1
Medium-chain fatty acids	PLCE1	rs41624917	T vs. C	0.424	0.006; 0.841	0.976	2.0
	ABCG2	rs41577868	T vs. G	0.364	0.023; 0.709	0.982	1.8
	ORL1	rs135588030	A vs. G	−0.550	−1.065; −0.008	0.979	2.9
Long-chain fatty acids	ABCG2	rs41577868	T vs. G	−0.383	−0.754; −0.009	0.978	2.5
	PRL	rs211032652	T vs. C	0.396	0.000; 0.789	0.975	2.5
	ORL1	rs135588030	A vs. G	0.650	0.080; 1.222	0.985	5.0
	LEP	rs110559656	A vs. G	0.498	0.035; 0.947	0.984	3.6
Branched-chain fatty acids	ORL1	rs135588030	A vs. G	0.039	0.001; 0.076	0.968	3.7
	PRL	rs110684599	A vs. C	0.041	0.001; 0.080	0.979	4.5
	STAT5A	rs109578101	T vs. C	−0.079	−0.148; −0.009	0.987	8.7
	GH1	rs41923484	C vs. G	0.032	0.001; 0.063	0.978	2.3
Trans fatty acids	CARD15	rs43710288	T vs. A	−0.041	−0.078; −0.004	0.984	2.6
Trans fatty acids 18:1	CARD15	rs43710288	T vs. A	−0.040	−0.078; −0.004	0.982	2.5
Unsaturation index							
C10:1/(C10:0 + C10:1)	CSN2	rs43703013	G vs. C	−0.003	−0.006; 0.000	0.984	<0.1
	GHR	rs109136815	T vs. C	−0.002	−0.004; 0.000	0.981	<0.1
C16:1/(C16:0 + C16:1)	PI	rs41257077	A vs. G	−0.001	−0.002; 0.000	0.993	<0.1
	LEP	rs110559656	A vs. G	0.001	0.000; 0.002	0.937	<0.1
	ADRB2	rs132839139	A vs. G	−0.004	−0.008; 0.000	0.981	<0.1
C18:1/(C18:0 + C18:1)	TLR2	rs43706434	A vs. G	−0.009	−0.018; 0.000	0.979	<0.1
	LEP	rs110559656	A vs. G	0.005	0.000; 0.010	0.978	<0.1
RA/(RA + VA)	TLR2	rs43706434	A vs. G	−0.011	−0.021; −0.001	0.984	<0.1

[1]Short-chain fatty acids included the C4:0, C6:0, C8:0 and C10:0 fatty acids; medium-chain fatty acids included all linear fatty acids from C11:0 to C16:1; long-chain fatty acids included all linear fatty acids from C17:0 to C24:0; *trans* fatty acids included all *trans* fatty acids; *trans* fatty acids C18:1 included all *trans* isomers of C18:1. RA = rumenic acid; VA = vaccenic acid.

[2]All gene symbols were defined in Cecchinato et al. (2014).

[3]http://www.ncbi.nlm.nih.gov/snp.

[4]SNP were considered to have a relevant effect on the trait when the posterior mean of the additive effect did not include zero in the HPD interval.

[5]HPD95 = lower and upper bound of the 95% highest posterior density region.

[6]PPN0 = the probability of the additive effect to be over or below zero.

[7]V_a = proportion (%) of genetic variance explained by each SNP.

a SNP in the *LEP* gene (g.1180C>T) and the analyzed fatty acid traits in 3 different bovine breeds.

Prolactin

As expected, the *PRL* gene on BTA23 showed relevant associations with some of the milk fatty acid traits considered (7 traits; Table 7). Prolactin plays a critical role in mammary gland development, lactogenesis, and galactopoiesis. The mechanism responsible for the effects of PRL on milk fatty acid composition is not completely known, but it seems to involve signal transducer and activator of transcription (**STAT**)5A, which

is regulated by PRL (Welte et al., 1994). However, an investigation of the PRL-mediated regulation of lipid biosynthesis genes in the mouse demonstrated that the expression of several transcription factors (including SREBP1) and lipogenic genes involved in de novo fatty acids synthesis, triglyceride synthesis, and β-oxidation were rapidly and coordinately decreased in lactating mammary gland cells via suppression of serum PRL. Moreover, PRL seems to be involved in cytosolic lipid droplet formation (Rudolph et al., 2011).

Here, we found relevant associations of 2 SNP of the *PRL* gene (rs110684599 and rs211032652) with the contents of 7 fatty acids. The *A* allele of rs110684599 was

positively associated with RA ($V_a = 16.2\%$), C14:0 *iso* ($V_a = 3.8\%$), and C15:0 *iso* ($V_a = 8.4\%$), whereas the *T* allele of rs211032652 was positively associated with C18:2 *trans*-9,*trans*-12 ($V_a = 1.5\%$), long-chain SFA [C18:0 ($V_a = 2.8\%$) and C20:0 ($V_a = 1.4\%$)], and C17:0 *anteiso* ($V_a = 6.7\%$). To our knowledge, this is the first time that a SNP of the *PRL* gene has been associated with RA, for which it explains more than 15% of genetic variance of the trait. We previously reported that another SNP of the *PRL* gene (rs109428015) was relevantly associated with some milk technological traits, such as the time required for curd firming and time to maximum curd firmness (Cecchinato et al., 2015). A previous study found a different *PRL* mutation, g.8398G>A, showing an effect (albeit not significant) on C10:0 and C12:1 in Dutch Holstein-Friesian cows (Schennink et al., 2009). On the other hand, studies on genome-wide associations with milk fatty acid traits in different populations of dairy cattle did not detect any QTL on BTA23 close to the *PRL* gene (Bouwman et al., 2011; Buitenhuis et al., 2014; Li et al., 2014). These contrasting results could be explained by a low frequency of SNP in *PRL* in Holsteins or by the presence of allele-specific populations.

It is worth mentioning that Nafikov and colleagues (2013a) found significant associations of haplotypes in the sterol regulatory element binding transcription factor 1 (**SREBF1**) with milk production and variations in lauric acid (12:0) and myristic acid (14:0) concentrations in milk. In particular, a tag SNP (*SREBF1*:g.13495T>C) caused the Pro852Leu mutation in the regulatory C-terminal domain of SREBP1, and Pro852 was found to be highly conserved among multiple mammalian species including cow. Despite this, the authors provided evidence for the prevalence of Leu852 in their population as a probable result of an-imal selection. Due to the putative functional relationships between PRL and SREBP1, we cannot exclude the possibility that the observed effects on milk fatty acids composition might be due to SNP in *SREBF1* rather than in *PRL*.

STAT5A

Signal transducer and activator of transcription 5A (*STAT5A*), which is located on BTA19, encodes a transcription factor that plays an important role in lactogenesis, as PRL imparts its effects on milk protein and lipid synthesis mainly through the Jak2/STAT5 signaling pathway (Rudolph et al., 2011). We found that 2 polymorphisms (rs109578101 and rs137182814) in *STAT5A* relevantly influenced 7 fatty acids, including some branched-chain fatty acids (**BCFA**). The *T* allele of rs109578101 negatively influenced C17:0 *anteiso* ($V_a = 18.8\%$), whereas the *C* allele of rs137182814 showed a positive association with C17:0 *iso* ($V_a = 12.8\%$) and negative associations with 2 PUFA: C18:3 *cis*-9,*trans*-11,*cis*-15 and C20:3 *cis*-8,*cis*-11,*cis*-14 ($V_a = 4.2$ and 14.5%, respectively). A negative association was also found between the *T* allele of rs109578101 and C22:5 *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 (Table 5). We previously reported a relevant association between *STAT5A* rs109578101 and asymptotic potential curd firmness (Cecchinato et al., 2015). In Dutch Holstein-Friesian cows, a *STAT5A* polymorphism (g.9501G>A) was found to be associated with medium-chain fatty acids (**MCFA**) and desaturase indices, with the *A* allele showing positive associations with C10:0 and C14:0, and negative associations with the monounsaturated MCFA and desaturase indices (Schennink et al., 2009). Interestingly, a major QTL for milk fat content and fatty acids in bovine milk was previously detected on

Table 7. List of the most representative candidate genes

Gene	No. of traits	BTA	Traits[1]
LEP	11	4	C6:0; C8:0; C10:0; C12:0; C14:0 *iso*; C17:1 *cis*-9; C18:1 *cis*-9; RA; C18:2 *trans*-11,*cis*-15; C16 index; C18 index
PRL	7	23	C14:0 *iso*; C15:0 *iso*; C17:0 *ante*; C18:0; C20:0; RA; C18:2 *trans*-9,*trans*-12
STAT5A	7	19	C11:0; C17:0 *ante*; C17:0 *iso*; C18:1 *trans*-4; C18:3 *cis*-9,*trans*-11,*cis*-15; C20:3 *cis*-8,*cis*-11,*cis*-14; C22:5 *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19
CCL3	7	19	C6:0; C8:0; C10:0; C10:1 *cis*-9; C12:0; C14:0; C17:1 *cis*-9
ACACA	6	19	C8:0; C10:0; C10:1 *cis*-9; C12:0; C17:1 *cis*-9; C20:5 *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17
GHR	6	20	C15:0; C15:0 *iso*; C17:0; C18:1 *trans*-10; C20:1 *cis*-9; C10 index
ADRB2	6	7	C15:0; C17:0; C17:1 *cis*-9; C20:0; C22:0; C16 index
LPIN1	5	11	C14:1 *cis*-9; C15:0; C16:1 *cis*-9; C18:2 *cis*-9,*cis*-12; C18:3 *cis*-9,*cis*-12,*cis*-15
STAT1	5	2	C18:1 *cis*-12; C18:1 *trans*-16; C18:1 *trans*-6–8; C20:3 *cis*-8,*cis*-11,*cis*-14; C20:4 *cis*-5,*cis*-8,*cis*-11,*cis*-14
FABP4	4	14	C6:0; C16:1 *trans*-9; C18:1 *trans*-4; C18:2 *cis*-9,*cis*-12
CSN2	4	6	C14:0; C18:1 *trans*-10; RA; C10 index

[1]RA = rumenic acid.

BTA19 in Holstein cows (Bouwman et al., 2011, 2012). In particular, significant associations were found for C4:0, C8:0 C10:0, C12:0, and C14:0 and SNP located in the region between 52.1 and 55.1 Mb on BTA19. Stoop et al. (2009) found a putative QTL close to *STAT5A* (which is located at around 43 Mb) that was positively associated with C14:0 content in a Dutch Holstein population. In the Brown Swiss breed, major chromosomal regions on BTA19 showed significant associations with RA, including significant SNP close to *STAT5A* (Strillacci et al., 2014).

Chemokine C-C Motif Ligand 3

The chemokine C-C motif ligand (*CCL*)3 gene on BTA19 encodes a protein that contributes to inflammatory responses by binding the receptors, chemokine (C-C motif) receptor (**CCR**)1, CCR4, and CCR5. Recently, CCL3 was also associated with obesity in humans (Muñoz and Costa, 2013). Here, we found a single SNP on *CCL3* (rs109686238) that was relevantly associated with 7 fatty acid traits. The *T* allele showed a positive association with the SCFA concentration (V_a = 11.8%), most prominently with C10:0 (V_a = 5.8%). A positive association was also observed with C12:0 and C14:0 (V_a = 4.6 and 5.2%, respectively). The *T* allele of rs109686238 of *CCL3* generally increased the content of de novo fatty acids from C6:0 to C14:0. Meanwhile, the *T* allele of this SNP was negatively associated with 2 products of the SCD enzyme: C10:1 *cis*-9 and C17:1 *cis*-9. We are unaware of any previous report examining *CCL3* gene polymorphisms and the effects on the inflammatory response, so we are unable to evaluate whether the positive association of the *T* allele with de novo fatty acids could also be associated with the main function of the gene.

Acetyl-CoA Carboxylase α

The acetyl-CoA carboxylase α (*ACACA*) gene on BTA19 encodes an enzyme that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, leading to the biosynthesis of de novo fatty acids from C4:0 to C16:0 in the mammary gland (Chilliard et al., 2000). Here, we report that rs110562092 of *ACACA* is associated with some de novo fatty acids. In particular, a negative association was found between the *A* allele and the concentrations of C8:0, C10:0, and C12:0 in milk fat (V_a = 5.2, 2.5, and 2.3%, respectively). A slight but relevant negative effect on the content of n-3 EPA (C20:5 *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17) was also detected (V_a = 0.2%). Interestingly, polymorphisms in *CCL3* and *ACACA* were found to affect largely the same fatty acids, and the 2 genes are located relatively close

together on BTA19 (around 14.6 and 13.8 Mb, respectively). However, analyses performed with Haploview software (Barrett et al., 2005) to check for the existence of linkage disequilibrium between the 2 SNP in these genes showed that they were completely independent in the sample population (data not shown). Matsumoto and colleagues (2012) showed that 3 SNP on *ACACA* influenced fatty acid composition in the milk of Holstein cows (in particular, the combined profile of C14:0, C16:0, and C18:0). Similarly, Li et al. (2014) found that a QTL in *ACACA* was significantly associated with C10:0 content in a Chinese Holstein population. Strillacci and colleagues (2014) found significant associations between SNP close to *ACACA* (13.7 Mb) and phenotypic variations in RA.

Growth Hormone Receptor

Growth hormone receptor (*GHR*) is located on BTA20 and encodes a member of the type I cytokine receptor family of growth hormone receptors. It is considered a strong positional and functional candidate gene for association with milk fatty acid traits because it is located very close to a QTL previously shown to affect milk production traits (Blott et al., 2003). Indeed, we found that the *T* allele of *GHR* rs109136815 was positively associated with the concentration of 5 fatty acids in milk fat. The most relevant effects were observed for C15:0 (V_a = 3.1%), C17:0 (V_a = 4.2%), and C18:1 *trans*-10 (V_a = 5.1%). The odd-chain fatty acids (such as C15:0 and C17:0) are produced by elongation of propionate or valerate by rumen microbes (Fievez et al., 2012), so the relationship with this *GHR* polymorphism is likely to be indirect. Rumen microbes are also responsible for the synthesis of C18:1 *trans*-10, which is an intermediate of the biohydrogenation of dietary PUFA. The role of C18:1 *trans*-10 in milk fat synthesis is not yet completely understood, but this fatty acid is likely to be involved (along with other rumen biohydrogenation intermediates) in modulating the expression of several genes involved in mammary lipid metabolism (Shingfield et al., 2013). To our knowledge, the literature does not contain any information regarding a relationship between C18:1 *trans*-10 and *GHR*. However, Li and colleagues (2014) found a significant association between one SNP located within *GHR* and the C18:0 content of milk from Chinese Holsteins.

Finally, the *GHR* polymorphism had only a trivial effect on the C10 index (V_a < 0.1%; Tables 6 and 7).

Adrenoceptor β2

Adrenoceptor β2 (*ADRB2*), which is located on BTA7, encodes the β2 adrenergic receptor. This recep-

tor is part of the adrenergic system, which stimulates lipid mobilization in adipose tissue (Kurokawa et al., 2008). The transcription of *ADBR2* has been shown to increase during the transition phase of dairy cows, as lipolysis is strongly related to the activation of ADRB2 via norepinephrine produced by the sympathetic nervous system (Sumner-Thomson et al., 2011).

In the present work, we observed that the *A* allele of *ADRB2* rs132839139 was negatively associated with the concentrations of some long-chain SFA in milk fat, such as C17:0 (V_a = 16.0%), C20:0 (V_a = 4.6%), and C22:0 (V_a = 1.9%). A relevant negative association was previously found between the same allele and MUN in Brown Swiss cows (Cecchinato et al., 2014). No association was found with respect to the main fatty acids involved in lipid mobilization, such as C16:0, C18:0, and C18:1. However, the *ADRB2* polymorphism showed a relevant negative association with the C16 index (V_a = 0.001%).

Lipin 1

The lipin 1 (*LPIN1*) gene on BTA11 encodes the enzyme that cleaves the P group of phosphatidic acid during its conversion to diacylglycerol. The *LPIN1* gene is the major isoform expressed in the bovine mammary gland, and its expression increased more than 3-fold during the first phase of lactation (Bionaz and Loor, 2008; Nafikov et al., 2014). It is known to be directly involved in milk fat synthesis and in regulating the transcription of other genes involved in milk fat synthesis (Bionaz and Loor, 2008). In the present work, we found that 2 SNP of *LPIN1* were associated with 5 fatty acids: C14:1 *cis*-9, C15:0, C16:1 *cis*-9, C18:2 *cis*-9,*cis*-12, and C18:3 *cis*-9,*cis*-12,*cis*-15 (Table 6). The *T* allele of rs136905033 was positively associated with C14:1 *cis*-9 (V_a = 3.5%), C15:0 (V_a = 12.3%), and C16:1 *cis*-9 (V_a = 6.6%), whereas it was negatively associated with α-linolenic acid (C18:3 *cis*-9,*cis*-12,*cis*-15; V_a = 8.2%). The *T* allele of the other SNP (rs137457402), which has a relatively high MAF (0.43; Cecchinato et al., 2014), was negatively associated with the concentration of linoleic acid in milk fat (C18:2 *cis*-9,*cis*-12; V_a = 4.1%). These alleles may thus negatively affect milk fatty acid composition through their associations with lower amounts of PUFA in milk fat. On the other hand, the positive associations of the *T* allele of rs136905033 with C14:1 *cis*-9 and C16:1 *cis*-9 suggest that this SNP may play a positive role in the biosynthetic activity of the mammary gland, where these 2 fatty acids are mainly produced (Mele et al., 2007). Results obtained in studies on human and animal models identified and characterized C16:1 *cis*-9 as a novel lipokine or lipid hormone (German, 2011).

Nafikov et al. (2014) did not observe any significant association between *LPIN1* haplotypes and the milk fatty acid composition in Holstein cows.

STAT1

The *STAT1* gene, which is located on BTA2, is constitutively expressed in the mammary gland at constant levels through pregnancy, lactation, and involution. The STAT1 protein is involved in the development and differentiation of the mammary gland, and it was shown to play a particularly important role in regulating the transcription of genes involved in milk protein synthesis and fat metabolism in Holstein cows (Cobanoglu et al., 2006).

We found that 2 SNP (rs43705173 and rs43706906) in the *STAT1* gene were associated with the concentration of 5 fatty acids. The *T* allele of rs43705173 and the *C* allele of rs43706906 were associated with slight reductions of C18:1 *trans*-16 (V_a = 3.0 and 1.8%, respectively), C18:1 *cis*-12 (V_a = 3.8 and 2.4%, respectively), C20:3 *cis*-8,*cis*-11,*cis*-14 (V_a = 1.2% for both SNP), and C20:4 *cis*-5,*cis*-8,*cis*-11,*cis*-14 (V_a = 1.1 and 1.2%, respectively). Given these effects on the same fatty acid traits, the existence of linkage disequilibrium between these SNP on *STAT1* gene was evaluated and confirmed by Haploview analyses (D′, the normalized linkage disequilibrium coefficient, = 0.98; data not shown). In the case of C18:1 *trans*-6–8, only rs43705173 had a relevant effect, showing a negative association with the *T* allele. The effect on *trans* fatty acids was probably only indirect, as this class of fatty acids originates in the rumen during the biohydrogenation process. In the cases of C20:3 *cis*-8,*cis*-11,*cis*-14 and C20:4 *cis*-5,*cis*-8,*cis*-11,*cis*-14, the 2 SNP for *STAT1* and the 1 SNP for *STAT5A* were the only SNP that had relevant effects. This suggests that elongation of n-3 and n-6 fatty acids is probably related to these 2 *STAT* genes. The literature includes few studies on the effects of *STAT1* polymorphisms on milk fatty acid composition. Two SNP located 0.50 Mb away from the *STAT1* gene were previously associated with the C18:0 and C18 index in Chinese Holstein (Li et al., 2014).

Fatty Acid Binding Protein 4

The fatty acid binding protein (*FABP*)*4* gene, which is located on BTA14, encodes an intracellular protein that binds nonesterified saturated and unsaturated fatty acids, eicosanoids, and other lipids to transport or store them inside a cell (Bionaz and Loor, 2008). Several papers have demonstrated significant associations between genetic polymorphisms in *FABP4* and the fatty acid composition in adipocytes (Michal et al.,

2006; Pannier et al., 2010). The *FABP4* gene is expressed in the mammary gland, along with *FABP3* and *FABP5*, but little information is available regarding the function of FABP4 in the mammary gland.

Our results showed that the *A* allele of *FABP4* rs110757796 induced a slight increase in the amount of linoleic acid (C18:2 *cis*-9,*cis*-12; $V_a = 6.7\%$). Interestingly, a previous study showed that the MAF was relatively low for this allele (0.16; Cecchinato et al., 2014), suggesting that it may be possible to develop breeding strategies to increase its frequency. A positive association was also observed with C6:0 ($V_a = 14.1\%$), and near-trivial positive associations were found for C16:1 *trans*-9 and C18:1 *trans*-4 ($V_a = 1.0$ and 0.4%, respectively). Marchitelli et al. (2013) found that *FABP4* was the most important gene affecting milk fatty acid composition in different bovine breeds. In particular, *FABP4* g.2989A>G showed negative effects on MCFA and positive effects on long-chain fatty acids (**LCFA**). A *FABP4* haplotype was found to be negatively associated with SFA, SFA:UFA ratio, C12:0, and C14:0, whereas it was positively associated with UFA and MUFA (Nafikov et al., 2013b).

β-Casein

The *CSN2* gene on BTA6 is highly polymorphic (Chessa et al., 2013) and encodes β-CN, one of the main caseins present in milk proteins. In the present study, we found that 2 SNP in *CSN2* had relevant effects on milk fatty acid composition: rs43703013 and rs43703011. In the first case, a negative association was revealed between the *G* allele and the concentration of RA in milk fat ($V_a = 9.2\%$). Bouwman et al. (2011) previously reported that a region of BTA6 between 111.2 and 115.6 Mb harbored SNP with a relevant effect on the concentration of RA in milk fat. The *A* allele of *CSN2* rs43703011 showed a negative effect on the concentration of C14:0 in milk fat ($V_a = 7.8\%$), a positive effect on the concentration of C18:1 *trans*-10 in milk fat ($V_a = 6.9\%$), and a relevant negative association with the C10 index ($V_a < 0.1\%$).

Polymorphisms in *CSN2* have been reported to have relevant effects on milk and protein yield. In dairy goats, *CSN2* haplotypes were shown to affect the milk fat percentage (Dagnachew and Ådnøy, 2014). Moreover, a previous genome-wide association suggested that a region on BTA6 close to candidate genes [e.g., peroxisome proliferator-activated receptor gamma and coactivator 1 α (*PARGC1A*)] harbored SNP that have significant effects on C6:0, C8:0 (Stoop et al., 2009), C12:1 *cis*-9, C14:1 *cis*-9, and C16:1 *cis*-9 (Bouwman et al., 2011).

Stearoyl-CoA Desaturase 1

Although *SCD1* was not included in the list of the most representative genes (in terms of number of relevant associations; Table 7), *SCD1* is another gene having a pivotal role in fatty acid metabolism and is a well-known candidate gene for association analyses with fatty acids. The *SCD1* gene on BTA26 encodes the SCD1 enzyme (also known as Δ^9-desaturase), which introduces a double bond at the Δ^9-position of a large spectrum of fatty acids, including acyl-CoA of C14:0, C16:0, C18:0, and C18:1 *trans*-11, which are converted into C14:1 *cis*-9, C16:1 *cis*-9, C18:1 *cis*-9, and RA, respectively (Mele et al., 2007). In the present study, the *T* allele of *SCD1* rs41255693, which causes an A-to-V amino acid substitution (*SCD1* A293V) at 21.1 Mb, was positively associated with the concentrations of both C22:0 and C20:1 *cis*-9 in milk fat ($V_a = 1.3$ and 2.1%, respectively). In a previous study from our group, the same SNP of the *SCD1* gene was also relevantly associated with an increase in fat percentage (Cecchinato et al., 2014). Moreover, several studies from other groups have reported polymorphisms in both exonic and intronic regions of the gene in Holsteins, and showed that they had some relevant effects on the desaturation index and the milk contents of RA and several SFA (Mele et al., 2007; Schennink et al., 2008). Stearoyl-CoA desaturase plays a major role in determining the milk contents of MUFA, primarily oleic acid, as well as the CLA content of milk fat. Surprisingly, we did not find any relevant association for additive genetic effect and the main products of SCD enzyme activity reported above. In a different Brown Swiss population, Conte et al. (2010) found that the same SNP had significant effects on C14 index and the milk content of C14:1 *cis*-9. However, that study was performed on a much smaller number of animals (351 vs. 1,158 in the present work) and using a different statistical approach. Besides, when specifically looking at the content of C14:1 *cis*-9 and the estimated C14 index, we observed similar values between the homozygous *AA* and the heterozygous *AV*, which both differed from the homozygous *VV*, suggesting the absence of an allele substitution effect. Interestingly, we observed relevant dominance effects (**d**) for rs136334180 on C18 and CLA indices (d = 0.01), C18:0 (d = −0.23), RA (d = 0.02), PUFA (d = 0.08), C20:1 *cis*-9 (d < 0.01) and for rs41255693 on C16:0 *iso* (d = −0.02) and C20:1 *cis*-9 (d = −0.01; data not shown), supporting the hypothesis that the effects of the *A* allele (rs136334180) and *T* allele (rs41255693) are not additive in the Brown Swiss population. The balance between the 2 alleles of SNP rs41255693 differed between Holsteins and our

Brown Swiss population, in which the SNP was nearly monomorphic (Conte et al., 2010; Cecchinato et al., 2014). Interestingly, the dominance effects of SNP in the *SCD* gene were found to be relevant for milk yield, fat yield, protein yield, and protein percentage in Chinese Holstein (Alim et al., 2012).

Other Genes

The results of our association analyses for other tested candidate genes are briefly reported below. Considering fatty acid groups and indices, relevant associations were found as follows: for SFA with SNP related to the genes encoding ATP-binding cassette sub-family G member 2 (*ABCG2*), 1-acylglycerol-3-phosphate O-acyltransferase 6 (*AGPAT6*), growth hormone 1 (*GH1*), and phospholipase C epsilon 1 (*PLCE1*); for MUFA with a SNP of *PLCE1*; for BCFA with polymorphisms of *GH1* and the gene encoding opiate receptor-like 1 (*ORL1*); for MCFA with polymorphisms of *ABCG2*, *ORL1* and *PLCE1*; for LCFA with SNP of *ABCG2*, the gene encoding toll-like receptor 2 (*TLR2*), and *ORL1*; for *trans* fatty acids with a SNP of *CARD15*; for the C16 index with a SNP of the gene encoding serpin peptidase inhibitor 1 (*PI*); and for the C18:0 and CLA indices with a polymorphism of *TLR2*.

Regarding individual fatty acids, relevant associations were found as follows: for C4:0 with polymorphisms of both the gene encoding liver X nuclear receptor α variant 1 (*LXRα*) and *PPARGC1A*; for C13:0 with SNP of the genes encoding CCL2 (*CCL2*) and CCR2 (*CCR2*); for C15:0 *iso* with a polymorphism of the gene encoding xanthine dehydrogenase (*XDH*); for C17:0 *iso* and C22:0 with a SNP of the gene encoding α_{S1}-CN (*CSN1S1*); for C18:1 *trans*-6–8 and C22:5 *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 with a SNP of the gene encoding fibroblast growth factor 2 (*FGF2*); for C20:0 with a SNP of the gene encoding κ-CN (*CSN3*); for C22:5 *cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19 with a polymorphism of the gene encoding diacylglycerol kinase gamma (*DGKG*); and for C20:5 *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17 with a SNP of the gene encoding leptin receptor (*LEPR*).

For many of these genes, the associations of SNP with milk technological and production traits were previously reported in the same population of Brown Swiss cows, such as the SNP of *CARD15* (MUN, % casein, % protein), *GH1* (rennet coagulation time), and *CSN3* (% lactose; Cecchinato et al., 2014, 2015). Several other associated SNP are located in genes previously identified as potential candidate genes in association analysis with milk fatty acids or in genes known to be involved in milk production or fat synthesis (e.g., *ABCG2*, *GH1*, *PPARGC1A*, *AGPAT6* and *FGF2*; Bionaz and Loor, 2008; Wang et al., 2008; Bouwman et al., 2011).

In our analyses, we did not include some well-known candidate genes for association analyses with milk fatty acids (i.e., *DGAT1* or *FASN*). In particular, *DGAT1* was not considered in the analysis because it is nearly monomorphic in Italian Brown Swiss (Conte et al., 2010), whereas *FASN* was included in the initial panel of tested SNP but was not genotyped due to a designability rank equal to 0.5 (moderate success rate, Supplemental Data file; http://dx.doi.org/10.3168/jds.2015-10420).

We found many relevant associations for LCFA, which are derived from circulating plasma lipids and originate from the diet, ruminal metabolic processes, and from endogenous lipids. However, when comparing the number of relevant SNP for fatty acids contents with <16C and >16C, balanced percentages on the total of SNP were found (42 and 58%, respectively; data not shown). It is worth noting that evidence of QTL for LCFA in bovine milk have been presented by Schennink et al. (2009), supporting the hypothesis that a genetic component exists that influences variation in these fatty acids by affecting their synthesis or metabolism (i.e., uptake, desaturation, esterification, biohydrogenation, and elongation). The existence of genetic variation for some LCFA was also evidenced by the high heritability values observed for C18:1 and CLA by Bouwman et al. (2014).

In this work, we used a targeted list of SNP in candidate genes, and sequence data are required to identify causal mutations responsible for the phenotype variations and thereby to potentially use these SNP in breeding programs. However, our results provide enough evidence to test these SNP in a genomic prediction approach based on the biological understanding of the phenotype compared with an "anonymous" approach without prior information.

CONCLUSIONS

In the present work, we used GC to perform association analyses between 51 previously selected SNP and 61 fatty acid traits in bovine milk samples. In terms of the fatty acid traits analyzed, this is the most comprehensive study performed to date on the milk fatty acid profile using a candidate gene approach. Most of the studied fatty acid traits (~81%) were relevantly associated with multiple SNP. The highest number of relevant associations was found mostly for SNP located in genes involved in fat synthesis or metabolism, linked to or contained in previously identified QTL for fat yield or content, or previously identified as candidate genes in association analyses with milk fatty acid profiles in other cow breeds. In particular, we found relevant associations with SNP located on chromosome

BTA19. Two candidate genes located on BTA19 (*CCL3* and *ACACA*) were relevantly associated with de novo SCFA and MCFA, thus explaining the high heritability values previously found for these fatty acids (with the exception of C6:0). Two other genes on BTA19 (*CCL2* and *GH1*) showed relevant association with SFA and BCFA. Among the candidate genes, *LEP* deserves particular attention, as it showed the greatest number of relevant associations (11). In addition, the 2 SNP of the *LEP* gene were associated with reductions of SFA and increases of MUFA and PUFA (including RA); according to current findings, this is likely to enhance milk quality. Recent studies have provided new insights into the importance and biological activity of specific fatty acids. Thus, it is important that the present study include macro-categories and representative fatty acids, as well as individual fatty acids that are present in small amounts but which may have potential beneficial effects for human health. Our identification of SNP in genes responsible for variations in bovine milk fat composition therefore provides worthwhile information that could be used in breeding programs to tailor the fatty acid content of bovine milk.

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