Poster Abstracts

Analyses of gene expression using combined ovine and bovine transcriptional profiling for identification of common lactation pathways

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A number of differentially expressed (DE) candidate genes have been proposed from expression levels obtained in individual microarray experiments for a number of species. The combination of information obtained from such datasets can lead to a list of differentially expressed genes that signify unique and conserved molecular processes that are functionally important. Such models are potentially more robust with greater confidence, and place less reliance on a single dataset. In this paper we combine two microarray datasets for three stages of lactation in both sheep and cattle and perform comparisons of heterogeneous data.

An Affymetrix bovine gene chip array containing about 24,000 probe sets was hybridized with RNA derived from mammary gland of pre, peak and late lactation stages in dairy sheep selected for high and low yield milk traits, providing a set of 12 chips. Data from an additional study using the same Affymetrix chip and experimental design, with 15 bovine biological replicates over corresponding pregnancy, lactation and involution stages was used. The combined data were corrected for background noise and normalised using the RMA normalisation method. Gene effects were estimated using a mixed model approach and a mixture model distribution was then fitted to the sets of estimated effects to obtain posterior probabilities for identification of differentially expressed genes. Clusters of top 20% of these genes for the two livestock species will show the DE genes that are co expressed in both representing conserved differential expression in the target mammary gland tissue.

Further analysis on exploring the contrasts and similarities between the DE genes of these two species will be explored by analysing the two datasets separately using the same quality control, normalisation and analysis processes. Gene expression pathway analysis results of the two studies can also be compared to identify common clusters of genes representing the same functions across both species.

Analysis of Microarray Gene Expression Profiles In Mammary Glands of CBA and QSI5 Mice

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The use of mouse models for mammary gland research has generated a number of discreet datasets. Comparing strains of mice, is also considered an effective approach for identifying key candidate genes. The QSi5 strain, developed at The University of Sydney, has demonstrated a superior lactation performance when compared to other inbred mouse strains. Comparison of gene expression profiles in this strain with other strains will help in identifying the genes responsible for the lactation performance phenotype, but this will be most effective using methods that permit inter-study comparison. Inguinal mammary glands were harvested from 5 animals in each of 2 strains at Day 12 of pregnancy. RNA isolation was performed using standard protocols and were hybridised to mouse Affymetrix gene expression arrays (430). A mixed model method of analysis was used to compare expression profiles of these microarrays and to compare to the Limma method. The analysis algorithm was modified to

compare the results to a previous study of gene expression in lactating mammary glands from a similar strain comparison. Microarray results were confirmed by qRT-PCR for selected genes. Differential gene expression and the effects of modulating probability on pathway analysis will be discussed.

Bovine Lactoferrin promotes the healing of corneal erosions in a mouse model

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Purpose: Previously we have found bovine lactoferrin (BLF) stimulated healing of alkali-induced-human corneal epithelial wounds *in vitro*. This study was to test the efficacy of BLF in promoting healing of alkali-induced corneal wounds using an animal model.

Methods: Six- to 8-week-old male BALB/c strain mice were used in this study. Corneal erosions were induced by alkali wounding by applying 1M NaOH pre-soaked filter disc for 2 minutes to the cornea. Following corneal wounding, BLF in PBS (62.5 µM), or control BSA (bovine serum albumin, 62.5µM) in PBS, or control vehicle (PBS only) was administered topically (two times daily for 7 days) to wounded and unwounded eyes. Animals were monitored daily and at the end of 7 days postwounding, the corneal wound/erosion was stained with fluorescein and photographed. Wound healing in response to the treatments was compared.

Results: Treatment with BLF was found to stimulate healing of the alkali-induced corneal wounds as compared to control groups. In the mouse corneal alkali-induced wounds, 65% mice treated with BLF healed completely 7 days after wounding,
whereas only 25% and 15% wounds healed in the PBS- and BSA-treated groups, respectively.

Conclusion: Bovine lactoferrin promoted healing of corneal erosion induced by alkali. The outcome of this in vivo study supports our *in vitro* findings.

Bovine mammary involution versus *Streptococcus uberis* induced mastitis: A microarray comparison

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Microarray analyses were performed to survey changes in gene expression in the bovine udder during both involution and mastitis. A total of 23,280 ESTs from the AgResearch bovine expressed sequence tag (EST) database which incorporates 203,000 bovine EST cDNAs derived from more than 50 tissue libraries were selected, amplified and arrayed on glass slides. The arrayed ESTs consisted of 13,522 different contigs, of which 9,499 were represented once. ~8,600 contigs matched entries in the SwissProt database. To examine gene expression changes during involution, total RNA from alveolar tissue was obtained from non-pregnant cows in mid-lactation slaughtered at 6 and 36h (n=6/group) after the last milking. The two time points were compared using a daisy chain design. To examine gene expression changes as a result of infection, two udder quarters of heifers (n=5, mid-late lactation) were infused with ~1000 colony-forming units of a clinical mastitis causing strain of *Streptococcus*

uberis. Total RNA from alveolar tissue from infected and control quarters was compared within udder. For each experiment RNA was labelled with Cy3 and Cy5 dyes and hybridised to the slides using dye swap replication. Log2 ratios of the background-corrected intensities of each probe were normalised employing a mixed model with spatial autocorrelation REML in GenStat and further analysed using Ingenuity Pathway Analysis. Widespread changes in mammary gene expression occurred within 36h post-milking and with infection. There were 76 genes in common which were up-regulated (≥1.5 fold, $P<0.01$) in both the involution and mastitis experiments. These genes are involved in acute phase response signalling, NRF mediated oxidative stress response pathways and the complement system. These included metallothionein 1A, superoxide dismutase 2, lactoferrin, serum amyloid A, and lipopolysaccharide binding protein. There were also 98 genes down-regulation (≥1.5 fold, $P<0.01$) in common to both experiments with the only significant pathway involving milk protein genes. As expected, these included αS1, β-, κ-casein and α-lactalbumin. A number of genes were differentially expressed between involution and mastitis. 192 genes (101 of which were up-regulated), many belonging to tight junction signalling and tissue development pathways, were regulated only in the involution experiment. Down-regulated genes in the involution experiment only, include STAT2 and SOCS2 and up-regulated genes include chemokine (C-X-C motif) ligand 3, CD68 and CD6. 766 genes (345 of which were down-regulated) predominantly belonging to immune response and apoptotic pathways were regulated only in the mastitis experiment. Histatherin, mannose receptor, C type 1, STAT5a and 6 were among the down-regulated genes in the mastitis experiment only, while prostaglandin D2 synthase and S100 calcium binding protein A9 were among the up-regulated genes. Our results demonstrate that in the bovine mammary gland, multiple immune responses and protective responses to oxidative stress occur during both involution and mastitis, while some responses are unique to each condition. It is possible that these diverse physiological responses (cessation of milking and infection) are linked through common signalling intermediates that are activated in both types of physiological perturbation. Further work will dissect the signalling pathways responsible for these effects.

Bovine Milk Proteomics and Glycomics

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Friesland Foods Domo, as operating company of the Dutch dairy cooperative Friesland Foods, is a global player on the infant nutrition and ingredients market. Current knowledge about the detailed composition of the protein fractions of bovine milk and whey, and the functional effects of variation in these fractions, is limited. For a wide array of applications, a proteomics and glycomics collaboration between the University of Groningen and Friesland Foods has been initiated. Research emphasis is on the analysis of quantitative and qualitative effects of natural variations (both genetic variations as well as environmentally-induced variations) and processing variations. It is expected that such information will enhance insight between product composition and functionality. A better understanding of the differences between human and bovine milk, in this manner, may stimulate new product development in the area of infant nutrition and process development.

Milk originating from typical Dutch dairy cows, most notably Friesian Holstein and Maas Rijn IJssel (MRY) cows, was analysed. To assess effects of processing variations, this milk was subjected to a

multi-step membrane filtration procedure. The resulting fractions were analyzed by 1D-GE/LC-MS/MS (Orbitrap, Q-star) and various glycan analysis methods, a.o. 1D-NMR, GC-MS, MALDI-TOF/TOF and LC. Preliminary results show over 100 identified proteins; in addition glycan analyses were performed. In conclusion, identification of the differences in (glyco)protein composition of the different (processing) fractions will lead to more possibilities for product development in the area of infant nutrition, food applications and process optimisation.

Chromium-containing milk powder ameliorates nonalcoholic fatty liver disease induced by high-fat diet in KK/HlJ mice

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Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized condition that may progress to end-stage liver disease. Insulin resistance and oxidative stress are key pathophysiological mechanisms in NAFLD. This study investigated the effects of chromium containing milk powder, a native chromium complex which utilized milk powder as chromium carrier, on hepatic insulin resistance, hepatic oxidative stress, and liver injury in NAFLD mice induced by high-fat diets for 8 weeks. Liver damage was evaluated by serological analysis, serum and hepatic lipid assay as well as hematoxylin-eosin staining in liver sections. Insulin sensitivity and oxidative stress were assessed by measuring insulin signaling molecules and malondialdehyde (MDA) in liver. The results showed that high fat diet induced insulin resistance, oxidative stress and extensive liver steatosis in KK/HlJ mice. Chromium-containing milk powder supplementation was effective in reducing hepatic steatosis with lowing serum alanine aminotransferase, aspartate aminotransferase, and levels of liver triglycerides in high fat diets mice. Moreover, chromium-containing milk powder supplementation significantly activated post-receptor insulin signaling such as increasing IRS1, p85α regulatory subunit of phosphatidylinositol 3-kinase (PI3-k), and glucose transporter 2 (Glut 2) expression, stimulating Akt activity, and decreased MDA levels in liver. This study shows that chromium-containing milk powder protective effects against fatty liver in mice induced by high-fat diet possibly through improving hepatic insulin resistance and oxidative stress.

Comparative genomics of *Bifidobacterium longum* strains identifies genes relevant for human 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 addition, Multi Locus Sequence Typing (MLST) was used to further classify these strains.

Preliminary analysis of the CGH data has revealed that the strains cluster into two clearly differentiated 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. A high-throughput analytical platform, combining microtiter plate analysis of microbial growth and MALDI-FTICR MS analysis of spent media was developed to obtain detailed, strain-specific, HMO consumption profiles. By combining CGH analyses with high throughput glycomics of HMO consumption, we have begun to identify a core set of genes, relevant for HMO utilization, that are shared across the *B. longum* clade.

Does Tgfβ signaling pathway play a role in secretory activation?

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The transition from pregnancy to lactation, termed secretory activation is characterized by the closure of the epithelial tight junction complexes that regulate the permeability of the paracellular pathway. Progesterone withdrawal together with prolactin and glucocorticoids is thought to trigger tight junction closure but the mechanism by which progesterone prevents tight junction closure during pregnancy is poorly understood. Previous microarray studies in our lab have identified Tgfβ3, Wnt5b and Igfbp5 as the possible paracrine factors mediating progesterone response during secretory activation [1]. We propose Tgfβ3 as the most plausible factor for the following two reasons. Firstly, Tgfβ3 responded immediately to the decline in progesterone 24 hours prior to parturition and secondly, Tgfβ has been implicated in tight junction regulation in other organs [2]. Further network analysis of the differentially expressed genes between late pregnancy (P19) and early lactation (L1) time points identified Tgfb3 to be interacting with known tight junction proteins – TJP1 (ZO1), CLDN3, JAM and PKC.

To delineate the role of Tgfβ in secretory activation, we obtained dominant negative TGFβII receptor (DNIIR) mice [3]. Histological examination of mammary glands from the DNIIR mice revealed precocious secretory activation with appearance of cytoplasmic lipid droplets as early as day 12 of pregnancy, which persisted until late pregnancy. There was decreased permeability of mammary tight junctions in DNIIR mice (1146.8 ± 195.4 cpm) compared to wild type (1836.3 ± 179.8 cpm) as demonstrated by the appearance of [^{14}C] sucrose in the blood after intraductal administration of the radiolabeled sucrose on day of parturition. Although DNIIR females were able to support their litters, the pup growth rate (3.63 ± 0.16 g) was significantly less compared to the WT females (4.21 ± 0.13 g) at day 9 of lactation.

These preliminary findings from DNIIR mice suggest that Tgfβ maintains tight junction permeability during pregnancy. We are currently doing immunohistochemical studies for localization of markers of secretory activation namely Glut1 as well as tight junction markers ZO-1, CLDN3 and CLDN8. Additionally, qRT-PCR assays are being done to assess any differences in expression patterns for selected Tgfβ downstream target genes Smad3, Tsc22 as well as the secretory activation markers. Further to corroborate the results from DNIIR mice, adenoviral constructs for over-expression of Tgfβ3 and DN-TgfβIIR are in preparation.

References
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Endothelial cell response to bovine serum albumin

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Serum albumin is found in relatively high concentrations in milk. Earlier work demonstrated a potent and highly specific anti-apoptotic activity in serum albumin for endothelial cells. More recently, we have reported that the as yet unidentified site in the albumin molecule responsible for the anti-apoptotic activity is partially cryptic, being further exposed by fragmentation of the molecule, and also that it operates through a PI3-Kinase G-Coupled protein dependent mechanism. Further studies indicate reduced intramolecular movement of albumin with non-enzymatic glycosylation, coupled with reduced anti-apoptotic activity. Utilising the cross-linking properties of glutaraldehyde, the aim of the current work was to investigate the role of intramolecular movement in the anti-apoptotic activity of albumin for endothelium.

In preliminary experiments, serum albumin was treated with glutaraldehyde at increasing concentrations and for up to 5 days before pressure dialysis in PBS and finally culture medium M199 for bioactivity determinations. Albumin treated with high levels of glutaraldehyde or for long periods of time were found to acquire a highly toxic activity for endothelium. The formation of albumin polymers by glutaraldehyde cross-linkage was revealed as laddered bands by SDS-PAGE. Nonetheless, low levels of glutaraldehyde treatment did not generate toxic activity and appeared not associated with the formation of polymerized material. Subsequent experiments in which albumin was treated with glutaraldehyde at low concentrations for times ranging from 6 Hr to 48 Hr revealed progressively reducing anti-apoptotic activity with increasing times of glutaraldehyde treatment. Work continues to characterize by fluorometry intramolecular movement in glutaraldehye treated albumin, while further work is planned to characterize the location of internal cross-linkages by MALDI-TOF analysis of CNBr and tryptic digests.

Data thus far are consistent with the proposition that the anti-apoptotic activity of albumin for endothelium is exposed by transient intramolecular conformational change, while further investigation is required to properly characterize the putative conformational changes.

Evolution of the major milk proteins in mammalian genomes

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Milk proteins are a major component of milk and are critical for the development of the neonate in mammals. Yet little is known about their sequence evolution. We set out to determine if certain milk proteins show more rapid evolution in certain lineages, in order to relate that to potential adaptive changes. We compared the milk proteins of 12 sequenced mammalian genomes, 10 of which are eutherian (Human, Chimp, Macaque, Rat, Mouse, Rabbit, Guinea pig, Cow, Dog, Cat, Horse), one metatherian (Oppossum), and one prototherian (Platypus). The milk proteins studied were alpha lactalbumin, beta lactoglobulin, alpha casein, beta casein, kappa casein, lactoferrin, MFGM,

butyrophilin, epidermal growth factor, xanthine dehydrogenase, episialin mucin 1, WDNM1, and adipose differentiation related protein. We observed a significantly higher (p-value<0.05) sequence divergence of most milk proteins compared to other proteins in the genomes of the 12 species. More importantly, we observe that certain species experienced higher sequence evolution than others. For example certain milk proteins are significantly accelerated in the ancestor of human and chimp subsequent to their speciation from macaque. This significant divergence specific to primates may reflect the changes in nutritional requirements arising from greater brain size, and more importantly their longer period separating birth and weaning. However, such accelerated change may also relate to other functional requirements that are species specific. We conclude from this comparative cross-genome study of milk proteins that changes in their protein sequences provide potential insights into the changing functional properties of milk.

Genetic parameters for milk protein composition of dairy cows

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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 objective of this study was to estimate genetic parameters of major milk proteins. From 1,940 first-parity Holstein-Friesian cows, one morning milk sample was collected in February or March 2005. Each sample was analyzed with capillary zone electrophoresis to determine the relative concentrations of the six major milk proteins. The results show that there is much genetic variation in the protein composition. The heritability, i.e. the proportion of phenotypic variation due to genetics, for the relative protein concentrations was high and ranged from 0.25 for β-casein to 0.80 for β-lactoglobulin. The heritability for the summed whey fractions (0.71) was higher than that for the summed casein fractions (0.41). For the caseins and α-lactalbumin, the proportion of phenotypic variation explained by herd was approximately 14%. For β-lactoglobulin, the proportion of phenotypic variation explained by herd was 5%, and considerably lower. Among the relative protein concentrations, most of the genetic correlations were low (between -0.38 and +0.45); α_{S1}-casein was negatively correlated with κ-casein (-0.56) and α_{S2}-casein (-0.49). Furthermore, β-lactoglobulin was negatively correlated with the summed casein fractions (-0.76) and casein index (-0.98), and positively correlated with the summed whey fractions (0.98), indicating that it is possible to change the relative proportion of caseins in milk. This study suggests that there are opportunities to change the milk protein composition in the cow's milk using selective breeding.

Genetic variation in bovine β-lactoglobulin gene unraveled

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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.

Milk-protein composition was determined of morning milk samples collected from 1912 Dutch Holstein Friesian cows in first lactation. β-lactoglobulin (β-LG) had a mean relative protein concentration of 8.4% (w/w) and a high heritability, i.e. proportion of phenotypic variation due to genetics, of 0.80.

A large part of the variation in amount of β-LG protein concentration is associated with β-LG protein variants A and B. In our population of 1912 cows, animals homozygous for β-LG protein variant A had a mean relative β-LG protein concentration of 9.5% (w/w), whereas animals homozygous for β-LG protein variant B had a mean relative β-LG protein concentration of 6.6% (w/w), a difference of 2.8%. This difference in amount of β-LG protein is of interest to the dairy industry because of its association with casein concentration and casein index, and, consequently, cheese yield.

To further investigate the genetic basis for the variation in relative β-LG protein concentration, genomic DNA was screened for polymorphisms in the coding and promoter region of the β-LG gene. In total 50 polymorphisms were detected.Two SNPs lead to amino acid changes and are the causal genetic polymorphisms of β-LG protein variants A and B. Forty-two polymorphisms were in complete linkage disequilibrium (LD) with β-LG protein variants A and B. Any of these 42 polymorphisms can be involved in the differential expression of the respective A and B alleles of the β-LG gene. One of the eight polymorphisms not in complete LD with β-LG protein variants A and B had a significant effect on adjusted β-LG protein concentration. This SNP segregated only within cows homozygous for β-LG protein variant A. Within these cows, adjusted β-LG protein concentration was reduced with 1.22 % in animals homozygous variant for the SNP compared with animals homozygous wild type for this SNP.

Investigation of the composition of porcine colostrum from multiparous sows and primiparous gilts.

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Colostrum, secreted 24-48hrs *post partum*, contains specific peptides, proteins and immunoglobulins that are essential for passive immune protection and for facilitating gastrointestinal development required to support post natal growth of the neonate. Despite the well-known role of colostrum and subsequent milk composition in neonatal growth there is very little information detailing the gross composition as well as detailed proteome of porcine colostrum. This is of particular significance in the intensively farmed pig production system as neonatal nutritional challenge or disease will significantly affect the efficiency of meat production and subsequent profitability.

Offspring born to primiparous gilts (no previous litters) are born lighter than those of multiparous sows and have lower weights at weaning which may suggest differences in the nutrition they receive *post partum* through to weaning. We are interested in investigating the effect of these differences on the gut development and subsequent growth of the newborn piglet initially through investigation of the composition of colostrum.

To investigate the compositional differences in colostrum between gilts (parity 0) and sows (parity 3) we hand collected colostrum immediately after the birth of the second piglet of the litter. In previous studies we identified differences in the gross composition of colostrum from sows and gilts including total protein and lactose concentrations although total fat concentration did not differ with parity number (Geale, et al 2007). We further characterised these proteins on 1-D PAGE gels to determine the molecular weight of the proteins present in porcine colostrum and have undertaken densitometric analysis of these gels as an initial survey of the potential differences in the protein composition between sow and gilt colostrum. Subsequently we sought to further elucidate the protein composition of sow and

gilt colostrum through additional analysis utilising 2-D PAGE gels. Importantly, we acknowledge that any differences in protein composition of colostrum may not be the only factor responsible for the observed differences in the neonatal growth of sow and gilt progeny.

Geale, P.F., Thomson, P.C., Wynn, P.C and Sheehy, P.A., (2007) Changes in Colostrum and Milk Composition Between Gilts and Sows. *Manipulating Pig Production XI:* Proc. Aust. Pig. Sci. Assoc (Inc) : 49

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Lactoferrin and lactoferricin covalently linked to biomaterial surfaces reduces bacterial attachment

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Corneal infection is a rare but severe complication of contact lens wear. However the large number of people using contact lenses means that even complications with low incidence may affect large numbers of individuals. There is consequently a need to develop surfaces which can reduce the rate of bacterial colonisation of the lens surface and consequent development of infection. Our previous research indicates that an excellent approach to reducing biomaterial associated infection is based on the milk product lactoferrin. In this project we have developed strategies for covalent linkage of lactoferrin and lactoferricin to contact lens surfaces and test their ability to prevent bacterial colonisation by the two most common types of bacteria associated with corneal infection *P. aeruginosa* and *S. aureus.*

Lactoferrin or lactoferricin was attached via the linker 4-azidobenzoic acid (ABA), the reaction scheme is illustrated in Figure 1 below:

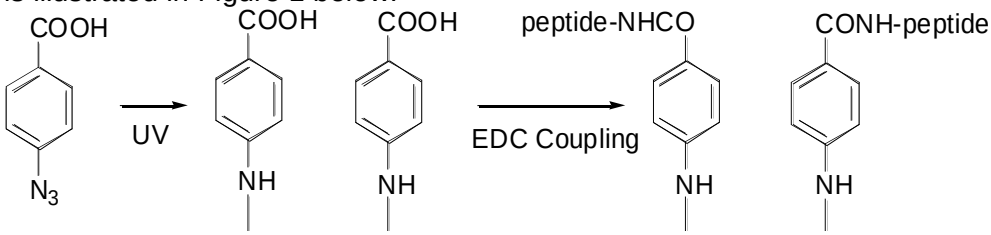

Figure 1: Schematic showing attachment to surface via the ABA azide linker

The surface attachment to glass and contact lenses was confirmed using X-ray Photoelectron Spectroscopy, and contact angle measurements. The efficacy of the modified contact lenses against bacterial adhesion and colonisation was elucidated *in vitro* through viable counts experiments and visualised through Live/Dead Fluorescence Staining and Scanning Electron Microscopy imaging of the modified glass samples. Activity against *P. aeruginosa* and *S. aureus* adhesion was seen for coatings on the contact lens for both peptides with approximately 1-log reduction compared to the control lenses. However for the direct EDC coupling on anionic lenses there was greater than 4-log reduction against *P. aeruginosa* and 2-log reduction was seen for *S. aureus*. *In vivo* testing of the modified lenses was carried out in a subcutaneous pocket model of biomaterial infection in mouse. The study showed a 30% reduction in the numbers of viable bacteria recovered.

Our studies have confirmed that lactoferrin and lactoferricin remains active when covalently linked to surfaces and are effective in reducing bacterial adhesion and viability. Strategies developed for coating antimicrobial contact lenses are also applicable to the biomedical device industry.

Lactoferricin increases the susceptibility of Pseudomonas aeruginosa to antibiotics

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Purpose. Pseudomonas aeruginosa is a major human opportunistic pathogen that has become increasingly resistant to antibiotic therapy. The aim of this research was to evaluate the ability of lactoferricin to increase susceptibility of *P. aeruginosa* to a range of antimicrobials, and to examine its mechanism of action.

Methods. Checkerboard synergism tests and time kill method were performed to investigate the combined effects of bovine lactoferricin and conventional antibiotics such as ciprofloxacin, ceftazidime and gentamicin against strains of *P. aeruginosa*. Scanning electron microscope was used to determine the changes of cellular morphology upon exposure to lactoferricin B and ciprofloxacin.

Results. Synergy and partial synergy between lactoferricin B and ciprofloxacin or caftazidime were identified in all five ciprofloxacin/ceftazidime resistant strains of *P. aeruginosa*, with MIC values reduced by 4-fold for ciprofloxacin and up to 8-fold for caftazidime. There was no synergistic effect seen between lactoferricin B and gentamicin against any of the strains of *P. aeruginosa*. In SEM images, sub-MIC of ciprofloxacin caused elongation of bacteria, and sub-MIC of lactoferricin B resulted in increases of filamentation and extracellular debris. When exposed to synergistic concentration of ciprofloxacin and lactoferricin B, test strains showed some degree of cell structural damage, e.g. lysed bacteria, increased cellular debris, increase filamentation and elongation of bacteria.

Conclusion. Our results have demonstrated that lactoferricin B enhances the activity of ciprofloxacin and ceftazidime *in vitro* against a range of *P. aeruginosa* isolates, particularly multi-drug resistant strains. The study suggests that lactoferricin B may have potential to be used as an adjunct agent to antibiotics in the treatment of corneal infection.

“LactoScan”: The French national program to detect and use QTL and/or major genes linked to ruminant milk composition

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The aim of this program is to develop reliable, high throughput, cheap and easy-to-use methods for individual proteins and fatty acids content measurement and to use these methods to identify the genetic and the environmental factors affecting these contents, for a better understanding of the milk synthesis in the mammary gland, for new opportunities in the definition of the breeding goals in selection, and for new tools in production management.

It includes six main tasks:
(1) adapt Mid Infra Red (MIR) spectrometers of the different milk analysis laboratories to export spectra, develop a dedicated data;
(2) develop calibration equations to predict fatty acid composition of milk in the three dairy species through MIR and chromatography data, from samples collected in four INRA farms;

(3) develop a reference method for protein measurement and derive calibration equations by the analysis of both reference and MIR data;
(4) collect MIR spectra, milk and blood samples, and diet and management information from 12,000 cows and 3,000 ewes, as well as complementary information from the national database; predict fatty acid and protein concentration from MIR spectra;
(5) genotype a part of these samples with a SNP chip and perform QTL fine mapping, determine the effect of management and feeding policies on fatty acids and protein profiles in milk.
(6) develop management and feeding strategies for general recommendation.

To carry out such a large collaborative program, we gather all scientific and economic stakeholders, from milk production (milk recording, analysis laboratories, cattle and sheep breeding, extension services) to milk-processing (federation of dairy industries).

We expect this program will provide advisors, producers and dairy industry with tools and knowledge to better direct genetic improvement of dairy ruminant milk quality-traits and to fulfill techno- and nutri-functional characteristics of milk for traditional or innovative processing.

Mapping tools for bovine genomics

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With the bovine genome assembly still in its infancy compared with the “mature” assemblies of human and mouse, there is an important need for tools that enable us to draw upon all conceivable mapping information that is independent of the assembly, to provide a backbone for, and a check against, the assembly. One very important task was to integrate the wide range of independent linkage, Rh and *in-situ* maps into a single best-bet map. This was first achieved by the Location DataBase (LDB) strategy and more recently by a consensus strategy. Another task has been to extract the wealth of mapping information that is inherent in sets of linkage-disequilibrium (LD) data. This has been achieved by regarding pair-wise LD data in the same way as geneticists traditionally regard pair-wise linkage data. Within a particular range of LD, it is possible to create genome-wide maps based on Locus Order from DisEquilibrium (LODE maps). Oxford grids are a powerful means of harnessing the ever-increasing power of comparative mapping to provide very useful independent mapping information. Each of these resources can be used to enhance and check the bovine genome assembly as it evolves.

Milk’s marvellous markers and macromolecules

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Biotechnology (Oxford University Press, 2007) is no ordinary text book. Practising scientists share their cutting edge research in a carefully constructed format. Unlocking the secrets of milk’s macromolecules and the marvels of markers demonstrate the scientific discovery process. The challenges and opportunities of translating these applications to the market place are presented including a model for analysing issues. The underlying message is that biotechnology in humane frameworks can help make the future more prosperous and sustainable for everyone.

Protein variants of β-lactoglobulin, β-casein and κ-casein show large effects on the protein composition of bovine milk

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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.

Relative concentrations of the six major milk proteins - α-lactalbumin (α-LA), α_{S1}-casein (α_{S1}-CN), α_{S2}-casein (α_{S2}-CN), β-lactoglobulin (β-LG), β-casein (β-CN) and κ-casein (κ-CN) - were measured by capillary zone electrophoresis in milk of 1,912 Dutch Holstein Friesian cows in their first lactation, sampled in February-March 2005. Screening of these major milk protein genes resulted, in addition to the detection of the common protein variants (α-LA B, α_{S1}-CN B and C, α_{S2}-CN B, β-LG A and B, β-CN A^1, A^2, A^3 and B, κ-CN A and B), in the detection of κ-CN E, which was not previously identified in Dutch Holstein Friesians. This study also shows that, over the past 15 years, allele frequencies of the protein variants have changed in the Dutch Holstein Friesian population.

We estimated the effects of protein variants of β-LG , β-CN and κ-CN on the concentrations of the six major milk proteins, and on milk production traits. The β-LG genotype was associated with the relative concentrations of β-LG (A >> B) and of α-LA, α_{S1}-CN, α_{S2}-CN, β-CN, and κ-CN (B > A), but not with any milk production trait. The β-CN genotype was associated with the relative concentrations of β-CN and α_{S2}-CN ($A^2 > A^1$) and of α_{S1}-CN and κ-CN ($A^1 > A^2$) and with protein yield ($A^2 > A^1$). The κ-CN genotype was associated with the relative concentrations of κ-CN (B > E > A), α_{S2}-CN (B > A), α-LA, and α_{S1}-CN (A > B) and with protein percentage (B > A). The results indicate that the effects are large: β-LG genotype explains about 90% of the genetic variation in β-LG, β-CN genotype explains about 54% of the genetic variation in β-CN, and κ-CN genotype explains about 29% of the genetic variation in κ-CN.

These results show opportunities to use milk protein variants for selective breeding of cows with a higher milk quality, e.g. for cheese production.

QTL for Milk Fat Composition: short,medium and long chain fatty acids

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One morning milk sample was collected on 1,933 Holstein-Friesian heifers located on 398 commercial herds in the Netherlands. Each sample was analyzed for total percentage of fat, and for fatty acid w/w percentages. Heritabilities ranged from approximately 0.25 for long chain fatty acids to approximately 0.60 for short and medium chain fatty acids. These results suggested that it is possible to genetically alter milk fat composition. A genome scan was performed to identify quantitative trait loci (QTL) for fatty acid w/w%. Seven half-sib families with in total 849 cows and their 7 sires were genotyped for 1,379 SNP. Phenotypes of the cows were pre-corrected for systematic effects. The daughter design was used for a QTL program with multi-marker interval mapping in a weighted across-family QTL regression. Thresholds and confidence intervals were set using 10,000 permutations and 1,000 bootstraps. The results showed significant QTL (P_{genome}<0.05) for 36 traits at 6 distinct chromosomal regions. Two of these QTL regions mapped to the approximate locations of the DGAT1 (BTA14) and the SCD1 gene (BTA26). Two of these QTL regions (BTA6 and BTA19) affected short and medium chain fatty acids,

whereas the last 2 (BTA15 and BTA16) affected long chain fatty acids. The identified QTL may be a first step in understanding physiology of milk fat synthesis, as well as providing the basis for the development of a selection tool to genetically improve milk fat composition.

Species-specific variations in casein phosphorylation

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The caseins are phosphoproteins common to all mammalian milks. They assemble into colloidal complexes, or micelles, with calcium phosphate. The assembly process is governed by hydrophobic interactions between the casein molecules in concert with ionic interactions between the phosphate groups, present on serine clusters of α and β-caseins, and calcium phosphate. Whereas the bovine caseins are highly phosphorylated, other species have more varied patterns of phosphorylation. We have used a proteomic approach to examine the casein phosphorylation pattern of human milk. The proteins were separated using 2-dimensional polyacrylamide gel electrophoresis and digested in-gel with trypsin. The peptides released were analysed by MALDI-TOF/TOF mass spectrometry to identify the proteins. Six gel spots were identified as β-casein and 9 as α_{s1}-casein. Tandem mass spectrometry was used to show the 6 β-casein spots differed in phosphorylation level with 0-5 phosphate groups. Similarly the α_{s1}-casein spots were shown to contain 0-8 phosphate groups. In contrast, the bovine proteins have 5 and 8 phosphates respectively. So although the maximum level of phosphorylation of each protein is the same for humans and cows, human milk also contains hypo-phosphorylated forms not seen in bovine milk. This is likely to have an impact on the micelle assembly process and the ability of the micelles to incorporate calcium phosphate, particularly with respect to the significant levels of the unphosphorylated forms of the proteins. The phosphorylated peptides released during digestion will also be different. This could have implications when bovine milk is fed to human infants, so further elucidation of the bioactivities of casein phosphopeptides would appear to be warranted.

The histatherin gene – a chimera of histatin and statherin in cattle, identified through targeted screening of an EST database

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Novel antimicrobial peptides and proteins in milk have the potential to circumvent current problems with resistance to current antibiotics. A transcriptomics approach was used to identify such proteins in bovines.

A database was constructed by AgResearch of over 200,000 bovine ESTs derived from 50 tissue-specific libraries and augmented with public ESTs. These were assembled into ~40,000 contigs. A proportion of these contigs had no close homologue in human and thus represent potential bovine specific genes. A subset was created which had at least some similarity to genes encoding known antimicrobial proteins. From these, a 614 bp contig was selected for further investigation which had 30-50% amino acid sequence identity to histatin and statherin. As no direct orthologue was found in the human refseq database or in the syntenic region of the human genome, the gene was therefore named histatherin.

Of the 49 ESTs in the contig, 33 were from a parotid salivary library and the rest from libraries derived from mammary tissue. The *in-silico* translated protein was 58 amino acids long, with a 19 amino acid secretion signal peptide. Histatherin appears to consist of 5 exons located between 88,340,106 and 88,352,956 bp on BTA6 near the CSN1S1, CSN1S2, CSN2, CSN3 and statherin genes. Northern analysis of 26 bovine tissues with histatherin cDNAs revealed a transcript of ~600 nt in parotid salivary and lactating mammary tissue. Microarray and RT-PCR analysis indicated an initial increase in expression followed by a decrease during mammary involution. The bovine upstream beta casein enhancer abuts the histatherin transcription start site so it is possible that this genomic arrangement has led to its expression and apparent lactogenic regulation in the mammary gland.

As the *in-silico* prediction of a translated sequence does not prove it's existence in nature, a peptide corresponding to the mature protein was synthesized and used to generate antibodies. Western blot analysis revealed a 6kD immunoreactive band in whey, casein and in a lactoferrin enriched fraction. This band could be demonstrated only when the blot was fixed in glutaraldehyde immediately after transfer.

As histatin is known to have anti-microbial activity, particularly against *C. albicans*, studies were initiated to elucidate the role and activities of histatherin. The peptide was tested for antimicrobial activity and found to kill *C. albicans* at 6 ug/ml and *E. coli* and *S. aureus* at ≤3 ug/ml. The peptide significantly affected membrane permeability in dye filled liposome assays, but did not stimulate TNFalpha production in monocytes, nor block an LPS response, suggesting that the peptide had no direct pro- or anti- inflammatory activity.

The results suggest that histatherin is a ruminant-specific gene that plays a role in host defence in the oral cavity and milk in cattle. An accurate description of when and how the gene first arose awaits further analysis. Given its relative abundance in the mammary gland, milk and saliva, it is conceivable that it may help prevent mastitis as well as infection in the newborn calf or play a role in establishing an optimal symbiotic relationship with rumen microflora.

Weight reduction induced changes in fatty liver metabolomics – the marked effect of dietary protein and calcium

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Background: Fatty liver is the key element linking obesity, insulin resistance and type 2 diabetes. Weight loss is one of the few efficient ways to decrease liver fat. Since lipids are a diverse class of molecules with various functions, it is clear that in addition to the quantity, the quality of lipids contributes to the pathophysiology of fatty liver. In this study we investigated the effect of weight loss on liver lipid and primary metabolite profile using metabolomics approach. We also show that the effect of weight loss can be further enhanced by modification of dietary protein and calcium.

Methods: The liver and serum metabolomic profile of lean and obese C57Bl/6J mice (n=10/group) were compared with two groups of weight-reduced mice. Weight loss was achieved by energy restriction on control diet and whey protein-based high-calcium diet. The metabolomics analyses were performed using the UPLC/MS based lipidomics platform and the HPLC/MS/MS based primary metabolite platform.

Results: Weight loss decreased the level of liver triacylglycerols, phospholipids and ceramides and increased the level of sphingomyelins, cholesterol esters and phosphatidylserines. However, even significant weight loss didn't restore the lipidomic profile to the level of lean animals. The effect of weight loss was significantly enhanced with high-calcium whey protein diet, which was also accompanied by marked upregulation of TCA cycle and poentose phosphate pathway metabolites in liver.

Conclusions: Weight loss induces marked changes in liver metabolic profile, but cannot restore the distribution of lipid species to the level of lean controls. The effect of weight loss is affected by the dietary protein source and calcium content.