

# Poster Abstracts

## $\alpha_{s1}$ -casein Polymorphism And Proteomics Of The Goat Milk Fat Globule Membrane: New Insights Into Milk Lipid Droplet Formation

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Fat is present in milk as droplets of neutral lipids surrounded by a complex membrane, called Milk Fat Globule Membrane (MFGM). Formation of lipid droplets is thought to occur by accumulation of lipids within the bilayer of the endoplasmic reticulum (ER). The lipid droplets dissociate from ER by budding and are transported to apical cell regions by as yet unknown mechanisms probably involving cytoskeletal components. These droplets are secreted in milk as fat globules by being progressively enveloped by apical plasma membrane.

The extensive genetic polymorphism described in the goat at the  $\alpha_{s1}$ -casein (CSN1S1) locus is associated with strong differences in milk protein and fat composition along with secretory disorders in the mammary epithelial cell (MEC). The goat model therefore represents a powerful tool to unravel mechanisms for milk fat secretion in cattle. However, only sparse studies exist on goat MFGM and little is known about the impact of  $\alpha_{s1}$ -casein genetic polymorphism on MFGM protein composition. To get more insights into the mechanisms involved in lipid droplet formation and their release as fat globules in milk, we have undertaken an original study to better characterize the fat globule in the goat milk by the means of biophysical and proteomic tools.

First, we brought evidence that the fat globule parameters (size and zeta potential) can be related to the genotype at the  $\alpha_{s1}$ -casein locus, and hence to milk fat content. Indeed, milk from animals with strong genotypes for  $\alpha_{s1}$ -casein (AA homozygous goats) display larger globules than milk from null genotypes (OO homozygous goats). In addition, the zeta potential (the global charge which reflects glycoprotein and/or glycolipid composition of the MFGM) was significantly lower in animals producing milk with reduced fat content. These results reveal the profound remodelling of the fat globule structure which occurs in  $\alpha_{s1}$ -casein null animals.

On the other hand, no genotype effect was observed for the main MFGM proteins: MUC-1, butyrophilin, and adipophilin. These data does not favour any active role for these proteins in regulating lipid droplet size and/or composition in the MEC. However, our proteomic studies based on a non conventional 2D approach (16 BAC/SDS-PAGE) combined with CyDye-labelling, showed that two MFGM-associated proteins were over expressed in  $\alpha_{s1}$ -casein null animals. Differential expression of these proteins in goats with extreme genotype for  $\alpha_{s1}$ -casein was confirmed by spectral counting (a label-free method based on total MS/MS spectra counts of peptides from a given protein allowing the measurement of relative abundance between proteins in complex mixtures), western blotting and quantitative PCR experiments on mammary tissues. Therefore, beyond the prevailing model of lipid droplet secretion

involving adipophilin, butyrophilin, and xanthine oxidase, our  $\alpha_{s1}$ -casein model suggests an active role for two additional proteins in regulating lipid droplet formation and/or extrusion from the MEC. Taken together, our results open new roads for regulation of milk secretion in mammals.

### **An Investigation On The Effect Of Feeding Systems On Bovine Milk Oligosaccharide Composition In Holstein Cows By Advanced Mass Spectrometry**

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It is well recognized that milk is important for the health of mammalian neonates. Evidence is accumulating that a basis for the evolutionary persistence of large amounts of oligosaccharides in mammalian milks is due to their prebiotic and anti-pathogenic properties. The most studied oligosaccharides are those present in human milk (both acidic and neutral oligosaccharides). Neutral oligosaccharides, especially, have been suggested to play an important role in prophylaxis against many pathogens. The health benefits that milk oligosaccharides provide for infants could also be available to humans of all ages, if the same structures and functions could be provided in the diet. There is thus an unmet need for alternative sources from which to obtain these molecules. Recent research has documented that bovine milk contains several structures analogous to human milk oligosaccharides, suggesting a similar protective action.

Owing to the structural complexity of oligosaccharides, the technologies for determining their structure have lagged far behind nucleic acids and proteins. In recent years, mass spectrometry (MS) has become the key tool for structural analysis of oligosaccharides because of its high resolution and sensitivity, and its ability to deliver compositional information necessary for oligosaccharide identification.

The objective of the present work was to investigate the effect of two different feeding systems (grazing treatment and total mixed ration or TMR) and lactation cycle on the composition of the neutral oligosaccharides in bovine milk. Fifteen Holstein cows and five heifers were assigned to each feeding system. In the grazing treatment, cows received grass silage ad-libitum plus minerals and vitamins during the dry period and they were turned out to pasture immediately after calving. In the TMR treatment cows were housed for the entire duration of the study and received a high-fiber/ low energy density TMR during the early dry period while a specific lactation TMR was provided from calving until the end of lactation. Milk samples from each treatment during the lactation cycles were analyzed by advanced mass spectrometry (MALDI FTICR) to reveal potential differences in the relative abundances and composition of oligosaccharides. Results showed that while identical oligosaccharide structures were present in milks from both feeding systems, differences in relative abundances of individual oligosaccharides were seen between the two feeding systems. The results of this study provide a technological pathway to the routine production of oligosaccharide-containing ingredients.

### **A Proteomic-Based Strategy To Identify Intestinotrophic Factors In The Milk Of PRM/Alf Mouse, A Model Of Intestinal Dolichomegaly**

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The PRM/Alf inbred mouse strain is characterized by a huge intestinal lengthening. The intestinal length of adult PRM/Alf mice is approximately 75 cm, compared to 55 cm in other inbred strains, such as DBA/2J, C3H/He, 129/Sv, and C57BL/6J. Since this lengthening takes place during the early post-natal growth, before weaning, we hypothesized that the milk should contain bioactive factors contributing to this remarkable phenotype. Thus, we investigated the milk protein fraction, using a proteomic approach, to compare milks from PRM/Alf and C57BL/6J (as a control) mouse lines and to highlight differences and identify such factors. Reverse Phase – High Performance Liquid Chromatography (RP-HPLC) was first carried out to analyse major milk proteins. The casein fraction shows similar chromatographic profiles between both milks. However, the relative contents of serum albumin and whey acidic protein were higher in PRM/Alf milks.

Another methodological approach, combining 2-D Differential In-Gel Electrophoresis (DIGE) and mass spectrometry, was then used to identify differential expression and/or structure of minor milk proteins between PRM/Alf and C57BL/6J. Four biological replicates from the two sub-groups (PRM/Alf vs. C57BL/6J) were used. Separations were performed on high resolution linear strips (pH range: 4-7). The total number of detected spots was 536. Using Same Spots Software, the statistical analysis (ANOVA) of DIGE data showed 73 differentially expressed spots (q-value < 0.05 and differentiation power > 0.8). The significant spots were automatically excised (ExQuest spot cutter, Biorad) and protein identification by LC-MS/MS mass spectrometry is in progress.

2D electrophoresis does not allow identification of very acidic (pH<3) and basic (pH>10-11) proteins, as well as the proteins with a low molecular mass. To overcome this limit, a complementary method based on 2D-chromatography ("ProteomeLab PF2D", Beckman Coulter) has been undertaken. Proteins are first separated by chromatofocusing (pI) and then separated by Reverse Phase chromatography (RP-HPLC) on a C18 column in the second dimension. As for 2D gel electrophoresis, separated proteins are subsequently analyzed using proteoview and deltaview softwares. Selected proteins are again identified by mass spectrometry.

## **A Requirement For Insulin In Prolactin-Stimulated Expression Of Milk Protein Genes In Mammary Explants From The Pregnant Mouse**

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The murine mammary explant culture model has been used for many years to mimic lactation and to examine the endocrine control of milk protein gene expression. Studies showed the expression of the milk protein genes requires the compliment of insulin (I), prolactin (P) and cortisol (F) in the culture media. Historically, mammary explants were cultured initially in IF and then P added to the media to induce expression of the milk protein genes, leading to the assumption that most mammary genes were prolactin-dependent. We have exploited this model using a mouse Affymetrix array to analyse the

individual contribution of insulin to expression of the milk protein genes as well as the synthesis of milk protein. Mammary explants from mid-pregnant mice were cultured in the hormone combinations FP, IF, IP and IFP. Explants were harvested after four days, RNA extracted and changes in mammary gene expression regulated by each of I, F and P in the presence of the other lactogenic hormones determined using Affymetrix microarray. The complement of I, F and P was required for maximal expression of the major casein and whey protein genes. The expression of 164 genes was responsive to insulin in the presence of FP and 18 of these genes were involved in milk protein synthesis. In addition to the milk protein genes, insulin stimulated the concomitant increase in expression of two key milk protein gene transcription factors, Elf5 and Stat5a, as well as expression of the folate receptor 1 (Folr1) gene highlighting an important role for the hormone in folate metabolism, a process which is emerging to be central for protein synthesis. This indicates insulin plays a crucial role in the transcription of many milk protein genes, and is important for milk protein synthesis at multiple levels within the mammary epithelial cell, including transcription, post-transcription and amino acid uptake and metabolism.

Of the total 354 genes that were responsive to IFP in the mammary explant culture system, 119 genes were largely regulated by any one of the hormones alone (prolactin regulated the expression of only two genes, hydrocortisone regulated expression of 65 genes and insulin alone regulated the expression of 52 genes, including Stat5a and Elf5). This analysis showed that the increase in gene expression of Stat5a and Elf5 had an absolute requirement for insulin, and, interestingly, these two milk protein transcription factors have both previously been identified as key components of the prolactin signaling pathway.

Subsequent culture experiments in HCII cells showed maximal expression of milk protein genes, as well as the synthesis of  $\beta$ -casein protein, required insulin in the presence of prolactin and dexamethasone. Furthermore, these studies confirmed that Stat5a and Elf5 gene expression could be induced in mammary epithelial cells cultured in the absence of prolactin but in the presence of insulin. Collectively, this data suggests that the requirement of insulin for milk protein gene expression may primarily be facilitated by Stat5a and Elf5. Whereas prolactin plays an essential role in phosphorylating and activating Stat5a, the gene for this protein is only expressed when insulin is present in culture media. This indicates that insulin plays a crucial role in the prolactin-induced expression of the milk protein genes. Therefore, study has begun to elucidate the molecular mechanisms of insulin underpinning the co-ordinate induction of milk protein synthesis.

### **Coevolution With Milk Has Influenced Innovation In The *Bifidobacterium Longum* Subsp. *Infantis* Genome To Utilize Sialylated And Fucosylated Milk Oligosaccharides**

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Following birth, the breast-fed infant gastrointestinal tract is rapidly colonized by a microbial consortium often dominated by bifidobacteria. This numerical advantage competitively excludes pathogen colonization in addition to conferring other health benefits to the developing neonate. To resolve the genesis of this milk-mediated enrichment, we have previously demonstrated that select bifidobacterial phylotypes utilize specific human milk oligosaccharides (HMOs) secreted early in lactation<sup>1</sup>. Accordingly, HMO ubiquity in the infant gut has profoundly influenced the remodeling of the *Bifidobacterium longum* subsp. *infantis* genome towards milk glycan utilization at the expense of plant-derived sugars. This includes the innovation of several catabolic processes previously unknown in the bifidobacteria<sup>2</sup>. Biochemical characterizations of these catabolic enzymes have confirmed that they are indeed active on sialylated and fucosylated oligosaccharides and their derivatives. Moreover,

bifidobacterial glycosidases exhibit unique expression profiles dependant on growth on lactose or HMO as a sole carbohydrate source. Temporal tracking of both neutral and acidic oligosaccharide consumption has revealed the specific compositions preferred by *B. longum* subsp. *infantis* while subsisting on these milk sugars. Furthermore, an array of evolutionarily divergent oligosaccharide transporters are employed in extracellular recognition and transmembrane import of specific milk glycan structures. Finally, next generation sequencing and chromosomal microarray analysis of additional bifidobacterial genomes has generalized these findings to a clade typically isolated from breast-fed infants. These studies have functionally verified, in part, the genomic mechanism by which *B. longum* subsp. *infantis* interacts with milk-borne molecules, and strongly supports a milk-dependant colonization strategy. Thus milk should no longer be regarded solely as consequential to the evolution of the mammalian genome, but as a selective imperative that has directed the fundamental structure of the mammalian microbiome as well.

1. LoCascio, R.G. et al. Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. *J Agric Food Chem* **55**, 8914-8919 (2007).
2. Sela, D.A. et al. The genome sequence of Bifidobacterium longum subsp. infantis reveals adaptations for milk utilization within the infant microbiome. *Proc Natl Acad Sci U S A* **105**, 18964-18969 (2008).

### **Comparative Genomics Of *Bifidobacterium Longum* Strains Identifies Genes Relevant For Human And Bovine Milk Oligosaccharides Utilization**

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Human milk oligosaccharides (HMO) are believed to provide protection against pathogens and prebiotic enrichment of beneficial commensals such as bifidobacteria. Bifidobacterium *longum* bv. *infantis* ATCC15697, a microorganism commonly present in the feces of breast-fed infants, preferentially consumes four milk oligosaccharides representing nearly 70% of all HMOs present in milk. Genome analysis of *B. infantis* ATCC15697 has revealed a number of gene clusters putatively associated with HMO consumption. This suggests that HMOs are a class of bioactive milk molecules capable of enriching the growth of specific bacterial populations in the gut of breastfeeding infants.

The purpose of this study was to use genomic and glycomic tools to investigate how the HMOs consumption patterns among several infant gut isolates of Bifidobacteria correlate with their genomic diversity. Using *B. infantis* ATCC15697 as a reference strain, comparative genomic hybridization (CGH) analyses was performed on fifteen additional strains having various HMO consumption profiles.

In 2008 I presented at this meeting the preliminary analysis of this work, the data analysis is being finalized and drafted as a manuscript for publication. The bacterial strain analyzed clearly cluster into two groups, the first containing exclusively *B. infantis* strains achieving high growth on HMO, and the second generally containing non-*B. infantis* or *B. longum* strains with moderate or little ability to grow on HMOs. The genomes of all the *B. infantis* analyzed in this study uniquely contain gene clusters associated with bovine and human milk oligosaccharides consumption and for adaptation for populating the breastfed infant gastrointestinal tract. Conversely, the *B. longum* group consistently lacks the

glycosyl hydrolases, oligosaccharide binding proteins and transporters and all other accessory proteins normally present in *B. infantis* and likely important for bovine and human milk consumption.

Findings of this study:

- *B. infantis* shows genomic and physiological adaptation of for optimal growth in milk oligosaccharides, and highlight the importance of this organism for use as a probiotic
- Oligosaccharides from bovine and human milk are likely to elicit very specific prebiotic responses only the genomes of *B. infantis* strains have the complex metabolic machinery needed to utilize them.
- CGH can reveal detailed, genome-wide probiotic strain diversity information which guide into rational selection of cultures food and dairy applications

## **Developmental Regulation Of Sialyltransferase Gene Expression In The Murine Mammary Gland**

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Sialoglycoconjugates are integral to various, ubiquitous biological phenomena, such as cell-cell interaction, cell migration, adhesion and metastasis. Interestingly, they seem to also have been co-opted by the mammary gland to fulfill a slightly different role. Sialoglycoconjugates found in milk can protect the gastrointestinal tract of the suckling neonate by competitively binding to invading pathogens and promoting growth of beneficial flora. They may also be important in protecting the involuting mammary gland from infection and their potential role in post-natal brain development is of particular interest in human infant nutrition. Sialyltransferases are a family of Golgi membrane-bound, glycosyltransferases that transfer sialic acid from CMP-sialic acid to carbohydrate acceptor groups of glycoproteins, glycolipids and free oligosaccharides, forming various sialoglycoconjugates. Their expression is primarily controlled at the level of transcription and is expedited through the use of several tissue/development-specific promoters giving rise to numerous transcripts divergent only in their 5' untranslated regions.

Thus far, 20 members of the mammalian sialyltransferase family have been cloned. Depending on the type of linkage formed and the type of sugar acceptor, they are traditionally segregated into four groups: ST6Gal, ST6GalNAc, ST3Gal and ST8Sia. There are one or more enzymes in each category and although some have broader acceptor specificities than others, the enzymes in each group only form one type of linkage between the sialic acid and the sugar acceptor.

Mining of mouse mammary development microarray data generated by Master et al. (2002), revealed differential expression of several sialyltransferase genes during pregnancy, lactation and involution. ST6GalNAc2 is dramatically up-regulated at day 12 of pregnancy and continues to increase in expression until day 2 of involution, when expression is abruptly reduced. ST3Gal1 is moderately up-regulated at day 12 of pregnancy but begins to gradually decline in expression from the first day of lactation, with a more significant decline after day 2 of involution. ST6Gal1 expression levels begin to slightly increase from the 12th day of pregnancy, followed by a dramatic jump at 18 hours into lactation and an equally dramatic drop at day 2 of involution. ST8Sia5 shows an inverted pattern of expression to that exhibited by ST6gal1, such that it is down-regulated by the same magnitude 18 hours into lactation and subsequently up-regulated at day 2 of involution. Expression of ST3Gal4 declines at day 9 of lactation but recovers at day 9 of involution, whilst ST3Gal2 levels are consistently lower in lactation than at day 6 of pregnancy, only recovering at day 7 of involution.

Lactation-specific regulation has already been demonstrated for ST6Gal1, where a mammary and lactation-specific 5'UTR exon, exon L, is recruited to generate a novel transcript and significantly increase ST6Gal1 gene expression at the onset of lactation. The existence of a lactation-specific promoter upstream of exon L was postulated. As preliminary analysis of the Master et al. (2002) microarray data demonstrates, several other sialyltransferase genes are also differentially expressed in the mammary gland during pregnancy, lactation and involution.

We propose that this could be evidence of a developmental regulatory mechanism for sialyltransferases, required to create an optimal profile of sialoglycoconjugates to cater for the needs of the neonate during lactation and the vulnerable state of the mammary gland during involution.

Master, S.R., Hartman, J.L., et al. (2002) Functional Microarray Analysis of Mammary Organogenesis Reveals a Developmental Role in Adaptive Thermogenesis. *Mol Endocrinol*, 16, 1185-1203.

### **Diet-Induced Obesity Leads To Early Mammary Gland Development During Pregnancy In Rabbit**

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Epidemiological and animal studies suggest that alterations to the hormonal and metabolic environments during puberty can induce both adverse effects on lactation and mammary tumorigenesis (de Assis *et al*, 2006). Obese women have an increased risk of failing to initiate successful breastfeeding and can experience a premature cessation of lactation (Riva *et al*, 1999; Rasmussen *et al*, 2004). The effects of obesity on lactogenesis were highlighted during early studies in the rat, where this pathological condition was shown to reduce the chances of a successful outcome regarding pregnancy and lactation (Rolls *et al*, 1984). More recent studies have suggested that impaired mammary gland development and lactogenesis are possible causes of lactation failures and the high mortality observed amongst the pups of obese dams. In cattle, evidence concerning the relationship between growth rate, mammary growth and milk yield led to the conclusion that increased growth rate due to high feeding levels before the onset of puberty could reduce pubertal mammary development and milk potential (Sejrsen, *et al*, 1997). Moreover, high energy feeding decreases mammary epithelial cell proliferation in areas of active ductal expansion at puberty (Davis Rinker *et al*, 2008).

We have further investigated the impact of obesity on mammary gland development. A rabbit model of diet-induced obesity was developed by feeding female rabbits with a high fat (HF) diet (+276% fat and +269% sugar when compared to the control (C) diet) from 8 weeks of age until mid-pregnancy (day 14 of pregnancy). Body weight gain between 21 weeks of age until day 14 of pregnancy was significantly higher (+10%) in HF animals, and was mainly associated with the development of more adipose tissue. By contrast, the number of fetuses did not differ between the two groups, although mean fetal weight was lower in HF rabbits than in controls.

Mammary gland morphology was altered in the HF group. On day 14 of pregnancy, the mammary ducts were made up of a cell monolayer. They were dilated and filled with dense products. Alveolar structures had invaded the entire fat pad, whereas they were more clustered in the control group. Electron microscopy analysis revealed that casein micelles were present in the lumen of alveolae. Moreover, numerous microvillousities were located in the apical region of mammary epithelial cells.

Immunohistochemical studies of HF mammary tissue revealed a more abundant accumulation of the major rabbit milk proteins, alphaS1-casein, kappa-casein and Whey Acidic Protein (WAP), in both the alveolar lumina and secretory ducts. As revealed by BodiPy staining, lipids had also accumulated in the lumens and ducts. Both analyses revealed an early secretory phenotype in the HF group.

Milk protein synthesis in mammary tissue extracts was quantified by Western blot analyses. A clear accumulation of milk proteins was observed in HF animals but was almost undetectable in controls.

These results show that diet-induced obesity, beginning before puberty, alters mammary gland development at mid-pregnancy in the rabbit. On day 14, obese mammary tissue displayed a morphological aspect and functional profile similar to those normally observed at the end of pregnancy. In rodents, similar precocious mammary development induced by WAP overexpression (Burdon *et al*, 1991) or the constitutive activation of PRL transduction pathways (Gourdou *et al*, 2004) have been associated with impaired lactation, so that lactation defects could be anticipated in HF animals. The effects on lactation of this early development are currently under study.

### **DGAT1 K232A Polymorphism Greatly Affects Mammary Tissue Activity And Milk Component Synthesis**

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Animal feeding, genetics and milking frequencies are the main factors affecting significantly milk biosynthesis and secretion in the healthy udder, with a direct impact on composition, techno-functional properties and nutritional value of milk. The non conservative K232A mutation occurring in the bovine DGAT1 gene, which encodes the enzyme catalyzing the last step of triacylglycerol biosynthesis, is responsible for a reduction in fat content (AA) and for a modification in fat composition. To determine cellular mechanisms underlying these differences, we have compared, using microarray, gene expression profiles from mammary tissue biopsies of cows reared in well defined and controlled experimental conditions. To each homozygous cow KK corresponded its full-sib (n=4) or half-sib (n=2) homozygous AA, at the same lactating stage and day of lactation. To compare gene expression in mammary tissues, we used two types of bovine microarrays: 22K oligoarrays spotted at CRB-GADIE (INRA, France) and commercial oligoarrays (4x44K Agilent technologies).

Our results confirmed a significant reduction of milk fat content (41.6 vs. 51.6 g/kg) and of unsaturated-middle chain fatty acid content (C14:1, C16:1), as well as a significant increases in saturated middle chain (C14:0) and in unsaturated-long chain (C18:1 *cis*11, C18:1 *cis*12, C18:2*n*-6 and C18:3*n*-3) fatty acid content, with AA genotype as compared to KK genotype. In addition, milk fat globules were significantly smaller with AA cows.

The first step of microarray data analysis was to create and validate a “super data set” by bringing together data set of each type of slide. “The super data set” was equivalent to a microarray on which 14 567 genes with reliable annotations are represented (about 55% of the whole bovine genome). Gene expression profiling performed on this new probe set revealed up-regulation of genes involved in lactose, lipid and protein biosynthesis for AA individuals. Genes of the lipid biosynthesis up-regulated

with AA genotype partially explain observed differences in fine milk fat composition by giving alternative pathways of diacylglycerol metabolism: triacylglycerol synthesis through DGAT2 enzyme and phospholipids synthesis with LRAT and PEMT enzymes.

### **Do Milk Proteins Affect the Enteric Nervous System?**

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The enteric nervous system (ENS) is intimately involved not only in gut motility, but also in aspects of gut function including immune regulation and abdominal sensations. The ENS undergoes significant maturational change during the neonatal period of milk intake. Many neurons of the ENS project towards the lumen sensing the chemical composition of the gut contents. Indeed, many milk proteins have known effects on neurons from the central or peripheral nervous systems.

Surprisingly the ENS has not been investigated as a major physiological target of milk. To investigate whether milk proteins have a role on the development or function of the neonatal ENS we have systematically cloned and expressed full-length cDNAs of milk proteins and have evaluated approximately 200 milk proteins for their ability to stimulate key intracellular signalling pathways in neuron-like cells and for their ability to stimulate neurite outgrowth of neuron-like cells.

Proteins that have shown effects on neural-like cell lines are being tested *in vitro* using a whole gut culture system to examine their effects on the ENS.

### **Effects Of Single Nucleotide Polymorphisms In Stearoyl Coa Desaturase On Milk Fatty Acid Profile In Lactating Holstein Cows Fed Diets Varying In Fat Content**

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Some components of milk fat have the potential to benefit human health. Conjugated Linoleic Acid (CLA) in particular has been shown to reduce the risk of cardiovascular disease and have anti-carcinogenic effects in animal models. The objective of this study was to determine the effect of single nucleotide polymorphisms (SNP) in the stearoyl CoA desaturase (SCD) gene on the ability of dairy cows to produce 9c, 11t-18:2 (CLA), and to determine whether the CLA to 11t- 18:1 (VCA) ratio can be used as a phenotypic indicator for CLA production capability. A SNP in SCD found on the fifth exon of chromosome 26, which encodes an amino acid change from alanine to valine, was evaluated in this study. Twelve lactating dairy cows in mid-lactation ( $180 \pm 44$  days in milk) were blocked by SCD genotype (AA, AV, and VV; n=4 for each genotype) and fed either a high or low fat diet in a cross-over design with 21-d periods. The high fat diet contained 5% sunflower seed oil on a dry matter basis, and dietary fat content was 3.2 and 8.3% for the low and high fat diet, respectively. Milk yield and milk fat concentration were not affected by dietary treatment or genotype, averaging 29.0 kg/d and 3.61%, respectively. The CLA concentration in milk fat was greater for cows fed the high fat diet compared with those fed the low fat diet (4.4 vs. 0.6 %;  $P < 0.0001$ ), but was not affected by genotype. The CLA-to-VCA ratio was 0.62 when AV and VV cows were fed the low fat diet, but decreased to 0.50 when they were fed the high fat diet. However, the CLA-to-VCA ratio was not affected by dietary fat content for AA cows, averaging 0.57 ( $P < 0.06$  for diet x genotype interaction). These results indicate that AA animals maintained consistent activity of SCD when they were fed the high fat diet. Because the CLA-to-VCA ratio is affected by dietary fat content, it may not be used as a phenotypic indicator to

identify animals with greater capability for CLA production in the field, in which animals are fed diets varying in dietary fat content.

## **Epithelial Stem Cells Endogenous To Human Breastmilk Give Rise To Three-Dimensional Differentiated Milk-secreting Units *In Vitro***

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Human breastmilk has previously been shown to contain a cellular complement that can generate differentiated epithelial lineages in primary culture. We have identified the generative population giving rise to these cultures in human milk based on the qualified stem cell marker p63, and shown that this population is enriched for the CD49f+/CD29hi/CD24hi phenotype shown to give rise to mammary outgrowths *in vivo*. The total cell population isolated from expressed breastmilk samples from 20 lactating women was immunofluorescently labeled for stem and differentiated epithelial cell markers prior to cultivation and at three day intervals for 21 days of growth in two- dimensional culture. We show that expansion of the stem cell population occurs in the floating cell population within the first five days of growth and depletes reciprocally with emergence of an adherent population homogenously expressing pan-epithelial markers. In the proceeding 16 days of growth sub-populations of adherent cells express myoepithelial and luminal epithelial markers in mutually exclusive colonies, indicating that lineage commitment occurs prior to adherence and differentiation. Further, we have used cells harvested from primary culture to cultivate alveolar milk-secreting structures (mammospheres) in a three-dimensional biomatrix. We also demonstrate using live-cell confocal imaging that tissue architecture in three dimensions is highly dependant on plating density, and that cultivation of cells at high density results in rapid (<12 hours growth) self-assembly into ductal structures that later develop terminal lumen-containing alveoli whilst plating at low density results in slow formation of single mammospheres. This approach provides a platform to study regulators of milk synthesis and mammary gland differentiation in an integrated parenchymal/extra- cellular matrix system, and for the first time allows experimentation on cells isolated non-invasively from the fully differentiated lactating epithelium.

## **From Genes To Function: Glycans From Invertebrates To Mammals**

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Glycosylation is universal in living organisms. Oligosaccharides are among the most diverse molecules in biological systems and play a crucial role in many processes such as cell-cell recognition, adhesion, division and differentiation. In the last decades, the identification of many glycoproteins in prokaryotic organisms demonstrated that protein glycosylation is not exclusively a prerogative of eukaryotes as previously thought.

Although a high degree polymorphism in glycan structures has been observed even within the same species (e.g. human milk free oligosaccharides), the core structure of the major glycan classes tends to be conserved across many species. As the genomes of new organisms become available, their sequence can be searched for genes encoding for the enzymes involved in oligosaccharides production.

Interestingly, recent studies proved that some bacteria and insects are able to glycosylate their proteins using the same processing pathways used by mammals.

Until now, studies have only focused on invertebrate glycans as immunogenic or virulent factors, while neglecting the possibility that some may have positive effects on human health. Various organisms, currently considered “undesired” in food-products, are actually capable interacting with our immune system and reduce proinflammatory responses. Research has revealed that autoimmune diseases, including allergies, inflammatory bowel diseases and arthritis, would be improved by reducing inflammatory processes.

For the first time, we conducted an investigation on selected artisanal dairy products containing unusual organisms, naturally present or deliberately introduced. Glycans were isolated and purified by solid-phase extraction and characterized by advanced mass spectrometry (MALDI-FTICR). Results showed that these organisms were able to produce glycans ex novo or to modify glycans present in the dairy milieu. As no information is available about the genome of the organisms studied, we are in the process of identifying genes encoding the enzymes involved in specific glycan pathways, validating their biological properties and the feasibility of scaling up for therapeutic applications.

## **Genome Mining For Discovery Of Milk Bioactives**

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Evaluation of milk derived proteins and peptides in tissue-specific bioassays is an essential step in the discovery and development of novel dairy bioactives. Milk contains components that provide critical nutritive elements, immunological protection, and biologically active substances to both neonates and adults. Beyond nutrition, there is an increasing amount of data and information to demonstrate a bioactive role for dairy components including a role in health benefits of individual components. With today's sophisticated Proteomic methodologies, biochemical and cell-biological research tools, the presence of many compounds with biological activity has been demonstrated. Functional activities of many peptides have been described, and the potential to engineer these peptides from milk derived protein fractions has benefited from advances in genomics. The objective of this study was to move forward with detailed screening and analysis of gene sets, with an increased focus on cow milk-derived peptides assessed in tissue specific assay and cross-research to proteomic data. This study has utilized a comparative functional genomics approach to analyse milk proteins and a database of gene sequences has been generated and interrogated for secreted proteins and peptides. Microarray based technology has been established from bovine systems which has yielded a comprehensive database containing gene and predicted polypeptides for in silico and in vitro analysis. Further in silico analysis of these proteins resulted in discovery of 500 candidates from a high throughput screening. To examine the relationship between genetic background and peptide functional variation, in vitro analysis of 26 proteins has been carried out. These proteins were found to involve in cell migration, proliferation and microvascular remodelling. Endothelial cell apoptosis contributes to microvascular remodelling in a variety of settings including involution embryogenesis, growth, chronic inflammation and wound healing. Bovine Serum albumin (BSA) one of the important constituent of milk was subjected to tissue specific study using Human Umbilical Vein Endothelial Cells. Despite the reported specificity for endothelial protection by BSA, we also demonstrated that protection is mediated by a partially cryptic albumin protein domain, which becomes more exposed and active following fragmentation. These results presents a strong case that dairy-derived proteins bioactive role in cell-cycle studies and may have a

strong application with regard to vascular remodelling and vascular disease states. While further investigation is required to properly characterize the putative conformational changes of these peptides and structural analysis for potential active sites. This approach has the capacity to unveil novel bioactivities, or to provide a strategy to reverse engineer bioactive peptides from milk proteins.

## **Identifying Rare Genetic Variants Of Milk Genes**

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In dairy cattle only a few specific genetic variants are known to affect the quantity and/or quality of milk and DNA-based selection for/against several of these variants has been successfully implemented. The gene variants known at present typically have large effects and are widespread across the cattle population. Rare genetic variants are likely to persist undiscovered even if they have large, either beneficial or detrimental effects and thereby affecting the variation in the technological qualities of milk. The occurrence of novel sequencing technologies where millions of sequences are performed in parallel now makes it possible to identify and exploit this particular type of variation.

Here we present an approach to detect both common and rare genetic variants of milk genes by targeted re-sequencing performed on pools of bovine DNA using the next-generation sequencing technology "454" provided by Roche. Targeted re-sequencing facilitates the detection of genetic variants in numerous animals simultaneously, while still retaining information about which breeds and individuals that carries the different genetic variants.

An experimental design for pooling of genomic DNA from >20.000 animals of different breeds has been developed and implemented on beta-lactoglobulin ( $\beta$ -LG). DNA isolated from bovine blood samples from 24 individuals of similar breed was pooled and amplicons covering coding sequence and intronic sequence of  $\beta$ -LG were amplified using specific primers. Subsequently amplicons were targeted according to a coded tag ligation protocol before sequencing on the Genome Sequencer FLX System (GS-FLX).

The GS Reference Mapper (Roche) was used for SNP detection and sequence data was analysed using bovine chromosome 11 (Btau 4.0) as a reference sequence. Called SNPs were validated by Sanger sequencing on individual animals. With this design we were able to detect a number of new as well as previously detected SNPs in both the coding region and in introns of  $\beta$ -LG.

## **Involution Of The Bovine Mammary Gland: Evidence Of Epigenetic Regulation**

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Milk protein gene expression in the mammary gland is regulated in a tissue-specific and temporal manner. During lactation in dairy cows, the transcription factor STAT5 is activated in response to prolactin and stimulates expression of milk protein genes by binding to target sequences in their promoters. At the end of lactation (i.e., the drying-off period induced by termination of milking) mammary gland involution is characterized by a rapid decrease in milk protein gene expression and an increase in apoptosis of milk secretory epithelial cells. This study examined the role of DNA methylation

in the silencing of milk protein gene expression in an attempt to understand the regulation of milk production at the molecular level. Alveolar tissue was obtained from non-pregnant cows in mid-lactation slaughtered at 0, 6, 12, 18, 24, 36, 72 and 192h (n=6/group) after the last milking. Quantitative real time RTPCR analysis of two STAT5 activated milk proteins,  $\alpha$ S1-casein and  $\alpha$ -lactalbumin, showed their mRNA levels were down-regulated 6.6-fold ( $P < 0.01$ ) and 3.5-fold ( $P < 0.05$ ), respectively, by 24 h post milking relative to 6 h and expression continued to decrease ( $P < 0.001$ ) until 192 h after the last milking. In situ end-labelling (ISEL) analysis showed the mammary sections from 0 to 36 h had very low levels of positive ISEL nuclei, and hence, a low level of apoptosis. By 72 and 192 h, the total number of ISEL apoptotic nuclei per alveolus was significantly increased (3.4-fold,  $P < 0.01$  and 5.5-fold,  $P < 0.001$ ; respectively) compared with 6 h post-milking. There were strong negative correlations of the level of  $\alpha$ S1-casein and  $\alpha$ -lactalbumin mRNA expression with the number of ISEL nuclei per alveolus ( $r = -0.78$ ,  $P < 0.001$  and  $r = -0.88$ ,  $P < 0.001$ , respectively). To elucidate the silencing mechanisms of these milk protein genes a quantitative MassARRAY methylation analysis was carried out.

This showed that during involution, there was an increase in methylation levels of the 3 CpG sites at a functional STAT5-binding site of the  $\alpha$ S1-casein-encoding gene. One site was increased from 15% to 32% ( $P < 0.05$ ), site 2 from 28% to 44% ( $P < 0.05$ ) and site 3 was increased from 28% to 53% methylation by 192 h compared to 18 h post milking. The level of methylation at each of these sites was negatively correlated with  $\alpha$ S1-casein mRNA expression (CpG site 1,  $r = -0.33$ ,  $P < 0.05$ ; site 2,  $r = -0.44$ ,  $P < 0.01$ ; site 3,  $r = -0.57$ ,  $P < 0.001$ ) and positively associated with apoptosis (CpG site 1,  $r = 0.55$ ,  $P < 0.01$ ; site 2,  $r = 0.63$ ,  $P < 0.001$ ; site 3,  $r = 0.8$ ,  $P < 0.001$ ). However, there was no change in methylation levels of the  $\alpha$ -lactalbumin promoter. Results suggest that alterations in methylation status at CpG sites at the functional STAT5 binding site in the  $\alpha$ S1-casein promoter are associated with the silencing of this gene during involution. Understanding the role of epigenetic mechanisms in regulating milk production may result in novel approaches and/or technologies for enhancing the lifetime lactation performance of dairy cows through manipulating epigenetic mechanisms.

## **Milk Proteins: The Quest For Balance**

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Milk is a hallmark of mammalian evolution: a unique food that has co-evolved with its producers to meet changing nutritional and developmental requirements. As milk is the only food source for newborn mammals, it has long been speculated that milk proteins should be enriched in essential amino acids. This belief has been supported by the comparison of essential amino acid content of milk proteins with other food sources. However no systematic analysis supports this assumption. Here we identify nine major milk proteins present in 13 mammalian species and compared them to a large group of other proteins from these same species. Our results indicate heterogeneity in amino acid composition of milk proteins as a food group, but also show a significant enrichment of essential amino acids in the milk-specific proteins (caseins and  $\alpha$ -lactalbumin), specifically by partitioning the 9 essential amino acids among these two different milk-specific protein groups. While high levels of certain amino acids appear to be consistently maintained, orthologous milk proteins display significant differences in amino acid composition across species. These observations are most consistent with temporal variation in the actions of selection, with periods of both positive and purifying selection. We argue that milk's amino acid composition is tailored to the species needs and capacities, balancing maternal nutritional supply and needs as well as the offspring's requirements. We conclude that the nutritional consequences of relying on milk sourced from other species may need to also address how the amino acid composition should be best modified to match that of human milk, and identify whether health benefits might accrue.

# NanoLC-MS/MS Reveals Dynamic Human Milk Protein Phosphorylation During The First Month Of Lactation

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## Introduction

Phosphorylation is a common, biologically relevant post-translational modification, which is complicated by several analytical hurdles, one of which is contamination by nonmodified peptides. Enriching phosphorylated peptides from a complex mixture has been shown to lessen the nonmodified peptide contamination. Titanium dioxide is an effective stationary phase for such enrichment. This work demonstrates that automated, integrated phosphopeptide enrichment is possible on a nano-LC scale by use of recently developed, chip-based phosphopeptide enrichment. Milk is a complex mixture, and contains several phosphoproteins that have developmental and immunomodulatory roles *in vivo*. It is known that protein content changes dramatically during lactation, although there have been essentially no studies on the dynamic behavior of protein phosphorylation during lactation.

## **Method**

Human milk from lactation days 2, 5, 10, 17 and 29 were used in this study. Milk proteins were isolated and 100 micrograms of protein was reduced, alkylated and digested with trypsin. Peptides were then desalted and dried. Aliquots of milk digests were then analyzed on an Agilent Chip-Q-TOF mass spectrometer. Samples were loaded onto a recently developed chip-based enrichment column that consists of a sandwiched C18/TiO<sub>2</sub>/C18 stationary phase. Nonphosphorylated peptides were released using a water/acetonitrile gradient and separated on an integrated C18 stationary phase for analysis. Next, the phosphopeptides were eluted from the enrichment column with an ammonium bicarbonate pH 9.0 buffer containing sodium orthovanadate and potassium fluoride. Both fractions were analyzed in data-dependant MS/MS mode with CID fragmentation.

## **Data**

On-line enrichment of phosphopeptides is demonstrated to be effective by two different metrics used in the study. A comparison of phosphopeptide and nonmodified peptide ion abundances in the flow-through fractions was used to determine the specificity of the separation. The chip was found to be over 95% selective for phosphopeptides under these conditions. Phosphopeptide enrichment efficiency, defined as the fraction of total phosphopeptide abundance occurring in the elution fraction, was greater than 65% for the highly complex human milk matrix. Contaminant peptides were either acidic or highly abundant, as expected. The enrichment chip isolates phosphorylated peptides despite a highly complicated peptide background, which eliminates the need for slower solid phase extraction based sample processing. Database searching was performed and the abundant phosphoproteins identified include alpha- and beta-casein, osteopontin, and polymeric Ig receptor. Specific phosphorylation sites were verified when possible using tandem MS, and their abundances were tracked during lactation. Variations in the degree of phosphorylation of specific milk proteins during lactation were shown. In several cases, specific sites within a phosphoprotein were shown to vary in a divergent fashion. Several other abundant whey proteins were simultaneously identified during these analyses, including lactoferrin, immunoglobulin A, and bile salt-stimulated lipase.

Compellingly, many of these changes occur during the first ten days of lactation, a time when the infant's immune and digestive systems are not fully matured, and when the infant's gut is being rapidly colonized by bacteria. As phosphorylation is known to affect cell-cell signaling and protein function,

these changes may suggest a yet undiscovered role for milk phosphoproteins in neonatal development of breastfed infants.

## **Natural Disease Resistance Parameters In Bovine Milk. A Potential For Antibodies?**

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Natural disease resistance of the cow can play a role in preventing and combating mastitis. Several immune parameters have been studied following experimental mammary infection with distinct pathogens. However, knowledge about the relationship between different immune parameters in an uninfected animal and its natural resistance to infection is limited. The hypothesis in this project is that certain immune parameters in milk correlate with the cow's capacity to resist mastitis. Identification of such parameters will enable the development of tests to objectively monitor the level of natural resistance to mastitis in practice in a non-invasive way. This would enable the farmer to take proper measures if needed. To investigate if the immune parameters, selected from literature, are useful to study natural resistance, in a pilot study, milk and blood samples were taken from twenty cows without clinical signs of diseases.

The goals of this experiment were to study; 1) the repeatability of the selected parameters within cows; 2) variation of these parameters between cows; and 3) the relation between these parameters in milk and blood. Subsequently, selected parameters, proven to be repeatable and variable, were tested in milk samples of the Dutch Milk Genomic Initiative. This project consist of ca. 2000 heifers with known pedigree, mastitis history and somatic cell count to study their heritability and relation with natural resistance to mastitis. The humoral parameters that were measured include (naturally occurring) antibody titres against keyhole limpet haemocyanin (KLH), a naïve antigen, and different pathogen associated molecular patterns (PAMPs), like lipopolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglycan (PGN) from bacteria. The antibodies showed repeatability within individual cows and variation between cows. Therefore they were suitable to be measured in the Milk Genomic Initiative samples of heifers, to study their heritability and relation with natural resistance against mastitis. Milk antibody levels to some of the antigens showed a moderate heritability, which is promising for breeding. A possible relation of the antibody titres with somatic cell count and mastitis incidence, is currently being analysed.

As a next step the observed immunological parameters are currently being analysed in conjunction with the established genotypes for these cows. Identification of quantitative trait loci (QTL) for milk composition were performed previously (Schopen et al. 2009, Stoop et al. 2009, Schennink et al. 2009). The genotypes consist of a set of 1536 single nucleotide polymorphism (SNP) randomly distributed across the bovine genome. The goal is to identify (QTL) for the measured immune parameters and to perform an association study to identify relevant genetic variation influencing the levels if these immune parameters.

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### **New Genetic Variants Influencing Bovine Milk Protein Composition**

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One of the aims of the Dutch Milk Genomics Initiative is to explore and exploit the variation in bovine milk-protein composition.

We have determined the protein composition in milk samples of 2000 Dutch Holstein Friesian cows, and established that a large part of the variation in milk-protein composition between cows can be attributed to genetics. To unravel this genetic variation, we estimated the effects of common genetic variants of  $\beta$ -lactoglobulin,  $\beta$ -casein and  $\kappa$ -casein on bovine milk-protein composition. We found that these genetic variants were associated with relative concentrations of all six major milk proteins. The common genetic variants of  $\beta$ -lactoglobulin,  $\beta$ -casein and  $\kappa$ -casein explain a considerable part of the genetic variation in bovine milk-protein composition. We have calculated that exploitation of this genetic variation in the Dutch dairy cattle population could increase the casein content of milk considerably and, consequently, improve cheese yield and profitability of the dairy industry.

To further investigate the genetic basis for the variation in bovine milk-protein composition, we screened genomic DNA for polymorphisms in the coding and promoter regions of all six major milk proteins. Many new polymorphisms were detected. One of these new genetic variants of  $\beta$ -lactoglobulin was shown to have a significant effect on  $\beta$ -lactoglobulin protein concentration and casein yield. We are currently analyzing two other genetic variants (in  $\beta$ -lactoglobulin and  $\beta$ -casein) for their association with milk-protein composition. Preliminary results indicate that these variants can also be applied to modulate bovine milk-protein composition, e.g. to increase casein content.

### **Nutrigenomics Strategy To Assess The Physiological Properties Of Dairy Products In Humans**

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Advances in molecular biology, microengineering and informatics, now allow one to holistically address how biological stimuli modulate the physiology of living organisms. Medicine and pharmacology have first applied 'functional omics' to investigate diseases and to develop drugs. The 21<sup>st</sup> century will witness the mutation of nutrition research from molecular nutrition to nutritional systems biology. Indeed, nutrigenomics is already deciphering the interaction of food with human organisms and nutrigenetics is evaluating the contribution of individual genetic configurations to this interaction.

Because of their cultural, nutritional, and economical importance, dairy products have always raised scientific and public debate. On one hand, a wealth of peer-reviewed publications highlights the broad physiological benefits of dairy products. On the other hand, some question the safety and relevance of the consumption of bovine milk by human adults. In this context, nutrigenomics provides a challenging analytical strategy to complement the information already available on these issues.

The potential of nutrigenomics for dairy research is first illustrated by presenting a nutritional study that investigates short-term global gene expression in blood of healthy human volunteers after ingestion of dairy products. We have conducted a randomized, controlled single-blinded crossover nutritional intervention trial. Six healthy male volunteers were set on a controlled diet devoid of dairy products and fermented food three days prior to the single ingestion of either 540g milk coagulated with glucono-delta lactone or yogurt. Blood samples were taken before (t=0h) and after (t=2h, 4h, 6h) ingestion of the dairy products. Total RNA was purified from whole blood and global gene expression was analyzed on DNA microarrays covering the entire human genome represented by 44'000 probes. Raw intensity values were normalized using SNOMAD (standardization and normalization of microarray data) R package. Analysis of variance was applied to normalized values and a kinetic analysis (linear contrast) was computed for all probesets. This approach identified 576 and 626 genes/probesets that respond differentially to the ingestion of milk and yogurt, respectively. Several pathways regulated by these genes are involved in the metabolism of nutrients present in milk, in particular unsaturated fatty acids, growth factors and hormones. Importantly, immune-related pathways, such as pathways triggered by Toll-like-receptors (TLR2, TLR4) and the transcription factor nuclear factor-kappa B (NF-kB) are down-regulated. These observations support anti-inflammatory properties of bovine milk in humans. Overall, the transcriptomic response of the volunteers following the ingestion of yogurt was comparable to the response observed with milk. However, genes and pathways were identified, which allow a differentiated discussion of the properties of milk and fermented dairy products.

We are currently applying nutrigenomics technologies to complement classical clinical endpoints in two further *in vivo* studies aimed at studying bioactive components in dairy products. The first study investigates the impact of *Lactobacillus casei* in a murine *E. coli* infection model. The second study compares the systemic effects in human volunteers of fat of dairy and vegetable origin.

At term, such studies will shed more light on the physiological properties of dairy products and eventually allow the identification of biomarkers that will lead to the selection of bacteria transforming milk into products with enhanced nutritional properties.

## **Prolactin-Release Inhibition And Once Daily Milking Affect Mammary Cell Activity At A Transcriptional Level**

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Inhibiting milking-induced prolactin (PRL) release and reducing milking frequency reduce the quantity of milk produced by dairy cows. Nevertheless, the mechanisms by which these two parameters modulate milk yield are not clear. To assess the regulation of the cell activity, by PRL and milking frequencies, five Holstein cows in early lactation received daily *i.m.* injection of 1 mg of Quinagolide, a suppressor of PRL release, for 9 weeks. Four control cows received the vehicle (water). During the last week of treatment, one half-gland was milked once and the other twice daily. Mammary biopsies were taken after 4 and 8 weeks of quinagolide treatment and epithelial cells were isolated from milk of both halves-udders in both groups of animals during the differential milking. Epithelial cells were prepared after milk

centrifugation and by purification using an anticytoketatin antibody bound to magnetic beads. RNA was extracted from mammary biopsies and milk purified epithelial cells. Milk protein mRNA expression were analysed by quantitative real time reverse transcription PCR. Quinagolide treatment induced a faster decline in milk production (- 5.3 kg/d compared to control during the last 4 weeks of treatments,  $P < 0.05$ ). Differential milking resulted in an important reduction of milk production in the half-gland once daily milked ( $P < 0.001$ ). In the half gland twice daily milked, the inhibitory effect of quinagolide was maintained ( $P < 0.05$ ), but was lost in the half-gland once daily milked ( $P > 0.15$ ). Levels of kappa casein ( $P < 0.05$ ) and alpha lactalbumin ( $P = 0.06$ ) mRNA were lower in mammary biopsies of Quinagolide-treated cows at week 4. Accordingly, the mRNA levels of kappa casein ( $P < 0.05$ ) and alpha lactalbumin ( $P = 0.06$ ) were also lower in milk purified epithelial cells from Quinagolide treated cows during the differential milking. Milking frequency also induced variations in milk protein mRNA levels in milk purified epithelial cells. Levels of kappa casein ( $P < 0.05$ ) and alpha lactalbumin ( $P < 0.01$ ) mRNA were downregulated in once daily milked udders compared to twice daily ones.

In conclusion, chronic administration of the prolactin-release inhibitor Quinagolide and once daily milking reduced milk production in dairy cows by affecting mammary cell activity at a transcriptional level. Moreover milk purified epithelial cells seem to be a valuable source of cells to study transcriptional regulations in mammary cells.

### **Occludin Disruption Leads To Epithelial Apoptosis And Cellular Extrusion: A Possible Protective Mechanism Against Pathogenic Organisms?**

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Occludin is a tight junction protein that can mediate formation of tight junctions; it is dispensable for the barrier functions of the tight junction as epithelia in occludin null mice behave essentially normally. Occludin interacts with a variety of pathogens including viruses and bacteria, an interaction that sometimes leads to extrajunctional localization of the molecule. To examine the function of extrajunctional occludin, we expressed a truncated form of the molecule in mammary epithelial monolayers or applied circularized LYHY, a function blocking peptide, to these monolayers. Both treatments led to extrajunctional localization of endogenous occludin, increased TUNEL staining, and cleavage of caspases 8 and 3. Apoptosing cells were extruded with no loss of epithelial resistance. Caspase 8 inhibition inhibited cleavage of caspase 3, implicating the extrinsic apoptotic pathway. At early times after peptide treatment endogenous occludin and the LYHY peptide were co-localized in extrajunctional patches, which were also shown to contain caspases 8 and 3, the death receptor FAS and the adaptor molecule FADD. These observations provide strong evidence that disruption of occludin function leads to activation of the death receptor pathway and extrusion of apoptotic cells. They also implicate occludin as a signaling molecule that may activates apoptotic pathways in response to infectious agents. Supported by PO1 HD38129 to MCN.

### **Rspo1 Is Essential To Normal Mammary Gland Development**

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The mammary gland, a mammalian specific organ, undergoes cycles of development and differentiation throughout lifespan. The Wnt/ $\beta$ -catenin pathway, found in many developmental processes such as the determination of cell and tissue polarity, stimulation of cell proliferation and differentiation and adult tissue homeostasis, is involved in the biology of the mammary gland.

Recently, its regulation by the R-Spondin (*Rspo*) genes has been reported. *Rspo* family genes, which were recently isolated in mouse, *Xenopus*, monotremes and human, encode secretory proteins containing an N-terminal signal peptide, a cysteine rich region with two furin like domains, a thrombospondin type 1 repeat motif and a C-terminal region comprised of positively charged amino acids. Four members exist in mice and human. In this context, we have studied the potential implication of these genes in the mice mammary gland biology. Their expression patterns show that they are quantitatively and qualitatively differentially expressed during the different developmental stages of the mammary gland, which suggests that they have different roles. *Rspo1* is the most expressed gene and has an expression pattern closely related to Wnt-4 expression pattern. By Immunohistochemistry, RSPO1 protein was found in the epithelial cell with a localization dependent on the mammary development stage. Indeed, a progressive relocalization of the protein from the cytoplasm to the basal membrane and the same basal is observed during the pregnancy.

This protein is further synthesized by the epithelial cells as it can be observed in the Golgi using electronic microscopy. In order to understand more precisely the potential function of *Rspo1*, studies were performed using two mice models: one over expressing *Rspo1* and one knockout for the gene. Over expression of *Rspo1* did not generate any mammary phenotype, while its invalidation induced a defect in the mammary gland development. Indeed, mammary gland from virgin (8-weeks) *Rspo1*<sup>-/-</sup> female mice revealed absence of tertiary side branching. In pregnancy, the branching defect persists and is associated to a defect of alveolar development (cells that secrete the milk). *Rspo1* gene seems to be a critical activator during the normal mammary gland development and differentiation.

## **Quantitative Trait Locus (QTL) Detection For Milk Protein Composition In Dairy Cattle**

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The Dutch Milk Genomics Initiative aims at identification of genes that contribute to natural genetic variation in quality traits of bovine milk, among which milk-protein composition. The heritabilities, i.e. the proportion of phenotypic variation due to genetics, for the major milk proteins were high and ranged from 0.25 for  $\beta$ -casein to 0.80 for  $\beta$ -lactoglobulin. The heritabilities show that genes contribute to the genetic variation of major milk proteins. However, it is not known which genes cause this genetic variation. The objective of this study, therefore, was to perform a whole genome scan to detect quantitative trait loci (QTL), i.e. DNA region on the genome which has an effect on a quantitative trait, for the milk protein composition in 1,912 Holstein-Friesian cows. One morning milk sample was taken between February and March 2005. All milk samples were analyzed for the major milk proteins using capillary zone electrophoresis. DNA was isolated from blood samples of 849 cows and semen samples

of seven bulls. As genetic markers, 1,341 single nucleotide polymorphisms (SNPs) were used. Ten significant chromosomal regions affecting milk protein composition were detected. The chromosomal regions most significantly related to the major milk proteins ( $P_{\text{genome}} < 0.05$ ) were found on *Bos Taurus* autosome (BTA) 6 and 11. The QTL on BTA6 was found at about 80 cM and affected  $\alpha$ S1-casein,  $\alpha$ S2-casein,  $\beta$ -casein and  $\kappa$ -casein. The QTL on BTA11 was found at 124 cM and affected  $\beta$ -lactoglobulin. The proportion of phenotypic variance explained by the QTL was 3.6% for  $\beta$ -casein and 7.9% for  $\kappa$ -casein on BTA6, and 28.3% for  $\beta$ -lactoglobulin on BTA11. The QTL affecting  $\alpha$ S2-casein on BTA6 and 17 showed a significant interaction. We investigated the extent to which the detected QTL on BTA6 and 11 could be explained by known polymorphisms in  $\beta$ -casein,  $\kappa$ -casein, or  $\beta$ -lactoglobulin genes. Correction for these known polymorphisms decreased the proportion of phenotypic variance explained by the QTL previously found on BTA6 and 11. Thus, several significant QTL affecting milk protein composition were found, of which some could partially be explained by known polymorphisms in milk protein genes. Fine mapping of the detected QTL regions to reduce confidence intervals of the detected QTL for milk protein composition, to facilitate new candidate genes that affect milk protein composition, is in progress by using the 60K bovine SNP chip.

### Structure And Function Of Marsupial Whey Acidic Protein

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Whey Acidic Protein (WAP), a major whey protein present in milk of a number of mammalian species belongs to the Kuntiz family of proteins which have characteristic cysteine-rich domains known as four-disulfide cores (4-DSC). Eutherian WAP, expressed in the mammary gland throughout lactation, has two 4-DSC domains, (DI-DII) while marsupial WAP, expressed only during mid-late lactation, contains an additional 4-DSC, (DIII) and has a DIII-D1-DII configuration. Recent studies have shown that mouse WAP added to culture medium of mouse HC11 cells is antiproliferative, possibly acting by an autocrine or paracrine mechanism. This is consistent with reports showing that over-expression of WAP in transgenic mice inhibits development of the mammary gland and secretion of milk. We have used *in vitro* models to examine the effect of tammar WAP (tWAP) on proliferation of a variety of cells types. tWAP was synthesised *in vitro* and added to culture medium. In contrast to the inhibitory action of mouse WAP on proliferation of HC11 cells, results suggest that tWAP stimulates proliferation of both HC11 cells and tammar mammary epithelial cells and increases expression of the cell cycle gene cyclin D1 and CDK-1 genes. However, tWAP does not induce proliferation of human embryonic kidney (HEK293) cells. tWAP was also observed to increase stimulation of mammosphere formation suggesting a role in stem cell lineage specificity. Expression and *in vitro* assay of individual tWAP domains shows proliferative activity is due solely to DIII. Earlier studies have shown that DNA synthesis in mammary tissue is higher in phase 2 than phase 3 which is consistent with a potential role of tWAP in this process. Therefore it is conceivable that tWAP plays a role in development of the tammar mammary gland and may be important to support the reproductive strategy of marsupials and monotremes. We have recently expressed and purified tWAP using His-tagged Baculovirus technology for crystallization and cell signaling studies. The significance of the loss of the third domain in eutherians remains unclear, but may be related to the requirement for mammary gland development during lactation in marsupials.

## **The Swedish-Danish Milk Genomics Initiative – Presentation Of The New Project**

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The project constitutes a Danish/Swedish initiative within the area of "milk genomics". The main content of the project is to identify genetically contingent differences in the nutritional, technological and health-related properties of the milk. Selected milk characteristics and properties including fatty acid and protein compositions, coagulation properties, small metabolites, vitamin and mineral content will be characterised using "omics" techniques (proteomics, metabonomics and peptidomics). Based on these variabilities candidate genes for the measured traits will be selected and sequenced to characterise variants. Furthermore, by linking phenotypic milk traits to genome scan new genetic markers for significant properties of milk are identified. The overall aim is thus to generate new insights into the complex genetic background of many milk quality traits, and the perspective is to identify genetic markers that can be used in future breeding to support development of differentiated milk types and specific dairy products of high, specific qualities. These products could eventually be targeted at consumer groups with specific nutritional or health related requirements.