Short communication: Confirmation of candidate causative variants on milk composition and cheesemaking properties in Montbéliarde cows

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ABSTRACT

In a previous study, we identified candidate causative variants located in 24 functional candidate genes for milk protein and fatty acid composition in Montbéliarde, Normande, and Holstein cows. We designed these variants on the custom part of the EuroG10K BeadChip (Illumina Inc., San Diego, CA), which is routinely used for genomic selection analyses in French dairy cattle. To validate the effects of these candidate variants on milk composition and to estimate their effects on cheesemaking properties, a genome-wide association study was performed on milk protein, fatty acid and mineral composition, as well as on 9 cheesemaking traits (3 laboratory cheese yields, 5 coagulation traits, and milk pH). All the traits were predicted from midinfrared spectra in the Montbéliarde cow population of the Franche-Comté region. A total of 194 candidate variants located in 24 genes and 17 genomic regions were imputed on 19,862 cows with phenotypes and genotyped with either the BovineSNP50 (Illumina Inc.) or the EuroG10K BeadChip. We then tested the effect of each SNP in a mixed linear model including random polygenic effects estimated with a genomic relationship matrix. We confirm here the effects of candidate causative variants located in 17 functional candidate genes on both cheesemaking properties and milk composition traits. In each candidate gene, we identified the most plausible causative variant: 4 are missense in the *ALPL*, *SLC26A4*, *CSN3*, and *SCD* genes, 7 are located in 5'UTR (*AGPAT6*), 3' untranslated region (*GPT*), or upstream (*CSN1S1*, *CSN1S2*, *PAEP*, *DGAT1*, and

PICALM) regions, and 6 are located in introns of the *SLC37A1*, *MGST1*, *CSN2*, *BRI3BP*, *FASN*, and *ANKH* genes.

Key words: Montbéliarde, cheesemaking properties, milk composition, candidate variants

Short Communication

Cheesemaking properties (**CMP**) are strongly related to bovine milk composition (Wedholm et al., 2006). In the PhénoFinlait project, we predicted protein and fatty acid milk composition using mid-infrared (**MIR**) spectrometry in the French Holstein, Montbéliarde, and Normande dairy cattle breeds (Ferrand-Calmels et al., 2014; Sanchez et al., 2017a). Genome-wide association study (**GWAS**) on whole-genome sequences imputed using data of run 4 of the 1,000 bull genome project (Daetwyler et al., 2014) led to the identification of candidate causative mutations in 24 candidate genes (Boichard et al., 2014; Sanchez et al., 2017b). To validate these mutations in an independent population, we designed them on the custom part of the EuroG10K Beadchip (Illumina Inc., San Diego, CA). The independent population, consisting of Montbéliarde cows, originated from the FROM'MIR project that studied CMP and milk composition (proteins, fatty acids, and minerals) from MIR spectra. In the present study, we tested effects of candidate causative variants evidenced in the PhénoFinlait project on both CMP and milk composition traits predicted from MIR spectra in 19,862 FROM'MIR Montbéliarde cows.

A total of 416 milk samples (246 individual cows, 100 herds, and 70 dairy vats) were used to develop prediction equations for CMP. The sampling was designed to maximize genetic diversity as well as diversity in milk composition. All the samples, collected in Protected Designation of Origin and Protected Geographi-

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cal Indication cheese area of Franche-Comté (eastern France), were aliquoted and analyzed within 24 h by MIR spectroscopy using MilkoScan FT6000 (Foss, Hillerød, Denmark) and by CMP reference methods for soft cheese (**SC**) and pressed cooked cheese (**PCC**) parameters. Three laboratory cheese yield, 13 milk rennet coagulation [Formoptic; adapted from a mechanical Formagraph (Foss Electric, Hillerød, Denmark) by Chr. Hansen (Horsholm, Denmark) and ENILBio (Poligny, France)], and 8 milk acidification by lactic acid bacteria parameters were measured. Equations of predictions were developed for all the 24 CMP traits using partial least squares regression. Accuracies of prediction (R^2) ranged from 0.04 to 0.89 according to traits (El Jabri et al., 2017; Laithier et al., 2017; Sanchez et al., 2018). Only 9 CMP traits with medium to high prediction accuracies ($0.54 \leq R^2 \leq 0.89$) were retained for genetic analyses (See definitions of the traits and their elementary statistics in Table 1). The 3 following laboratory curd yields (**CY**): $CY_{\text{FRESH}} = 100 \times (\text{g of curd/g of milk})$, $CY_{\text{DM}} = 100 \times (1 - \text{g of DM whey/g of DM milk})$, and $CY_{\text{FAT-PROT}} = (\text{protein content} + \text{fat content}) \times (\text{g of milk/g of curd})$ were measured in

duplicate using the method of Hurtaud et al. (1993, 1995) that applies centrifugation ($2,700 \times g$, 15 min, 32°C); laboratory **CY** are therefore expected to be higher than actual **CY** obtained in dairy industry. Five coagulation traits measured with a Formoptic device adapted from a mechanical Formagraph (Foss) by Chr. Hansen and ENILBio assessing curd firming and rennet coagulation time (**K10** and **RCT**, respectively) or curd firmness [at **RCT** and at 2 times **RCT** (**a** and **a2**, respectively)]; **K10/RCT** were measured for both **SC** and **PCC**, whereas **a2** was measured for only the **SC**. Finally, we retained milk pH for **PCC**. Full details on reference measurements, MIR prediction equations as well as cheesemaking traits can be found in Sanchez et al. (2018).

Moreover, 15 milk composition traits (Table 1) were predicted from MIR spectra using equations of prediction previously developed in PhénoFinlait (Ferrand et al., 2012; Ferrand-Calmels et al., 2014; Sanchez et al., 2017a) and Optimir (Gengler et al., 2016) projects. These included 6 proteins (α -LA and β -LG whey proteins and α_{S1} -CN, α_{S2} -CN, β -CN and κ -CN), 4 fatty acids (total SFA, UFA, MUFA, and PUFA), and 5 min-

Table 1. Midinfrared predictions of cheesemaking properties and milk composition ($n = 1,442,371$), mean, SD, and CV, and performances of prediction equations, R^2 and root mean square error (RMSE), in a validation set

Trait		Mean	SD	CV	R^2_{val}	RMSE _{val}
Cheesemaking properties ¹						
CY_{FRESH}	$100 \times (\text{g of curd/g of milk}), \%$	38.2	7.2	18.9	0.86	2.78
CY_{DM}	$100 \times (\text{g of DM of curd/g of DM of milk}), \%$	67.1	4.8	7.2	0.89	1.67
$CY_{\text{FAT-PROT}}$	$(\text{g of milk fat} + \text{g of milk protein})/\text{kg of curd}, \text{g/kg}$	186.1	21.8	11.7	0.54	14.50
a_{PCC}	Curd firmness at rennet coagulation time (RCT), firm index (FI)	18.8	2.5	13.3	0.72	1.44
$K10/RCT_{\text{PCC}}$	Curd organization index standardized for RCT	0.40	0.09	24.7	0.62	0.06
a_{SC}	Curd firmness at RCT, FI	19.1	2.7	13.9	0.73	1.52
$a2_{\text{SC}}$	Curd firmness at $2 \times \text{RCT}$, FI	23.0	2.1	9.1	0.64	1.37
$K10/RCT_{\text{SC}}$	Curd organization index standardized for RCT	0.36	0.10	27.9	0.62	0.071
$\text{pH}_{0_{\text{PCC}}}$	Started value of pH	6.5	0.07	1.1	0.65	0.035
Milk protein composition, g/100 g of protein						
α -LA	α -lactalbumin	4.0	0.35	8.8	0.59	0.22
β -LG	β -lactoglobulin	12.3	1.6	13.2	0.74	0.82
α_{S1} -CN	α_{S1} -casein	32.3	0.27	0.8	0.88	0.094
α_{S2} -CN	α_{S2} -casein	9.7	0.34	3.5	0.82	0.14
β -CN	β -casein	29.8	1.1	3.7	0.92	0.31
κ -CN	κ -casein	8.7	0.43	5.0	0.8	0.19
Milk fatty acid composition, g/100 g fat						
SFA	Saturated fatty acids	70.0	7.1	10.1	0.995	0.37
MUFA	Monounsaturated fatty acids	26.9	5.4	20.1	0.97	0.95
UFA	Unsaturated fatty acids	30.5	5.2	17.1	0.98	0.70
PUFA	Polysaturated fatty acids	3.3	1.0	30.3	0.76	0.044
Milk mineral composition, mg/kg of milk						
Na	Sodium	340	46.2	13.6	0.44	34.6
Ca	Calcium	1,162	95.8	8.3	0.82	40.6
P	Phosphorous	1,008	81.0	8.0	0.75	40.5
Mg	Magnesium	100	7.4	7.3	0.77	3.55
K	Potassium	1,476	106.9	7.2	0.68	60.5

¹For pressed cooked cheese (PCC) and soft cheese (SC). CY = curd yield.

erals (Na, Ca, P, Mg, and K). Equations of prediction were applied on about 6 million MIR spectra collected from 330,000 Montbéliarde cows in the Franche-Comté region. Data from cows with at least 3 test-day records during the first lactation (1,442,371 test-day records from 189,817 cows) were adjusted for nongenetic effects using a mixed model. Herd $\times$ test-day $\times$ spectrometer and stage of lactation were included in this model as fixed effects, whereas animal genetic and permanent environment effects were assumed random. Data adjusted for fixed effects were then averaged per cow. A subset of 19,862 FROM-MIR cows were genotyped for the BovineSNP50 BeadChip (6,505 cows; Illumina Inc.) or for the customized low-density EuroG10K BeadChip (13,357 cows mainly for versions 1 to 5) for genomic selection purpose. All genotypes were imputed at 50K SNP density to the custom part SNP of version 7 of the EuroG10K BeadChip with FImpute software (Sargolzaei et al., 2014) using 177,736 cows genotyped for the BovineSNP50 or EuroG10K (versions 1 to 7) BeadChips. Mean squared correlations (R^2) between imputed and true genotypes reached 91.6% in a validation set for variants with minor allele frequency (MAF) $\geq 1\%$. Single-trait association analyses were performed between all the polymorphic variants with MAF $\geq 1\%$ (45,120 SNP) and the 24 traits (9 CMP and 15 milk composition traits). A mixed linear model was applied with the GCTA software (Yang et al., 2011), including a mean, the additive fixed effect of the candidate variant, and the random polygenic effects of animals estimated with the genomic relationship matrix calculated from the genotypes of the BovineSNP50 BeadChip. The SNP effect was considered significant if its $-\log_{10}(P)$ value estimated assuming a Student distribution was higher than 6 (5% threshold after Bonferroni correction, 0.05/45,120).

Significant effects were found on at least 1 of the 24 traits analyzed for 162 of the 194 candidate variants (MAF $\geq 1\%$) previously selected in the PhénoFinlait project for milk protein or fatty acid composition (Boichard et al., 2014; Sanchez et al., 2017b). We confirmed effects on both CMP and milk composition traits (proteins, fatty acids, and minerals) for 13 of the 14 candidate regions. We found the well-known regions of caseins [BTA6], *PAEP* (BTA11), or *DGAT1* (BTA14) genes as well as other regions on BTA1, 2, 4, 5, 17, 19, 20, 26, 27, and 29. In these regions, candidate variants of the EuroG10K custom chip were systematically more significant than the SNP of the BovineSNP50 BeadChip. We targeted 24 genes (1 to 5 per region) found to be the best candidates in the PhénoFinlait project (Table 2). For each gene, 1 to 33 candidate variants were present in the custom part of the EuroG10K

BeadChip (i.e., 245 in total). Among them, 162 with MAF higher than 1% had significant effects on at least 1 CMP or milk composition trait and 110 were ranked among the 10 most significant variants (top 10) for at least 1 of the traits analyzed. Effects of variants located in *BOP1* (7), *MROH1* (13), and *CYPB11* (1) genes on BTA14 were not tested because they had too low MAF (<0.01). Four other candidate genes could be excluded because all their polymorphisms had no significant effects (*ABCG2* and *DHX37*) or because significant variants were not located in the top 10 of the peak (*GPSM1* and *RECQL4*).

Seventeen candidate genes, each containing between 1 to 27 candidate variants, ranked in the top 10 of peaks, were kept for further investigation. Ranks of each variant were then examined in peaks for all traits to find the best candidate causative variant in each gene. In most of the genes, 1 variant, reported in Table 2, was ranked at the top of the peak for several traits analyzed in this study. Four of these variants were missense in *ALPL*, *SLC26A4*, *CSN3*, and *SCD* genes. In the *CSN3* gene, the candidate variant, located at 87,390,612 bp, is the missense variant responsible for the κ -CN A/B polymorphism. In the GWAS peaks, it was ranked first and second for 2 cheese yield traits (*CY_{FAT-PROT}* and *K10/RCT_{PCC}*, respectively) and first to eighteenth for protein contents (Table 3). In *SCD* on BTA26, we observed a missense variant at 21,144,708 bp that was previously found with effects on milk fatty acid composition (Li et al., 2016). In our study, it was the variant with the most significant effects for *CY_{FRESH}*, *CY_{DM}*, and α_{S1} - and α_{S2} -CN contents. We found 7 other candidate variants located in 5'UTR (*AGPAT6*), 3'UTR (*GPT*), or upstream (*CSN1S1*, *CSN1S2*, *PAEP*, *DGAT1*, and *PICALM*) regions. Finally, 6 variants were located in introns of the *SLC37A1*, *MGST1*, *CSN2*, *BRI3BP*, *FASN*, and *ANKH* genes. Surprisingly, polymorphisms previously found as causal variants in *PAEP* (Ganai et al., 2009) and *DGAT1* (Grisart et al., 2002) genes were not the most significant for any traits in our study. In each of these genes, we identified an upstream variant that was the best candidate. The *K232A* mutation in the *DGAT1* gene was excluded from the final results because it had an MAF lower than 1% in the Montbéliarde cows (0.9%). Nevertheless, effects associated with this mutation presented $-\log(P)$ values higher than those of the candidate variant located in the upstream region of the *DGAT1* gene (e.g., 143 against 125 for fat content). Thus, we cannot exclude that the mutation described by Grisart et al. (2002) is the causative variant for the effects observed in our study. In contrast, despite a high MAF in Montbéliarde cows for the 2 causal variants identified by Ganai et

Table 2. Best candidate variants (VAR) in 24 candidate genes (R^2 = imputation accuracy estimated in a validation population)

BTA	Gene	Total VAR ¹	VAR MAF ² >1%	Sign. VAR ³	Top 10 VAR ⁴	Best candidate VAR (bp)	Functional annotation	MAF	R ²
1	<i>SLC37A1</i>	14	13	12	10	144,398,764	Intronic	0.45	98.4
2	<i>ALPL</i>	8	8	7	7	131,812,821	Missense	0.38	95.8
4	<i>SLC26A4</i>	4	4	2	2	48,990,317	Missense	0.28	99.1
5	<i>MGST1</i>	26	22	19	7	93,945,738	Intronic	0.07	98.9
6	<i>ABCG2</i>	5	3	0	0	—	—	—	—
6	<i>CSN1S1</i>	11	9	9	4	87,141,456	Upstream	0.30	98.3
6	<i>CSN1S2</i>	10	7	7	4	87,261,372	Upstream	0.30	98.3
6	<i>CSN2</i>	14	13	10	8	87,187,426	Intronic	0.20	93.7
6	<i>CSN3</i>	21	18	17	10	87,390,612	Missense	0.40	95.6
11	<i>GPSM1</i>	3	3	3	0	—	—	—	—
11	<i>PAEP</i>	33	31	31	27	103,298,431	Upstream	0.45	1
14	<i>RECQL4</i>	3	3	3	0	—	—	—	—
14	<i>GPT</i>	2	2	2	2	1,623,927	3'UTR ⁵	0.48	96.3
14	<i>DGAT1</i>	9	1	1	1	1,795,176	Upstream	0.48	97.7
14	<i>BOP1</i>	7	0	0	0	—	—	—	—
14	<i>MROH1</i>	13	0	0	0	—	—	—	—
14	<i>CYP11B1</i>	1	0	0	0	—	—	—	—
17	<i>BRI3BP</i>	12	12	9	9	53,072,959	Intronic	0.06	98.5
17	<i>DHX37</i>	6	6	0	0	—	—	—	—
19	<i>FASN</i>	10	10	8	5	51,386,735	Intronic	0.37	94.7
20	<i>ANKH</i>	20	17	11	3	58,427,343	Intronic	0.07	98.8
26	<i>SCD</i>	6	6	5	5	21,144,708	Missense	0.46	96.2
27	<i>AGPAT6</i>	4	4	4	4	36,212,352	5'UTR	0.50	96.9
29	<i>PICALM</i>	3	2	2	2	9,611,304	Upstream	0.22	95.5

¹Number of total variants in the gene in the custom part of the EuroG10K BeadChip (Illumina Inc., San Diego, CA).

²Number of variants with minor allele frequency (MAF) ≥ 0.01 .

³Number of variants with MAF ≥ 0.01 and $-\log(P\text{-value}) \geq 6$.

⁴Number of variants ranked in the top 10 for at least 1 trait.

⁵UTR = untranslated region.

al. (2009) in the *PAEP* gene (45% for both variants located at 103,303,475 and 103,304,757 bp), they were never ranked at the top of the peak. Moreover, on average for all the traits we analyzed, the ranks of the 2 *PAEP* causal variant mutations were respectively 27.6 and 17.6, whereas it was 9.7 for the candidate mutation located at 103,298,431 bp in the upstream region of the *PAEP* gene. This latter variant could be located in a regulatory region of the *PAEP* gene and could modulate its expression.

Analyses of both CMP and milk composition traits show that variants with significant effects on CMP were also significant on milk protein, fatty acids, or mineral composition. This result confirms the genetic links, previously described via genetic correlations, between CMP and milk composition and in particular with protein composition (Wedholm et al., 2006). Moreover, considering all results together can help to establish the functional link existing between these traits and candidate genes. For example, the best candidate variant in the *SLC37A1* gene, which encodes a glucose-6-phosphate transporter, had significant effects on 3 CMP traits [$8 \leq -\log(P) \leq 30$] and 5 milk composition traits [$13 \leq -\log(P) \leq 167$] with the most significant effects obtained on phosphorous. Similarly, the best

candidate variant in the *ANKH* gene, encoding an inorganic pyrophosphate transport regulator that helps to prevent the deposition of Ca and P in bones, had significant effects on 5 CMP [$7 \leq -\log(P) \leq 46$] and 9 milk composition traits [$30 \leq -\log(P) \leq 175$], with the most significant effects found for α -LA that exhibit a high affinity to Ca. These 2 examples illustrate the interest to consider fine-scale phenotypes in complement to complex phenotypes, such as CMP.

We confirmed the effects of 13 genomic regions, previously identified on milk composition, on CMP and milk protein, fatty acids, and mineral composition predicted from MIR spectra in an independent population of 19,862 Montbéliarde cows. We showed that simultaneously analyzing fine-scale phenotypes and traits of interest can facilitate the identification of functional candidate genes. We reported candidate causative variants in 17 genes that have functional links with traits studied. To explore other genomic regions and to find other candidate variants, a GWAS will be performed on whole-genome sequence variants after imputations with the run 6 of the 1,000 bull genome project. Pleiotropic GWAS results will then be exploited to search for a set of interacting genes co-associated with CMP and milk composition.

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Table 3. Ranks of the 17 best candidate variants in the significant peaks identified for each milk cheesemaking and composition trait defined in Table 1

BTA	Gene	Location of the best candidate variant (bp)	Cheesemaking properties					Protein composition					Fatty acid composition					Mineral composition				
			CY _{FRESH}	CY _{DM}	CY _{FAT-PROT}	K10/ RCT _{POC}	K10/ RCT _{ISC}	a _{PPC}	α-LA	β-LG	α _{SI} ⁺ CN	β-CN	κ-CN	SFA	MUFA	UFA	PUFA	Na	Ca	P	Mg	K
1	<i>SLC37A1</i>	144,398,764			5			3			4							4		2	1	3
2	<i>ALPL</i>	131,812,821					2					5		4					2			
4	<i>SLC36A4</i>	48,990,317							1	1		1		1				1				
5	<i>MGST1</i>	93,945,738			5	18					5											
6	<i>CSN1S1</i>	87,141,456	32	26	47	37	42	51		19	28	5		26		1	1	1				
6	<i>CSN1S2</i>	87,261,372	2	3	28	3	4	5	3	58	50	66	50	38					9	8		40
6	<i>CSN2</i>	87,187,426	51	50	48	38	48	28	45	20	18	4	19					4				46
6	<i>CSN3</i>	87,390,612	31	28	1	11	2	50	13	5	2	1	18	3	2				4			41
11	<i>PAEP</i>	103,298,431	1	1	7	5	21	5	3	1	12	23	2	18	2	10						9
14	<i>GPT</i>	1,623,927			46	77	77			85	45	34				7	5	5	6			7
14	<i>DGAT1</i>	1,795,176												68	1	1	1	1		68	77	11
17	<i>BR13BP</i>	53,072,959				2		2					3	4	1							1
19	<i>FASN</i>	51,386,735														1	1	1	1			1
20	<i>ANKH</i>	58,427,343			2		1			1		1	2	1						1		1
26	<i>SCD</i>	21,144,708	1	1					1			1	1			12	11	12	15			2
27	<i>AGPAT6</i>	36,212,352	1	1	4											1	1	1	1			4
29	<i>PICALM</i>	9,611,304									2	2							2	2		

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