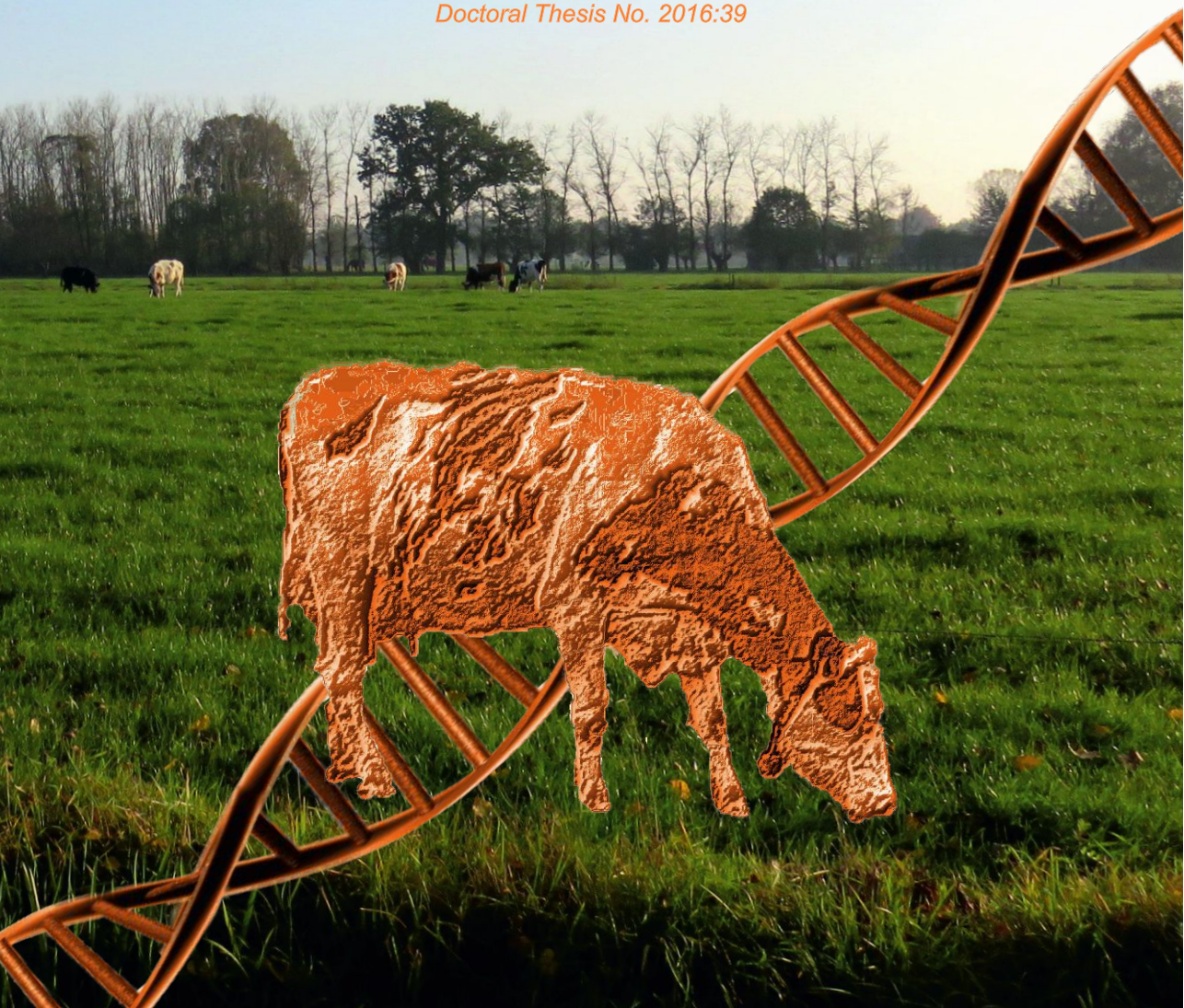
Mapping and fine-mapping of genetic factors affecting bovine milk composition

Sandrine Isolde Duchemin

Acta Universitatis Agriculturae Sueciae
Doctoral Thesis No. 2016:39



Propositions

1. Imputation is the limiting factor for detection of rare-variant quantitative trait loci in traditional genome-wide association studies.
(this thesis)

2. Good annotation of the cattle genome is crucial for gene discovery.
(this thesis)

3. The real CRISPR/Cas9 revolution is the editing of human somatic cells, not the editing of human germ-line cells.

4. Diseases in animals as dynamic events are best modelled, diagnosed and treated by veterinarians.

5. Women who accept a gender quota are in fact agreeing they are less than men.

6. In science sand grains from publications build up to mountains of knowledge.

Propositions belonging to the thesis, entitled:
"Mapping and fine-mapping of genetic factors affecting bovine milk composition"

Sandrine Isolde Duchemin
Wageningen, 30 May 2016

Mapping and fine-mapping of genetic factors affecting bovine milk composition

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The research presented in this doctoral thesis was conducted under the joint auspices of the Swedish University of Agricultural Sciences and the Graduate School Wageningen Institute of Animal Sciences of Wageningen University and is part of the Erasmus Mundus Joint Doctorate program “EGS-ABG".

Mapping and fine-mapping of genetic factors affecting bovine milk composition

Sandrine Isolde Duchemin

ACTA UNIVERSITATIS AGRICULTURAE SUECIAE
DOCTORAL THESIS № 2016:39

Thesis

submitted in fulfillment of the requirements for the degree of doctor from
Swedish University of Agricultural Sciences
by the authority of the Board of the Faculty of Veterinary Medicine and Animal Science and from
Wageningen University
by the authority of the Rector Magnificus, Prof. Dr. A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board of Wageningen University and the Board of the Faculty of Veterinary Medicine and Animal Science at the Swedish University of Agricultural Sciences
to be defended in public
on Monday May 30, 2016
at 4.00 p.m. in the Aula of Wageningen University

ISSN 1652-6880
ISBN (print version) 978-91-576-8580-3
ISBN (electronic version) 978-91-576-8581-0
ISBN 978-94-6257-730-5
DOI:10.18174/370103

Duchemin, S.I.
Mapping and fine-mapping of genetic factors affecting bovine milk composition.

Joint PhD thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden and Wageningen University, the Netherlands (2016)
With references, with summary in English

Abstract

Duchemin, S.I. (2016). Mapping and fine-mapping of genetic factors affecting bovine milk composition. Joint PhD thesis, between Swedish University of Agricultural Sciences, Sweden and Wageningen University, the Netherlands

Bovine milk is an important source of nutrients in Western diets. Unraveling the genetic background of bovine milk composition by finding genes associated with milk-fat composition and non-coagulation of milk were the main goals of this thesis. In ***Chapter 1***, a brief description of phenotypes and genotypes used throughout the thesis is given. In ***Chapter 2***, I calculated the genetic parameters for winter and summer milk-fat composition from ~2,000 Holstein-Friesian cows, and concluded that most of the fatty acids (**FA**) can be treated as genetically the same trait. The main differences between milk-fat composition between winter and summer milk samples are most likely due to differences in diets. In ***Chapter 3***, I performed genome-wide association studies (**GWAS**) with imputed 777,000 single nucleotide polymorphism (**SNP**) genotypes. I targeted a quantitative trait locus (**QTL**) region on Bos taurus autosome (**BTA**) 17 previously identified with 50,000 SNP genotypes, and identified a region covering 5 mega-base pairs on BTA17 that explained a large proportion of the genetic variation in de novo synthesized milk FA. In ***Chapter 4***, the availability of whole-genome sequences of keys ancestors of our population of cows allowed to fine-mapped BTA17 with imputed sequences. The resolution of the 5 mega base-pairs region substantially improved, which allowed the identification of the LA ribonucleoprotein domain family, member 1B (***LARP1B***) gene as the most likely candidate gene associated with de novo synthesized milk FA on BTA17. The LARP1B gene has not been associated with milk-fat composition before. In ***Chapter 5***, I explored the genetic background of non-coagulation of bovine milk. I performed a GWAS with 777,000 SNP genotypes in 382 Swedish Red cows, and identified a region covering 7 mega base-pairs on BTA18 strongly associated with non-coagulation of milk. This region was further characterized by means of fine-mapping with imputed sequences. In addition, haplotypes were built, genetically differentiated by means of a phylogenetic tree, and tested in phenotype-genotype association studies. As a result, I identified the vacuolar protein sorting 35 homolog, mRNA (***VPS35***) gene, as candidate. The VPS35 gene has not been associated to milk composition before. In ***Chapter 6***, the general discussion is presented. I start discussing the challenges with respect to high-density genotypes for gene discovery, and I continue discussing future possibilities to expand gene discovery studies, with which I propose some alternatives to identify causal variants underlying complex traits in cattle.

For my family

"Flatter me, and I may not believe you.
Criticize me, and I may not like you.
Ignore me, and I may not forgive you.
Encourage me, and I will not forget you.
Love me and I may be forced to love you."

William Arthur Ward, writer, 1921-1994.

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1

General Introduction

1.1 Milk

Milk has fascinated mankind since the beginning of the ages. A clear example of this fascination is the Milky Way galaxy, which contains our Planet Earth. The Milky Way galaxy has its roots in the Greek-Roman Mythology. The word galaxy originates from galas, which is a synonym for milk in Greek language. According to the Mythology, the Milky Way galaxy was "drops of milk" spelt by goddess Hera, when breastfeeding Hercules, the bastard son of Zeus (Larousse encyclopedia, 2015). "The origin of the Milky Way" has been immortalized by the renaissance artist Jacopo Tintoretto circa 1575-1580 (National Gallery, London, UK; Figure 1.1), and the "Birth of the Milky Way" by the Flemish artist Peter Paul Rubens in 1637 (Museo del Prado, Madrid, Spain). In many civilizations, the Milky Way galaxy has been used as a metaphor for a splash of milk in the dark skies of our Universe. Essentially, this metaphor is a way of expressing the importance of milk for mankind. It is so important that from the very beginning of life, an infant receives milk as the primary source of nutrients.

Figure 1.1 – "The origin of the Milky Way" by Jacopo Tintoretto circa 1575-1580 (exposed in the National Gallery, London, UK)

The fascination exerted by Universe on mankind is understandable. By contemplating stars, mankind loses notion of time allowing deeper lessons to be learnt. When G. Galilei (*in:* Galilei and Van Helden, 1989) first observed the Milky Way galaxy through his telescope in 1610, he discovered that it was formed by many smaller groups of stars. Following the steps of G. Galilei (*in:* Galilei and Van Helden, 1989) a deeper look into the splash of milk in the dark skies might give us insights

into the composition of milk. The splash might represent the fluid part of milk. The small groups of stars composing this splash might represent the main components in milk, such as proteins and fatty acids. The interstellar dust accompanying these stars might represent the minerals in milk. In just a few instants, the composition of milk is described as an (scientific) idea that has been transmitted throughout centuries by a simple metaphor.

Metaphors with our Universe do not stop at the Milky Way galaxy. Mankind named constellations after species of animals (e.g., Taurus, Aries, and Pisces), just like cave men have represented wild animals in their cave drawings. From stone-age to modern times, domestication of animals has been one of the drivers for men's transition from hunters to farmers. During this process, the role of cattle was undeniable. By domesticating cows, mankind preserved through time important resources, such as the genetic variation of bovine species. The preservation of this genetic variation has important consequences for the current technological development of mankind. It is so important that from the beginning of every life, genetic variation will determine the future of all species.

By using metaphors, such as Milky Way galaxy and names of constellations, mankind transmitted more than just a simple image from cave to modern men. As intrinsic parts of the Milky Way galaxy, cave and modern men would be united forever as one student. For mankind, these metaphors have engraved in our collective memories a deep respect for our Planet Earth and its scarce resources. Resources beyond genetic variation have been translated. In our modern times, this deep respect is taught by uniting human needs (milk as a nutrient) and animal resources (genes affecting bovine milk composition) through Animal Breeding and Genetics.

The scope of my thesis was to investigate the genetic background of bovine milk composition. More specifically, my thesis focuses on the composition of milk-fat, and on non-coagulation of milk.

1.2 Milk-fat composition

Bovine milk fat is an important source of energy for mankind. The main bioactive lipids in bovine milk are fatty acids (**FA**). According to Jensen (2002), bovine milk-fat is composed of more than 400 individual FA, most occurring in amounts less than 1%. The individual FA in bovine milk-fat are organized in chain of carbons that vary in length from 4 to 22 carbons. According to their chain-lengths, these individual FA

are grouped as short-chain (C4:0 – C12:0), medium-chain (C14:0 – C16:0) and long-chain (C18:0 – C22:0) FA. In addition, individual FA can be either saturated or unsaturated. FA are saturated when a carbon is connected by a single bond to an adjacent carbon in the chain, and FA are unsaturated when a carbon is connected to an adjacent carbon in the chain by double or triple bonds. Differences in FA regarding their saturation are shown in Figure 1.2.

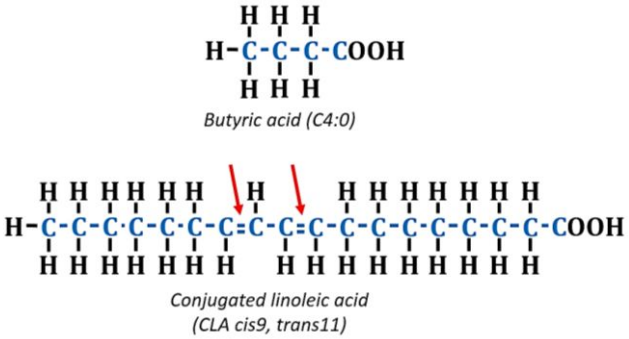

Figure 1.2 – Representation of fatty acids (FA). Butyric acid representing saturated FA, and conjugated linoleic acid representing unsaturated FA. Arrows in red point out the double bonds between adjacent carbons.

The biosynthesis of milk-fat occurs in the mammary gland of a cow. Individual FA in the mammary gland arise from circulating blood lipids and *de novo* synthesis. Circulating blood lipids originate from the feed of the cow or from the cow's body fat. Through the *de novo* synthesis, FA are elongated from precursors by adding C2:0. These precursors can be either acetate (C2:0), propionate (C3:0) or butyrate (C4:0). C2:0 and C3:0 originate from lipids in circulating blood, while C4:0 may either originate from blood lipids or the *de novo* synthesis itself (e.g., Craninx et al., 2008). Depending on the precursor, FA synthesized de novo may terminate at either C16:0 or C17:0. It is assumed that *de novo* synthesis produces the short-chain FA, C14:0 and 50% of C16:0 in milk, whereas the remaining 50% of C16:0 and the long-chain FA come from the lipids in circulating blood.

FA in bovine milk are relevant for human health. According to Calder et al. (2015), FA are essential for the well-being of humans, and they have important biological activities regarding the cell and tissue metabolism, as well as responsiveness to hormones and other signals in human cells. Stoop et al. (2008) indicated that FA in bovine milk are heritable, with heritability estimates between 0.22 and 0.71. These heritability estimates suggest that milk-fat composition can be improved by

breeding. In addition, Tzompa-Sosa et al. (2014) showed that increases in long-chain saturated FA can influence the thermal properties of milk-fat, which can lead to important changes in the quality of milk-fat derived products. Moreover, breeding could be used to reduce the concentration of certain FA in bovine milk-fat. For instance, low concentrations of C16:0 in bovine milk-fat would best meet infant requirements regarding the consumption of milk-fat derived products (e.g., Tzompa-Sosa et al., 2014). Therefore, increasing the biological knowledge regarding bovine milk-fat composition can be of great interest to the dairy industry.

1.3 Non-coagulation of milk

In addition to FA, bovine milk is an important source of proteins for mankind. The main proteins in bovine milk are the caseins, which account for almost 80% of the proteins in milk. There are four caseins in bovine milk: α_{s1}-,α_{s2}-, β-, and κ-casein. Most of these caseins are organized in micelles. These micelles are not soluble in water and can precipitate in the presence of rennet. This property is used in cheese production to induce coagulation of milk. In 2013, almost 30% of the total production of bovine milk in Sweden was destined to cheese production (LRF Dairy Sweden, 2015).

Besides the caseins, whey proteins account for the remaining 20% of the proteins in milk, of which β-lactoglobubin and α-lactalbumin are the most important ones. The whey proteins are considered by-products of cheese production. In contrast to caseins, whey proteins are soluble in water, and can only be denatured by heat. When heated, whey proteins can produce products such as ricotta and whey butter.

It is economically relevant for the cheese industry to reduce time and losses while producing cheese. In this sense, if caseins in bovine milk do not coagulate after rennet addition, the entire chain of cheese production is delayed, generating losses for this industry. Consequently, non-coagulation of milk can be considered as a new phenotype that accounts for the needs of the cheese industry. Non-coagulation (**NC**) of milk is prevalent among several dairy cattle breeds, such as Swedish Red, Finnish Ayrshire, Holstein-Friesian, and Italian Brown Swiss, to name a few (e.g., Frederiksen et al., 2011; Cecchinato et al., 2011, Gustavsson et al., 2014). The prevalence of NC milk varies among these breeds ranging from 4% in Italian Brown Swiss (Cecchinato et al., 2009) up to 13% in Finnish Ayrshires (Ikonen et al., 2004). A recent study reported the prevalence of NC milk at 18% in the Swedish Red cows (Gustavsson et al., 2014).

1.4 Genomic regions influencing bovine milk composition

Many genomic regions of the cattle genome have been associated with milk composition. While many of these genomic regions have not been studied in detail yet, some genes have been associated with milk-fat composition and non-coagulation of milk.

For bovine milk-fat composition, the main identified genes are: diacylglycerol O-acyltransferase 1 (*DGAT1*) located on *Bos taurus autosome* (**BTA**) 14, stearoyl-CoA desaturase 1 (*SCD1*) located on BTA26, acyl-CoA synthase short-chain family member 2 (*ACSS2*) located on BTA13, fatty acid synthase (*FASN*) located on BTA19, and 1-Acylglycerol-3-Phosphate O-Acyltransferase 6 (*AGPAT6*) located on BTA27. The association of the *DGAT1* and *SCD1* genes with milk-fat composition has been studied e.g., by Schennink et al. (2007, 2008). The association of the ACSS2, FASN and AGPAT6 genes with milk-fat composition has have been studied e.g., by Bouwman et al. (2011) and LittleJohn et al. (2014). The involvement of each of these genes occurs at different stages in the synthesis of milk-fat in the mammary gland of a cow: intracellular FA activation (*ACSS2*), fatty acid synthesis (*FASN*), unsaturation of FA (*SCD1*), and triacylglycerol synthesis (*AGPAT6*, *DGAT1*).

For bovine milk protein composition, the six major proteins in milk are encoded on the following chromosomes: α-lactalbumin on BTA5, the α_{s1}-,α_{s2}-, β-, and κ-caseins on BTA6, and β-lactoglobubin on BTA11. However, other chromosomal regions have been associated with milk protein composition (Schopen et al., 2011). These chromosomal regions encoding milk proteins seem to influence milk coagulation properties including non-coagulation of milk. Studies by Jensen et al. (2012) and by Gregersen et al. (2015) suggest that poor- and non-coagulation of milk are influenced by the milk protein variants of the k-casein gene. In contrast, study by Tyrisevä et al. (2008) and Gregersen et al. (2015) revealed that non-coagulation of milk can be influenced by other parts of the cattle genome too.

Promising genomic regions across the cattle genome in association with the desired trait can be identified with genetic markers. It is expected that associations with FA or non-coagulation of milk can be targeted to smaller chromosomal regions with sequences as compared to other panels of genetic markers, such as 50,000 (**50k**) and 777,000 (**777k**) single nucleotide polymorphism (**SNP**) markers. Sequences should contain all of the causal variants (Meuwissen and Goddard, 2010) that are believed

to be associated with the studied phenotype. The use of sequences for association studies has been enabled by the availability of an increasing number of sequenced animals (bulls and cows) from projects like the 1000Bull Genome Consortium (Daetwyler et al., 2014).

1.5 Aim and outline of this thesis

The present thesis aims at unraveling the genetic background of bovine milk composition by finding genes associated with milk-fat composition and non-coagulation of milk in targeted chromosomal regions. Throughout this thesis, there is a consistent increase in the number of genotypes analyzed, which have been useful to increase the resolution of some interesting genomic regions associated with bovine milk composition. In **Chapter 2**, we calculated the genetic correlations between the composition of bovine milk fat in winter and summer, and *DGAT1* and *SCD1* by season interactions. The conclusions of this work were further explored in Chapters 3 and 4. In **Chapter 3**, a quantitative trait locus on *Bos taurus autosome* (**BTA**) 17 explaining a large proportion of the genetic variation in de novo synthesized milk FA is mapped. In **Chapter 4**, we fine-mapped this QTL associated with de novo synthesized milk FA on BTA17 using imputed sequences. In **Chapter 5**, a similar fine-mapping methodology was used for the identification of a QTL on BTA18 associated with non-coagulation of milk in Swedish Red cows. In **Chapter 6**, challenges regarding the substantial increase in the number of genotypes used in this thesis, and the future possibilities to expand gene discovery are discussed.

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2

Genetic correlation between composition of bovine milk fat in winter and summer, and *DGAT1* and *SCD1* by season interactions

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Journal of Dairy Sciences (2013) 96:592-604

Abstract

Milk fat composition shows substantial seasonal variation, most of which is probably caused by differences in the feeding of dairy cows. The present study aimed to know whether milk fat composition in winter is genetically the same trait as milk fat composition in summer. For this purpose, we estimated heritabilities, genetic correlations, effects of acyl-CoA: diacylglycerol acyltransferase1 (***DGAT1***) K232A and stearoyl-CoA desaturase1 (***SCD1***) A293V polymorphisms for milk fat composition in winter and summer, and tested for genotype by season interactions of *DGAT1* K232A and *SCD1* A293V polymorphisms. Milk samples were obtained from 2,001 first lactation Dutch Holstein Friesian cows, most of which with records in both winter and summer. Summer milk contained higher amounts of unsaturated fatty acids (**FA**) and lower amounts of saturated FA compared to winter milk. Heritability estimates were comparable between seasons: moderate to high for short and medium chain FA (0.33 to 0.74) and moderate for long chain FA (0.19 to 0.43) in both seasons. Genetic correlations between winter and summer milk were high, indicating that milk fat composition in winter and in summer can largely be considered as genetically the same trait. *DGAT1* K232A and *SCD1* A293V polymorphisms effects were similar across seasons for most FA. *DGAT1* 232A allele in winter as well as in summer milk samples was negatively associated with most FA with less than 18 carbons, SFA, SFA to UFA, and C10 to C16 unsaturation indices, and was positively associated with C14:0, unsaturated C18, UFA, and C18 and CLA unsaturation indices. *SCD1* 293V allele in winter as well as in summer milk samples was negatively associated with C18:0, C10:1 to C14:1cis-9, C18:1trans-11, and C10 to C14 unsaturation indices, and positively associated with C8:0 to C14:0, C16:1cis-9, and C16 to CLA unsaturation indices. In addition, significant *DGAT1* K232A by season interaction was found for some FA and *SCD1* A293V by season interaction was only found for C18:1trans-11. These interactions were due to scaling of genotype effects.

Key words: genetic correlation, seasonal variation, *DGAT1*, *SCD1*

2.1 Introduction

Milk is an important source of lipids, proteins, vitamins and minerals in many Western human diets. Among the milk produced by the main dairy species (e.g., cows, goats and sheep), bovine milk is economically the most important. Bovine milk fat contains essential nutrients including fat soluble vitamins and bio-active lipids (German & Dillard, 2006) and is pointed out by FAO (2008) as being the main source of saturated fatty acids (**SFA**) in human diets.

Genetic factors can influence milk fat composition, and its genetic variation has been reported in previous studies (e.g., Soyeurt et al., 2006; Schennink et al., 2007). Stoop et al. (2008) concluded that short and medium chain fatty acids (**FA**) synthesized de novo are more affected by genetic factors than long chain FA that originate from the cow's diet or from mobilization of body fat (Chilliard et al., 2000; Palmquist, 2006). Moreover, polymorphisms in *DGAT1* and *SCD1* genes have been recognized as having large effects on milk fat composition (Moioli et al., 2007; Schennink et al., 2007; 2008).

In addition, nutrition of dairy cows can considerably alter milk fat composition (e.g., Palmquist et al., 1993; Lock & Bauman, 2004; Chilliard et al., 2007). It is well established that feeding dairy cows with polyunsaturated fatty acids (**PUFA**) that originate from forages results in a reduction of de novo synthesized FA and in an increase of long chain FA in milk fat (e.g., Chilliard et al., 2001; Bauman and Griinari, 2003). Furthermore, there are indications that nutrition affects mammary lipogenic gene expression (Bernard et al., 2008; Mach et al., 2011).

Substantial seasonal variation in milk fat composition has been found in European countries (Precht and Molketin, 2000; Thorsdottir et al., 2004; Heck et al., 2009). The main cause for this seasonal variation seems to be the differences in diets: in winter cows in Northern Europe are usually kept inside and fed silage whereas in summer cows are mainly on pasture and fed with fresh grass. These considerable differences in diets might affect the genetic background of milk fat composition. However, at present no information is available of possible genotype by season interaction on milk fat composition. Therefore, our aim was to study whether winter milk fat composition is genetically the same trait as summer milk fat composition. For this purpose, we estimated heritabilities, genetic correlations, effects of *DGAT1* K232A and *SCD1* A293V polymorphisms for milk fat composition in winter and summer, and

tested for genotype by season interactions of *DGAT1* K232A and *SCD1* A293V polymorphisms.

2.2 Materials and methods

This study is part of the Dutch Milk Genomics Initiative, which was initiated to identify opportunities to change milk composition through breeding. Based on data collected in this project, heritability estimates for milk fat composition based on winter milk samples have been published by Stoop et al. (2008) and effects of polymorphisms in the *DGAT1* and *SCD1* genes on milk fat composition based on winter samples have been published by Schennink et al. (2007; 2008). In the present study, heritability estimates for milk fat composition in winter and summer were obtained using a bivariate approach. Furthermore, to test whether winter milk fat composition is genetically the same trait as summer milk fat composition, we estimated genetic correlations between milk fat composition in winter and summer and, more specifically, we tested for *DGAT1* and *SCD1* by season interactions.

2.2.1 Animals

Data were available on 2,001 first lactation Holstein Friesian cows from 398 commercial herds in the Netherlands. Winter records were available from 1,905 cows, with each cow between 63 and 282 days in lactation. Summer records were available from 1,795 cows, with each cow between 97 and 335 days in lactation. A total of 1,699 cows had both a winter and a summer record, 206 animals had only a winter milk sample and 96 animals had only a summer sample. Details about the experimental design can be found in Stoop et al. (2008). In total 3,700 records on milk fat composition were available.

2.2.2 Phenotypes

One milk sample of 500 mL per cow per season was collected during morning milking between February and March 2005 (“winter”) and between May and June 2005 (“summer”). Sample bottles contained sodium azide (0.03 w/w%) for conservation. Fat percentage (**fat%**) was measured by infrared spectroscopy using a MilkoScan FT6000 (Foss Electric, Hillerod, Denmark) at the Milk Control Station (Qlip, Zutphen, the Netherlands). Milk fat composition was measured by gas chromatography (**GC**) at the COKZ laboratory (Qlip, Leudsen, the Netherlands), as described by Schennink et al. (2007). The fatty acids were identified and quantified by comparing the methyl ester chromatograms of the milk fat samples with the chromatograms of pure FA

methyl ester standards (Stoop et al., 2008), and were measured as weight proportion of total fat (%w/w). In this study, results are shown for individual FA: C4:0 to C18:0, C10:1 to C18:1*cis-9*, C18:1*trans-11*, C18:2*cis-9,trans-11* (**CLA**), C18:2*cis-9,12* and C18:3*cis-9,12,15*. For C10:1 and C12:1, it could not be ascertained, if the *cis*-double bond occurred at the carbon 9 position. Because of coelution associated with the GC extraction method, C14:1*cis-9* represents the sum of C14:1*cis-9* and C15:0*iso,* and C18:1*cis-9* represents the sum of C18:1*cis-9* and C18:1*trans-12*. The groups of saturated FA (**SFA**), unsaturated FA (**UFA**) and the ratio SFA to UFA are described in Table 2.1. SFA and UFA sum to approximately 94 % w/w of total fat.

Table 2.1 - Trait definition: groups of fatty acids

Group	Content
SFA	C4:0, C5:0, C6:0, C7:0, C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0 and C18:0.
UFA	C10:1, C12:1, C14:1*cis-9*[1], C16:1*cis-9*, C18:1*trans-4-8*[2], C18:1*trans-9*, C18:1*trans-11*, C18:1*cis-9*[3], C18:1*cis-11*, C18:2*cis-9,12*, C18:2*cis-9,trans-11* (CLA) and C18:3*cis-9,12,15*.
SFA to UFA	saturated to unsaturated FA ratio.

[1]C14:1cis-9 due to coelution associated with the GC extraction method represents the sum of C14:1cis-9 and C15iso.
[2]C18:1trans-4-8 due to coelution associated with the GC extraction method represent the sum of C18:1trans-4, C18:1trans-5, C18:1trans-6, C18:1trans-7 and C18:1trans-8.
[3]C18:1cis-9 due to coelution associated with the GC extraction method represents the sum of C18:1cis-9 and C18:1trans-12.

Fatty acid unsaturation indices were defined as described by Kelsey et al. (2003):

$$\frac{unsaturated\ cis{-}9}{unsaturated\ cis{-}9{+}saturated} * 100, \text{ e.g., } C14index = \frac{c14{:}1\ cis{-}9}{c14{:}1\ cis{-}9{+}c14{:}0} * 100$$

Indices were calculated for the following product and substrate pairs: C10:1 and C10:0 (**C10index**); C12:1 and C12:0 (**C12index**); C14:1*cis-9* and C14:0 (**C14index**); C16:1*cis-9* and C16:0 (**C16index**); C18:1*cis-9* and C18:0 (**C18index**); CLA and C18:1*trans-11* (**CLAindex**).

2.2.3 Genotypes

Blood samples for DNA isolation were collected between April and June 2005. Genotyping of the *DGAT1* K232A polymorphism was performed with a TaqMan® allelic discrimination assay (Applied Biosystems, Foster city, CA), according to Schennink et al. (2007). For the *DGAT1* K232A polymorphism 1,692 animals were

genotyped, whereas for 103 animals no genotypes were available either because no DNA was available (N = 92) or because the genotyping was ambiguous (N = 11). Genotypes for the *SCD1* A293V polymorphism were assayed with the SNaPshot® single base primer extension method (Applied Biosystems, Foster city, CA), according to Schennink et al. (2008). For the *SCD1* A293V polymorphism 1,637 animals were genotyped, whereas for 158 animals no genotypes were available either because no DNA was available (N = 92) or the sample was genotyped ambiguously (N = 66).

2.2.4 Statistical Analyses

Variance and covariance components were estimated by bivariate analyses between a trait in winter and the same trait in summer milk samples using an animal model in ASReml (Gilmour et al., 2002), as described by Stoop et al. (2008):

$$y_{ijklmn} = \mu + b_1 * dim_{ijklmn} + b_2 * e^{-0.05*dim_{ijklmn}} + b_3 * afc_{ijklmn} + b_4 * afc^2_{ijklmn} + season_k + scode_l + herd_m + a_n + e_{ijklmn} \quad [1]$$

where y_{ijklmn} is the dependent variable; μ is the overall mean; b_1 and b_2 are the regression coefficients relative to dim_{ijklmn}; dim_{ijklmn} is the covariate describing the effect of days in milk, modeled with a Wilmink curve (Wilmink, 1987); b_3 and b_4 are the regression coefficients relative to afc_{ijklmn}; afc_{ijklmn} is the covariate describing the effect of age at first calving; $season_k$ is the fixed effect of calving season (June – August 2004, September – November 2004, or December 2004 – February 2005); $scode_l$ is the fixed effect accounting for differences in genetic level between groups of proven bull daughters and young bull daughters; $herd_m$ is the random effect of herd; a_n is the random additive genetic effect of animal; and e_{ijklmn} is the random residual effect.

The variance-covariance structure of [1] was defined as: $Var(a_n) = \mathbf{A}\sigma_a^2$, where **A** is the matrix of additive genetic relationships between individuals and σ_a^2 is the additive genetic variance; $Var(herd_m) = \mathbf{I}\sigma_{herd}^2$, where **I** is the identity matrix and σ_{herd}^2 is the herd variance and $Var(e_i) = \mathbf{I}\sigma_e^2$, where *I* is the identity matrix and σ_e^2 is the residual variance.

Intraherd heritability was calculated (Heringstad et al., 2006) to make heritability estimates comparable with other studies that considered the effect of herd as fixed, and was defined as: $h^2 = \frac{\sigma_a^2}{\sigma_a^2+\sigma_e^2}$

The fraction of variance due to herd reflects the relative importance of herd effects such as feed and management practices, and was defined as: $herd = \frac{\sigma^2_{herd}}{\sigma^2_a+\sigma^2_{herd}+\sigma^2_e}$.

Phenotypic, genetic, herd and residual correlations between a trait in winter and the same trait in summer milk samples were calculated as: $r = \frac{\sigma_{Tw,Ts}}{\sqrt{(\sigma^2_{Tw}*\sigma^2_{Ts})}}$, where $\sigma_{Tw,Ts}$ = covariance between the same trait measured in winter and summer milk samples; σ^2_{Tw} = variance of the trait in winter samples and σ^2_{Ts}= variance of the trait in summer samples. The genetic correlation between a trait measured in two different environments can be used to assess genotype by environment interaction (e.g. Falconer and Mackay, 1996). We followed this approach to assess whether milk fat composition in winter and summer milk is genetically the same trait. Significance of genetic correlations was based on the likelihood ratio test, in which the likelihood of the full model was compared to the likelihood of a model with restricted genetic correlation of 0.995. A value of 0.995 was chosen because restricting the genetic correlation to 1 leads to singularity. Significance of the likelihood ratio test was based on a Chi-Square distribution with one degree of freedom.

Model [1] was extended with a fixed genotype effect to estimate effects of *DGAT1* (KK, KA or AA genotypes) or *SCD1* (AA, AV or VV genotypes), and to estimate *DGAT1* or *SCD1* by season interactions. Animals with missing genotypes were assigned to a separate genotype class. Missing genotypes appeared to be randomly distributed across other effects in the model.

2.3 Results

2.3.1 Milk-fat composition in winter and summer

Phenotypic means for fat composition in winter and summer milk samples are shown in Table 2.2. In summer milk, short chain FA (C4:0 to C12:0) contributed 13.67% to total fat, medium chain FA (C14:0 and C16:0) contributed 40.32% and C18:0 contributed 9.88%. Among the unsaturated C18 FA, the largest fraction was C18:1*cis-9* (20.56%). Fat% was slightly higher in winter (4.36) as compared to summer milk (4.26; P=2.4e-5). The largest differences in summer compared to winter milk were a 3.42%w/w decrease in C16:0 (P<0.001), a 2.38%w/w increase in C18:1*cis-9* (P<0.001) and a 1.16%w/w increase in C18:0 (P<0.001). Furthermore, relatively large increases could also be seen for C18:1*trans-11* (+0.45%w/w), CLA (+0.17%w/w) and C18:3*cis-9,12,15* (+0.07%w/w; P<0.001). In addition, a 3.39%w/w decrease in SFA and a

3.00%w/w increase in UFA were observed (P<0.001). Among unsaturation indices, increases for C14index (+0.49%w/w) and C16index (+0.37%w/w), and a decrease in CLAindex (2.10%w/w) were seen in summer compared to winter milk (P<0.001). Standard deviations of unadjusted FA were on average 20% larger in summer than in winter milk.

2.3.2 Heritability estimates and variance components

Heritability (h^2), the fraction of variance due to herd ($herd$), and the ratios of phenotypic, genetic and herd variances for milk fat composition in winter and summer are shown in Table 2.3. In winter milk, moderate to high heritability estimates were found for fat%, short chain FA (C4:0 to C12:0), medium chain FA (C14:0 and C16:0), C12:1, C16:1*cis-9*, CLA, and C12 to C18 unsaturation indices. In summer milk, moderate to high heritability estimates were found for fat%, short chain FA (C4:0 to C12:0), medium chain FA (C14:0 and C16:0), C10:1 to C18:1*cis-9,* and C10 to C14 unsaturation indices. In general, heritability estimates for winter and summer milk were very similar.

Fraction of variance due to herd ($herd$) in winter milk was moderate to high for C12:0, and most unsaturated C18 FA. $Herd$ in summer milk was moderate to high for C12:0, C16:0, unsaturated C18 FA, and groups of FA. In general, $herd$ was higher in summer compared to winter milk for most FA, groups of FA, and all unsaturation indices.

Differences in h^2 and $herd$ for milk fat composition between winter and summer can either be the result of changes in additive genetic, herd or residual variance. Therefore, we also compared the magnitude of the individual variance components in winter and in summer milk. In summer, σ_a^2 was considerably higher for C18:1*trans-11* and CLA compared to winter milk. For most FA, σ_{herd}^2 was substantially higher in summer compared to winter milk, especially for C18:1*trans-11*, CLA, and SFA.

2.3.3 Correlations between milk-fat composition in winter and summer

The phenotypic, genetic, herd and residual correlations between winter and summer milk fat composition are shown in Table 2.4. The phenotypic correlations ranged from 0.29 for C18:1*trans-11* to 0.69 for C18:2*cis-9,12* and C14index, indicating that phenotypic correlation between winter and summer milk for individual FA is in the same order of magnitude as the phenotypic correlation for fat% (0.63). Genetic

correlations were higher than 0.90 for most FA and unsaturation indices. For C8:0 (0.93), C10:0 (0.95), C14:0 (0.94), C16:0 (0.76), C18:1*trans-11* (0.70), CLA (0.80), C18:3*cis-9,12,15* (0.79), SFA (0.77), UFA (0.82) and SFA to UFA (0.79), genetic correlations were significantly different from 1 (P<0.05). Herd correlations were lower than 0.42 (C6:0) for most FA, groups of FA and unsaturation indices, except for herd correlations of 0.54 for C12:0 and 0.76 for C18:2*cis-9,12*.

2.3.4 *DGAT1* effects on milk-fat composition

Estimated effects for *DGAT1* K232A polymorphism on milk fat composition in winter and summer milk samples are shown in Table 2.5. The 232A allele was associated with lower fat% in both winter and summer milk. In winter as well as in summer milk, the 232A allele was negatively associated with most FA with less than 18 carbons, SFA, SFA to UFA, and C10 to C16 unsaturation indices, and was positively associated with C14:0, unsaturated C18, UFA, and C18 and CLA unsaturation indices. In general, effects of *DGAT1* K232A polymorphism were very similar in winter and in summer milk.

Significant *DGAT1* by season interaction was found for C4:0 to C14:0, C16:1*cis-9,* C18:1*cis-9*, CLA, C18:3*cis-9,12,15,* SFA, UFA, and C14 and C16 unsaturation indices (P ≤ 0.05). Significant *DGAT1* by season interactions seem to be due to scaling rather than re-ranking: genotype effects in both seasons were in the same direction but of a different magnitude. Figure 2.1 shows an example of scaling of the genotype effects on C18:1*cis-9.*

2.3.5 *SCD1* effects on milk-fat composition

Estimated effects for *SCD1* A293V polymorphism on milk fat composition in winter and summer milk samples are shown in Table 2.6. *SCD1* A293V polymorphism had no significant effects on fat% in winter as well as in summer milk. In winter milk, the 293V allele was negatively associated with C18:0, C10:1 to C14:1*cis*-9, C18:1*trans-11*, C18:3*cis-9,12,15*, and C10 to C14 unsaturation indices, and positively associated with C8:0 to C14:0, C16:1*cis-9*, CLA*,* and C16 to CLA unsaturation indices. In summer milk, the 293V allele was negatively associated with C18:0, C10:1 to C14:1*cis*-9, C18:1*trans-11*, CLA, and C10 to C14 unsaturation indices, and positively associated with C8:0 to C14:0, C16:1*cis-9*, C18:3*cis-9,12,15,* and C16 to CLA unsaturation indices. In general, effects of *SCD1* A293V polymorphism were very similar in winter and in summer milk. Significant *SCD1* by season interaction was found only for C18:1*trans-11* (P = 0.03). The 293V allele was negatively associated with C18:1*trans-11* and this negative effect was larger in summer than in winter milk (Figure 2.2).

2.4 Discussion

Heritability estimates for fat composition in winter and summer milk were very similar, and estimates of winter milk are comparable with results published by Stoop et al. (2008) , which are based on univariate analyses. Intraherd heritability estimates in our study are higher than estimates reported by others (Renner and Kosmack, 1974; Karijord et al., 1982, Soyeurt et al., 2008). This might be because these studies used different methods to measure FA, or studied different breeds.

Genetic correlations between winter and summer milk were high for all FA, indicating that milk fat composition in winter and in summer can be largely considered as genetically the same trait. Effects of *DGAT1* K232A and *SCD1* A293V polymorphisms on milk fat composition in winter and in summer were similar and their effects in summer milk confirm the results of Schennink et al. (2007; 2008) for winter milk. The results also showed several differences between winter and summer milk, which will be discussed in more detail.

2.4.1 Effects of season on milk-fat composition

Summer milk contained larger proportions of C18:0 and unsaturated C18, and smaller proportions of short and medium chain FA compared to winter milk, which is in agreement with literature (Palmquist et al., 1993; Soyeurt et al., 2008; Heck et al., 2009). Differences between winter and summer milk fat in our study could be partly due to differences in lactation stage, as cows in summer were on average 80 days later in lactation than in winter (247 versus 167 days). Effects of lactation stage were accounted for in the statistical analysis and are known to be relatively small (Kelsey et al., 2003; Stoop et al., 2008). Therefore, we expect that it has not influenced our results.

Table 2.2 - Phenotypic mean ± standard deviation for fat%, individual fatty acids, groups of fatty acids and unsaturation indices based on 1,905 winter milk samples and 1,795 summer milk samples.

Trait	Winter[1]	Summer	-Log (P)[2]
Milk production trait			
Fat %	4.36±0.70	4.26±0.73	4.6***
Individual fatty acids[3]			
C4:0	3.50±0.27	3.52±0.35	1.3^{ns}
C6:0	2.22±0.17	2.17±0.21	15.0***
C8:0	1.37±0.14	1.32±0.17	22.0***
C10:0	3.03±0.43	2.87±0.46	26.6***
C12:0	4.11±0.69	3.79±0.73	40.9***
C14:0	11.61±0.92	11.15±1.06	43.2***
C16:0	32.59±2.83	29.17±3.50	203.8***
C18:0	8.72±1.42	9.88±1.77	99.3***
C10:1	0.37±0.07	0.35±0.07	17.7***
C12:1	0.12±0.03	0.11±0.03	23.7***
C14:1*cis-9*	1.36±0.26	1.38±0.28	1.6*
C16:1*cis-9*	1.45±0.32	1.40±0.30	6.0***
C18:1*cis-9*	18.18±2.04	20.56±2.80	170.4***
C18:1*trans-11*	0.78±0.22	1.23±0.61	174.3***
C18:2*cis-9,trans-11* (CLA)	0.39±0.11	0.56±0.28	120.4***
C18:2*cis-9,12*	1.20±0.29	1.12±0.25	16.7***
C18:3*cis-9,12,15*	0.42±0.11	0.49±0.16	59.8***
Groups of fatty acids[3]			
SFA	69.08±2.80	65.69±4.02	162.1***
UFA	25.03±2.42	28.03±3.39	158.5***
SFA / UFA	2.79±0.37	2.39±0.43	159.7***
Unsaturation indices[4]			
C10 index	10.89±1.91	11.00±1.82	1.1^{ns}
C12 index	2.74±0.54	2.76±0.56	0.6^{ns}
C14 index	10.51±1.84	11.00±1.84	15.1***
C16 index	4.24±0.82	4.61±0.92	36.4***
C18 index	67.62±3.74	67.60±3.89	0.1^{ns}
CLA index	33.72±4.06	31.62±3.96	57.0***

[1]Data based on winter milk samples for fat%, C4:0 to C18:0, C18:1cis-9, C18:1trans-11, CLA, C18:2cis-9,12, C18:3cis-9,12,15, and SFA to UFA have been published by Stoop et al. (2008).
[2]Significance levels were assessed by a t-test considering winter and summer milk samples as independent traits, and -Log(P) represent the –Log(P-values) of the difference between seasons, where **P-value < 0.001, **P-value < 0.01, * P-value ≤ 0.05 and ns = non-significant, i.e., P > 0.05.
[3]Expressed in % w/w.
[4]Unsaturation indices calculated as unsaturated/(unsaturated + saturated)x100.

Table 2.3 - Heritability (h^2), fraction of variance due to herd ($herd$), phenotypic(σ_p^2), genetic(σ_a^2) and herd(σ_{herd}^2) variances and ratios of phenotypic, genetic and herd variances for fat%, individual fatty acids, groups of fatty acids and unsaturation indices based on 1,905 winter milk samples and 1,795 summer milk samples

Trait	h^2 winter[1]	h^2 summer[1]	$herd$ winter[2]	$herd$ summer[2]	σ_p^2 summer[3]	σ_a^2 summer	σ_{herd}^2 summer	σ_p^2summer/ σ_p^2winter[3]	σ_a^2summer/ σ_a^2winter	σ_{herd}^2 summer/ σ_{herd}^2winter
Milk production trait										
Fat %	0.57	0.63	0.06	0.11	0.58	0.33	0.06	1.12	1.16	1.92
Individual fatty acids										
C4:0	0.43	0.38	0.16	0.24	0.13	0.04	0.03	1.63	1.29	2.39
C6:0	0.48	0.41	0.16	0.18	0.04	0.01	0.01	1.56	1.29	1.80
C8:0	0.62	0.41	0.20	0.19	0.03	0.01	0.01	1.42	0.96	1.35
C10:0	0.74	0.55	0.23	0.19	0.22	0.10	0.04	1.11	0.88	0.90
C12:0	0.64	0.51	0.43	0.40	0.55	0.17	0.22	1.10	1.16	1.92
C14:0	0.58	0.51	0.17	0.34	1.15	0.39	0.39	1.29	0.90	2.55
C16:0	0.37	0.36	0.30	0.51	12.40	2.23	6.28	1.51	1.06	2.58
C18:0	0.24	0.19	0.19	0.30	3.15	0.41	0.95	1.59	1.07	2.56
C10:1	0.33	0.47	0.10	0.25	5.11E-3	1.80E-3	1.29E-3	1.15	1.36	2.87
C12:1	0.37	0.48	0.21	0.30	0.95E-3	0.32E-3	0.29E-3	1.21	1.39	1.77
C14:1cis-9	0.33	0.46	0.07	0.15	0.08	0.03	0.01	1.23	1.54	2.72
C16:1cis-9	0.42	0.39	0.07	0.09	0.09	0.03	0.01	0.90	0.80	1.29
C18:1cis-9	0.27	0.37	0.29	0.35	7.79	1.88	2.69	1.86	2.30	2.26
C18:1trans-11	0.29	0.20	0.58	0.64	0.38	0.03	0.25	8.28	4.91	9.10
C18:2cis-9,trans11(CLA)	0.43	0.28	0.51	0.58	0.08	0.01	0.05	6.09	3.32	7.02
C18:2cis-9,12	0.20	0.23	0.50	0.57	0.07	0.01	0.04	0.82	0.84	0.93
C18:3cis-9,12,15	0.26	0.22	0.64	0.63	25.94E-3	2.15E-3	16.30E-3	2.19	1.96	2.14

(continuation)

Trait	h^2 winter[1]	h^2 summer[1]	$herd$ winter[2]	$herd$ summer[2]	σ_p^2 summer[3]	σ_a^2 summer	σ_{herd}^2 summer	σ_p^2summer/ σ_p^2winter[3]	σ_a^2summer/ σ_a^2winter	σ_{herd}^2 summer/ σ_{herd}^2winter
Groups of fatty acids										
SFA	0.30	0.34	0.29	0.44	15.88	3.06	6.94	2.00	1.83	3.02
UFA	0.30	0.32	0.29	0.40	11.34	2.20	4.55	1.93	1.78	2.66
SFA to UFA	0.29	0.31	0.29	0.42	0.18	0.03	0.08	1.33	1.14	1.91
Unsaturation indices										
C10 index	0.31	0.43	0.06	0.13	3.29	1.22	0.44	0.94	1.21	1.98
C12 index	0.36	0.51	0.06	0.15	0.31	0.14	0.05	1.12	1.44	2.82
C14 index	0.44	0.52	0.06	0.07	3.36	1.64	0.22	1.05	1.25	1.08
C16 index	0.48	0.33	0.06	0.13	0.89	0.26	0.12	1.28	0.83	2.68
C18 index	0.35	0.31	0.06	0.11	15.38	4.18	1.72	1.09	0.89	2.17
CLA index	0.26	0.25	0.08	0.17	16.03	3.39	2.69	0.96	0.85	2.00

[1] $h^2 = \sigma_a^2/(\sigma_a^2+\sigma_e^2)$. Standard errors between 0.01 and 0.12

[2] $herd = \sigma_{herd}^2/(\sigma_a^2+\sigma_{herd}^2+\sigma_e^2)$. Standard errors between 0.02 and 0.08

[3] $\sigma_p^2 = \sigma_a^2+\sigma_{herd}^2+\sigma_e^2$.

Seasonal variation in milk fat composition seems to be the result of pasture grazing of dairy cows in summer compared to winter (Precht and Molketin, 2000; Thorsdottir et al., 2004). Grazing or availability of fresh cut grass in summer will result in a different dietary supply of FA, because fresh cut grass contains more PUFA than conserved forages which are affected by decreases in the leaf/stem ratio during the maturation period (Dewhurst et al., 2001). It is well known that supply of PUFA through the diet of dairy cows decreases *de novo* synthesized FA and increases long chain FA in milk fat (e.g., Chilliard et al., 2001; Agenas et al, 2002; Bernard et al, 2008). Therefore, our observation that summer milk had higher amounts of long chain FA and lower amounts of *de novo* synthesized FA compared to winter milk is probably because about 50% of the cows in our experiment had access to pasture in summer (3.5 to 24 hours/day), whereas all cows were kept indoors in winter.

Differences in dietary supply of FA between winter and summer are also reflected by our relatively low herd correlations between milk fat composition in winter and summer milk. This suggests that effect of herd, of which diet is part, on milk fat composition is not constant over the year. This might be related to the considerably higher herd variances in summer compared to winter milk found in our results. Variation due to herd might be due to several factors, however, differences in feeding regimes between and within herds play a major role. Larger herd variances in summer are most likely due to larger differences in feeding strategies between herds as well as within a herd: apparently the quantity and composition of forages, either fresh or conserved, varies more between herds and within a herd in summer compared to winter.

In contrast, herd correlations found in our study for C12:0 and for C18:2*cis-9,12* were higher than for other FA, probably because the supply of these FA on a herd were relatively constant during the year. Most concentrate feed supplied to Dutch dairy cows have high concentration of C12:0, due to the presence of ingredients such as palm kernel expeller (47%) and extracted coconut (48%) both rich in C12:0 (Grummer, 1991; Heck et al., 2009). The high herd correlation for C12:0 might be because on a herd the same type of concentrate is fed to cows in both winter and summer. C18:2*cis-9,12* is one of the major PUFA found in maize silage (Chilliard et al., 2001, Khanal et al., 2008). The high herd correlation for this FA suggest that herds that feed maize silage do this in winter as well as in summer.

Table 2.4 Phenotypic (r_p), genetic (r_a), herd (r_{herd}), and residual (r_e) correlations (SE in parentheses) for fat%, individual fatty acids, groups of fatty acids and unsaturation indices between 1,905 winter milk samples and 1,795 summer milk samples.

Trait	r_p	$r_a^{\mathbf{1}}$	r_{herd}	r_e
Milk production trait				
Fat %	0.63 (0.02)	0.99 (0.04)^{ns}	0.19 (0.15)	0.40 (0.09)
Individual fatty acids				
C4:0	0.48 (0.02)	0.94 (0.06)^{ns}	0.31 (0.08)	0.25 (0.09)
C6:0	0.55 (0.02)	0.95 (0.05)^{ns}	0.42 (0.08)	0.29 (0.09)
C8:0	0.52 (0.02)	0.93 (0.05)*	0.40 (0.08)	0.16 (0.14)
C10:0	0.56 (0.02)	0.95 (0.03)*	0.41 (0.07)	-0.03 (0.26)
C12:0	0.54 (0.02)	0.98 (0.03)^{ns}	0.54 (0.05)	-0.06 (0.21)
C14:0	0.52 (0.02)	0.94 (0.04)*	0.37 (0.07)	0.14 (0.15)
C16:0	0.42 (0.03)	0.76 (0.11)**	0.21 (0.06)	0.47 (0.07)
C18:0	0.45 (0.02)	0.90 (0.10)^{ns}	0.26 (0.08)	0.41 (0.05)
C10:1	0.44 (0.02)	0.99 (0.04)^{ns}	0.31 (0.10)	0.15 (0.10)
C12:1	0.49 (0.02)	1.00 (0.03)^{ns}	0.37 (0.07)	0.21 (0.10)
C14:1*cis-9*	0.61 (0.02)	1.00 (0.02)^{ns}	0.16 (0.14)	0.46 (0.06)
C16:1*cis-9*	0.67 (0.02)	0.97 (0.03)^{ns}	0.19 (0.17)	0.53 (0.06)
C18:1*cis-9*	0.41 (0.03)	0.91 (0.08)^{ns}	0.19 (0.07)	0.33 (0.07)
C18:1*trans-11*	0.29 (0.03)	0.70 (0.17)**	0.26 (0.05)	0.22 (0.07)
C18:2*cis-9,trans-11* (CLA)	0.36 (0.03)	0.80 (0.11)**	0.30 (0.05)	0.25 (0.08)
C18:2*cis-9,12*	0.69 (0.02)	0.96 (0.07)^{ns}	0.76 (0.03)	0.52 (0.04)
C18:3*cis-9,12,15*	0.44 (0.03)	0.79 (0.13)**	0.41 (0.05)	0.40 (0.05)
Groups of fatty acids				
SFA	0.42 (0.03)	0.77 (0.11)**	0.23 (0.07)	0.42 (0.06)
UFA	0.40 (0.03)	0.82 (0.10)*	0.17 (0.07)	0.38 (0.06)
SFA to UFA	0.40 (0.03)	0.79 (0.11)**	0.17 (0.07)	0.42 (0.06)
Unsaturation indices				
C10 index	0.55 (0.02)	0.97 (0.09)^{ns}	0.16 (0.15)	0.53 (0.03)
C12 index	0.58 (0.02)	1.00 (0.02)^{ns}	0.05 (0.16)	0.39 (0.08)
C14 index	0.69 (0.02)	0.99 (0.02)^{ns}	0.15 (0.20)	0.50 (0.07)
C16 index	0.62 (0.02)	0.93 (0.05)^{ns}	0.22 (0.15)	0.50 (0.06)
C18 index	0.60 (0.02)	0.99 (0.03)^{ns}	0.30 (0.16)	0.45 (0.05)
CLA index	0.56 (0.02)	0.97 (0.04)^{ns}	0.23 (0.13)	0.49 (0.04)

[1]Supercripts indicate whether the genetic correlation differs significantly from 0.995, where **P-value < 0.01, * P-value ≤ 0.05 and ns = non-significant, i.e., P > 0.05

Table 2.5 Effects of the *DGAT1* K232A polymorphism (SE in parentheses) on fat%, individual fatty acids, groups of fatty acids and unsaturation indices based on 1,905 winter milk samples and 1,795 summer milk samples

Trait	-Log(P) *DGAT1* X season interaction[1]	Winter			Summer		
		KA[2] (N=829)	AA[3] (N=644)	-Log (P)[4]	KA[2] (N=773)	AA[3] (N=592)	-Log (P)[4]
Milk production trait							
Fat %	1.2[ns]	-0.46 (0.04)	-0.99 (0.04)	126.9***	-0.46 (0.04)	-0.95 (0.05)	126.8***
Individual fatty acids							
C4:0	1.5*	-0.01 (0.02)	0.01 (0.02)	0.3[ns]	0.01 (0.02)	0.00 (0.02)	0.2[ns]
C6:0	5.1***	-0.02 (0.01)	-0.06 (0.01)	13.4***	-0.04 (0.01)	-0.12 (0.01)	14.1***
C8:0	5.0***	0.00 (0.01)	-0.03 (0.01)	9.2***	-0.02 (0.01)	-0.08 (0.01)	10.0***
C10:0	5.1***	0.07 (0.03)	0.02 (0.03)	3.2***	-0.03 (0.03)	-0.14 (0.03)	3.7***
C12:0	2.7**	0.13 (0.04)	0.10 (0.04)	1.0[ns]	-0.01 (0.04)	-0.07 (0.04)	1.0[ns]
C14:0	4.0***	0.44 (0.06)	0.80 (0.06)	33.4***	0.30 (0.07)	0.52 (0.07)	32.6***
C16:0	0.1[ns]	-1.05 (0.16)	-2.56 (0.17)	65.0***	-1.14 (0.17)	-2.63 (0.18)	65.6***
C18:0	0.0[ns]	-0.16 (0.09)	-0.07 (0.10)	0.7[ns]	-0.16 (0.11)	-0.11 (0.12)	0.7[ns]
C10:1	0.7[ns]	0.00 (0.00)	-0.02 (0.00)	8.4***	-0.01 (0.00)	-0.03 (0.00)	8.9***
C12:1	1.0[ns]	0.23E-3 (1.76E-3)	-4.88E-3(1.89E-3)	3.0***	-3.85E-3 (1.82E-3)	-6.59E-3 (1.97E-3)	3.0***
C14:1*cis-9*	0.3[ns]	-0.01 (0.020	-0.04 (0.02)	1.3*	-0.03 (0.02)	-0.04 (0.02)	1.3*
C16:1*cis-9*	1.9*	-0.14 (0.02)	-0.32 (0.02)	53.2***	-0.12 (0.02)	-0.27 (0.02)	53.7***
C18:1*cis-9*	2.6**	0.66 (0.12)	1.73 (0.13)	61.0***	1.01 (0.15)	2.34 (0.16)	62.8***
C18:1*trans-11*	0.4[ns]	-0.01 (0.01)	0.03 (0.01)	3.5***	0.02 (0.03)	0.05 (0.03)	3.9***
C18:2*cis-9,trans-11* (CLA)	2.3**	0.02 (0.01)	0.05 (0.01)	16.0***	0.04 (0.01)	0.09 (0.01)	15.2***
C18:2*cis-9,12*	0.4[ns]	0.06 (0.01)	0.13 (0.02)	28.2***	0.07 (0.01)	0.15 (0.01)	29.0***
C18:3*cis-9,12,15*	1.3*	0.01 (0.00)	0.04 (0.01)	23.5***	0.01 (0.01)	0.06 (0.01)	22.8***

(continuation)

Trait	-Log(P) *DGAT1* X season interaction[1]	Winter			Summer		
		KA[2] (N=829)	AA[3] (N=644)	-Log (P)[4]	KA[2] (N=773)	AA[3] (N=592)	-Log (P)[4]
Groups of fatty acids							
SFA	2.8**	-0.72 (0.17)	-2.00 (0.18)	44.3***	-1.20 (0.21)	-2.84 (0.22)	46.6***
UFA	3.0**	0.62 (0.14)	1.68 (0.15)	42.4***	1.04 (0.18)	2.43 (0.19)	44.7***
SFA / UFA	0.4^{ns}	-0.11 (0.02)	-0.26 (0.02)	44.8***	-0.14 (0.02)	-0.30 (0.02)	46.2***
Unsaturation indices							
C10 index	1.1^{ns}	-0.31 (0.12)	-0.55 (0.13)	2.3**	-0.20 (0.12)	-0.26 (0.13)	2.0**
C12 index	1.1^{ns}	-0.09 (0.03)	-0.20 (0.04)	5.5***	-0.09 (0.04)	-0.13 (0.04)	5.3***
C14 index	1.4*	-0.49 (0.11)	-0.98 (0.12)	12.8***	-0.47 (0.12)	-0.75 (0.13)	12.6***
C16 index	1.8*	-0.26 (0.05)	-0.58 (0.06)	21.2***	-0.20 (0.06)	-0.41 (0.07)	21.7***
C18 index	0.6^{ns}	1.18 (0.24)	2.23 (0.26)	23.5***	1.40 (0.25)	2.71 (0.27)	23.3***
CLA index	0.7^{ns}	1.09 (0.27)	1.82 (0.29)	15.0***	1.27 (0.26)	2.36 (0.28)	15.3***

[1]-Log(P) *DGAT1* x season interaction represents -log(P-values) of the interaction between *DGAT1* genotypes in winter milk samples and *DGAT1* genotypes in summer milk samples, where ***P-value<0.001, **P-value <0.01,* P-value≤0.05 and ns=non-significant, i.e., P >0.05.
[2]Estimated contrast of KA - KK genotypes, where KK is set to zero, obtained using model [1] extended with *DGAT1* K232A as a fixed genotype effect.
[3]Estimated contrast of AA - KK genotypes, where KK is set to zero, obtained using model [1] extended with *DGAT1* K232A as a fixed genotype effect.
[4]Significance levels are represented by -log (P-values) of the effects of *DGAT1* K232A polymorphism in winter and summer milk samples, respectively. Nominal P-values are reported.

Table 2.6 Effects of the *SCD1* A293V polymorphism (SE in parentheses) on fat%, individual fatty acids, groups of fatty acids and unsaturation indices based on 1,905 winter milk samples and 1,795 summer milk samples.

Trait	-Log(P) *SCD1* x season interaction[1]	Winter			Summer		
		VA[2] (N=689)	VV[3] (N=117)	-Log (P)[4]	VA[2] (N=653)	VV[3] (N=103)	-Log (P)[4]
Milk production trait							
Fat %	0.7^{ns}	0.00 (0.03)	0.05 (0.07)	0.1^{ns}	-0.02 (0.04)	0.04 (0.07)	0.1^{ns}
Individual fatty acids							
C4:0	0.5^{ns}	-0.02 (0.01)	0.01 (0.03)	1.1^{ns}	-0.01 (0.02)	0.05 (0.03)	1.1^{ns}
C6:0	0.7^{ns}	0.01 (0.01)	0.02 (0.02)	1.0^{ns}	0.01 (0.01)	0.06 (0.02)	0.7^{ns}
C8:0	0.8^{ns}	0.01 (0.01)	0.02 (0.01)	1.7*	0.02 (0.01)	0.05 (0.02)	1.5*
C10:0	0.3^{ns}	0.10 (0.02)	0.15 (0.04)	8.1***	0.09 (0.02)	0.20 (0.04)	7.5***
C12:0	0.1^{ns}	0.09 (0.03)	0.14 (0.06)	2.3**	0.05 (0.03)	0.13 (0.06)	2.3**
C14:0	0.9^{ns}	0.22 (0.04)	0.40 (0.09)	6.5***	0.13 (0.05)	0.30 (0.09)	6.5***
C16:0	0.6^{ns}	-0.14 (0.13)	-0.26 (0.25)	0.4^{ns}	-0.12 (0.13)	0.22 (0.27)	0.3^{ns}
C18:0	0.3^{ns}	-0.29 (0.07)	-0.43 (0.13)	5.5***	-0.24 (0.08)	-0.64 (0.16)	6.0***
C10:1	0.5^{ns}	-0.03 (0.00)	-0.06 (0.01)	42.7***	-0.03 (0.00)	-0.05 (0.01)	42.9***
C12:1	0.0^{ns}	-0.01 (0.00)	-0.02 (0.00)	25.0***	-0.01 (0.00)	-0.02 (0.00)	24.1***
C14:1*cis-9*	0.0^{ns}	-0.17 (0.01)	-0.32 (0.02)	78.8***	-0.17 (0.01)	-0.33 (0.02)	77.7***
C16:1*cis-9*	0.2^{ns}	0.16 (0.02)	0.34 (0.03)	47.6***	0.15 (0.01)	0.35 (0.03)	48.4***
C18:1*cis-9*	0.5^{ns}	0.09 (0.09)	0.20 (0.18)	0.3^{ns}	0.17 (0.12)	-0.04 (0.24)	0.4^{ns}
C18:1*trans-11*	1.6*	-0.01 (0.01)	-0.04 (0.02)	2.1**	-0.07 (0.02)	-0.11 (0.04)	2.3**
C18:2*cis-9,trans-11*(CLA)	0.4^{ns}	0.02 (0.00)	0.02 (0.01)	2.5**	-0.07 (0.02)	-0.11 (0.04)	1.7*
C18:2*cis-9,12*	0.4^{ns}	0.01 (0.01)	-0.02 (0.02)	0.9^{ns}	0.01 (0.01)	-0.04 (0.02)	1.4*
C18:3*cis-9,12,15*	1.1^{ns}	0.01 (0.00)	-0.01 (0.01)	1.5*	0.02 (0.01)	0.00 (0.01)	2.2**

(continuation)

Trait	-Log(P) *SCD1* x season interaction[1]	Winter			Summer		
		VA[2] (N=689)	VV[3] (N=117)	-Log (P)[4]	VA[2] (N=653)	VV[3] (N=103)	-Log (P)[4]
Groups of fatty acids							
SFA	0.2^{ns}	-0.02 (0.13)	0.05 (0.25)	0.0^{ns}	-0.06 (0.16)	0.29 (0.32)	0.0^{ns}
UFA	0.5^{ns}	0.04 (0.11)	0.08 (0.22)	0.0^{ns}	0.07 (0.14)	-0.21 (0.28)	0.1^{ns}
SFA to UFA	0.3^{ns}	-0.01 (0.02)	-0.01 (0.03)	0.0^{ns}	0.00 (0.02)	0.03 (0.03)	0.0^{ns}
Unsaturation indices							
C10 index	0.1^{ns}	-1.18 (0.09)	-2.15 (0.17)	70.8***	-1.11 (0.08)	-2.11 (0.17)	69.2***
C12 index	0.0^{ns}	-0.29 (0.02)	-0.55 (0.05)	51.5***	-0.29 (0.03)	-0.53 (0.05)	50.8***
C14 index	0.0^{ns}	-1.34 (0.08)	-2.59 (0.16)	98.4***	-1.31 (0.08)	-2.59 (0.16)	97.0***
C16 index	0.2^{ns}	0.47 (0.04)	0.98 (0.08)	56.7***	0.49 (0.04)	1.05 (0.09)	58.7***
C18 index	0.1^{ns}	0.85 (0.19)	1.51 (0.37)	6.6***	0.75 (0.20)	1.47 (0.39)	7.0***
CLA index	0.3^{ns}	1.29 (0.20)	2.43 (0.40)	14.3***	1.14 (0.20)	2.13 (0.39)	15.0***

[1]-Log(P) *SCD1* x season interaction represents -log(P-values) of the interaction between *SCD1* genotypes in winter milk samples and *SCD1* genotypes in summer milk samples, where ***P-value<0.001, **P-value <0.01,* P-value≤0.05 and ns=non-significant, i.e., P >0.05.
[2]Estimated contrast of VA - AA genotypes, where AA is set to zero, obtained using model [1] extended with *SCD1* A293V as a fixed genotype effect.
[3]Estimated contrast of VV - AA genotypes, where AA is set to zero, obtained using model [1] extended with *SCD1* A293V as a fixed genotype effect.
[4]Significance levels are represented by -log (P-values) of the effects of *SCD1* A293V polymorphism in winter and summer milk samples, respectively. Nominal P-values are reported.

It is well established that the supply of FA reaching the mammary gland of a cow for milk fat synthesis can be indirectly affected by processes that occur in the rumen known to convert PUFA into SFA (e.g., Chilliard et al., 2001, Jenkins et al., 2008). These processes are dependent on many factors that include: quantity and composition of microbiota (Haarfoot & Hazlewood, 1997; Lock & Bauman, 2004), the proportion of forages and concentrates in a cow's diet (Dewhurst et al., 2006) and the source of the PUFA supplied to dairy cows (Sterk et al., 2011). Therefore, part of the observed differences in milk fat composition between winter and summer milk can also be attributed to dietary effects on processes in the rumen, which are known to affect the amounts of C18:1*trans-11* and CLA reaching the mammary gland of a cow (Mach et al., 2011).

2.4.2 Effects of polymorphisms in *DGAT1* and in *SCD1*

Some studies indicate that nutrition affects mammary expression of lipogenic genes (Bernard et al., 2008; Mach et al., 2011). Therefore, effects of polymorphisms in *DGAT1* and *SCD1* on milk fat composition might differ between winter and summer. In the present study, significant *DGAT1* by season interactions were found on many FA, and *SCD1* by season interaction was found only on C18:1*trans-11*. However, estimated genotype effects suggest that these interactions are due to scaling rather than to re-ranking (Figures 2.1 and 2.2). High genetic correlations between milk fat composition in winter and summer as well as similar genotypic effects in winter and summer support the idea that mainly the same genes are involved in milk fat composition in winter and in summer.

DGAT1. Is the gene encoding acyl-CoA: diacylglycerol acyltransferase1 (*DGAT1*; *EC: 2.3.1.20*), which is an enzyme responsible for the fixation of FA to the third position of triacylglycerol (**TAG**) (Cases et al., 1998; Palmquist, 2006; Yen et al., 2008). The K232A polymorphism causes an amino acid change (Lysine > Alanine at position 232 of the protein) that might alter the activity or specificity of the enzyme. In our study, the *DGAT1* 232A allele was associated with a lower milk fat%, which agrees with previous research (e. g., Grisart et al., 2002; Winter et al., 2002; Thaller et al., 2003). *DGAT1* shows a preference to esterify short chain and UFA to the third position of a TAG (Kinsella, 1976; Morand et al., 1998; Mistry and Medrano, 2002). In winter, the *DGAT1* 232A allele was negatively associated with most FA with less than 18 carbons and was positively associated with all unsaturated C18. In summer milk, higher amounts of UFA were found compared to winter milk. This larger supply seems to increase the effect of the *DGAT1* K232A polymorphism, especially for UFA for which

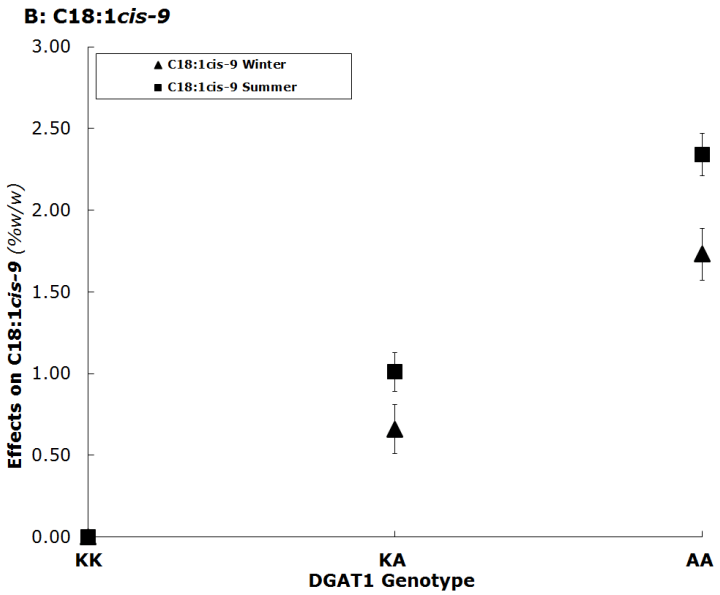

Figure 2.1 Estimated effects of *DGAT1* K232A polymorphism in winter and summer samples represented by the contrasts of AA-KK and KA-KK genotypes, where KK is set to zero. These contrasts illustrate the significant *DGAT1* K232A by season interaction on C18:1cis-9. SE are shown as error bars.

it has preference, because the effects of *DGAT1* 232A allele on most unsaturated C18 and UFA were larger in summer compared to winter milk and resulted in *DGAT1* by season interaction.

SCD1. Is the gene encoding stearoyl-CoA desaturase1 (*SCD1*; *EC: 1.14.19.1*) and the A293V polymorphism causes an amino acid change (Alanine > Valine at position 293 of the protein) which might affect the catalytic function of the enzyme, responsible for the insertion of a *cis*-double bond between carbon 9 and 10 of a FA (Pereira et al., 2003). In the present study, *SCD1* A293V polymorphism had no significant effects on fat%. These results are in line with Schennink et al. (2008).

Unsaturation indices have been suggested as indicators to indirectly measure the desaturation activity of the *SCD1* enzyme (e.g., Peterson et al., 2002). In both winter and summer, high means for C18 and CLA unsaturation indices (Table2.2) indicate that C18:0 and C18:1*trans-11* are unsaturated to a higher extent than C10:0, C12:0, C14:0 and C16:0. These results are in line with Enoch et al. (1976) who suggest that *SCD1* has preferences in unsaturating longer chain FA. In addition, the *SCD1* 293V

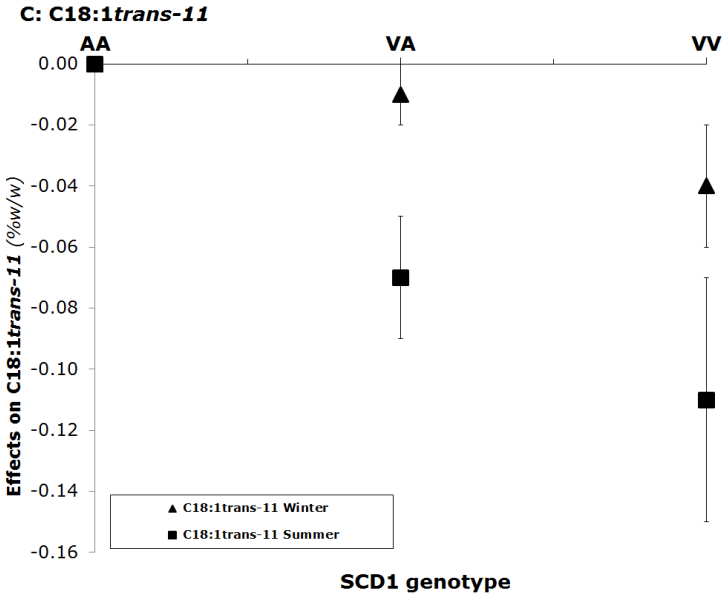

Figure 2.2 Estimated effects of *SCD1* A293V polymorphism in winter and summer samples represented by the contrasts of VV-AA and VA-AA genotypes, where AA is set to zero. These contrasts illustrate the significant *SCD1* A293V by season interaction on C18:1trans-11. SE are shown as error bars.

allele was positively associated with C16 to CLA indices compared to the *SCD1* 293A allele in both winter and summer (Table 2.6). These associations suggest that the *SCD1* 293V allele might have a higher affinity or specificity to unsaturate longer chain FA (e.g., C18:0 or C18:1*trans-11*) than other available FA (e.g., C10:0 or C14:0).

2.5 Conclusions

Milk fat composition in winter and in summer can be largely considered as genetically the same trait, because of the very high genetic correlations found between winter and summer milk fat composition. Differences in milk fat composition between winter and summer can probably be attributed to differences in the diets of cows between the two seasons rather than to genetic differences. Effects of *DGAT1* K232A and *SCD1* A293V polymorphisms on fat composition are similar in winter and in summer milk. Significant *DGAT1* and *SCD1* by season interactions were found for some fatty acids, and these interactions seem to be due to scaling of the genotype effects.

2.6 Acknowledgements

This study is part of the Dutch Milk Genomics Initiative, funded by Wageningen University, NZO (Dutch Dairy Association, Zoetermeer, the Netherlands), Cooperative Cattle Improvement organization CRV (Arnhem, the Netherlands), and the Dutch technology foundation STW (Utrecht, the Netherlands). The authors thank the owners of the herds for their help in collecting the data.The first author expresses her gratitude for having benefitted from academic and financial support of the Erasmus Mundus program "European Master in Animal Breeding and Genetics (EM-ABG)", and the Koepon Foundation.

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3

A quantitative trait locus on *Bos taurus* autosome 17 explains a large proportion of the genetic variation in de novo synthesized milk fatty acids

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Journal of Dairy Science (2014) 97:7276-7285

Abstract

A genomic region associated with milk fatty acid (**FA**) composition has been detected on Bos Taurus Autosome (**BTA**) 17 based on 50k SNP genotypes. The aim of our study was to fine-map BTA17 with imputed 777k single nucleotide polymorphism (**SNP**) genotypes in order to identify candidate genes associated with milk FA composition. Phenotypes consisted of gas chromatography measurements of 14 FA based on winter and summer milk samples. Phenotypes and genotypes were available on 1,640 animals in winter milk, and on 1,581 animals in summer milk samples. Single-SNP analyses showed that several SNP in a region located between 29.0 and 34.0 mega base-pairs were in strong association with C6:0, C8:0, and C10:0. This region was further characterized based on haplotypes. In summer milk samples, for example, these haplotypes explained almost 10% of the genetic variance in C6:0, 9% in C8:0, 3.5% in C10:0, 1.8% in C12:0, and 0.9% in C14:0. Two groups of haplotypes with distinct predicted effects could be defined, suggesting the presence of one causal variant. Predicted haplotype effects tended to increase from C6:0 to C14:0, however, the proportion of genetic variance explained by the haplotypes tended to decrease from C6:0 to C14:0. This is an indication that the quantitative trait locus (**QTL**) region is either involved in the elongation process or in early termination of de novo synthesized FA. Although many genes are present in this QTL region, most of these genes on BTA17 have not been characterized yet. The strongest association was found close to the progesterone receptor membrane component 2 (**PGRMC2**) gene. This gene has not been associated to milk FA composition. Therefore, no clear candidate gene associated with milk FA composition could be identified for this QTL.

Key words: milk fatty acid composition, dairy cattle, candidate genes, high-density genotyping.

3.1 Introduction

Bovine milk-fat is composed of more than 400 different fatty acids (**FA**), many of which are still un-identified (Jensen, 2002). FA may differ in the number of carbons and this difference can be related to the origin of the FA. Most short-chain FA are FA of less than 12 carbons that are mainly elongated from acetate by de novo synthesis in the mammary gland of a cow (e.g., Palmquist, 2006). Medium-chain FA are FA of 14 and 16 carbons and, while C14:0 mainly originates from de novo synthesis, C16:0 originates from two sources: approximately 50% from de novo synthesis and 50% from the diet of a cow. Most long-chain FA are FA of 18 or more carbons that mainly originate from the cow's diet, or from body fat mobilization (e.g., Chilliard et al., 2000). In addition to differences in the number of carbons, FA may also differ in their degree of saturation. On average, more than 70% of the identified FA in milk consist of saturated FA, and the remaining consist of unsaturated FA.

Variation in the content of several FA in milk is affected by genetic factors. Stoop et al. (2008) reported that individual milk FA have heritability estimates that range from 0.22 to 0.71. Some well characterized genes are recognized as having large effects on milk-fat and FA composition, such as acyl-CoA: diacylglycerol acyltransferase1 (***DGAT1***) located on BTA14, and stearoyl-CoA desaturase1 (***SCD1***) located on BTA26 (e.g., Schennink et al., 2007, Schennink et al., 2008). In addition, several regions of the bovine genome have been identified as having effects on milk-fat and FA composition but have not been characterized yet (e.g., Bouwman et al, 2012). By fine–mapping these regions, it is possible to identify candidate genes (Ishii et al., 2013) associated with milk FA composition. Further insights into the biosynthesis of milk-fat and FA are relevant if the aim is to change milk FA composition by means of breeding (Boichard and Brochard, 2012) or feeding strategies.

Fine-mapping allows to refine genomic regions by testing a large number of single nucleotide polymorphism (**SNP**) that are likely associated with a quantitative trait locus (**QTL**) (Hinds et al., 2005). Recently, a genomic region associated with short-chain FA in milk has been detected on BTA17 (Bouwman et al., 2012). However, no candidate gene or causal variant has been identified so far. The aim of our study was to fine-map BTA17 with imputed 777k SNP genotypes in order to identify candidate genes associated with milk FA composition.

3.2 Material and Methods

This study is part of the Dutch Milk Genomics Initiative that aims at exploring the possibilities to modify milk FA composition through breeding. Bouwman et al. (2012) performed a genome-wide association study (**GWAS**) using 50k SNP genotypes based on milk FA composition of winter and summer milk samples. In the present study, we re-analyzed the same phenotypes, and fine-mapped BTA17 using imputed 777k SNP genotypes.

3.2.1 Animals and phenotypes

Morning milk samples of 500mL per cow were retrieved from 2,001 first-lactation Holstein-Friesian cows from 398 herds throughout the Netherlands. At least three cows per herd were sampled in two distinct seasons: February-March 2005 (which will be referred to as "Winter" samples) and May-June 2005 (which will be referred to as "Summer" samples). The milk samples were taken from the same cows during the same lactation. Some cows sampled in winter were no longer lactating when summer milk samples were taken. Additional cows were sampled from the same herds to guarantee milk samples from at least three cows per herd. A total of 1,905 cows had phenotypic records in the winter, with each cow lactating between 63 and 282 days (see Stoop et al., 2008). A total of 1,795 cows had phenotypic records in the summer, with each cow lactating between 97 and 335 days (see Duchemin et al, 2013). About 50% of the cows in our experiment had access to pasture in summer (3.5 to 24 h/d), whereas all cows were kept indoors and fed silage in winter. Further details about the experimental design can be found in Stoop et al. (2008).

Milk FA composition was measured by gas chromatography at the COKZ laboratory (Qlip, Leudsen, Netherlands). Milk-fat was extracted from the milk samples, and fatty acid methyl esters were prepared from fat fractions, as described by Schennink et al. (2007). The FA were identified and quantified by comparing the methyl ester chromatograms of the milk fat samples with the chromatograms of pure FA methyl ester standards (Stoop et al., 2008). FA included in this study were measured as weight proportion of total fat (%wt/wt) and are described in Table 3.1. In addition, an indicator of de novo synthesized milk FA was created by combining C6:0 through C14:0 individual FA in the index referred to as "C6:0-C14:0" (Table 3.2).

3.2.2 Genotypes and imputation

A blood sample from each cow and semen from each bull were used to extract DNA. The DNA of 55 sires and 1,813 daughters belonging to our experimental population was genotyped with a 50k SNP chip. This chip was designed by CRV (Arnhem, Netherlands), and was used to genotype the animals with the Infinium assay (Illumina, San Diego, CA).

A reference population of 1,333 animals belonging to CRV and including the 55 sires with offsprings in our data was additionally genotyped with a 777k SNP chip (Illumina, San Diego, CA). This information on the reference population was used to impute the genotypes of our experimental population from 50k to 777k SNP. This imputation was done using Beagle version 3.2.2 (Browning and Browning, 2009), and resulted in a total of 1,736 animals being imputed to 777k SNP. From these 1,736 animals, 12 animals were excluded because of pedigree inconsistencies and, subsequently, three animals were excluded because their herds no longer met the requirement of a minimum of three animals sampled per herd. As a consequence, 1,721 animals with imputed 777k SNP genotypes were available for this study. Imputation of BTA17 increased the number of SNP genotypes from 1,562 (i.e., 50k) to 22,240 (i.e., 777k). The positions of the imputed SNP were based on the bovine genome assembly UMD 3.1. (Zimin et al., 2009)

3.2.3 Fine-mapping of BTA17

The fine-mapping of BTA17 was performed separately for winter and summer milk samples by using imputed 777k SNP genotypes and the 14 FA described in Table 3.1. For each season, animals were included in the analyses if both phenotypic and genotypic data were available. Therefore, a total of 1,640 animals were available for winter milk, and a total of 1,581 animals were available for summer milk samples.

Single SNP analyses were performed using the following animal model:

$$y_{ijklmno} = \mu + b_1 * dim_i + b_2 * e^{-0.05 * dim_i} + b_3 * afc_j + b_4 * afc_j^2 + season_k + scode_l + SNP_m + herd_n + a_o + e_{ijklmno} \quad (1)$$

where $y_{ijklmno}$ is the dependent variable; μ is the overall mean; *b1* and *b2* are the regression coefficients related to dim_i; dim_i is the covariate describing the effect of days in milk, modeled with a Wilmink curve (Wilmink, 1987); b3 and b4 are the regression coefficients related to afc_j; afc_j is the covariate describing the effect of age at first calving; $season_k$ is the fixed effect of calving season (June – August 2004,

September – November 2004, or December 2004 – February 2005); $scode_l$ is the fixed effect accounting for differences in genetic level between groups of proven bull daughters and young bull daughters; SNP_m is the fixed effect of SNP genotype ; $herd_n$ is the random effect of herd, and is assumed to be distributed as ~N(0, $\mathbf{I}\sigma^2_{herd}$), for which **I** is the identity matrix, and is the herd variance; a_o is the random additive genetic effect of animal, and is assumed to be distributed as ~N(0, $\mathbf{A}\sigma^2_a$), where **A** is the additive genetic relationships matrix which consisted of 12,548 animals, and σ^2_a is the additive genetic variance; and $e_{ijklmno}$ is the random residual effect, and is assumed to be distributed as ~N(0, $\mathbf{I}\sigma^2_e$), for which **I** is the identity matrix, and σ^2_e is the residual variance.

Additive genetic and herd variances were estimated without the inclusion of SNP information, and the resulting estimates were fixed within model (1).

Heritability estimates were calculated from univariate analyses based on model (1) without the inclusion of SNP effects as follows: $h^2 = \frac{\sigma^2_a}{\sigma^2_a + \sigma^2_e}$. Analyses were performed separately for winter and summer milk samples. All statistical analyses were performed using ASReml 3.0 (Gilmour et al., 2009).

3.2.4 Construction of haplotypes

Haplotypes were constructed to further characterize a genomic region on BTA17, and these were constructed separately for winter and summer milk samples. This construction started with the identification of promising SNP by single SNP analyses using model (1). The SNP with the highest significance was defined as "QTagSNP1". Subsequently, we corrected for the effect of QTagSNP1, by including QTagSNP1 as a fixed effect in model (1). This correction allowed to run a second round of single SNP analyses, and to retrieve remaining significant SNP. After this second round of analyses, if another SNP still was significant, it was defined as "QTagSNP2". In these analyses, a SNP was considered to be still significant if $-\log_{10}(P\text{-value}) \geq 3$. Next, we corrected for the effects of QTagSNP2 in the model already extended with QTagSNP1, by further including QTagSNP2 as a fixed effect. This methodology was repeated until no additional significant SNP were retrieved. Linkage disequilibrium (**LD**) was estimated as r^2 between all the identified QTagSNP using PLINK version 1.07 (Purcell et al., 2007). After the identification of QTagSNP, haplotypes were constructed based on the identified QTagSNP.

Effects of haplotypes were estimated with the following animal model:

$$y_{ijklnpqr} = \mu + b_1 * dim_i + b_2 * e^{-0.05 * dim_i} + b_3 * afc_j + b_4 * afc_j^2 + season_k + scode_l + haplo1_p + haplo2_q + herd_n + a_r^* + e_{ijklnpqr} \quad (2)$$

where variables are as previously described for model (1), and: *haplo1*$_p$ is the random effect of the first haplotype; *haplo2*$_q$ is the random effect of the second haplotype, and they are both assumed to be distributed as N ~ (0, $\mathbf{I}\sigma^2_{haplo}$), for which **I** is the identity matrix, and σ^2_{haplo} is the haplotype variance. The first and second haplotypes were jointly used to estimate one haplotype variance (σ^2_{haplo}) and one effect for each haplotype. This was achieved by combining the design matrices of both haplotypes in ASReml. a_r^* is the random additive genetic effect of animal estimated without the inclusion of haplotypes, and is assumed to be distributed as N ~ (0,$\mathbf{A}\sigma^2_{a^*}$), for which **A** is the additive genetic relationships matrix which consisted of 12,548 animals, and $\sigma^2_{a^*}$is the additive genetic variance that remains after accounting for haplotype effects. The total additive genetic variance was defined as:$\sigma^2_a = \sigma^2_{a^*} + \sigma^2_{haplo}$. The fraction of genetic variance explained by haplotypes was defined as:$\sigma^2_{haplo}/\sigma^2_a$.

Additionally, we tested whether predicted haplotype effects differed from each other. Significance levels of the differences between predicted effects of haplotypes were assessed using Student's t-tests, as implemented in ASReml. The predicted effect of a haplotype was considered significantly different from another haplotype if P-value ≤ 0.05.

3.3 Results

3.3.1 Phenotypic means and heritability estimates

Phenotypic means and heritability estimates for milk FA composition in winter and summer milk samples are shown in Table 3.1. Winter milk had higher contents of short-chain FA than summer milk samples (14.2% vs. 13.7%), higher contents of medium-chain FA (44.2% vs. 40.4%), and lower contents of long-chain FA, such as C18:0 (8.7% vs. 9.9%) and cis-9 C18:1 (18,2% vs. 20.5%). Phenotypic variances were higher in summer as compared to winter milk samples, but genetic variances were similar in both seasons. A detailed discussion on differences between winter and summer milk samples can be found in our previous study (Duchemin et al., 2013).

Table 3.1 Phenotypic means (SD), and heritability estimates (h^2)[1] for individual fatty acids (FA) based on 1,640 winter milk samples and 1,581 summer milk samples

	Winter		Summer	
Individual FA (% wt/wt)	Mean (SD)	h^2	Mean (SD)	h^2
Saturated FA:				
C4:0	3.51 (0.27)	0.47	3.52 (0.35)	0.41
C6:0	2.23 (0.16)	0.46	2.17 (0.21)	0.39
C8:0	1.36 (0.14)	0.59	1.32 (0.17)	0.35
C10:0	3.02 (0.43)	0.73	2.87 (0.45)	0.48
C12:0	4.12 (0.70)	0.62	3.79 (0.72)	0.48
C14:0	11.62 (0.92)	0.62	11.16 (1.05)	0.54
C16:0	32.62 (2.84)	0.47	29.20 (3.49)	0.40
C18:0	8.71 (1.39)	0.28	9.86 (1.77)	0.19
Unsaturated FA:				
C10:1[2]	0.37 (0.07)	0.35	0.35 (0.07)	0.50
C12:1[2]	0.12 (0.03)	0.38	0.11 (0.03)	0.47
cis-9 C14:1[3]	1.36 (0.25)	0.35	1.38 (0.28)	0.43
cis-9 C16:1	1.45 (0.32)	0.44	1.40 (0.30)	0.38
cis-9 C18:1[4]	18.18 (2.05)	0.22	20.53 (2.76)	0.35
cis-9, trans-11 C18:2 (CLA)	0.39 (0.11)	0.55	0.56 (0.27)	0.27

[1] $h^2 = \sigma_a^2/(\sigma_a^2 + \sigma_e^2)$, where h^2 is the heritability estimate, σ_a^2 is the additive genetic variance and σ_e^2 is the residual variance; SE between 0.01 and 0.12 for winter samples, and between 0.02 and 0.08 for summer samples.

[2] For C10:1 and C12:1, the cis double bond could not be ascertained at the carbon 9 position.

[3] *cis*-9 C14:1 represents the sum of *cis*-9 C14:1 and *iso* C15 due to co-elution associated with the gas chromatography (**GC**) extraction method.

[4] *cis*-9 C18:1 represents the sum of *cis*-9 C18:1 and *trans*-12 C18:1 due to co-elution associated with the GC extraction method.

3.3.2 Fine-mapping of BTA17

Results of the fine-mapping of BTA17 for winter and summer milk samples are shown in Additional File 1. For both seasons, we analyzed the associations between 22,240 imputed SNP and each of the 14 FA. In a region between 29.0 and 34.0 mega base-pairs (Mbp), multiple SNP showed highly significant associations with C6:0, C8:0, and C10:0. Moreover, multiple SNP showed associations both in winter and summer milk samples (Additional file 1). Previously, Bouwman et al. (2012) identified associations of multiple regions on BTA17 with C6:0, C8:0, C10:0, C14:1, and C16:1. Detailed analyses in the current study focused on the region between 29.0 and 34.0 Mbp

because here the strongest and most consistent associations were found across winter and summer milk samples.

Figure 3.1A illustrates the strongest associations found with the imputed 777k SNP genotypes for C8:0 in summer milk samples. Additionally in Figure 3.1A, these associations were overlaid with the associations found by Bouwman et al. (2012) using 50k SNP genotypes, which was mainly the same data as used in the current study. Within the marked region (figure 3.1A), 10 significant SNP were found with the 50k SNP whereas 83 significant SNP were found with the imputed 777k SNP. The most significant SNP identified based on the imputed 777k SNP ($-\log_{10}$(P-value) = 7.93) was not present on the 50k SNP array. The most significant SNP identified based on the 50k SNP genotypes was less significant ($-\log_{10}$(P-value) = 6.21; Bouwman et al., 2012) than the most significant SNP identified in the present study. The location of the QTL could be refined to the genomic region located between 29.0 and 34.0 Mbp on BTA17 (figure 3.1A). Figure 3.1B shows the results of the associations for five FA in summer milk samples for this region.

3.3.3 Construction of haplotypes

The construction of haplotypes was based on the identified QTagSNP in the fine-mapping of BTA17. These SNP, QTagSNP1 and QTagSNP2, were different for winter and summer milk samples. For winter milk samples, QTagSNP1 was BovineHD1700008470 (***rs109426433***) located at 29.92 Mbp, and with minor allele frequency (**MAF**) of 0.47. QTagSNP1 was associated with C6:0 ($-\log_{10}$(P-value) = 4.90), C8:0 ($-\log_{10}$(P-value) = 6.28), C10:0 ($-\log_{10}$(P-value) = 4.03) and C12:0 ($-\log_{10}$(P-value) = 1.33). QTagSNP2 was BovineHD1700009150 (***rs135934524***) located at 32.90 Mbp, with MAF of 0.44. QTagSNP2 was associated with C6:0 ($-\log_{10}$(P-value) = 2.76), C8:0 ($-\log_{10}$(P-value) = 3.27), and C10:0 ($-\log_{10}$(P-value) = 2.24). QTagSNP1 and QTagSNP2 showed the strongest associations with C8:0. LD between QTagSNP1 and QTagSNP2 was $r^2 = 0.04$.

For summer milk samples, QTagSNP1 was BovineHD1700008490 (***rs109290136***) located at 30.08 Mbp (Figure 3.1B), with MAF of 0.44. QTagSNP1 was associated with C6:0 (-log10(P-value) = 6.82), C8:0 (-log10(P-value) = 7.93), C10:0 (-log10(P-value) = 6.13) and C12:0 (-log10(P-value) = 3.35). QTagSNP2 was BovineHD1700008967 (rs135465158) located at 32.17 Mbp (Figure 3.1C), with MAF of 0.14. QTagSNP2 was associated with C6:0 (-log10(P-value) = 2.82), C8:0 (-log10(P-value) = 3.19), and C10:0 (-log10(P-value) = 1.84). QTagSNP1 and QTagSNP2 showed the strongest associations with C8:0. LD between QTagSNP1 and QTagSNP2 was $r^2 = 0.07$. LD

between QTagSNP1 and all other markers in the fine-mapped region as well as significance of association with C8:0 is represented in Additional File 3.2 – figure A. LD between QTagSNP2 and all other markers in the fine-mapped region as well as significance of association with C8:0 is represented in Additional file 3.2 - figure B. LD between QtagSNP1 for winter milk samples and QtagSNP1 for summer milk samples was $r^2 = 0.56$; LD among other combinations of QTagSNP based on winter or on summer milk samples was low ($r^2 < 0.10$). For both winter and summer milk samples, two QTagSNP were identified. These two QTagSNP were used for haplotype construction, and this construction resulted in four haplotypes. As QTagSNP were not the same in winter and in summer milk samples, different haplotypes were constructed for both seasons.

3.3.4 Predicted effects of haplotypes

Predicted effects of haplotypes are shown in Table 3.2. For winter samples, frequencies of haplotypes were 0.33 for A-A, 0.21 for A-G, 0.12 for C-A, and 0.35 for C-G. While A-A haplotypes were associated with higher contents of C6:0, C8:0, C10:0, C12:0, C14:0 and the index C6:0-C14:0, C-G haplotypes were associated with lower contents of these FA and index. The absolute difference between one copy of the most contrasting haplotypes (A-A and C-G) was 0.040 for C6:0, 0.039 for C8:0, 0.090 for C10:0, 0.054 for C12:0, 0.065 for C14:0, and 0.239 for the index C6:0-C14:0. The fraction of genetic variance explained by haplotypes was 2.7% for C6:0, 2.8% for C8:0, 1.4% for C10:0, 0.5% for C12:0, 0.3% for C14:0, and 0.7% for the index C6:0-C14:0. Effects of the C-A haplotype did not differ from effects of the A-G haplotype for C6:0, C8:0 and C10:0, while they differed significantly (P-value ≤ 0.05) from effects of the C-G haplotype for C8:0. These results suggest that there are two groups of haplotypes with distinct effects (A-A, and A-G/C-A/C-G) for C6:0 and C10:0, and there are three groups of haplotypes with distinct effects (A-A, C-G, and A-G/C-A) for C8:0.

For summer samples, frequencies of haplotypes were 0.44 for A-G, 0.12 for A-A, 0.01 for C-A, and 0.42 for C-G. While C-G haplotypes were associated with higher contents of C6:0, C8:0, C10:0, C12:0, C14:0, and the index C6:0-C14:0, A-G haplotypes were associated with lower contents of these FA and index. The absolute difference between one copy of the most contrasting haplotypes (C-G and A-G) was 0.048 for C6:0, 0.043 for C8:0, 0.102 for C10:0, 0.101 for C12:0, 0.106 for C14:0, and 0.495 for the index C6:0-C14:0. The fraction of genetic variance explained by haplotypes was 0.3% for C4:0, 9.7% for C6:0, 9% for C8:0, 3.5% for C10:0, 1.8% for C12:0, 0.9% for C14:0, and 5.0% for the index C6:0-C14:0.

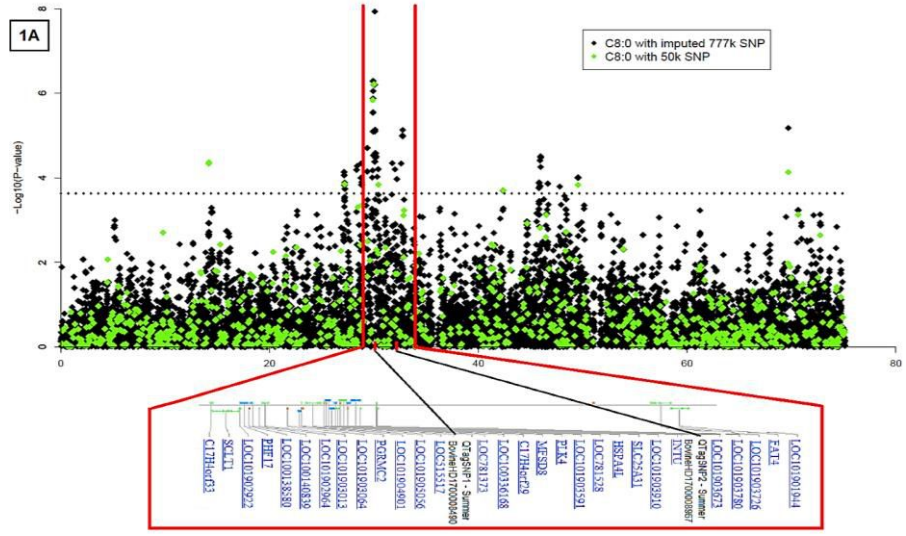
In summer samples, predicted effects of the A-G haplotype differed significantly (P-value ≤ 0.05; table 3.2) from effects of A-A, C-G and C-A haplotypes for C6:0, C8:0, C10:0, and the index C6:0-C14:0. Additionally, effects of the A-G haplotype differed significantly (P-value ≤ 0.05) from effects of the C-G haplotype for C12:0, and C14:0. Effects of the C-G haplotype did not differ from the effects of C-A and A-A haplotypes for any of the traits. These results suggest that there are two groups of haplotypes with distinct effects (A-G, and A-A/C-A/C-G) for C6:0, C8:0, C10:0, C12:0, C14:0, and the index C6:0-C14:0.

3.4 Discussion

In the present study, we refined the location of a QTL first described by Bouwman et al. (2012). This QTL seems to influence multiple de novo synthesized FA. We fine-mapped BTA17 by using imputed 777k SNP genotypes, and by using winter and summer milk FA composition. To further characterize the effects associated with this genomic region, we constructed haplotypes for each season.

3.4.1 Fine-mapping of BTA17

The fine-mapping of BTA17 combined high-density SNP genotyping with imputation. Imputation was based on a large reference population genotyped with 777k SNP. Additionally, the 55 sires belonging to our experimental population were genotyped with both 50k and 777k SNP. Our experimental population, which is composed of the daughters of the 55 sires, was imputed from 50k to 777k SNP genotypes using Beagle (Browning and Browning, 2009). The estimated error of this imputation was below 1%. Pausch et al. (2013) showed that imputation to high-density genotypes largely depends on the size of the reference population. An imputation accuracy of about ~99% can be obtained when a reference population of more than 400 animals is used (Pausch et al., 2013). This is in line with the imputation accuracy obtained in the current study. When imputation accuracy is high, GWAS based on imputed genotypes can assist in fine-mapping because imputation provides a high-resolution view of an associated region, and increases the chance that a causal SNP can be directly identified (Marchini and Howie, 2010). In the present study, the number of SNP increased by at least 10 times with the imputation of BTA17 from 50k to 777k SNP genotypes.


1A
C8:0 with imputed 777k SNP
C8:0 with 50k SNP
−Log10(P−value)
0
20
40
60
80
C17H16orf53
SGLT1
LOC101902922
PHF17
LOC100138580
LOC100140830
LOC101903964
LOC101928013
LOC101928064
PGRMC2
LOC101904901
LOC101928356
LOC515317
QTagSNP1 - Summer
Bovine+ED70000486
LOC781373
LOC100336168
C17H4orf29
MFSD8
ELK4
LOC101903591
LOC781528
HSPA4L
SLC35A1
LOC101928210
LOC101903673
QTagSNP2 - Summer
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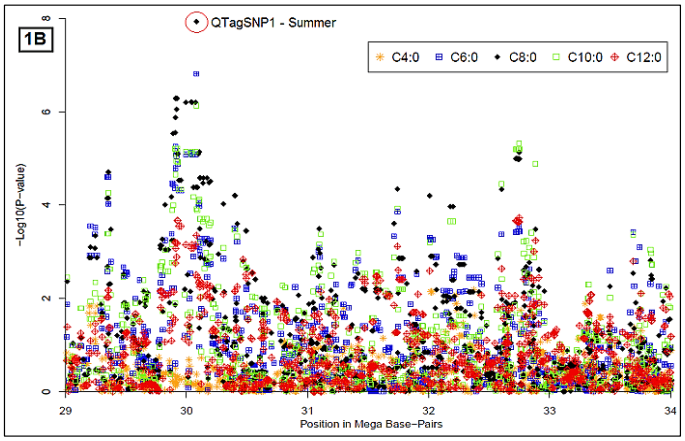
ENTPD
LOC101903780
LOC101903726
EAF1
LOC101901944


1B
QTagSNP1 - Summer
C4:0
C6:0
C8:0
C10:0
C12:0
−Log10(P−value)
0
2
4
6
8
29
30
31
32
33
34
Position in Mega Base−Pairs

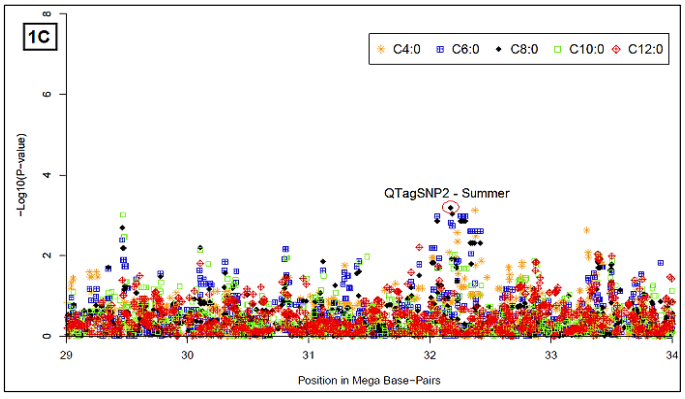

1C
C4:0
C6:0
C8:0
C10:0
C12:0
QTagSNP2 - Summer
−Log10(P−value)
0
2
4
6
8
29
30
31
32
33
34
Position in Mega Base−Pairs

Figure 3.1. **(A)** Fine-mapping of BTA17 for C8:0 in summer milk samples showing genome-wide association of imputed 777k (777,000) SNP overlaid with genome-wide association of 50k (50,000) SNP genotypes done by Bouwman et al. (2012). The black dotted line is the genome-wide significance level based on 50k SNP genotypes at a false discovery rate of 0.05 [$-\log_{10}$(*P*-value) = 3.63]. A list of candidate genes was added as well as an indication of the location of SNP, with the highest significance referred to QTagSNP1 and the SNP with the second highest significance referred to QTagSNP2. **(B)** Fine-mapping of candidate region from 29.0 to 34.0 Mbp associated with C4:0 to C12:0 on BTA17 (results represent summer samples only). Circle indicates QTagSNP1. **(C)** Fine-mapping of candidate region on BTA17 after the correction for QTagSNP1 (results represent summer samples only). Circle indicates QTagSNP2.

GWAS by Bouwman et al. (2012) with 50k SNP genotypes identified a QTL associated with milk FA composition on BTA17. By fine-mapping BTA17 with the imputed 777k SNP genotypes, additional SNP were found to be significantly associated with milk FA, and these were more significant than the SNP found by Bouwman et al. (2012). In addition, multiple FA showed associations with the same genomic region on BTA17, both in winter and in summer milk samples (Additional File 1). We focused on the strongest and most consistent associations found in both winter and summer milk samples. These associations were identified in this region located between 29-34 Mbp. Additional analyses in which we extended the region (26- 34 Mbp) showed results that were comparable to the ones presented in this paper.

Within this genomic region, summer milk showed more pronounced associations than winter milk samples. Duchemin et al. (2013) reported strong genetic correlations between winter and summer milk-fat composition of de novo synthesized FA (e.g., 0.95 for C6:0, 0.93 for C8:0, and 0.95 for C10:0). These strong genetic correlations suggest that de novo FA in winter and in summer milk are genetically the same trait. In addition, GWAS by Bouwman et al. (2012) showed that many genomic regions associated with milk FA in winter milk could be confirmed in summer milk samples (e.g., BTA17). Therefore, it is likely that milk FA composition is influenced by similar groups of genes. When studying the effects of *DGAT1* polymorphism on milk-fat composition in winter and summer milk samples, Duchemin et al. (2013) concluded that genotypic effects were in the same direction, but some of the genotypic effects were larger in summer as compared to winter.

Table 3.2 Predicted effects of haplotypes (frequency given in parenthesis after each haplotype) for de novo synthesized milk fatty acids based on 1,640 winter milk samples and 1,581 summer milk samples.

Trait	Winter milk samples				$\sigma^2_{haplo}/\sigma^2_a$
	A-A (0.33)	A-G (0.21)	C-A (0.12)	C-G (0.35)	(%)[1]
C4:0	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.000 ± 0.000[a]	0.0%
C6:0	0.021 ± 0.010[a]	0.002 ± 0.010[b]	-0.004 ± 0.011[bc]	-0.019 ± 0.010[c]	2.7%
C8:0	0.020 ± 0.009[a]	0.002 ± 0.009[b]	-0.002 ± 0.010[b]	-0.019 ± 0.009[c]	2.8%
C10:0	0.045 ± 0.023[a]	0.005 ± 0.023[b]	-0.005 ± 0.025[bc]	-0.045 ± 0.023[c]	1.4%
C12:0	0.026 ± 0.020[a]	0.004 ± 0.020[ab]	0.001 ± 0.022[ab]	-0.028 ± 0.020[b]	0.5%
C14:0	0.037 ± 0.028[a]	0.003 ± 0.028[ab]	-0.012 ± 0.031[ab]	-0.028 ± 0.028[b]	0.3%
C6:0-C14:0	0.106 ± 0.084[a]	0.027 ± 0.085[ab]	0.000 ± 0.091[ab]	-0.133 ± 0.084[b]	0.7%
	Summer milk samples				
	A-G (0.44)	A-A (0.12)	C-A (0.01)	C-G (0.42)	
C4:0	-0.009 ± 0.009[a]	0.002 ± 0.010[a]	0.006 ± 0.011[a]	0.000 ± 0.009[a]	0.3%
C6:0	-0.043 ± 0.021[a]	-0.009 ± 0.022[b]	0.046 ± 0.027[c]	0.005 ± 0.021[bc]	9.7%
C8:0	-0.035 ± 0.016[a]	-0.003 ± 0.017[b]	0.030 ± 0.021[b]	0.008 ± 0.016[b]	9.0%
C10:0	-0.068 ± 0.031[a]	0.003 ± 0.033[b]	0.030 ± 0.043[b]	0.034 ± 0.032[b]	3.5%
C12:0	-0.049 ± 0.032[a]	0.003 ± 0.035[ab]	-0.006 ± 0.045[ab]	0.052 ± 0.033[b]	1.8%
C14:0	-0.055 ± 0.039[a]	0.004 ± 0.044[ab]	0.000 ± 0.054[ab]	0.051 ± 0.041[b]	0.9%
C6:0-C14:0	-0.329 ± 0.168[a]	-0.050 ± 0.180[b]	0.213 ± 0.237[b]	0.166 ± 0.172[b]	5.0%

[a-c] For each trait (i.e., within a row), different letters indicate a significant difference between haplotypes at $P \leq 0.05$, using Student's t-test.

[1] $\sigma^2_a = \sigma^2_{a^*} + \sigma^2_{haplo}$, where σ^2_a is the total additive genetic variance, $\sigma^2_{a^*}$ is the additive genetic variance that remains after accounting for haplotype effects, and σ^2_{haplo} is the haplotype variance

Duchemin et al. (2013) concluded that differences between winter and summer milk-fat composition were likely due to differences in the diets of the cows, and that the effects of *DGAT1* were scaled. This scaling resulted in significant *DGAT1* by season interaction, especially for short-chain FA (C4:0 to C14:0). In the present study, similar scaling effects might explain the more pronounced associations found in summer as compared to winter milk samples.

3.4.2 Construction of haplotypes

Haplotypes were constructed by first retrieving the most significant SNP within the fine-mapped region. This SNP, QTagSNP1, was associated with C8:0. Most of the variation in the region was explained by QTagSNP1, but not all. The remaining variation was accounted for by QTagSNP2 (results shown for summer samples, Figure 3.1B and 3.1C). After adjusting for both QTagSNP, no other significant SNP was found. Based on the two QTagSNP, a total of four haplotypes were constructed. In summer milk samples, these haplotypes explained almost 10% of the genetic variance in C6:0, 9% in C8:0, 3.5% in C10:0, 1.8% in C12:0, and 0.9% in C14:0 (Table 3.2). When these FA were combined into an index, haplotypes explained 5% of the genetic variance in de novo synthesized milk FA (C6:0-C14:0; Table 3.2).After testing for differences between these haplotypes, we concluded that estimated effects in summer milk for three out of four haplotypes did not differ from each other. Therefore, our four haplotypes could be divided in two groups with distinct effects on C6:0, C8:0, C10:0, C12:0, C14:0, and the index C6:0-C14:0: A-G versus the remaining haplotypes. The existence of two groups of haplotypes with distinct effects can be explained by one causal variant, i.e., one QTL. However, we cannot exclude the presence of multiple causal variants in strong LD.

The QTL region is associated with multiple de novo synthesized FA. The de novo synthesis occurs within the mammary gland of a cow, and is a process that elongates precursors by adding C2:0. These precursors originate from blood lipids and can be either acetate (C2:0), propionate (C3:0) or butyrate (C4:0). Butyrate in milk may originate from de novo synthesis or directly from β-hydroxybutyrate derived from the blood (e.g., Craninx et al., 2008). Depending on the precursor, the elongation process ends either at C16:0 or at C17:0. Results of the current study show that predicted effects of haplotypes increase from C6:0 to C14:0, however, the proportion of genetic variance explained by haplotypes decreases from C6:0 to C14:0. This increase of haplotype effects tends to be more pronounced in summer than in winter milk samples (Table 3.2). These results suggest that our candidate gene is involved in the elongation of FA or the early termination of this process

(Barber et al., 1997), and it might be up-regulated in summer as compared to winter milk samples.

Interestingly, in other species, such as humans, macaques and pigs, this genomic region is highly conserved. Further, in dairy cattle breeds, two studies suggested that this region on BTA17 contains signatures of selection: Qanbari et al. (2011) identified signatures of selection in a region close to the progesterone receptor membrane component 2 (*PGRMC2* at 29.8Mb) gene; and Stella et al. (2010) in a region close to the sprout homolog1, antagonist of FGF signaling (Drosophila) (*SPRY1* at 34.7 Mbp) gene. Possibly this genomic region is related to a highly conserved evolutionary mechanism.

3.4.3 Candidate genes

Information on candidate genes possibly associated with de novo synthesized FA was retrieved from the National Center for Biotechnology Information (**NCBI**) website. The QTL region on BTA17 contains 29 genes, but 18 of these genes have not been characterized yet Between QTagSNP1 and QTagSNP2 in summer samples, there are 11 genes of which five have been characterized (Figure 3.1A).

The gene that has been characterized and is closest to the most significant association is *PGRMC2*, which is located between 29.87 and 29.89 Mbp. This gene belongs to the Superfamily cytochrome b5-like heme/steroid binding domain. This Superfamily is involved in the fatty acid metabolic process, and oxido-reductase activity. In humans, this gene has been associated with breast adenocarcinoma (Causey et al., 2011), and it was pointed out as a regulator of cytochrome P450 enzyme activity (Wendler and Wehling, 2013). By sequencing the mRNA found in milk fat layer, Lemay et al. (2013) showed that *PGRMC2* is expressed in humans throughout the lactation, which included colostrum, transitional and mature milk.
In cattle, *PGRMC2* has been associated with fertility. Kowalik et al. (2013) showed that expression of *PGRMC2* mRNA in the bovine endometrium was higher in the first trimester of pregnant cows as compared to cyclic animals. However, the translation of *PGRMC2* mRNA in protein within the bovine endometrium was not different between cyclic and pregnant cows. In our study, cows in winter and summer sampling period were in a different stage of lactation (average of 166 days in winter and average of 247 days in summer samples), and probably at different stages of pregnancy. This might be a reason for the more pronounced associations found in summer milk samples. Therefore, we performed additional analyses in which we investigated interactions between stage of lactation and our QTagSNPs in both

seasons. None of these interactions were significant (results not shown). Bionaz et al. (2012) showed that *PGRMC2* is expressed during lactation in bovine mammary tissue. *PGRMC2* has not been associated with milk FA composition in dairy cattle.

Of the genes located within our QTL region, Bionaz et al. (2012) showed that four other genes are highly expressed during lactation in bovine mammary tissue: UPF0462 protein C4orf33-like (***LOC513251***), sodium channel and clathrin linker 1 (***SCLT1***), la-related protein 1B-like (***LOC515517***), and chromosome 17 open reading frame, human C4orf29 (***C17H4orf29***). The location in Mbp for these genes is between 29.10-29.12 for *LOC513251*, between 29.12-29.35 for *SCLT1*, between 30.03-30.07 for *LOC515517,* and between 30.10-30.13 for *C17H4orf29*. By sequencing the mRNA found in milk fat layer, Lemay et al. (2013) showed in humans that ***C17h4orf33*** (validated *LOC513251* gene in humans), ***LARP1B*** (validated *LOC515517* gene in humans), and ***C17H4orf29*** are expressed during all stages of lactation. These four genes have not yet been associated to milk FA composition.

In the present paper, we refined the location of a QTL, which is associated with multiple de novo synthesized milk FA, to a region between 29.0 and 34.0 Mbp on BTA17. We characterized the effects associated with this region by constructing haplotypes, and identified candidate genes possibly related to this QTL.

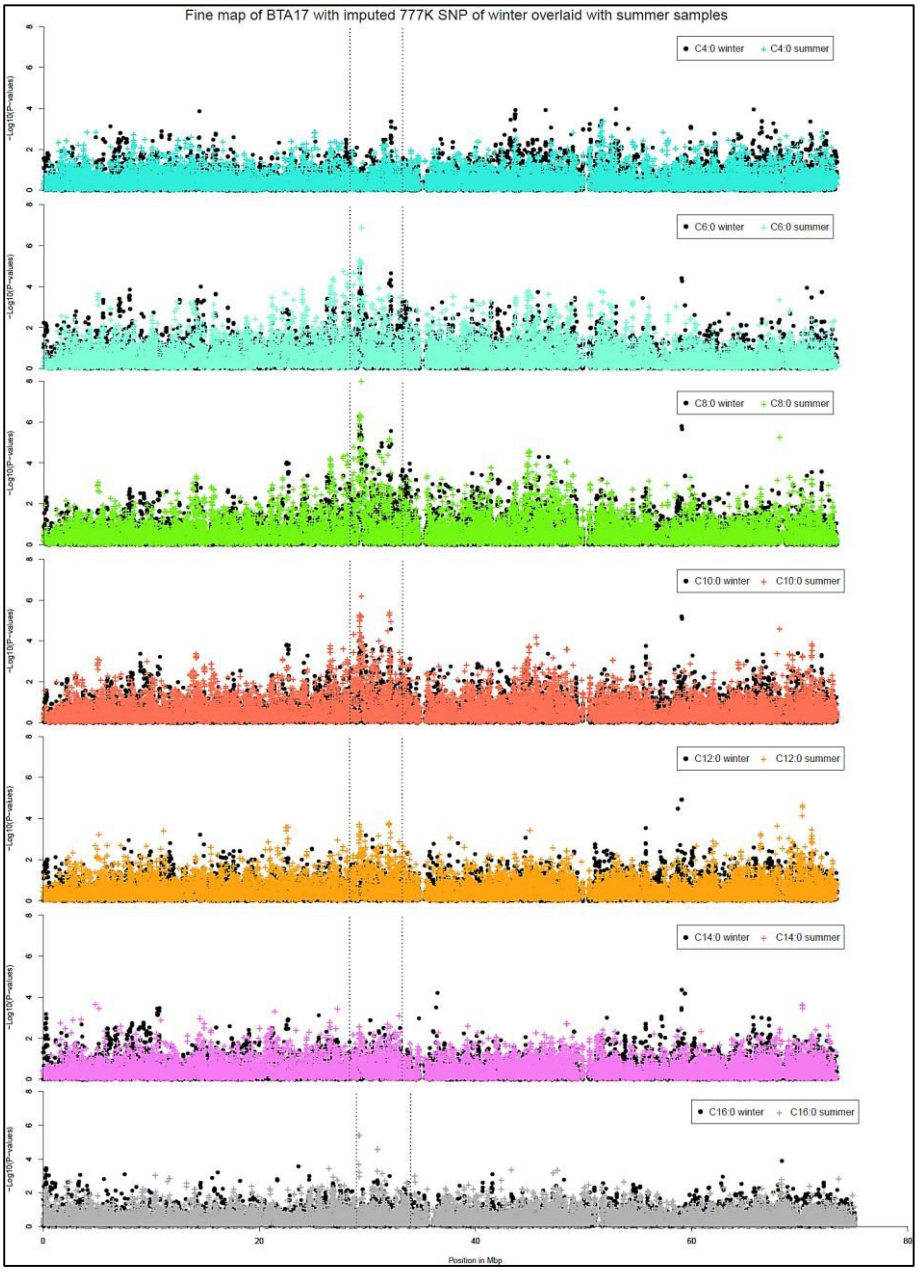
3.5 Conclusions

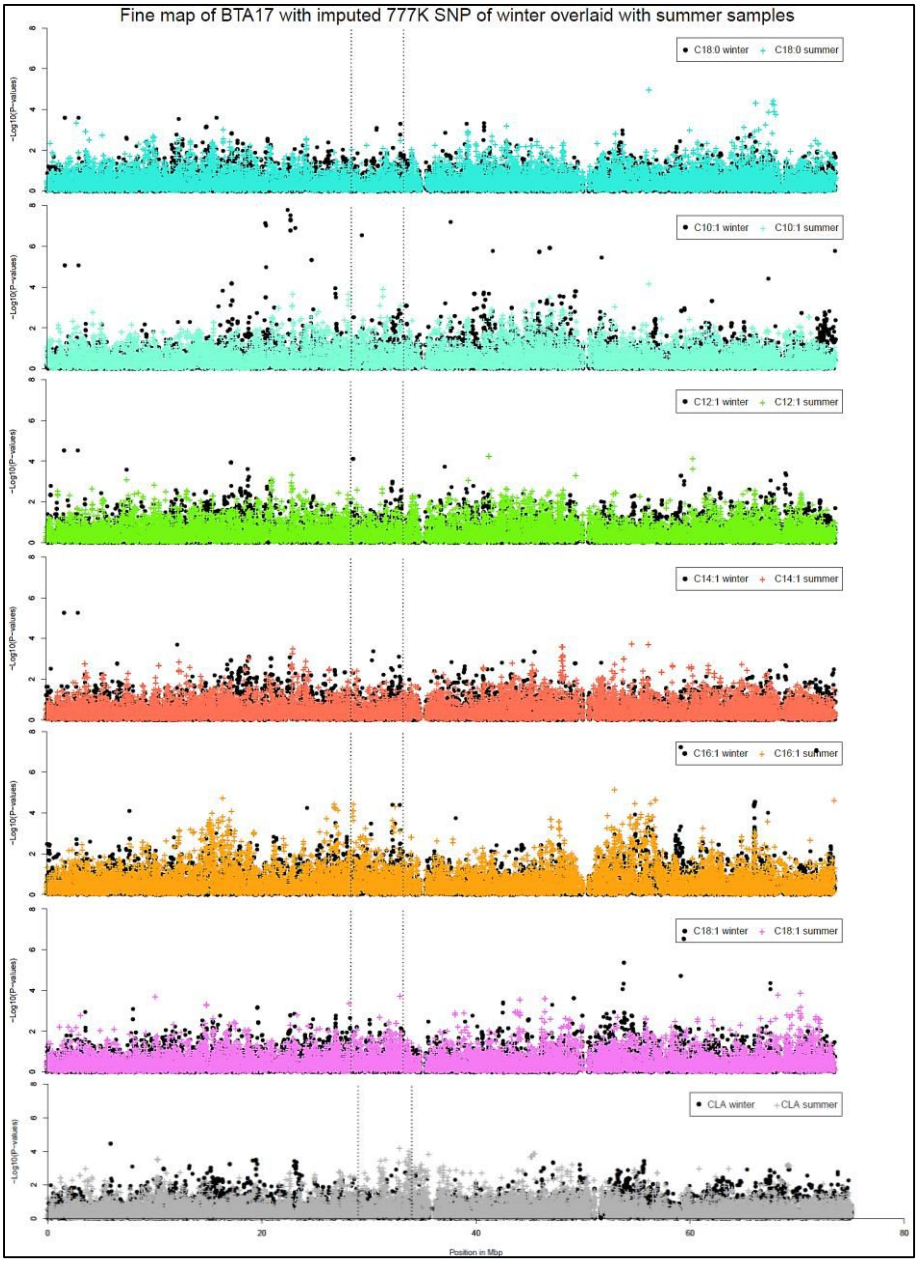
The fine-mapping of BTA17 improved the location of a QTL associated with multiple de novo synthesized milk FA. In summer milk samples, this QTL region explained a large proportion of the genetic variance in these FA individually (e.g., 10% in C6:0). When all de novo synthesized milk FA were combined into an index, this QTL region explained 5% of the genetic variance. This QTL region seems to be involved in either the elongation process of the de novo FA synthesis or in the early termination of this process. In addition, the effects of this QTL region are bigger in summer as compared to winter milk samples. Candidate genes associated with milk FA composition could not be clearly identified for this QTL because the QTL region on BTA17 is still being characterized. A characterized gene that might be of interest within the QTL region is *PGRMC2*.

3.6 Acknowledgements

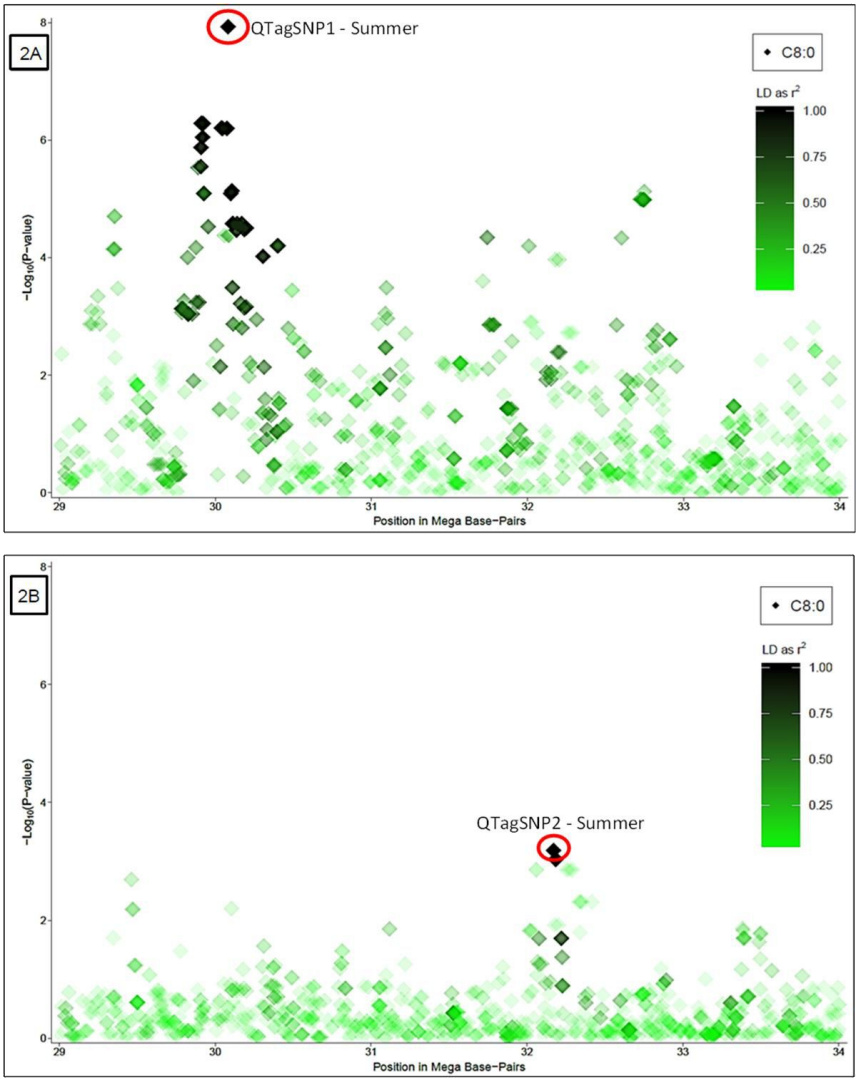
This study is part of the Dutch Milk Genomics Initiative, funded by Wageningen University, the Dutch Dairy Association (NZO), Cooperative Cattle Improvement Organization (CRV; Arnhem, the Netherlands), and the Dutch Technology Foundation (STW). We would like thank Chris Schrooten from CRV for the imputation of the 777k SNP genotypes. The first author currently benefits from a joint grant from the European Commission (within the framework of the Erasmus-Mundus joint doctorate “EGS-ABG”) and Breed4Food (a public-private partnership in the domain of animal breeding and genomics and CRV).

3.7 Supplementary files





Supplementary Figure 3.1. Fine-mapping of BTA17 with imputed 777k SNP genotypes overlaid between winter and summer samples for 14 FA. The marked region between black dotted lines (29.0 to 34.0 Mbp) is the region we focused on to refine the location of the QTL.



Supplementary Figure 3.2. (A) Fine-mapping of candidate region from 29.0 to 34.0 Mbp associated with C8:0 on BTA17 (results represent summer samples only). Circle indicates QTagSNP1. Linkage disequilibrium (**LD**), measured as r^2, between QTagSNP1 and all other markers for the trait is represented as a gradient of colors**. (B)** Fine-mapping of candidate region associated with C8:0 on BTA17, after the correction for QTagSNP1 (results represent summer samples only). Circle indicates QTagSNP2. LD, measured as r^2, between QTagSNP2 and all other markers for the trait is represented as a gradient of colors.

3.8 References

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4

Fine-mapping of BTA17 using imputed sequences for associations with de novo synthesized fatty acids in bovine milk

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(Manuscript in preparation)

Abstract

A genomic region associated with milk fatty acids on *Bos taurus* autosome (**BTA**) 17 has been discovered with 50,000 (**50k**) SNP and characterized with imputed 777,000 (**777k**) SNP genotypes. The aim of this study was to characterize this genomic region using imputed whole-genome sequences (**WGS**) and identify candidate genes associated with milk fatty acids (**FA**) composition on BTA17. Phenotypes and genotypes were available for 1,905 cows sampled in winter, and for 1,795 cows sampled in summer. Phenotypes consisted of gas chromatography measurements of 6 FA in winter and in summer milk samples. Genotypes consisted of imputed 777k SNP, and 89 sequenced founders of our population of cows. In addition, 450 WGS from the 1,000 bull genome consortium were available. Using 495 Holstein-Friesians sequences as reference population, we imputed the imputed 777k SNP genotyped cows to sequence level. Single-marker analyses were run with an animal model, and many significant associations with C6:0, C8:0, C10:0, C12:0 and C14:0 were identified. For example, for C8:0, a total of 1,182 significant associations in winter milk samples, and a total of 1,943 significant associations in summer milk samples were identified. Similar results were identified for all 6 FA. For C8:0 in summer milk samples, the genomic region located between 29 and 34 mega base-pairs on BTA17 revealed a total of 608 significant associations. The most significant association ($-\text{Log}_{10}$(P-value) = 7.66) was found for 8 SNP in perfect linkage disequilibrium. After fitting one of these 8 SNP as a fixed effect in the model, and re-running the single-marker analyses, no further significant associations were found. In the QTL region located between 29 and 34 mega base-pairs, a total of 14 genes could be identified. Six out of the 8 SNP in perfect LD were located in the LA ribonucleoprotein domain family, member 1B (***LARP1B***) gene. This primary candidate gene has not been associated with milk-fat composition yet.

Key words: QTL, candidate genes, sequences, LARP1B

4.1 Introduction

Bovine milk-fat is an important source of energy in human diets. The main bioactive lipids in bovine milk are fatty acids (**FA**). FA from bovine milk have important biological activities regarding the cell and tissue metabolism, as well as responsiveness to hormones and other signals in human cells (Calder, 2015). Previous studies on milk FA composition have indicated that amounts of individual FA in bovine milk are heritable (e.g., Duchemin et al., 2013). Heritability estimates range from 0.22 to 0.71 in Dutch Holstein-Friesian cows (Stoop et al., 2008). These findings suggested there is high genetic variability in the content of many individual FA in bovine milk.

Supporting these findings, polymorphisms in the acyl-CoA: diacylglycerol (***DGAT1***) and in the stearoyl-CoA desaturase1 (***SCD1***) genes have been associated with milk FA composition (e.g., Moioli et al, 2007; Schennink et al., 2007, 2008). In addition, Bouwman et al. (2012) identified many promising genomic regions associated with individual FA in bovine milk, when performing a genome-wide association study (**GWAS**) with 50,000 single-nucleotide polymorphism (**SNP**) genotypes. One of these regions located on Bos taurus autosome (**BTA**) 17 was fine-mapped with imputed 777,000 SNP (**777k**) genotypes, and significant associations with short-chain de novo synthesized FA have been identified (Duchemin et al., 2014). Furthermore, other studies have helped characterize BTA17. In Danish Holsteins, Buitenhuis et al. (2014) performed a GWAS identifying a QTL on BTA17 associated with conjugated linoleic acid (**CLA**). In Fleckvieh cattle breed, Pausch et al. (2012) performed a GWAS identifying a genomic region on BTA17 associated with supernumerary teats, and this genomic region has been associated with the absence of teats in Japanese Black cattle (Ihara et al., 2007). In *Bubalus bubalis,* Venturini et al. (2014) performed a GWAS on milk production traits and identified significant associations with milk production traits (i.e., milk yield, fat yield and protein yield) on BTA17 (note: BTA17 is used as a one-to-one correspondence to BBU17 in buffaloes). Despite the attempts to characterize BTA17 with a limited annotation of the cattle genome and genetic markers separated by more than 4 mega-base pairs in most cases, it is still difficult to identify the causal variants underlying the identified QTL.

With the advent of whole-genome sequences (**WGS**) in cattle, causal variants underlying QTL should be identified more easily with GWAS. WGS should contain the polymorphisms causing the genetic differences between individuals (Meuwissen and Goddard, 2010). To overcome the high-costs associated with WGS, Druet et al.

(2014) proposed to sequence influential ancestors of a population, and impute the rest of this population to sequence level. A GWAS using imputed WGS was first implemented by Daetwyler et al. (2014). Their study successfully mapped previously identified QTL affecting milk production traits and curly coat in cattle. Therefore, GWAS using imputed WGS can be used successful in (fine) mapping complex traits.

GWAS by Bouwman et al. (2012) identified a QTL region on BTA17 influencing C6:0, C8:0 and C10:0 FA. This genomic region was further characterized by Duchemin et al. (2014), and their findings suggested that this QTL region influenced multiple short-chain FA (C6:0 to C12:0) in a similar location on BTA17. Although candidate genes have been suggested for this QTL region, no causal variant for this QTL has been identified yet. The aim of this study was to use imputed WGS to identify the causal variant underlying the QTL on BTA17 associated with multiple short-chain FA previously identified by Bouwman et al. (2012), and fine-mapped by Duchemin et al. (2014).

4.2 Material and Methods

4.2.1 Animals and phenotypes

Morning milk (500mL/cow) was sampled from 2,001 primiparous Holstein-Friesian cows belonging to 398 herds throughout the Netherlands. These samples were collected in two periods: February-March 2005 (referred to as winter samples) and May-June 2005 (referred to as summer samples). For each herd, most of the cows were sampled in both periods. However, some cows sampled in winter were no longer in lactation in summer. Consequently, additional cows were sampled in summer to ensure that at least 3 cows per herd were sampled in both periods. For winter milk samples, phenotypes were available on 1,905 cows, and their lactation stages ranged from 63 to 282d (see Stoop et al., 2008). For summer milk samples, phenotypes were available on 1,795 cows, and their lactation stages ranged from 97 to 335d (see Duchemin et al., 2013). During the winter, all cows were kept indoors and fed silage, while in summer 50% of the cows could graze pasture (3.5 to 24h/d). More information on the experimental design is available in Stoop et al. (2008).

Milk FA were measured by gas chromatography at the COKZ laboratory (Qlip, Leudsen, Netherlands). The milk FA included in this study were C4:0, C6:0, C8:0, C10:0, C12:0, and C14:0, and they were expressed as weight proportion of total fat (%wt/wt). For more information regarding phenotypes, see Stoop et al. (2008).

4.2.2 Genotypes and variant calling

Blood from cows and semen from bulls were sampled to retrieve DNA for genotyping purposes. First, a total of 55 sires (**founders**) and 1,813 cows (**experimental population**) were genotyped with a 50k SNP chip designed by CRV (Arnhem, the Netherlands) with the Illumina Infinium array (Illumina Inc., San Diego, CA). Second, 777k SNP genotypes were imputed for the 1,813 cows, based on their 50k SNP genotypes and a reference population of 1,333 animals including the 55 founder sires genotyped with the 777k SNP chip (Illumina). See Duchemin et al., (2014) for details. The imputation resulted in 1,736 cows imputed to 777k SNP genotypes. From these 1,736 animals, some animals were removed from the data: 12 animals because of pedigree inconsistencies, and, subsequently, 3 animals that did not meet the criteria of a minimum of 3 animals sampled per herd. Therefore, 777K SNP genotypes were available for 1,721 cows. For BTA17, the target of the present study, the data consisted of a total of 22,240 imputed SNP genotypes for each of the 1,721 cows. Third, the 55 founder sires and 34 influential ancestors (grand-sires) of the experimental population (**MGI**) were sequenced. These 89 ancestors were sequenced with the HiSeq® 2000 Sequencing System (Illumina Inc., San Diego, CA). All downstream analyses were performed according to the protocols described by Daetwyler et al., (2014). Multi-sample variant calling was done using the UnifiedGenotyper implemented in GATK, following the procedures as explained by Daetwyler et al., (2014). The resulting raw VCF files were filtered for exclusion of duplicates, resulting in 854,779 called sites for BTA17.

In addition, 450 WGS from Holstein-Friesian cows and bulls were available from Run5 of the 1000 Bull Genome Consortium (**RUN5**; Daetwyler et al., 2014). These 450 WGS included the re-sequenced 44 out of the 55 founder sires. All positions of the variants on sequences were aligned to the bovine genome assembly UMD3.1 (Zimin et al., 2009). SNP and indels at same base-pairs positions were excluded because of alignment and sequencing problems. For further details on alignment, variant calling and filtering, see Daewytler et al. (2014). For BTA17, a total of 1,157,678 sites were available for each of the 450 sequenced animals.

4.2.3 Imputation

We created a reference population containing both MGI and RUN5 WGS. This reference population consisted of imputing the 45 MGI WGS to the level of the 450 RUN5 WGS to equalize the number of sites. Comparison of called sites for BTA17 between the 45 MGI and the 450 RUN5 WGS showed that 495,726 called sites

overlapped, and 661,952 sites in the RUN5 WGS were not called in MGI WGS. These 661,952 sites were set to missing in the 45 MGI WGS and imputed based on the 450 RUN5 WGS. Imputation was done using Beagle version 4.0 (Browning and Browning, 2007). After imputation, the 45 MGI WGS were combined with the 450 RUN5 WGS, resulting in a reference population of 495 Holstein-Friesian animals with 1,157,678 sites for BTA17.

Inconsistencies between 777K SNP genotypes and WGS sites of the reference population were checked using the Conform-gt software (https://faculty.washington.edu/browning/conform-gt.html). Three hundred and eighty three SNP were inconsistent sites due to strand problems, and 1,481 SNP showed different positions between the 777k SNP genotypes and in the WGS. These inconsistencies were set to missing and imputed to WGS. All BTA17 WGS sites were imputed for the 1,721 cows with Beagle version 4.0 based on their imputed 777K SNP genotypes and the reference population of 495 animals with WGS. The accuracy of imputation for each marker was provided by Beagle as the bi-allelic r^2 (**AR2**). Only polymorphic markers with an AR2 ≥ 0.8 were retained for the remaining analyses.

4.2.4 Fine-mapping of BTA17 with imputed sequences

The fine-mapping of BTA17 with imputed sequences was performed in Asreml 4.0 (beta version, Gilmour et al., 2009), and consisted of two steps. For the first step, we ran single-variant analyses for each FA with all polymorphic variants imputed with an AR2 ≥ 0.8, using the following animal model:

$$y_{ijklmno} = \mu + b_1 * dim_{ijklmno} + b_2 * e_i^{-0.05*dim_{ijklmno}} + b_3 * afc_{ijklmno} + b_4 * afc^2_{ijklmno} + season_k + scode_l + variant_m + herd_n + a_o + e_{ijklmno} \quad [1]$$

where, $y_{ijklmno}$ is the phenotype; b_1 and b_2 are the regression coefficients regarding $dim_{ijklmno}$; $dim_{ijklmno}$ is the fixed effect of days in milk modelled by a Wilmink's curve (Wilmink, 1987); b_3 and b_4 are the regression coefficients regarding $afc_{ijklmno}$; $afc_{ijklmno}$ is the fixed effect of age at first calving; $season_k$ is the fixed effect of calving season (June-August 2004, September-November 2004 or December 2004 – February 2005); $scode_l$ is the fixed effect accounting for genetic differences between groups of proven bull daughters and young bull daughters; $variant_m$ is the fixed effect of a variant; $herd_m$ is the random effect of herd assumed to be distributed as $N \sim (0, \boldsymbol{I}\sigma^2_{herd})$, where **I** is the identity matrix and σ^2_{herd} is the herd variance; a_n is the random additive genetic effect of animal

assumed to be distributed as $N \sim (0, \boldsymbol{A}\sigma_a^2)$, where **A** is the additive relationship matrix based on 12,548 animals and σ_a^2 is the additive genetic variance; and e_{ijklmn} is the random residual effect assumed to be distributed as $N \sim (0, \boldsymbol{I}\sigma_e^2)$, where **I** is the identity matrix and σ_e^2 is the residual variance.

Variance components were estimated based on model [1] prior to the inclusion of information on genetic markers, and these variance component estimates were subsequently fixed within model [1].

The strongest association found in the first step was named as "TagSNP1". For the second step, TagSNP1 was added as a fixed effect in model [1], and single-variant analyses were re-run for each FA with all polymorphic variants imputed with an AR2 ≥ 0.8.

Manhattan plots illustrating significance of associations were produced in R (R Core Team, 2015). In addition, linkage disequilibrium (B) between TagSNP1 and all polymorphic SNP imputed with an AR2 ≥ 0.8 was calculated using PLINK version 1.9 (Purcel et al., 2007).

4.2.5 Candidate genes and causal variants

Candidate genes were assessed with the online tool variant effect predictor (Ve!P; McLaren et al., 2010) available through Ensembl (http://www.ensembl.org). This tool determines the effects of SNP, insertions, deletions, copy number variants and structural variants on either genes, transcripts, proteins or regulatory regions.

4.3 Results

4.3.1 Descriptive statistics

The phenotypic means and heritability estimates for the 6 studied FA are presented in Table 4.1. In both samples, C14:0 was the most abundant FA. Heritability estimates were higher in winter milk samples in comparison with summer milk samples, especially for C8:0 and C10:0. Phenotypic means and heritability estimates of these 6 FA in winter and summer milk samples have been discussed in detail by Duchemin et al. (2013).

4.3.2 Imputation

To enable combining the MGI WGS with the RUN5 WGS into one reference population, the 661,952 sites that were not called in the 45 MGI WGS were imputed

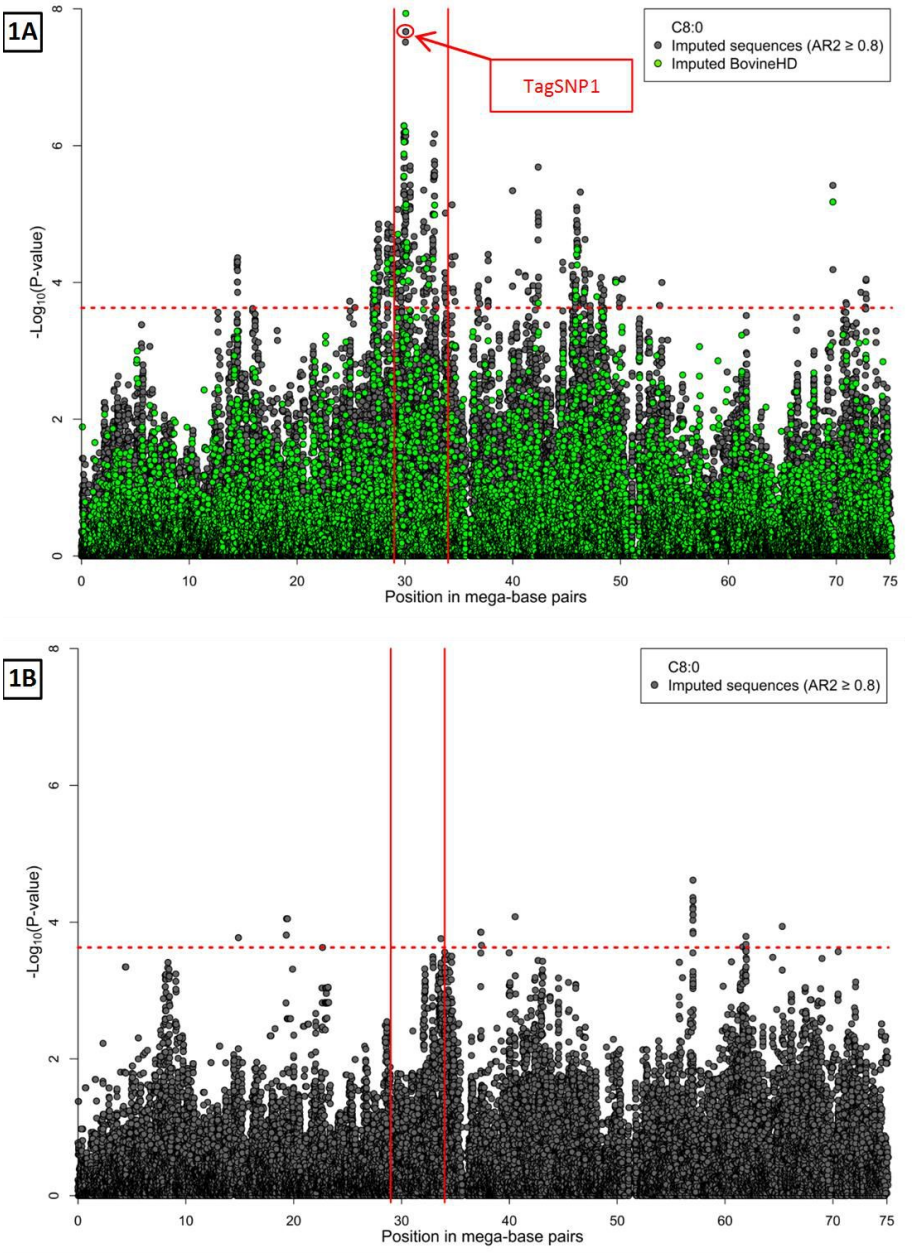

Figure 4.1 – (**A**) Fine-mapping of BTA17 for C8:0 in summer milk samples showing the genome-wide association of imputed sequences with an accuracy of imputation (AR2) ≥ 0.8 overlaid with imputed 777k (777,000) SNP genotypes done by Duchemin et al. (2014). The red dotted line is the genome-wide significance level based on 50,000 SNP genotypes at a false discovery

rate of 0.05 [-Log_{10}(P-value)=3.63]. The vertical red lines indicate the location of the QTL region previously identified by Duchemin et al. (2014). The SNP with the highest significance is referred to as TagSNP1. (**B**) Fine-mapping of BTA17 for C8:0 in summer milk samples showing the genome-wide association of imputed sequences with AR2 ≥ 0.8 after correction for TagSNP1.

based on the 450 RUN5 WGS. The average accuracy of imputation for these 661,952 sites was equal to 0.97. Based on the reference population of 495 WGS, all 1,157,678 sites on BTA17 were imputed for the 1,721 cows. As Table 4.2 shows, 58.6% of these sites were monomorphic variants in our data set, and have been excluded from our analyses. The remaining 41.4% were polymorphic variants. From these 41.4% polymorphisms, a total of 356,044 (30.8%) were imputed with AR2 ≥ 0.8 (average accuracy = 0.96). All polymorphisms imputed with AR2 ≥ 0.8 were considered for our fine-mapping of BTA17 with imputed sequences.

4.3.3 Fine-mapping of BTA17 with imputed sequences

Associations were analyzed for each of the 6 FA separately for winter and summer milk samples. We analyzed these phenotypes for both samples combined with all 356,044 imputed sequence variants of BTA17 imputed with AR2 ≥ 08 (supplementary figure 4.1 A, B, C and D). We focus on C8:0 because associations were found in a similar location for all 6 FA in both samples, and the strongest of these associations was identified with C8:0. For C8:0, an association was significant at $-Log_{10}$(P-value)= 3.63. This threshold was defined by Bouwman et al. (2012), and corresponds to the genome-wide significance level based on the 50k SNP genotypes at a false discovery rate (**FDR**) of 5%. For C8:0 in winter milk samples, we identified 1,182 significant associations on BTA17 at a $-Log_{10}$(*P*-value) > 3.63.

For C8:0 in summer milk samples, we identified 1,943 significant associations on BTA17 ($-Log_{10}$(*P*-value) > 3.63). Of these significant associations, 608 were located within the previously defined QTL region (Duchemin et al., 2014) between 29 and 34 MBP (Figure 4.1A). A set of 8 SNP in perfect LD showed the strongest association with C8:0 in summer milk samples at a $-Log_{10}$(*P*-value) = 7.66. One of these 8 SNP was defined as TagSNP1. TagSNP1 (rs110127535) had a MAF of 0.44 and was imputed with an AR2 = 0.98. TagSNP1 was added as fixed effect in model 1, and associations were analyzed again between each of the 6 FA in both winter and summer samples and all 356,044 imputed sequence variants of BTA17 with AR2 ≥ 0.8. No significant associations were found after adjusting for the TagSNP1 for any

of the 6 FA in winter or summer milk samples (Figure 4.1B, Supplementary figure 4.1B).

4.3.4 Candidate genes

The QTL region located between 29 and 34 MBP on BTA17 contains 14 genes based on the current annotation of the cattle genome (table 4.3). These 14 genes are: chromosome 4 open reading frame 33 (***C4orf33***) gene, sodium channel and clathrin linker 1 (***SLCT1***) gene; jade family PHD finger 1 (***JADE1***) gene; progesterone receptor membrane component 2 (***PGRMC2***) gene; LA ribonucleoprotein domain family, member 1B (***LARP1B***) gene; U2 spliceosomal RNA (***U2***) gene, the abhydrolase domain containing 18 (***ABHD18*** former ***C4orf29***) gene; small nucleolar RNA SNORA42/SNORA80 family (***SNORA42***) gene; major facilitator superfamily domain containing 8 (***MFSD8***) gene, the polo-like kinase 4 (***PLK4***) gene; solute carrier family 25 member 31 (***SLC25A31***) gene; inturned planar cell polarity protein (***INTU***) gene; and FAT atypical cadherin 4 (***FAT4***) gene. In addition, Ve!P was used on the 608 variants including TagSNP1, and these variants were distributed as indicated in figure 4.2.

Of the 8 SNP that showed the strongest association: 2 SNP were intergenic, 3 SNP were upstream gene variants of the *LARP1B* gene, and 3 SNP were intron variants in the *LARP1B* gene. The splice-region variant (rs110862734) is located at 29.94 MBP in the *LARP1B* gene [-Log_{10}(*P*-value) for association with C8:0 in summer milk = 6.18; LD with 8 most significantly associated SNP = 0.92].

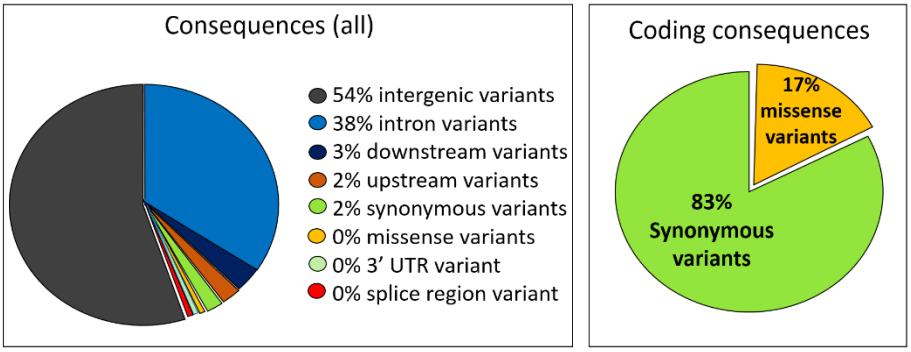

Figure 4.2 – Distribution of the 608 significant variants according to their functions and their coding consequences.

4.4 Discussion

The fine-mapping of BTA17 was first performed by Duchemin et al. (2014), in which the de novo synthesized FA in winter and summer milk samples were analyzed with imputed 777k SNP genotypes. Their study identified two intergenic SNP associated with multiple FA in a QTL region located between 29 and 34 mega base-pairs on BTA17. In the present work, 6 of the FA studied by Duchemin et al. (2014) have been considered. Our goal was to identify the causal variant underlying this QTL region, and to characterize this QTL region with recent information on candidate genes.

4.4.1 Fine-mapping of BTA17 with imputed sequences

The fine-mapping of BTA17 with imputed sequences identified many significant associations with the 6 studied FA. In agreement with Duchemin et al. (2014), multiple FA showed strong signals at a similar location on BTA17. These multiple FA were the de novo synthesized FA in the mammary gland of a cow. It is assumed that de novo synthesis elongates FA by adding C2:0 to precursors, such as C2:0, C3:0 and C4:0. Depending on which precursors, the elongation of FA is assumed to end at either C16:0 or C17:0. The origin of these precursors in the mammary gland of a cow varies: C2:0 and C3:0 are originated mainly from blood lipids, while C4:0 can either arise from blood lipids or be de novo synthesized (e.g., Craninx et al, 2008). In the present study, C4:0 does not seem to be influenced by this QTL region, while this QTL region seems to influence the other 5 FA. With imputed sequences, it was possible to observe that the QTL region does not only influences C6:0, C8:0 and C10:0, but also C12:0 and C14:0 (Supplementary file 1). Although the signals were weaker for C12:0 and C14:0, their signals overlap with the remaining FA in the QTL region for winter and summer milk samples (Supplementary file 1A and B).

Whole-genome sequences (**WGS**) should contain all of the causal variants underlying complex traits. On BTA17, the density of markers increased by more than 20 times from imputed 777k SNP genotypes to sequence level. With this increased density, more associations became significantly associated with the 6 studied FA: from less than 100 using imputed 777k SNP genotypes to more than a thousand with sequence data. For instance, 8 SNP were in perfect LD, and represent our strongest associations with C8:0 in summer milk samples. Duchemin et al. (2014) using imputed 777k SNP genotypes identified only one top SNP, and at a higher significance level than the eight SNP identified by this study. In the present study, we imputed to sequence level the already imputed 777k SNP genotypes. With sequence data, the extent of LD among SNP is conversed at 5-10kb in Bos Taurus breeds (e.g.,

Gibbs et al., 2009). According to Weiss and Terwilliger (2000), the distribution of LD shows stochastic variance, which tends to be highly skewed under certain conditions, as described by Terwilliger (2001). As a consequence, some parts of the genome will exhibit regions of long LD, while most SNP will exhibit less LD than predicted by lower density panels of genetic markers. If this is the case, it is possible that the imputation overestimated the extent of LD between genetic markers, and therefore, the effect of the top SNP with imputed 777k SNP is likely overestimated in comparison with sequence data. Other GWAS using imputed sequences have identified a considerable number of significant variants closely linked to each other (e.g., Daetwyler et al., 2014; Sahana et al., 2014). In addition, single-marker analyses assume that each marker contributes independently to the genetic variance. Taken together, these findings might explain why Duchemin et al. (2014) obtained better significance level for their top SNP than in the present study.

TagSNP1 seems to explain most of the genetic variation in a region distributed over almost 20 MBP on BTA17 (Figure 4.1B). The QTL region identified in the present study is wider than the 5 MBP QTL region narrowed by Duchemin et al. (2014). The present study confirms the multiple FA that were found associated with a similar QTL region and the strongest association that was found with C8:0, both by Duchemin et al. (2014). Study conducted by Govignon-Gion et al. (2014) found a QTL region on BTA17 associated with C4:0 and C6:0 when performing GWAS with imputed 500K SNP genotypes for three different breeds of dairy cattle. This QTL region was present in the three breeds, and the strongest associations were identified with C4:0. In our study, C4:0 in winter and summer milk samples was not significantly associated with our QTL region. The main difference between Govignon-Gion et al. (2014) and the present study is the method of measurement of fatty acids. Our 6 studied FA were measured by gas chromatography for both winter and summer milk samples, whereas the FA studied by Govignon-Gion et al. (2014) were measured by mid-infrared spectrometry. This might explain the observed differences between the studies.

Previously for the same QTL region, Bouwman et al. (2012) identified 10 significant SNP with 50k SNP genotypes, and Duchemin et al. (2014) identified 83 significant SNP with imputed 777k SNP genotypes for C8:0 in summer samples. From the 50K to the imputed 777k SNP genotypes, there was no overlap of associations. From imputed 777k SNP to imputed sequences, 70 associations found by the imputed 777k SNP genotypes were also found among the 608 significant associations with imputed sequences. In addition, the 8 SNP include the strongest SNP (***rs109290136***) identified

by Duchemin et al. (2014) with imputed 777k SNP genotypes. However, the significance of the top SNP in Duchemin et al. (2014) was higher [$-Log_{10}$(*P*-value) = 7.93] than the significance of the strongest SNP identified with imputed sequences (Figure 4.1A).

4.4.2 Candidate genes

Six of our eight strongest associations are located within the *LARP1B* gene. According to genecards (http://www.genecards.org/), the *LARP1B* gene encodes a protein containing domains found in the La related protein of Drosophila melanogaster. The *LARP1* family was first described in Drosophila melanogaster (Chauvet et al., 2000), where the Drosophila *LARP1* gene is required for spermatogenesis, embryogenesis and cell cycle progression (e.g., Ichihara et al., 2007). Study by Blagden et al. (2009) showed that the Drosophila *LARP1* gene interacts with poly A binding protein (PABP), and suggested that the phenotype observed in *LARP1* mutants could be the result of defective mRNA translation or regulation. In Caenorhabditis elegans, the *CeLARP1* gene was identified as an RNA-binding protein (Nykamp et al., 2008). In yeast, the mRNA-dependent LA-related proteins family (*LARP1*) when in association with *SLF1* promotes copper detoxification (Schenk et al., 2012). In viruses, the *LARP1B* gene has the biological process of mitophagy in response to mitochondrial depolarization (Orvedahl et al., 2011). In Arabidopsis, the overexpression of the *LARP1B* gene causes a premature leaf yellowing phenotype, and leaf senescence (Zhang et al., 2012).

According to Stavraka and Blagden (2015), la related proteins family 1 (*LARP1*) genes in humans have two paralogues: *LARP1A* and *LARP1B*. *LARP1A* (or simply *LARP1*) is positioned at chromosome 5q34, encoding 1096 amino acid proteins. *LARP1B* (or *LARP2*) is positioned at chromosome 4q28, encoding 914 amino acid proteins. According to Stavraka and Blagden (2015), *LARP1A* and *LARP1B* are similar (60% homology and 73% of positivity). Burrows et al. (2010) showed that *LARP1A* is more abundant than *LARP1B*, therefore *LARP1B* has been less studied. According to Uniprot (www.uniprot.org; accessed on 11/21/2015), the gene ontology regarding the molecular function of the *LARP1B* gene in humans is the poly(A) RNA binding, i.e., the very same as *LARP1A*. Review by Bousquet-Antonelli, and Deragon (2009) suggested that members of the same family are functional homologs and/or share a common molecular mode of action on different RNA baits.

Interestingly, in mammalian cells, Tcherkezian et al. (2014) found that the *LARP1A* gene associates with the mTOR complex 1 (***mTORC1***) and is required for global protein synthesis as well as cell growth and proliferation. This implicates the *LARP1A*

gene as an important regulator of cell growth and proliferation. Bionaz et al. (2012) reviewed the role of *mTORC1* relating it to the regulation of protein synthesis, particularly translation in the mammary tissue. Interestingly, *mTORC1* was considered to be the missing link between nutrition and milk protein synthesis (Bionaz et al., 2012). According to Bionaz et al. (2012), insulin regulates the amount of translation of the *mTORC* pathway that will influence milk protein synthesis. Gomes and Blenis (2015) suggest that, through various mechanisms, *mTORC1* stimulate mRNA translation, aerobic glycolysis, glutamine anaplerosis, lipid synthesis, the pentose phosphate and pyrimidine synthesis, thus producing the major components necessary for cell growth and proliferation. Although less studied as compared with the *LARP1A* gene, the *LARP1B* gene possess the same molecular function as *LARP1A* gene. We cannot exclude that the *LARP1B* might play a role regarding cell growth and proliferation in the mammary gland of a cow.

Furthermore, the *LARP1B* gene contains a splice-region variant. According to Sammeth et al. (2008) splice-region variants generate different mature transcripts from the same primary RNA sequence. Although no further information is available on the possible transcripts generated by the *LARP1B* gene, this gene is highly expressed in bovine mammary tissue (Bionaz et al., 2012), and it is expressed in all stages of lactation in humans (Lemay et al., 2013). Yet, the *LARP1B* gene has not been associated to milk FA composition or milk-fat synthesis.

Previously, the candidate gene identified by Duchemin et al. (2014) was the *PGRMC2* gene. The *PGRMC2* gene is still among the genes associated with the QTL region that influences multiple FA on BTA17 (Table 4.3). However, the *PGRMC2* gene was assigned as the most likely candidate gene because it was the closest gene to the strongest association found by Duchemin et al. (2014). At that time, there were no associations found in the *PGRMC2* gene. In addition, the identified *LOC515517* was the gene closest to the strongest association on BTA17. However, because of limited annotation available on BTA17 at that time, *LOC515517* was identified as a suggestive candidate gene while *PGRMC2* was suggested as primary candidate gene. Since then, *LOC515517* has been annotated in the cattle genome as *LARP1B* gene.

4.5 Conclusions

The fine-mapping of BTA17 with imputed sequences identified a substantial number (in the thousands) of significant associations with de novo synthesized milk FA (C6:0

to 14:0). With imputed sequences, the resolution of the QTL region influencing multiple milk FA improved compared to previous studies. The strongest associations were identified with C8:0 in summer milk samples. With imputed sequences, the number of candidate genes in this QTL region was reduced from 29 to 14. Among these 14 candidate genes, 6 out of 8 SNP in strong LD were identified in the *LARP1B* gene. The *LARP1B* gene is expressed in bovine mammary tissue. Nonetheless, the *LARP1B* gene has not been associated with milk FA composition at present.

4.6 References

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4.7 Tables

Table 4.1 - Phenotypic means (**SD**), and heritability estimates (h^2)[1] for individual fatty acids (**FA**) based on 1,640 winter milk samples and 1,581 summer milk samples

	Winter		Summer	
Individual FA (% wt/wt)	Mean (SD)	h^2	Mean (SD)	h^2
C4:0	3.51 (0.27)	0.47	3.52 (0.35)	0.41
C6:0	2.23 (0.16)	0.46	2.17 (0.21)	0.39
C8:0	1.36 (0.14)	0.59	1.32 (0.17)	0.35
C10:0	3.02 (0.43)	0.73	2.87 (0.45)	0.48
C12:0	4.12 (0.70)	0.62	3.79 (0.72)	0.48
C14:0	11.62 (0.92)	0.62	11.16 (1.05)	0.54

[1] $h^2 = \sigma_a^2/(\sigma_a^2 + \sigma_e^2)$, where σ_a^2 is the additive genetic variance and σ_e^2 is the residual variance. SE between 0.01 and 0.12 for winter samples, and between 0.02 and 0.08 for summer samples.

Table 4.2 - Distribution of the average accuracy of imputation (**AR2**) stratified per ranges of minor allele frequency (**MAF**), and the number of markers (as counts and in percentage) for the 45 sequences of Milk Genomics Initiative (**MGI**) and the 450 sequences of the 1000Bull Genome Consortium (**RUN5**)

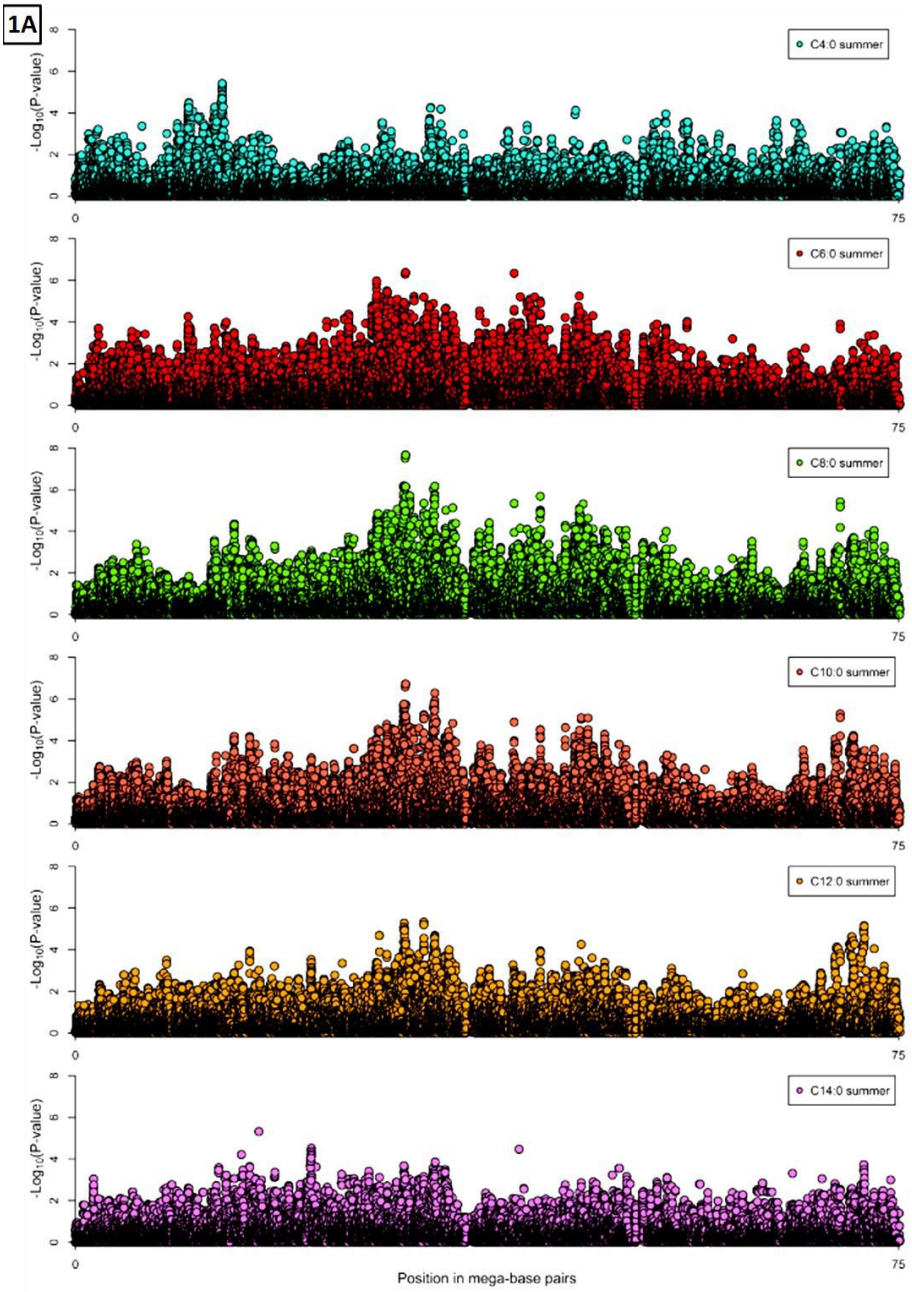
		MGI45				RUN5				
MAF	AR2	AR2	typed	imputed	Total (%)	AR2	typed	imputed	total	Total (%)
0	all	0.92	64,564	614,367	58.6%	0.00	64,564	614,367	678,931	58.6%
	≥0.8	0.97	41,601	531,552	49.5%	-	0	0	0	0.0%
0-0.1	all	0.97	158,733	42,979	17.4%	0.73	158,733	42,979	201,712	17.4%
	≥0.8	0.99	154,028	37,790	16.6%	0.94	102,738	17,003	119,741	10.3%
0.1.2	all	0.98	90,821	1,549	8.0%	0.89	90,821	1,549	92,370	8.0%
	≥0.8	0.99	90,000	765	7.8%	0.96	76,582	313	76,895	6.6%
0.2-0.3	all	0.98	68,423	965	6.0%	0.91	68,423	965	69,388	6.0%
	≥0.8	0.99	67,830	511	5.9%	0.97	60,442	223	60,665	5.2%
0.3-0.4	all	0.98	54,352	934	4.8%	0.92	54,352	934	55,286	4.8%
	≥0.8	0.99	53,803	462	4.7%	0.97	48,559	210	48,769	4.2%
0.4-0.5	all	0.96	58,833	1,158	5.2%	0.86	58,833	1,158	59,991	5.2%
	≥0.8	0.99	56,074	426	4.9%	0.97	49,823	151	49,974	4.3%
Total	all	0.97	495,726	661,952	100.0%	0.72	495,726	661,952	1,157,678	100.0%
	≥0.8	0.99	463,336	571,506	89.4%	0.96	338,144	17,900	356,044	30.8%

Table 4.3 - Details about candidate genes identified in the QTL region located between 29 and 34 mega base-pairs on BTA17

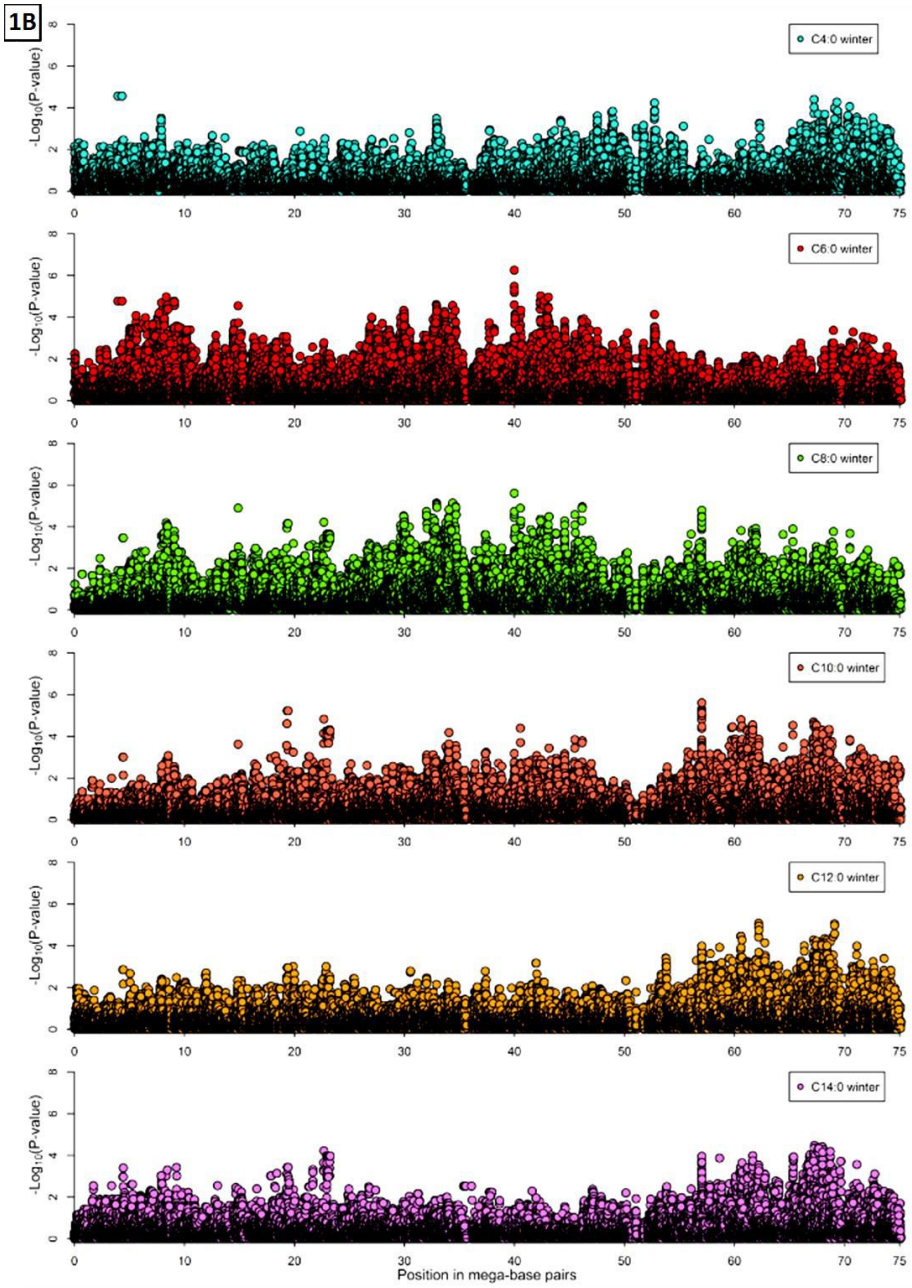
Genes	Identifier	Location	Numbers of variants
C4orf33	ENSBTAG00000044159	Chr17:29,105,309-29,116,531	2
SLCT1	ENSBTAG00000013611	Chr17:29,190,572-29,354,595	23
JADE	ENSBTAG00000017493	Chr17:29,368,416-29,421,827	5
PGRMC2	ENSBTAG00000010843	Chr17:29,872,406-29,890,867	8
LARP1B	ENSBTAG00000012135	Chr17:29,938,416-30,073,786	83
U2	ENSBTAG00000043806	Chr17:30,096,344-30,096,524	4
C4orf29 (ABHD18)	ENSBTAG00000010630	Chr17:30,106,834-30,143,868	11*
SNORA42	ENSBTAG00000042423	Chr17:30,118,538-30,118,671	1
MFSD8	ENSBTAG00000044058	Chr17:30,144,105-30,181,831	11**
PLK4	ENSBTAG00000039552	Chr17:30,185,756-30,202,777	4
SLC25A31	ENSBTAG00000012826	Chr17:30,291,318-30,319,495	2
INTU	ENSBTAG00000012824	Chr17:30,324,842-30,404,702	3
FAT4	ENSBTAG00000003345	Chr17:32,712,712-32,889,849	109

*one intron variant of the ABHD18 gene overlaps with the MFSD8 gene, for which it is an upstream gene variant.
**one downstream variant of the MFSD8 gene overlaps with the PLK4 gene, for which it is an downstream gene variant.

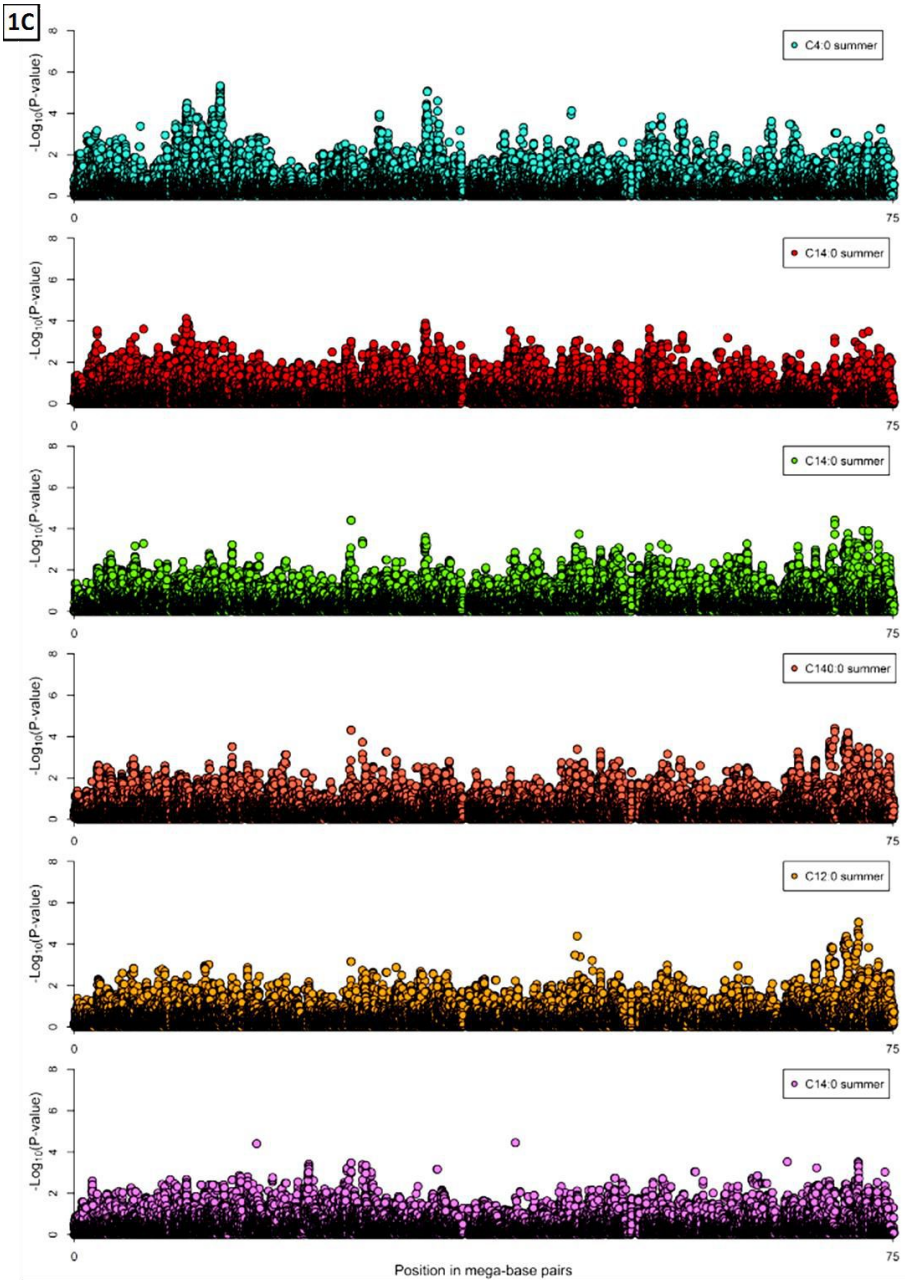
4.8 Supplementary figures



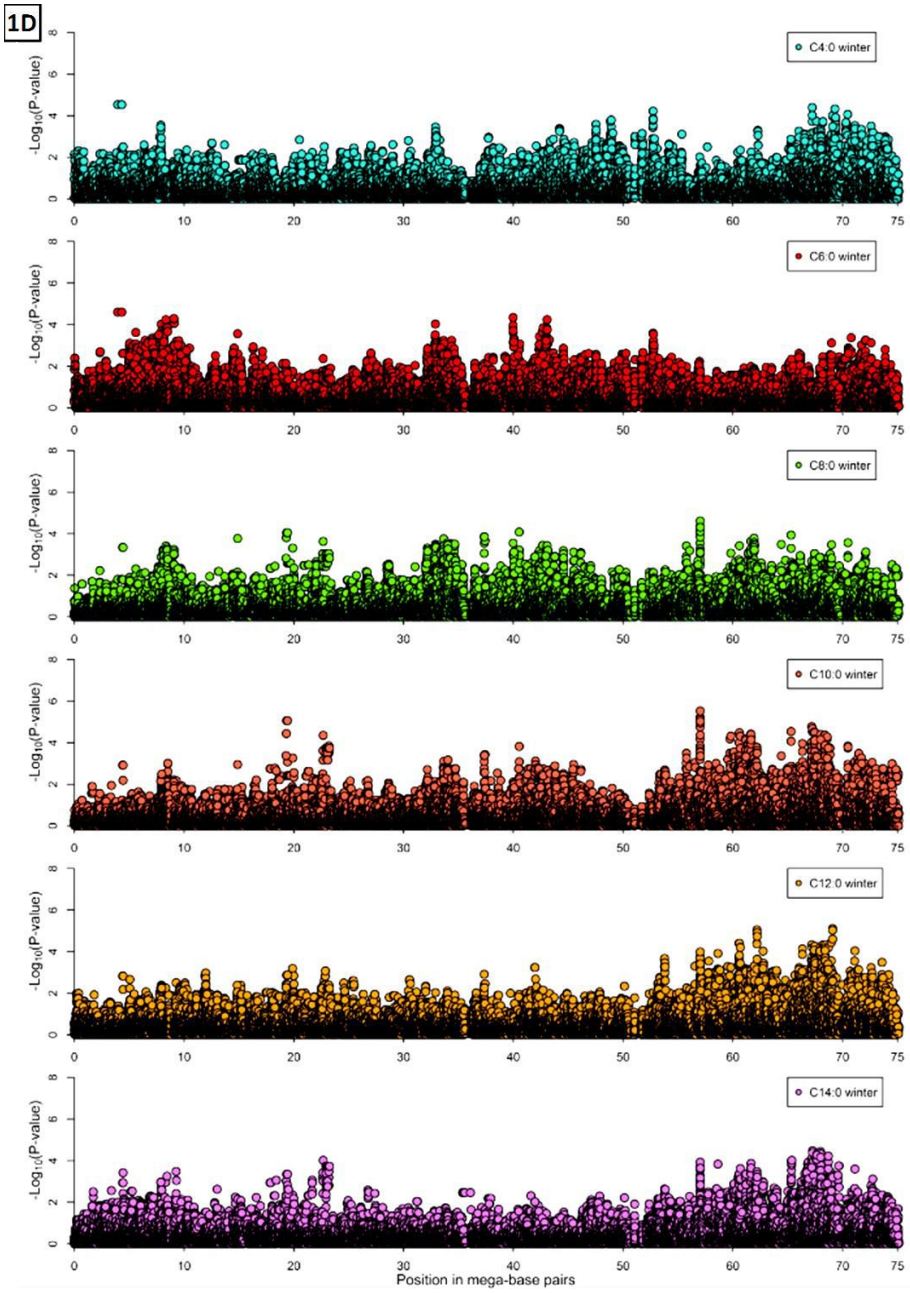
Supplementary Figure 4.1 (**A**) Fine-mapping of BTA17 with an accuracy of imputation equal and greater than 0.8 (AR2 ≥ 0.8) showing summer milk samples for 6 fatty acids.



Supplementary Figure 4.1 (**B**) Fine-mapping of BTA17 with an accuracy of imputation equal and greater than 0.8 (AR2 ≥ 0.8) showing winter milk samples for 6 fatty acids.



Supplementary Figure 4.1 (**C**) Fine-mapping of BTA17 with an accuracy of imputation equal and greater than 0.8 (AR2 ≥ 0.8) showing summer milk samples for 6 fatty acids, after fitting the SNP with the highest significance.



Supplementary Figure 4.1 (**D**) Fine-mapping of BTA17 with an accuracy of imputation equal and greater than 0.8 (AR2 ≥ 0.8) showing winter milk samples for 6 fatty acids, after fitting the SNP with the highest significance.

5

Identification of QTL on chromosome 18 associated with non-coagulating milk in Swedish Red cows

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Frontiers in Genetics: Livestock Genomics (2016) 7:57.

Abstract

Non-coagulating (**NC**) milk, defined as milk not coagulating within 40 min after rennet-addition, can have a negative influence on cheese production. Its prevalence is estimated at 18% in the Swedish Red (**SR**) cow population. Our study aimed at identifying genomic regions and causal variants associated with NC milk in SR cows, by doing a GWAS using 777k SNP genotypes and using imputed sequences to fine map the most promising genomic region. Phenotypes were available from 382 SR cows belonging to 21 herds in the south of Sweden, from which individual morning milk was sampled. NC milk was treated as a binary trait, receiving a score of one in case of non-coagulation within 40 minutes. For all 382 SR cows, 777k SNP genotypes were available as well as the combined genotypes of the genetic variants of αs1-β-κ-caseins. In addition, whole–genome sequences from the 1000Bull Genome Consortium (**Run 3**) were available for 429 animals of 15 different breeds. From these sequences, 33 sequences belonged to SR and Finish Ayrshire bulls with a large impact in the SR cow population. Single-marker analyses were run in ASReml using an animal model. After fitting the casein loci, 14 associations at $-\text{Log}_{10}(\textit{P-value}) > 6$ identified a promising region located on BTA18. We imputed sequences to the 382 genotyped SR cows using Beagle 4 for half of BTA18, and ran a region-wide association study with imputed sequences. In a 7 mega base-pairs region on BTA18, our strongest association with NC milk explained almost 34% of the genetic variation in NC milk. Since it is possible that multiple QTL are in strong LD in this region, 59 haplotypes were built, genetically differentiated by means of a phylogenetic tree, and tested in phenotype-genotype association studies. Haplotype analyses support the existence of one QTL underlying NC milk in SR cows. A candidate gene of interest is the *VPS35* gene, for which one of our strongest association is an intron SNP in this gene. The *VPS35* gene belongs to the mammary gene sets of pre-parturient and of lactating cows.

Key words: non-coagulating milk, sequences, dairy, cheese production, haplotypes, *VPS35*.

5.1 Introduction

Non- or poor-coagulating milk is an undesirable characteristic of milk with a negative influence on cheese production. Non-coagulating (**NC**) milk is prevalent among several dairy cattle breeds, such as Swedish Red (**SR**), Finnish Ayrshire (**FAY**), Holstein-Friesian (**HF**) and Italian Brown Swiss, to name a few (e.g., Frederiksen et al., 2011; Cecchinato et al., 2011, Gustavsson et al., 2014a). The prevalence of NC milk varies among these breeds ranging from 4% in Italian Brown Swiss (Cecchinato et al., 2009) up to 13% in FAY (Ikonen et al., 2004). A recent study has estimated the prevalence of NC milk, defined as milk not coagulating within 40 min after rennet-addition, at 18% in the SR cow population (Gustavsson et al., 2014a). Targeted research on NC milk can help geneticists develop breeding programs to modify milk composition and technological properties of milk and thus reduce the prevalence of NC milk.

Bittante et al. (2012) suggested that effects of herd have little influence on milk coagulation properties (**MCP**) including NC milk, although several factors can influence the composition of bovine milk (e.g., breed, a cow's diet, age of a cow, and the stage of lactation; Chilliard et al., 2001). In addition, MCP seem be influenced by many factors, such as SCC (e.g., Ikonen et al., 2004; Cassandro et al., 2008), titratable acidity (e.g., Penasa et al., 2010), casein composition (Okigbo et al., 1985b), pH (Nájera et al., 2003), stage of lactation (Okigbo et al., 1985a; Ostersen et al.,1997), and breed (e.g., Auldist et al., 2004; De Marchi et al., 2007, Bittante et al., 2012), among many other factors. Heritability estimates for MCP and NC milk range from 0.26 in FAY (Ikonen et al., 2004) to 0.45 in SR cows (Gustavsson et al., 2014a). These heritability estimates suggest that breeding could effectively reduce the prevalence of NC milk. In Sweden, the breeding program includes production traits to guarantee the increase in both protein and fat contents (Nordic Cattle Genetic Evaluation, 2013). The negative genetic correlations between NC milk and protein content estimated by Gustavsson et al. (2014a) suggest that breeding for higher protein content in the Swedish Red cows can lead to an increase in the prevalence of NC milk. In Sweden, 41% of SR cows produce milk for the dairy industry, and more than 30% of total milk production is used for cheese production (LRF Dairy Sweden, 2015). Since total milk production is about 3 million tons per year (LRF Dairy Sweden, 2015) and the market price of milk produced is about 0.28 euros per kg, the problem of NC milk affects milk with a value of approximately 63 million euros per year. Frederiksen et al. (2011) has estimated in 25% the proportion of NC milk in a batch of well-coagulating milk that is sufficient to deteriorate the MCP of well-coagulating milk.

Van Hooydonk et al. (1986) showed that the addition of calcium would restore coagulation of NC milk but not to the level of well–coagulating milk according to Hallén et al. (2010). Furthermore, addition of calcium above 0.04% have been reported to produce a bitter flavour (Schwarz and Mumm, 1948) which could be detrimental to cheese production. Therefore, it is important to the Swedish industry to reduce the frequency of NC milk.

It is well established that MCP, including NC milk, are strongly influenced by variable proportions, and genetic variants of milk protein fractions (especially of κ-casein (**CN**); Bittante et al., 2012). In poor- and non-coagulating milk samples of Danish Jerseys and HF cows, Jensen et al. (2012) showed that BB-A^2A^2-AA was the predominant combined genotype of α_{S1}-, β-, and κ-CN associated with NC milk. Hallén et al. (2007) and Gustavsson et al. (2014b) showed that some of these genotypes (especially β-, and κ-CN genotypes A^2A^2-AA) segregate in SR cows. Besides these genetic variants of milk protein fractions in the cattle genome, other undiscovered genes might play a role in the prevalence of NC milk. These genes can be identified by genome-wide association studies (**GWAS**) using high-density genotyping techniques.

High-density genotyping techniques, such as whole-genome sequences (**WGS**), can help GWAS increase the power and the precision of quantitative trait loci (**QTL**) mapping. Whole-genome sequences are expected to contain most of the polymorphisms causing the genetic differences between individuals (Meuwissen and Goddard, 2010). When an entire population is sequenced, WGS are independent of linkage disequilibrium (**LD**) between polymorphisms and the causal variant (Druet et al., 2014) compared with a lower panel of markers. However, sequencing an entire population can be expensive, and a cost-effective strategy consists of sequencing key ancestors of a population, and imputing to sequence level the rest of this population (Druet et al., 2014). To demonstrate this approach, Daetwyler et al. (2014) imputed dairy cattle populations that were genotyped with 777k SNP (**BovineHD**) to sequence level using WGS from the 1000 Bull Genome Project. Their study targeted some known genomic regions where QTL affecting milk production and curly coat had previously been identified, and they successfully identified the causal variants underlying these QTL. Therefore, GWAS using imputed sequences could assist in the identification of causal variants.

A recent GWAS on SR cows used BovineHD as genotypes and MCP as phenotypes (Gregersen et al., 2015). However, their GWAS did not include NC milk in the

analyses. The aim of our study was to identify genomic regions and causal variants associated with NC milk in SR cows. For this purpose, firstly we ran a GWAS using BovineHD genotypes to identify the most promising genomic region associated with NC milk, and secondly we fine-mapped this genomic region using imputed sequences.

5.2 Material and Methods

5.2.1 Animals and phenotypes

Morning milk samples were retrieved from 382 SR cows belonging to 21 herds in the southern part of Sweden. Cows were kept indoors, were fed according to standard practices, and were milked 2 or 3 times a day. Cows were daughters of 160 sires, and were chosen to be as genetically unrelated as possible. Cows were multiparous, ranging from 1 through 3 parturitions, and were in different lactations stages, ranging from 2.5 through 61 weeks in lactation.

Milk samples were collected in two distinct periods: April through May 2010, and September 2010 through April 2011. Directly after collection, milk samples were cooled and transported to Lund University (Lund, Sweden), where samples were defatted by centrifugation (at 2,000 x g for 30 min) to reduce the number of factors influencing coagulation properties. Fresh skimmed milk samples were preserved by adding bronopol (Sigma-Aldrich, Schnelldorf, Germany) solution of 17% wt/vol (2µL/mL), as described in Hallén et al. (2007). For rheological measurements, these milk samples were stored at +4ºC, but no longer than 3d. Skimmed milk samples were heated to 32ºC for 30min, after which chymosin (0.44mL/L Chy-Max Plus, 205 international milk clotting units (IMCU)/mL, Chr. Hansen A/S Hørsholm, Denmark) was added, and the resulting solution was gently stirred. The addition of the chymosin represented time zero. Measurements, such as rennet gel strength, rennet coagulation time, and yield stress of rennet-induced gels, were done and described by Gustavsson et al. (2014a). Some samples were unable to coagulate within 40 min after rennet-addition, and were defined as non-coagulating (**NC**) milk samples. When observed, NC milk was scored as one, while coagulating milk was scored as zero. Of the 382 cows that had available phenotypes on coagulation properties, 18% of these cows had NC milk.

5.2.2 Genotypes

A blood sample of each of the 382 SR cows was collected for genotyping purposes. These cows were genotyped for 777,963 SNP using the Illumina BovineHD BeadChip

(Illumina Inc., San Diego, CA). Quality controls of the data were performed using the R-package GenAbel (Aulchenko et al., 2007), and consisted of a minimum of 95% of non-missing SNP per called genotypes (**call rate**) and minor allele frequency (**MAF**) of a minimum of 1% for a called SNP. All SNP without a map position on the UMD 3.1 genome assembly (Zimin et al., 2009) as well as SNP on the sex chromosome were discarded. After these edits, a total of 624,302 SNP were available for further analyses.

In addition, blood samples were used to extract DNA to genotype all cows for genetic variants of αs1-, β- and κ-caseins (**CN**) using TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA), as described in Gustavsson et al. (2014b). For these variants, the assays were distinguished among the following: αs1-CN variant A, B, C, D, and F; β-CN variants A^1, A^2, A^3, B and I; and κ-CN variants A, B and E). In their study, combined genotypes were created by combining the genetic variants of αs1-β-κ-CN. These combined genotypes were used in the present study, and are referred to as "CNcluster".

Whole–genome sequences were available for 428 bulls and for 1 cow from 15 different breeds (Run 3 of the 1000 Bull Genomes consortium; Daetwyler et al., 2014), representing a multi-breed reference population. Among these sequences, 33 belonged to SR and FAY bulls with a large impact in the SR cow population. All positions of the variants on sequences were aligned to the bovine genome assembly UMD3.1 (Zimin et al., 2009). Within this multi-breed reference population, positions containing both a SNP and an indel were excluded because of possible problems with alignment and sequencing.

5.2.3 GWAS on BovineHD genotypes

Single-marker analyses were run in ASReml 4.0 (Beta version; Gilmour et al., 2009) using the following animal model:

$$y = \mu + herd + parity + wim + e^{-0.05*wim} + CNcluster + Marker + a + e \quad [1]$$

where *y* is the dependent variable; μ is the overall mean, *herd* is the covariate that describes the effect of a cow belonging to a specific herd; *parity* is the covariate that describes the effect of number of parities per cow; *wim* is the covariate that describes the effect of weeks in milk, modeled as a Wilmink curve (Wilmink, 1987); *CNcluster* is the covariate describing the effect of the combined genotypes; *Marker*

is the fixed effect of a variant genotype; a is the random effect of animal and is assumed to be distributed as $N \sim (0, \boldsymbol{G}\sigma_a^2)$, where $\boldsymbol{G}$ is the genomic relationship matrix based on 382 animals and σ_a^2 is the additive genetic variance. We calculated the G-matrix based on the BovineHD genotypes using the software calc_grm (Calus, 2013). σ_a^2 was estimated with a model excluding the effect of *Marker,* and was fixed in model 1. e is the random residual effect and is assumed to be distributed as $N \sim (0, \boldsymbol{I}\sigma_e^2)$, where **I** is the identity matrix and σ_e^2 is the residual variance.

The most promising genomic region with multiple signals at $-\text{Log}_{10}$ (P-value) ≥ 6 was imputed from the BovineHD genotypes to sequence level, and a region-wide association study (**RWAS**) was performed.

5.2.4 Imputation

Imputation started by checking the BovineHD against the sequenced reference population for inconsistencies using the Conform-gt software (http://faculty.washington.edu/browning/conform-gt.html). After this check, the 382 cows were imputed from BovineHD genotypes to sequence level for half of a chromosome using Beagle version 4.0 (Browning and Browning, 2007). Beagle version 4 was run with the following settings: 50 for phase iterations, 50 for nthreads, and 100 for imputation iterations. To account for the nature of the different variants, we ran three imputations based on different reference populations. These imputations were named as follows: “Nordic-red-specific”, “Dairy-specific”, and “Common”. For the imputation of the “Nordic-red-specific”, the reference population used consisted of the 33 sequences belonging to SR and FAY breeds. For the imputation of the “Dairy-specific”, the reference population used consisted of the 284 sequences belonging to dairy breeds (8 breeds). For the imputation of the “Common”, the reference population used consisted of 429 sequences belonging to Nordic-red, dairy and beef breeds (15 breeds). Following this approach, each variant was imputed three times based on the three different reference populations, which resulted in different imputation accuracies (Beagle allelic-r2, **AR2**) for each variant. The genotype with the highest imputation accuracy across the three imputations was selected as the best-imputed genotype.

We calculated the average concordance between the imputed genotypes across the three different scenarios of imputation, as implemented in VCFtools version 0.1.12b (Danecek et al., 2011). Subsequently, we combined the best-imputed genotypes into one data set that was used in the RWAS.

5.2.5 RWAS on imputed sequences for half a chromosome

A RWAS with imputed sequence data for the most promising region on half a chromosome was run using model 1. The imputed sequences were filtered to remove poorly imputed genotypes: only variants that were imputed with an AR2 ≥ 0.2 were included in the RWAS. Single-marker analyses were run using model 1 with one modification: the variance of the genetic effect *a* was assumed to be distributed as $N \sim (0, \mathbf{G1}\sigma_{a^*}^2)$, where **G1** is the genomic relationship matrix based on 382 animals and $\sigma_{a^*}^2$ is the additive genetic variance. The G1-matrix was calculated using the software calc_grm (Calus, 2013). The BovineHD genotypes of half chromosome that were used in the imputation to sequence level were not included in the G1-matrix calculations. $\sigma_{a^*}^2$ was calculated before the inclusion of Marker, and was fixed in model 1.

The most significant association from the first RWAS (coined **TagSNP1**) was subsequently included as a fixed effect in model 1, and a second RWAS was run. For this second RWAS, only the variants with an AR2 ≥ 0.8 were re-analyzed and considered for further analyses, such as linkage disequilibrium (**LD**) calculations and haplotype analyses.

5.2.6 Haplotype analyses

The construction of haplotypes started by selecting the SNP moderately to highly correlated with TagSNP1 (LD > 0.5). LD was calculated as the squared correlation between TagSNP1 and all other SNP using PLINK version 1.9 (Purcell et al. 2007). An LD plot was produced using the R-package ggplot2 (Wickham, 2009). Next, we combined these correlated SNP into haplotypes.

For the haplotypes, we produced a phylogenetic tree using the molecular evolutionary genetics analysis (**MEGA6**) software, version 6.0. The MEGA6 software was developed for comparative analyses of DNA and protein sequences that aim at inferring the molecular evolutionary patterns of genes, genomes, and species over time (Kumar et al. 1994; Tamura et al. 2013). To build the phylogenetic tree, we applied the Neighbor-Joining statistical method (Saitou and Nei, 1987) with a substitution model based on the proportion of nucleotide substitutions per site between nucleotides of loaded sequences. Alignment gaps and missing information gaps were accounted for with the partial-deletion option implemented in the software, and gaps were removed when the number of ambiguous sites ≥ 0.95.

Subsequently, the phylogenetic tree, all phenotypes and 2 copies of each haplotypes per cow were supplied to TreeScan software, version 1.0 (Templeton et al., 2005). TreeScan uses the phylogenetic tree built from haplotypes in phenotype-genotype association studies. With its iterative approach, TreeScan cuts in two parts a branch of the phylogenetic tree. For part 1, all haplotypes are grouped, and treated as a single allele, say A. For part 2, all haplotypes are grouped, and treated as a single allele, say B. These alleles allow different combinations of genotypes: AA, AB and BB. Subsequently, associations between phenotypes and genotypes (AA, AB and BB) are statistically tested with the F-statistics of a one-way ANOVA. This iterative approach is repeated until all the branches of the phylogenetic tree have been tested. The null hypothesis considered for the inference of branches (i.e., haplotypes) is of no association between a partition and the trait of interest, which in our case was NC milk. In addition, the following settings were used in TreeScan: the number of simulations to obtain P-values for the ANOVA tests p=5,000; the significance level α =0.05, and the minimum number of individuals required in each observed genotypic class c=2. A bipartition was considered as significantly associated to NC milk at P-value <0.05.

5.2.7 Bioinformatics and candidate genes

We used the variant effect predictor (**Ve!P**) online tool (at http://www.ensembl.org/info/docs/tools/vep/index.html; McLaren et al., 2010) to determine the effect of the variants (SNPs, insertions, deletions, CNVs or structural variants) on genes, transcripts, and protein sequence, as well as regulatory regions.

5.3 Results

5.3.1 GWAS on BovineHD genotypes

The GWAS on BovineHD genotypes identified many significant SNP associated with NC milk after fitting the casein loci (Supplementary Figures 5.1A, 5.1B, 5.1C, and 5.1D). The accompanying QQ-plot indicated that a small proportion of SNP were deviating from the x=y line. This smaller proportion of SNP represented the most likely associated SNP among the thousands of non-associated SNP with NC milk. In addition, no important deviations from the x=y line were observed, suggesting no obvious signs of population stratification (Supplementary Figure 5.2). Fourteen of the many significant associations had $-\text{Log}_{10}$(Pvalue) larger than 6, and they are located on BTA11, BTA13 and BTA18 (Table 5.1). The most promising region was located on BTA18, and was distributed over a region of 7 mega base-pairs (**MBP**). Because BTA18 showed the most significant association with NC milk after fitting the

casein loci, we focused on this chromosome by running a RWAS using imputed sequence data.

Table 5.1 Most significant SNP from genome-wide association study with NC milk† based on BovineHD genotypes in 382 Swedish Red cows.

Chromosome	SNP	position	-Log$_{10}$(Pvalue)	σ^2_{marker} §	$\sigma^2_{marker}/\sigma^2_p$ *
11	rs136987882	55787730	6.29	0.01	0.07
13	rs136185829	47744740	6.15	0.01	0.07
13	rs109492822	47749851	6.15	0.01	0.07
13	rs134756836	47754335	6.15	0.01	0.07
18	rs137544086	9179722	6.19	0.01	0.07
18	rs41865365	11166809	8.77	0.01	0.09
18	rs110267892	13136171	6.65	0.01	0.07
18	rs109208214	13934856	10.18	0.02	0.11
18	rs135171892	13939170	10.18	0.02	0.11
18	rs137827420	13943440	10.18	0.02	0.11
18	rs137429187	13960525	10.18	0.02	0.11
18	rs132908573	13967910	10.18	0.02	0.11
18	rs110637786	15017982	9.35	0.01	0.10
18	rs110615481	15047675	6.54	0.01	0.08

†NC milk as binary trait where 0 = coagulating milk and 1= non-coagulating milk

§σ^2_{marker} = marker's variance, computed for each marker as 2 times major allele frequency times minor allele frequency times the square of the allele substitution effect

* $\sigma^2_{marker}/\sigma^2_p$ = proportion of phenotypic variance explained by a marker

5.3.2 Imputation for half of BTA18

Before imputation, the inconsistencies between the BovineHD genotypes and the sequence data were strand problems (i.e., 1 for Nordic-red-specific, 815 for Dairy-specific, and 927 for commons), and 7 SNP from the BovineHD genotypes not present in the sequence data. All these inconsistencies were set to missing in the BovineHD data, and imputed.

After imputation, the total number of variants in the region between 0-30 MBP on BTA18 increased from 7,873 SNP on the BovineHD to 562,432 variants on the sequence level, representing an increase of 71 times in the total number of variants. From the 562,432 imputed variants, 69.3% were monomorphic (MAF=0), 24.5% were polymorphic and AR2 ≥ 0.2, and 14.3% were polymorphic and AR2 ≥ 0.8 (Table 5.2). After filtering out the monomorphic variants, 137,949 polymorphic variants imputed with an AR2 ≥ 0.2 were left. This is an increase of more than 17 times in the total number of variants from BovineHD genotypes (N=7,873 SNP) to sequence level (N=137,949 sites). These 137,949 variants originated from the three scenarios as

Table 5.2 Distribution of the average accuracy of imputation (AR2) per ranges of minor allele frequency (MAF), and the number of markers (as counts and in percentage) for the three scenarios of imputation

MAF	AR2[†]	Nordic-red specific		Dairy-specific		Common		Total number of variants	
		average AR2	N1[§]	average AR2	N2[*]	average AR2	N3[c]	N[¥]	(%)
0	All	0.00	389,518	0.00	94	0.00	54	389,666	69.3%
	≥ 0.2	-	0	0.31	4	0.31	0	4	0.0%
	≥ 0.8	-	-	-	-	-	-	-	-
0-0.1	All	0.42	28,346	0.37	17,720	0.34	9,547	55,613	9.9%
	≥ 0.2	0.72	16,772	0.62	11,266	0.59	5,861	33,899	6.0%
	≥ 0.8	0.94	9,467	0.91	2,748	0.90	1,082	13,297	2.4%
0.1-0.2	All	0.69	23,425	0.63	6,951	0.61	2,774	33,150	5.9%
	≥ 0.2	0.76	20,922	0.70	6,136	0.67	2,462	29,520	5.2%
	≥ 0.8	0.94	12,994	0.92	2,329	0.91	761	16,084	2.9%
0.2-0.3	All	0.75	22,765	0.70	4,593	0.68	1,467	28,825	5.1%
	≥ 0.2	0.80	21,235	0.75	4,075	0.73	1,291	26,601	4.7%
	≥ 0.8	0.94	14,609	0.92	2,049	0.92	412	17,070	3.0%
0.3-0.4	All	0.78	21,900	0.74	4,422	0.71	1,205	27,527	4.9%
	≥ 0.2	0.83	20,429	0.80	3,759	0.78	991	25,179	4.5%
	≥ 0.8	0.95	15,189	0.93	2,186	0.92	392	17,767	3.2%
0.4-0.5	All	0.67	22,731	0.62	3,854	0.59	1,066	27,651	4.9%
	≥ 0.2	0.83	18,794	0.79	3,154	0.78	798	22,746	4.0%
	≥ 0.8	0.95	13,937	0.93	1,779	0.92	272	15,988	2.8%
Total	All	0.55	508,685	0.51	37,634	0.49	16,113	562,432	100.0%
	≥ 0.2	0.79	98,152	0.73	28,394	0.71	11,403	137,949	24.5%
	≥ 0.8	0.94	66,196	0.92	11,091	0.91	2,919	80,206	14.3%

[†]where: All considers all imputed animals, ≥0.2 considers animals imputed with an AR2 equal and higher than 0.2, and ≥ 0.8 considers animals imputed with an AR2 equal and higher than 0.8. [§]N1 = total number of markers for the Nordic-Red specific scenario. [*]N2 = total number of markers for the Dairy-specific scenario. [c]N3= total number of markers for the Common scenario. [¥]N= sum of markers for all three imputation scenarios (N1+N2+N3).

follows: 98,152 variants from the Nordic-red-specific scenario, plus 28,394 variants from the Dairy-specific scenario, plus 11,403 variants from the common scenario. In addition, the 98,152 variants from the Nordic-red-specific scenario are composed of 91,363 SNP, 6,753 indels, and 36 multi-allelic variants. The 28,394 variants from the Dairy-specific scenario are composed of 27,113 SNP, 1,253 indels, and 28 multi-allelic variants. The 11,403 variants from the common scenario are composed of 10,989 SNP, 401 indels, and 13 multi-allelic variants.

The average concordance was calculated by comparing genotypes imputed in the three different scenarios, and reported sites were alleles in exact match between files. Results indicated that 97.0% of the imputed genotypes from the Nordic-red-specific scenario were concordant with the Dairy-specific scenario; 96.8% of the imputed genotypes from the Nordic-red-specific scenario were concordant with the common scenario; and 98.9% of the imputed genotypes from the Dairy-specific scenario were concordant with the common scenario.

5.3.3 RWAS on imputed sequences for half of BTA18

A RWAS based on imputed sequences was run for half of BTA18, which corresponds to a genomic region of 30 MBP running from position 0 on bovine genome built UMD 3.1. Throughout this region, a total of 205 variants were significantly associated with NC milk at $-Log_{10}(Pvalue) > 6$ and imputed with AR2 ≥ 0.8 (Supplementary Table 5.1). The most significant variants were 1 indel and 2 SNP. The indel was rs385975260 occurring at 15.03 MBP, and was imputed with AR2 = 0.87. The first SNP was rs525335650 located at 15.03 MBP, and was imputed with AR2 = 0.87. The second SNP was rs379827811 located at 15.04 MBP, and was imputed with AR2 = 0.42. These 2 SNP and 1 indel are in perfect LD with each other. We chose rs525335650 among these three imputed variants, since it was the best imputed variant, and tagged it as TagSNP1 (Figure 5.1A).

After including TagSNP1 as a fixed effect in model 1, a total of 80,206 variants with an AR2 ≥ 0.8 were re-analyzed. We re-analyzed these 80,206 imputed variants instead of the 137,949 imputed variants to reduce potential false-positive associations with NC milk caused by imputation errors. After accounting for TagSNP1 in model 1 as fixed effect, no remaining associations were found (Figure 5.1B).

5.3.4 Haplotype analyses

A total of 129 SNP plus 17 indels in LD with TagSNP1 (Figure 5.2) were combined into 59 haplotypes. These 59 haplotypes were the basis to build a phylogenetic tree, for

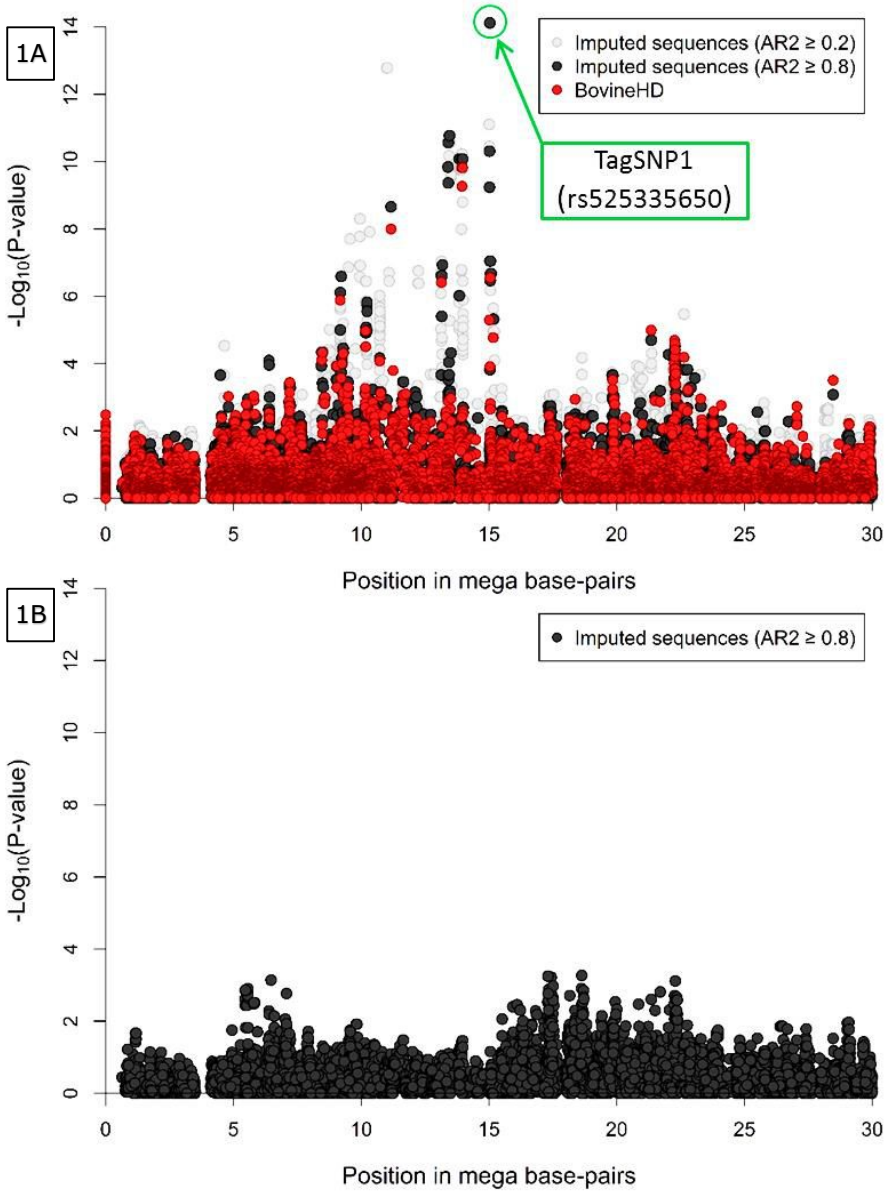

Figure 5.1 Region-wide association study (**RWAS**) with non-coagulating (**NC**) milk in 382 Swedish Red cows. **Figure 5.1A** RWAS based on 137,949 polymorphic imputed variants overlaid with the BovineHD genotypes for half of BTA18. In light gray, imputed variants with accuracy of imputation (AR2) ≥ 0.2. In black, imputed variants with AR2 ≥ 0.8. "TagSNP1" as most significant association. **Figure 5.1B** RWAS after correcting for TagSNP1. In black, imputed variants with AR2 ≥ 0.8 (N=80,206 variants).

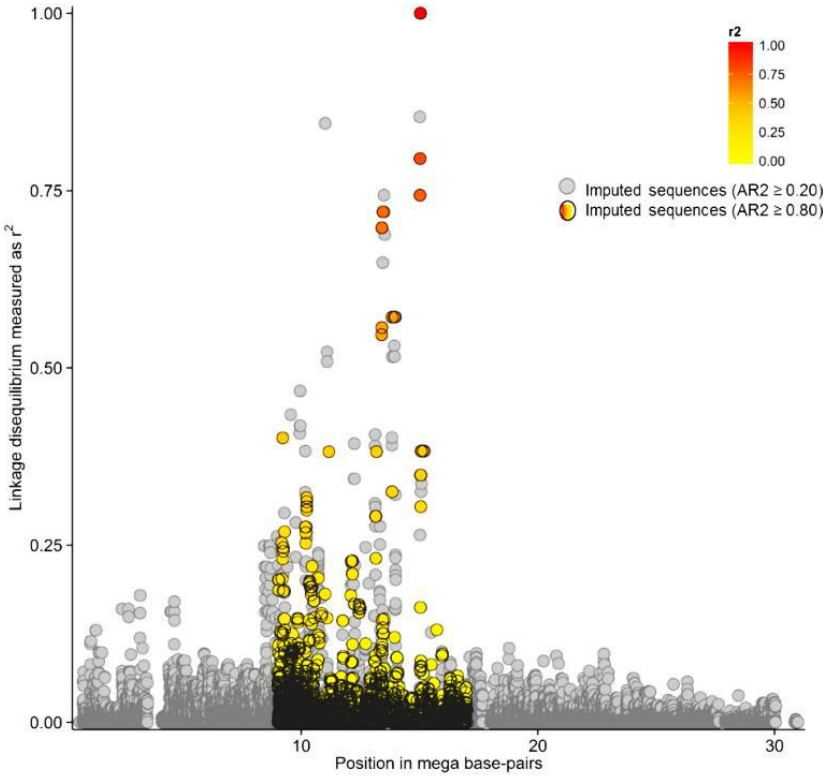

Figure 5.2 Linkage disequilibrium in the QTL region. In the colored region, pairwise linkage disequilibrium as the squared correlation between the most significant association, "TagSNP1", and all other markers. In light gray, imputed variants with accuracy of imputation (AR2) ≥ 0.2. In black, imputed variants with AR2 ≥ 0.8.

which each branch represented one unique haplotype segregating in the SR cow population (Figure 5.3A). The iterative inference of haplotypes using TreeScan occurred by, for example, cutting the phylogenetic tree in two parts at branch "A", where haplotypes 38 and 58 were grouped in one part, while all other haplotypes were grouped in the other part. The parts were then tested against each other. After all branches of the tree were tested, associations with NC milk were: branch "A" at P-value = 0.002; haplotype 38 at P-value = 0.03; and, haplotype 58 at P-value =0.03 (Figure 5.3A). Next, we scrutinized in depth the sequences of haplotypes 38 and 58, and we found they have 3 SNP in common. When comparing haplotypes 38 and 58 with haplotypes 13, 20, 29 and 39 (Figure 5.3B), haplotypes 38 and 58 differed from the other haplotypes at these exact same 3 SNP. Interestingly, these 3 SNP shared by haplotypes 38 and 58 are quite close to our TagSNP1 (Figure 5.3B).

5.3.5 Bioinformatics and candidate genes

According to Ve!P, the 129 SNP plus the 17 indels, which included our TagSNP1, were distributed as follows: 32% of intron variants; 26% of downstream gene variants, 25% of upstream gene variants; 12% of intergenic variants; 4% of 3'UTR variants; 1% of synonymous variants, and 1% of missense variants. In summary, 67% of these 129 SNP plus 17 indels were synonymous variants without changes to the encoded amino acids. The remaining 33% were missense variants with changes in one or more bases to the encoded amino acid.

In addition, Ve!P showed that our QTL region on BTA18 contains 7 genes (Table 5.3), of which 1 is a validated gene and 6 are provisional genes. These 7 genes are: validated carbonic anhydrase VA, mitochondrial (***CA5A***) gene; BTG3 associated nuclear protein (***BANP***) gene; cytochrome b-245, alpha polypeptide (***CYBA***) gene; the mevalonate (diphospho) decarboxylase, mRNA (***MVD***) gene; snail family zinc finger 3 (***SNAI3***) gene; ring finger protein 166 (***RNF166***) gene; and, vacuolar protein sorting 35 homolog, mRNA (***VPS35***) gene. In addition, the *CA5A* gene is located within a copy number variation.

Table 5.3 Details about candidate genes identified in the QTL region

Genes	Identifier	Location	Numbers of variants
CA5A	ENSBTAG00000010151	chr18:13,356,215-13,445,854	8
BANP	ENSBTAG00000023745	chr18:13,425,303-13,493,366	3
CYBA	ENSBTAG00000003895	chr18:13,931,107-13,938,075	40
MVD	ENSBTAG00000012059	chr18:13,938,827-13,945,489	72*
SNAI3	ENSBTAG00000017528	chr18:13,958,995-13,964,622	36**
RNF166	ENSBTAG00000020942	chr18:13,969,303-13,977,633	3
VPS35	ENSBTAG00000002493	chr18:15,038,821-15,066,463	2

*40 of these 72 variants in the *MVD* gene overlap with variants in the *CYBA* gene. These are: 26 downstream variants in the *MVD* gene corresponding to 16 introns, 1 synonymous, and 9 upstream variants in the *CYBA* gene; and seven 3' UTR, 1 synonymous, 5 intron, and 1 missense variants in the *MVD* gene corresponding to upstream variants in the CYBA gene.
**5 of these 36 hits are downstream gene variants in the *SNAI3* gene that correspond to upstream gene variants in the *RNF166* gene.

The genomic position of the 3 strongest associations with NC milk on BTA18 are shown in Supplementary Figure 5.3A. Of these associations, rs379827811 is an intron variant in the *VPS35* gene. According to Ve!P, rs379827811 is upstream to 14 missense variants, 1 synonymous variant, 1 stop gained variant and 1 splice region variant (Supplementary Figure 5.3B).

The 3 SNP shared by haplotypes 38 and 58 identified in the haplotype analyses are intergenic variants located between 20.5 and 31.2 kilo base-pairs (**kbp**) downstream to the *VPS35* gene.

5.4 Discussion

In the present study, we used the same phenotypes and BovineHD genotypes as in Gustavsson et al. (2014a) to perform a GWAS with NC milk, and we further fine-mapped a genomic region on half of BTA18 using imputed sequences. This genomic region is distributed over 7 MBP on BTA18, and is strongly associated with NC milk. At least one QTL could be fine-mapped using imputed sequences. In addition, we conducted haplotype analyses to disentangle the occurrence of multiple QTL in strong LD within this region. At last, we identified potential candidate genes within this QTL region.

5.4.1 GWAS on BovineHD genotypes

The GWAS on BovineHD genotypes showed significant associations with NC milk distributed over 7 mega base-pairs (**MBP**) on BTA18 (Table 5.1). These 7 MBP explain large fractions of the phenotypic variation in NC milk, ranging from 7% to 11%. Tyrisevä et al. (2008) performed a genome scan to map non-coagulation of milk in 477 genotyped FAY cows. Their study used microsatellite markers and identified a QTL located around 17 MBP on BTA18. Their QTL is very close to the 7 MBP region identified in our study. The methodology used by Tyrisevä et al. (2008) is different from the present study. It is important to note that the study by Tyrisevä et al. (2008) is based on a linkage study within sire families with pooled DNA of cows with extreme phenotypes, and our study is based on an association analysis of genotyped cows with scored phenotypes. Both methodologies have the common goal of pointing out the potential candidate genes associated with a trait of interest, and, despite the differences between both studies, a similar genomic region was associated with NC milk.

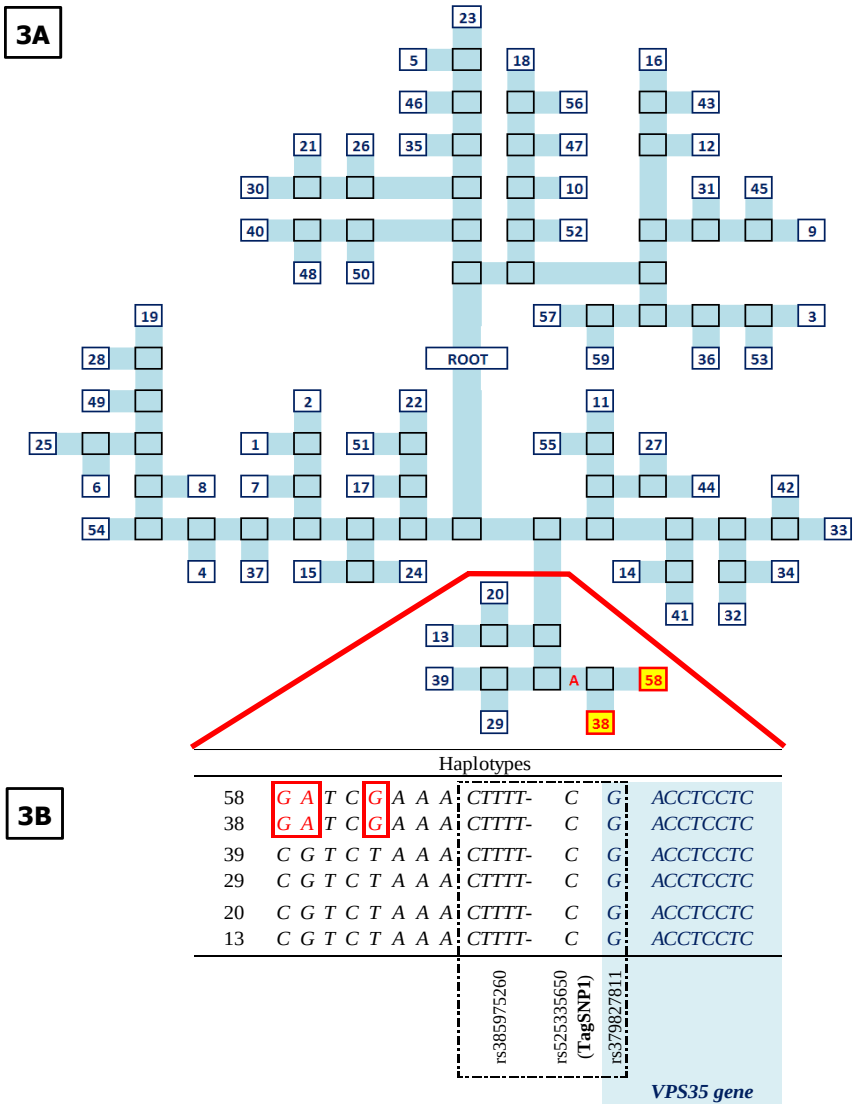

Figure 5.3 Haplotypes analyses characterizing the QTL region in SR cows. **Figure 5.3A** Phylogenetic tree of the 59 unique haplotypes, numbered in blue. In light blue, a branch of the tree. In black borders, bipartitions. In red and yellow, significant haplotypes at P-value <0.05. **Figure 5. 3B** relevant part of the sequences of significant versus other haplotypes. In red, differences between haplotypes. Dashed in black, strongest associations including TagSNP1. In light blue, the *VPS35* gene.

Eleven significant associations found by our GWAS were in agreement with associations found by the GWAS of Gregersen et al. (2015), who studied MCP properties but not NC milk. This agreement occurred with the following two traits: rennet gel strength measured 30 minutes after chymosin addition (**G'30**), and rennet coagulation time (**CTrennet**). For G'30, associations agreed on BTA1, BTA13, BTA18, and BTA22. More specifically, these associations were: 4 SNP located between 70.75 and 70.90 MBP on BTA1; 5 SNP located between 58.10 and 58.14 MBP on BTA13; 1 SNP at 13.13MBP on BTA18; and 1 SNP located at 19.35 MBP on BTA22. The strong negative genetic correlation between NC milk and G'30 (-0.82; Gustavsson et al., 2014a) is likely to explain the agreement of results between both studies regarding G'30. For CTrennet, associations agreed on BTA18, and these were: 1 SNP located at 11.16 MBP, and 1 SNP located at 11.65 MBP. Gregersen et al. (2015) used (log-transformed) CTrennet in their GWAS, whereas we analyzed NC milk, a trait derived from CTrennet. Despite the use of different but CTrennet-related traits, it was unexpected to find only two associations in agreement between both studies. A reason for this little agreement might be caused by our approach to analyze NC milk, which dealt with the right-censored nature of coagulation time in a more suitable way (Cecchinato and Carnier, 2011).

An important aspect of our GWAS on BovineHD genotypes was the analyses of NC milk as a normally distributed trait despite its binary nature. Cecchinato and Carnier (2011) were the first authors to suggest this approach because NC milk samples have been consistently excluded from most analyses when observed (e.g., Ikonen et al., 2004; Gregersen et al., 2015). Cecchinato and Carnier (2011) showed that statistical models have difficulties to correctly account for NC milk, and suggested to score NC milk as a binary trait and include it as a normally distributed trait in linear mixed models. This option allows for analyses of NC milk without the exclusion of information. Following this approach, Gustavsson et al. (2014a) included NC milk as a binary trait in their analyses, and estimated genetic parameters for rennet-induced coagulation properties in SR cows. In addition, the inclusion of NC milk as a binary trait in our study could be one of the reasons why little overlap was found with the study by Gregersen et al. (2015) regarding CTrennet.

Besides their GWAS, Gregersen et al. (2015) found a suggestive QTL for the log-transformed G'30 trait by haplotypes analyses. This suggestive QTL was found in the interval located between 11.65 and 22.34 MBP on BTA18. Although not significant in their study, this suggestive QTL interval is in agreement with 9 out of 10 of our most significant SNP associated with NC milk on BTA18 (Table 5.1). In addition, the top

SNP indicated by Gregersen et al. (2015) at 11.16 MBP is among our most significant SNP associated with NC milk.

Breeding for higher protein content in SR cows might lead to problems in the foreseeable future, suggested by the moderate, yet unfavorable genetic correlation between NC milk and protein content (Gustavsson et al., 2014a). Our main goal was to disentangle the effects of genetic variants of milk protein fractions from other genetic variants associated with NC milk. For this reason, we included a multi-locus genotype that combined the genetic variants of the main milk protein fractions (i.e., αs1-β-κ-CN; "**CNcluster**") in our model. Bittante et al. (2012) reviewed the most important genetic factors that affect MCP, indicating that MCP, including NC milk, are strongly influenced by variable proportions, and genetic variants of milk protein fractions (especially of κ-CN). These milk protein fractions, mainly representing caseins, are encoded on BTA6 and thus, the recombination among alleles is small (Bittante et al., 2012). In contrast, Tyrisevä et al. (2008) did not find significant associations between NC milk and the casein loci. In the present study, the casein loci were included as part of the design of our GWAS with NC milk, resulting in significant associations that are independent from the casein loci. This means that genes found by our study represent a new set of genes compared with the genes of the casein loci known to influence the prevalence of NC milk (e.g., Jensen et al., 2012; Gustavsson et al., 2014b).

5.4.2 Imputation

Imputation of SR cows was quite challenging because most of the variants were poorly imputed at sequence level when directly using the 429 WGS as reference population. As pointed out by Bouwman and Veerkamp (2014), breed-specific variants are best imputed by using a large single-breed reference population. This suggestion would mean that only 33 out of the 429 WGS would be of interest to impute our 382 SR cows to sequence level. The challenge of imputing a small breed like SR was overcome by running three different scenarios of imputation, and each time with a different reference population. The genotype that had the best imputation accuracy across the three scenarios was selected as the best-imputed genotype. The average accuracies of imputation using our approach were 0.79 for variants imputed with AR2 ≥ 0.2, and 0.93 for variants imputed with AR2 ≥ 0.8. While this is a slightly ad-hoc approach, there was good concordance between the three imputation scenarios and our subsequent focus on variants with AR2 ≥ 0.8 adds further rigor to our analyses.

5.4.3 RWAS on imputed sequences for half of BTA18

The RWAS on imputed sequences for half of BTA18 revealed many significant associations with NC milk (Supplementary Table 5.1). One of our three strongest associations, TagSNP1, explained almost 34% of the genetic variation and 14% of the phenotypic variance in NC milk (Figures 5.1A and 5.1B). This large fraction of genetic variance explained by TagSNP1 is independent of the casein loci on located on BTA6. Altogether, these findings strongly suggest the existence of at least one causal variant in our focus region distributed over 7 MBP associated with NC milk. It might be plausible that one causal variant, i.e., 1 QTL is associated with NC milk in our focus region, although we cannot exclude the presence of multiple QTL in strong LD associated with NC milk in our focus region. Similar findings were found by Daetwyler et al. (2014) and Sahana et al. (2014). In their GWAS with imputed sequences, the considerable number of significant variants closely linked to each other increased the complexity of identifying a causal variant. In our study, we performed haplotype analyses to answer whether one or multiple QTL were present in the 7 MBP.

5.4.4 Haplotype analyses

Among the many advantages of haplotype over single-variant analyses (Balding, 2006), two of them are: a) haplotype analyses naturally account for the correlated structure between variants because all the genetic variation in a population is transmitted from parent to offspring through haplotypes (Clark, 2004); and b) haplotype analyses reduce the number of parameters tested in association studies as compared with single-variant analyses (e.g., Clark, 2004; Balding, 2006). In contrast, a "tagging" strategy would reduce the power gained from using haplotypes *per se* (Balding, 2006). In our study, this limitation was dealt with by using the TreeScan approach (Templeton et al., 2005). TreeScan considered two aspects simultaneously: the correlated structure of variants closely linked to each other, and the origin of this haplotype in the population through a phylogenetic tree. Using the TreeScan approach, 2 out of the 59 haplotypes were found to be associated with NC milk in our QTL region (Figure 5.3A). The two significant haplotypes had 3 SNP in common, and these SNP are located from 13.7 to 24.4 kbp apart from TagSNP1 (Figure 5.3B). These findings support the presence of one QTL influencing NC milk in our focus region. Nonetheless, the task of identifying the causal variant remains challenging. According to Vasemägi and Primmer (2005), when an association between TagSNP1 and the causal variant is found, other linked associations can be responsible for the variation in the trait of interest. This might be our case since TagSNP1 was one out of three closely linked variants strongly associated with NC milk.

5.4.5 Bioinformatics and candidate genes

Our three strongest association with NC milk are composed of 1 indel and 2 SNP. One of the 2 SNP (rs379827811) is an intron variant located between 15.04 MBP within the *VPS35* gene (Figures 5.3B, Supplementary Figure 5.3A, and Supplementary Figure 5.3B). In humans, the *VPS35* gene is a component of the retromer complex that mediates endosome-to-Golgi retrieval of membrane proteins such as the cation-independent mannose 6-phosphate receptor. According to Malik et al. (2015), cargo-selective sorting is important for the correct sub-cellular destination of membrane proteins. The retromer complex mediated by *VPS35* gene seems to promote the recycling of specific membrane proteins, such as β2-adrenergic receptor and the glucose transporter *GLUT1*, directly back to plasma membrane (Seaman et al., 2013). It is important to mention that *GLUT1* is the major glucose transporter in the basal membrane of epithelial cells and, in the mice mammary gland, its expression was increased when greater demand for glucose for the synthesis of lactose was needed (Anderson et al., 2007). If the recycling mechanism of the retromer complex is defective, it is possible that not enough membrane proteins are recycled, and in turn, are not available for milk synthesis.

A mutation in the *VPS35* gene has been associated with Parkinson's disease (Zavodszky et al., 2014). In mice-models for Parkinson's disease, a *VPS35* deficiency could contribute to retinal ganglion neuro-degeneration, leading to the blindness of many retinal degenerative disorders (Liu et al., 2014). In addition, Lemay et al. (2013) shows that the *VPS35* gene is expressed throughout lactation in humans, which include colostrum, transitional, and mature milk, after they sequenced the mRNA found in milk fat layer. In Arabidopsis, the *VPS35* gene has been associated with protein sorting and is involved in the plant growth and leaf senescence (Yamazaki et al., 2008). In addition, Munch et al. (2015) shows that a dysfunction in the *VPS35* gene can contribute to immune-associated cell death in Arabidopsis. In cattle, Lemay et al. (2009) classified the mammary gene sets according to their condition and their developmental specific-stage, and showed that the *VPS35* gene belonged to the mammary gene sets of pre-parturient and of lactating cows. The VPS35 gene has not been associated to non-coagulating milk yet.

5.5 Conclusions

The GWAS on BovineHD genotypes found significant associations with NC milk distributed over 7 MBP on BTA18 for SR cows. These 7 MBP contained 14 SNP that

explained from 7% to 11% of phenotypic variation in NC milk. This large proportion of explained phenotypic variance is independent of the casein loci. To further characterize these 7 MBP, we ran a region-wide association study with imputed sequences. The significance of the associations increased from $-Log_{10}$(P-value) =10.18 on BovineHD genotypes to $-Log_{10}$(Pvalue) = 14.12 on imputed sequences. NC milk in SR cows was influenced by at least one QTL within these 7 MBP. A haplotype analyses identified 2 haplotypes that differed from the other 57 haplotypes at 3 SNP. These 3 SNP were located near to the strongest association identified by the region-wide association study with imputed sequences. For BTA18, haplotype analyses support the existence of one QTL underlying NC milk in SR cows. A candidate gene of interest is the *VPS35* gene, for which one of our strongest association is an intronic SNP in this gene. It has been suggested that the VPS35 gene is involved in the recycling of specific membrane proteins, such as β2- adrenergic receptor and the glucose transporter GLUT1. The *VPS35* gene belongs to the mammary gene sets of pre-parturient and of lactating cows, and has not been associated to NC milk yet.

5.6 Author Contributions

MG and MP coordinated the data collection and analysis of milk samples. MG, MP, WFF & DJK designed and supervised the study. SID, DJK & WFF analyzed the data and interpreted the results. SID, MG, DJK, MP, WFF wrote the manuscript. All authors revised and accepted the final version of the manuscript.

5.7 List of abbreviations

AR2 – beagle's accuracy of imputation;
BovineHD – 777,963 SNP genotypes
BTA – *Bos taurus* autosome
CN – caseins
CTrennet - rennet coagulation time
FAY – Finnish Ayrshire
G'30 – rennet gel strength measured 30 minutes after chymosin addition
GWAS – genome-wide association study
LD – linkage disequilibrium
MAF – minor allele frequency
MBP – mega base-pair
MCP – milk coagulation properties

NC – non-coagulating
QTL - quantitative trait loci
RWAS – region-wide association study
SR - Swedish Red
TagSNP1- most significant association retrieved from RWAS
Ve!P – variant effect predictor
WGS – whole-genome sequences

5.8 Acknowledgements

SID currently benefits from a joint-grant from the European Commission [within the framework of the Erasmus-Mundus joint doctorate "EGS-ABG" (Paris, France) and Breed4Food (a public-private partnership in the domain of animal breeding and genomics and CRV]. Further, the authors wish to thank the Swedish Farmer's Foundation for Agricultural Research (SLF), Stockholm, Sweden for financial support as well as Dr. Frida Gustavsson, Lund University, Sweden for milk collection and analyses of coagulation data. DJK & WFF acknowledges Mistra Biotech, a research program financed by Mistra – Stiftelsen för miljoöstrategisk forskning and SLU.

5.9 Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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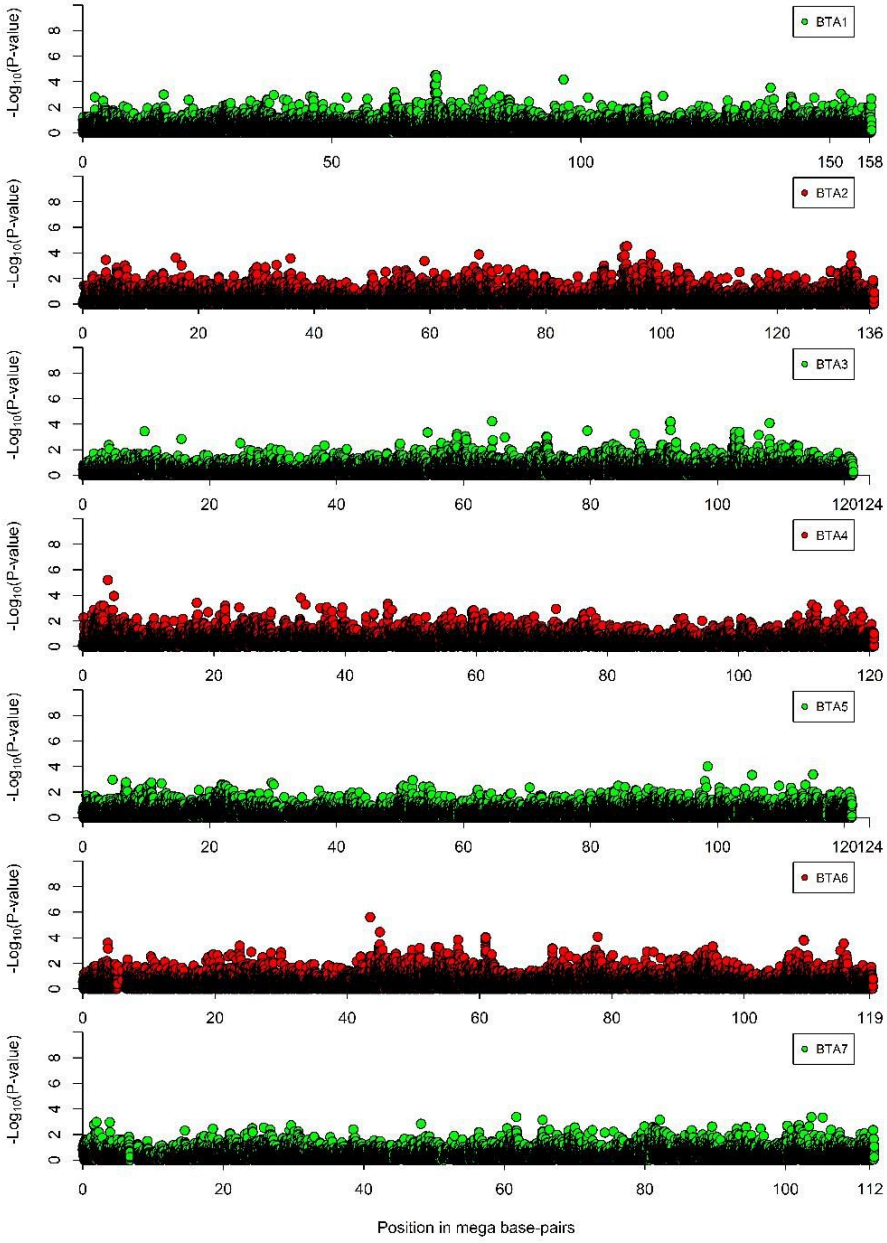
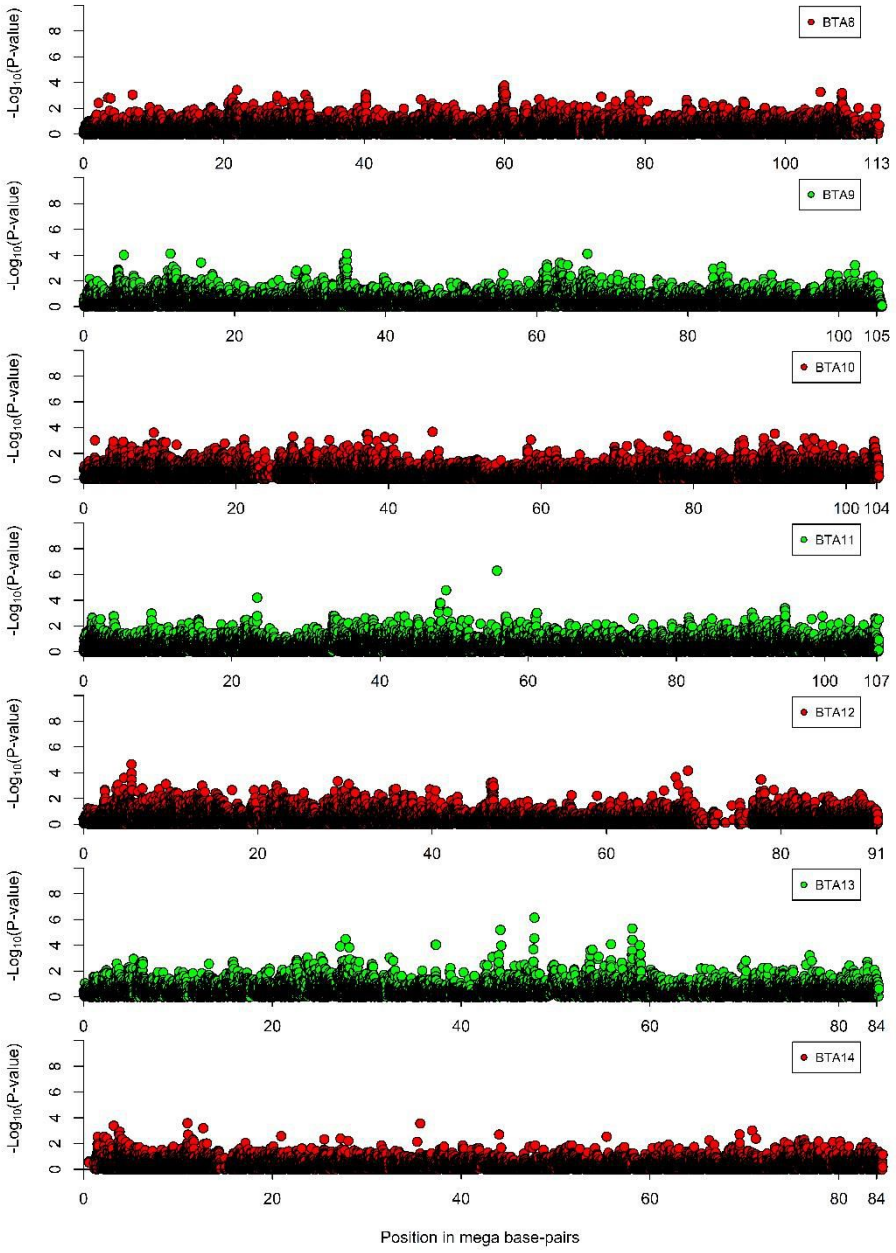
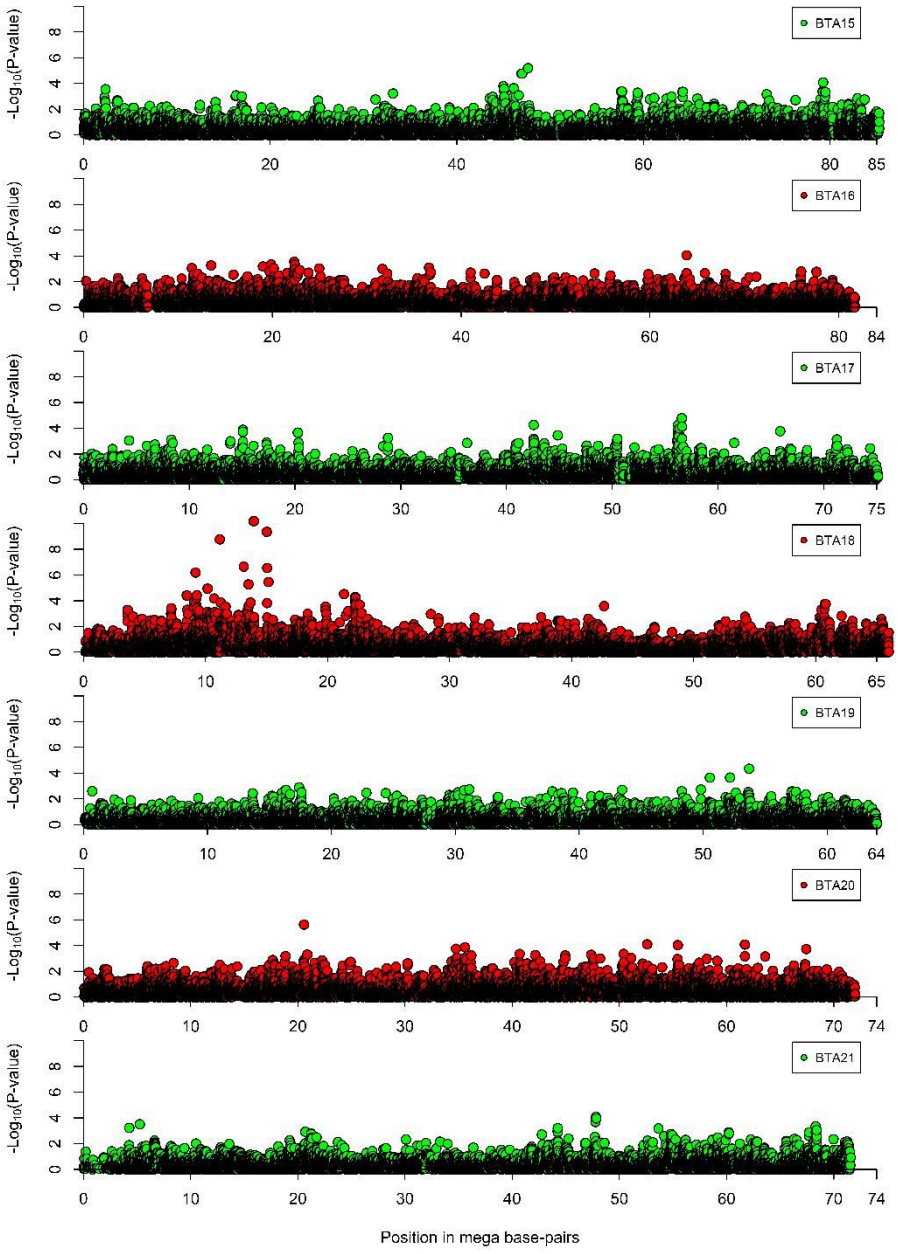
5.11 Supplementary Files



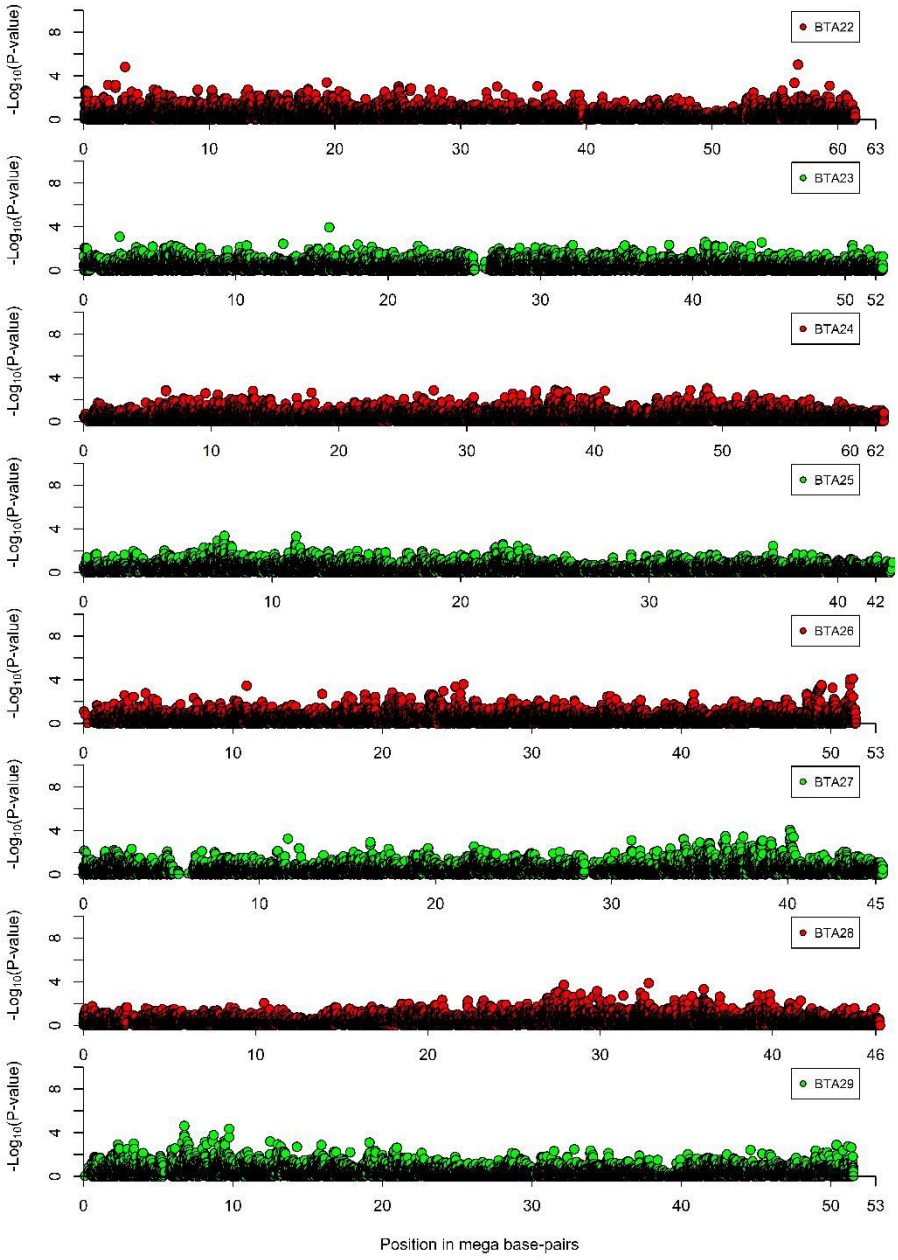
Supplementary Figure 5.1 A Genome-wide association study using 777,963 SNP genotypes affecting non-coagulating milk in Swedish Red cows for BTA1 through BTA7



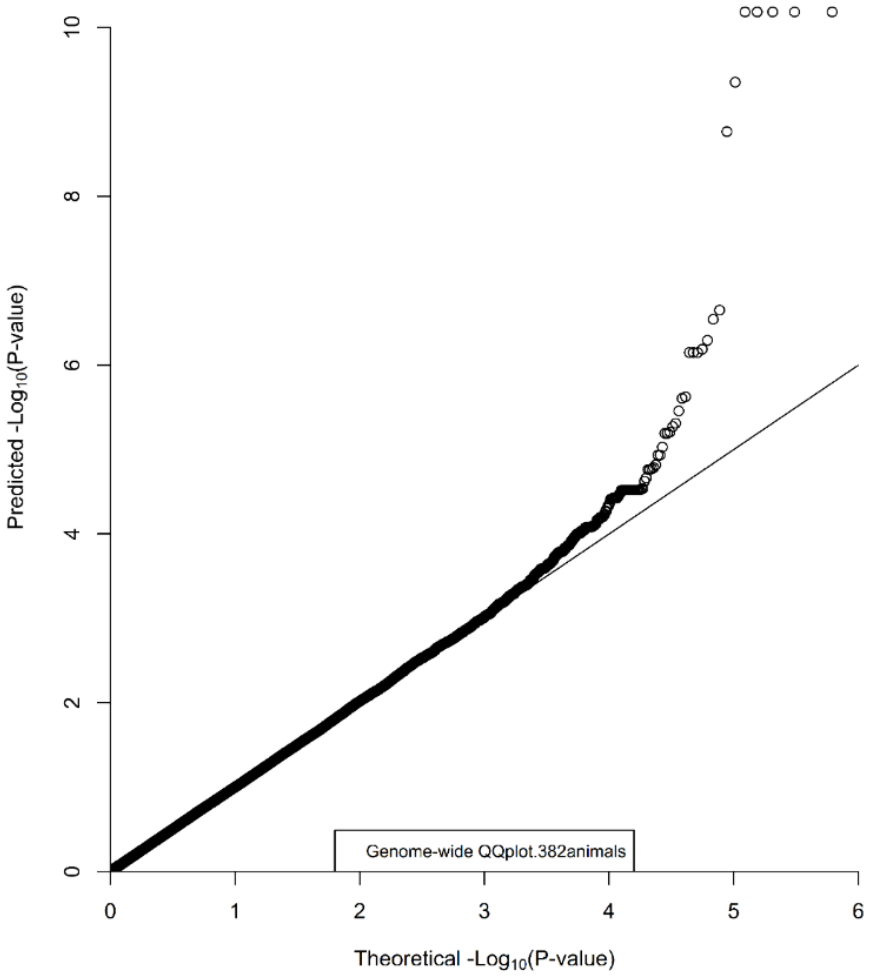
Supplementary Figure 5.1 B Genome-wide association study using 777,963 SNP genotypes affecting non-coagulating milk in Swedish Red cows for BTA8 through BTA14.



Supplementary Figure 5.1 C Genome-wide association study using 777,963 SNP genotypes affecting non-coagulating milk in Swedish Red cows for BTA15 through BTA21.



Supplementary Figure 5.1 D Genome-wide association study using 777,963 SNP genotypes affecting non-coagulating milk in Swedish Red cows for BTA22 through BTA29.



Supplementary Figure 5.2 Genome-wide QQ-Plot for the GWAS with NC milk based on 777,963 SNP genotypes and 382 Swedish Red Cows.

Supplementary Figure 5.3. Views from Ensembl (http://www.ensembl.org) of strongest associations. **(A)** Genomic location of rs385975260, rs525335650 (TagSNP1), and rs379827811. **(B)** rs379827811 as intron variant to the *VPS35* gene.

Supplementary Table 5.1. Region-wide association study: list of most significant variants associated with non-coagulating (**NC**)[†] milk in Swedish Red cows

Chromosome	Name of variant	-Log_{10} (Pvalue)	AR2[§]	σ^2_{marker}[¢]	$\sigma^2_{marker}/\sigma^2_p$[*]
18	18:9179338	6.11	0.80	0.01	0.07
18	18:9179437	6.11	0.82	0.01	0.07
18	18:9179455	6.11	0.83	0.01	0.07
18	18:9179462	6.11	0.83	0.01	0.07
18	18:9179471	6.11	0.81	0.01	0.07
18	18:9179491	6.11	0.82	0.01	0.06
18	18:9179500	6.11	0.82	0.01	0.06
18	18:9179561	6.11	0.91	0.01	0.06
18	18:9179563	6.11	0.91	0.01	0.06
18	18:9179722	6.11	1.00	0.01	0.07
18	18:9179819	6.11	0.99	0.01	0.07
18	18:9179826	6.11	1.00	0.01	0.07
18	18:9179834	6.11	1.00	0.01	0.07
18	18:9180145	6.11	0.99	0.01	0.07
18	18:9180426	6.11	0.99	0.01	0.07
18	18:9180513	6.11	0.99	0.01	0.07
18	18:9180543	6.11	0.99	0.01	0.07
18	18:9180617	6.11	0.99	0.01	0.07
18	18:9180637	6.11	0.99	0.01	0.07
18	18:9181238	6.11	0.99	0.01	0.07
18	18:9181315	6.11	0.99	0.01	0.07
18	18:9181629	6.11	0.99	0.01	0.07
18	18:9181646	6.11	0.99	0.01	0.07
18	18:9182405	6.11	0.81	0.01	0.08
18	18:9214353	6.59	0.85	0.01	0.08
18	18:9215169	6.59	0.96	0.01	0.07
18	18:9215335	6.59	0.86	0.01	0.08
18	18:9215376	6.59	0.96	0.01	0.07
18	18:9215787	6.59	0.96	0.01	0.07
18	18:9215948	6.59	0.96	0.01	0.07

(continuation)

Chromosome	Name of variant	-Log_{10} (Pvalue)	AR2[§]	σ^2_{marker}[¢]	$\sigma^2_{marker}/\sigma^2_p$[*]
18	18:9216194	6.59	0.96	0.01	0.07
18	18:11166809	8.66	1.00	0.01	0.09
18	18:13136070	6.61	0.83	0.01	0.07
18	18:13136171	6.61	1.00	0.01	0.07
18	18:13137293	6.61	0.95	0.01	0.07
18	18:13138676	6.61	0.95	0.01	0.07
18	18:13142955	6.61	0.90	0.01	0.07
18	18:13145923	6.61	0.90	0.01	0.07
18	18:13146013	6.61	0.90	0.01	0.07
18	18:13146020	6.61	0.90	0.01	0.07
18	18:13146999	6.61	0.90	0.01	0.07
18	18:13147063	6.61	0.90	0.01	0.07
18	18:13147747	6.61	0.90	0.01	0.07
18	18:13149017	6.61	0.90	0.01	0.07
18	18:13149305	6.61	0.90	0.01	0.07
18	18:13151402	6.61	0.86	0.01	0.07
18	18:13151967	6.61	0.86	0.01	0.07
18	18:13152843	6.61	0.86	0.01	0.07
18	18:13155943	6.61	0.83	0.01	0.07
18	18:13175633	6.93	0.90	0.01	0.07
18	18:13175950	6.93	0.89	0.01	0.07
18	18:13391752	9.84	0.83	0.02	0.11
18	18:13391841	9.84	0.83	0.02	0.11
18	18:13393733	9.84	0.85	0.01	0.11
18	18:13403337	10.57	0.83	0.02	0.11
18	18:13403968	9.37	0.87	0.01	0.10
18	18:13405460	9.37	0.87	0.01	0.10
18	18:13408106	10.57	0.85	0.02	0.11
18	18:13409996	10.57	0.84	0.01	0.10
18	18:13450556	10.77	0.80	0.02	0.11
18	18:13453819	10.77	0.82	0.01	0.10

(continuation)

Chromosome	Name of variant	-Log_{10} (Pvalue)	AR2[§]	σ^2_{marker} [¢]	$\sigma^2_{marker}/\sigma^2_p$ [*]
18	18:13454607	10.77	0.95	0.02	0.11
18	18:13839520	10.08	0.80	0.01	0.09
18	18:13840950	6.02	0.86	0.01	0.07
18	18:13934348	10.08	0.84	0.02	0.11
18	18:13934429	10.08	0.82	0.01	0.10
18	18:13934546	10.08	0.87	0.02	0.11
18	18:13934657	10.08	0.93	0.01	0.10
18	18:13934670	10.08	0.95	0.02	0.11
18	18:13934856	10.08	1.00	0.01	0.11
18	18:13934858	10.08	0.97	0.02	0.11
18	18:13934872	10.08	0.97	0.01	0.11
18	18:13934903	10.08	0.94	0.02	0.11
18	18:13934926	10.08	0.95	0.02	0.11
18	18:13935065	10.08	0.95	0.02	0.11
18	18:13935102	10.08	0.94	0.02	0.11
18	18:13935106	10.08	0.94	0.02	0.11
18	18:13935269	10.08	0.92	0.01	0.10
18	18:13935300	10.08	0.93	0.01	0.11
18	18:13935356	10.08	0.84	0.01	0.11
18	18:13935590	10.08	0.90	0.02	0.11
18	18:13938211	10.08	0.86	0.02	0.11
18	18:13938277	10.08	0.90	0.01	0.10
18	18:13938283	10.08	0.90	0.01	0.10
18	18:13938291	10.08	0.91	0.01	0.10
18	18:13938461	10.08	0.85	0.01	0.09
18	18:13938602	10.08	0.99	0.02	0.11
18	18:13938614	10.08	0.99	0.02	0.11
18	18:13938680	10.08	0.99	0.02	0.11
18	18:13938708	10.08	0.95	0.01	0.10
18	18:13938871	10.08	0.99	0.02	0.11
18	18:13938963	10.08	1.00	0.02	0.11

(continuation)

Chromosome	Name of variant	-Log_{10} (Pvalue)	AR2[§]	σ^2_{marker}[¢]	$\sigma^2_{marker}/\sigma^2_p$[*]
18	18:13939032	10.08	0.95	0.01	0.10
18	18:13939085	10.08	0.91	0.01	0.10
18	18:13939109	10.08	0.96	0.01	0.10
18	18:13939170	10.08	1.00	0.01	0.11
18	18:13939213	10.08	0.96	0.01	0.10
18	18:13939414	10.08	1.00	0.01	0.11
18	18:13939492	10.08	0.96	0.01	0.10
18	18:13939541	10.08	0.89	0.01	0.10
18	18:13940296	10.08	0.96	0.02	0.11
18	18:13941584	10.08	0.89	0.01	0.10
18	18:13941841	10.08	0.91	0.01	0.11
18	18:13942012	10.08	0.90	0.01	0.10
18	18:13943200	10.08	0.93	0.02	0.11
18	18:13943440	10.08	1.00	0.01	0.11
18	18:13944067	10.08	0.95	0.01	0.11
18	18:13944341	10.08	0.95	0.01	0.11
18	18:13944359	10.08	0.95	0.01	0.11
18	18:13944405	10.08	0.95	0.01	0.11
18	18:13944426	10.08	0.94	0.01	0.11
18	18:13944487	10.08	0.94	0.01	0.11
18	18:13944678	10.08	0.94	0.02	0.11
18	18:13944759	10.08	0.93	0.01	0.11
18	18:13944979	10.08	0.97	0.02	0.11
18	18:13945037	10.08	0.92	0.01	0.11
18	18:13945704	10.08	0.88	0.02	0.12
18	18:13945860	10.08	0.85	0.02	0.11
18	18:13945962	10.08	0.86	0.02	0.12
18	18:13946128	10.08	0.88	0.02	0.11
18	18:13946143	10.08	0.87	0.02	0.11
18	18:13946439	10.08	0.85	0.01	0.10
18	18:13947029	10.08	0.84	0.02	0.12

(continuation)

Chromosome	Name of variant	-Log_{10} (Pvalue)	AR2[§]	σ^2_{marker} [¢]	$\sigma^2_{marker}/\sigma^2_p$ [*]
18	18:13947133	10.08	0.84	0.02	0.12
18	18:13947135	10.08	0.84	0.02	0.12
18	18:13947191	10.08	0.84	0.02	0.12
18	18:13947229	10.08	0.83	0.02	0.12
18	18:13948757	10.08	0.86	0.02	0.11
18	18:13949676	10.08	0.83	0.02	0.11
18	18:13949754	10.08	0.85	0.02	0.11
18	18:13949853	10.08	0.85	0.02	0.12
18	18:13949912	10.08	0.85	0.02	0.12
18	18:13950098	10.08	0.85	0.02	0.12
18	18:13950100	10.08	0.85	0.02	0.12
18	18:13950384	10.08	0.85	0.02	0.12
18	18:13950481	10.08	0.85	0.02	0.12
18	18:13950512	10.08	0.85	0.02	0.12
18	18:13950714	10.08	0.86	0.02	0.12
18	18:13951417	10.08	0.90	0.02	0.11
18	18:13951454	10.08	0.90	0.02	0.11
18	18:13951584	10.08	0.83	0.02	0.12
18	18:13952060	10.08	0.90	0.02	0.11
18	18:13952858	10.08	0.90	0.02	0.11
18	18:13953290	10.08	0.87	0.02	0.12
18	18:13953846	10.08	0.86	0.02	0.12
18	18:13953980	10.08	0.86	0.02	0.12
18	18:13954496	10.08	0.90	0.02	0.11
18	18:13955270	10.08	0.91	0.01	0.10
18	18:13955479	10.08	0.87	0.02	0.12
18	18:13956152	10.08	0.87	0.02	0.12
18	18:13956601	10.08	0.90	0.02	0.11
18	18:13956677	10.08	0.90	0.01	0.11
18	18:13956796	10.08	0.90	0.02	0.11
18	18:13956954	10.08	0.90	0.02	0.11

(continuation)

Chromosome	Name of variant	-Log_{10} (Pvalue)	AR2[§]	σ^2_{marker}[¢]	$\sigma^2_{marker}/\sigma^2_p$ [*]
18	18:13957123	10.08	0.90	0.02	0.11
18	18:13957548	10.08	0.86	0.02	0.12
18	18:13957651	10.08	0.90	0.02	0.11
18	18:13957672	10.08	0.87	0.02	0.12
18	18:13958100	10.08	0.86	0.02	0.12
18	18:13958151	10.08	0.84	0.02	0.12
18	18:13958362	10.08	0.91	0.02	0.11
18	18:13958364	10.08	0.91	0.02	0.11
18	18:13958689	10.08	0.92	0.02	0.11
18	18:13958726	10.08	0.92	0.02	0.11
18	18:13959429	10.08	0.92	0.02	0.11
18	18:13959552	10.08	0.92	0.02	0.11
18	18:13959862	10.08	0.92	0.02	0.11
18	18:13959864	10.08	0.92	0.02	0.11
18	18:13960117	10.08	0.93	0.02	0.11
18	18:13960334	10.08	0.94	0.02	0.11
18	18:13960525	10.08	1.00	0.01	0.11
18	18:13961532	10.08	0.91	0.01	0.11
18	18:13962136	10.08	0.97	0.02	0.11
18	18:13962696	10.08	0.96	0.02	0.11
18	18:13962940	10.08	0.96	0.02	0.11
18	18:13962990	10.08	0.93	0.01	0.11
18	18:13963215	10.08	0.96	0.02	0.11
18	18:13964657	10.08	0.88	0.01	0.11
18	18:13965595	10.08	0.93	0.02	0.11
18	18:13967836	10.08	0.94	0.02	0.11
18	18:13967910	10.08	1.00	0.01	0.11
18	18:13968028	10.08	0.93	0.02	0.11
18	18:13970606	10.08	0.80	0.02	0.12
18	18:13970771	10.08	0.80	0.01	0.10
18	18:13971413	10.08	0.86	0.02	0.11

(continuation)

Chromosome	Name of variant	-Log$_{10}$ (Pvalue)	AR2[§]	σ^2_{marker} [ç]	$\sigma^2_{marker}/\sigma^2_p$ [*]
18	18:15017933	10.31	0.99	0.01	0.11
18	18:15017982	9.24	1.00	0.01	0.10
18	18:15018610	9.24	0.99	0.01	0.10
18	18:15019735	10.31	0.99	0.01	0.11
18	18:15024959	10.31	0.83	0.01	0.10
18	18:15029101	14.12	0.88	0.02	0.14
18	18:15032047	14.12	0.88	0.02	0.14
18	18:15038074	7.05	0.85	0.01	0.07
18	18:15046094	7.05	0.89	0.01	0.07
18	18:15047436	6.46	0.99	0.01	0.07
18	18:15047675	6.46	1.00	0.01	0.07
18	18:15047877	6.46	0.99	0.01	0.07
18	18:15047927	6.46	0.99	0.01	0.07
18	18:15049190	7.05	0.84	0.01	0.08
18	18:15051124	7.05	0.86	0.01	0.08
18	18:15055682	7.05	0.84	0.01	0.08
18	18:15056537	7.05	0.87	0.01	0.08
18	18:15064047	6.68	0.89	0.01	0.08
18	18:15081850	6.68	0.96	0.01	0.08
18	18:15083765	6.68	0.96	0.01	0.08

[†]NC milk as binary trait where 0= coagulating and 1=non-coagulating

[§]AR2 =accuracy of imputation obtained from Beagle 4.0

[ç]σ^2_{marker} = marker's variance, computed for each marker as 2 times major allele frequency times minor allele frequency times allele substitution effect

[*]$\sigma^2_{marker}/\sigma^2_p$ = phenotypic variance explained by a marker

6

General discussion

6.1 Introduction

In this thesis, the genetic backgrounds of milk-fat composition and of non-coagulation of milk have been explored. Firstly, for bovine milk-fat composition, we investigated how genetic differences between winter and summer milk contributed to the observed phenotypic differences (Chapter 2). We showed that winter and summer milk-fat composition are largely genetically the same trait. Phenotypic differences between winter and summer milk-fat composition were mainly caused by dietary differences rather than by genetic differences. Furthermore, for most fatty acids (**FA**), no significant *DGAT1* and *SCD1* by season interactions were found. In case significant interactions were present, we showed that these interactions were likely caused by the scaling of the genotype effects. Secondly, for bovine milk-fat composition and for non-coagulation (**NC**) of milk, we explored their genetic variation by means of genome-wide association studies (**GWAS**). Through GWAS (in Chapters 3 and 5), we characterized promising chromosomal regions associated with the phenotypes. Subsequently, in Chapters 3, 4 and 5, these promising regions were fine-mapped with imputed 777k SNP genotypes and imputed sequence data. The fine-mappings refined the location of quantitative trait loci (**QTL**), and contributed to the identification of candidate genes for these QTLs.

In this general discussion, I discuss different perspectives regarding gene discovery in cattle. I had the opportunity to use a substantial number of genetic markers for gene discovery, and encountered some challenges. Therefore, firstly, I discuss the challenges with respect to high-density genotypes for gene discovery. Secondly, I discuss future possibilities to expand gene discovery studies, and I propose some alternatives to identify causal variants underlying complex traits in cattle.

6.2 Challenges with high-density genotypes for gene discovery

The two main challenges for gene discovery were the imputation to high-density genotypes and the annotation of the cattle genome. In general, the attainment of high-density genotypes (and herein, I include sequences as high-density genotypes) requires several expensive steps, such as genotyping DNA samples in laboratories, using bioinformatic tools plus programmers to handle the huge data sets, and storing data. In recent years in cattle, imputation has been used to reduce costs and to accelerate the attainment of these high-density genotypes for large groups of

animals. A recognized imputation strategy consists in genotyping influential ancestors in a population, and imputing the rest of the population to a higher density of genetic markers (e.g., Druet et al., 2014). After using imputation in Chapters 3, 4 and 5, the density of genetic markers increased while the distance between genetic markers decreased. Regarding the distance between genetic markers, it was reduced from 10 mega base-pairs (**bp**) with 50k SNP to ± 4 mega bp with (imputed) 777k SNP genotypes (Chapter 3), and to a few kilo bp with (imputed) sequences (Chapters 4 and 5). GWAS and fine-mapping using these imputed genotypes resulted in a substantial increase in the number of significant associations (in the thousands) with the phenotypes (Chapters 4 and 5). As a consequence, it became more difficult to identify among the thousands of significant associations which one is the causal mutation.

After finding thousands of significant associations with the phenotypes, the next step consisted in identifying candidate genes underlying these phenotypes. For this purpose, the annotation of the cattle genome is an important tool to pin-point candidate genes. The annotation of genomes including cattle is a dynamic process, hence, constantly changing over time. Currently, important developments regarding the assembly and the annotation of genomes including cattle are on their way. These developments, more specifically the FAANG Consortium (Andersson et al., 2015), will contribute to identify candidate genes and regulatory elements more efficiently than at present.

I will discuss in more detail the two challenges for gene discovery: imputation to high-density genotypes and the annotation of the cattle genome.

6.2.1 Imputation of high-density genotypes

A key feature in using GWAS with imputed high-density genotypes is the accurate imputation of genotypes. According to Marchini and Howie (2010), genotype imputation is a statistical method of predicting (i.e., imputing) genotypes in a sample based on a reference population (**RefPop**). The sample is a representation of a population, typically genotyped for a lower density of genetic markers (e.g., 50k SNP genotypes), and this sample has not been assayed for a higher density of genetic markers (e.g., 777k SNP genotypes). The RefPop consists of individuals that are related to the sampled population and that have been genotyped for a higher density of genetic markers (e.g., 777k SNP genotypes). Based on the RefPop, the sampled population is imputed to a higher density of genetic markers (see figure 6.1). The accuracies of the resulting imputed genotypes range from 0 (poorly imputed) to 1

(correctly imputed). In most cases, genotypes are imputed at accuracies lower than 1. Imputation accuracy is influenced by factors, such as the size of the RefPop, the genetic distance between the sampled population and the RefPop, the minor allele frequency (**MAF**), and the linkage disequilibrium (**LD**) between genetic markers (e.g., Zhang and Druet, 2010; Van Raden et al., 2013; Pausch et al., 2013; and Uemoto et al., 2015).

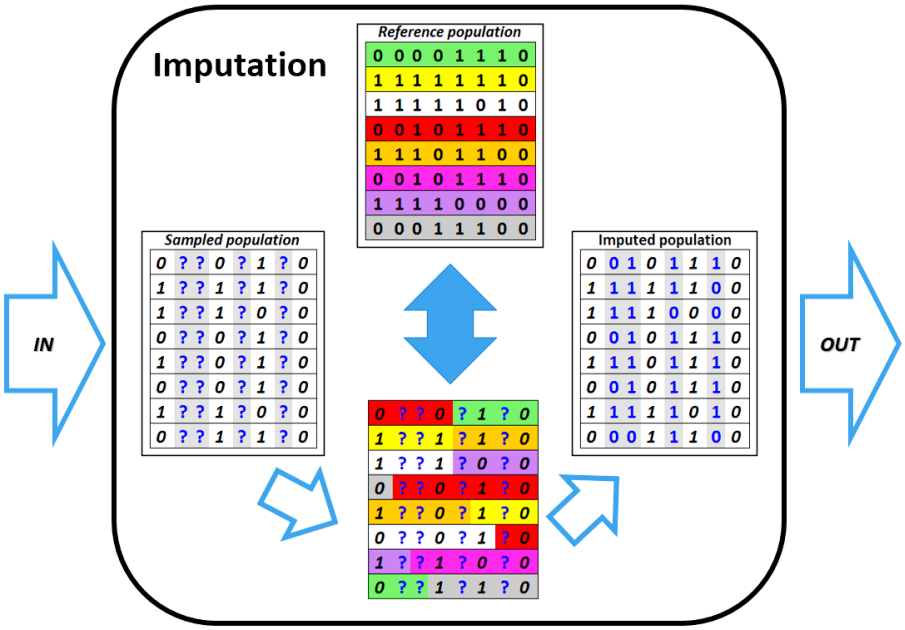


Figure 6.1 – Schematic representation of how imputation works. The sampled population is genotyped at a lower density of genetic markers. The reference population (**RefPop**) contains individuals related with the sampled population that are genotyped at a higher density of genetic markers. Based on the RefPop, the sampled population is imputed to a higher marker density.

Size of the reference population and the genetic distance between the sampled and the reference population. The 1000 Bulls Genome Consortium (Daetwyler et al., 2014) is a world-wide collaborative initiative that aims at sequencing animals from the cattle population, and at creating a multi-breed RefPop. Using this multi-breed RefPop, a substantial increase in the density of genetic markers is currently available for imputation giving the opportunity to impute genotypes to whole-genome sequences (**WGS**). The WGS are available for more than 15 breeds, and each breed is represented by a number of key sequenced influential ancestors. Recently the 1,000 Bull Genome Consortium increased the

number of sequenced animals, and has included sequences of influential cows in this multi-breed RefPop. By accounting for influential cows and bulls, more relationships between the sampled population and the RefPop are considered. Consequently, the accuracy of imputed genotypes should increase.In this multi-breed RefPop, the Hostein-Friesian (**HF**) breed is well represented with 450 HF sequenced ancestors (the latest *Run5*). In contrast, the Swedish Red (**SR)** breed is represented with 16 SR sequenced ancestors and the Finnish Ayrshire (**FAY**) breed is represented with 17 FAY sequenced ancestors.

In Chapter 4, we aimed at imputing the imputed 777k SNP genotypes of HF cows to WGS level. Therefore, only HF sequences (N=450) from the multi-breed RefPop were used to impute genotypes to WGS level, and due to the size of the RefPop, at high accuracies (> 0.9). In contrast, in Chapter 5, a rather limited number (N=33) of sequenced SR and FAY were available for the imputation to WGS level. The 33 sequenced SR and FAY bulls have a large impact in the SR cow population. To make the best possible use of the multi-breed RefPop, our approach in Chapter 5 consisted of imputing a variant three times, each time with a different RefPop (33 SR and FAY sequences, 284 dairy-breeds sequences, and 429 beef- and dairy-breeds sequences). Subsequently, we were able to impute the genotypes of SR cow population to WGS. Based on the findings of Chapter 5, the accuracies of imputed genotypes in smaller breeds (e.g., SR) will only improve if the addition of sequenced animals in the multi-breed RefPop is tailored toward smaller breeds.

Minor Allele Frequency. According to Daetwyler et al. (2014) imputation errors for low MAF (< 0.05) genetic markers are high when imputing a cow population to WGS level. If an allele segregates at low MAF, then there is a relatively small number of sequenced ancestors in the RefPop carrying this low MAF variant. Hence, the imputation of this low MAF variant in the sampled population will be more difficult, and there is a high probability that this variant will be poorly imputed. Therefore, the interpretation of GWAS findings needs more caution when significant associations concern imputed low MAF variants. GWAS detects QTL with genetic markers at a certain power. This detection occurs under the assumption that a genetic marker is correlated with the QTL. MAF at the QTL is an important determinant of power because the heritability of a QTL is directly proportional to the frequencies of the alleles at the QTL locus (Sham and Purcell, 2014). In this context, the power of detecting a QTL segregating at low MAF is low. In addition, the power of detecting this QTL becomes even lower when using imputed low MAF variants, especially if their imputation accuracy is low. If a variant has low MAF, low imputation accuracy

and is strongly correlated with the QTL, this implies that QTL effect size needs to be sufficiently high to be detected by GWAS. In Chapter 4, the 8 strongest associations with milk-fat composition segregate at a MAF=0.44. For the findings of Chapter 4, the imputation accuracy of low MAF variants was not an important issue. In Chapter 5, the 3 strongest associations with NC milk were segregating at a MAF=0.03 and explained more than 10% of the phenotypic variance. This strong signal, which was first detected in SR cows genotyped for 777k SNP genotypes, can be explained by a large QTL effect of more 1 phenotypic standard deviation. This illustrates that rare variants should not by default be considered sequencing errors and therefore excluded from GWAS.

The inclusion of pedigree information can improve the accuracy of imputation of low MAF genetic markers. This approach focuses on imputing identical-by-descent genetic markers that segregate from parents to offspring instead of using information on LD between genetic markers. However, this approach is computationally time-consuming. Some examples of softwares with implemented algorithm that account for simple pedigree information (i.e., duos and trios) are Beagle, fastPHASE, and Fimpute. Recently, a method that imputes SNP combining LD and identical-by-descent information has been proposed (iBLUP, Yang et al., 2014). In general, accounting for pedigree information is expected to impute low MAF genetic markers more accurately than without pedigree information.

Linkage disequilibrium. The non-random association between two loci is defined as LD. Two sampling processes cause LD to arise in a population according to Hill and Weir (1980). First, the sampling of gametes from parents to offspring, and this process depends on the effective population size. Second, the number of individuals sampled from a finite population. In the case of cattle, crossbreeding, mutation, drift, and small population size are events that create LD. Imputation uses LD present in the RefPop to impute the genotypes of the sampled population. One of the problems is that LD can exist between an (imputed) marker and QTL in one family but not in other families (Goddard and Hayes, 2012). For Chapters 3 and 4, the sires of the sampled population of HF cows were included in the RefPop, and in Chapter 5, this was also the case with the 33 sequenced ancestors of SR and FAY. However, in Chapter 5, we also used two other imputation scenarios that included different breeds, for which the sequenced SR and FAY have no common ancestors. In this case, LD in SR and FAY breeds can be different than LD in other breeds. LD across-breeds is expected to be smaller than LD within a breed because more recombination events separate individuals from different breeds (De Roos et al., 2008). Therefore,

imputation accuracy is probably influenced by the differences in LD within- and across- breeds, which might result in lower imputation accuracies for genotypes in small breeds compared with large breeds.

In both cases, for milk-fat composition and for NC milk, imputation to high-density genotypes was challenging. The factors affecting imputation and their consequences on the interpretation of GWAS and fine-mapping results cannot be solved with the data at hand. Only through validation studies it will be possible to confirm the findings reported in this thesis. Validation studies would further help to ascertain if the strongest associations identified in Chapters 3, 4 and 5, and thus the most likely candidate genes, can be confirmed. If a validation study would be based on multiple breeds and these associations persist across breeds, the genetic markers are likely to be very close to the QTL, because of the limited extent of LD across-breeds (e.g., De Roos et al., 2008; Goddard and Hayes, 2012). However, we cannot exclude the possibility that the QTL might not segregate in other breeds (Goddard and Hayes, 2012). Nonetheless, by attempting to validate our associations, it would lead us closer to the identification of the causal variants for the QTL identified in Chapters 3, 4 and 5.

6.2.2 Annotation of the cattle genome

The second major challenge encountered in Chapters 3, 4 and 5 was the limited, hence, incomplete annotation of the cattle genome. The cattle genome contains the genetic information organized in chromosomes, which include the genes for the protein coding regions, and the DNA sequences for the non-protein coding regions. The genome annotation attaches to these genes and DNA sequences the biological information of an organism (Stein, 2001). In Chapter 3, the QTL region located between 29 and 34 mega bp on BTA17 contained 29 genes. A total of 18 out of the 29 genes had not been annotated yet. Among these 18 genes, the non-annotated *LOC515517* was the gene closest to our strongest association on BTA17, and was pointed out as a suggestive candidate gene in Chapter 3. *LOC515517* was assigned this symbol because the investigation of all orthologs for this gene was incomplete. Orthologs are genes in different species that evolved from a common ancestral gene by speciation. The full determination of orthologs assist in the annotation of a gene. Two years later, this QTL region was re-analyzed with imputed sequences (Chapter 4). In these two years, the non-annotated *LOC515517* has been annotated as the *LARP1B* gene in the cattle genome. In Chapter 4, the *LARP1B* gene became our primary candidate gene because 6 out of the 8 strongest associations were located in this gene. In two years, a clear improvement has been made on the annotation of

genes and their biological functions, at least for BTA17. The lesson taken from Chapters 3 and 4 is that the limited annotation of the cattle genome should not be a reason to discard suggestive candidate genes.

The annotation of the genome of domesticated animal species is a slow and complex process. In the last decade, the annotation of the genome of domesticated animal species has been extrapolated from the annotation of the human genome, through actions such as the encyclopedia of DNA elements (**ENCODE**). ENCODE is a global initiative to identify functional variants in high-quality sequences of humans. It is the aim of ENCODE to improve the annotation of structural and regulatory variants as well as non-coding genes in humans. The ENCODE initiative has been very successful in humans, and was expanded to other species like mouse (Shen et al., 2012; Yue et al., 2014). However, the idea of extrapolating gene-expression and its regulation network from human to mouse was not successful because of substantial divergence between these two species (Yue et al., 2014). This genetic diversity between species contributes to the complexity and the slow annotation of the domesticated animal species genomes.

The genetic diversity of domesticated animal species is the focus of the recently started functional annotation of the animal genomes (**FAANG**) consortium. The FAANG consortium aims at identifying all functional elements in the genome of domesticated animal species (Andersson et al., 2015), and involves a collaboration between several research groups worldwide. In a first stage, many different tissues across domesticated animal species will be sampled, such as skeletal muscle, adipose and liver tissues, and in addition, samples of reproductive, immune and nervous systems will be collected. These sampled tissues and systems are necessary to perform functional studies. These studies enable the prediction of the function encoded in sequences. Andersson et al. (2015) argue that filling the genotype-to-phenotype gap requires functional genome annotation of species with substantial phenotype information. The FAANG initiative aims at improving the annotation of the genome of domesticated animal species by creating standardized protocols for sampling, storing, and analyzing the information among the participating research groups (Clarke et al., 2015). The samples will be analyzed by some of the following protocols: transcribed loci (using RNA sequencing), chromatin accessibility and architecture (the link between gene-expression and nuclear organization of cells), and histone modification marks (to identify regulatory elements; Andersson et al., 2015). In a second stage, other tissues will be sampled, such as rumen tissues from ruminant species, mammary tissue from mammals, among others (Andersson et al.,

2015). As pointed out by Zhou et al. (2015), the genomes of chicken, cow and pig have been assembled, but limited information is available on the enhancers, promoters, and other elements of the genome of these species. The identification of these elements and their biological roles will improve the annotation of these three genomes. I expect that it will take some time (> 5 years) to gather and analyze all this information, in order to produce a comprehensive and better annotated genome for each of the domesticated animal species, including cattle. Therefore, the identification of candidate genes will be more efficient in the near future.

6.3 From GWAS to causal variants

The typical outcomes of GWAS are large chromosomal regions, and many polymorphisms that are statistically associated with phenotypes. In Chapter 3, GWAS with imputed 777k SNP genotypes identified a QTL region covering 5 mega bp that contained 29 genes. Subsequent fine-mapping with imputed sequences (Chapter 4) refined the QTL region and reduced the number of candidate genes from 29 to 14. Although this characterization of chromosomal regions associated with our phenotypes (Chapters 3, 4 and 5) was successful, what remains unclear from GWAS and subsequent fine-mapping is whether a polymorphism is the actual causal variant. For complex traits, such as bovine milk composition, it would be interesting to identify causal variants. It would increase biological knowledge, and specifically, help to understand how these causal variants influence our phenotypes. Consequently, it would be possible to predict potential pleiotropic effects on non-(routinely) recorded traits with consequences on the selection of the next-generation of cows. According to Falconer and Mackay (1996), quantitative genetic theory will become more realistic when the numbers and the properties of genes are known because it would improve the methods to studying complex traits. If this is the case, we need to find causal variants to confirm that the identified genes influence the phenotypes. Therefore, in this section, I propose several possibilities to identify causal variants. In more detail, I explore the possibilities of using targeted gene-expression studies, gene-editing, and gene knockouts in livestock to identify causal variants.

6.3.1 Exploring alternatives to identify causal variants

As indicated by Das et al. (2011), the causality of a polymorphism is difficult to be determined by GWAS and fine-mapping. In practice, when GWAS and fine-mapping identify significant associations with the phenotype, the associated variants can be located within protein-coding regions. When this happens, the gene is declared a

candidate gene and the polymorphism might be a causal variant. If the variant is causal, it is possible to predict changes to the encoded-protein, thus predicting functional changes to the phenotype (e.g., Freedman et al., 2011). Consequences on the phenotype can be straightforward for monogenic diseases in humans, such as the Duchenne muscular dystrophy. This disease is caused by large deletions of one or more exon(s) in the dystrophin gene causing severe muscular dystrophy in about 60% of male infants (Hoffman et al., 1987). However, consequences on complex traits are more difficult to interpret than for monogenic diseases. In Chapters 4 and 5, many associations with milk FA composition and with NC milk were identified within and outside protein-coding regions. In Chapters 4 and 5, the *LARP1B* and the *VPS35* genes were nominated as positional candidate genes, after these genes were found expressed in bovine mammary tissue (Bionaz et al., 2012), and during different stages of lactation in humans (Lemay et al., 2013). Figure 6.2 (A and B) illustrates the strongest associations with milk FA composition in the LARP1B gene and with NC milk in the *VPS35* gene. Although we limited the number of candidate genes to only 2, the interpretation of possible functional changes of these 2 genes on milk FA composition and on NC milk are unclear.

Furthermore, two other complications arise. First, the strongest identified associations with milk FA composition and with NC milk are in strong LD (figure 6.2-A and B). Hence, we cannot disentangle which of these associations would promote changes to the phenotypes. Second, some of these correlated associations are intron variants in these candidate genes (figures 6.2 A and B). Particularly in livestock species, there might be a bias in declaring candidate genes toward well–annotated genes (Taşan et al., 2015) because non-coding protein regions still need to be characterized (Andersson et al., 2015). Consequently, associations identified in non-protein coding regions are often ignored. To understand the possible changes to the phenotypes, I hypothesize that the causal variants are among one of the significant associations with the *LARP1B* and *VPS35* genes. If this is the case, this hypothesis can serve as research question for further studies, such as targeted gene-expression studies.

6.3.2 Targeted gene-expression studies

Gene-expression is the process by which functional gene products are formed. Gene products have been studied in many species including mice, rats and humans, and in different cell types (e.g., de Koning et al., 2007; Civelek and Lusis, 2014). Gene

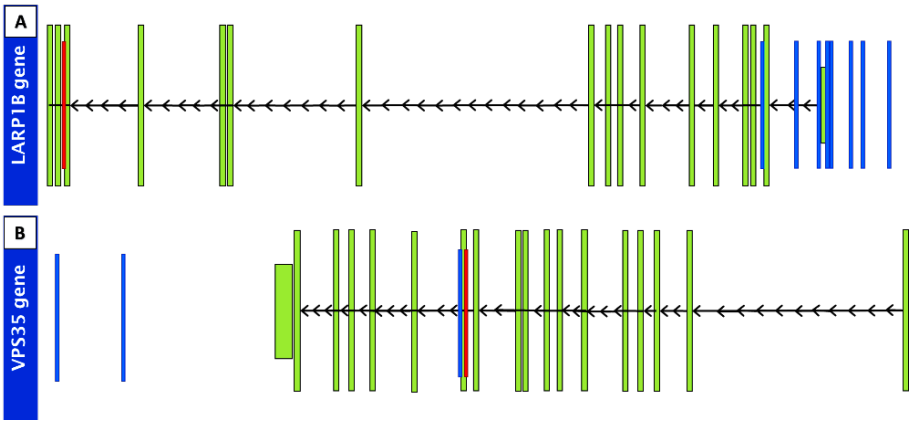


Figure 6.2 – Schematic view of the *LARP1B* and the *VPS35* genes. The green boxes represent the exons connected by a black line and small arrows showing the protein coding direction of the genes. Blue boxes represent the location of the strongest associations, and the red boxes represent the splice region variants. (**A**) The *LARP1B* gene, and its eight strongest associations with multiple fatty acids on Bos Taurus Autosome (BTA) 17 [at –log10 (P-value) = 7.66, and linkage disequilibrium between the eight markers = 1]. (**B**) The *VPS35* gene, and its three strongest associations with non-coagulation of milk on BTA18 [at –log10 (P-value) = 14.12, and linkage disequilibrium between the three markers = 1].

products can be transcripts of genes (mRNA) but equally protein abundance and metabolite levels. The most often analyzed gene products are mRNA rather than protein abundance or metabolite levels (e.g., Albert and Kruglyak, 2015). Typically, the mRNA expression is constantly changing over time (e.g., Jiang et al., 2013). After establishing that most genes are quantitatively expressed, Jansen and Nap (2001) proposed the "genetical genomics" approach. G*enetical genomic*s combines the (quantitative) gene-expression and the genetic variation from related individuals in segregating populations (as a representation of genetic markers).

In *genetical genomics* (or its equivalent genome-wide association of gene-expression studies – **eQTL**), the mRNA abundance is treated as the quantitative phenotype, and the genomic regions influencing gene-expression result in the detection of eQTL (e.g., Jansen and Nap, 2001; Jansen, 2003). According to Jansen and Nap (2001), the eQTLs can act in two ways: a) in *cis* by influencing the expression of the closest gene nearby (also known as *locale* QTL); or b) in *trans* by influencing the expression of genes in other parts of the genome (also known as *distant* eQTL). In animal breeding, Kadarmideen et al. (2006) indicated that eQTLs contribute to the refinement of the identified traditional QTL, candidate gene and SNP discovery. Furthermore, de Koning et al. (2007) combined eQTL and fine-mapping to reduce the confidence

interval of functional trait loci in poultry. As a consequence, the chromosomal region under investigation and the number of candidate genes were reduced. This targeted eQTL approach allows the identification of cis-acting eQTL rather than trans-eQTL. Targeted eQTL are especially important when there is no obvious biological reason supporting a significant association with the phenotypes. The reason being that eQTL can provide further insights into the function, regulation and pathways of genes underlying a complex trait (e.g., Jansen, 2003; de Koning et al., 2007; Lowe and Reddy, 2015). For instance, the *LARP1B* and the *VPS35* genes have not been associated to bovine milk composition before the present thesis. Further insights into the function, regulation and pathways would clarify the functional role of the *LARP1B* and the *VPS35* genes in relation to their respective phenotypes.

According to Hassan and Saeij (2014), if a genetic variant influences the mRNA abundance of a nearby gene, which in turn modulates a complex trait, this cis-eQTL can co-localize with the QTL identified by traditional GWAS. When a common chromosomal region identified by cis-eQTL co-localizes with the QTL from traditional GWAS at the same genetic variant, it provide strong evidence that the underlying candidate gene is correctly identified (Schadt et al., 2005). In addition, this co-localization (if observed) would suggest that the causal variant is associated with the gene-expression and with the phenotype simultaneously (Schadt et al., 2005). Based on these findings, targeted eQTL focused on the expression of the *LARP1B* and the *VPS35* genes would help confirm that the candidate genes were correctly assigned, and help determine the most likely causal variants for these phenotypes.

Nonetheless, targeted eQTL on the expression of *LARP1B* and *VPS35* genes can point out variants in regulatory elements. In humans, some studies have suggested that multiple correlated associations can influence the activity of multiple enhancers (regulatory elements). When the activity of these regulatory elements is coordinated, their effects can alter gene-expression (e.g., Corrandin et al., 2014; Lowe and Reddy, 2015). Albert and Kruglyak (2015) indicated that many polymorphisms identified in human GWAS are over-represented in regulatory regions. In addition, Parikshak et al. (2015) indicated that these regulatory elements are located in non-protein coding regions of the genome. In our case, multiple significant associations with the *LARP1B* and the *VPS35* genes are in strong LD and are located in non-protein coding regions (figure 6.2 A and B). I would investigate if the co-localization of the cis-eQTL with the QTL from a traditional GWAS would occur at one of the variants located in the non-protein coding regions of *LARP1B* and *VPS35* genes. If this would happen, the position of the regulatory element showing the cis-

eQTL effect could be accurately determined based on the sequence data. One limitation, however, is that the regulatory elements of the cattle genome are not annotated yet. In summary, it is possible that the significant associations in strong LD for the *LARP1B* and the *VPS35* genes are regulatory elements.

A step further from targeted eQTL would be to investigate the proteins encoded by the genes directly. This approach would be interesting because of a highly regulated mechanism known as alternative splicing (Hassan and Saeij, 2014). Through this process, introns and exons in genes are re-arranged creating the opportunity for mRNA to synthesize different protein variants (isoforms) that may have different cellular functions (Wang et al., 2008). This process occurs at a specific site known as splice junction (or splice variant). Interestingly, the *LARP1B* and the *VPS35* genes contain splice-region variants (figure 6.2 A and B). Using RNA-sequencing technology, it is possible to distinguish between the transcript abundance from alternative splicing and regular transcript abundance (Trapnell et al., 2010). According to Wickramasinghe et al. (2014), RNA-sequencing technology is the method of choice for studying RNA transcripts, and this technology shows great ability in studying allele-specific expression and non-coding RNA. In a further study, it might be worth investigating the different isoforms resulting from the splice-variants found in the *LARP1B* and the *VPS35* genes with RNA-sequencing.

The contribution of RNA-sequencing is not limited to studying gene-expression. RNA-sequencing can also be used for SNP and gene discovery, as well as gene ontology and pathway analysis. The RNA-sequencing approach is different than genetical genomics. Using RNA-sequencing and gene-expression of bovine milk retrieved from somatic cells, the different isoforms of interesting genes are tested for associations directly with the phenotypes. When a significant association is identified, if this association is identified within the isoforms, then SNP and candidate genes can be identified. Several studies have used this approach to identify candidate genes associated with bovine milk composition (e.g., Cánovas et al.,2010; Wickramasinghe et al., 2012; and Cánovas et al., 2013). It is important to acknowledge the substantial contribution of the RNA sequencing technology for studying bovine milk composition.

6.3.3 Gene-editing and gene knockouts in livestock

A complementary approach to gene-expression studies is targeting genes in mouse models. Targeting a gene in mouse models means to disrupt a specific gene in the genome of a mouse, thus creating a knockout mouse for that specific gene. In the

last 50 years, gene targeting by means of homologous recombination combined with the refinement of protocols (e.g., microinjection of purified DNA, electroporation, and positive selection enrichments) and the subsequent transmission to mouse germlines have led to knockout more than 7,000 genes in transgenic mouse models (Capecchi, 2005). The "principles for introducing specific gene modifications in mice by the use of embryonic stem cells" have made Dr. Capecchi, Dr. Evans and Dr. Oliver winners of the Nobel Prizes in Physiology or Medicine in 2007. This refinement of methods and protocols has substantially accelerated the biological knowledge of genes, and has led to the development of gene-editing.

Gene-editing. Although gene targeting has required the introgression of exogenous DNA into the genome of a mouse, gene-editing with site-specific nucleases is an alternative to target specific genes without the introgression of exogenous DNA (e.g., Capecchi, 2005; Carlson et al., 2014). According to Cappechi (2005), the use of these site-specific nucleases allow to target a series of alleles in the same gene, thus manipulating any chosen allele in mouse models. There are at least three known site-specific nucleases: the zinc-finger nucleases (Kim et al., 1996), the transcription activator-like effector nucleases (Boch et al., 2009; Moscou and Bogdanove, 2009), and the clustered regularly interspaced short palindromic repeats associated endonuclease cas9 (**CRISPR/Cas9**; Cong et al., 2013; Mali et al., 2013). My focus will be on the most recent, the CRISPR/Cas9 system.

The CRISPR/Cas9 system is part of the protection mechanism against viruses that has been identified from the immune system of bacteria. The CRISPR/Cas9 was first described by Cong et al. (2013) and by Mali et al. (2013), as a RNA-guided site-specific DNA cleavage technique. According to Cong et al. (2013), the Cas9 nuclease can direct short RNAs to induce precise cleavage at DNA loci, facilitating the knockout of targeted genes. Initially, the CRISPR/Cas9 technique was intended to understand genes, their regulation and their biological functions because of its easiness of programmability and of usage (Cong et al., 2013). Gene-editing has the potential of targeting a single gene as well as multiple genes simultaneously. Gene-editing can be used to obtain cell-specific knockdown (one copy of the gene inactivated) or knockout (both copies of a gene inactivated) as well as gene specific mutations using rodent models (Shalem et al., 2015). For this reason, it has become an important ally to study genes underlying complex traits, such as bovine milk composition. For bovine milk composition, gene-editing has the potential to accelerate knowledge discovery (about genes, their biological function, and their influence at the

phenotypic level). On this regard, gene-editing is substantially contributing to improve the annotation of domesticated animal species genomes, including cattle.

Gene knockouts in livestock. With gene-editing, some gene knockouts in livestock have been successfully produced. With the zinc-finger nuclease, the knockout of the *PPARγ* gene in pigs (Yang et al., 2011) and of the β-*LG* gene in cattle (Yu et al., 2011) was possible. However, Carlson et al. (2014) indicated that proprietary algorithms were responsible for impeding the use of this zinc-finger nuclease. With the transcription activator-like effector nucleases, Proudfoot et al. (2015) reports the gene-editing of the myostatin (**MSTS**) gene in sheep and in cattle with successful results. In the future, using gene-editing with the CRISPR/Cas9 technique, knockout cows are likely to be produced. The resulting (functional) changes will be interpretable at the phenotypic level. It would be useful to understand the extent of changes from one or multiple genes on bovine milk composition, but also on the important physiologic changes faced by cows at parturition. For phenotypes such as bovine milk, I foresee in the coming future gene knockout cows being widely produced, kept and challenged in a commercial environment. I can also foresee the knockdown of one or multiple alleles in the *LARP1B* and the *VPS35* genes, as well as the knockout of these genes in gene-edited cows.

While gene-editing with the CRISPR/Cas9 technique will become widely used in the future, functional changes in bovine milk composition can already be studied using a lactating bovine mammary epithelial cell (**bMEC**) model. Zhao et al. (2010) and Jedrzejczak and Szatkowska (2014) indicated that bMEC models are suitable to study bovine milk synthesis. Instead of using bMEC sampled from tissues through biopsy, Boutinaud et al. (2002) isolated mRNA directly from somatic cells, which are naturally released in milk during lactation. Using RNA sequencing, Medrano et al. (2010) and Cánovas et al. (2014), both concluded the viability of using milk somatic cells and milk fat globules to study mammary gland expression. For bovine milk composition, functional changes to be phenotypes can already be assessed by studying the gene-expression of *LARP1B* and the *VPS35* genes directly from milk samples. In addition, it is also a possibility to target one or multiple alleles in a single gene (e.g., the *LARP1B* and the *VPS35* genes) using bMEC models.

In summary, there are many opportunities to transform the significant associations identified from traditional GWAS and fine-mapping in research questions for further studies. All the approaches discussed in this section would, a priori, help to identify causal variants underlying complex traits such as bovine milk composition, and a

posteriori, help to understand the function of genes and their biological role in bovine milk.

6.4 References

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Summary

Summary

The present thesis aims at unraveling the genetic background of bovine milk composition by finding genes associated with milk-fat composition and non-coagulation of milk. The fine-mapping was realized by increasing the number of genotypes analyzed in the targeted chromosomal regions. This allowed to increase the resolution for these genomic regions and pin-point candidate genes associated with bovine milk composition.

In **Chapter 2**, we analyzed milk fat composition in winter and summer and estimated in both seasons' genetic parameters, the effects of acyl-CoA: diacylglycerol acyltransferase1 (***DGAT1***) K232A and stearoyl-CoA desaturase1 (***SCD1***) A293V polymorphisms. Furthermore, we estimated genetic correlations between winter and summer milk fatty acids and tested for genotype by season interactions of *DGAT1* K232A and *SCD1* A293V polymorphisms. Phenotypes consisted of gas chromatography measurements (%w/%w) of seventeen individual fatty acids (C4:0 to C18:0, C10:1 to C18:1cis-9, C18:1trans-11, C18:2cis-9,trans-11 (**CLA**), C18:2cis-9,12 and C18:3cis-9,12,15), groups of fatty acids (saturated FA (**SFA**), unsaturated FA (**UFA**) and the ratio SFA to UFA), and six unsaturation indices (C10 index – CLAindex). These phenotypes were available for 2,001 cows in winter and in summer milk samples. We showed that the genetic correlations between winter and summer milk FA were very high, and these indicated that milk-fat composition in winter and in summer can largely be considered as genetically the same trait. We showed that effects of *DGAT1* K232A and *SCD1* A293V polymorphism were very similar in winter and in summer milk for most FA. At last, we tested for genotype by season interactions, and demonstrated significant *DGAT1* K232A by season interaction for some FA. A *SCD1* A293V by season interaction was only found for C18:1trans-11. These genotype by season interactions were due to scaling of genotype effects.

In **Chapter 3** and in **Chapter 4**, we used a subset of the fatty acids analyzed in **Chapter 2**. This subset consisted of six individual FA (C4:0 - C14:0) were available for winter and for summer milk samples.

In **Chapter 3**, a quantitative trait locus (**QTL**) on *Bos taurus autosome* (**BTA**) 17 explaining a large proportion of the genetic variation in de novo synthesized milk FA was fine-mapped. This QTL region has been identified previously using 50k SNP genotypes. We fine-mapped this QTL region with imputed 777k single nucleotide polymorphism (**SNP**) genotypes to identify candidate genes associated with milk FA composition. Single-SNP analyses showed that several SNP in a region located

between 29.0 and 34.0 mega base-pairs were in strong association with C6:0, C8:0, and C10:0. This region was further characterized based on haplotypes, and these analyses suggested the presence of one causal variant. Although many genes are present in this QTL region on BTA17, the strongest association was found close to the progesterone receptor membrane component 2 (***PGRMC2***) gene. This gene has not been associated previously to milk FA composition.

In **Chapter 4**, the chromosomal region associated with de novo synthesized milk FA on BTA17 was further re-fined using imputed whole-genome sequences (**WGS**). WGS were available for 450 Holstein-Friesian (**HF**) animals (the 1000 bull genome consortium (*Run5*) and 45 HF sequenced animals from the Dutch Milk Genomics Initiative. Based on these 495 HF sequences, all cows were imputed from (imputed) 777k SNP genotypes to sequence level. Single-marker analyses identified many significant associations (in the thousands) with c6:0, c8:0, c10:0, c12:0 and c14:0. Most significant associations were detected in a region covering 5 mega base-pairs and in this region a total of 14 genes could be identified. Six out of the 8 SNP that showed the strongest associations were located in the LA ribonucleoprotein domain family, member 1B (***LARP1B***) gene. This candidate gene has not been associated with milk-fat composition before.

In **Chapter 5**, firstly, we performed a GWAS using 777k SNP genotypes to identify the most promising genomic regions associated with non-coagulation (**NC**) of milk in Swedish Red cows. Secondly, we fine-mapped the most promising genomic region using imputed sequences. Individual morning milk samples were available for the 382 Swedish Red cows that were also genotyped using a 777k SNP array. Using 429 sequences from the 1000 bull genome consortium (*Run 3*), all cows were imputed from 777k to sequence level. Single-marker analyses identified 14 associations with NC milk in a 7 mega base-pairs region on BTA18. For this region, our strongest association explained almost 34% of the genetic variation in NC milk. Haplotypes were built, genetically differentiated by means of a phylogenetic tree, and tested in phenotype-genotype association studies. A candidate gene is the vacuolar protein sorting 35 homolog, mRNA (***VPS35)*** gene, for which one of our strongest association is an intron SNP in this gene. The *VPS35* gene belongs to the mammary gene sets of pre-parturient and of lactating cows, and has not been associated to milk composition yet.

In **Chapter 6**, the general discussion is presented. Firstly, I discuss the imputation to high-density genotypes and the annotation of the cattle genome. I discuss what

imputation is, the factors which affect imputation accuracy, and the consequences of using imputed genotypes for GWAS and fine-mapping studies. Regarding the annotation of the cattle genome, I discuss the major difficulties in finding candidate genes with the current annotation, and discuss future initiatives that will contribute for a better annotation of genomes in the future.

Secondly, the future possibilities to expand gene discovery are discussed. In this section, the discussion starts with the importance of identifying causal variants underlying complex traits. The discussion continues by exploring possibilities, such as targeted gene-expression studies, eQTL, gene editing and knockout cows, to identify the causal variants underlying complex traits

Training and education

Training and Supervision Plan

The Basic Package (9 ECTS)	year	credits*
Welcome to EGS-ABG	2011	2.0
WIAS Introduction Course	2011	1.5
Course on philosophy of science and/or ethics	2011	1.5
EGS-ABG Summer Research School Aarhus/Denmark	2012	2.0
EGS-ABG Summer Research School SLU/Sweden	2014	2.0
Scientific Exposure (13.0 ECTS)	**year**	**credits***
International conferences (4.5 ECTS)		
63th EAAP Annual Meeting, Bratislava, Slovak Republic	2012	1.2
9th International Symposium on Milk Genomics and Human Health, Wageningen, Netherlands	2012	0.6
11th World Conference in Animal Breeding and Genetics, Vancouver, Canadá	2014	1.5
66th EAAP Annual Meeting, Warsaw, Poland	2015	1.2
Seminars and workshops (4.0 ECTS)		
Nutrition and fat metabolism in dairy cattle	2011	0.3
WIAS Science Day (2012,2013, 2016)	2012	0.9
Workshop on Techniques for Measuring Milk Phenotypes	2012	0.6
WIAS Seminar: Aspects of sow and piglet performance	2013	0.3
Symposium Genetics of Social Life: Agriculture Meets Evolutionary Biology	2013	0.3
Mini-symposium: How to write a world-class paper	2013	0.3
WIAS Seminar Genomic selection for novel traits	2013	0.3
Seminar series HGEN at SLU, Uppsala, Sweden	2014	1.0
Presentations (6.0 ECTS)		
WIAS Science day2012, Wageningen, Netherlands - poster	2012	1.0

63th EAAP Annual Meeting, Bratislava, Slovak Republic - oral	2012	1.0
9th International Symposium on Milk Genomics and Human Health, Wageningen, Netherlands - poster	2012	1.0
9th International Symposium on Milk Genomics and Human Health, Wageningen, Netherlands - oral	2012	1.0
11th World Conference in Animal Breeding and Genetics, Vancouver, Canadá - oral	2014	1.0
66th EAAP Annual Meeting, Warsaw, Poland - oral	2015	1.0

In-Depth Studies (21.0 ECTS)	**year**	**credits***
Disciplinary and interdisciplinary courses (20.5 ECTS)		
Identity By Descent (IBD) approaches to genomic analysis of genetic traits, Wageningen, Netherlands	2012	1.2
Fatty acids in dairy cattle in relation to product quality and health, Gent, Belgium	2012	3.0
Advanced methods and algorithms in animal breeding with focus on genomic selection, Wageningen, Netherlands	2012	1.5
Social Genetics Effects: Theory and Genetic Analysis, Wageningen, Netherlands	2013	0.9
Advanced statistical and genetic analysis of complex data using ASReml 4, Wageningen, Netherlands	2014	1.5
Advanced Quantitative Genetics for Animal Breeding, Mustiala, Finland	2014	3.0
Bioinformatics approaches to Identify causative sequence variants in farm animals, Uppsala, Sweden	2014	1.5
EpiNOVA: Advanced Course - Data Quality, Tallinn, Estonia	2014	3.5
Introduction to theory and implementation of Genomic Selection, Wageningen, Netherlands	2014	1.35
Linear Models in Animal Breeding, Lofoten, Norway	2015	3.0

PhD students' discussion groups (1 ECTS)		
Quantitative Genetic Discussion Group (2011-2013, 2015)	2011	1.0
Professional Skills Support Courses (9.0 ECTS)	**year**	**credits***
Techniques for Writing and presenting a Scientific Paper	2012	1.2
Course Supervising MSc thesis work	2012	1.0
Project and Time Management	2013	1.5
Scientific Writing	2013	1.8
Writing Grant Proposals	2015	2.0
Social Dutch for employees	2013	1.8
Research Skills Training (2.0 ECTS)	**year**	**credits***
External training period at SLU, Sweden	2014	2.0
Management Skills Training (6 ECTS)	**year**	**credits***
Organization of seminars and courses (2.0 ECTS)		
Advanced methods and algorithms in animal breeding with focus on genomic selection	2012	2.0
Membership of boards and committees (4.0 ECTS)		
WAPS council member (2012-2013)	2012	2.0
EGS-ABG student representative (2011-2013)	2011	2.0
Education and Training Total (60 ECTS)		

* one ECTS credit equals a study load of approximately 28 hours

Curriculum vitae

About the author

Sandrine Isolde Duchemin is born on the 4th August 1975 in Vendôme, France. When she was 5 years old, her family emigrated to Brazil. She obtained her first bachelor in Economic Sciences at *Pontifícia Universidade Católica do Rio de Janeiro* (PUC-RJ) in 1998. After a few years, she changed her career orientation and, in 2009, Sandrine became Doctor in Veterinary Medicine (DVM). Her bachelor thesis was entitled "Utilização de embriões F1 produzidos *in vitro* em rebanhos leiteiros comerciais e em rebanho controlado". In August 2009, she started the European Masters in Animal Breeding and Genetics (EM-ABG). This program gave her the opportunity to stay one year in the Netherlands, and one year in France. During these two years, she wrote two major theses. The first major thesis was written in the Netherlands, entitled "Effects of polymorphisms in *DGAT1* and *SCD1* on milk-fat composition of summer milk samples", and the second major thesis was written in France, entitled "Genomic selection in Lacaune dairy sheep". In August 2011, she received her double-degree Masters in Animal Breeding and Genetics. In September 2011, she started her PhD, which is part of the European Graduate School in Animal Breeding and Genetics (EGS-ABG). While most of her PhD was done at Wageningen (Netherlands), she had the opportunity to spend one year at Uppsala (Sweden). The results of her PhD are presented in this thesis entitled "Mapping and fine-mapping of genetic factors affecting bovine milk composition."

Peer-reviewed publications

Duchemin, S. I., Colombani, C., Legarra, A., Baloche, G., Larroque, H., Astruc, J.-M., Barillet, F., Robert-Granié, C., and E. Manfredi. 2012. Genomic selection in the French Lacaune dairy sheep breed. *J Dairy Sci* 95:2723-2733.

Duchemin, S. , H. Bovenhuis, W. M. Stoop, A. C. Bouwman, J. A. M. van Arendonk, and M. H. P. W. Visker. 2013. Genetic correlation between composition of bovine milk fat in winter and summer, and *DGAT1* and *SCD1* by season interactions. *J Dairy Sci* 96:592-604.

Duchemin, S. I., Visker, M.H.P.W., Van Arendonk, J.A.M., and Bovenhuis, H. 2014. A quantitative trait locus on *Bos taurus autosome* 17 explains a large proportion of the genetic variation in de novo synthesized milk fatty acids. *J Dairy Sci* 97: 7276-7285.

Duchemin, S. I., Glantz, M., de Koning, D-J., Paulsson, M., and W.F. Fikse. 2016. Identification of QTL on chromosome 18 associated with non-coagulating milk in Swedish Red cows. *Front Genet* 7:57. doi: 10.3389/fgene.2016.00057.

Manuscripts in preparation

Duchemin, S. I., Bovenhuis, H., Megens, H-J., Van Arendonk, J. A. M., and M. H. P. W. Visker. Fine-mapping of BTA17 using imputed sequences for associations with de novo synthesized fatty acids in bovine milk.

Conference papers

Robert-Granié, C., **Duchemin, S.**, Larroque, H., Baloche, G., Barillet , F., Moreno-Romieux, C., Legarra, A.,and E. Manfredi. A comparison of various methods for the computation of genomic breeding values in French Lacaune dairy sheep breed. In: 62th Annual Meeting of the European Federation of Animal Science (EAAP), Stavanger, Norway in August 2011.

Duchemin, S. I., Bovenhuis, H., Stoop, W. M., Bouwman, A. C., van Arendonk, J. A. M., and Visker, M. H. P. W. Genetic relation between composition of bovine milk fat in winter and summer. The 9th International Symposium Milk Genomics and Human Health, Wageningen, The Netherlands, October 2012.

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Acknowledgements

Acknowledgements

To God: Thank you for this third opportunity.

To my friends and colleagues: *Acknowledgements are always a very difficult task to write. And throughout this PhD, lots of people have contributed directly and indirectly to this achievement. I would like to say thank you to each and every one of you who contributed, but in a different way.*

This is the year 2009 and I am decided to make some changes. Yet, I have no idea what is to come. Guided by my will, this idea grows stronger and stronger inside my heart. After a few clicks and a directed search on the internet, I find EM-ABG. The advertisement seem too good to be true. Never mind: I subscribe. The road ahead is unknown, and one of the most important journeys of my life is about to start.

Exactly three days after I subscribed, I receive an e-mail from the captain of ABGC, Johan Van Arendonk, asking me if I would like to apply for a scholarship that would cover my living expenses while on board. I will never forget that I really thought it was a phishing attempt. After successfully getting the scholarship, I travel to this far distant new world called the Netherlands. In my luggage, some pieces of clothes and a heart full of hope and eager for adventure. After 26 hours of travel, I finally arrive to this beautiful place called Wageningen Bay.

What an exciting first view! Beyond the main deck of ABGC, I can see Forum Building as the harbor that connects all the other ships. The joy and the excitement are suddenly cut by the voice of the captain: "You have the opportunity and the privilege to be part of this diverse and multicultural team. Enjoy the training, the trip, and have fun!". After a few introductions, EM-ABG are sent to the hold of ABGC ship, where during two years, me and my colleagues will struggle with codes, cleaning data and learning all aspects of the genetic architecture of traits in Animal Breeding and Genetics. As final exam, I am challenged to sail across these beautiful and calm waters of Wageningen Bay. The final result is priceless! After two unforgettable years, the training is completed.

I would like to kindly thank Johan, Dieuwertje, Patricia, Marleen, Aniek, Ada, Gerda, Piet, Eduardo, Christelle, Andrés, Guillaume and all the teachers for their support, guidance and friendship during EM-ABG. I would kindly thank the Koepon family for the amazing opportunity that they offered me.

This is the year 2011, and new challenges have been announced: there is a possibility of subscribing to EGS-ABG. The catchy advertisement comes with a difficult mission: sailing to the North in the open sea. Without hesitation, I subscribe. ☺ ."All on

board", shouts Captain Johan! EGS-ABG gathers together for the first time. The main deck is a huge promotion for most of us. Some came with more experience than others, and the group is very diverse. At first sight, this is going to be challenging. The main deck is indeed a huge responsibility. But we are not alone, at least we think so! All PhD receive specific jobs, but our destination remains unknown. Only the captain and his crew know the direction ABGC ship is heading for. The sails are lifted, and in no time, we leave the quiet and calm waters of Wageningen Bay!

Under the supervision of Colonel Henk and Major Marleen, I happily start my task. After a few months at sea, the excitement has been replaced by a tedious and continuous routine. Asreml, Excel, Linux and R are just part of the job, which is complemented with endless meetings with Colonel Henk and Major Marleen. To keep the spirit alive, some strategic stops are planned, like harbors Pub-Quiz, WE-day and ABGC day-outs. Ahead of us, the first storm in sight: the huge storm coined "Paper One". Paper One Storm soon brings lots of bumpy waves and strong winds. Winds from the North and South reviewers that seemed to battle endlessly with us on the main desk. I almost was thrown out of the main deck. Colonel Henk shouting endless orders, followed by obedient Major Marleen, and a beaten up PhD Sandrine. "Pull the sails down!" shouts Colonel Henk, "The reviewers are angry", he continues. "We need to hold ourselves, 'cause these winds are too strong!!!!". Milk Genomics meetings, presentations, minutes, discussions, posters, endless shift hours, few sets of brilliant ideas, a list of new suggestions, and frustration stepping in at high speed. These were unusual times for me, and all my expectations changed. Would I be able to continue? At these times, the excellent team of PhDs is like an island of comfort in these troubled waters. After discussing and sharing our deepest fears and frustrations, the morale of the PhDs substantially improves. Motivated as I have never been before, I think: "Let's go through this storm, let's do this!". Welcome meetings, presentations, minutes, discussions, posters, QDG, TLMs! Finally, Paper One Storm has passed; and I remember thinking: "OUF, I survived!".

I would like to kindly thank Johan, Henk and Marleen for their guidance and support throughout the PhD. Yes, I do not come with a manual, but neither do you. ☺. I would kindly thank CRV for their financial support for the last year of my PhD. I would kindly say thank you to Erik Mullaart for your constant interest in my work, Daylan, Elsa, Kasper, and Hein for the nice discussions within Milk Genomics. I would like to say thank you to Mahlet, Marzieh, Yogesh, Hooiling, Troncg, Susan, Ewa, Katrijn, Naomi, Gabriel, Marcos, Hamed, Mirte, Bert, Kimberly, Sabine, Tessa, Jovana, Sonia, Maria, Zih-Hua, Anoop, Maulik, Vinicius, Coralia, Amabel, Mathijs, Claudia, Kasper, Saskia, Mathieu, Floor, Qiuyu, Mandy, Wosseni, Robert, Haibo, Shuwen, Yvonne, Esther, Ilse,

Anouk, Aniek, Jérémie, Alex, Rosilde and Maya. I would also like to say thank you to Pim, Henry, Jan, Piter, Liesbeth, John, Richard, Martin, and all the other staff members for all the discussions at QDG and at lunch breaks.

In subsequent years, ABGC ship came across some other important storms. I can say Paper One Storm prepared me for the next storms that were still to come. However, nothing was as frightening as in 2013 when the sea started shaking so much that I was sea-sick. This has never happened before. After receiving a lot of help from my good friend Marshall Dieuwertje, I discover that I have to go back to Rio de Janeiro Bay and stay some time recovering while on land. Before I left, Captain Johan was very supportive "Sandrine", he said, "Take your time, health is more important than anything. When you are fully recovered you come back." How grateful I am to have this kind of support. I leave ABGC ship thinking: "I will be back before you know it".

A few months later, I return to ABGC ship. A part of me is excited. I miss being at ABGC, I miss the EGS-ABG gang, all the other PhDs, I miss the Marshalls, the nice friends and colleagues, and I miss the blue Sea of Knowledge that lies in front of ABGC ship. The other part of me is different. I have deeply changed after the sickness, and things do not look the same. It seems that time has continued for everyone, and it has stopped for me. Caught in my thoughts, I hear this voice behind me, "Oh dear, don't be sad, everything is going to be fine". I look back, and see Marshall Ada. She continues: "Your program has been upgraded. You just need time to get used to it. All will be fine at the end. You will see, relax, and no worries". I am so grateful to be hearing this. And Marshall Lisette adds a little more: "No worries, we, 1975 are the best! I am sure you will recover in no time. Hey girl, we are '75s! Uh-u!". My heart is feeling lighter again, and I think proudly to myself: "Yes, 'mam. I am a '75s. Go for it!".

Dieuwertje, I will never forget how much you helped me. Thank you! For all the support and help on this difficult phase, I acknowledge Dr. Cafure and his family, my family, Johan, Marleen, and Henk. I would like to say thank you for the amazing support and hard work that Ada and Lisette did. "Lieve Dames, dank jullie wel!"

This is the year 2014, and on this very sunny day, Captain Johan, Colonel Henk and Major Marleen altogether announce my final destination: "Sandrine", said captain Johan, "You are going to the North Pole. There, you will spend some time in a ship called SLU. The captain is a good friend of mine and you can learn lots of things from him and his crew. I argued back: "Captain, my Captain! These are dangerous waters. I am going to freeze to death!" "Naja", says Captain Johan, "You just need some good clothes, then it will be OK!". Colonel Henk watching me worried, says "Sandrine, keep

an eye on polar bears. Beware of sliding bears! They can swipe you out of the deck!". "Safe trip! "said Major Marleen. After waving goodbye to all colleagues and friends, and gathering nice tips from my fellow PhD Dianne, my puzzlement was replaced by the eagerness of discovering this new boat, place and crew.

It is on a summer sunny day when I finally reach ship SLU. This boat was somewhat surprising; the main deck was round. I was a little lost at first, especially because so many people around me were saying "Fiiiikka!". I could not stop thinking: "What a strange language!". "AH, AH", says this voice at the far end of the deck. "You made it! Welcome, welcome to the main deck of the SLU ship. By the way, I am Captain DJ and this is my crew: Major Freddy, Lieutenants Fernando and Lisa. You also know Nancy and André!". It was so nice to see these familiar faces. Very supportive PhDs Nancy and André helped me settling in very fast. In no time, the round deck became a very familiar place. But there was that dark side of the deck. I turn to André, and ask: "Hey bro, what is on that dark side of the deck?". "Sandrine, follow me", he said. In no time, we step into the dark side, and André says: "Meet the SLU Mafia!". "Hey, bro! Who is THAT? You are not supposed to bring strange people in." says this PhD to André. She turns to me and says: "My name is Agnese, and I am sort of the leader of the SLU mafia! And these are Merina, Chrissy, Bingjie, Ahmed, Thu, Shizhi, Xiaowei, and all the others! This is where all the PhD gather and organize many parties and all sorts of activities! You are most welcome to join! By the way, Fiiiiikkka." I thought "And here we go again". ☺

It was mid-October 2014 and strong winds were bringing very dark clouds that marked the beginning of the winter. The forecast was announcing light snow for the evening, and at the main deck, I noticed that the days were getting shorter quite rapidly. Captain DJ in his usual good shoes was sort of inspired: "Sandrine, the weather is not an issue, we are inside the ship. For some months, the main deck will remain closed, and we will be stuck in the North Pole until spring, next year." I say: "WHAT????? Spring is in April, we are gonna die!" Major Freddy and Lieutenant Fernando started their usual jokes "Ah, Ah, we are gonna die indoors, so we will go out to ski, ice-skate and all sorts of nice things! It will be fun! You will see!". The next morning the weatherman announces: "Yesterday, it only snowed one meter of snow." "Whow, this winter is gonna be promising", I thought.

I would like to say thank DJ and Freddy for all the support that you gave me while in Sweden and afterwards. Thank you Maria and Marie for all the nice comments. A special thank you to Lisa because you let me stay two months in your house, and I am really grateful for this. Fernando, Karl and Cano thanks for keeping me smiling. A

special thank you to the SLU Mafia. Thank you for all the amazing stuff we did together: "Guys, you rock!". Thanks Sofia, Kim, Emilie, Thomas, Eva and Valentina for the nice discussions.

Thank you Dianne for the tips before I went to Sweden, especially the one to go to Kiruna! It was fantastic! Nancy and André: Hey you two! I shall never forget you! After a PhD, we shared so many moments! I can only say: thanks for everything.

This is the year 2015 and spring makes its way in this rather dark room. This new ship ABGC 2.0 is located in the middle of this rather dark forest. A change that I notice, especially after spending sometime at the North Pole. This is the last chapter of this tremendous adventure called EGS-ABG to me. I have experienced so much, and many PhD have harvested their thesis already. The direction set for me now is towards the sun. I am heading full speed towards the final stage of every training: the Aula. This period of time is intense, and everything has to be ready before spring 2016. Courses have to be finalized, all the Storm Papers are mastered by now, and the final challenge makes its entrance in no time: Hurricane General Discussion. Winds much stronger than expected and waves just look like mountains of waters in front of ABGC 2.0 ship. Everything is so dark, and suddenly caught off guards, I fell in the sea. "Woman at Sea", shouts the Captain. I am safe and sound. I am quite lucky because new EGS-ABG and PhDs have started their training.

So nice to meet them with their high spirits and hearts full of determination. The nice and quiet main deck is suddenly taken by their voices, bringing a new sense of hope. They do not realize, but they came to the rescue right on … "DRING, DRING", I am immediately transposed at the computer behind my desk at Radix building. "DRING, DRING", insists the phone. "Bonjour Maman, Bonjour Papa!"…

Para minha Família: *Merci Maman et Papa! Merci pour tous ce que vous avez fait pour moi et de m'avoir enseignée ce que l'amour inconditionnel est. Je vous aime! Merci Yvan et Stéphane, pour les visites, voyages et vos soucis. Obrigada Maria-Claudia e Sophia pelo carinho. Obrigada à tia Carmen, tio Reimar , Alexandra, Simão, Felipe, Mariana, Fernando e à falecida tia Margitte por todo o carinho, interesse e apoio.*

Afinal, um PhD não é fruto de coincidências. É fruto de muito trabalho, e dedicação. Por isso, estendo os meus agradeçimentos a todos os meus professores da FAA-Valença/RJ, e em especial à minha amiga Aparecida e ao meu amigo Generoso.

Colophon

Colophon

The work performed in Chapters 2, 3 and 4 are part of the Dutch Milk Genomics Initiative, funded by Wageningen University, the Dutch Dairy Association (NZO), Cooperative Cattle Improvement Organization (CRV; Arnhem, the Netherlands), and the Dutch Technology Foundation (STW). The work performed in Chapter 5 was financed by the Swedish Farmer's Foundation for Agricultural Research (SLF), Stockholm, Sweden.

The author was supported by the European Commission (within the framework of the Erasmus-Mundus joint doctorate “EGS-ABG”) and Breed4Food (a public-private partnership in the domain of animal breeding and genomics and CRV).

The cover of this thesis was designed by Sandrine I. Duchemin.

The thesis was printed by Digiforce | Proefschriftmaken.nl, De Limiet 26, 4131NC, Vianen, the Netherlands.