SLUTRAPPORT
NR. 2017-148

Enestående oligosakkarider: Oligosakkaridprofiler af komælk for optimering af sundhedsgavnlige egenskaber

Mejeribrugets
ForskningsFond

NOVEMBER 2017

Final report
for collaborative projects funded via the Danish Dairy Research Foundation (DDRF)

1. Title of the project

Enestående oligosakkarider*:* Oligosakkaridprofiler af komælk for optimering af sundhedsgavnlige egenskaber.
Superior oligosaccharides: Oligosaccharide profiles of cow's milk for the optimization of health promoting attributes.

2. Project manager

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3. Other project staff

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4. Sources of funding

DDRF

Arla Foods Ingredients

5. Project period

Project period with DDRF funding: January, 2015 – December, 2016

Revised, if necessary: January 2015 – July 2017

6. Project summary

Det overordnede mål med projektet er at undersøge potentialet for at udnytte naturligt forekommende frie oligosakkarider i komælk og muligheden for at øge disse gennem avl. Undersøgelser har vist, at de frie oligosakkarider ikke kan nedbrydes i mennesket, men fordøjes af gavnlige bakterier i tarmen, der dermed stimuleres og er med til at opretholde et sundt mave-tarmsystem, styrke immunforsvaret, samt stimulere udviklingen af hjernen hos nyfødte. Frie oligosakkarider findes i rig udstrækning i modermælk, men både kompleksiteten og koncentrationen af disse er lavere i komælk. Idet modermælkserstatning er baseret på komælk, er de sundshedsgavnlige egenskaber af de frie *humane* oligosakkarider fraværende i modermælkserstatning, som derimod indeholder de oligosakkarider, der findes i komælk. Indledende undersøgelser har vist, at der findes strukturelle- og koncentrationsfor-

skelle i oligosakkarider i komælk af forskellige racer. Formålet i projektet er at profilere oligosakkarider i dansk mælk indsamlet i Milk Genomics-projektet fra to danske malkeracer og påvise særlige oligosakkarider, der indtil videre er ukendte i komælk, samt undersøge muligheden for at fremavle et højere niveau af ønskelige oligosakkarider i komælk. Denne viden vil i fremtiden bidrage til, at der kan produceres modermælkserstatninger med oligosakkaridernes sundhedsgavnlige egenskaber – lavet på komælk. Projektet har udviklet en ny metode til at kvantificere oligosakkarider in komælk. Desuden er der generelt dokumenteret høje arvbarheder for frie oligosakkarider i mælk, hvilket betyder, at der er et betydeligt potentiale for at øge indholdet af disse gennem avl. Identifikation af solide kandidatgener underbygger disse resultater.

The overall goal with the project is to characterize the free oligosaccharides that are present as a natural part of cow's milk and examine the potential for increasing these through selective breeding. Research has shown that humans are unable to digest free oligosaccharides and have to rely on gut bacteria which are able to digest oligosaccharides thereby being stimulated and ensure a healthy digestive system. Furthermore, oligosaccharides enhance the immune system and can also stimulate brain development of newborns. Free oligosaccharides are abundantly present in human colostrum and milk. However, the complexity and the abundance of oligosaccharides in cow's milk are lower than in human milk. Infant formula is predominantly based on cow's milk. Thus, the beneficial *human* oligosaccharides are absent and it contains only minute amounts of bovine oligosaccharides, and the health promoting effects are thus minimized. Preliminary investigations have shown that there are structural and concentration differences between cow's milk from different breeds. The aim of the project is to elucidate oligosaccharides in milk from Danish dairy breeds collected within the Milk Genomics project, to identify oligosaccharides currently unknown in cow's milk, and to reveal opportunities for increasing desirable oligosaccharides in bovine milk. In the future, this knowledge can be used to produce infant formula with the health promoting effects of oligosaccharides – based on cow's milk. Remarkable achievements from this project include the development of a novel tagging method for bovine milk oligosaccharides and the identification of moderate to high heritabilities for oligosaccharides. Taken together, these results suggest a very promising potential for increasing bovine milk oligosaccharides through accurate characterization and selective breeding. Identification of candidate genes involved in oligosaccharide metabolism support these findings.

7. Project aim

Formålet med forskningsprojektet er at undersøge variationen i oligosakkarider i mælk fra individuelle køer indenfor og mellem de to mest anvendte danske malkeracer med henblik på at forstå betydningen af oligosakkariders koncentration og sammensætning. Endvidere ønskes det på baggrund af denne profilering at undersøge muligheden for gennem avl at øge mængden af sundhedsgavnlige oligosakkarider i komælk og dermed forbedre modermælkserstatninger.

Mål

- At profilere oligosakkarider i komælk i forhold til koncentration og sammensætning, herunder mulig identifikation af nye oligosakkarider
- At undersøge potentialet for at øge mængden af specifikke oligosakkarider gennem avl

Oligosakkarider i mælk vil blive profileret ved hjælp af avancerede MS teknikker og sammenholdt med køernes genetiske baggrund baseret på eksisterende genotypninger fra den bovine højdensitet SNP chip.

The overall aim of the present research project is to evaluate the content and composition of the oligosaccharide fraction of bovine dairy milk and evaluate the potential for genetic improvement of these through selective breeding.

8. Background for the project

Free oligosaccharides (OS) are bioactive molecules present in mammalian milk, which offer numerous benefits beyond providing the neonate essential nutrients. Studies on human milk OS have shown multiple beneficial roles played by these molecules including stimulating growth of selected beneficial bacteria in the gut, participating in development of the brain and exerting anti-pathogenic activity by preventing the pathogen binding to intestinal epithelial cells. However, the concentration of OS is significantly lower in bovine milk compared with both human colostrum and mature human milk. Additionally, more than 200 human milk OS have been identified, compared with only about 40 identified bovine milk OS (Barile et al., 2010; Ninonuevo et al., 2006; Tao et al., 2008). Due to the beneficial effects of OS and in order to be able to produce a healthier infant formula based on bovine milk, it is important to enhance the complexity and increase the concentration of beneficial OS in cow's milk. Characterization of the complexity and abundances of OS across bovine breeds and lactation stages are crucial for identification of viable sources for purification that could lead to infant formula with improved OS functionality. In the present research project, OS will be purified and analyzed from milk samples within and across breeds. The samples will be screened for OS and differences in OS profiles.

Previous work on characterization of oligosaccharides in milk has mainly been performed on colostrum (Tao et al., 2008), and while this makes extraction and subsequent analyses easier due to the higher colostrum concentrations of OS, it does not reveal the OS profile of mature bovine milk. Studies on mature bovine milk reveal decreased OS complexity and abundance (Tao et al., 2009). Preliminary studies from our lab have shown overall differences in OS complexity when comparing small amounts of samples from two Danish breeds (Sundekilde et al., 2012). **Thus, as infant formulas are manufactured predominantly with mature bovine milk components, investigations of OS in mature milk of bovine origin are very important and the present project has a large number of milk samples to investigate differences in OS abundance and composition both within and between breeds.**

The potential for improving bovine OS through breeding has to our knowledge not been documented before. Initial genetic estimation can be used to evaluate whether traits are heritable and identify underlying SNP markers affecting OS variation in bovine breeds in line with previous work conducted on milk metabolites (Buitenhuis et al., 2013). **Thus, the potential for changing milk composition towards highly valuable OS milk through selective breeding will be evaluated through genetic parameter estimation.**

Barile D, Marotta M, Chu C, Mehra R, Grimm R, Lebrilla CB, & German JB. (2010) Journal of Dairy Science, 93, 3940-3949.

Buitenhuis AJ, Sundekilde UK, Poulsen NA, Bertram HC, Larsen LB, & Sørensen P (2013) Journal of Dairy Science 96, 3285-3295.

Ninonuevo MR., Park Y., Yin H., Zhang J., Ward RE., Clowers BH., German JB., Freeman SL., Killeen K., Grimm R., & Lebrilla CB. (2006), Journal of Agricultural and Food Chemistry, 54, 7471–7480.

Sundekilde, UK., Barile, D., Meyrand, M., Poulsen, N., Larsen, LB., Lebrilla, CB., German, JB., & Bertram, HC. (2012). Journal of Agricultural and Food Chemistry, Submitted.

Tao, N., Depeters, EJ., Freeman, S., German, JB., Grimm, R., & Lebrilla, CB. (2008) Journal of Dairy Science, 91, 3768-3778.

Tao, N., Depeters, EJ., Freeman, S., German, JB., Grimm, R., & Lebrilla, CB. (2009) Journal of Dairy Science, 92, 2991-3001.

9. Sub-activities in the entire project period

The present project utilizes milk samples and genetic material collected within the Danish/Swedish Milk Genomics Initiative funded by the Danish strategic research council (www.milkgenomics.dk). In total, almost 700 samples from individual cows have been profiled for milk phenotypes and genotyped from the two major Danish dairy breeds. The following analyses will be conducted:

- Characterization of bovine milk OS profiles of the two breeds by high-performance liquid chromatography chip quadrupole time-of-flight mass spectrometry (HPLC-Chip/TOF MS).
- Estimation of heritabilities and genome wide association studies of bovine milk OS

***WP1**. Characterization of the OS profiles in bovine milk samples by high-performance liquid chromatography chip quadrupole time-of-flight mass spectrometry:*
Participants: Aarhus University, Department of Food Science (AU-FOOD), University of California, Davis (UC Davis)

Samples will be selected from the milk bank and forwarded to UC Davis from AU Food. OS will be isolated and purified from skimmed milk samples using an extraction protocol adapted from the literature. Oligosaccharide profiling in 700 milk samples will be performed on an Agilent 6500 series HPLC-Chip/TOF MS in collaboration with researchers at University of California, Davis.

Outcome: OS profiles of 700 milk samples showing individual and breed specific differences.

***WP 2**. Genetic analyses; heritability and global association studies performed using the bovine HD SNP-chip:*
Participants: AU-FOOD, Aarhus University, Department of Molecular Biology and Genetics (AU-MBG)

Genotyping has been performed using the bovine HD SNP-chip in the Danish/Swedish Milk Genomics Initiative project. Genetic parameters (heritability and genetic correlations) of bovine milk OS will be estimated within two Danish dairy breeds using a multivariate mixed model. Furthermore, for both Danish dairy breeds a genome wide association study (GWAS) will be performed based on the bovine milk OS identified in this project using either a single SNP approach or a Bayesian approach. Identification of genetic markers requires extensive sample information, data on farm management, feeding, lactation stage, cell count etc., which together with milk samples will be provided from AU-FOOD.

Outcome: Genetic QTL markers and heritabilities of important bovine milk OS will be determined, and breed specific differences in bovine milk OS profiles will be

explained on the basis of genetic differences, which can be used for optimizing the breeding programs.

***WP 3**. Data analysis and publications:*
Participants: AU-FOOD, AU-MBG, UC Davis

The OS profiling obtained in WP1 will affect the results achieved in WP2. All primary data from the project will be published in international peer-reviewed scientific journals and presented at international conferences. Finally, the aim is that main conclusions will be published in popular science magazines towards the end of the project period.

10. Project results

Initially, a novel high-throughput method for OS extraction and relative quantification via mass spectrometry was developed. To reduce the time needed for instrumental analysis for such a large dataset, extracted OS were labeled with a series of isobaric tags and multiplexed prior to analysis by mass spectrometry, as is commonly done in proteomics experiments. Upon tandem fragmentation, these commercially-available tags produce a reporter ion with a unique mass that allows deconvolution of the multiplexed data and serves as a basis for relative OS quantification. Each tag contains a unique arrangement of ^{13}C and ^{15}N isotopes, which causes each reporter ion variant to generate a distinct mass spectral peak when a set of multiplexed samples is analyzed during tandem mass spectrometry (MS/MS) experiments (Figure 1). A total of five samples can be multiplexed into each vial, therefore reducing analysis time by a factor of five compared to traditional relative quantification techniques. This technique should minimize the confounding effects of drifts in instrumental performance. Prior to OS quantification of the Milk Genomics samples, an instrumental method for analysis of isobarically labeled OS was optimized for bovine milk. The method was validated through the analysis of a variety of OS typically found in bovine milk, as shown in Table 1.

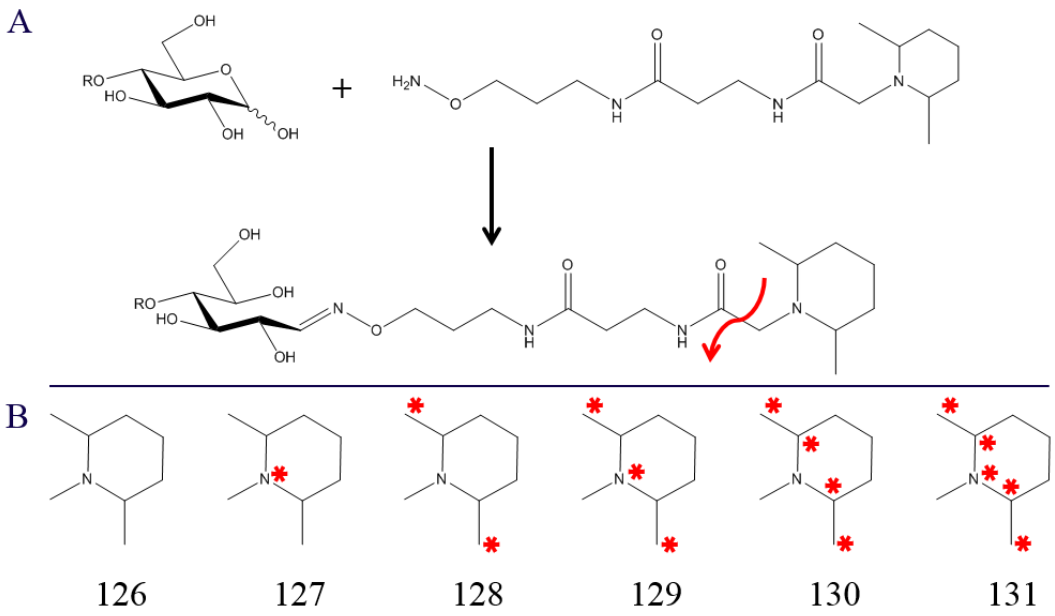

Figure 1. Tandem mass tag structure and labeling reaction (A). Using collision-induced dissociation, tags fragment along the red line shown above. Each tag variant contains a unique number of ^{13}C and ^{15}N isotopes (denoted by red asterisks in part B), which causes each variant to generate a unique mass spectral peak upon tandem fragmentation. The nominal mass of the hydrogen-ion adduct of each reporter ion is shown below the respective structures in part B.

Table 1. Measured relative abundances of 15 bovine milk oligosaccharides, labeled in a 2:1 ratio with two unique isobaric tags and analyzed in four replicate injections.

Composition				Experimental ratio		Coefficient of variation
Hex	HexNAc	Fuc	NeuAc	Tag 1 abundance	Tag 2 abundance	
3	3	1	0	2	1.030	17.2%
3	6	1	0	2	1.042	5.6%
4	5	1	0	2	0.981	19.1%
5	4	1	0	2	1.092	4.5%
2	0	0	1	2	1.122	1.4%
2	0	0	1	2	1.337	11.0%
2	0	0	2	2	1.283	19.4%
2	1	0	0	2	1.319	1.7%
3	2	0	0	2	1.187	1.8%
3	3	0	0	2	1.178	9.4%
4	1	0	0	2	1.156	1.0%
4	2	0	0	2	1.116	3.5%
4	2	0	0	2	1.153	7.4%
5	4	0	0	2	1.113	3.4%
8	0	0	0	2	1.205	13.5%

In total, 15 bovine milk oligosaccharides were quantified by the optimized LC-MS/MS method in milk samples from 334 Danish Holstein and 300 Jersey cows. This included quantification of several fucosylated OS compositions, a category of OS which are theorized to possess beneficial bioactivities. Fucosylated OS in bovine milk do not normally generate a strong signal in mass spectrometry-based experiments and are therefore quantified with difficulty. However, isobaric labeling has proven to increase the ionization of these OS, which will strengthen the potential impact of the study by allowing more fucosylated OS to be quantified than would be possible otherwise. The results confirmed previous findings that milk from Danish dairy breeds differs in their oligosaccharide profile. Significant differences in the abundance were identified between the breeds, with most OS having a greater abundance in the Jersey samples including several complex fucosylated OS (Figure 2). Although Jersey milk had higher average OS abundances, the breed also demonstrated much higher variation between animals in OS production. In both breeds, correlations were observed between the abundance of specific OS pairs, which could provide clues into the underlying synthetic pathways. Especially, 4 Hex

1 HexNac and 3 Hex 2 HexNAc as well as Lacto-N-Hexaose and Lacto-N-tetraose displayed were strong positive phenotypic correlations in both breeds.

Individual milk samples were collected from 40 farms and the selected cows were within their first three parities and in mid-lactation. In both breeds, parity significantly affected several oligosaccharides, whereas days in milking was mainly affecting variation in milk oligosaccharides in Danish Holstein. Furthermore, herd or management effects were also found to play a role for specific oligosaccharides.

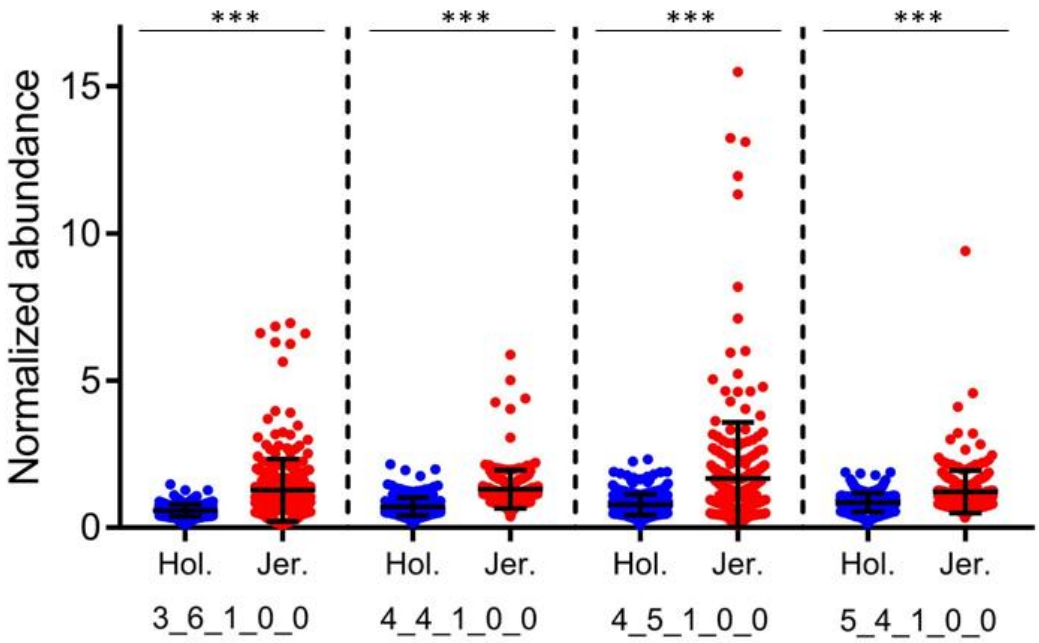

Figure 2. Effect of breed on fucosylated OS abundances (*** P<0.001). Oligosaccharide composition (Hex_HexNAc_Fuc_NeuAc_NeuGc).

All Holstein and Jersey cows were genotyped using the bovine high-density single nucleotide polymorphism (SNP) array. Genomic DNA was extracted from ear tissue. The Restricted Maximum Likelihood (REML) approach in DMU was used to perform a genome wide association study and estimate variance components. The SNPs on the bovine HD chip were mapped to the Btau4.0 assembly. In Danish Holstein, heritabilities ranged from 0 for 2 Hex 2 NeuAc and 4 Hex 4 HexNAc 1 Fuc to 0.67 for 3 Hex 2 HexNAc and 0.68 for 4 Hex 1 HexNAc. With an average heritability of 0.36. For Danish Jersey, heritability ranged from 0 for 2 Hex 1 HexNAc (isomer 2) and 2 Hex 2 NeuAc to 0.92 for 4 Hex 2 HexNAc (Lacto-N-hexose). With an average heritability of 0.42. Variation in heritabilities among OS reflected the same pattern between the breeds, and generally, there was a tendency for acidic OS to have low heritabilities in both breeds (Figure 3). However, most bovine OS display moderate to high heritabilities, which indicate a very strong genetic influence underlying bovine milk OS and a good potential for increasing these through selective breeding.

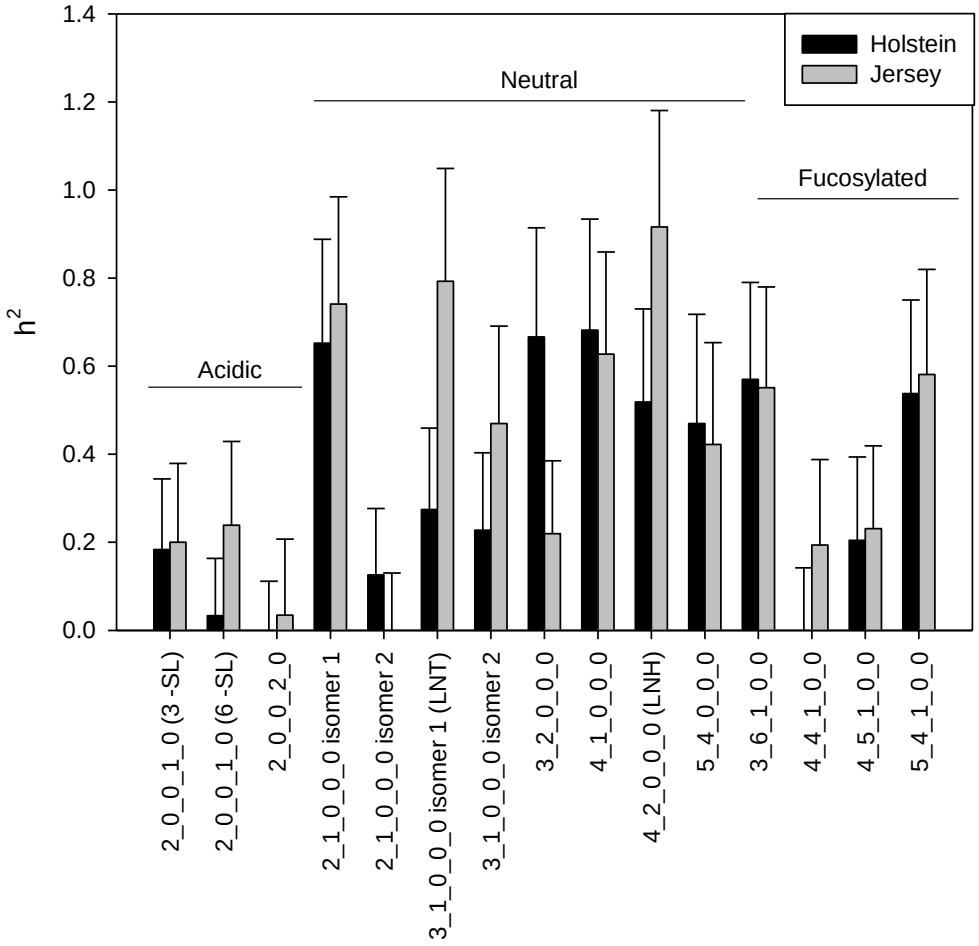

Figure 3. Heritability estimates (mean ± standard error) for individual acidic, neutral and focusylated OS in Danish Holstein and Danish Jersey. Oligosaccharide composition (Hex_HexNAc_Fuc_NeuAc_NeuGc).

A genome wide association study identified 1770 significant SNP markers (FDR < 0.10) associated with the bovine milk oligosaccharides in Danish Holstein and 7290 significant SNPs in Danish Jersey. The number of overlapping SNPs between the breeds were just 10. No significant SNPs were identified for acidic OS in Holstein and Jersey. In Holstein, the SNP markers were aligned to five neutral and one fucosylated OS. Most significant SNPs were detected for Lacto-N-Hexaose (n = 736). Of these 289 were located on BTA 1, where the most significant SNPs were BOVINEHD0100024179 and BOVINEHD0100024184 with –log10(P-value) of 20.36 and 20.77, respectively. Very interestingly BOVINEHD0100024179 was also the most significant SNP for lacto-N-tetraose (LNT, –log10(P-value) = 19.77). This quantitative trait locus (QTL) contains the gene *B3GNT5* which encodes UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5, an enzyme involved in glycan synthesis. Likewise, an overlapping SNP between lacto-N-tetraose and Lacto-N-Hexaose was found for ALG3 encoding alpha-1.3-mannosyltransferase. This gene is also located on BTA1, and the encoding enzyme is related in N-linked glycosylation. Other candidate genes of interest detected were B3GALNT2 encoding beta-1.3-N-acetylgalactosaminyltransferase 2 on BTA28 (5 Hex 4 HexNAc), GLT6D1 encoding glycosyltransferase 6 domain containing 1 on BTA11 (4 Hex 1 HexNAc) and LOC520336 encoding N-acetyllactosaminide beta-1.6-N-acetylglucosaminyl-transferase isoform C on BTA23 (lacto_N_Hexaose). In

total 226 SNPs were overlapping between Lacto-N-Hexaose and Lacto-N-tetraose suggesting a common genetic influence of these OS.

In Jersey, the SNP markers were aligned to seven neutral and four fucosylated bovine OS. More than 1000 significant SNPs were assigned to Lacto-N-Hexaose and Lacto-N-tetraose. 388 of these SNPs were overlapping between the traits and a very significant QTL on BTA2 included overlapping SNPs for MAN1C1 encoding mannosidase alpha class 1C member 1 and PIGV encoding phosphatidylinolsitol glycan anchor biosynthesis class V, which are genes involved in glycan metabolism. Nineteen of the most significant SNP (-log10(P-value) ~ 53) on BTA11 for 2 Hex 1 HexNAc (isomer 1) in Jersey were assigned to the ABO gene, which encodes for the different transferases related to the ABO blood type system (Figure 4). As one of the SNPs is a non-synonymous mutation resulting in an amino acid change, this candidate gene is very interesting for OS synthesis in milk. The QTL on BTA11 also included other SNPs assigned to candidate genes of interest including ST6GALNAC6 encoding N-acetylgalactosaminide alpha-2-6-sialyltransferase and GLT6D1 encoding glycosyltransferase 6 domain containing 1. Furthermore, a large number of overlapping SNPs were found for fucosylated OS suggesting a common synthesis pathway for these OS. Candidate genes of interest including LFNG encoding O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase and an overlapping SNP for this gene was found in 5 Hex 4 HexNAc 1 Fuc and 4 Hex 4 HexNAc 1 Fuc.

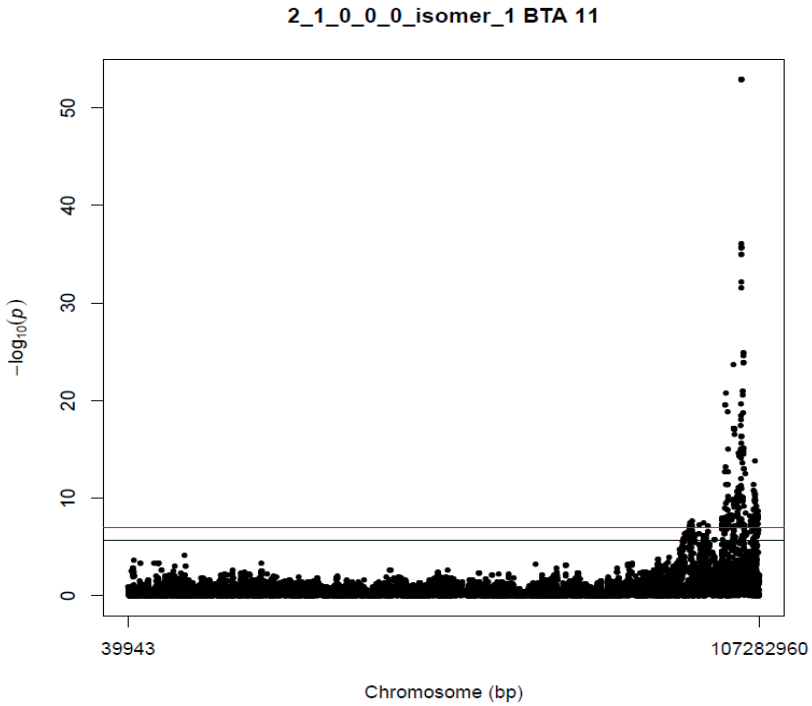

Figure 4. QTL on BTA11 for 2 Hex 1 HexNAc (isomer 1) in Jersey. Top SNPs are assigned to the ABO gene.

In conclusion, with an average heritability of 0.36 in Danish Holstein and 0.42 in Danish Jersey, most bovine oligosaccharides display moderate to high heritabilities, which indicate a very strong genetic influence underlying bovine milk oligosaccharides and a good potential for increasing these through selective breeding. The project has provided novel insights into OS (co)variation and identified a number of interesting candidate genes involved in the metabolism of bovine milk OS.

11. Deviations

The high affinity of the tags for carbonyl groups has resulted in some unspecific tagging of low molecular weight contaminants from lab equipment and containers. These contaminants don't conflict with OS profiling using traditional techniques, but are detrimental to the more sensitive quantitative data in experiments using carbonyl-reactive tags. Therefore, a systematic investigation was required to determine the sources of all contaminants and prevent their interaction with the samples. This issue caused some delay in the analysis of milk samples. Furthermore, the difference in OS abundancy between the breeds required additional method optimization and further delayed the analysis of the Jersey samples. All milk samples have now been quantified and the genetic analyses are ongoing. This resulted in a six months extension of the project.

12. The relevance of the results, including relevance for the dairy industry

To our knowledge, this is the first study documenting a solid breeding potential for bovine milk oligosaccharides. This can be of high value for the dairy ingredient industry, where isolation of bovine milk oligosaccharides can be valuable for improved infant formulae.

13. Communication and knowledge sharing about the project

Papers in international journals:

Robinson R, NA Poulsen, D Barile. Multiplexed bovine milk oligosaccharide analysis with aminoxy Tandem Mass Tags. Submitted to PLoS One.

Robinson, RC, Colet E, Tian T, Poulsen NA, Barile D. An improved method for the purification of milk oligosaccharides by graphitized carbon-solid phase extraction. Submitted to *International Dairy Journal*.

Easily read papers:

Carbohydrates in milk come under scrutiny. Newsletter, November 2014. DCA – Danish Centre for Food and Agriculture.

Kulhydrater fra mælk under lup. Mælkeritidende 2014, 25-26:12.

Mælkens enestående kulhydrater - Optimering af komælkens sundhedsgavnlige egenskaber gennem avl. Mælkeritidende 2015, 12: 10-11

Mælk indeholder sunde stoffer. Perspektiv, Årsberetning 2014. DCA – Nationalt Center for Fødevarer og Jordbrug, s. 32-33

Oral presentations at scientific conferences, symposiums etc.:

Robinson, RC, NA Poulsen, LB Larsen, D Barile. 2017. Comparative analysis of bioactive oligosaccharide production in dairy cows using novel analytical techniques. IC-FOODS Conference – building the internet of food. Nov. 6-8 2017, Davis, California. Abstract and poster.

Robinson, RC, NA Poulsen, LB Larsen, D Barile. 2017. Comparative analysis of bioactive oligosaccharide production in dairy cows using novel analytical techniques. International Symposium on Milk Genomics and Human Health. Sep. 26-28 2017, Quebec City, Canada. Abstract, poster and oral presentation.

Poulsen, NA, B Buitenhuis, RC Robinson, D Barile, LB Larsen. 2017. Genetic parameter estimation for bovine milk oligosaccharides in Danish Holstein. International Symposium on Milk Genomics and Human Health. Sep. 26-28 2017, Quebec City, Canada. Abstract and oral presentation.

Robinson RC, Poulsen NA, Larsen LB, and Barile D. 2016. A novel method for high-throughput analysis of bovine milk oligosaccharides. Abstract and oral presentation at the 13th International Symposium on Milk Genomics and Human Health. September 27-29. Davis, CA. This poster was also presented at the First International Conference for Food Ontology, Operability, Data and Semantics, UC Davis November 7-9. 2016, and was awarded the first place in the poster competition (category: Food & Health).

Robinson, RC and Barile, D. 2016. A novel method for high-throughput analysis of bioactive oligosaccharides. HPLC 2016. June 19-24. San Francisco, CA.

Robinson, RC and Barile, D. 2015. A novel method for high-throughput analysis of bioactive oligosaccharides. 2015 Graduate Student Research Poster Competition at the University of California, Davis. December 10. Davis CA. Awarded 3rd Place by a panel of judges from industry.

Oral presentations at meetings:

Randall RC. 2017. Comparative analysis of bioactive oligosaccharide production in dairy cows using novel analytical techniques. Presentation at the Milk Bioactive Group meeting, FFHI, UC Davis, California, November 3.

14. Contribution to master and Ph.D. education

The project has been part of PhD project of Randall Robinson, UC Davis, CA.

15. New contacts/projects

As part of the project Assistant Professor Nina A. Poulsen was on a 6-month research stay at Daniela Barile's lab in UC Davis, CA.

16. Signature and date

The project is formally finalised when the project manager and DDRF-representative (e.g. steering committee leader) have signed this final report.

Date: ___30.10 2017__Signature, Project manager:_Lotte Bach Larsen_____

Date: 30.10.2017 Signature, DDRF-representative: Grith Mortensen