Genetic and genomic analyses of latent variables related to the milk fatty acid profile, milk composition, and udder health in dairy cattle

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ABSTRACT

The aim of this study was to perform genetic, genome-wide association (GWAS), and gene-set enrichment analyses with latent variables related to milk fatty acid profile (i.e., fatty acids factor scores; FAF), milk composition, and udder health in a cohort of 1,158 Italian Brown Swiss cows. The phenotypes under study were 12 FAF previously identified through factor analysis and classified as follows: de novo FA (F1), branched-chain FA-milk yield (F2), biohydrogenation (F3), long-chain fatty acids (F4), desaturation (F5), short-chain fatty acids (F6), milk protein and fat contents (F7), odd fatty acids (F8), conjugated linoleic acids (F9), linoleic acid (F10), udder health (F11) and vaccelenic acid (F12). (Co)variance components were estimated for factor scores using a Bayesian linear animal model via Gibbs sampling. The animals were genotyped with the Illumina BovineSNP50 BeadChip v.2 (Illumina Inc., San Diego, CA). A single marker regression model was fitted for GWAS analysis. The gene-set enrichment analysis was run on the GWAS results using the Gene Ontology and the Kyoto Encyclopedia of Genes and Genomes pathway databases to identify the ontologies and pathways associated with the FAF. Marginal posterior means of the heritabilities of the aforementioned FAF ranged from 0.048 for F12 to 0.310 for F5. Factors F1 and F6 had the highest number of relevant genetic correlations with the other traits. The genomic analysis detected a total of 39 significant SNP located on 17 *Bos taurus* autosomes. All latent variables produced signals except for F2 and F10. The traits with the highest number of significant associations were F11 (17) and F12 (7). Gene-set enrichment analyses identified significant pathways (false discovery rate 5%) for F3 and F7. In particular, systemic lupus erythematosus was enriched

for F3, whereas the MAPK (mitogen-activated protein kinase) signaling pathway was overrepresented for F7. The results support the existence of important and exploitable genetic and genomic variation in these latent explanatory phenotypes. Information acquired might be exploited in selection programs and when designing further studies on the role of the putative candidate genes identified in the regulation of milk composition and udder health.

Key words: factor analysis, fatty acids, udder health, genome-wide association study (GWAS), gene-set enrichment

INTRODUCTION

Fat is the main energy component of milk and it is known to contain more than 400 individual fatty acids (FA; Månsson et al., 2008). Milk FA derive from de novo synthesis and from the uptake of preformed FA from diet, rumen metabolism, or lipid depot mobilization (Lock and Bauman, 2004). They affect the physical and sensory properties of dairy products (Chen et al., 2004) and contain functional food components that may have beneficial effects for maintaining health and preventing disease (Lock and Bauman, 2004). Milk FA are currently used as biomarkers of different metabolic patterns in animals (e.g., enteric methane emission, ketosis and acidosis status, feed efficiency, lipid mobilization). The possibility of altering milk fat composition through dietary manipulation or selective breeding has, therefore, many implications for the dairy cattle industry. Modification of the milk FA profile in dairy cattle by nutrition, management, or selective breeding may be challenged, however, by the pattern of phenotypic and genetic correlations among individual milk FA (Heck et al., 2012; Pegolo et al., 2016; Hein et al., 2018). This complex network reflects the interrelationships between the various molecular and metabolic pathways involved in the regulation of lipid metabolism in the bovine mammary gland (Bionaz and Looor, 2008; Pegolo et al., 2017).

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Furthermore, FA concentration in milk is strongly related to milk yield and composition and to udder health. Indeed, IMI (e.g., mastitis) generally cause a decrease in milk fat percentage and affect milk fat composition (Schultz, 1977; Kitchen, 1981; Needs and Anderson, 1984). Somatic cell count is currently the gold standard to screen for IMI, although it is affected by numerous factors (e.g., stress, management, the cow's immune system, genetics; Pyörälä, 2003) and does not always correlate with udder infection (Lakic et al., 2009). Various proposals have, therefore, been made to complement SCC with other indicators (e.g., milk lactose, pH, electrical conductivity, minerals) to detect subclinical mastitis (Macciotta et al., 2012; Egger-Danner et al., 2015; Bobbo et al., 2017), and these could be incorporated into selection indices for genetic improvement.

Statistical approaches based on data reduction methods, such as principal components analysis (**PCA**) and multivariate factor analysis (**MFA**), may be adopted to elucidate the detailed structure of the pattern of correlations among measured traits and to extract fewer latent explanatory variables, phenotypically independent among them, that could be included as new phenotypes in breeding programs (Macciotta et al., 2004). With respect to dairy cattle, PCA and MFA have been used to study milk FA profile (Fievez et al., 2003; Conte et al., 2016; Mele et al., 2016), milk composition, milk protein profile, and cheese-making properties (Macciotta et al., 2012; Dadousis et al., 2018b). In addition, the genetic parameters of latent variables extracted from milk major components and protein fractions have been estimated, and the genomic regions underlying their variability have been identified (Macciotta et al., 2012; Dadousis et al., 2017, 2018a), providing evidence for their potential use in dairy cattle breeding. A pathway-based approach in combination with standard single-marker genome-wide association studies (**GWAS**) has revealed that some of the genes associated with the extracted latent variables are part of complex, highly connected pathways or biological functions (Dadousis et al., 2017).

Previously, we applied GWAS and network analysis to individual milk FA to explore the genetic basis controlling milk FA variability and identify key regulators for milk FA profile (Pegolo et al., 2017). Herein, we carried out genetic and genomic analyses on latent variables that were previously extracted using MFA from those original phenotypes plus milk production traits and indicators of udder health (**FAF**; Mele et al., 2016). We hypothesized that using a few synthetic variables might allow us to simplify the interpretation of the relationships among the traits while maintaining

the genetic makeup and biological significance of the original phenotypes. Moreover, because factor analysis aims to explain the covariance of a system, extracted latent variables are able to represent whole pathways of FA. Thus, a GWAS carried out using factor scores instead of single FA measurements may provide wider knowledge on the genetic background of FA metabolism. Therefore, the aims of the present study were to (1) estimate the genetic variation and heritabilities for the FAF; (2) infer the additive genetic and herd-date correlations among the FAF; (3) identify the genomic regions associated with the FAF through GWAS analysis; and (4) complement single-marker GWAS with gene-set enrichment analyses to identify the putative biological pathways underlying the variation in the FAF.

MATERIALS AND METHODS

Animals and Sampling

This study is part of the Cowability-Cowplus project. Briefly, milk samples were collected once during the evening milking from 1,158 Brown Swiss cows kept in 85 herds located in Trento Province (northeastern Italy) as described in Cipolat-Gotet et al. (2013). The milk samples (with no preservative added) 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 Padua (Legnaro, Padua, Italy) for subsequent analyses.

Phenotypes

Fatty Acids. Details about the FA analyses were reported in Pegolo et al. (2016). Briefly, FAME were prepared by the direct extraction and alkali-catalyzed trans-methylation procedure, previously described by Feng et al. (2004). The FA composition was determined using a ThermoQuest gas chromatograph (Thermo Electron 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). The operating conditions of the GC apparatus were as follows: helium carrier gas at a flow rate of 1 mL/min; split ratio: 1:80; oven temperature: 60°C held for 1 min, increased to 173°C at 2°C/min and held for 30 min, increased to 185°C at 1°C/min and held for 5 min, and finally increased to 220°C at 3°C/min and held for 19 min; injector temperature: 270°C; detector temperature: 300°C.

Table 1. Proposed factor names and variables (with proper signs) loaded in each factor (F) according to the factor analysis reported in Mele et al. (2016)¹

Latent factors	Traits loaded ¹ (sign)
F1: De novo fatty acids (FA)	8:0 (+), 10:0 (+), 12:0 (+), 14:0 (+), C17:0 (−), C17:1 <i>c</i> 9 (−), C18:1 <i>c</i> 9 (−)
F2: Branched FA-milk yield	Milk yield (−), 14:0 <i>iso</i> (+), 15:0 <i>ante</i> (+), 15:0 <i>iso</i> (+), 16:0 <i>iso</i> (+), 17:0 <i>ante</i> (+)
F3: Biohydrogenation	18:1 <i>c</i> 12 (+), 18:1 <i>t</i> 10 (+), 18:1 <i>t</i> 16 (+), 18:1 <i>t</i> 6+ <i>t</i> 8 (+), 18:1 <i>t</i> 9 (+), 18:2 <i>c</i> 9, <i>c</i> 12 (+)
F4: Long-chain FA	22:0 (+), 18:1 <i>t</i> 4 (+), 20:3 <i>c</i> 8, <i>c</i> 11, <i>c</i> 14 (+), 20:4 <i>c</i> 5, <i>c</i> 8, <i>c</i> 11, <i>c</i> 14 (+), 20:5 <i>c</i> 5, <i>c</i> 8, <i>c</i> 11, <i>c</i> 14, <i>c</i> 17 (+), 22:5 <i>c</i> 7, <i>c</i> 10, <i>c</i> 13, <i>c</i> 16, <i>c</i> 19 (+)
F5: Desaturation	18:0 (−), 10:1 <i>c</i> 9 (+), 14:1 <i>c</i> 9 (+), 16:1 <i>c</i> 9 (+)
F6: Short-chain FA	4:0 (+), 6:0 (+), 8:0 (+)
F7: Milk fat protein	Fat % (+), Protein % (+), Casein % (+)
F8: Odd FA	11:0 (+), 13:0 (+), 15:0 (+)
F9: CLA	18:1 <i>t</i> 11 (+), 18:2 <i>c</i> 9, <i>t</i> 11 (+)
F10: Linolenic	18:3 <i>c</i> 9, <i>c</i> 12, <i>c</i> 15 (+)
F11: Udder health	Lactose % (−), SCS (+)
F12: Vaccelenic acid	18:2 <i>t</i> 11, <i>c</i> 15 (+)

¹*c* = *cis*, *t* = *trans*.

MFA. An MFA was carried out on the correlation matrix of 53 variables consisting of 47 individual FA and 6 milk production and quality traits (milk yield, contents of milk fat, protein, casein, and lactose, and SCS) measured in the 1,158 cows, as detailed in Mele et al. (2016). In brief, the number of extracted factors was selected based on eigenvalues (>1), the relationships with the original variables, and the amount of explained variance. Only variables exhibiting correlations ≥0.60 (absolute value) were included in each factor. In total, 12 latent variables were extracted, which accounted for about 75% of the total variance. These factors represented specific groups of FA or biological processes (Table 1): de novo FA (**F1**), branched FA-milk yield (**MY**) (**F2**), biohydrogenation (**F3**), long-chain fatty acids (**F4**), desaturation (**F5**), short-chain fatty acids (**F6**), milk protein and fat contents (**F7**), odd FA (**F8**), CLA (**F9**), linoleic acid (**F10**), udder health (**F11**), and vaccelenic acid (**F12**).

Genotypes

The Illumina BovineSNP50 BeadChip v.2 (Illumina Inc., San Diego, CA) was used to genotype 1,152 cows (blood samples were not available for all phenotyped animals). Quality control filtered markers that did not meet the following criteria: (1) call rate >95%, (2) minor allele frequency ≥0.01%, and (3) no extreme deviation from Hardy-Weinberg proportions ($P > 0.001$, Bonferoni corrected). In total, 37,568 SNP were considered for subsequent analyses. After filtering for low call rate (<95%), 1,011 cows were retained.

Genetic Analyses

Genetic analyses for the 12 FAF were carried out using the following hierarchical model:

$$\mathbf{y} = \mathbf{X}\mathbf{b} + \mathbf{Z}_1\mathbf{h} + \mathbf{Z}_2\mathbf{a} + \mathbf{e}, \tag{1}$$

where **y** is the vector of phenotypic records with dimension *n*; **X**, **Z**₁, and **Z**₂ are appropriate incidence matrices for systematic effects (**b**), herd-date effects (**h**; *n* = 85), and polygenic additive genetic effects (**a**), respectively. Included in **b** are the nongenetic effects of DIM (classes of 30 d each) and parity (classes 1, 2, 3, ≥4). All analyses were conducted using a standard Bayesian approach. The priors for **b** and the variance components were assumed to be flat.

The a priori distributions of **h** and **a** were assumed multivariate normal, as follows:

$$p(\mathbf{h} \mid \sigma_h^2) \sim N(0, \mathbf{I}\sigma_h^2) \text{ and } p(\mathbf{a} \mid \sigma_a^2) \sim N(0, \mathbf{A}\sigma_a^2),$$

where **I** is an identity matrix with dimensions equal to the number of elements in **h**, **A** is the numerator relationship matrix between individuals (Wright, 1922; the pedigree file contained data on 3,378 animals), and σ_h^2 and σ_a^2 are the herd-date and additive genetic variances, respectively.

Genetic and herd-date correlations between the variables were estimated through a set of bivariate analyses that implemented model [1] in its multivariate version. In this case, traits were assumed to jointly follow a multivariate normal distribution along with the additive genetic, herd-date, and residual effects.

Marginal posterior distributions of all unknowns were estimated using the Gibbs sampling algorithm [the TM program (<http://snp.toulouse.inra.fr/~alegarra>) was used for all Gibbs sampling procedures]. Chain lengths and burn-in period were assessed by visual inspection of the trace plots. After a preliminary analysis, we kept the chains of 850,000 samples and used a burn-in pe-

riod of 50,000, after which 1 in every 100 subsequent samples was retained.

The posterior mean was used as the point estimate for all parameters. Lower and upper bounds of the highest 95% probability density regions (HPD95) were obtained from the estimated marginal densities and used to estimate heritability. For the correlations, in addition to the means of each marginal posterior distribution, we also estimated the probability of each mean being greater than 0 when the mean is positive, or lower than 0 when the mean is negative (P). All estimates with $P > 95\%$ were considered “relevant” correlations. A more detailed description of these features can be found in Blasco (2005).

Intra-herd heritability (h^2) was computed as

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

and herd-date contribution (h_{herd}) was computed as

$$h_{\text{herd}} = \frac{\sigma_h^2}{\sigma_a^2 + \sigma_h^2 + \sigma_e^2}.$$

GWAS and Pathway Analyses

GWAS. Genome-wide association analyses were conducted variable by variable using a single-marker regression model implemented in the GenABEL R package (GenABEL, 2013) and the genome-wide association using mixed model and regression-genomic control (**GRAMMAR-GC**) approach (Amin et al., 2007). First, an additive polygenic model with a genomic relationship matrix was fitted:

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{a} + \mathbf{e}, \quad [2]$$

where $\mathbf{y}$ is a vector of FAF; $\boldsymbol{\beta}$ is a vector with fixed effects of DIM (classes of 30 d each), parity of the cow (classes 1, 2, 3, ≥ 4) and herd-date ($n = 85$); and $\mathbf{X}$ is the incidence matrix that associates each observation with specific levels of the factors in $\boldsymbol{\beta}$. The 2 random terms included in the model were animal and residuals, which were assumed to be normally distributed as $\mathbf{a} \sim N(0, \mathbf{G}\sigma_g^2)$ and $\mathbf{e} \sim N(0, \mathbf{I}\sigma_e^2)$, where $\mathbf{G}$ is the genomic relationship matrix, $\mathbf{I}$ is an identity matrix, and σ_g^2 and σ_e^2 are the additive genomic and residual variances, respectively. In a second step of the GRAMMAR-GC, the residuals obtained in model [2] were regressed on the SNP (single marker regression) to test for associations. Finally, the genomic control (GC) approach

corrects for the conservativeness of the GRAMMAR procedure and estimates the marker effects (Svishcheva et al., 2012). A P -value threshold of 5×10^{-5} was adopted to determine significant associations (Burton et al., 2007). The r^2 statistic (squared correlation coefficient) was chosen to predict the extent of linkage disequilibrium (**LD**) using the R package LDheatmap (Shin et al., 2006).

Gene-Set Enrichment Analysis. A pathway analysis was performed to capture the weaker but related single-variant signals that were missed by standard GWAS because of its stringent P -value threshold. It builds on the assumption that complex traits may be controlled by changes in biological pathways or cellular functions in which many highly coordinated genes might each play a modest role.

First, we selected from the GWAS results the relevant SNP based on a nominal P -value (< 0.05). Then, if these SNP were located within a gene or at a distance < 15 kb from the coding region (Pickrell et al., 2010) based on the Ensembl *Bos taurus* UMD 3.1 assembly, they were assigned to the gene using the BiomaRt R package (Durinck et al., 2005, 2009). We performed an enrichment analysis using the Fisher's exact test with false discovery rate (**FDR**) correction in the goseq R package (Young et al., 2010) to identify significantly overrepresented Gene Ontology (**GO**) terms (Ashburner et al., 2000) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathways (Ogata et al., 1999; $\text{FDR} < 0.05$). Only terms with > 10 and $< 1,000$ genes were included in the analyses to avoid testing broad or narrow functional categories.

RESULTS

Heritabilities of FAF and Herd Effects

The means of the marginal posterior densities of variance components, as well as the estimates of heritabilities and effects of herd-date (calculated as the proportion of total phenotypic variance) for the 12 FAF are summarized in Table 2. Factors F5 (desaturation; 0.310) and F7 (milk fat protein; 0.271) had the highest heritabilities, whereas F4 (long-chain FA; 0.055), F9 (CLA; 0.056), and F12 (vacceletic; 0.048) had the lowest. The other FAF had low heritabilities ranging from 0.125 (F8: odd FA) to 0.214 (F2: branched FAMY). Moreover, the amount of variance explained by herd-date was generally moderate or large, with the highest values (> 0.50) obtained for F2 (branched FAMY), F3 (biohydrogenation), F4 (long-chain FA), F6 (short-chain FA), F9 (CLA), and F10 (linolenic) and the lowest for F11 (udder health).

Additive Genetic and Herd-Date Correlations Among FAF

Point estimates of additive genetic and herd-date correlations among the 12 FAF are shown in Table 3. Overall, 28 genetic correlations (out of the 66 assessed) presented a posterior probability above (positive estimates) or below (negative estimates) 0, which are considered relevant correlations within a Bayesian framework. Factors F1 (de novo FA) and F6 (short-chain FA) exhibited the highest number of relevant genetic correlations with the other traits. In particular, F1 was strongly and negatively correlated with F3 (biohydrogenation; -0.734) and F5 (desaturation; -0.707); F1 was also correlated negatively, although to a lesser extent, with F8 (odd FA) and F10 (linolenic) and positively with F4 (long-chain FA), F6 (short-chain FA), and F12 (vacceletic). Factor F6 was positively correlated with F10 (linolenic), F11 (udder health), and F12 (vacceletic), and negatively with F4 (long-chain FA), F5 (desaturation), F8 (odd FA), and F9 (CLA). Interestingly, F9 was strongly and negatively correlated with F11 (udder health; -0.728). In addition, F11 had moderate negative correlations with F3 (biohydrogenation) and F8 (odd FA) and a moderate positive correlation with F4 (long-chain FA).

Few relevant herd-date correlations were found. In particular, F2 (branched FA-MY) was negatively correlated with F1 (de novo FA) and F7 (milk fat protein). Furthermore, F5 (desaturation) was negatively correlated with F12 (vacceletic) (Table 3).

GWAS and Pathway Analysis

GWAS. A summary of the results of the GWAS for the 12 FAF is presented in Table 4. In total, we detected 39 significant SNP located on 17 *Bos taurus* autosomes. No signal was common to all FAF. The P -values ranged from $4.90\text{E-}05$ to $7.80\text{E-}13$. Signals were produced by all latent variables except F2 (branched FA-MY) and F10 (linolenic). The traits with highest number of significant associations were F11 (udder health; 17) and F12 (vacceletic; 7). The chromosomes with the highest number of significant SNP were BTA3 (8) and BTA8 (5). Significant signals were found on BTA3 for 4 traits: F3 (biohydrogenation; at ~ 74.75 Mb), F8 (odd FA; at ~ 72.74 Mb), F11 (udder health; at ~ 44.36 Mb and at ~ 84.83 to 84.89 Mb) and F12 (vacceletic; at ~ 108.26 – 110.10 Mb). On the other hand, all signals located on BTA8 (at ~ 64.09 , ~ 81.47 , ~ 103.28 – 104.48 , and ~ 109.06 Mb) were significant for F11 (udder health). Moreover, 3 significant associations were found on BTA26 (at ~ 24.60 , ~ 33.58 , and ~ 51.54 Mb) and on BTA28 (at ~ 35.81 and ~ 43.14 – 43.18 Mb) for F11. A region of consecutive SNP (>2) was detected on BTA6 (~ 81.65 – 82.71 Mb) for F7 (milk fat protein). Signals were detected on BTA15 for both F4 (long-chain FA; at ~ 40.51 Mb) and F6 (short-chain FA; at ~ 52.44 – 52.47 Mb). Only 1 SNP was detected on BTA19 (at ~ 11.40 Mb) and this was significantly associated with F9 (CLA). Significant associations on BTA20 were detected for both F1 (de novo FA; at ~ 57.92 Mb) and F5 (desaturation; at ~ 51.14 Mb). The highest signals

Table 2. Estimates¹ of genetic variance (σ_a^2), herd-date variance (σ_h^2), residual variance (σ_e^2), heritability (h^2), and herd-date variance as proportion of total variance (h_{herd}) for the latent factors (F)²

Item	Variance			Heritability ³		
	σ_a^2	σ_h^2	σ_e^2	h^2	HPD95	h_{herd} ⁴
F1: De novo fatty acids (FA)	0.102 (0.05)	0.197 (0.04)	0.606 (0.05)	0.144	0.03; 0.30	0.216 (0.05)
F2: Branched FA-milk yield	0.084 (0.03)	0.561 (0.09)	0.308 (0.03)	0.214	0.08; 0.37	0.584 (0.04)
F3: Biohydrogenation	0.079 (0.03)	0.614 (0.10)	0.329 (0.03)	0.193	0.05; 0.36	0.596 (0.04)
F4: Long-chain FA	0.024 (0.01)	0.610 (0.10)	0.415 (0.02)	0.055	0.00; 0.14	0.577 (0.4)
F5: Desaturation	0.245 (0.07)	0.118 (0.03)	0.541 (0.06)	0.310	0.13; 0.49	0.129 (0.02)
F6: Short-chain FA	0.076 (0.03)	0.509 (0.08)	0.405 (0.03)	0.157	0.03; 0.32	0.511 (0.04)
F7: Milk fat protein	0.149 (0.04)	0.164 (0.03)	0.398 (0.04)	0.271	0.12; 0.45	0.229 (0.03)
F8: Odd FA	0.088 (0.04)	0.311 (0.05)	0.611 (0.04)	0.125	0.02; 0.26	0.305 (0.04)
F9: CLA	0.027 (0.01)	0.536 (0.09)	0.463 (0.02)	0.056	0.00; 0.14	0.518 (0.04)
F10: Linolenic	0.097 (0.04)	0.542 (0.09)	0.382 (0.03)	0.201	0.05; 0.39	0.526 (0.04)
F11: Udder health	0.118 (0.06)	0.073 (0.02)	0.731 (0.06)	0.138	0.02; 0.30	0.081 (0.02)
F12: Vacceletic acid	0.036 (0.02)	0.258 (0.05)	0.721 (0.03)	0.048	0.00; 0.10	0.251 (0.03)

¹Estimates are the mean of the marginal posterior density of the parameter. Standard deviations are given within parentheses.

²Latent variable defined according to Mele et al. (2016).

³Where $h^2 = \sigma_a^2 / (\sigma_a^2 + \sigma_e^2)$, and HPD95 = the highest 95% probability density regions.

⁴Where $h_{\text{herd}} = \sigma_h^2 / (\sigma_h^2 + \sigma_e^2)$.

Table 3. Estimates of additive genetic (above diagonal) and herd-date (below diagonal) relationships among latent factors (F)¹

Factor ²	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
F1: De novo fatty acids (FA)												
F2: Branched FA-milk yield	-0.435											
F3: Biohydrogenation	-0.092	-0.357										
F4: Long-chain FA	0.043	-0.207	-0.058									
F5: Desaturation	0.047	0.187	-0.192	0.092								
F6: Short-chain FA	-0.109	0.139	0.193	0.169	0.359							
F7: Milk fat protein	0.354	-0.421	0.13	-0.137	-0.145	0.006						
F8: Odd FA	-0.236	0.085	-0.137	0.07	0.11	0.11	0.015					
F9: CLA	0.125	0.238	-0.04	0.201	0.189	0.059	-0.253	-0.363				
F10: Linolenic	0.282	-0.174	-0.059	0.023	0.027	-0.078	0.014	-0.044	0.139			
F11: Udder health	-0.164	0.055	0.029	0.156	-0.058	-0.058	-0.228	-0.103	0.166	-0.093		
F12: Vaginal acid	0.131	0.009	-0.109	-0.019	-0.462	0.008	-0.069	-0.115	0.008	0.197	0.276	

¹Estimates are the mean of the marginal posterior density of the parameter. Boldface indicates additive genetic correlations with >95% of posterior probability accumulated above 0 (positive estimates) or below 0 (negative estimates).

²Latent variable defined according to Mele et al. (2016) (Table 1).

were detected for F11 (udder health) and these corresponded to the markers ARS-BFGL-NGS-33061 ($P = 7.80\text{E-}13$) and ARS-BFGL-NGS-15117 ($P = 1.47\text{E-}08$), which were located on BTA3 (~44.34 Mb) and BTA8 (~81.47 Mb), respectively. The effects were relatively large for these SNP, around 1.3 to 1.5 standard deviations from the mean (Table 4).

Gene-Set Enrichment Analyses. For each trait, ~850 SNP identified from the GWAS results ($P < 0.05$) were assigned, on average, to ~700 genes, which were mined in the goseq package to identify the putative biological functions and pathways that helped explain variability in the FAF. Significantly enriched terms ($\text{FDR} < 0.05$) were detected only for 2 FAF (Table 5): F3 (biohydrogenation) and F7 (milk fat protein). In particular, KEGG analysis revealed that a set of genes pertaining to systemic lupus erythematosus was significantly enriched for F3 ($\text{FDR} = 0.00023$), whereas overrepresentation of MAPK signaling pathway was shown for F7 ($\text{FDR} = 0.00018$).

DISCUSSION

In this study, we used 12 explanatory latent independent variables that were previously extracted from factor analysis of 53 original inter-correlated traits related to milk composition and udder health (Mele et al., 2016) as synthetic phenotypes for genetic and genomic analyses. This approach substantially reduced computational time and simplified interpretation of the biological significance of the results.

Genetic Analyses

Overall, heritability estimates of the FAF agreed with those of the individual traits comprising each factor. For instance, the heritability of F1 (de novo FA; 0.144) was in line with the estimates for 8:0, 10:0, 12:0, and 14:0 (0.097–0.243) obtained in a previous work (Pegolo et al., 2016). The moderate heritability of F5 (desaturation; 0.310) was comparable to that obtained for 14:1 *cis-9* ($h^2 = 0.360$; Pegolo et al., 2016), likely reflecting the activity of stearoyl-CoA desaturase 1 (SCD1). Indeed, in the bovine mammary gland, almost all 14:1 *cis-9* originates from SCD1 activity (Bernard et al., 2006). The moderate h^2 of F7 (milk fat protein; 0.271) was likely due to the strong genetic background of the protein and casein contents (Schopen et al., 2009; Buitenhuis et al., 2016; Pegolo et al., 2018). The low heritabilities found for F4 (long-chain FA; 0.055) and F9 (CLA; 0.056) were in line with estimates for the individual FA comprising these factors (Pegolo et al., 2016), the variability of which in milk fat is largely

dependent on environmental effects and particularly on dietary content of these FA (Heck et al., 2012).

Overall, the pattern of genetic correlations was consistent with the biological pathways represented by the 12 FAF; for example, the negative correlation between F4 (long-chain FA) and F6 (short-chain FA) or the positive correlation between F1 (de novo FA) and F6 (short-chain FA). Factor F1 (de novo FA) exhibited strong

negative genetic correlations with F3 (biohydrogenation) and F5 (desaturation). Specific biohydrogenation products have been found to be potent inhibitors of milk fat synthesis (Bauman and Griinari, 2001). It has been reported that 18:1 *trans*-10 strongly reduces the expression of fatty acid synthase (*FASN*), *SCD1*, and sterol regulatory element binding transcription factor 1 (*SREBF1*) in bovine mammary epithelial cells, pro-

Table 4. Summary results of the genome-wide association analysis on the extracted latent factors (F)¹

Trait/SNP	BTA	Location ² (bp)	MAF ³	P-value	SNPEff ⁴
F1: De novo fatty acids (FA)					
ARS-BFGL-BAC-2466	20	57,916,773	0.49	4.85E-05	0.148
F3: Biohydrogenation					
Hapmap55103-rs29011390	3	74,744,528	0.11	4.12E-05	−0.191
Hapmap38390-BTA-17559	12	22,979,469	0.45	3.42E-05	0.121
F4: Long-chain FA					
ARS-BFGL-BAC-27771	15	40,508,622	0.01	3.54E-06	0.659
ARS-BFGL-NGS-58720	27	38,699,737	0.22	4.06E-05	−0.143
ARS-BFGL-NGS-67720	29	17,876,279	0.06	2.77E-05	0.247
F5: Desaturation					
BTB-00787333	20	51,143,018	0.28	3.24E-05	0.197
F6: Short-chain FA					
ARS-BFGL-NGS-5022	15	52,440,792	0.49	7.58E-06	−0.148
ARS-BFGL-NGS-73444	15	52,465,901	0.49	5.68E-06	−0.150
F7: Milk fat protein					
BTA-110240-no-rs	6	81,652,194	0.20	4.90E-05	0.174
Hapmap56688-rs29025335	6	81,767,374	0.19	1.83E-05	0.191
Hapmap53172-rs29012675	6	82,706,745	0.05	3.60E-07	0.412
F8: Odd FA					
BTB-00135284	3	72,743,814	0.01	3.58E-05	0.873
UA-IFASA-4222	5	10,511,028	0.23	3.97E-05	−0.194
F9: CLA					
ARS-BFGL-NGS-102654	19	11,401,381	0.38	3.45E-07	0.168
F11: Udder health					
ARS-BFGL-NGS-33061	3	44,364,191	0.01	7.80E-13	1.424
ARS-BFGL-NGS-107651	3	84,825,930	0.01	4.20E-05	1.229
BTB-00141133	3	84,888,306	0.02	5.87E-06	1.518
Hapmap41750-BTA-109512	4	70,452,369	0.01	9.91E-06	0.657
ARS-BFGL-NGS-69854	8	64,089,209	0.01	4.83E-07	1.280
ARS-BFGL-NGS-15117	8	81,464,865	0.05	1.47E-08	1.348
ARS-BFGL-NGS-103223	8	103,278,121	0.05	8.56E-06	0.445
ARS-BFGL-NGS-62416	8	104,484,489	0.02	4.91E-06	0.483
Hapmap59395-rs29023511	8	109,059,409	0.01	1.37E-05	0.680
BTA-88360-no-rs	12	20,610,785	0.03	1.05E-05	1.208
ARS-BFGL-NGS-113197	17	3,119,640	0.01	1.30E-05	0.540
BTA-60940-no-rs	26	24,604,029	0.02	4.06E-06	0.834
ARS-BFGL-NGS-42871	26	33,583,861	0.01	4.33E-05	0.595
ARS-BFGL-NGS-26253	26	51,539,404	0.01	4.91E-06	1.127
ARS-BFGL-NGS-94161	28	35,810,609	0.13	8.10E-06	0.889
BTA-64483-no-rs	28	43,135,873	0.13	1.76E-05	0.278
BTB-00993386	28	43,179,395	0.13	1.76E-05	0.278
F12: Vaccelenic acid					
ARS-BFGL-NGS-28282	3	108,257,297	0.31	5.56E-06	0.104
ARS-BFGL-NGS-70647	3	108,283,132	0.39	3.13E-05	0.090
Hapmap34322-BES8_Contig446_1023	3	110,104,792	0.28	7.34E-07	0.117
Hapmap35103-BES3_Contig455_1055	11	92,297,411	0.23	3.64E-06	0.118
ARS-BFGL-NGS-36373	11	97,092,757	0.23	6.30E-06	0.116
ARS-BFGL-NGS-27118	16	4,305,200	0.44	2.70E-05	−0.088
ARS-BFGL-NGS-15267	24	31,609,408	0.38	3.28E-05	−0.091

¹Latent variables defined according to Mele et al. (2016).

²Location = SNP position on the chromosome based on the Ensembl *Bos taurus* UMD3.1 assembly.

³MAF = minor allele frequency.

⁴Effect of the significant SNP; factor scores are standardized with mean of 0 and SD of 1.

viding support for its potential antilipogenic properties (Kadegowda et al., 2009). In addition, postruminal infusions of a mixture of 18:1 FAME providing 92.1 g/d of 18:1 *trans*-10 to lactating cows reduced the milk fat content and output by approximately 20% (Shingfield et al., 2009). Several relevant genetic correlations were found between F11 (udder health) and the other FAF, which might provide new insights into the inflammatory response in the bovine mammary gland. Factor F11 was indeed included in this study for consistency with the previous work (Mele et al., 2016) but also because the original phenotypes, together with milk FA, contribute to explain the complexity of the relationships between milk quality and composition, and mammary gland functionality. Factor F11 showed a strong negative genetic correlation with F9 (CLA) and, to a lower extent, with F3 (biohydrogenation) and F8 (odd FA). At the same time, a positive genetic correlation was found between F11 and F4 (long-chain FA) and F6 (short-chain FA). High-genetic-merit cows are usually more susceptible to negative energy balance and mastitis, especially in the first part of lactation (Van Knegsel et al., 2014). Therefore, high-grain diets are often administered to cover the higher requirements of net energy for lactation. Previous studies have highlighted that metabolic disorders related to high-grain diets are usually associated with increases in the concentrations of LPS in the rumen and peripheral blood, because of the translocation of LPS from the digestive tract (Khafipour et al., 2009). The inflammatory response induced by circulating LPS has been also associated with several diseases, including mastitis (Zimov et al., 2011). Circulating LPS released from the cell wall of

gram-negative bacteria (including bacteria involved in udder infection) or experimentally infused in blood have been proposed as potent modulators of some key genes involved in lipid metabolism such as *SREBP1* and *SCD1* (Xu et al., 2016; Wang et al., 2018a). More precisely, LPS downregulates both *SCD1* and *SREBP1* expression, indirectly also affecting the metabolic steps regulated by the genes reported above. Interestingly, F11 was strongly negatively associated with F9 (CLA), which, in turn, was positively associated with the pair 18:1 *trans*-11 and 18:2 *cis*-9,*trans*-11. It is well known that the content of 18:2 *cis*-9,*trans*-11 in milk fat strongly depends on mammary desaturation activity. In fact, the endogenous synthesis of 18:2 *cis*-9,*trans*-11 from 18:1 *trans*-11 explains almost 80% of total 18:2 *cis*-9,*trans*-11 in milk fat (Corl et al., 2001). In the case of *SREBP1*, the downregulating effect of LPS has been associated with a reduction in transcription levels of acetyl-CoA carboxylase-1 and fatty acid synthase in mammary gland. Because these enzymes are responsible for the de novo synthesis of milk FA starting from acetate and butyrate, mastitis and, indirectly, milk SCC should be associated with a reduction of the elongation process of acetate and butyrate to palmitate. This might explain the positive genetic correlation observed between F11 and F6. It is also worth mentioning the positive association between F11 and F4, which is in turn positively associated with very long chain PUFA (both n-3 and n-6). Also, in this case, this pattern of association could be related to metabolic changes in mammary gland induced by mammary infection. In fact, the inflammatory response of mammary gland includes a specific group of potent lipid-derived inflam-

Table 5. Significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using genes associated with the extracted latent factors¹

Trait ² and category ³	Description	FDR ⁴	SGInCat ⁵	GenesInCat ⁵	Significant genes ⁶
F3: Biohydrogenation bta05322	Systemic lupus erythematosus	0.00023	11	78	<i>C7, H2AFY, ACTN1, HIST1H3I, HIST1H2AG, LOC505183, LOC526789, LOC614970, LOC529277, HIST1H2BB, HIST1H2AJ</i>
F7: Milk fat protein bta04010	MAPK signaling pathway	0.00018	25	159	<i>MAP4K2, PRKCA, MAP2K6, CACNA1B, LAMTOR3, MAP3K5, MAPKAPK5, KRAS, MAPK13, NTRK2, CACNG2, MAP2K4, FGF12, TAOK3, PLA2G4A, PPP3CA, MAPK10, PTPN5, NFKB1, TGFB1, ARRB1, MAPK14, CACNG6, CACNA2D4, MAP2K1</i>

¹Latent variables defined according to Mele et al. (2016).

²Only the traits showing significantly enriched terms are reported (false discovery rate <0.05).

³Category = Kyoto Encyclopedia of Genes and Genomes (KEGG) ID.

⁴False discovery rate.

⁵SGInCat = number of significant genes in the category; GenesInCat = number of genes in the category.

⁶SNP ($P < 0.05$) were assigned to genes if they were located within the gene or in a flanking region of 15 kb up- and downstream of the gene based on the Ensembl *Bos taurus* UMD3.1 assembly.

matory mediators called eicosanoids or oxylipids. These molecules are lipid metabolites derived from n-6 and n-3 PUFA, which are oxygenated through both enzymatic and nonenzymatic pathways (Sordillo, 2018).

The negative genetic correlations among F11 and F3 (biohydrogenation) and F8 (odd FA), which are associated with bacterial FA such as *trans* FA and odd-chain FA are more difficult to explain and deserve further investigation. Lipopolysaccharides are associated with changes in ruminal bacterial microbiota and disruption of ruminal metabolism in dairy cattle (Jing et al., 2014). Because FA associated with F3 (biohydrogenation) and F8 (odd FA) derived from rumen metabolism, genetic correlations among F11, F3, and F8 might suggest potential interactions between environment and genetic factors.

GWAS, Gene-Set Enrichment, and Pathway-Based Analyses

Overall, the results obtained overlapped to an extent with previous GWAS analyses carried out on individual milk FA. For instance, the marker ARS-BFGL-BAC-2466, which was significantly associated with F1 (de novo FA) was located at 57.91 Mb on BTA20, close to a region (49.86–55.43 Mb) reported to be associated with 12:0 and 14:0 contents in milk fat (Li et al., 2014). The marker ARS-BFGL-NGS-67720, significant for F4 (long-chain FA), was previously found to be associated with 20:4 *cis*-5, *cis*-8, *cis*-11, *cis*-14 (Pegolo et al., 2017). The 2 significant SNP detected for F8 (odd FA) on BTA3 (BTB-00135284) and BTA5 (UA-IFASA-4222) were previously found to be associated with odd-chain FA (Pegolo et al., 2017). Factor F7 (milk fat protein) generated signals on BTA6, likely confirming the effect of QTL for milk fat and protein (Freyer et al., 2003; Buitenhuis et al., 2016; Pegolo et al., 2018).

The search for genes within the 1-Mb region surrounding the significant SNP detected in the GWAS allowed us to identify putative positional candidate genes for the FAF. For instance, both the markers BTA-110240-no-rs and Hapmap56688-rs29025335, which mapped to BTA6, were significant for F7 (milk fat protein; Table 4) and might have an effect on *trans*-2,3-enoyl-CoA reductase like (*TECRL*). In particular, BTA-110240-no-rs corresponded to an intron variant of *TECRL*, and Hapmap56688-rs29025335 was located 0.1 Mb from the gene. These SNP were in high LD ($r^2 = 0.94$). It has been suggested that this gene plays a role in FA and lipid metabolism, according to the GO and Reactome pathway databases. The marker ARS-BFGL-NGS-67720, which was associated with F4 (long-chain FA), was located on BTA29 at 17.88 Mb and corresponded to an intron variant of the GRB2-

associated binding protein 2 (*GAB2*). The GAB family includes scaffolding/adaptor proteins that recruit cytoplasmic signaling proteins to transduce and integrate signals emanating from growth factors and signaling proteins [e.g., epidermal growth factor (EGF), insulin, and IGF-1; Nishida and Hirano, 2003; Nakaoka and Komuro, 2013], regulating mammary gland growth as well as glucose and lipid metabolism (Bionaz and Looor, 2008, 2011; Rezaei et al., 2016). In addition, FA, in particular UFA, stimulate mammary epithelial cells growth and enhance the effects of other growth factors such as IGF-I and EGF (Hovey et al., 1999). Interestingly, 4 out of the 5 long-chain FA included in F4 (long-chain FA) were unsaturated, the exception being 22:0.

The marker BTB-00787333, associated with F5 (desaturation), mapped 0.4 Mb from the cadherin-12 precursor (*CDH12*). In Holstein cows, this gene is associated with 14:0 and 18:0 contents in milk and with the Wnt signaling pathway (Li et al., 2014), which is reported to control adipogenesis and metabolism (Ross et al., 2000).

The results obtained for F11 (udder health) support the usefulness of this synthetic indicator as a tool for monitoring udder health in dairy cow. Indeed, several significant markers mapped close to genes involved in the immune/stress response or previously associated with mastitis and mastitis indicators (Table 4). For instance, the strong signal detected on BTA8 (ARS-BFGL-NGS-15117) was located 50 kb from growth arrest-specific 1 (*GAS1*), a positional candidate gene for clinical mastitis in dairy cows (Naderi et al., 2018). The marker ARS-BFGL-NGS-26253 was located on BTA26, 2 kb from serine/threonine kinase 32C (*STK32C*). This gene is located within a copy variant region significantly associated with EBV for SCS in Holstein cows (Durán Aguilar et al., 2017). The marker ARS-BFGL-NGS-94161 was located on BTA28, 3 kb from the pulmonary surfactant-associated protein D (*SFTPD*) and 30 kb from the pulmonary surfactant-associated protein A (*MBL1*), 2 genes that are involved in the innate immune response. Interestingly, a polymorphism in *MBL1* has also been associated with SCS in dairy cows, suggesting this gene may play a role in the host's response to mastitis (Wang et al., 2011). The marker ARS-BFGL-NGS-69854, located on BTA8, corresponded to a coding synonymous variant in γ -aminobutyric acid (GABA) B receptor, 2 (*GABBR2*); GABA is reported to regulate proliferation and apoptosis as well as LPS-induced inflammation in bovine mammary epithelial cells through the toll-like receptor (*TLR*)4 signaling pathway (Wang et al., 2018c). Accordingly, we detected another significant association on BTA8 (Hapmap59395-rs29023511) located 0.2 Mb from the TLR-4 precursor (*TLR4*). Moreover, a poly-

morphism in *TLR4* has been associated with mastitis resistance in dairy cow (Wang et al., 2007). Finally, the markers ARS-BFGL-NGS-107651 and BTB-00141133 (located at ~84.8 Mb on BTA3) and BTA-64483-no-rs (located at 43.14 Mb in BTA28) mapped close to a QTL for SCS according to the Cattle QTL database. When searching for the nearest genes, we found that ARS-BFGL-NGS-107651 and BTB-00141133 (which were in high LD, $r^2 = 0.92$) corresponded to intron variants of the nuclear factor IA (*NFIA*) and BTA-64483-no-rs mapped at 25 Kb from mitogen-activated protein kinase 8 (*MAPK8*), genes that play a role in immune system (Fazi et al., 2005; Hillreiner et al., 2017).

Pathway analyses showed that systemic lupus erythematosus was significantly enriched for F3 (biohydrogenation). In this disease, autoimmunity and inflammation are associated with pronounced changes in lipid and lipoprotein metabolism, and, in particular, with higher levels of triglycerides and very low density lipoprotein cholesterol (VLDL-C) and lower levels of high-density lipoprotein cholesterol (HDL-C; Borba et al., 2006). Several core histone-coding genes were associated with this pathway; histones are important regulators of gene expression, cell cycle progression, and genome stability (Mei et al., 2017). Variations in the expression of histone variants have been observed in fat-accumulating tissues in mouse models and humans (reviewed by Borghesan et al., 2014). The present results are consistent with a previous GWAS and pathway-based analysis on individual milk FA, which showed that the systemic lupus erythematosus pathway was enriched for *trans* FA 18:1 (Pegolo et al., 2017). However, the possible link between variation in the genes associated with this pathway and *trans*-FA metabolism in dairy cows requires further investigation.

Finally, the MAPK signaling pathway was overrepresented for F7 (milk fat protein). The MAP kinases and their downstream targets are involved in mediating cellular responses to extracellular stimuli; they directly mediate the metabolic effects of insulin (Gehart et al., 2010), which plays a crucial role in controlling lipogenesis and protein synthesis in the bovine mammary gland (Bionaz and Loor, 2008, 2011). Accordingly, the MAPK pathway has been associated with the regulation of milk fat and protein metabolism in dairy cattle (Lu et al., 2012; Pegolo et al., 2017; Wang et al., 2018b).

CONCLUSIONS

The results of the present study show MFA to be an effective tool for reducing complexity in genetic and genomic studies of interrelated phenotypes, while maintaining the biological significance and behavior of the original variables. The genetic variation found in the

12 FAF suggests their possible use as new phenotypes in breeding programs aimed at improving bovine mammary gland functionality and milk quality to reduce the number of traits. Before that, however, further research on a larger population and different breeds is required to validate the results obtained in this study. Finally, in addition to confirming previously identified signals and genes, we found that factors represent a source of novel information about genomic regions and putative candidate genes for milk FA (e.g., *TECRL*, *GAB2*) and udder health traits (e.g., *GBBR2*, *MAPK8*, *NFIA*), which are still coherent with the biology of the traits. This information provides a valuable resource for designing further studies on the function of these candidate genes.

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