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Candidate gene association analysis for milk yield, composition, urea nitrogen and somatic cell scores in Brown Swiss cows

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*The aim of this study was to investigate 96 single-nucleotide polymorphisms (SNPs) from 54 candidate genes, and test the associations of the polymorphic SNPs with milk yield, composition, milk urea nitrogen (MUN) content and somatic cell score (SCS) in individual milk samples from Italian Brown Swiss cows. Milk and blood samples were collected from 1271 cows sampled once from 85 herds. Milk production, quality traits (i.e. protein, casein, fat and lactose percentages), MUN and SCS were measured for each milk sample. Genotyping was performed using a custom Illumina VeraCode GoldenGate approach. A Bayesian linear animal model that considered the effects of herd, days in milk, parity, SNP genotype and additive polygenic effect was used for the association analysis. Our results showed that 14 of the 51 polymorphic SNPs had relevant additive effects on at least one of the aforementioned traits. Polymorphisms in the glucocorticoid receptor DNA-binding factor 1 (*GRLF1*), prolactin receptor (*PRLR*) and chemokine ligand 2 (*CCL2*) were associated with milk yield; an SNP in the stearoyl-CoA desaturase (*SCD-1*) was related to fat content; SNPs in the caspase recruitment domain 15 protein (*CARD15*) and lipin 1 (*LPIN1*) affected the protein and casein contents; SNPs in growth hormone 1 (*GH1*), lactotransferrin (*LTF*) and *SCD-1* were relevant for casein number; variants in beta casein (*CSN2*), *GH1*, *GRLF1* and *LTF* affected lactose content; SNPs in beta-2 adrenergic receptor (*ADRB2*), serpin peptidase inhibitor (*PI*) and *SCD-1* were associated with MUN; and SNPs in acetyl-CoA carboxylase alpha (*ACACA*) and signal transducer and activator of transcription 5A (*STAT5A*) were relevant in explaining the variation of SCS. Although further research is needed to validate these SNPs in other populations and breeds, the association between these markers and milk yield, composition, MUN and SCS could be exploited in gene-assisted selection programs for genetic improvement purposes.*

Keywords: milk quality, somatic cell, urea, gene locus, association analysis

Implications

Additive associations of allelic variants from 51 single-nucleotide polymorphisms (SNPs) with milk yield, milk composition, urea nitrogen content and somatic cell score were investigated in Brown Swiss cows. Of the 51 polymorphic SNPs tested, 14 were associated with at least one of the tested traits. This information may be useful in marker-assisted selection or a related technique, such as genomic selection placing greater prior emphasis on known quantitative trait loci (QTLs), to increase the accuracy of selection (especially for quality and health traits) and increase genetic gain.

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Introduction

Until recently, the majority of international dairy breeding programs were selected mainly for increased milk production (Meredith *et al.*, 2012). However, breeding goals must diversify to include milk quality, health and functional traits if we hope to minimize and reverse genetic declines in these traits. Fat, protein and casein content are of great importance for the milk industry. Mastitis, which is commonly measured using the somatic cell score (SCS) as an indicator trait, is one of the most important and costly production diseases in the dairy industry. Finally, milk urea nitrogen (MUN) is an interesting trait with remarkable environmental implications; milk urea is synthesized as consequence of an imbalance between dietary nitrogen and energy in the rumen, and reflects inefficient protein synthesis. As the main

non-protein source of nitrogen in milk, MUN reflects the efficiency of nitrogen utilization and the output of nitrogen to the environment.

Several studies have identified genetic variations in milk quality (Ikonen *et al.*, 2004; Cecchinato *et al.*, 2011), MUN (Miglior *et al.*, 2007; Stoop *et al.*, 2007) and SCS (Rupp *et al.*, 2009). However, selection for improved milk production, better quality traits and reduced SCS (indicating increased mastitis resistance) can be potentially enhanced through the identification of quantitative trait loci (QTL), which can help geneticists infer and comprehend the genetic and molecular mechanisms underlying these traits.

Here, in an effort to increase the number of single-nucleotide polymorphisms (SNPs) known to be related to health and quality traits in cattle, we investigated a number of SNPs that have previously been associated with milk traits as well as a subset of genes that were annotated in dbSNP but had not previously been included in an association study. The aims of the present study were to: (i) evaluate allelic and genotypic frequencies of 96 candidate gene polymorphisms; and (ii) investigate the associations between these polymorphisms and milk yield, composition, MUN and SCS in Brown Swiss cows.

Material and methods

Field data

A total of 1271 Brown Swiss cows from 85 herds located in Trento Province (Italy) were sampled once. The dairy systems, land use, feeding strategies, management practice and milk destination of the investigated area have been described by Sturaro *et al.* (2013). Within a given day, only one herd was sampled. Two milk subsamples per cow were collected and immediately refrigerated at 4°C without any preservative. One random subsample was transported to the Milk Quality Laboratory of the Breeders Federation of Trento Province (Trento, Italy) for composition analysis. Data on the cows and herds were provided by the Breeders Federation of Trento Province (Italy). Pedigree information was supplied by the Italian Brown Swiss Cattle Breeders Association (ANARB, Verona, Italy); we included cows for which phenotypic records were available for the investigated traits and those of all their known ancestors (8845 records in the pedigree file).

Analysis of milk quality

Individual milk samples were analyzed for fat, protein, casein, lactose (expressed in %) and MUN (expressed as mg/100 g) using a MilkoScan FT6000 (Foss, Hillerød, Denmark). Somatic cell count values were obtained with a Fossomatic FC counter (Foss) and converted to SCS by means of logarithm transformation.

DNA extraction and quality control

Peripheral blood samples were collected from each animal in 5 ml Vacutainer tubes containing sodium citrate as an anticoagulant, and stored at −20°C until analysis. DNA extraction was carried out with a DNeasy® 96 Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) starting with 100 μl of whole blood. For quality control, DNA was resolved by 1% agarose gel electrophoresis and stained with SYBR Safe® (Invitrogen, Carlsbad, CA, USA). All DNA samples were quantified with a QBit system (Invitrogen).

Gene and SNP selection

Candidate gene selection was carried out using two different approaches. First, we used a functional candidate gene approach by selecting genes known to be involved in the synthesis of proteins, fatty acids and components of the immune system. Second, we used a positional candidate gene approach by querying public databases to identify genes located in chromosomal regions that have been associated with milk quality and technological properties.

From within the chosen genes, we selected 113 SNPs for use in building a 96-SNP custom Oligo Pool Assay. Little information was available on their frequency in the Brown Swiss when the SNPs were selected. Thus, we chose most of the SNPs on the basis of their variations in other breeds, and verified their frequency in our population, expecting some differences owing to different selection strategies in the different breeds. Moreover, using SNPs found polymorphic in different breeds could also be useful, as an SNP with the same effect in different breeds could be the causative mutation for a specific trait. Anyway, the estimate of the effects of SNP on production traits has to be verified in any new population in which this information is planned to be used in marker-assisted selection.

As the SNP were going to be genotyped with the Illumina GoldenGate Assay (Illumina, San Diego, CA, USA), they were all submitted for scoring by the Illumina assay design tool and the most suitable for the chosen technology were selected: 89 SNP having scores >0.6 (designability rank = 1, high success rate) and 7 having scores between 0.5 and 0.6 (designability rank = 0.5, moderate success rate; data not shown). The 96 selected SNPs were located in 54 genes and included synonymous mutations, non-synonymous mutations and promoter region mutations. They were genotyped using the GoldenGate system (Illumina) according to the manufacturer's protocol. Automatic allele calling was carried out using the GeneCall software (Illumina) with a CG threshold of 0.25.

Statistical analysis

Allele frequencies, genotype frequencies and Hardy–Weinberg equilibrium were determined using Genepop program (version 1.2; Raymond and Rousset, 1995). The association studies for all investigated genes were carried out using the following mixed linear animal model:

$$y_{ijkl} = \mu + \mathrm{DIM}_i + \mathrm{Parity}_j + h_k + a_l + x_{lm}\beta_m + \varepsilon_{ijkl} \qquad (1)$$

where y_{ijkl} was the phenotypic record for the analyzed trait, DIM_i was the effect of the ith class of days in milk

(DIM; $i = 1$ to 10; 30 days for each class, with class 1 being <30 days and class 10 being >300 days); parity$_j$ was the effect of the jth parity of the cow ($j = 1$ to 5 or more); h_k was the effect of the kth herd ($k = 1$ to 85); a_l was the infinitesimal genetic effect of individual l; x_{lm} (0,1,2) reflected the number of copies of the minor allele at the mth SNP of subject l; β_m was the additive effect of the lth SNP; and ε_{ijkl} was the random residual term.

All the models were analyzed under a standard Bayesian approach. The joint distribution of all parameters in the model $p(\mathbf{b}, \mathbf{h}, \mathbf{a}, \sigma_e^2, \sigma_h^2, \sigma_a^2 \mid \mathbf{y})$ was proportional to:

$$p(\mathbf{b}, \mathbf{h}, \mathbf{a}, \sigma_e^2, \sigma_h^2, \sigma_a^2 \mid \mathbf{y}) \propto p(\mathbf{y} \mid \mathbf{b}, \mathbf{h}, \mathbf{a}, \sigma_e^2) p(\sigma_e^2) p(\mathbf{b}) \times p(\mathbf{h} \mid \sigma_h^2) p(\sigma_h^2) p(\mathbf{a} \mid \mathbf{A}, \sigma_a^2) p(\sigma_a^2),$$

where $\mathbf{y}$ was the vector of phenotypic records; $\mathbf{b}$ the vector of systematic effects; $\mathbf{h}$ the vector of herd effects; and $\mathbf{a}$ the vector of polygenic additive genetic effects. More specifically, $\mathbf{b}$ included the systematic effects of SNP, DIM and parity. Moreover, $\mathbf{A}$ was the numerator relationship matrix between individuals and σ_e^2, σ_h^2 and σ_a^2 were the residual, herd and additive genetic variances, respectively. For all univariate analyses, bounded uniform priors were used for the environmental variables, and $\mathbf{a}$ and $\mathbf{h}$ were assumed *a priori* to be independent and normally distributed, as:

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

and

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

where $\mathbf{I}$ was the identity matrix. Gibbs samples of parameters of concern were obtained as implemented in the TM program (available at http://cat.toulouse.inra.fr/~alegarra/). In the present work, the Gibbs sampler ran with a single chain of 1 000 000 points, and the first 50 000 were discarded as burn-in, previously tested by the Raftery and Lewis (1992) methodology. Samples were saved every 100 iterations. Owing to the autocorrelations between successive samples, convergence was tested using the Geweke's *Z*-criterion (Geweke, 1992), and Monte Carlo sampling errors as well as the effective sample size (ESS) were computed using the time-series procedures described by Geyer (1992). The parameters of concern were the dispersion parameters and the additive effect of SNPs, as defined by Falconer and Mackay (1996). The posterior mean was used as a point estimate for the parameters of concern. The lower and upper bounds of the 95% highest posterior probability density regions (HPD95) for the additive effects 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/trait). SNPs were considered to have a relevant effect on the trait when the posterior means of the additive effect did not include 0 in the HPD interval. Moreover, as suggested by Ramírez *et al.* (2014), we computed PPN0, which was the posterior probability of the estimated effect being lower than 0 (for negative effects) or greater than 0 (for positive effects). Only relevant SNPs are presented in the tables. The genetic variance explained by an SNP (V_a) was calculated from the estimated genotype effects and the observed genotype frequencies. The result was expressed as a percentage of the total additive genetic variance obtained from model 1 without the genotype effect.

Intra-herd heritability, which was computed without considering the effect of SNPs in the model, was defined as:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2}$$

where σ_a^2 and σ_e^2 were the additive genetic and residual variances, respectively.

Results and discussion

Descriptive statistics

The descriptive statistics for the investigated traits are reported in Table 1. The single test-day milk production, fat content, protein content and casein content were all representative of the Italian Brown Swiss population (Cecchinato *et al.*, 2011). In our study, the average MUN (25.99 mg/100 g) was slightly higher than that found by Butler *et al.* (1996), who reported MUN values of 22.8 mg/dl for non-pregnant cows, 21.3 mg/dl for cows later identified as pregnant, and overall mean values of 22.3. More recently, MUN levels of 17.9 mg/dl (Rius *et al.*, 2010) and 15.5 mg/dl have been reported. MUN levels are influenced by several different factors, including the sampling time, season, breed, nutritional factors, inefficient ruminal degradation of proteins, less efficient protein synthesis in the mammary gland and changes in conversion processes. The published data were mostly obtained from Holstein–Friesian cows, and the fed diets should be considered; thus, a detailed comparison is impossible. We measured MUN/100 g, which is slightly less than the 1 dl studied by the other authors, but the two can be considered to reflect approximately the same unit.

Table 1 *Descriptive statistics of milk yield, composition, MUN and SCS (N = 1271)*

Trait	Mean	s.d.	P1	P99
Milk yield (kg/day)	24.62	7.81	9.20	45.30
Milk composition (%)				
Fat	4.21	0.72	2.58	6.28
Protein	3.69	0.42	2.86	4.71
Casein	2.88	0.32	2.25	3.67
Lactose	4.85	0.19	4.30	5.22
Casein number	0.78	0.01	0.74	0.80
MUN (mg/100 g)	25.99	8.19	9.00	46.55
SCS (U)	2.92	1.84	– 0.47	7.54

PP1 = 1st percentile; P99 = 99th percentile; MUN = milk urea nitrogen; SCS = somatic cell score.

Table 2 *Features of marginal posterior densities of additive genetic variance (σ_a^2) and heritability (h^2) for milk yield, composition, MUN and SCS*

Trait	σ_a^2	Heritability	
	Estimate[1]	Estimate[1]	HPD95
Milk yield (kg/day)	4.124	0.182	0.07; 0.37
Milk composition (%)			
Fat	0.051	0.122	0.03; 0.26
Protein	0.022	0.279	0.13; 0.47
Casein	0.013	0.282	0.13; 0.47
Lactose	0.003	0.170	0.05; 0.34
Casein number	0.004	0.151	0.04; 0.30
MUN (mg/100 g)	6.954	0.356	0.20; 0.52
SCS (U)	0.254	0.096	0.02; 0.23

HPD95 = lower and upper bound of the 95% highest posterior density region; MUN = milk urea nitrogen; SCS = somatic cell score.
[1]Mean of the marginal posterior density of the parameter.

Finally, the average concentration of lactose was 4.85 (0.19), which was similar to the percentages obtained by Miglior *et al.* (2006) and Stoop *et al.* (2007) in the Holstein breed.

Variance components and heritability

The point estimates and features of the marginal posterior densities obtained for the additive genetic variance and trait heritability (without considering the effects of SNPs) are reported in Table 2. The genetic variances and heritability estimates for milk composition (i.e. protein, casein and fat content) were similar to those obtained by Cecchinato *et al.* (2011) in Brown Swiss cows. Ikonen *et al.* (2004) estimated heritabilities of 0.29 for protein content and 0.18 for fat content in a Finnish Ayrshire population. The heritability of casein content in the present paper (0.28) was lower than the estimates of 0.35 and 0.31 obtained by Ikonen *et al.* (2004) and Samorè *et al.* (2007), respectively. For SCS, our heritability estimate (0.096) was slightly higher than those of Ikonen *et al.* (2004) and Samorè *et al.* (2007) (0.06 and 0.07, respectively), but very similar to the 0.09 estimated in Ikonen *et al.* (1999). The heritability values of milk yield, protein and casein content, and SCS were all within the mean ± s.d. of the estimates reviewed by Bittante *et al.* (2012). In our study, the posterior estimate of heritability for MUN was 0.356, with an HPD95 interval varying from 0.20 to 0.52. In the literature, the heritability estimates for MUN have ranged between 0.14 and 0.44 (Wood *et al.*, 2003; Mitchell *et al.*, 2005; Miglior *et al.*, 2007), depending on the utilized instrument and the cow's parity. For example, Mitchell *et al.* (2005) estimated a heritability of 0.22 for first-parity cows using IR spectroscopy, but obtained an estimate of 0.14 using wet chemistry techniques; Wood *et al.* (2003) estimated a higher heritability for IR-determined MUN (0.44) using random regression analysis; and Miglior *et al.* (2007) reported that the average daily heritabilities of MUN varied from 0.384 to 0.414, depending on the parity. Our heritability estimate for lactose percentage was 0.151, which was much lower than the values of 0.50 found by Stoop *et al.* (2007) and Miglior *et al.* (2007) in different breeds and using different statistical models. The between-study inconsistencies in the estimated heritabilities may reflect various factors, including the breed, study procedures, conditions for trait recording, utilized models and methods of estimation.

Allele frequencies

Of the 96 selected SNPs, a total of 76 SNPs in 44 genes were successfully genotyped, with call rates between 0.833 and 0.999. The remaining 20 SNPs suffered from insufficient intensity or failure of cluster separation. Not all of the investigated SNPs had been previously analyzed in the Brown Swiss breed. Of the 76 genotyped SNPs, 25 were monomorphic in our population (Table 3), confirming the need to test molecular markers in different breeds before using them in marker-assisted selection.

Some alleles that had been positively and significantly associated with different milk traits in other breeds were not found in our population, including the A variant of the *POU1F1* gene and the T allele of *PPARGC1A* rs109579682 (Khatib *et al.*, 2007). In contrast, others were fixed in our population, including the A variant of *ABCG2* rs43702337, the C allele of *OPN* rs11093045 and the G allele of *GHR* rs109231659 (Waters *et al.*, 2011). With respect to the polymorphic loci, *ACACA* rs110562092 and *STAT5A* rs137182814 showed perfectly balanced allelic frequencies, whereas *ABCG2* rs41577868, *PLCB1* rs41624761, *LxR-alpha* rs134390757, *FGF2* rs110937773, *GRLF1* rs41572288 and *SCD-1* rs136334180 showed very nearly balanced frequencies. In terms of MAF, 23 SNPs had frequencies between 0.5 and 0.3, 28 were between 0.28 and 0.05, and only 7 were lower than 0.10. Thus, all of the successfully genotyped polymorphic SNPs were subjected to association studies.

For many of the SNPs, such as those in the *LPIN1*, *XDH*, *PLCB1*, *LIPE*, *CCL3*, *PLIN*, *AGPAT1*, *PLCE1* and *AGPAT6* genes, the allele frequencies were not previously known in Brown Swiss cows. For others, the minor allele in our population was the same as described in another population, but the allelic frequencies differed. This was the case for *STAT1* rs43705173, where the T allele had a frequency of 0.37 in our population compared with 0.33 in the Holstein breed (Cobanoglu *et al.*, 2006); *LEP* rs29004508, where the T allele had frequencies of 0.17 and 0.25, respectively, in our population and in the Dutch Holstein–Friesian breed (Liefers *et al.*, 2004); *CARD15* rs43710288, where the T allele had frequencies of 0.38 and 0.46, respectively, in our population and the Holstein–Friesian breed (Pant *et al.*, 2007); and *CCR2* rs41257559, where the T allele had a frequency of 0.31 in the present population and 0.46 in the Canadian Holstein breed (Leyva-Baca *et al.*, 2007). For *CCL2*, rs41255714 had the same minor allele as that reported by Leyva-Baca *et al.* (2007) in the Canadian Holstein breed (G, with frequencies of 0.35 and 0.44, respectively), whereas the minor allele was T (0.23) in our population and C (0.32) in the Canadian

Table 3 *List of the successfully genotyped SNPs including chromosome position (referring to Bos taurus UMD_3.1 assembly) and MAF*

Gene	Chromosome	Position	dbSNP	SNP	MAF
POU1F1 (POU class 1 homeobox 1)	1	35013926		A/C	A = 0
		35014129	rs109007595	C/T	C = 0.35
DGKG (Diacylglycerol kinase, gamma)	1	81589478	rs41608610	C/T	C = 0.16
STAT1 (Signal transducer and activator of transcription 1-alpha/beta)	2	79888611	rs43705173	T/C	T = 0.37
		79923716	rs43706906	C/G	C = 0.42
LEPR (Leptin receptor)	3	80092003	rs43349286	T/C	T = 0.26
LEP (Leptine)	4	93249281	rs29004170	C/G	C = 0.43
		93263979	rs29004508	T/C	T = 0.17
		93257549	rs110559656	A/G	G = 0.31
OLR1 (Oxidized low-density lipoprotein receptor 1)	5	100247877	rs133629324	A/C	C = 0.10
		100253752	rs135588030	A/G	G = 0.22
ABCG2 (ATP-binding cassette. sub-family G member 2)	6	37983812	rs41577868	T/G	G = 0.48
		38027010	rs43702337	A/C	C = 0
CSN1S1 (alpha s1 casein)	6	87141416	rs109817504	A/G	G = 0.10
		87155366	rs110981354	C/G	G = 0
		87157262	rs43703010	A/G	G = 0.10
CSN1S2 (alpha s2 casein)	6	87266180		T/C	C = 0
CSN2 (Beta casein)	6	87181453	rs43703013	G/C	C = 0.16
		87181501	rs43703012	A/C	C = 0
		87181542	rs109299401	A/C	A = 0
		87181619	rs43703011	A/C	A = 0.23
		87182992		T/C	T = 0
CSN3 (kappa casein)	6	87390458	rs110870535	A/G	G = 0
		87390576	rs43703015	T/C	C = 0.22
PPARGC1A (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha)	6	44857081		A/C	C = 0.05
		44875251	rs109579682	T/C	T = 0
		44875315	rs133669403	A/G	G = 0
SPP1 (Secreted phosphoprotein 1)	6	38121192	rs133929040	A/G	A = 0
		38122665	rs110930453	T/C	T = 0
ADRB2 (Beta-2 adrenergic receptor)	7	62220606	rs132839139	A/G	G = 0.05
LPL (Lipoprotein lipase)	8	67487606	rs110590698	T/A	T = 0
		67497852	rs133043641	T/G	T = 0
TLR4 (Toll-like receptor 4)	8	108834063	rs8193048	A/G	G = 0
		108838612	rs8193066	A/G	G = 0
LPIN1 (Lipin 1)	11	86056573	rs137457402	T/G	T = 0.43
		86129986	rs136905033	T/C	T = 0.10
XDH (Xanthine dehydrogenase)	11	14191183	rs42890834	A/G	G = 0.39
PLCB1 (Phospholipase C beta 1)	13	1278678	rs110270855	T/C	T = 0.18
		1655502	rs41624761	T/C	C = 0.45
FABP4 (Fatty-acid-binding protein 4)	14	46834401	rs135425060	A/C	A = 0
		46835065	rs110757796	A/G	A = 0.16
LxR-alpha (Oxysterols receptor LXR-alpha)	15	78324597	rs134390757	T/C	T = 0.46
FGF2 (Fibroblast growth factor 2)	17	35247491	rs110937773	A/G	A = 0.48
TLR2 (Toll-like receptor 2)	17	3952556	rs43706433	A/G	A = 0.36
		3952732	rs43706434	A/G	A = 0.15
CARD15 (Caspase recruitment domain 15 protein)	18	19210671	rs43710288	T/A	T = 0.38
GRLF1 (Glucocorticoid receptor DNA-binding factor 1)	18	54450227	rs41572288	T/C	C = 0.49
LIPE (hormone-sensitive lipase)	18	51214707	rs110137537	A/C	A = 0.26
ACACA (Acetyl-CoA carboxylase alpha)	19	13794520	rs133999659	T/A	T = 0
		13887927	rs110562092	A/G	G = 0.5
CCL2 (Chemokine ligand 2)	19	16233476	rs41255714	A/G	G = 0.35
		16234934	rs41255713	T/C	T = 0.23
CCL3 (Chemokine ligand 3)	19	14673538	rs109686238	T/C	C = 0.37
GH1 (growth hormone 1)	19	48768916	rs41923484	C/G	C = 0.23
STAT5A (Signal transducer and activator of transcription 5A)	19	43045807	rs137182814	C/G	C = 0.5
		43054393	rs109578101	T/C	T = 0.11
GHR (Growth hormone receptor)	20	31891078	rs109136815	T/C	T = 0.30
		32146186	rs109231659	T/G	G = 0

Table 3: (*Continued*)

Gene	Chromosome	Position	dbSNP	SNP	MAF
PRLR (Prolactin receptor)	20	39115344	rs135164815	A/G	G = 0
		39132325	rs109428015	T/C	T = 0.24
PI (Serpin peptidase inhibitor)	21	59580932	rs136294648	A/C	A = 0
		59582394	rs41257077	A/G	A = 0.23
PLIN (Perilipin 1)	21	21504687	rs134625550	C/G	C = 0
CCR2 (Chemokine receptor 2)	22	53613730	rs41257559	T/C	T = 0.31
LTF (Lactotransferrin)	22	53538186	rs43765462	T/G	G = 0
		53538807	rs43765461	T/C	C = 0.10
PPARG (Peroxisome proliferative activated receptor gamma)	22	57432122		A/G	A = 0
AGPAT1 (1-acylglycerol-3-phosphate O-acyltransferase 1)	23	27017243	rs137499341	C/T	A = 0.28
PRL (Prolactin)	23	35106206	rs211032652	C/T	A = 0.37
		35114464	rs110684599	A/C	A = 0.25
PLCE1 (Phospholipase C epsilon 1)	26	15383866	rs41624917	T/C	T = 0.27
SCD-1 (Stearoyl-CoA desaturase)	26	21144708	rs41255693	T/C	T = 0.15
		21149234	rs136334180	A/G	A = 0.47
AGPAT6 (1-acylglycerol-3-phosphate O-acyltransferase 6)	27	36212557	rs110454169	T/C	T = 0.38
		36220692	rs109913786	T/C	T = 0.17
FADS2 (Fatty-acid desaturase 2)	29	41078894	rs109043635	T/C	C = 0

SNP = single-nucleotide polymorphism; MAF = minor allele frequency.

Table 4 *Features of the estimated marginal posterior densities of additive effects for the relevant SNP[1] on milk yield, composition, MUN and SCS*

Gene	Trait	Allele	Estimate[2]	HPD95	PPN0	V_a (%)
ACACA (rs110562092)	SCS (U)	A *v.* G	− 0.191	− 0.35; − 0.02	0.989	7.18
ADRB2 (rs132839139)	MUN (mg/100 g)	A *v.* G	− 2.262	− 4.47; − 0.054	0.978	6.99
CARD15 (rs43710288)	Protein (%)	T *v.* A	0.032	0.00; 0.06	0.990	2.19
	Casein (%)	T *v.* A	0.028	0.00; 0.05	0.994	9.24
CCL2 (rs41255714)	Milk yield (kg/day)	A *v.* G	0.651	0.14; 1.16	0.991	4.68
CSN3 (rs43703015)	Lactose (%)	T *v.* C	− 0.029	− 0.06; − 0.01	0.981	9.95
GH (rs41923484)	Casein number	C *v.* G	− 0.003	− 0.005; − 0.0008	0.997	0.08
	Lactose (%)	C *v.* G	− 0.026	− 0.04; − 0.005	0.995	8.26
GRLF1 (rs41572288)	Milk yield (kg/day)	T *v.* C	0.556	0.08; 1.03	0.991	3.75
	Lactose (%)	T *v.* C	0.019	0.00; 0.04	0.990	6.01
LPIN1 (rs137457402)	Protein (%)	T *v.* G	0.027	0.00; 0.05	0.979	1.62
	Casein (%)	T *v.* G	0.021	0.00; 0.04	0.980	1.66
LTF (rs43765461)	Casein number	T *v.* C	0.007	0.0004; 0.013	0.982	0.22
	Lactose (%)	T *v.* C	0.077	0.007; 0.144	0.986	35.57
PI (rs41257077)	MUN (mg/100 g)	A *v.* G	− 0.676	− 1.29; − 0.06	0.985	2.33
PRLR (rs109428015)	Milk yield (kg/day)	T *v.* C	0.786	0.15; 1.45	0.991	5.46
SCD-1 (rs136334180)	Fat (%)	A *v.* G	0.069	0.01; 0.12	0.990	4.65
SCD-1 (rs41255693)	Fat (%)	T *v.* C	0.194	0.02; 0.375	0.984	18.82
	MUN (mg/100 g)	T *v.* C	1.908	0.78; 3.05	0.996	13.35
	Casein number	T *v.* C	0.005	0.001; 0.009	0.990	0.16
STAT1 (rs43706906)	SCS (U)	C *v.* G	0.218	0.06; 0.376	0.998	9.12

HPD95 = lower and upper bound of the 95% highest posterior density region; PPN0 = the posterior probability of the additive effect to be over or below zero; V_a = proportion of genetic variance explained by each SNP; SCS = somatic cell score; MUN = milk urea nitrogen; SNP = single-nucleotide polymorphism.
[1]SNPs were considered having a relevant effect on the trait when the posterior means of the additive effect did not include 0 in the HPD interval.
[2]Mean of the marginal posterior density of the parameter.

Holstein. For *FABP4* rs110757796, the minor allele was A (0.16), whereas Cho *et al.* (2008) reported that the minor allele was G (0.375). In the latter case, the difference might be because of selection differences between Brown Swiss dairy cattle and Native Korean beef cattle. Finally, *GHR* rs109136815, an SNP in exon 10 that determines a silent mutation in amino acid 545 and was previously associated with milk yield (Blott *et al.*, 2003), was found to have a minor allele (C) frequency twofold higher in Brown Swiss compared with the values found for five other breeds by Waters *et al.* (2011).

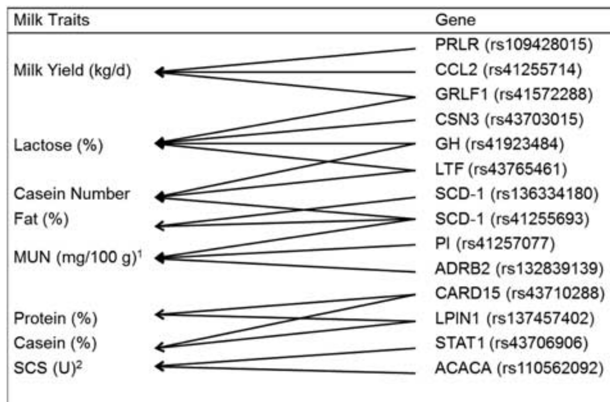

Figure 1 Schematic relationship between the relevant SNP and milk yield, composition, MUN and SCS. SNP = single-nucleotide polymorphism; MUN = milk urea nitrogen; SCS = somatic cell score.

Association analysis

The features of the marginal posterior densities of the additive effects for the relevant SNPs on milk yield, composition, MUN and SCS are reported in Table 4. All Monte Carlo s.e.'s were small, and the Geweke *Z*-test did not detect any lack of convergence. Moreover, the ESS values were high for all of the tested SNPs (data not shown). The marginal posterior distributions of the additive effects were approximately normal.

Milk composition and properties are determined by many factors, among which exist a complicated relation that is rather difficult to interpret. We found associations with milk traits for 14 of the 51 polymorphic genes analyzed (Table 4). Of these SNPs, six (*GRLF1* rs41572288, *GH* rs41923484, *LTF* rs43765461, *SCD-1* rs41255693, *CARD15* rs43710288 and *LPIN1* rs137457402) were found in association with multiple traits, whereas the remaining eight (*PRLR* rs109428015, *CCL2* rs41255714, *CSN3* rs43703015, *SCD-1* rs136334180, *PI* rs41257077, *ADRB2* rs132839139, *ACACA* rs110562092 and *STAT1* rs43706906) had effects only on one trait, as shown in Figure 1.

Considering milk yield *PRLR* rs109428015 (T *v.* C = 0.786; PPN0 = 0.991; $V_a = 5.46\%$) and *CCL2* rs41255714 (A allele = + 0.65 kg of milk; PPN0 = 0.991; $V_a = 6\%$) were associated only with this trait, whereas allele A of *GRLF1* rs41572288 was found to be positively associated with both milk yield and lactose percentage (T allele = + 0.56 kg of milk and + 0.019% of lactose; PPN = 0.99; and $V_a = 4\%$ and 6.01%, respectively). *PRLR* was already associated with milk yield in the Finnish Ayrshire breed and *CCL2* in the Canadian Holstein (Leyva-Baca *et al.*, 2007). As for *GRLF1* gene, two SNPs had previously been associated with feed intake and feed conversion rate, indicating that this gene is involved in the production of energy in cattle, and thus potentially explaining its relation with lactose percentage. The association with milk yield can be indirectly due to its well-known association with lactose percentage. However, additional research is warranted to examine the role of this gene in milk production.

Lactose percentage was also influenced by *CSN3* rs43703015, where the estimated substitution effect of the T allele was equal to − 0.029 (PPN0 = 0.981) with almost 10% of additive genetic variance explained by the SNP. Interestingly, this was the only association involving casein variants, confirming their modest effect on milk composition (Penasa *et al.*, 2010). Other genes had SNP in associations with milk composition: *GH* rs41923484 and *LTF* rs43765461, together with lactose percentage, also influenced casein number. In particular, the C allele of *GH* rs41923484 reduced both lactose percentage (−0.026; PPN = 0.995; $V_a = 8.26\%$) and casein number (−0.003; PPN0 = 0.997; $V_a = 0.08$), whereas the T allele of *LTF* rs43765461 was positively associated with both traits with a striking effect on lactose percentage (+0.077; PPN0 = 0.986), explaining a very high proportion of additive genetic variance (35.57%). The effect of each SNP on casein number was very limited, even considering *SCD-1* rs41255693 (T *v.* C = + 0.005; PPN0 = 0.990; $V_a = 0.16\%$).

SCD-1 polymorphisms were previously associated with fat, protein and/or casein contents in Belgian Blue Red and White, Jersey, Montbeliarde, Normande (Soyeurt *et al.*, 2008) and Brown Swiss (Soyeurt *et al.*, 2008; Cecchinato *et al.*, 2012) cows. The associations with fat percentage and casein number were confirmed in our population, with a high effect on fat percentage (T *v.* C = + 0.194; PPN0 = 0.984; $V_a = 18.82\%$). *SCD-1* rs41255693 was also associated with MUN (T *v.* C = + 1.908; PPN0 = 0.996) and it explained more than 13% of the additive genetic variances of the trait.

Another interesting association was found between *ADRB2* rs132839139 and *PI* rs41257077 with respect to MUN. For *ADRB2* rs132839139, the estimated effect for the A allele was −2.262 mg/100 g (PPN0 = 0.978; $V_a = 6.99\%$). For *PI* rs41257077, the corresponding estimate for the A allele was −0.676 mg/100 g (PPN0 = 0.985; $V_a = 2.33\%$). As milk urea is synthesized as a consequence of an imbalance between dietary nitrogen and energy in the rumen, we speculate that the effect of the *ADRB2* gene may be related to the involvement of β-adrenergic receptors in lipolysis and the regulation of muscle growth to the detriment of fat deposition. Although stimulation of β-adrenergic receptors in the bovine mammary gland has been shown to affect milk characteristics, including milk yield, little genetic information is yet available for cattle. Cows with greater genetic merit in terms of milk production were found to have increased adipose tissue lipolysis, increased responses to β-adrenergic stimulation, increased hormone-sensitive lipase (LIPE) activity and decreased lipogenesis, compared with animals of average genetic merit. Thus, the relationship of these genes with energy balance and milk traits should further be investigated. The bovine *PI* gene is located in a QTL associated with milk production and health traits (Khatib *et al.*, 2005). The primary role of *PI* is to protect tissues against proteolytic digestion by neutrophil elastase. Khatib *et al.* (2005) discovered several polymorphisms in this gene. Here, we found associations only for *PI* rs41257077, previously associated with decreased SCS, with MUN. In the human

mammary gland, PI may affect the survival of milk proteins, such as lactoferrin and lysozyme. Thus, an SNP that influences unfavorably the protection of these proteins could increase MUN.

The last genes influencing simultaneously different traits were *CARD15*, involved in recognizing gram-positive and gram-negative bacteria, and thus acting as a general sensor of bacterial infection, and *LPN1*, which play crucial roles during adipose tissue development and triacyl–glycerol accumulation (Phan and Reue, 2005). In our population, *CARD15* rs43710288 was associated with the casein and protein percentages (T *v.* A = 0.032 and T *v.* A = 0.028, respectively) and was responsible for 2.19% and 9.24% of the additive genetic variances of these traits. The SNP c.3020A > T (*CARD15* rs43710288) was found to be associated with estimated breeding values for SCS, udder depth, milk yield and protein yield, whereas SNP c.4500A > C was associated with milk, fat and protein yields in Canadian Holstein bulls (Pant *et al.*, 2007). The authors concluded that these two SNPs, together with other gene polymorphisms, could be used to genetically select for mastitis resistance and production, and our study confirms their contention that *CARD15* rs43710288 is a candidate for further functional studies.

LPIN1 rs137457402, whose posterior probabilities of the additive effect of the T allele were equal to 0.027 (PPN0 = 0.979) and 0.021 (PPN0 = 0.98) for protein and casein percentage, respectively, explained roughly 2% of the additive genetic variances of both traits. Recent studies have shown that lipin proteins, particularly LPIN1, play crucial roles during adipose tissue development and triacyl–glycerol accumulation (Phan and Reue, 2005). Furthermore, the expression levels of the lipin genes have been shown to influence lactation, with *LPIN1* predominating during lactation. Thus, this gene is clearly involved in modifying the composition of milk during lactation. Finck *et al.* (2006) demonstrated that LPIN1 is essential for *PPARa* activation, suggesting that *LPIN1* may be involved in regulating the transcription of other genes involved in milk fat synthesis. However, additional research will be necessary to clearly delineate its role in milk production.

Finally, two genes showed significant effects on SCS. The T allele of *ACACA* rs110562092 (our unique polymorphic ACACA SNP of the two directly chosen from dbSNP) was unfavorably associated with SCS (−0.191; PPN0 = 0.989). It explained a relevant proportion of the additive genetic variance of SCS (7.18%). Interestingly, *STAT1* rs43706906 was also relevant in explaining variation of SCS in our population (the T allele increase the trait by 0.218 U; PPN0 = 0.998; V_a = 9.18%). *STAT1* is a signal transducer and activator of transcription that is activated by numerous cytokines, growth factors and hormones, and is involved in the development and differentiation of the mammary gland. Thus, an association with SCS could be expected. The *STAT1* rs43705173 SNP, which was not associated with any milk trait in our Brown Swiss population, was previously correlated with SCS and other milk traits in the Holstein breed by Cobanoglu *et al.* (2006). Thus, the effects of *STAT1* rs43706906 might reflect linkage disequilibrium between this mutation and a causative mutation located in the gene. Notably, *STAT1* expression is tightly correlated with lipid accumulation, and *ACACA* encodes a key enzyme in the regulation of fatty-acid synthesis. Fatty acids are essential for the formation of cell membranes, and are used to synthesize fat for storage in adipose tissue or secretion into milk by the mammary gland. Thus, our results may suggest the presence of a complex relationship between *STAT1* and *ACACA*.

Conclusions

Polymorphisms in 51 SNPs were tested for their associations with milk production, composition, MUN and SCS in Brown Swiss cows. SNPs in 14 genes (*ACACA*, *ADRB2*, *CARD15*, *CCL2*, *CSN3*, *GH*, *GRLF1*, *LPIN1*, *LTF*, *PI*, *PRLR*, *SCD-1*, *SCD-1* and *STAT1*) were found to be associated with at least one of the aforementioned traits. In particular, the most striking effects were found for: *LTF* rs43765461 on lactose percentage (35.57% of additive genetic variance; T allele positively associated) with also a small positive effect on casein number; *SCD-1* rs41255693 on fat percentage (18.82% of additive genetic variance; T allele positively associated) with also more than 13% of the additive genetic variances of MUN (T allele = +1.908), and a small effect on casein number; *CSN3* rs43703015 with almost 10% of additive genetic variance explained by the SNP (allele T negatively associated); *CARD15* rs43710288 responsible for 9.24% and 2.19% of the additive genetic variance of protein and casein percentages, respectively (allele T positively associated), and was responsible for and of the additive genetic variance of these traits.

These information may be useful in marker-assisted selection or a related technique (e.g. genomic selection placing greater prior emphasis on known QTLs), with the goal of increasing the accuracy of selection, especially for quality and health traits, and increasing genetic gains.

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