Genetic and nongenetic factors contributing to differences in α_S -casein phosphorylation isoforms and other major milk proteins

Z. H. Fang,*† H. Bovenhuis,† A. Delacroix-Buchet,* G. Miranda,* D. Boichard,* M. H. P. W. Visker,† and P. Martin*¹

*Génétique Animale et Biologie Intégrative, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France

†Animal Breeding and Genomics Centre, Wageningen University and Research, PO Box 338, 6700 AH Wageningen, the Netherlands

ABSTRACT

Relative concentrations of α_S -casein (α_S -CN) phosphorylation isoforms vary considerably among milk of individual cows. We aimed to explore to what extent genetic and other factors contribute to the variation in relative concentrations of α_S -CN phosphorylation isoforms and the phosphorylation degree of α_S -CN defined as the proportion of isoforms with higher degrees of phosphorylation. We also investigated the associations of genetic variants of milk proteins and casein haplotypes with relative concentrations of α_S -CN phosphorylation isoforms and with the phosphorylation degree of α_S -CN in French Montbéliarde cattle from the cheese production area of Franche-Comté. Detailed milk protein composition was determined by liquid chromatography coupled with electrospray ionization mass spectrometry from 531 test-day morning milk samples. Parity, lactation stage, and genetic variation of cows contributed to the phenotypic variation in relative concentrations of individual α_S -CN phosphorylation isoforms and in the phosphorylation degree of α_S -CN. As lactation progressed, we observed a significant increase for relative concentrations of α_S -CN isoforms with higher degrees of phosphorylation (α_{S1} -CN-9P, α_{S2} -CN-13P, and α_{S2} -CN-14P) as well as for the phosphorylation degree of both α_{S1} -CN and α_{S2} -CN. Furthermore, the β -CN I variant was associated with a greater proportion of isoforms with lower degrees of phosphorylation (α_{S1} -CN-8P, α_{S2} -CN-10P, and α_{S2} -CN-11P); the β -CN B variant was associated with a greater proportion of isoforms with higher degrees of phosphorylation (α_{S1} -CN-9P, α_{S2} -CN-12P to α_{S2} -CN-14P). The heritability estimates were low to moderate for relative concentrations of α_{S2} -CN phosphorylation isoforms (0.07 to 0.32), high for relative concentrations of α_{S1} -CN-8P (0.84) and α_{S1} -CN-9P

(0.56), and moderate for phosphorylation degrees of α_{S1} -CN (0.37) and α_{S2} -CN (0.23). Future studies investigating relations between the phosphorylation degree of α_S -CN and technological properties of milk will be beneficial for the dairy industry.

Key words: casein haplotype, genetic variant, lactation stage, French Montbéliarde

INTRODUCTION

Detailed milk protein composition exhibits high heterogeneity because of quantitative variation in the content of the different milk proteins, numerous genetic variants, and isoforms with different degrees of post-translational modifications such as glycosylation in κ -CN and phosphorylation in all caseins (Caroli et al., 2009; Holland and Boland, 2014). Phosphorylation of caseins is one of the key factors responsible for constructing and stabilizing casein micelles (De Kruif and Holt, 2003). Although all 4 caseins (α_{S1} -CN, α_{S2} -CN, β -CN, and κ -CN) are phosphoproteins, α_{S1} -CN and α_{S2} -CN are more phosphorylated, and their phosphorylation profiles are more heterogeneous than those of β -CN and κ -CN. In bovine milk, α_{S1} -CN accounts for about 35% of the total casein and has 2 common phosphorylation isoforms: α_{S1} -CN-8P and α_{S1} -CN-9P (where P indicates phosphate group attached); α_{S2} -CN accounts for about 10% of the total casein and is present with isoforms from 10 to 14P and occasionally with 9P or 15P (Holland and Boland, 2014; Fang et al., 2016). Relative concentrations of α_S -CN phosphorylation isoforms vary considerably among milk of individual cows (Bijl et al., 2014a; Fang et al., 2016).

The phosphorylation degree (**PD**) of α_S -CN is one of the factors affecting technological properties of milk. Bijl et al. (2014b) demonstrated that high α_{S1} -CN-8P concentration in bovine milk is a great benefit for the production of uncooked curd cheese because α_{S1} -CN-8P is hydrolyzed more efficiently by chymosin during ripening. Additionally, 2 studies have shown that poorly or noncoagulating milk was associated with a greater

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¹Corresponding author: patrice.martin@inra.fr

proportion of α_S -CN isoforms with higher degrees of phosphorylation (e.g., α_{S1} -CN-9P, α_{S2} -CN-12P, and α_{S2} -CN-13P) when compared with well-coagulating milk, although the numbers of observations were limited (Frederiksen et al., 2011; Jensen et al., 2012). Recently, Fang et al. (2016) suggested that α_S -CN isoforms with lower degrees of phosphorylation (e.g., α_{S1} -CN-8P, α_{S2} -CN-10P, and α_{S2} -CN-11P) might be regulated differently compared with α_S -CN isoforms with higher degrees of phosphorylation (e.g., α_{S1} -CN-9P, α_{S2} -CN-12P to α_{S2} -CN-14P). Therefore, it is of great interest to explore to what extent genetic and other factors contribute to the variation in α_S -CN phosphorylation profile. Bijl et al. (2014a) and Buitenhuis et al. (2016) reported the genetic parameters for relative concentrations of α_{S1} -CN-8P and α_{S1} -CN-9P in Dutch Holstein Friesians and in Danish Holstein and Jersey, respectively. However, no information is available regarding α_{S2} -CN phosphorylation profile as well as detailed milk protein composition in the Montbéliarde breed.

The objective of this study was to investigate the genetic and nongenetic sources of variation in the phosphorylation degree of α_S -CN, and in relative concentrations of α_S -CN phosphorylation isoforms and other major milk proteins in French Montbéliarde cattle from Franche-Comté cheese production area. We also investigated the associations of genetic variants of milk proteins and casein haplotypes with detailed milk protein composition.

MATERIALS AND METHODS

Milk Samples

Test-day morning milk samples from 531 Montbéliarde cows were collected from 430 commercial herds across 3 French departments (239 herds in Doubs, 160 herds in Jura, and 31 herds in Haute-Saône) located in the production area of protected designation of origin (PDO) cheeses: Comté, Morbier, Mont d'Or, and Bleu de Gex, and of protected geographical indication (PGI) French Gruyère cheese. The sampling periods were during October–December 2014 and April–July 2015. The objective of the sampling was to maximize genetic diversity and milk content diversity to obtain optimal representation of the variation in milk protein composition from the current French Montbéliarde cattle population in the Franche-Comté region. For this purpose, we sampled cows across different parities (1–5) and lactation stages (7–652 d), and based on paternal pedigree and on protein and calcium content in milk from previous lactation records. Cows descended from 191 sires and 68 paternal grandsires, 52 of which were

also maternal grandsires. Milk (25 mL) was preserved with Bronopol after collection, transported on ice to the laboratory, and then frozen at -20°C until analyzed by liquid chromatography (LC)/electrospray ionization (ESI)-MS.

Milk Protein Profiling

Milk protein composition was determined by LC/ESI-MS method developed at INRA as described in detail by Fang et al. (2016). Briefly, milk proteins were separated by reversed-phase HPLC using an Ultimate LC 3000 system (Thermo Fisher Scientific, Waltham, MA) with a Discovery BIOWide Pore (Supelco, Bellefonte, PA) C5 column (150×2.10 mm, $300 \text{ \AA}$). Genetic variants and isoforms of the 6 major milk proteins (α_{S1} -CN, α_{S2} -CN, κ -CN, β -CN, α -LA, and β -LG) were identified using an ESI-TOF mass spectrometer microTOF II focus (Bruker Daltonics, Wissembourg, France). Relative concentrations of individual milk proteins and of α_{S1} -CN phosphorylation isoforms were estimated based on dividing the peak area of an individual protein by the total integrated peak area in the chromatogram (absorbance at 214 nm) of an individual milk sample. Mass signal intensity obtained from mass spectrometry was used to estimate the proportion of each α_{S2} -CN phosphorylation isoform as a fraction of total α_{S2} -CN. The relative concentration of each α_{S2} -CN phosphorylation isoform was estimated using the following equation:

$$\frac{\text{mass signal intensity of isoform}}{\sum \text{mass signal intensity of isoform}} \times \text{total } \alpha_{S2}\text{-CN (\% wt / wt)}. \quad [1]$$

The phosphorylation degrees of α_{S1} -CN and α_{S2} -CN were defined as the proportion of isoforms with higher degrees of phosphorylation, which were calculated as α_{S1} -CN PD = (α_{S1} -CN-9P/total α_{S1} -CN) $\times$ 100 and α_{S2} -CN PD = $[(\alpha_{S2}$ -CN-12P + α_{S2} -CN-13P + α_{S2} -CN-14P)/total α_{S2} -CN] $\times$ 100.

Statistical Analyses

To estimate variance components and genetic parameters, the following animal model was used:

$$y_{ijklm} = \mu + \text{region}_i + \text{parity}_j + \text{lstage}_k + \text{season}_l + \text{animal}_m + e_{ijklm}, \quad [2]$$

where y is the observation of the trait of interest; μ is the overall mean; region_i is the fixed effect of the i th region class (3 classes for the 3 French departments: Doubs, Jura, and Haute-Saône); parity_j is the fixed effect of the j th parity class (3 classes: 1st, 2nd, ≥ 3 rd parity); lstage_k is the fixed effect of the k th lactation stage class [0–50 DIM ($n = 95$), 51–100 DIM ($n = 73$), 101–150 DIM ($n = 69$), 151–200 DIM ($n = 76$), 201–250 DIM ($n = 82$), 251–300 DIM ($n = 66$), >300 DIM ($n = 68$)]; season_l is the fixed effect of the l th season class (2 classes for 2 sampling seasons: October–December 2014 and April–July 2015); animal_m is the random additive genetic effect of animal m and is assumed to be distributed as $N(\mathbf{0}, \mathbf{A}\sigma_a^2)$, where $\mathbf{A}$ is the additive genetic relationships matrix consisting of 5,546 animals with pedigree traced back for 5 generations, and σ_a^2 is the additive genetic variance; e_{ijklm} is the random residual effect and is assumed to be distributed as $N(\mathbf{0}, \mathbf{I}\sigma_e^2)$, where $\mathbf{I}$ is the identity matrix, and σ_e^2 is the residual variance. The heritability was defined as

$$h^2 = \frac{\sigma_a^2}{\sigma_p^2},$$

where the phenotypic variance $\sigma_p^2 = \sigma_a^2 + \sigma_e^2$.

To estimate the effects of genetic variants of milk proteins on detailed milk protein composition, model [2] was extended with a genotype effect where the genotypes of the milk proteins were inferred from the genetic variants obtained from LC-ESI-MS analysis. The genotype classes containing less than 5 observations were excluded from the analysis. The proportion of additive genetic variance explained by the genetic variants was calculated as

$$\frac{\sigma_{\text{genotype}}^2}{\sigma_a^2} \times 100,$$

where $\sigma_{\text{genotype}}^2$ is calculated based on the estimated genotype effects obtained from the extended model [2] and the observed frequencies of genetic variants, and σ_a^2 is the additive genetic variance estimated from model [2].

The $\alpha_{\text{S1}}\text{-}\beta\text{-}\kappa\text{-CN}$ haplotypes were inferred with Beagle 4.1 (Browning and Browning, 2007) adapted to multiallelic variants from $\alpha_{\text{S1}}\text{-CN}$, $\beta\text{-CN}$, and $\kappa\text{-CN}$ genetic variants. A multiallelic locus with n alleles ($n > 2$) was replaced by $(n - 1)$ virtual fully linked biallelic loci. The allele coded as 1 from the i th biallelic virtual locus ($1 \leq i < n$) corresponded the i th allele of the original

locus and the allele coded as 2 corresponded to any other allele.

To estimate the effects of $\alpha_{\text{S1}}\text{-}\beta\text{-}\kappa\text{-CN}$ haplotypes on detailed milk protein composition, the following model was used:

$$y_{ijklmno} = \mu + \text{region}_i + \text{parity}_j + \text{lstage}_k + \text{season}_l + \text{animal}_m + \text{haplo1}_n + \text{haplo2}_o + e_{ijklmno}; \quad [3]$$

model [3] was model [2] extended with haplo1_n and haplo2_o , where haplo1_n is the effect of the first $\alpha_{\text{S1}}\text{-}\beta\text{-}\kappa\text{-CN}$ haplotype, and haplo2_o is the effect of the second $\alpha_{\text{S1}}\text{-}\beta\text{-}\kappa\text{-CN}$ haplotype. The 2 haplotypes per animal were randomly assigned to haplo1_n or haplo2_o , and the design matrix for haplo1_n was added to the design matrix for haplo2_o . The haplo1_n and haplo2_o were modeled as fixed effects to estimate the effect of having 1 copy of each haplotype. The haplotype classes containing less than 5 observations were excluded from the analysis. The proportion of additive genetic variance explained by haplotypes was calculated as

$$\frac{\sigma_{\text{haplotype}}^2}{\sigma_a^2},$$

where haplotypes were modeled as random effects assumed to be distributed as $N(\mathbf{0}, \mathbf{I}\sigma_{\text{haplo}}^2)$. All statistical analyses were performed using ASReml (Gilmour et al., 2009).

RESULTS

Genetic Parameters

Table 1 summarizes the means, standard deviations, and genetic parameters for relative concentrations of the 6 major milk proteins and $\alpha_{\text{S}}\text{-CN}$ phosphorylation isoforms, and for the phosphorylation degree of $\alpha_{\text{S}}\text{-CN}$ ($\alpha_{\text{S}}\text{-CN PD}$). Heritability estimates for relative concentrations of the 6 major milk proteins were moderate to high and ranged from 0.22 ($\alpha\text{-LA}$) to 1.00 ($\alpha_{\text{S1}}\text{-CN}$). The standard error of the heritability estimate for $\alpha_{\text{S1}}\text{-CN}$ concentration could not be approximated accurately as the estimate was at the boundary of the parameter space. Likelihood ratio test suggested that the 95% confidence interval of the heritability estimate for $\alpha_{\text{S1}}\text{-CN}$ concentration ranged from 0.75 to 1.00. For $\alpha_{\text{S}}\text{-CN}$ phosphorylation isoforms, heritability estimates for relative concentrations of $\alpha_{\text{S1}}\text{-CN-8P}$ (0.84) and $\alpha_{\text{S1}}\text{-CN-9P}$ (0.56) were high, and for $\alpha_{\text{S1}}\text{-CN PD}$ (0.37) was moderate. The heritability estimates for relative

Table 1. Mean, SD, phenotypic variance¹ (σ_p^2), and h^2 for the 6 major milk proteins, the individual phosphorylation isoforms of α_{S1} -CN and α_{S2} -CN, and the phosphorylation degrees (PD)² of α_{S1} -CN and α_{S2} -CN measured on test-day morning milk samples from 531 Montbéliarde cows (SE in parentheses)

Trait	Mean	SD	σ_p^2	h^2
Major milk protein (% wt/wt)				
α_{S1} -CN	32.92	2.18	4.51	1.00 (–)
α_{S2} -CN	8.22	0.88	0.74	0.29 (0.14)
κ -CN	9.03	0.86	0.73	0.62 (0.20)
β -CN	28.14	2.69	7.48	0.75 (0.19)
α -LA	3.54	0.70	0.32	0.22 (0.12)
β -LG	12.16	1.87	3.41	0.73 (0.19)
Phosphorylation isoform (% wt/wt)				
α_{S1} -CN-8P	25.27	2.08	3.60	0.84 (0.18)
α_{S1} -CN-9P	7.65	0.96	0.84	0.56 (0.18)
α_{S1} -CN PD	23.27	2.80	6.19	0.37 (0.15)
α_{S2} -CN-10P	0.72	0.30	0.08	0.11 (0.09)
α_{S2} -CN-11P	3.04	0.55	0.28	0.32 (0.14)
α_{S2} -CN-12P	2.68	0.34	0.11	0.09 (0.12)
α_{S2} -CN-13P	1.57	0.31	0.07	0.07 (0.11)
α_{S2} -CN-14P	0.40	0.14	0.02	0.14 (0.13)
α_{S2} -CN PD	57.05	8.36	55.94	0.23 (0.12)

¹Phenotypic variance after adjusting for systematic effects: sampling region and season, parity, and lactation stage.

² α_{S1} -CN PD = (α_{S1} -CN-9P/total α_{S1} -CN) $\times$ 100; α_{S2} -CN PD = [(α_{S2} -CN-12P + α_{S2} -CN-13P + α_{S2} -CN-14P)/total α_{S2} -CN] $\times$ 100.

concentrations of α_{S2} -CN phosphorylation isoforms and for α_{S2} -CN PD were low to moderate (0.07 to 0.32).

Effects of Parity and Lactation Stage on Detailed Milk Protein Composition

Parity significantly affected relative concentrations of β -CN and α -LA (all $P < 0.001$; Table 2). For α_S -CN phosphorylation isoforms, parity significantly affected α_{S1} -CN PD, α_{S2} -CN PD, and relative concentrations of all α_S -CN phosphorylation isoforms except α_{S2} -CN-11P and α_{S2} -CN-12P. Lactation stage significantly affected α_{S1} -CN PD, α_{S2} -CN PD, and relative concentrations of all α_S -CN phosphorylation isoforms except α_{S2} -CN-12P (all $P < 0.001$, Figure 1). The changes in relative concentrations of individual α_S -CN phosphorylation isoforms during lactation are shown in Figure 1. The magnitude of lactation stage effects is expressed as a fold change in respective phenotypic standard deviations of relative concentrations of α_S -CN isoforms (as given in Table 1) to facilitate comparison across isoforms. The magnitude of the effects varied from 0.01 to 1.5 phenotypic standard deviation. As lactation progressed, we observed a significant decrease in relative concentrations for the group of isoforms with lower degrees of phosphorylation (α_{S1} -CN-8P, α_{S2} -CN-10P, and α_{S2} -CN-11P) and a significant increase in relative concentrations for the group of isoforms with higher degrees of phosphorylation (α_{S1} -CN-9P, α_{S2} -CN-13P, and α_{S2} -CN-14P) as well as a significant increase in both α_{S1} -CN PD and α_{S2} -CN PD.

Effects of Genetic Variants and Casein Haplotypes on Detailed Milk Protein Composition

We investigated the effects of genetic variants of milk proteins and α_{S1} - β - κ -CN haplotypes on relative concentrations of the 6 major milk proteins and α_S -CN phosphorylation isoforms, and α_S -CN PD. Two genetic variants were detected for α_{S1} -CN (B and C) and α_{S2} -CN (A and D), 3 for κ -CN (A, B, and E) and β -LG (A, B, and D), and 4 for β -CN (A1, A2, B, and I) in the sampled population (Fang et al., 2016).

Effects of α_{S1} -CN Variants. The α_{S1} -CN genotypes affected relative concentrations of α_{S1} -CN, κ -CN, β -CN, and α -LA (all $P < 0.001$; Table 3); the α_{S1} -CN genotype explained 22% of additive genetic variance of α_{S1} -CN concentration. The C variant was positively associated with α_{S1} -CN and α -LA concentrations and negatively associated with κ -CN and β -CN concentrations. For α_S -CN phosphorylation profile, α_{S1} -CN genotypes affected relative concentrations of all α_S -CN phosphorylation isoforms except α_{S2} -CN-12P and α_{S2} -CN-14P ($P < 0.001$ to $P = 0.046$; Table 3). The C variant was positively associated with α_{S1} -CN-8P, α_{S1} -CN-9P, and α_{S2} -CN-10P concentrations and negatively associated with α_{S2} -CN-11P and α_{S2} -CN-13P concentrations. The α_{S1} -CN genotypes did not significantly affect α_S -CN PD.

Effects of β -CN Variants. The β -CN genotypes affected relative concentrations of all 6 major milk proteins ($P < 0.001$ to $P = 0.003$, Supplemental Table S1; <https://doi.org/10.3168/jds.2016-12338>); the β -CN

Table 2. Effects of parity on the 6 major milk proteins, individual phosphorylation isoforms of α_{S1} -CN and α_{S2} -CN, and phosphorylation degrees (PD)¹ of α_{S1} -CN and α_{S2} -CN measured on test-day morning milk samples from 531 Montbéliarde cows (SE in parentheses)

Trait	Parity			−Log ₁₀ (<i>P</i>)
	1 (n = 64)	2 (n = 197)	≥3 (n = 269)	
Major milk protein (% wt/wt)				
α _{S1} -CN	0	−0.19 (0.15)	−0.23 (0.15)	0.5 ^{NS}
α _{S2} -CN	0	−0.09 (0.14)	−0.11 (0.14)	0.1 ^{NS}
κ-CN	0	0.14 (0.13)	0.14 (0.14)	0.2 ^{NS}
β-CN	0	−0.54 (0.42)	−1.45 (0.44)	3.7***
α-LA	0	−0.16 (0.09)	−0.13 (0.09)	31.9***
β-LG	0	−0.14 (0.29)	0.16 (0.30)	0.6 ^{NS}
Phosphorylation isoform (% wt/wt)				
α _{S1} -CN-8P	0	−0.47 (0.29)	−0.72 (0.30)	1.3*
α _{S1} -CN-9P	0	0.04 (0.15)	0.26 (0.15)	1.5*
α _{S1} -CN PD	0	0.45 (0.40)	1.20 (0.41)	3.0***
α _{S2} -CN-10P	0	−0.03 (0.05)	−0.11 (0.05)	2.4**
α _{S2} -CN-11P	0	−0.04 (0.08)	−0.09 (0.08)	0.4 ^{NS}
α _{S2} -CN-12P	0	0.08 (0.05)	0.09 (0.05)	0.7 ^{NS}
α _{S2} -CN-13P	0	0.02 (0.04)	0.14 (0.04)	5.3***
α _{S2} -CN-14P	0	0.00 (0.02)	0.05 (0.02)	3.6***
α _{S2} -CN PD	0	1.87 (1.22)	4.30 (1.22)	3.8***

¹ α_{S1} -CN PD = (α_{S1} -CN-9P/total α_{S1} -CN) × 100; α_{S2} -CN PD = [(α_{S2} -CN-12P + α_{S2} -CN-13P + α_{S2} -CN-14P)/total α_{S2} -CN] × 100.
P* < 0.05, *P* < 0.01, ****P* < 0.001.

genotypes explained 81% of additive genetic variance of β -CN concentration. The associations of individual genetic variants of β -CN with relative concentrations of the 6 major milk proteins are present in Table 4 where the number of alleles was modeled as a class variable. The β -CN B variant showed positive association with β -CN concentration and negative association with α_{S1} -CN, α_{S2} -CN, κ -CN, α -LA, and β -LG concentrations, whereas the A2 variant showed opposite associations.

The A1 variant was positively associated with β -CN concentration and negatively associated with α_{S2} -CN, α -LA, and β -LG concentrations. The I variant was positively associated with α_{S2} -CN, κ -CN, and β -LG concentrations and negatively associated with β -CN concentration.

For α_S -CN phosphorylation profile, β -CN genotypes affected relative concentrations of all α_S -CN phosphorylation isoforms (*P* < 0.001 to *P* = 0.037; Supplemen-

Figure 1. Effects of lactation stage on α_{S1} -CN (A) and α_{S2} -CN (B) phosphorylation profile and their phosphorylation degree (PD) throughout lactation. The x-axis shows days in milk. The y-axis shows the effect of lactation stage expressed as a fold change in phenotypic standard deviation (σ_p) of the relative concentration of each phosphorylation isoform. α_{S1} -CN PD = (α_{S1} -CN-9P/total α_{S1} -CN) × 100; α_{S2} -CN PD = [(α_{S2} -CN-12P + α_{S2} -CN-13P + α_{S2} -CN-14P)/total α_{S2} -CN] × 100. ****P* < 0.001.

Table 3. Effects of α_{S1} -CN genotypes on the 6 major milk proteins, individual phosphorylation isoforms of α_{S1} -CN and α_{S2} -CN, and phosphorylation degrees (PD)¹ of α_{S1} -CN and α_{S2} -CN measured on test-day morning milk samples from 531 Montbéliarde cows (SE in parentheses)

Trait	α_{S1} -CN genotype			-Log ₁₀ (P)
	BB (n = 469)	BC (n = 57)	CC (n = 5)	
Major milk protein (% wt/wt)				
α_{S1} -CN	0	2.75 (0.25)	5.80 (0.85)	29.0***
α_{S2} -CN	0	-0.17 (0.12)	0.39 (0.40)	0.6 ^{NS}
κ -CN	0	-0.87 (0.11)	-1.35 (0.37)	14.0***
β -CN	0	-2.26 (0.36)	-4.01 (1.21)	9.7***
α -LA	0	0.23 (0.08)	0.73 (0.26)	3.1***
β -LG	0	-0.12 (0.25)	-1.71 (0.86)	0.9 ^{NS}
Phosphorylation isoform (% wt/wt)				
α_{S1} -CN-8P	0	2.21 (0.23)	5.17 (0.79)	23.6***
α_{S1} -CN-9P	0	0.55 (0.13)	0.71 (0.42)	4.4***
α_{S1} -CN PD	0	-0.22 (0.36)	-1.61 (1.17)	0.5 ^{NS}
α_{S2} -CN-10P	0	0.05 (0.04)	0.29 (0.13)	1.3*
α_{S2} -CN-11P	0	-0.26 (0.07)	-0.05 (0.23)	2.8**
α_{S2} -CN-12P	0	0.00 (0.04)	0.04 (0.14)	0.0 ^{NS}
α_{S2} -CN-13P	0	-0.10 (0.04)	-0.12 (0.13)	1.6*
α_{S2} -CN-14P	0	-0.03 (0.02)	-0.03 (0.06)	0.7 ^{NS}
α_{S2} -CN PD	0	-0.89 (1.07)	-4.61 (3.50)	0.5 ^{NS}

¹ α_{S1} -CN PD = (α_{S1} -CN-9P/total α_{S1} -CN) $\times$ 100; α_{S2} -CN PD = [(α_{S2} -CN-12P + α_{S2} -CN-13P + α_{S2} -CN-14P)/total α_{S2} -CN] $\times$ 100.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

tal Table S2; <https://doi.org/10.3168/jds.2016-12338>) and α_{S2} -CN PD ($P < 0.001$; Supplemental Table S2). The associations of individual genetic variants of β -CN with α_S -CN phosphorylation profile are present in Table 5. The B variant showed positive associations

with both α_{S1} -CN PD and α_{S2} -CN PD, whereas the I variant showed opposite associations.

Effects of κ -CN Variants. The κ -CN genotypes showed highly significant effects on milk protein composition, especially on κ -CN concentration (Table 6);

Table 4. Associations of β -CN genotypes with the 6 major milk proteins measured on test-day morning milk samples from 531 Montbéliarde cows¹ (SE in parentheses)

β -CN genotype ²	α_{S1} -CN	α_{S2} -CN	κ -CN	β -CN	α -LA	β -LG
B						
0 (n = 210)	0	0	0	0	0	0
1 (n = 271)	-1.75 (0.15)	-0.31 (0.08)	-0.22 (0.07)	2.95 (0.19)	-0.18 (0.05)	-0.40 (0.16)
2 (n = 50)	-3.46 (0.26)	-0.95 (0.13)	-0.94 (0.13)	5.29 (0.32)	-0.42 (0.09)	-0.02 (0.28)
-Log ₁₀ (P)	39.2***	11.1***	11.3***	61.9***	5.4***	1.5*
A2						
0 (n = 180)	0	0	0	0	0	0
1 (n = 279)	1.63 (0.16)	0.34 (0.08)	0.16 (0.08)	-2.49 (0.20)	0.13 (0.05)	0.27 (0.17)
2 (n = 72)	3.89 (0.24)	0.25 (0.13)	0.47 (0.12)	-5.33 (0.31)	0.41 (0.09)	0.20 (0.27)
-Log ₁₀ (P)	46.8***	3.5***	3.1***	53.8***	5.3***	0.5 ^{NS}
A1						
0 (n = 437)	0	0	0	0	0	0
1 (n = 93)	-0.18 (0.23)	-0.51 (0.10)	-0.10 (0.10)	2.15 (0.30)	-0.14 (0.07)	-0.57 (0.21)
-Log ₁₀ (P)	1.3 ^{NS}	6.4***	0.5 ^{NS}	11.8***	1.5*	2.2**
I						
0 (n = 368)	0	0	0	0	0	0
1 (n = 153)	0.02 (0.20)	0.67 (0.08)	0.35 (0.08)	-1.50 (0.26)	0.10 (0.05)	0.35 (0.18)
2 (n = 10)	-0.36 (0.61)	1.22 (0.26)	0.44 (0.26)	-2.74 (0.79)	0.05 (0.18)	1.03 (0.55)
-Log ₁₀ (P)	0.1 ^{NS}	16.9***	4.1***	8.8***	0.7 ^{NS}	1.4*

¹One cow with A1A1 genotype was excluded from the analysis.

²The number below the β -CN genotype indicates the number of alleles (0, 1, or 2) that a cow carried.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 5. Associations of β -CN genotypes with individual phosphorylation isoforms of α_{S1} -CN and α_{S2} -CN, and the phosphorylation degree (PD)¹ of α_{S1} -CN and α_{S2} -CN measured on test-day morning milk samples from 531 Montbéliarde cows² (SE in parentheses)

β -CN genotype ³	α_{S1} -CN				α_{S2} -CN						
	8P	9P	PD		10P	11P	12P	13P	14P	PD	
B											
0 (n = 210)	0	0	0		0	0	0	0	0	0	
1 (n = 271)	-1.55 (0.14)	-0.23 (0.08)	0.53 (0.23)		-0.04 (0.03)	-0.08 (0.05)	-0.13 (0.03)	0.05 (0.03)	0.03 (0.01)	1.76 (0.67)	
2 (n = 50)	-3.05 (0.25)	-0.45 (0.14)	1.12 (0.40)		-0.14 (0.05)	-0.28 (0.08)	-0.17 (0.05)	0.06 (0.04)	0.06 (0.02)	6.52 (1.18)	
-Log ₁₀ (P)	35.9***	2.9**	2.1**		2.1**	2.7**	5.7***	1.1 ^{NS}	2.7**	6.5***	
A2											
0 (n = 180)	0	0	0		0	0	0	0	0	0	
1 (n = 279)	1.22 (0.15)	0.41 (0.08)	0.10 (0.24)		-0.03 (0.03)	-0.05 (0.05)	0.11 (0.03)	0.02 (0.03)	0.00 (0.01)	-1.00 (0.73)	
2 (n = 72)	3.11 (0.23)	0.81 (0.13)	-0.26 (0.37)		-0.02 (0.04)	-0.19 (0.07)	0.22 (0.04)	-0.04 (0.04)	-0.03 (0.02)	-0.29 (1.10)	
-Log ₁₀ (P)	34.2***	9.4***	0.3 ^{NS}		0.2 ^{NS}	1.4*	6.2***	0.5 ^{NS}	0.9 ^{NS}	0.4 ^{NS}	
A1											
0 (n = 437)	0	0	0		0	0	0	0	0	0	
1 (n = 93)	-0.04 (0.21)	-0.13 (0.11)	-0.25 (0.29)		0.01 (0.03)	-0.04 (0.06)	-0.11 (0.04)	-0.08 (0.03)	-0.03 (0.01)	1.09 (0.88)	
-Log ₁₀ (P)	0.6 ^{NS}	0.8 ^{NS}	0.4 ^{NS}		0.2 ^{NS}	0.3 ^{NS}	2.5**	1.9*	0.9 ^{NS}	0.7 ^{NS}	
I											
0 (n = 368)	0	0	0		0	0	0	0	0	0	
1 (n = 153)	0.21 (0.18)	-0.23 (0.09)	-0.62 (0.25)		0.11 (0.03)	0.35 (0.05)	0.06 (0.03)	0.00 (0.00)	-0.01 (0.01)	-4.85 (0.72)	
2 (n = 10)	-0.04 (0.56)	-0.47 (0.28)	-1.12 (0.78)		0.19 (0.09)	0.64 (0.15)	0.23 (0.10)	0.04 (0.09)	-0.10 (0.04)	-5.07 (2.29)	
-Log ₁₀ (P)	0.3 ^{NS}	1.7*	1.6*		4.0***	13.8***	1.2 ^{NS}	0.1 ^{NS}	0.3 ^{NS}	9.8***	

¹ α_{S1} -CN PD = (α_{S1} -CN-9P/total α_{S1} -CN) $\times$ 100; α_{S2} -CN PD = [(α_{S2} -CN-12P + α_{S2} -CN-13P + α_{S2} -CN-14P)/total α_{S2} -CN] $\times$ 100.

²One cow with A1A1 genotype was excluded from the analysis.

³The number below the β -CN genotype indicates the number of alleles (0, 1, or 2) that a cow carried.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 6. Effects of κ -CN genotypes on the 6 major milk proteins, individual phosphorylation isoforms of α_{S1} -CN and α_{S2} -CN, and phosphorylation degrees (PD)¹ of α_{S1} -CN and α_{S2} -CN measured on test-day morning milk samples from 531 Montbéliarde cows² (SE in parentheses)

Trait	κ -CN genotype			-Log ₁₀ (P)
	AA (n = 144)	AB (n = 268)	BB (n = 116)	
Major milk protein (% wt/wt)				
α_{S1} -CN	-0.15 (0.21)	0	0.82 (0.21)	3.9***
α_{S2} -CN	-0.40 (0.09)	0	0.44 (0.09)	12.7***
κ -CN	-1.12 (0.07)	0	0.25 (0.07)	53.0***
β -CN	1.87 (0.24)	0	-2.10 (0.25)	33.3***
α -LA	-0.12 (0.06)	0	0.14 (0.06)	2.6**
β -LG	-0.08 (0.19)	0	0.49 (0.19)	1.6*
Phosphorylation isoform (% wt/wt)				
α_{S1} -CN-8P	-0.18 (0.19)	0	0.78 (0.19)	4.4***
α_{S1} -CN-9P	0.06 (0.10)	0	0.05 (0.10)	0.1 ^{NS}
α_{S1} -CN PD	0.25 (0.26)	0	-0.42 (0.28)	0.9 ^{NS}
α_{S2} -CN-10P	0.01 (0.03)	0	0.01 (0.03)	0.0 ^{NS}
α_{S2} -CN-11P	-0.22 (0.05)	0	0.17 (0.05)	8.1***
α_{S2} -CN-12P	-0.01 (0.03)	0	0.17 (0.03)	5.9***
α_{S2} -CN-13P	0.02 (0.03)	0	0.02 (0.03)	0.4 ^{NS}
α_{S2} -CN-14P	0.00 (0.01)	0	-0.02 (0.01)	0.3 ^{NS}
α_{S2} -CN PD	2.49 (0.79)	0	-1.01 (0.83)	3.2***

¹ α_{S1} -CN PD = (α_{S1} -CN-9P/total α_{S1} -CN) $\times$ 100; α_{S2} -CN PD = [(α_{S2} -CN-12P + α_{S2} -CN-13P + α_{S2} -CN-14P)/total α_{S2} -CN] $\times$ 100.

²One cow with AE genotype and 2 cows with BE genotype were excluded from the analysis.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

the κ -CN genotype explained 65% of additive genetic variance of κ -CN concentration. The B variant was positively associated with relative concentrations of all major milk proteins except β -CN concentration for which the association was negative. For α_S -CN phosphorylation profile, κ -CN genotypes affected α_{S1} -CN-8P, α_{S2} -CN-11P and α_{S2} -CN-12P concentrations ($P < 0.001$; Table 6), and the B variant was positively associated with relative concentrations of aforementioned isoforms. The κ -CN genotypes significantly affected α_{S2} -CN PD, and the A variant was positively associated with α_{S2} -CN PD.

Effects of β -LG Variants. The β -LG genotypes significantly affected relative concentrations of all major milk proteins except α_{S2} -CN concentration ($P < 0.001$ to $P = 0.002$; Table 7); the β -LG genotypes explained 95% of additive genetic variance of β -LG concentration. The difference in β -LG concentration between AA and BB genotypes was about 4%. The B variant was positively associated with α_{S1} -CN, κ -CN, β -CN, and α -LA concentrations and negatively associated with β -LG concentration. For α_S -CN phosphorylation profile, β -LG genotypes affected α_{S1} -CN-8P and α_{S1} -CN-9P concentrations ($P < 0.001$; Table 7), and the B variant was positively associated with both isoforms' concentrations. The β -LG genotypes did not significantly affect α_S -CN PD.

Effects of Casein Haplotypes. Inferring α_{S1} - β - κ -CN casein haplotypes resulted in 10 haplotypes: CA2A, BA2A, BA1A, BBA, BA1B, BA2B, BIB, BBB, BIA,

and BA1E (frequencies provided in Supplemental Table S3; <https://doi.org/10.3168/jds.2016-12338>). The α_{S1} -CN C variant occurred only with the β -CN A2 and κ -CN A variants. The κ -CN E variant occurred only with the β -CN A1 variant. The β -CN I variant occurred predominately with the κ -CN B variant. The α_{S1} - β - κ -CN haplotypes significantly affected relative concentrations of the 6 major milk proteins ($P < 0.001$ to $P = 0.0025$); the proportions of additive genetic variance explained by haplotypes were high (0.46–0.89) for the 4 caseins and low to moderate (0.04–0.22) for the 2 whey proteins (Table 8). The haplotype carrying the α_{S1} -CN C variant was positively associated with α_{S1} -CN concentration. The haplotypes carrying the β -CN A2 and I variants were negatively associated with β -CN concentration and positively associated with α_{S1} -CN and α_{S2} -CN concentrations. The haplotypes carrying the κ -CN B variant were positively associated with κ -CN concentration. For the α_S -CN phosphorylation profile, the α_{S1} - β - κ -CN haplotypes significantly affected both α_{S1} -CN PD and α_{S2} -CN PD, and relative concentrations of all α_S -CN phosphorylation isoforms ($P < 0.001$ to $P = 0.03$); the proportion of additive genetic variance explained by haplotypes was as high as 0.72 for α_{S1} -CN-8P concentration, and ranged from 0.04 to 0.39 for relative concentrations of the other α_S -CN phosphorylation isoforms and α_S -CN PD (Table 9). The haplotype carrying the β -CN I variant showed negative associations with both α_{S1} -CN PD and α_{S2} -CN

Table 7. Effects of β -LG genotypes on the 6 major milk proteins, individual phosphorylation isoforms of α_{S1} -CN and α_{S2} -CN, and phosphorylation degrees (PD)¹ of α_{S1} -CN and α_{S2} -CN measured on test-day morning milk samples from 531 Montbéliarde cows² (SE in parentheses)

Trait	β -LG genotype			$-\text{Log}_{10}(P)$
	AA (n = 103)	AB (n = 259)	BB (n = 167)	
Major milk protein (% wt/wt)				
α_{S1} -CN	−0.84 (0.21)	0	1.32 (0.18)	3.6***
α_{S2} -CN	0.01 (0.10)	0	0.17 (0.09)	0.9 ^{NS}
κ -CN	−0.27 (0.09)	0	0.33 (0.08)	7.2***
β -CN	−0.84 (0.30)	0	0.41 (0.26)	3.0**
α -LA	−0.09 (0.07)	0	0.21 (0.06)	4.3***
β -LG	1.88 (0.11)	0	−2.41 (0.10)	135.9***
Phosphorylation isoform (% wt/wt)				
α_{S1} -CN-8P	−0.61 (0.20)	0	0.99 (0.17)	11.4***
α_{S1} -CN-9P	−0.29 (0.10)	0	0.32 (0.09)	6.1***
α_{S1} -CN PD	−0.21 (0.29)	0	0.05 (0.25)	0.2 ^{NS}
α_{S2} -CN-10P	0.01 (0.03)	0	0.03 (0.03)	0.3 ^{NS}
α_{S2} -CN-11P	0.02 (0.06)	0	0.08 (0.05)	0.5 ^{NS}
α_{S2} -CN-12P	0.03 (0.04)	0	0.01 (0.03)	0.3 ^{NS}
α_{S2} -CN-13P	−0.01 (0.03)	0	0.03 (0.03)	0.4 ^{NS}
α_{S2} -CN-14P	0.01 (0.01)	0	0.01 (0.01)	0.3 ^{NS}
α_{S2} -CN PD	0.14 (0.87)	0	−0.61 (0.76)	0.2 ^{NS}

¹ α_{S1} -CN PD = (α_{S1} -CN-9P/total α_{S1} -CN) $\times$ 100; α_{S2} -CN PD = [(α_{S2} -CN-12P + α_{S2} -CN-13P + α_{S2} -CN-14P)/total α_{S2} -CN] $\times$ 100.

²Two cows with BD genotype were excluded from the analysis.

** $P < 0.01$, *** $P < 0.001$.

PD, whereas the haplotype carrying the β -CN B variant showed opposite associations.

DISCUSSION

We report heritability estimates for detailed milk protein composition and explored factors affecting detailed milk protein composition, especially for α_S -CN phosphorylation isoforms and the phosphorylation degree of α_S -CN (α_S -CN PD). Accurate quantification of relative concentrations of α_{S2} -CN phosphorylation isoforms has

been a challenge due to limitations of the analytical techniques. As a result, limited information was available about factors contributing to the variation in relative concentrations of α_{S2} -CN phosphorylation isoforms and the phosphorylation degree of α_{S2} -CN.

Heritability Estimates

To our knowledge, this is the first study to report heritability estimates for relative concentrations of individual α_{S2} -CN phosphorylation isoforms (0.07–0.32) and for

Table 8. Effects of α_{S1} - β - κ -CN haplotypes and proportions of additive genetic variance explained by haplotypes (h_{haplo}) for the 6 major milk proteins measured on test-day morning milk samples from 531 Montbéliarde cows¹ (SE in parentheses)

Item	α_{S1} -CN	α_{S2} -CN	κ -CN	β -CN	α -LA	β -LG
α_{S1} - β - κ -CN haplotype ²						
CA2A (n = 31)	4.08 (0.18)	0.11 (0.10)	−0.59 (0.09)	−3.81 (0.22)	0.38 (0.07)	−0.26 (0.24)
BA2A (n = 34)	2.87 (0.20)	0.23 (0.11)	−0.17 (0.09)	−3.04 (0.23)	0.24 (0.08)	0.22 (0.26)
BA1A (n = 26)	1.98 (0.21)	−0.07 (0.12)	−0.14 (0.10)	−0.24 (0.25)	0.07 (0.09)	0.14 (0.28)
BBA (n = 178)	0	0	0	0	0	0
BA1B (n = 22)	0.73 (0.24)	−0.45 (0.13)	0.35 (0.12)	−0.08 (0.28)	0.17 (0.10)	−0.52 (0.31)
BA2B (n = 141)	1.91 (0.12)	0.42 (0.07)	0.68 (0.06)	−3.29 (0.14)	0.21 (0.05)	0.24 (0.15)
BIB (n = 94)	0.91 (0.13)	0.81 (0.08)	0.53 (0.06)	−2.94 (0.16)	0.19 (0.06)	0.49 (0.18)
$-\text{Log}_{10}(P)$	85.7***	27.0***	45.8***	106.7***	7.1***	2.6**
h_{haplo} ³	0.69 (0.15)	0.46 (0.20)	0.89 (0.21)	0.76 (0.14)	0.22 (0.15)	0.04 (0.04)

¹Cows that carried BIA, BBB, and BA1E haplotypes were excluded from the analysis due to low numbers of observations.

²The effects of haplotypes are estimated by modeling the haplotypes as fixed effects, and the estimate is the effect of having 1 copy of that haplotype.

³The proportion of additive genetic variance explained by haplotypes, where the haplotypes are modeled as random effects.

** $P < 0.01$, *** $P < 0.001$.

Table 9. Effects of α_{S1} - β - κ -CN haplotypes and proportion of additive genetic variance explained by haplotypes (h_{haplo}) for individual phosphorylation isoforms of α_{S1} -CN and α_{S2} -CN, and phosphorylation degrees (PD)¹ of α_{S1} -CN and α_{S2} -CN measured on test-day morning milk samples from 531 Montbéliarde cows² (SE in parentheses)

Item	α_{S1} -CN			α_{S2} -CN						
	8P	9P	PD	10P	11P	12P	13P	14P	PD	
α_{S1} - β - κ -CN haplotype ³										
CA2A (n = 31)	3.39 (0.18)	0.69 (0.12)	-0.70 (0.34)	0.10 (0.04)	-0.15 (0.06)	0.07 (0.04)	-0.13 (0.04)	-0.05 (0.02)	-2.60 (0.96)	
BA2A (n = 34)	2.30 (0.20)	0.58 (0.13)	-0.28 (0.36)	0.10 (0.04)	-0.13 (0.07)	0.20 (0.04)	-0.11 (0.04)	-0.06 (0.02)	-1.82 (1.03)	
BA1A (n = 26)	1.78 (0.21)	0.22 (0.14)	-0.76 (0.39)	0.08 (0.04)	0.01 (0.07)	-0.05 (0.05)	-0.10 (0.04)	-0.05 (0.02)	-2.30 (1.12)	
BBA (n = 178)	0	0	0	0	0	0	0	0	0	
BA1B (n = 22)	0.72 (0.23)	0.00 (0.15)	-0.47 (0.44)	0.01 (0.05)	-0.01 (0.08)	0.02 (0.05)	-0.15 (0.05)	-0.06 (0.02)	0.91 (1.26)	
BA2B (n = 141)	1.60 (0.11)	0.32 (0.07)	-0.37 (0.21)	-0.00 (0.02)	0.08 (0.04)	0.14 (0.03)	0.00 (0.02)	-0.02 (0.01)	-1.72 (0.60)	
BIB (n = 94)	0.96 (0.13)	-0.06 (0.09)	-0.83 (0.25)	0.12 (0.03)	0.37 (0.05)	0.10 (0.03)	-0.02 (0.03)	-0.03 (0.01)	-5.28 (0.71)	
$-\text{Log}_{10}(P)$	64.9***	9.5***	1.6*	4.9***	14.6***	7.7***	4.1***	3.1*	9.9***	
h_{haplo}^4	0.72 (0.17)	0.20 (0.12)	0.04 (0.04)	0.39 (0.43)	0.36 (0.19)	0.38 (0.33)	0.21 (0.19)	0.12 (0.12)	0.32 (0.23)	

¹ α_{S1} -CN PD = (α_{S1} -CN-9P/total α_{S1} -CN) $\times$ 100; α_{S2} -CN PD = [$(\alpha_{S2}$ -CN-12P + α_{S2} -CN-13P + α_{S2} -CN-14P)/total α_{S2} -CN] $\times$ 100.

²Cows that carried BIA, BBB, and BAIE haplotypes were excluded from the analysis due to low numbers of observations.

³The effects of haplotypes are estimated by modeling the haplotypes as fixed effects, and the estimate is the effect of having 1 copy of that haplotype.

⁴The proportion of additive genetic variance explained by haplotypes, where the haplotypes are modeled as random effects.

* $P < 0.05$, *** $P < 0.001$.

α_{S2} -CN PD (0.23). Information about the 6 major milk proteins is scarce as well because their quantification is time consuming and costly. A few studies have reported heritability estimates for relative concentrations of the 6 major milk proteins (Ikonen et al., 1997; Schopen et al., 2009; Boichard et al., 2014; Buitenhuis et al., 2016), and only 2 studies have reported heritability estimates for α_{S1} -CN-8P and α_{S1} -CN-9P concentrations (Bijl et al., 2014a; Buitenhuis et al., 2016). In our study, we did not adjust for the herd effect because most of the cows were located in different herds. Consequently, we could not estimate the proportion of phenotypic variance due to differences between herds, so heritability estimates of this study were expected to be lower than the intra-herd heritability estimates reported in previous studies. However, differences between herds contribute only a relatively small part of phenotypic variances of the 6 major milk proteins, α_{S1} -CN-8P and α_{S1} -CN-9P concentrations according to previous studies (Schopen et al., 2009; Bonfatti et al., 2011; Bijl et al., 2014a).

The heritability estimates for relative concentrations of the 6 major milk proteins were similar to or higher than those reported by Boichard et al. (2014), who analyzed major protein fractions predicted by mid-infrared spectra in the Montbéliarde breed. In comparison with other breeds, the heritability estimates for α_{S1} -CN (1.00) and β -CN (0.75) in our study were higher than those reported by Schopen et al. (2009; 0.47 for α_{S1} -CN and 0.25 for β -CN in Dutch Holstein-Friesian) and by Buitenhuis et al. (2016; 0 for α_{S1} -CN and 0.05 for β -CN in Danish Holstein; 0.05 for α_{S1} -CN and 0.29 for β -CN in Danish Jersey). The heritability estimate for κ -CN (0.62) in our study is similar to those reported by Schopen et al. (2009; 0.64) and Buitenhuis et al. (2016; 0.77 for Danish Holstein) but higher than the one for Danish Jersey (Buitenhuis et al., 2016; 0.29). The heritability estimate for β -LG (0.73) in our study is similar to the one reported by Schopen et al. (2009; 0.80) but higher than those reported by Buitenhuis et al. (2016; 0.58 for Danish Holstein and 0.16 for Danish Jersey). As shown in previous studies (Bobe et al., 1999; Heck et al., 2009; Schopen et al., 2009), the α_{S1} -CN, κ -CN, β -CN, and β -LG genotypes contributed a major part of the genetic variation in α_{S1} -CN, κ -CN, β -CN, and β -LG concentration, respectively.

For α_{S1} -CN phosphorylation profile, the heritability estimate for α_{S1} -CN-8P concentration (0.84) in our study is higher than those reported by Bijl et al. (2014a; 0.48 in Dutch Holstein-Friesian) and Buitenhuis et al. (2016; 0.01 in Danish Holstein and 0.41 in Danish Jersey). The heritability estimate for α_{S1} -CN-9P concentration (0.56) in our study is within the range of the one reported by Bijl et al. (2014a; 0.76) and is higher than those reported by Buitenhuis et al. (2016;

0.25 in Danish Holstein and 0.23 in Danish Jersey). Discrepancies between studies might be due to genetic differences between breeds, limited sample sizes in the study of Buitenhuis et al. (2016), and in this study, and use of different analytical methods. In terms of analytical methods, Schopen et al. (2009) and Bijl et al. (2014a) measured protein fractions by capillary zone electrophoresis, so β -CN was measured together with glycosylated and multi-phosphorylated κ -CN, and κ -CN was only measured as the mono-phosphorylated isoform (Heck et al., 2008). Furthermore, protein fractions measured with the same analytical method, such as LC (as used by Buitenhuis et al., 2016), could differ because of differences in separation conditions.

Effects of Genetic Variants and Casein Haplotypes on the 6 Major Milk Proteins

We investigated the associations of genetic variants of milk proteins and casein haplotypes with detailed milk protein composition, and this is the first study of such association analysis done with the Montbéliarde breed. Our study confirms the effect of the κ -CN B variant on κ -CN concentration and the effect of the β -LG B variant on β -LG concentration, which seem to be the true effects as they are consistent across studies and breeds (Van Eenennaam and Medrano, 1991; Bobe et al., 1999; Graml and Pirchner, 2003; Hallén et al., 2008; Heck et al., 2009; Bonfatti et al., 2010). Discrepancies for other associations among studies could be due to reasons mentioned above (i.e., genetic differences between breeds, limited sample sizes in some studies, and use of different analytical methods). Regarding genetic differences between breeds, the casein loci are in close proximity, and linkage disequilibrium between genetic variants has been reported (Ikonen et al., 1999; Heck et al., 2009; Bonfatti et al., 2010). Differences in linkage disequilibrium between breeds will affect the associations of the genetic variants with detailed milk protein composition. Therefore, investigating the associations of casein haplotypes with detailed milk protein composition provides better insights into the associations of individual genetic variants.

α_{S1} -CN. Effects of α_{S1} -CN genotypes on milk protein composition are rarely reported due to the low frequency of the C variant in western cattle breeds such as Dutch Holstein-Frisian, Italian Simmental, Danish Holstein and Swedish Red (Heck et al., 2009; Bonfatti et al., 2010; Gustavsson et al., 2014). The C variant was associated with higher α_{S1} -CN concentration and lower β -CN concentration, which is in line with the results of Graml and Pirchner (2003). However, we did not detect the dominance effect reported by those authors. This could be due to the small number of homozygous

CC cows in our population, genetic differences between breeds, or differences in the analytical methods.

β -CN. For the 6 major milk proteins, the associations of the B and A2 variants were in opposite directions, which agrees with associations reported by Visker et al. (2011). Positive association of the B variant with β -CN concentration and negative association of the B variant with α_{S1} -CN and α_{S2} -CN concentrations are consistent with previous studies (Bonfatti et al., 2010; Visker et al., 2011). However, we found a negative association of the B variant with κ -CN concentration, whereas Visker et al. (2011) reported a positive association and Bonfatti et al. (2010) found no association. Positive association of the I variant with α_{S2} -CN concentration is in line with association reported by Visker et al. (2011). As for the effect of the I variant on α_{S1} -CN concentration, we found no association, whereas Bonfatti et al. (2010) and Visker et al. (2011) reported negative associations.

κ -CN. Besides a favorable effect of the κ -CN B variant on κ -CN concentration, we also report a favorable effect of the κ -CN B variant on α_{S2} -CN concentration, which is similar to what Heck et al. (2009) reported, whereas Bobe et al. (1999) found no effect of κ -CN genotypes on α_{S2} -CN concentration. Contrary to previous studies, we found a positive association of the B variant with α_{S1} -CN concentration and a negative association of the B variant with β -CN concentration, whereas other authors reported a negative association of the B variant with α_{S1} -CN concentration and no association with β -CN concentration (Bobe et al., 1999; Hallén et al., 2008; Heck et al., 2009; Bonfatti et al., 2010).

β -LG. We report that the β -LG genotypes accounted for 95% of genetic variation of β -LG concentration, which agrees with the results reported by Bobe et al. (1999) and Heck et al. (2009). These authors concluded that β -LG genotypes predominately regulate β -LG concentration in total milk protein. We show a favorable effect of the B variant on α_{S1} -CN, κ -CN, β -CN, and α -LA concentrations, and a negative effect on β -LG concentration. These findings agree with those from previous studies, as the B variant of β -LG decreases the proportion of β -LG which results in increased proportions of the other milk proteins (Bobe et al., 1999; Hallén et al., 2008; Heck et al., 2009; Bonfatti et al., 2010). Note that the effect of the β -LG BB genotype on β -LG concentration in this study was about 1.5 times larger than the one reported by Heck et al. (2009). One explanation for this difference might be that the observed genotype effect is the combination of the effect of β -LG genotypes and the effect of one or multiple linked causal mutations. Multiple genetic polymorphisms in the coding and promoter regions have been detected in the β -LG gene, and many of them are in linkage

disequilibrium with the β -LG genotypes (Ganai et al., 2009). The effect of a linked causal mutation might vary across breeds and populations due to differences in linkage disequilibrium with the β -LG genotypes.

Casein Haplotypes. The frequency and effects of the CA2A haplotype on detailed milk protein composition are reported for the first time in this study in French Montbéliarde cows due to the low frequency of the α_{S1} -CN C variant in other breeds. The β -CN B variant occurred predominantly with the κ -CN A variant in French Montbéliarde cows as reported for Simmental cows (Bonfatti et al., 2010), whereas the β -CN B variant occurred only with the κ -CN B variant in Dutch Holstein-Friesian cows (Visker et al., 2011).

Haplotype analysis confirmed positive association of the α_{S1} -CN C variant with α_{S1} -CN concentration, positive association of the κ -CN B variant with κ -CN concentration, and positive association of the β -CN I variant with α_{S2} -CN concentrations. These results are consistent across studies and breeds, which seem to be direct associations of the genetic variants or causal mutations that are closely linked to the genetic variants (Van Eenennaam and Medrano, 1991; Bobe et al., 1999; Graml and Pirchner, 2003; Hallén et al., 2008; Heck et al., 2009; Bonfatti et al., 2010; Visker et al., 2011). We report a positive association of the β -CN I variant with α_{S1} -CN concentration, whereas Visker et al. (2011) reported a negative association. Due to its low frequency, the BIA haplotype was excluded from the association analysis. Hence, it is not straightforward to infer if associations of the BIB haplotype resulted from the β -CN I variant or from the κ -CN B variant. Positive associations of the BIB haplotype with κ -CN and α -LA concentrations most likely resulted from associations of the κ -CN B variant because associations of BA1B, BA2B, and BIB haplotypes with κ -CN and α -LA concentrations were in the same direction and of about similar magnitude.

Factors Affecting Phosphorylation of α_{S1} -CN and α_{S2} -CN

Effects of Parity and Lactation Stage. We show changes of α_S -CN phosphorylation profile across parity and lactation. Cows in parity 3 or higher produced milk with higher degrees of phosphorylation of α_S -CN as relative concentrations of α_{S1} -CN-9P, α_{S2} -CN-13P, and α_{S2} -CN-14P increased. Furthermore, both α_{S1} -CN PD and α_{S2} -CN PD increased as lactation progressed, whereas the total α_{S1} -CN and α_{S2} -CN concentrations were not affected by lactation stage. Note that relative concentrations of the group of the isoforms with lower degrees of phosphorylation (α_{S1} -CN-8P, α_{S2} -CN-10P, and α_{S2} -CN-11P) changed in the opposite direction compared

with those of the group of isoforms with higher degrees of phosphorylation (α_{S1} -CN-9P, α_{S2} -CN-13P, and α_{S2} -CN-14P) during lactation. This observation supports the hypothesis that different sets of genes regulate phosphorylation of α_S -CN (Bijl et al., 2014a; Fang et al., 2016). Such different sets of genes involved in phosphorylation of caseins could show different expression throughout lactation as the mammary transcriptome is known to vary at different lactation stages due to physiological changes such as pregnancy (Bionaz et al., 2012; Wickramasinghe et al., 2012). Moreover, Bijl et al. (2014a) detected association between the *DGAT1* gene and α_{S1} -CN-9P concentration, and Bovenhuis et al. (2015) showed that the magnitude of the *DGAT1* effect on milk production traits changes during lactation. The *DGAT1* A allele is almost fixed in the French Montbéliarde breed (0.96, Gautier et al., 2007), so the K232A polymorphism in this gene is not responsible for the observed change during lactation in the French Montbéliarde. Nevertheless, the effects of other genes involved in the phosphorylation mechanism of caseins may show similar variation throughout lactation.

Effects of Genetic Variants and Casein Haplotypes. Phosphorylation of caseins occurs in the Golgi apparatus after the synthesis of polypeptide chains. If casein-phosphorylating enzymes are not saturated, the associations of genetic variants with individual phosphorylation isoforms can be ascribed to the associations of genetic variants with total α_S -CN concentrations. This is supported by the observation that the effects of genetic variants on α_{S1} -CN-8P, α_{S1} -CN-9P, and total α_{S1} -CN concentration were in the same direction and of similar magnitude. The same explanation applies to the associations of genetic variants with individual α_{S2} -CN phosphorylation isoforms. This is confirmed by estimating the effects of genetic variants on the phosphorylation degree of α_{S1} -CN and α_{S2} -CN. Only the negative effect of the β -CN I variant and the positive effects of the κ -CN A variant and the β -CN B variant seem to be direct effects on α_{S1} -CN PD and α_{S2} -CN PD. These effects of the β -CN I and B variant are also confirmed by haplotype analysis as the BIB haplotype showed positive associations with α_{S1} -CN-8P, α_{S2} -CN-10P, and α_{S2} -CN-11P concentrations and a negative association with α_S -CN PD, and the BBA haplotype showed opposite associations. In contrast, Bijl et al. (2014a) reported negative associations of β - κ -CN haplotype IB with both α_{S1} -CN-8P and α_{S1} -CN-9P concentrations in Dutch Holstein-Friesian cows. Combining the negative association of β - κ -CN haplotype IB with α_{S1} -CN concentration reported by Visker et al. (2011), the associations of the IB haplotype with α_{S1} -CN-8P and α_{S1} -CN-9P concentrations could be ascribed to its association with total α_S -CN concentration as dis-

cussed above. Moreover, Bijl et al., (2014a) showed that β -LG genotypes affect only α_{S1} -CN-8P concentration, whereas we showed that β -LG genotypes affected both α_{S1} -CN-8P and α_{S1} -CN-9P concentrations. Inconsistencies between the 2 studies might be due to the reasons mentioned above (i.e., genetic differences between the 2 breeds and differences in linkage disequilibrium between the β -LG genotypes and other causal variants across breeds).

CONCLUSIONS

We report the difference in relative concentrations of α_S -CN phosphorylation isoforms and in the phosphorylation degree of α_S -CN between cows due to systematic environmental effects (parity and lactation stage) and genetic variation. We show that α_S -CN phosphorylation profile changed across parity and lactation, and exploitable genetic variation for the phosphorylation degrees of α_{S1} -CN and α_{S2} -CN exists in the French Montbéliarde cattle. Furthermore, the β -CN I variant is associated with a greater proportion of isoforms with lower degrees of phosphorylation (α_{S1} -CN-8P, α_{S2} -CN-10P, and α_{S2} -CN-11P); the β -CN B variant is associated with a greater proportion of isoforms with higher degrees of phosphorylation (α_{S1} -CN-9P, α_{S2} -CN-12P to α_{S2} -CN-14P). Currently, knowledge regarding the effects of the phosphorylation degree of α_S -CN on technological properties of milk is limited and requires further investigation.

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