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Association of candidate gene polymorphisms with milk technological traits, yield, composition, and somatic cell score in Italian Holstein-Friesian sires

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ABSTRACT

Advances in DNA-based marker technology have enabled the identification of genomic regions underlying complex phenotypic traits in livestock species. The incorporation of detected quantitative trait loci into genetic evaluation provides great potential to enhance selection accuracies, hence expediting the genetic improvement of economically important traits. The objective of the present study was to investigate 96 single nucleotide polymorphisms (SNP) located in 53 candidate genes previously reported to have effects on milk production and quality traits in a population of highly selected Holstein-Friesian bulls. A total of 423 semen samples were used to genotype the bulls through a custom oligo pool assay. Forty-five SNP in 32 genes were found to be associated with at least 1 of the tested traits. Most significant and favorable SNP trait associations were observed for polymorphisms located in *CCL3* and *AGPAT6* genes for fat yield (0.037 and 0.033 kg/d, respectively), *DGKG* gene for milk yield (0.698 kg/d), *PPARGC1A*, *CSN1S1*, and *AGPAT6* genes for fat percentage (0.127, 0.113, and 0.093%, respectively), *GHR* gene for protein (0.064%) and casein percentage (0.053%), and *TLR4* gene for fat (0.090%), protein (0.066%), and casein percentage (0.050%). Somatic cell score was favorably affected by *GHR* (−0.095) and *POU1F1* (−0.137), and interesting SNP-trait associations were observed for polymorphisms located in *CSN2, POU1F1*, and *AGPAT6* genes for rennet coagulation time (−0.592, −0.558, and −0.462 min, respectively), and *GHR* and *CSN2* genes for curd firmness 30 min after rennet addition (1.264 and 1.183 mm, respectively). In addition to the influence of individual SNP, the effects of composite genotypes constructed by grouping SNP according to their individual effects on traits considered in the analysis were also examined. Favorable and significant effects on milk traits were observed for 2 composite genotypes, one including 10 SNP and the other 4 SNP. The former was associated with an increase of milk (0.075 kg/d), fat (0.097 kg/d), protein (0.083 kg/d), and casein yields (0.065 kg/d), and the latter was associated with an increase of fat (0.244%), protein (0.071%), and casein percentage (0.047%). Although further research is required to validate the identified SNP loci in other populations and breeds, our results can be considered as a preliminary foundation for further replication studies on gene-assisted selection programs.

Key words: candidate gene, milk coagulation trait, milk yield and composition, somatic cell count, Holstein bulls

INTRODUCTION

In recent decades, interest has been growing in the global quality and technological aspects of livestock products. In the dairy industry, contemporary breeding goals have broadened to include, along with milk production characteristics, health and functional traits in an effort to improve the overall functionality of the dairy cow. In many milk-producing countries, a large fraction of the milk is used for cheesemaking. In Italy, for example, more than 70% of the overall milk production is used to manufacture cheese; thus, milk technological traits are of great importance for the national dairy industry (Cassandro et al., 2008; Tiezzi et al., 2013). Important milk traits, which include milk yield, composition (fat, protein, and casein content), and milk coagulation properties (**MCP**), mainly described by rennet coagulation time (**RCT**; min) and curd firmness 30 min after rennet addition to milk ($\mathbf{a_{30}}$; mm), have therefore gained considerable interest worldwide. Milk coagulation properties have been reported to improve the efficiency of the cheesemaking process; in fact, milk with a high capacity to properly react to rennet and to produce a firm curd results in greater cheese yield (Bynum and Olson, 1982; Riddell-Lawrence and Hicks, 1989). Pretto et al. (2013) demonstrated that a_{30} has a positive effect on Grana Padano cheese yield, which

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in turn is expected to result in greater income for the dairy industry. The economic relevance of MCP has been recently reported by Cassandro et al. (2016), who estimated the economic values for RCT and a_{30} of milk destined to produce Grana Padano cheese in the Italian Holstein cattle population. Moreover, indirect selection for resistance to mastitis through the reduction of SCC is another important goal to be considered for improving milk production and quality.

Exploitable additive genetic variation for milk coagulation ability has been assessed in several cattle breeds in various countries. Heritability estimates of MCP are greater compared with milk yield and similar to other quality traits, ranging from 15 to 40% (Lindström et al., 1984; Ikonen et al., 2004; Penasa et al., 2010; Vallas et al., 2012). Moreover, the assessment of MCP can be routinely performed through mid-infrared spectroscopy, a technology widely used in milk recording programs to predict milk quality traits (Dal Zotto et al., 2008; De Marchi et al., 2008).

Traditionally, selection to improve profitability of livestock production has been based on EBV calculated from phenotypic records and pedigree, and on knowledge of the heritability of each trait (Goddard and Hayes, 2009). Nevertheless, several authors demonstrated the feasibility of improving cheesemaking-related traits through the identification of QTL affecting the traits (Ogorevc et al., 2009; Meredith et al., 2012; Cecchinato et al., 2014, 2015).

In recent years, due to their abundance and variability, SNP have been largely used in dairy cattle as powerful markers to identify loci underlying phenotypic variation in association studies. The candidate gene strategy allows focusing the analysis on particular genes involved in key metabolic pathways or physiological processes, which are probable to be involved in the traits of interest. The availability of many thousands of SNP has led to the development of genomic selection (Meuwissen et al., 2001). The advantage of this approach over traditional selection is that animals can be selected accurately early in life, based on their genomic predictions, and for traits that are difficult or expensive to measure. Currently, genomic selection is widely used in several countries, especially in the Holstein breed. Costs for analysis of high-density SNP genotyping have decreased dramatically in recent years, but they are still prohibitively high for expanding the analysis to the population level. The selection of a panel of few associated genes for a specific trait can be a viable strategy to reduce the cost of analysis for preselection and within-family selection of young bulls, especially for new traits for which national genetic evaluation is not yet performed, such as MCP. The objective of the present study was to evaluate the effect of 96 SNP and 3 composite genotypes within 53 candidate genes on milk production and composition traits, SCS, and MCP in Italian Holstein-Friesian sires.

MATERIALS AND METHODS

Sampling and Analysis of Milk Quality

From October 2011 to September 2014, a total of 292,007 individual milk samples from 45,115 Holstein-Friesian cows reared in Veneto Region (northeast Italy) and daughters of 4,531 sires, were collected during monthly test-day milk recording. Milk samples were collected according to the International Committee for Animal Recording (ICAR, 2009) guidelines and analyzed in the laboratory of the Breeders Association of Veneto region (Padova, Italy) using Milko-Scan FT6000 (Foss Electric A/S, Hillerød, Denmark). Traits recorded were fat (**FP**), protein (**PP**), and casein (**CP**) percentages, RCT, and a_{30}. Mid-infrared spectroscopy models were implemented for routine prediction of MCP, as reported by De Marchi et al. (2013). In addition to quality traits and MCP, information on daily yields (kg/d) of milk (**MY**), fat (**FY**), protein (**PY**), and casein (**CY**) were available. Casein-to-protein (**C/P**; %) and protein-to-fat (**P/F**; %) ratios were also calculated. Values of SCC were determined with Cell Fossomatic 250 (Foss Electric A/S) and transformed to SCS to achieve normality and homogeneity of variances according to the formula of Wiggans and Shook (1987): $SCS = 3 + \log_2(SCC/100{,}000)$.

Sires DNA Extraction and Genotyping

Semen samples were collected from 423 sires and DNA extraction was carried out using the DNeasy Blood & Tissue Kit (catalog no. 69506, Qiagen, Valencia, CA), following the manufacturer's instructions. The extracted DNA was quantified with the Qubit System (Invitrogen, Carlsbad, CA) and assessed for integrity by 1% agarose gel electrophoresis.

Candidate gene selection was carried out using both a functional approach and a positional approach. In the functional approach, candidate genes were chosen on the basis of evidence of physiological or biochemical processes related to milk production and quality traits and involved in the immune system. For the positional approach, the identification of candidate genes was mainly based on the physical linkage information in chromosomal regions associated with milk composition and technological properties.

A first panel of 96 SNP was selected within 53 genes using either information available in the literature or in silico, after a database interrogation on NCBI dbSNP

(https://www.ncbi.nlm.nih.gov/projects/SNP/). All samples were genotyped with the Illumina GoldenGate Assay (Illumina Inc., San Diego, CA) using a 96-SNP custom oligo pool assay. Automatic allele calling was carried out using the GeneCall software (Illumina) with a GCscore threshold of 0.25. The GCscore is a quality measures on the genotype calls from the genotyping assay.

Statistical Analysis

Step 1: Estimation and Deregression of Breeding Values. Breeding values were estimated using the following linear animal model:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z_h h} + \mathbf{Z_p p} + \mathbf{Z_a a} + \mathbf{e},$$

where $\mathbf{y}$ is the vector of phenotypic records for the analyzed traits (MY, FY, PY, CY, FP, PP, CP, SCS, RCT, a_{30}, C/P, and P/F); $\mathbf{b}$ is the vector of fixed effects of parity (3 classes: parity 1, 2, and 3–9) and stage of lactation (12 monthly classes: 6–35, 36–65, 66–95, 96–125, 126–155, 156–185, 186–215, 216–245, 246–275, 276–305, 306–335, and 336–365 d); $\mathbf{h}$ is the vector of solutions for herd-test-date random effect $h \sim N\left(0, \mathbf{I}\sigma^2_h\right)$; $\mathbf{p}$ is the vector of solutions for cow permanent environmental effect $p \sim N\left(0, \mathbf{I}\sigma^2_p\right)$; $\mathbf{a}$ is the vector of solutions for animal additive genetic effect $a \sim N\left(0, \mathbf{A}\sigma^2_a\right)$; and $\mathbf{e}$ is the vector of random residuals $e \sim N\left(0, \mathbf{I}\sigma^2_e\right)$. $\mathbf{X}$, $\mathbf{Z_h}$, $\mathbf{Z_p}$, and $\mathbf{Z_a}$ represent the corresponding incidence matrices linking the phenotypic records to the appropriate random effects, $\mathbf{I}$ is an identity matrix of appropriate order, and $\mathbf{A}$ is the additive genetic relationship matrix among individuals. Variance components σ^2_h, σ^2_p, σ^2_a, and σ^2_e were from Tiezzi et al. (2013), and EBV and their standard errors were estimated using the BLUPF90 program (Misztal et al., 2002).

As bull EBV includes pedigree information, there is a risk that SNP could result associated with the trait due to the parental information rather than phenotype. To eliminate the contribution of information from relatives, EBV were deregressed according to Garrick et al. (2009) methodology. The deregressed EBV were used as phenotypic records for the bulls, as their use is proven to be reliable when different number of progeny per bull is available (Garrick et al., 2009).

Step 2: Single-Marker Association Analysis. The SNP that fulfilled the following criteria were included in the association analysis: (1) call rate ≥95%, (2) minor allele frequency (**MAF**) ≥5%, and (3) no extreme deviation from Hardy-Weinberg equilibrium ($P > 0.001$). After the quality control, 96 SNP, distributed across 43 genes, were retained. Names of genes, their chromosome position referring to *Bos taurus* UMD_3.1 (http://www.ensembl.org/index.html) assembly, and information about each SNP are given in Table 1. The single-marker regression model was

$$\mathbf{y} = \mathbf{X}b^m + \mathbf{Z}_a a + \frac{\mathbf{e}}{\mathbf{w}},$$

where $\mathbf{y}$ is the vector of pseudo-phenotypes; b^m are the mean and mth marker fixed effect solutions; a is the individual additive genetic effect $a \sim N\left(0, \mathbf{A}\sigma^2_a\right)$; $\mathbf{e}$ is the vector of residuals $e \sim N\left(0, \mathbf{I}\sigma^2_e\right)$; $\mathbf{w}$ is the vector of weights for the pseudo-phenotypes; $\mathbf{X}$ is the 2-columns incidence matrix reporting a vector of 1s (mean) and the number of copies of the minor allele (0, 1, or 2) for the mth marker over the individuals; and $\mathbf{Z}$ is the incidence matrix for the animal effect. Variance components σ^2_a and σ^2_e were estimated for each trait by marker model, and $\mathbf{I}$ and $\mathbf{A}$ are defined as in step 1. The model provides an estimate of the regression coefficient of the pseudo-phenotype on the number of copies of the minor allele that is to be interpreted as the average allele substitution effect (i.e., the average change in phenotype when 1 copy of the major allele is substituted with a copy of the minor allele). Individual records were weighted by the reliability of the respective deregressed breeding value. A total of 780 models were run (65 markers by 12 traits). Association analyses were run using the BGLR R-package (Pérez and de Los Campos, 2014).

Step 3: Composite Genotype Association Analysis. Composite genotypes were constructed by grouping SNP according to their individual effects on traits considered in the present study. Using relevant SNP, a total of 3 groups were defined as reported in Table 2. Group 1 included 4 SNP with significant effects for 6 or more different milk traits; group 2 included 10 SNP with significant effects for 5 or more different milk traits; and group 3 included 4 SNP with significant positive effects for only MY. Within each group, only composite genotypes showing a frequency of at least 10 were selected for testing. Composite genotypes were named by collapsing the number of copies of the minor allele for the SNP included in the group (e.g., genotype AA-Bb-Cc-dd would be shown as 0.1.1.2). Composite genotypes belonging to the same group were tested in the same model as

$$\mathbf{y} = \mathbf{X}b^c + \mathbf{Z}_a a + \frac{\mathbf{e}}{\mathbf{w}},$$

Table 1. List of the successfully genotyped SNP including chromosome (Chr) position referring to *Bos taurus* UMD_3.1 (http://www.ensembl.org/index.html) and minor allele frequency (MAF)

Gene	Gene name	Chr	Position	SNP	MAF
POU1F1	POU class 1 homeobox 1	1	35013926	No rs	0.15
			35014129	rs109007595	0.25
ETS2	Protein C-ets-2	1	152886878	rs135514413	0.05
DGKG	Diacylglycerol kinase gamma	1	81589478	rs41608610	0.24
STAT1	Signal transducer and activator of transcription 1-α/β	2	79888611	rs43705173	0.30
			79923716	rs43706906	0.44
LEPR	Leptin receptor	3	80092003	rs43349286	0.11
LEP	Leptin	4	93249281	rs29004170	0.48
			93257549	rs110559656	0.32
			93262003	rs29004487	0.25
ORL1	Oxidized low density lipoprotein (lectin-like) receptor 1	5	100247877	rs133629324	0.08
			100253752	rs135588030	0.26
PPARGC1A	PPARG coactivator 1-α	6	44857081	No rs	0.09
			44875251	rs109579682	0.07
			44875315	rs133669403	0.22
ABCG2	ATP-binding cassette sub-family G member 2	6	37983812	rs41577868	0.41
SPP1	Secreted phosphoprotein 1	6	38121192	rs133929040	0.44
CSN1S1	Casein α-S1	6	87141416	rs109817504	0.09
CSN2	Casein β	6	87181501	rs43703012	0.07
			87181542	rs109299401	0.37
			87182992	No rs	0.18
CSN3	Casein kappa	6	87390576	rs43703015	0.34
			87390479	rs43706475	0.33
			87390632	rs43703017	0.25
ADRB2	Beta-2 adrenergic receptor	7	62220606	rs132839139	0.10
TLR4	Toll-like receptor 4	8	108834063	rs8193048	0.12
LPL	Lipoprotein lipase	8	67487606	rs110590698	0.06
LPIN1	Phosphatidate phosphatase lipin 1	11	86056573	rs137457402	0.21
			86129986	rs136905033	0.10
XDH	Xanthine dehydrogenase/oxidase	11	14191183	rs42890834	0.39
PLCB1	Phospholipase C - β 1	13	1278678	rs110270855	0.37
			1655502	rs41624761	0.24
LBP	Lipopolysaccharide-binding protein	13	67875446	rs41704669	0.36
FABP4	Fatty acid-binding protein 4	14	46834401	rs135425060	0.49
LxR-α	Oxysterols receptor LXR-α	15	78324597	rs134390757	0.48
TLR2	Toll-like receptor 2	17	3952556	rs43706433	0.31
			3952732	rs43706434	0.19
FGF2	Fibroblast growth factor 2	17	35247491	rs110937773	0.36
CARD15	Caspase recruitment domain protein 15	18	19210671	rs43710288	0.48
GRLF1	Glucocorticoid receptor DNA binding factor 1	18	54450227	rs41572288	0.34
LIPE	Hormone-sensitive lipase	18	51214707	rs110137537	0.40
CCL2	Chemokine (C-C motif) ligand 2	19	16233476	rs41255714	0.48
			16234934	rs41255713	0.33
ACACA	Acetyl-coenzyme A carboxylase α	19	13887927	rs110562092	0.33
STAT5A	Signal transducer and activator of transcription 5A	19	43045807	rs137182814	0.37
			43054393	rs109578101	0.41
CCL3	C-C motif chemokine 3	19	14673538	rs109686238	0.18
GHR	Growth hormone receptor	20	31891078	rs109136815	0.16
			32146186	rs109231659	0.44
			—	No rs	0.20
PRLR	Prolactin receptor	20	39132325	rs109428015	0.14
PI	Protease inhibitor 2	21	59582394	rs41257077	0.38
PLIN	Perilipin 1	21	21504391	rs109363579	0.39
LTF	Lactotransferrin	22	53538186	rs43765462	0.26
			53538807	rs43765461	0.23
CCR2	Chemokine (C-C motif) receptor 2	22	53613730	rs41257559	0.45
PRL	Prolactin	23	35106206	rs211032652	0.49
			35114464	rs110684599	0.21
BTN1A1	Butyrophilin subfamily 1 member A1	23	31363023	rs43706495	0.21
LPAAT	Lysophosphatidic acid acyltransferase	23	80092003	rs43349286	0.09
SCD-1	Stearoyl-coenzyme A desaturase 1	26	21144708	rs41255693	0.24
			21149234	rs136334180	0.33
PLCE1	Phospholipase C. epsilon 1	26	15383866	rs41624917	0.40
AGPAT6	Glycerol-3-phosphate acyltransferase 6	27	36212557	rs110454169	0.21
			36220692	rs109913786	0.19

Table 2. List of SNP (dbSNP within parentheses) used for the construction of composite genotypes for 3 groups

Group 1[1]	Group 2[2]	Group 3[3]
PPARGC1A (rs133669403)	*DGKG* (rs41608610)	*DGKG* (rs41608610)
CSN1S1 (rs110981354)	*PPARGC1A* (rs133669403)	*STAT1* (rs43705173)
CSN3 (rs43703015)	*CSN1S1* (rs110981354)	*CSN2* (rs43703013)
GHR (rs41923484)	*CSN2* (rs109299401)	*FABP4* (rs110757796)
	CSN2 (rs43703013)	
	CSN3 (rs43703015)	
	GHR (rs41923484)	
	AGPAT6 (rs109913786)	
	STAT5A (rs109578101)	
	LPL (rs110590698)	

[1]Group of SNP with significant effects on 6 or more traits.
[2]Group of SNP with significant effects on 5 or more traits.
[3]Group of SNP with significant positive effects on milk yield.

where $\mathbf{y}$, a, $\mathbf{e}$, $\mathbf{w}$, and $\mathbf{Z}_a$ are defined as above; and $\mathbf{X}$ and $\mathbf{b}^c$ are the incidence matrix and vector of solutions for the mean and the composite genotypes, respectively, used to test for each group as presented in Table 2. Variance components σ_a^2 and σ_e^2 were estimated for each trait by group model. A total of 36 models were run (3 groups by 12 traits).

RESULTS AND DISCUSSION

Descriptive Statistics

Descriptive statistics and additive genetic variance used for breeding value estimation of the investigated traits are provided in Table 3. Milk yield, FY, PY, and CY averaged 29.81, 1.12, 1.00, and 0.78 kg/d, respectively, which are slightly lower than findings of previous studies on the same dairy cattle breed (Cassandro et al., 2008; Tiezzi et al., 2013). Means of FP, PP, and CP were 3.83, 3.42, and 2.68%, respectively, in agreement with values reported for the Holstein-Friesian cow (Cassandro et al., 2008; Tiezzi et al., 2013). The average SCS was 3.05, which is in the physiological range and in line with the value (3.08) reported by Cassandro et al. (2008). Regarding MCP, RCT occurred at 22.22 min and a_{30} was 23.49 mm. Rennet coagulation time was slightly longer than values reported in previous studies, which ranged from 20.2 to 20.7 min (Tiezzi et al., 2013; Penasa et al., 2014; Cassandro et al., 2015). The average of a_{30} was similar to results reported by Penasa et al. (2014) and Cassandro et al. (2015), but greater than a_{30} (21.71 mm) of Tiezzi et al. (2013). Possible differences between the current and previous studies for MCP include sampling size and time as well as breed and parity of the cow.

Table 3. Descriptive statistics of milk production and composition traits, SCS, and milk technological traits, and estimates of additive genetic variance (σ_a^2) used for breeding value estimation

Trait	Mean	SD	σ_a^2
Milk production, kg/d			
Milk yield	29.81	9.42	7.0907
Fat yield	1.12	0.36	0.0089
Protein yield	1.00	0.28	0.0063
Casein yield	0.78	0.22	0.0041
Milk composition, %			
Fat	3.83	0.78	0.0944
Protein	3.42	0.42	0.0256
Casein	2.68	0.35	0.0179
SCS	3.05	1.88	0.3076
Milk technological trait[1]			
RCT, min	22.22	19.09	2.8863
a_{30}, mm	23.49	5.44	17.6962
Casein/protein, %	78.27	10.30	0.2754
Protein/fat, %	92.30	2.03	35.5581

[1]RCT = rennet coagulation time; a_{30} = curd firmness at 30 min after rennet addition.

Allele Frequencies

Of the 96 selected SNP, a total of 65 SNP in 43 candidate genes located on 21 chromosomes were successfully genotyped in 423 sires. The MAF ranged from 0.05 to 0.49. Thirty SNP had minor allele frequencies between 0.31 and 0.49, 24 between 0.12 and 0.30, and 11 lower than 0.12 (Table 1). For the other remaining SNP excluded from the analysis, 12 SNP failed in the genotyping process (insufficient intensity for cluster separation or poor cluster definition), 15 SNP had MAF <5% and 4 SNP were genotyped for a lower sample size (number of bulls <423; Supplemental Table S1, https://doi.org/10.3168/jds.2017-12666).

Single-Marker Association Analysis

Through the use of deregressed EBV it was possible to estimate the association between SNP and phenotypic traits. A total of 45 SNP, located in 32 candidate genes, were significantly associated ($P < 0.05$) or exhib-

ited a statistical trend for association ($P < 0.10$) with at least 1 of the milk traits investigated (Table 4), indicating putative pleiotropic effects and confirming the positive or negative genetic correlation between traits. Significant SNP were spotted on 18 chromosomes, with 11 SNP (24.4%) identified on BTA chromosome 6. Of the 32 candidate genes identified, 23 were found in association with several traits, whereas the remaining 9 had effects only on 1 trait. Most relevant SNP milk trait associations were observed for *CCL3*, *AGPAT6*, *DGKG*, and *CSN3* with milk yield traits; *PPARGC1A*, *AGPAT6*, *CSN1S1*, *GHR*, *TLR4*, *LPL*, and *DGKG* with milk composition traits; and *GHR* with SCS. Interesting genes associated with MCP were *CSN2*, *CSN3*, *POU1F1*, *GHR*, and *AGPAT6*.

Milk Yield Traits. Considering milk yield traits (MY, FY, PY, and CY), we identified significant associations for 13 SNP in 12 genes ($P < 0.05$; Table 4). Polymorphisms *DGKG* rs41608610 ($P < 0.01$), *STAT1* rs43705173, and *CSN2* rs43703013 ($P < 0.05$) resulted in an increase of MY ranging from 0.473 to 0.698 kg/d, whereas *PPARGC1A* rs133669403 (−0.693 kg/d; $P < 0.05$) and *TLR4* rs8193066 (−0.669 kg/d; $P < 0.05$) negatively affected this trait. Polymorphisms associated with FY increment were *CCL3* rs109686238 (0.037 kg/d; $P < 0.001$), *AGPAT6* rs109913786 (0.033 kg/d; $P < 0.01$), and *CSN1S1* rs110981354 (0.026 kg/d; $P < 0.05$), whereas *LPL* rs110590698 (−0.037 kg/d; $P < 0.05$) and *FGF2* rs110937773 (−0.018 kg/d; $P < 0.05$), both known to be involved in milk production or fat synthesis (Keso et al., 2001; Wang et al., 2008), were associated with a reduction of daily FY (Table 4). An increase of both PY and CY was reported for *CSN2* rs109299401 (0.027 and 0.023 kg/d, respectively; $P < 0.05$) and *GHR* rs41923484 (0.017 and 0.014 kg/d, respectively; $P < 0.05$), whereas *CCL3* rs109686238 affected only PY (0.017 kg/d; $P < 0.05$). Genetic variants of the β-CN gene (*CSN2*) were associated with greater milk, protein, and casein yields, further confirming a major role of the casein gene cluster in affecting production traits (e.g., Nilsen et al., 2009; Huang et al., 2012; Russo et al., 2012). In contrast, the polymorphism rs43703015 of the bovine κ-CN gene (*CSN3*), which is associated with higher milk quality (Boettcher et al., 2004; Caroli et al., 2009) and FY (Mancini et al., 2013), reduced PY and CY (−0.020 and −0.017 kg/d, respectively, $P < 0.01$; Table 4). This result is in line with other studies reporting a decrease of milk yield traits (Kučerová et al., 2006) or no significant association for polymorphisms in the *CSN3* region and milk traits in different cattle breeds (Cecchinato et al., 2014; Fontanesi et al., 2015; Rahmatalla et al., 2015).

Milk Composition Traits. Several genetic factors influence the composition of milk. Here we observed significant association for at least 1 of the milk composition traits (FP, PP, and CP) for 20 SNP located in 17 genes ($P < 0.05$; Table 4). The polymorphism rs133669403, located in the *PPARGC1A* gene, was associated with an increase of FP (0.127%; $P < 0.001$). This was in contrast to SNP association results previously reported for the German Holstein population (Weikard et al., 2005), in which an increment of milk FY but a reduction of FP were observed, indicating that the *PPARGC1A* gene might be involved in genetic variation underlying the QTL for milk fat synthesis on BTA6. Significant associations with FP were also found in *DGKG* rs41608610 (−0.108%; $P < 0.001$) and *LPL* rs110590698 (−0.148%; $P < 0.001$), which decreased milk FP (Table 4). Moreover, a significant positive influence on FP was reported for polymorphisms *CSN1S1* rs110981354, *TLR4* rs8193066, and *AGPAT6* rs109913786, with estimated effects ranging from 0.090 to 0.113% ($P < 0.01$), and for *ORL1* rs135588030, *GHR* rs41923484, *LTF* rs43765462, and *PLCE1* rs41624917 from 0.050 to 0.064% ($P < 0.05$).

Considering polymorphisms of the casein genes, known to be relevant in relation to milk production traits and milk protein quality, the variants rs109299401 and rs43703011 of the *CSN2* gene increased PP by 0.056 and 0.033% and FP by 0.050 and 0.027%, respectively ($P < 0.05$). Opposite effects on those 2 traits were observed for another *CSN2* polymorphism (rs43703013), which decreased PP by 0.030% and FP by 0.023% ($P < 0.05$). The effect observed in our study for *CSN2* rs109299401 on PP was opposite to findings of Fontanesi et al. (2014) in Holstein sires. Moreover, PP was negatively affected (−0.024%; $P < 0.05$) by *CSN3* (rs43703017), which is known to affect PP in different dairy populations (Boettcher et al., 2004). However, our result is in line with the moderate to negligible effect of this gene on milk composition traits reported in Holstein-Friesian (Penasa et al., 2010) and in Brown Swiss cows (Cecchinato et al., 2014). Finally, SNP *CSN1S1* rs110981354 increased PP (0.046%; $P < 0.05$) and FP (0.113%; $P < 0.01$).

Besides the casein genes, 5 other polymorphisms enhanced milk PP: *TLR4* rs8193066 (0.066%; $P < 0.001$), *GHR* rs41923484 (0.064%; $P < 0.001$), and *PPARGC1A* rs133669403 and rs109579682, and *PRLR* rs109428015 (0.032–0.044%; $P < 0.05$). Milk CP was positively affected by *TLR4* rs8193066 (0.050%; $P < 0.001$) and *GHR* rs41923484 (0.053%; $P < 0.001$), and by *CSN2* rs109299401 and rs43703011, *PPARGC1A* rs109579682, *PRLR* rs109428015, *LPIN1* rs137457402, and *CCL2* rs41255713 (0.025–0.050%; $P < 0.05$). The *GHR* gene, in particular, is considered a strong candidate gene affecting MY and composition, as it is in close proximity to QTL previously shown to influence

Table 4. Association analysis of informative SNP markers with milk production and composition traits, SCS, and milk technological traits in Holstein-Friesian sires[1]

			Trait											
Gene	SNP	Chr	MY	FY	PY	CY	FP	PP	CP	SCS	RCT	a_{30}	C/P	P/F
POU1F1	No rs	1								−0.137*	−0.558**			
	rs109007595	1						−0.030†	−0.026*		−0.531**	0.793†		
ETS2	rs135514413	1					−0.095*	−0.044†	−0.035†					2.394*
DGKG	rs41608610	1	0.698**				−0.108***	−0.049***	−0.040**					1.669*
STAT1	rs43705173	2	0.538*											
ORL1	rs133629324	5											−0.130†	
	rs135588030	5					0.057*				−0.383*	0.874*		−1.440*
PPARGC1A	rs133669403	6	−0.693*				0.127***	0.044*	0.031†				−0.116†	−2.552**
	No rs	6						−0.031†		0.113†				
	rs109579682	6						0.032*	0.026*					
SPP1	rs133929040	6									−0.357*	0.721*		
CSN1S1	rs110981354	6		0.026*			0.113**	0.046*	0.030†				−0.122†	−2.587**
CSN2	rs109299401	6			0.027*	0.023*		0.056*	0.050*				0.174*	
	rs43703011	6						0.033*	0.027*		0.285†			
	rs43703013	6	0.473*					−0.030*	−0.023*		−0.592***	1.183***		
CSN3	rs43706475	6	−0.447†		−0.013†	−0.011†				0.114†				
	rs43703015	6	−0.364†	−0.016†	−0.020**	−0.017**					0.523***	−1.647***	−0.078†	
	rs43703017	6						−0.024*	−0.019†	0.093*	−0.229†			
TLR4	rs8193066	8	−0.669*				0.090**	0.066***	0.050***					
LPL	rs110590698	8		−0.037*			−0.148***	−0.073***	−0.058**					2.683*
LPIN1	rs137457402	11						0.029†	0.027*			0.700†	0.110*	
PLCB1	rs41624761	13												1.087†
	rs110270855	13								−0.092†	−0.334*			
FABP4	rs110757796	14	0.405†		0.011†	0.009†								
LxR.α	rs134390757	15		0.017†									0.079†	
TLR2	rs43706434	17												−1.678*
FGF2	rs110937773	17	−0.427†	−0.018*										
LIPE	rs110137537	18												−1.148†
CCL2	rs41255714	19				0.011†							0.120*	
	rs41255713	19						0.028†	0.025*					
STAT5A	rs109578101	19	−0.345†		−0.011†	−0.008†	0.041†							−1.042†
CCL3	rs109686238	19		0.037***	0.017*	0.014†								−2.062**
GHR	rs41923484	20			0.017*	0.014*	0.064*	0.064***	0.053***			1.264**		
	rs109231659	20								−0.095**				
	rs109136815	20						−0.026†	−0.019†		−0.318†			
PRLR	rs109428015	20					0.051†	0.038*	0.029*					
PI	rs41257077	21		0.018†	0.012†						−0.314†			
LTF	rs43765462	22		0.017†			0.058*							−1.221†
CCR2	rs41257559	22									−0.281†			
PRL	rs110684599	23												−1.222*
BTN1A1	rs43706495	23	−0.432†				0.051†							−1.269†
LPAAT	rs43349286	23					0.063†							
PLCE1	rs41624917	26					0.050*				−0.340*	0.761*		−1.337*
AGPAT6	rs110454169	27									−0.335†			
	rs109913786	27		0.033**			0.093**				−0.462**	0.970*		−1.982**

[1]MY = milk yield (kg/d); FY = fat yield (kg/d); PY = protein yield (kg/d); CY = casein yield (kg/d); FP = fat percentage (%); CP = casein percentage (%); PP = protein percentage (%); RCT = rennet coagulation time (min); a_{30} = curd firmness at 30 min after rennet addition (mm); C/P = casein to protein ratio (%); P/F = protein to fat ratio (%); Chr = chromosome.

†$P < 0.10$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

those traits in Holstein dairy breed (Arranz et al., 1998; Blott et al., 2003; Rahmatalla et al., 2011; Waters et al., 2011; Meredith et al., 2012) and Finnish Ayrshire breed (Viitala et al., 2006). According to Cecchinato et al. (2014), *LPIN1* rs137457402 affected CP, resulting in an increase of this trait (0.027%; $P < 0.05$). On the contrary, SNP *DGKG* rs41608610 and *LPL* rs110590698 were associated with a significant reduction of PP (−0.049 and −0.073%, respectively; $P < 0.001$) and CP (−0.040 and −0.058%, respectively; $P < 0.01$). Interestingly, these 2 SNP were both found to reduce PP and CP of Holstein cow milk in the present study, whereas they had no effect on the same traits in Brown Swiss milk (Cecchinato et al., 2014).

SCS. Interesting results were obtained for SNP within the *GHR* (rs109231659) and *POU1F1* (no rs) genes, which determined a reduction of the SCS by 0.095 and 0.137, respectively ($P < 0.05$; Table 4). As reported in other Holstein populations (Gengler et al., 2008; Rahmatalla et al., 2011; Waters et al., 2011), our study confirmed the significant effect of *GHR* on the reduction of the SCS, which is an interesting candidate for selection to improve resistance against mastitis. Nevertheless, in the Brown Swiss cattle breed investigated by Cecchinato et al. (2014), this polymorphism had no effect on SCS. The SNP rs43703017, located in the κ-CN gene (*CSN3*), was associated with an increase of SCS (0.093, $P < 0.05$; Table 4).

Milk Technological Traits. We obtained interesting results for the improvement of MCP for 9 and 8 polymorphisms associated with RCT and a_{30}, respectively (Table 4). Significant polymorphism associations with MCP were observed in the casein genes, *CSN2* and *CSN3*, according to previous findings (Caroli et al., 2009; Penasa et al., 2010). In particular, in our population, β-CN gene polymorphism, *CSN2* rs43703013, was associated with shorter RCT (−0.592 min; $P < 0.001$) and greater a_{30} (1.183 mm; $P < 0.001$), resulting in the increased suitability of milk for processing and cheesemaking (Cassandro et al., 2008). Association with *AGPAT6* rs109913786 polymorphism resulted in a better coagulation of milk, with shorter RCT (−0.462 min; $P < 0.01$) and greater a_{30} (0.970 mm; $P < 0.05$). Moreover, favorable associations with both MCP analyzed were found for SNP rs135588030, rs133929040, and rs41624917 located in *ORL1*, *SPP1*, and *PLCE1*, respectively, with an estimated effect for RCT ranging from −0.383 to −0.340 min ($P < 0.05$) and for a_{30} from 0.721 to 0.874 mm ($P < 0.05$; Table 4).

The polymorphism of the κ-CN gene, *CSN3* rs43703015, has shown to negatively affect MCP, as it was associated with longer RCT (0.523 min; $P < 0.001$) and lower a_{30} (−1.647 mm; $P < 0.001$). The polymorphism *POU1F1* rs109007595 improved RCT (−0.531 min; $P < 0.01$). A positive effect for cheesemaking ability was observed also for SNP *POU1F1* (no rs) and *PLCB1* rs110270855, which were associated with the reduction of RCT by 0.558 min ($P < 0.01$) and 0.334 min ($P < 0.05$), respectively. Finally, the polymorphism *GHR* rs41923484 had a positive effect on a_{30} (1.264 mm; $P < 0.01$).

Milks with greater C/P ratio generally produce firmer curds and lead to less moisture in cheeses; this ratio has been used as an indicator of the suitability of milks for cheesemaking (Auldist et al., 2002). In the present study, the polymorphisms *CSN2* rs109299401, *LPIN1* rs137457402, and *CCL2* rs41255714 increased this trait (0.110–0.174%; $P < 0.05$). Four polymorphisms, namely *PPARGC1A* rs133669403, *CSN1S1* rs110981354, *CCL3* rs109686238, and *AGPAT6* rs109913786, were significantly associated with a less favorable P/F ratio for cheesemaking in our population (−2.587 to −1.982%; $P < 0.01$), whereas *DGKG* rs41608610, *ETS2* rs135514413, and *LPL* rs110590698 were associated with a higher P/F ratio (1.669–2.683%, $P < 0.05$; Table 4).

Composite Genotype Association Analysis

The associations of composite genotypes with milk yield and composition traits, SCS, and milk technological traits are presented in Table 5. Composite genotypes were constructed by grouping SNP in 3 groups according to their individual effects on traits as reported in Table 4. Two, 4, and 6 significant composite genotypes were detected for group 1, group 2, and group 3, respectively.

The composite genotypes 2.1.1.2 of group 1 (MY, −0.069 kg/d; FY, −0.086 kg/d; PY, −0.079 kg/d; CY, −0.054 kg/d; Table 5) and 0.2.2.0.0.2.2.2.1.0 of group 2 (MY, −0.049 kg/d; FY, −0.060 kg/d; PY, −0.056 kg/d; CY, −0.038 kg/d; Table 5) were unfavorably associated with milk yield traits, whereas a positive significant effect on all milk yield traits was observed for the composite genotype 0.2.2.0.0.2.2.1.0.0 of group 2 (MY, 0.075 kg/d; FY, 0.097 kg/d; PY, 0.083 kg/d; CY, 0.065 kg/d; Table 5). Milk composition was negatively influenced by 4 composite genotypes of group 2, exerting a reduction of FP (−0.216 to −0.115%), and by 4 genotypes of group 3, which decreased FP (−0.188 to −0.098%) and CP (−0.034%). The only exception was the genotype 0.2.0.0 of group 3, which was associated with a positive effect on those traits (FP, 0.244%; PP, 0.071%; CP, 0.047%; Table 5). An increase of SCS was observed for 1 composite genotype of group 1 (0.669) and 2 of group 3 (0.651 and 0.656). The only significant association with MCP was observed for the genotype 0.2.0.1 (group 3), which decreased a_{30} (−0.155 mm),

Table 5. Estimated effects and SE of significant composite genotypes on the studied traits

Trait[1]	Gene group	Genotype[2]	Frequency[3]	Estimate	SE	HPD95[4]
MY	Group 1	2.1.1.2	14	−0.069	0.038	−0.007; −0.131
	Group 2	0.2.2.0.0.2.2.2.1.0	22	−0.049	0.028	−0.003; −0.096
	Group 2	0.2.2.0.0.2.2.1.0.0	10	0.075	0.039	0.139; 0.011
FY	Group 1	2.1.1.2	14	−0.086	0.045	−0.011; −0.161
	Group 2	0.2.2.0.0.2.2.1.0.0	10	0.097	0.054	0.186; 0.009
	Group 2	0.2.2.0.0.2.2.2.2.1.0	22	−0.060	0.034	−0.004; −0.116
PY	Group 1	2.1.1.2	14	−0.079	0.041	−0.011; −0.146
	Group 2	0.2.2.0.0.2.2.1.0.0	10	0.083	0.047	0.161; 0.005
	Group 2	0.2.2.0.0.2.2.2.2.1.0	22	−0.056	0.030	−0.006; −0.105
CY	Group 1	2.1.1.2	14	−0.054	0.030	−0.004; −0.104
	Group 2	0.2.2.0.0.2.2.1.0.0	10	0.065	0.034	0.122; 0.009
	Group 2	0.2.2.0.0.2.2.2.2.1.0	22	−0.038	0.023	−0.001; −0.075
FP	Group 2	0.2.2.0.0.2.2.2.1.0.0	10	−0.175	0.100	−0.011; −0.339
	Group 2	0.2.2.0.0.2.2.2.2.0.0	11	−0.216	0.088	−0.071; −0.362
	Group 2	0.2.2.0.0.2.2.2.2.1.0	22	−0.115	0.069	−0.001; −0.229
	Group 2	1.2.2.0.1.1.2.2.1.0	17	−0.171	0.075	−0.047; −0.294
	Group 3	0.1.0.0	22	−0.188	0.070	−0.073; −0.303
	Group 3	0.1.0.2	14	−0.159	0.084	−0.021; −0.297
	Group 3	0.2.0.0	16	0.244	0.076	0.368; 0.119
	Group 3	0.2.0.1	47	−0.098	0.053	−0.011; −0.185
	Group 3	1.2.1.1	21	−0.135	0.070	−0.020; −0.251
PP	Group 3	0.2.0.0	16	0.071	0.034	0.127; 0.014
CP	Group 3	0.2.0.0	16	0.047	0.029	0.094; 0.000
	Group 3	0.2.0.1	47	−0.034	0.019	−0.004; −0.065
SCS	Group 1	2.2.0.2	20	0.669	0.298	1.160; 0.179
	Group 3	0.1.0.0	22	0.651	0.279	1.110; 0.192
	Group 3	0.2.0.2	17	0.656	0.339	1.213; 0.098
a_{30}	Group 3	0.2.0.1	47	−0.155	0.073	−0.035; −0.275
C/P	Group 3	0.2.0.1	47	−0.482	0.251	−0.069; −0.894
P/F	Group 2	0.2.2.0.0.2.2.1.0.0	10	−0.085	0.035	−0.027; −0.143
	Group 2	0.2.2.0.0.2.2.2.0.0	11	−0.086	0.036	−0.027; −0.146
	Group 2	0.2.2.0.0.2.2.2.1.0	22	−0.061	0.024	−0.021; −0.102
	Group 2	1.2.2.0.1.1.2.2.1.0	17	−0.057	0.028	−0.011; −0.102
	Group 3	0.1.0.0	22	−0.077	0.027	−0.033; −0.121
	Group 3	0.2.0.0	16	0.054	0.030	0.103; 0.005

[1]MY = milk yield (kg/d); FY = fat yield (kg/d); PY = protein yield (kg/d); CY = casein yield (kg/d); FP = fat percentage (%); PP = protein percentage (%); CP = casein percentage (%); RCT = rennet coagulation time (min); a_{30} = curd firmness at 30 min after rennet addition (mm); C/P = casein to protein ratio (%); P/F = protein to fat ratio (%).

[2]Composite genotypes were named as collapsing the number of copies of the minor allele for the SNP included in the group (e.g., genotype AA-Bb-Cc-dd would be shown as 0.1.1.2). SNP included in composite genotypes are arranged according to the order reported in Table 2 for each group.

[3]Frequency is the number of bulls exhibiting that composite genotype.

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

resulting in the worsening of milk clotting characteristics. Composite genotypes had negative effects on P/F (−0.086 to −0.057%) and C/P (−0.482%) ratios, except for genotype 0.2.0.1 for P/F (0.054%; Table 5).

CONCLUSIONS

The present research is the first study carried out in the Italian Holstein-Friesian breed with the aim of identifying genomic regions putatively associated with milk technological traits. Forty-five SNP in 32 genes were associated with at least 1 of the analyzed traits (milk production and composition, SCS, and MCP). In particular, favorable associations with MCP were observed for *CSN2*, *POU1F1*, *GHR*, *AGPAT6*, *ORL1*, *SPP1*, *PLCB1*, and *PLCE1*. Other genes did positively associate with milk yield traits (*CCL3*, *AGPAT6*, *DGKG*, *STAT1*, *CSN1S1*, *CSN2*, *GHR*), composition traits (*PPARGC1A*, *AGPAT6*, *CSN1S1*, *GHR*, *TLR4*, *ORL1*, *CSN2*, *CCL2*, *PRLR*, *LTF*, *PLCE1*), and SCS (*GHR* and *POU1F1*). In addition, favorable effects on milk traits were observed for 2 composite genotypes: 1 genotype (0.2.2.0.0.2.2.1.0.0) of group 2 exerted an increment of milk yield traits and 1 genotype (0.2.0.0) of group 3 was associated with higher milk composition. Information on composite genotypes may be useful in preselection of young bulls for several traits, including MCP. Although further experimentation is required to validate the identified SNP loci in other populations and breeds, results of the present study can be con-

sidered as a preliminary foundation for future research on gene-assisted selection programs in the Holstein-Friesian breed.

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