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Integrated proteomics and biological assays for characterization of non-nutritional effects of butyric acid on intestinal cells*

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ABSTRACT

The non-nutritional effects of butyric acid on human intestinal epithelial cells were characterized by integration of biological assays and 2-dimensional gel-based proteomics for characterization of cellular protein expression. Human colonic adenocarcinoma (Caco-2) cells were cultured with sodium butyrate (NaB), foetal calf serum (fast-growing cells) or basal medium alone (control cells). Biological assays showed that butyric acid decreased cell proliferation and induced apoptosis. Principal component analysis of proteomic results showed that protein expression in NaB-treated cells was different from both control and fast-growing cells. Significantly different expressed proteins were characterized by mass spectrometry.

KEY WORDS: butyrate, milk, cell proliferation, apoptosis, proteomics

INTRODUCTION

A number of nutritionally non-essential components present in the diet have been discovered to have beneficial effects for general health and in prevention of a number of diseases, including cancer. One such nutrient, the short chain fatty acid, butyric acid, can be derived in large quantities from bacterial fermentation of dietary fibre in the bowel, and is also a component of bovine milk (Jensen, 2002). Several animal studies have demonstrated that fibres producing the highest levels of butyrate in the colon are correlated with a greater protection against colon carcinogenesis (Archer and Hodin,

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1999). However, the cellular effects of butyrate are complex, especially since those observed in normal colon epithelial cells may be the opposite of those in transformed colon epithelial cells. In colon epithelial cells, proliferation may be stimulated by butyrate, while in transformed colon epithelial cells proliferation may be inhibited and differentiation promoted (Dzierzewicz et al., 2002).

The Caco-2 cell line is a well-established model of human colorectal tumour. It represents a selected population of colon cancer cells that have adapted to conditions *in vitro*, and have been widely used in studies of differentiation and regulation of intestinal functions.

Proteomics is a tool for the simultaneous determination of the protein composition of complex mixtures, like cell lysates. One of the tools in proteomics is to combine the separation power of two-dimensional gel electrophoresis (2DE) with multivariate statistics and mass spectrometry (MS) for identification of differentially expressed proteins in response to different treatments. The objective of this study was to investigate the non-nutritional effects of NaB on intestinal cells by integrated biological and proteomic methods.

MATERIAL AND METHODS

Cell proliferation

Human colonic adenocarcinoma (Caco-2) cells were cultured in Dulbecco's Minimal Essential Medium (DMEM with 25 mM Hepes) supplemented with foetal calf serum (FCS, 10%), L-glutamine (2 mM), and antibiotic solution for 24 h (pre-treatment period). The treatments included medium containing FCS (0.625%; control cells), NaB in different concentrations (0.1, 1, 10 and 50 mM), or FCS (10%; fast-growing cells) added for incubation periods of 48 h. Cell proliferation was measured by alamarBlue reduction (BioSource International Inc.).

Preparation of cell lysates for apoptosis and proteomics

For measurement of cell apoptosis and for proteomic analysis of protein expression, lysates of Caco-2 cells were produced by addition of RIPA lysis buffer. The treatments included: medium containing FCS (0.625%; control cells), 10 mM NaB (apoptotic cells), or FCS (10%; fast-growing cells) added for incubation periods of 48 h. Cell lysates were frozen at -80°C until proteomic analysis or measurement of apoptosis as caspase 3/7 activity (Promega Corporation). The cell lysates for proteomic studies were prepare from two different experiments: at day one two replicate gels of each of the three different treatments were analysed, while at day 2 four replicate gels of each treatment were used, giving a total of 18 gels in the experiment.

Two-dimensional gel electrophoresis

The 2DE was carried out essentially as described by Lametsch and Bendixen (2001) with the following modifications. A Dodeca cell (BioRad) allowing the simultaneous analysis of 12 gels was used. The first dimension of protein separation was carried out in immobilized 11 cm IPG strips (pH 5-8; BioRad), with 12.5% gradient Criterion gels (BioRad) in the second dimension. For analytical gels subjected to image analysis a volume of cell lysates corresponding to 50 µg as determined by BCA assay (BioRad) was applied, whereas for preparative gels for MS analysis a volume of sample corresponding to 370 µg protein was applied. Analytical gels were silver stained according to Lametsch and Bendixen (2001), while preparative gels for MS were stained according to Shevchenko et al. (1996).

In gel digestion, desalting and concentration of protein spots

Protein spots of significance were subjected to in-gel digestion by addition of trypsin essentially as described by Jensen et al. (1998), and the peptide mixture was desalted and concentrated as described by Lametsch et al. (2002). The peptides were eluted in 0.5 µl matrix solution (15-20 g/L of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile) directly onto the MALDI target plate (Bruker Daltonics GmBH).

Identification of proteins by MALDI-TOF mass spectrometry

Mass spectra were obtained using a Bruker Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonik GmBH) in reflection mode. The ion accelerating voltage was 25 kV with a delay time of 40 ns. The laser frequency was 50 Hz and 200 laser shots were accumulated for each spectrum. Mass searches were carried out in the data base Swiss Prot (Swiss Institute of Bioinformatics) using the MS ion search programme Mascot (Matrix Science).

Image analysis and multivariate statistics

The 2DE gels (n=18) were analysed using ImageMaster 2D analyser software (Amersham Pharmacia). The relative spot volumes were calculated for each separate gel to overcome gel-to-gel variations in spot intensities and sample loadings. Data was evaluated by principal component analysis (PCA) and discriminant PLS (D-PLS) using the multivariate statistical software package Unscrambler ver. 9.2 (Camo Process A/S). Standardized (centred: µ=0, and normalized: 1/SD) variables and full cross validation was used. The PCA was

carried out for control cells, cells treated with 10 mM NaB and fast-growing cells. The D-PLS regression was carried out for proteome data from growing cells and apoptotic cells to look for differentially regulated protein spots. Protein spots contributing least to the D-PLS model were removed by Jack-knifing through variable selection, and based on the remaining spots significant ($P<0.05$) regression coefficients were identified.

RESULTS

Cell cultures

The microscopic appearance of Caco-2 cells treated with NaB (10 mM), FCS (10%), or in basal medium alone is shown in Figure 1. Cells treated with NaB (10 mM) appeared apoptotic as indicated by condensed chromatin and fragmented nuclei (Figure 1c). Cells cultured in 10% FCS showed a very dense cell layer leaving essentially no space in between cells (Figure 1b) compared with control cells (Figure 1a).

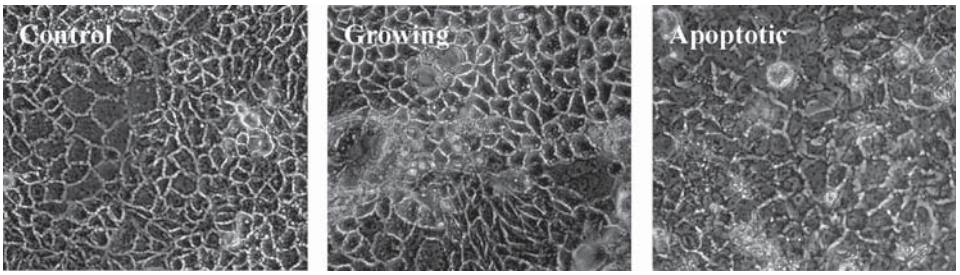

Figure 1. Microscopic appearance of human adenocarcinoma (Caco-2) cells cultured for 72 h in medium containing 0.625% FCS (control), 10% FCS (growing) or 10 mM Na-butyrate (apoptotic)

Increasing NaB caused a biphasic response in cell proliferation (Figure 2). Concentrations of 0.1 and 1mM of NaB increased cell proliferation by 18 and 34%, respectively, while concentrations of 5, 10 and 50 mM decreased cell proliferation to 77, 41 and 12%, respectively, compared with proliferation in control cells. Treatment of cells with 10% FCS increased cell proliferation by 55% compared with the control cells (data not shown). Cell apoptosis measured as caspase 3/7 activity was lower in cells treated with 10% FCS compared with control cells (365±11 vs 515±15 AU), while apoptosis in cells treated with 10 mM NaB was increased by a factor more than 8 relative to apoptosis in control cells (4444±34 vs 515±15 AU).

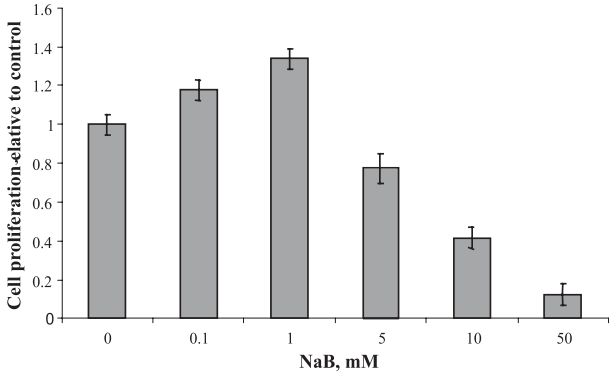

Figure 2. Effect of Na-butyrate (NaB) on proliferation of human adenocarcinoma (Caco-2) cells measured by alamarBlue reduction. Cells were cultured for 72 h in medium containing different concentrations of NaB. Values are least square means obtained from 2 culture experiments with 4 replicates and presented as relative to proliferation obtained in basal medium

Proteomics

A typical gel of the separated proteins in the Caco-2 cell lysates is shown in Figure 3. Image analysis of the 2D-gels in the analysis included annotation of 571 individual protein spots.

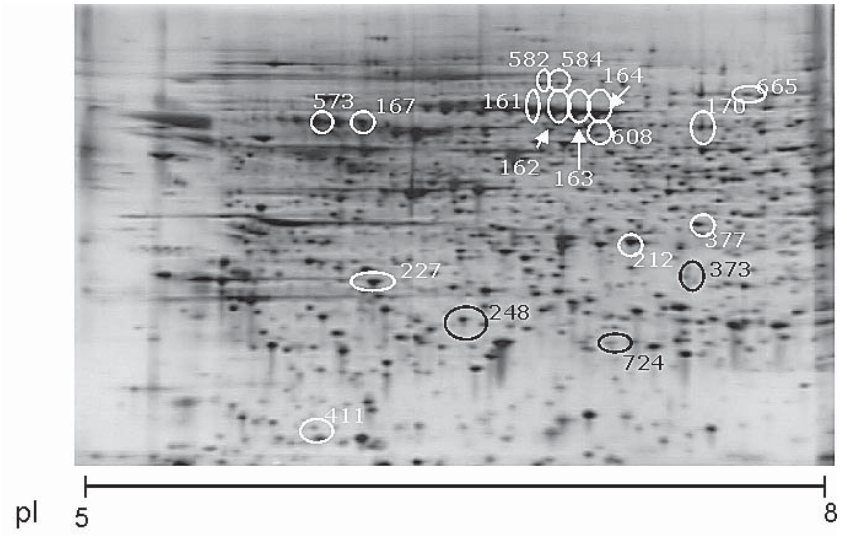

Figure 3. 2-DE of proteins in Caco-2 cell lysates. The pH gradient of the first dimension is indicated. The ID's of proteins up-regulated in NaB treated cells (black) and fast-growing cells (white) according to the image analysis are indicated

Multivariate statistics using PCA of relative spot values showed that the protein expression in NaB-treated cells was different along the PC2 axis from both control cells and fast-growing cells, which in turn were more similar in their protein expressions (Figure 4). The grouping along the PC1 axis was mainly due to variations between two different experiments.

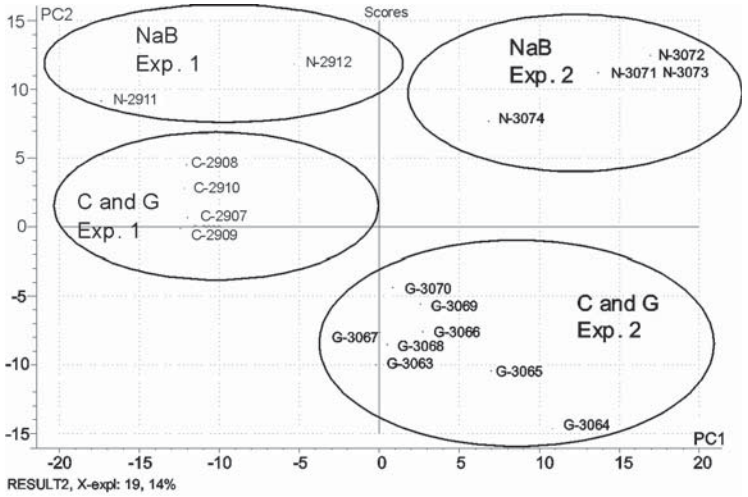

Figure 4. PCA score plot of 2DE-gel samples along the first two PCs. N indicates NaB treated samples, while C and G indicate control cells and fast growing cells, respectively. The numbers are ID's of 2DE gels

The differences in protein expression between cells treated with NaB and 10% FCS (fast-growing cells) were further analysed by D-PLS. The result of this analysis is indicated in Figure 3. A total of 18 protein spots were significantly different between the two treatments. Of these, 15 were down-regulated in the NaB-treated cells, while 3 were up-regulated after NaB treatment. These significant protein spots were excised from preparative 2D gels, and subjected to MALDI-TOF MS for identification.

By this analysis the following proteins were identified. The accession numbers in SwissProt are indicated. Down-regulated at NaB treatment: spot 167 and 573: α-tubulin (P68361); spot 170: T-complex protein γ-subunit (P49368); spot 212: 60 S acidic ribosomal protein (Q95140); spot 227: chloride intracellular protein (O00299); spot 377: transaldolase (P37837); spot 584: ATP dependent DNA helicase 2, subunit 2 (P13010); spot 608: Ser/Thr protein kinase (Q8CIN4) and spot 665: stress-induced prosphoprotein 1 (P31948). Up-regulated at NaB treatment: spot 248: ubiquitin C-terminal hydrolase (P09936); spot 373: translin associated protein X (Trax) (Q99598). For two spots, one down-regulated by NaB

treatment (spot 411) and one up-regulated by NaB (spot 724) it was not possible to obtains MS results for identification.

DISCUSSION

The ability of specific nutrient components to modify the risk of a number of diseases, including cancer, has been the subject of intense studies by nutritional, medical and food researchers. One such nutrient, butyrate, can be derived in large quantities from bacterial fermentation of dietary fibre in the bowel, and can also be found in milk. Since butyrate has been shown to induce apoptosis in cancer cells, it may contribute to the decreased incidence of bowel cancer that has been associated with fibre intake.

The effects of butyrate on the epithelial cells of colon have been examined in a number of *in vitro* cell-based models. These studies have shown that the cellular effects of butyrate are complex, especially since those observed in normal and transformed (cancerous) colon epithelial cells might be opposite (Archer and Hodin, 1999; Dzierzewicz et al., 2002). Using integrated proteomics and biological assays, we characterized the effect of butyrate on human colonic adenocarcinoma (Caco-2) cells.

The biological assays showed that butyrate decreased cell proliferation in agreement with previous studies (Dzierzewicz et al., 2002; Fu et al., 2004). This decrease was seen at physiological concentrations of NaB (i.e. 5 and 10 mM) and at higher concentrations of NaB (i.e. 50 mM). However, lower concentrations of NaB (i.e. 0.1 and 1 mM), increased cell proliferation compared with control cells. As the concentration of butyrate in the human colon varies from 5-25 mM (Bergman, 1990; Fusunyan et al., 1998), an inhibitory effect of butyrate on colon cells might be suggested in human colonic epithelial tissue. Butyrate has furthermore been shown to be a differentiating and apoptotic agent in Caco-2 cells (Dzierzewicz et al., 2002). In the present study, apoptosis, as measured by caspase 3/7 activity, was increased substantially by NaB added to cells in a concentration of 10 mM. These results suggest that butyrate, as a luminal component, may influence the balance between cell proliferation, differentiation and apoptosis, required for maintaining the health of the large bowel epithelium.

Using proteomics as a tool, we took the characterization of the effects of butyrate on the protein expression patterns one step further. The protein expression pattern of NaB-treated Caco-2 cells was compared with protein expression in fast-growing and control Caco-2 cells. The identified proteins down-regulated at NaB treatment included cytoskeleton proteins, enzymes, chaperones and regulatory proteins. A protein involved in ubiquitination of proteins for the degradation by proteasomes was up-regulated in the apoptotic NaB-treated cells. However, translin

associated protein X (Trax), a protein essential for cellular proliferation (Yang and Hecht, 2004), was surprisingly up-regulated by NaB treatment. The present data are in accordance with the suggested mechanism for effect of butyrate on cells, where the cellular cytoskeleton has been identified as a major target by modifying microfilament and microtubule assembly (Hague and Paraskeva, 1995).

CONCLUSIONS

The present study shows that butyrate inhibit proliferation and induce apoptosis in human adenocarcinoma (Caco-2) cells. These data are in line with the epidemiological and experimental data suggesting that a diet high in fibres protects against the development of colon cancer. Proteomics showed that the protein expression was significantly affected by NaB treatment, and that cytoskeletal proteins and chaperones were higher in fast-growing cells, while a protein involved in protein degradation by chaperones was up-regulated by NaB. It is concluded that NaB has significant effects on proliferation and apoptosis of human intestinal cancer cells and that these effects are reflected in the protein expression patterns of the cells.

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High-throughput measurements for functional genomics of milk

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ABSTRACT

Recent developments in analytical technology have simplified a detailed characterization of milk and milk-based samples. A range of powerful new instrumentation types have recently been installed at various institutes at Campus Ås (Norway). At the campus we have recently implemented efficient, multi-channel instrumentation for genomics, transcriptomics, proteomics, biospectroscopy, metabolomics and various quality assessments. The present paper gives an informal outline of various modern analytical tools for characterization of various milk and milk-based samples.

KEY WORDS: biochemometrics, biostatistics, analytical chemistry, functional genomics, transcriptomics, proteomics, metabolomics, biospectroscopy

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INTRODUCTION

Functional genomics offers new opportunities in milk science research. Phenotypic properties can be related to genotypic information statistically, in order to quantify expected relationships and to discover unexpected relationships. Detailed genomic studies can then reveal the causal genetic basis for these relationships, and lead to more efficient animal breeding as well as improved farm management, animal feeding and milk processing.

In order to obtain relevant results with sufficient statistical reliability, it is advantageous to be able to choose cost-effective measurement techniques: On one hand, low-cost high-speed screening methods based on e.g., multivariate Fourier Transform Infra Red (FTIR) biospectroscopy, can be applied to millions of milk samples or thousands of individual animals in order to identify particularly interesting samples or individuals. On the other hand, the most interesting samples or animals can be submitted to higher-cost detailed studies, e.g., genome-wide characterization of Singe Nucleotide Polymorphism (SNP) for tens of thousands of genetic markers, or 2D gel proteome-wide electrophoresis. Economically important but more time-consuming quality assessments, such as consumer studies or feeding experiments, can finally be used for a small set of particularly interesting samples or animals. In between, a number of different types of measurements can also be put to use, to reveal systematic patterns of variation.

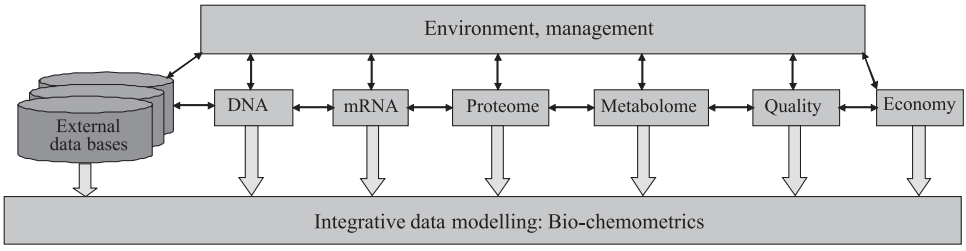


Figure 1. Integrative functional genomics: Relating traditional genetic data (breeding, management, production) to DNA data (genome), mRNA data (transcriptome), protein composition (proteome), metabolic profile (metabolome), as well as animal or product quality data and production economy, using advanced mathematical and statistical tools, such as bio-chemometrics

Figure 1 illustrates the conventional causality in functional genomics. The right-pointing arrows linking the boxes show that the genomic information, representing diversity in the DNA sequence of the animals, is transcribed into mRNA to varying degrees. The transcriptome in turn defines the proteome, which includes the enzymes that produces the variety of metabolites - the so-called metabolome. Together, the proteome and metabolome affect the quality and quantity (animal

productivity and - health components, taste, smell and appearance, etc.), which in turn affect the over-all economy of the agricultural production. But the left-pointing arrows linking the boxes in the figure illustrate that a range of regulatory feedback mechanisms complicates this functionality track.

The figure also outlines how data, obtained at different stages along this causality track, can be related to external data - from environment, farm management or existing data bases in animal breeding/-health/-production, etc.

At Campus Ås, the Norwegian University of Life Sciences (UMB), the Norwegian Food Research Institute (Matforsk) and several other institutions have made a concerted effort over the last couple of years to ensure sufficient measurement capacity at all stages along this functional genomics causality track.

Several milk- and meat-related research projects, using these new facilities, have recently been financed and initiated on the campus. At this stage it is too early to report new research findings. The present paper represents only a brief progress report for some of these measuring techniques. Since the concept is highly cross-disciplinary, the basic principles behind each of the measuring techniques will be outlined.

RESULTS

The bovine genome

The sequencing of the bovine genome is expected to be completed in 2007. This information will of course be important in basic biological studies - for comparison to other species. Moreover, it will help us understand genetic effects in bovine milk. The information in the genome is overwhelming, but is now becoming accessible with a combination of advanced measuring techniques and advanced computer science.

The bovine genome consists of 30 chromosomes, and each animal has two copies of each. Except for the sex chromosome, the two copies are rather similar. A chromosome represents a sequence of the four possible DNA nucleotides: adenine, cytocine, guanidine and thymine (abbreviated by letters A, C, G and T), linked into a DNA strand. “The bovine genome” represents the DNA sequences of the 30 bovine chromosomes.

However, within the bovine species, the DNA sequences of the different families are slightly different. Most of these differences represent single-letter changes at certain locations, called Single Nucleotide Polymorphisms (SNPs, e.g., a C has been changed into a G at position # 1000 along the DNA of chromosome 10). Once upon a time these SNPs may have arisen by random mutation. But unless lethal, these mutations are then passed on from generation to generation,

just like spelling mistakes in a medieval manuscript. These variations of the DNA sequence of a given SNP are called its "alleles". Each animal has two alleles at each SNP location - one inherited from its mother and one from its father.

If we hope to explain why different animals grow differently or produce different milk qualities, we need to measure which SNP-alleles are found in which breeding lines. At the Centre for Integrative Genetics (CIGENE), Norwegian University of Life Sciences (UMB), we have several up-to-date types of equipment for SNP detection in e.g., semen- or blood samples from individual animals. Methods that monitor diversity in a whole range of genes or gene marker positions may be called "genomic".

Figure 2 (left plot) illustrates such a methodology - the output from the new 22.6K bovine gene marker micro-array chip from the company Affymetrix (Affymetrix, Santa Clara, CA, USA). CIGENE has been involved in the development of this chip. It delivers allele characterization for each animal at more than 22608 different gene marker positions along the DNA sequence, across all chromosomes, in terms of microscopic spots, as shown in the Figure.

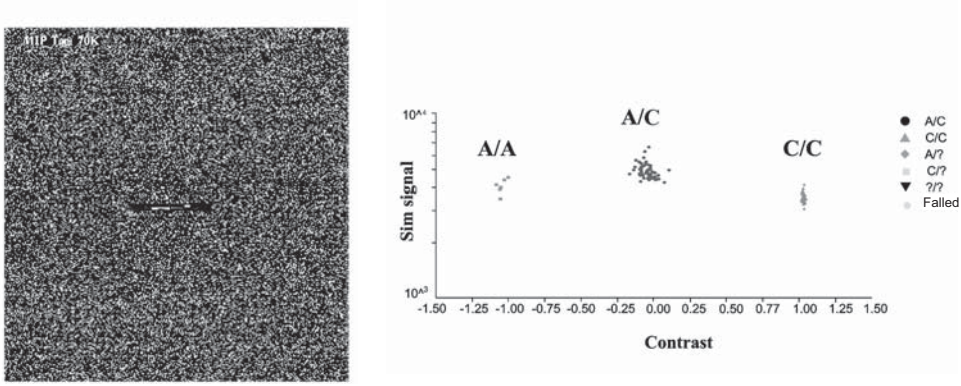


Figure 2. Overview of raw data SNP detection for 23000 gene markers (bovine Affymetrics chip) for one bull (left plot). The right plot provides data from one of these gene markers, for a set of NRF bulls, showing the three combinations of the two alleles for this SNP (C or A)

Figure 2 (right plot) illustrates the output for just one of these gene markers for a certain set of cows: some animals are homozygous A/A for this marker (the animal has inherited allele A from both its mother and its father), while others are homozygous C/C or heterozygous (one copy of A and one copy of C). For each animal, such results are delivered for each of 22608 gene markers.

With this type of massively parallel chip-technology, the cost of per individual SNP is rather low. But each animal requires one chip each, so it calls for a selection of particularly informative animals. At present, about 1500 sires previously or presently involved in the national breeding programme, are the first

to be characterized with this technique. Since each of these bulls have a high number off daughters, phenotypes measured for individual cows - e.g., milk composition - can be averaged into stabile estimates of sire averages. However, we also characterize some of the individual dams that are used in animal feeding studies at the university farm.

In CIGENE we also have other genomics instrument - e.g., lower-cost Sequenom mass-spectrometry based SNP analysis for a few (typically, <50) SNPs at a time, and equipment for re-sequencing of selected DNA sequences of selected animals, for further SNP identification and - verification.

As Figure 1 illustrated, traditional genetics, employing large data bases of breeding information about which bulls have sired which cows etc., can now be supplemented with detailed genomic information.

The DNA sequence information of a given, typical gene consists of its regulatory sequence followed by a coding sequence. The gene regulation sequence consists of DNA patterns that control how this gene is to be utilized at a given point in time, depending e.g., on the presence of gene products from other genes. The coding sequence consists of a series of coding regions (exons) whose information can be transcribed into messenger RNA (mRNA), interspersed with non-coding regions (introns), whose information cannot be translated into mRNA, although they might have other roles - this is an open question at present. When the gene's regulatory sequence is activated, its coding regions are transcribed into one contiguous pre-mRNA sequence, containing both introns and exons. Afterwards the intronic sequences are spliced out, leaving a mature mRNA that diffuses out from the cell kernel. In many cases, but not all, the mRNA transcript can then be subsequently translated into the amino acid sequence of a corresponding protein, because three and three adjacent nucleic acids defines a given amino acid. This protein can, in turn, serve as enzymatic catalyst in metabolic reactions, as structure-building proteins, as gene regulation factors, etc. Due to so-called alternative splicing one DNA sequence can give rise to two or more proteins.

With genotypic and phenotypic data available for sufficiently many individual animals with sufficiently large genetic variation, it is possible to find so-called Quantitative Trait Loci (QTL) - DNA regions in the chromosomes that correlate statistically to a phenotypic trait. Fine mapping combined with functional studies can ultimately reveal the underlying causal mutation(s).

These research processes, in turn, pose several data analytical challenges - both in terms of cross-disciplinary communication, of mathematical data-modelling that is cognitively accessible, and of statistical testing that is sufficiently robust against false positives, etc. One simplifying aspect of this is to combine the DNA data about the many individual SNPs into a lower number of so-called "haplotypes" - groups of adjacent gene markers that are inherited together.

The transcriptome

The complex, dynamic processes of gene regulation, metabolic regulation etc., which control the biological activity (cell differentiation, ageing, response to environment, etc.), need to be studied if we want to bring our understanding of milk production on a causal biological and chemical basis. For this purpose it is important to have cost-effective techniques to monitor the genetic DNA diversity as well as the gene transcription into mRNA, proteins and subsequent metabolites along the functional genomics causality chain (Figure 1). Methods that monitor the expression of a whole range of genes may be called "transcriptomic".

The micro-array technique illustrated in Figure 2 can also be used for quantifying how each of a high number of genes are transcribed into mRNA in individual animals at given points in time. Alternatively, to monitor a lower number of genes, but at a lower price per animal, our Sequenom mass-spectrometric instrument can be used instead (Ding and Cantor, 2003), as illustrated for a casein gene marker in Figure 3. To monitor e.g., how casein production genes are used by a goat, a biopsy is taken from udder and quickly conserved to stop chemical modification. After various automated mRNA robot-based clean-up stages, the sample is split into e.g., 12 aliquotes, and various known concentrations of an artificial "competitor" mRNA are added. By the so-called PCR reaction both the unknown and the known mRNAs are amplified proportionally to concentrations measurable in the maldi-tof mass spectrometer.

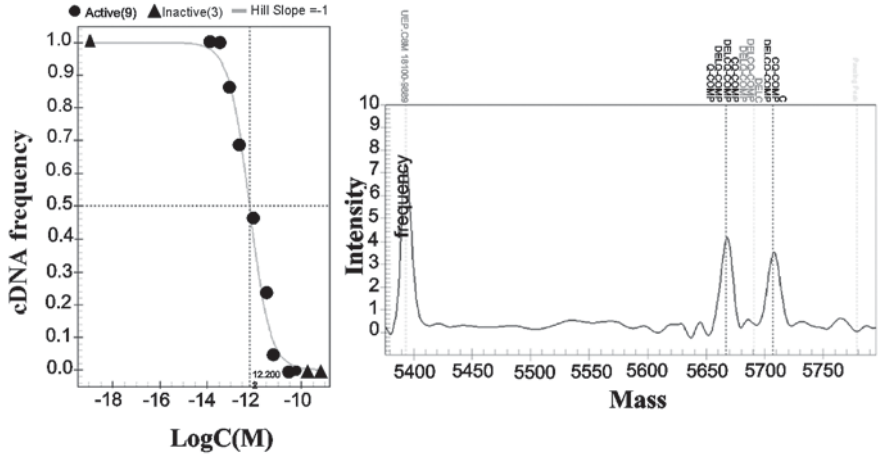


Figure 3. Gene expression determined by MALDI-TOF mass spectrometry: Quantification of two alleles of a given caprine casein gene marker. Left plot: Competitor titration curve to find the concentration of mRNA in this udder biopsy. Right plot: MALDI-TOF MS "spectrogram" raw data for a specific (targeted) gene for one single goat sample at one given competitor concentration. Screenshots from the Sequenom software

In the left plot of Figure 3 the relative concentration of the mRNA for this gene is plotted against known concentrations of an artificial "competitor" mRNA. The white arrows indicate how the unknown mRNA concentration of this gene in this udder biopsy is determined as the competitor concentration that gives the same mass spectrometric peak height. The right plot of Figure 3 illustrates the mass spectrometric raw data at one of these known concentrations.

With this technique we can study how the mRNA expression of various genes, e.g., the synthesis of the four major caseins, vary with their DNA allele structure, with lactation cycle, feeding and stress, etc.

The proteome

The degree to which the produced mRNA of a gene results in actual proteins is also informative. Methods that monitor a whole range of proteins can be called "proteomic".

Figure 4 (left plot) illustrates the "classical" proteomics technique - 1D gel electrophoresis (isoelectric focusing), for goat milk samples. Several different milk samples are applied side by side, and the proteins - caseins and whey proteins - are then separated into bands according to their electric charge. In order to be useful in functional genomics, these proteomic images need to be converted into quantitative data. This involves scanning the gels as digital images and tracing the individual tracks, as illustrated by two samples.

Examples of quantified electrophoresis raw data traces from two animals are shown at the top of Figure 4 (right plot). In order to compare them, two types of normalization are then required: horizontal spatial alignment (middle), to correct

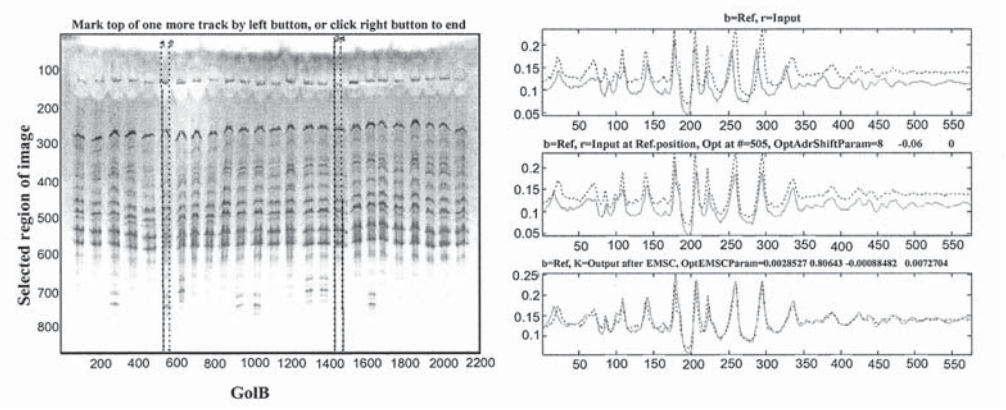


Figure 4. Proteomics of milk proteins. Left plot: 1D electrophoresis image with two samples (tracks) marked for quantification. Right plot: normalization of 1D electrophoresis data from one track (solid) relative to a reference track (dotted): Top: raw data. Middle: data aligned spatially. Bottom: aligned, baseline- and scale-corrected data

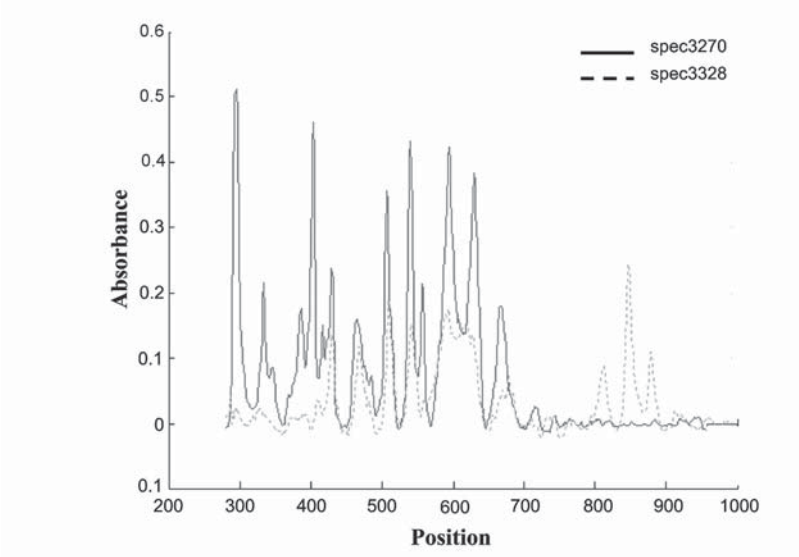


Figure 5. 1D electrophoresis of milk from two goats differing in αS1-casein

for physical variations in the electrophoresis gel structure, and vertical intensity correction (bottom), consisting of a subtraction of baseline background and a division by an overall scaling factor.

Figure 5 shows the results after normalization of the milk proteins from two animals representing two of the most typical Norwegian goat genotypes. Animal no. 3328 was previously found by DNA assessment (Figure 2) to be heterozygous for a certain position for αS1 casein in exon 12 - the animal has one normal (G) allele and one allele called "null" which is a deletion (0) that results in no production of the normal αS1 casein. In contrast, animal no. 3270 is homozygous in the αS1-casein (00) - it totally lacks expression of the normal allele. As expected, the proteomic results in Figure 5 indicate that the latter lacks the normal αS1-casein.

The proteomic measurements can also reveal other details. For instance, several different phosphorylated forms of the αS1 casein are evident. However, 1D electrophoresis is somewhat time-and labour-consuming.

Figure 6 illustrates how similar proteomic "fingerprint" can be obtained with MALDI-TOF analysis, which is faster and less labour-intensive. With this technique, the skim-milk samples are simply diluted, mixed with a light-absorbing matrix compound, spotted and dried on a steel plate and measured. Each plate takes 96 different sample spots. The plate is then bombarded in vacuum with shots of intense laser light on each spot, which vaporizes and ionizes the proteins. An electrical field then drives the ionized proteins towards a mass spectrometric detector; smaller proteins arrive before the larger proteins. Figure 6 shows the

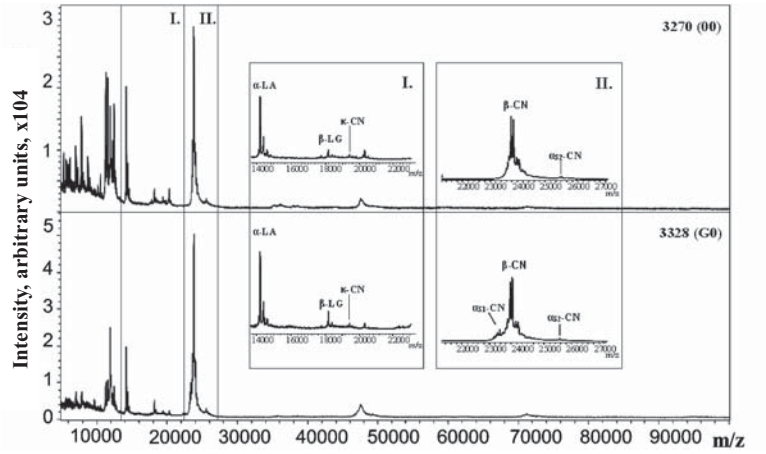


Figure 6. Proteomic MALDI-TOF mass spectrometry of two caprine milk samples

milk proteome of the same two individual goats as in Figure 5, no. 3270 (00) and no. 3328 (G0). For more information, see e.g., Chianese et al. (1993) and Miranda et al. (2004).

The metabolome - chromatography

The enzymatic activities from the proteome catalyse the metabolic processes in the cell. Methods that monitor a whole range of metabolic components or aspects can be called "metabolomics". The classical metabolic measurement principle is chromatography, in which the different chemical components in a sample are separated from each other by passing the sample through a chromatographic column.

Figure 7 shows the fatty acid profile of a bovine milk sample, based on gas chromatography with mass spectrometric detection. The fatty acids can be quantified from the areas or their respective peaks, after horizontal alignment of retention time and vertical subtraction of baseline and scaling for general size factor. A number of different fatty acids can thus be quantified, with different chain lengths, different degrees and patterns of unsaturation, etc.

This type of fatty acid profile data, measured for a large number of related milk samples, can be related to genomic, transcriptomic or proteomic data for the same samples, as well as to other known variables such as feeding strategy, time of year, etc. Multivariate analysis of these data can then reveal their common underlying biological production mechanisms. Chromatographic metabolome analyses can of course also be performed for other compositional aspects of milk, blood, urine, etc., e.g., hormones, oxidation products, anti-oxidants. For instance, GC-MS is

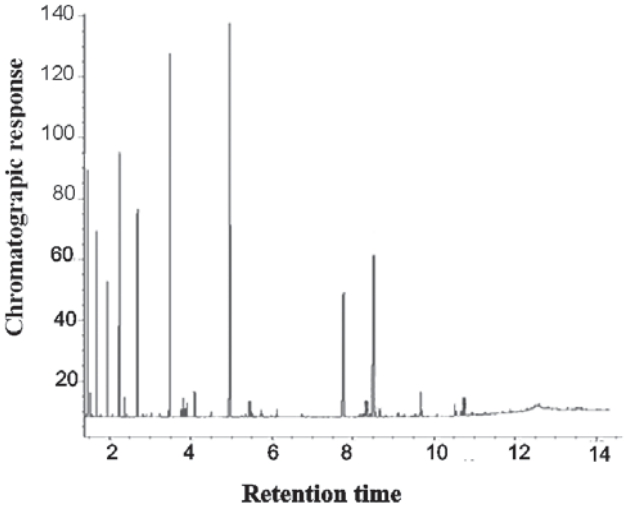


Figure 7. Metabolomics of milk: GC-MS determination of fatty acid profile of a bovine milk sample

a suitable technique for analysing volatile oxidation products of milk and milk products. GC with fluorescence detection is a suitable tool for the qualitative and quantitative analysis of tocopherols.

The biological structure detection - biospectroscopy

While chromatography is generally a mature technology delivering reliable results, faster metabolome alternatives are available, based on spectrophotometry.

Photons are affected by milk samples in various ways, and several principally different spectrophotometric techniques are used for the qualitative and quantitative analysis of milk samples. Most of these techniques are cheap, fast and precise, and yield several types of information about the samples at the same time.

Figure 8 shows the infrared absorption spectra of a set of bovine milk samples, in terms of the absorbance as a function of wavenumber (the wavenumber convention is just the inverse of the wavelength). The data were obtained in one of our modern Fourier-Transform Infra Red spectrometers (Bruker), after simply applying a drop of milk on a zinc selenide surface, drying off most of the water, and recording how much light survives through the sample at different wavelengths in the infrared range, i.e. at wavelengths longer than that visible to the human eye. The figure illustrates that different milk constituents - proteins, lipids, carbohydrates and remaining (bound) water, absorb light at more or less distinct wavenumber regions, because their different chemical bond types (-C-H_3, -O-H, (-N-H_2, etc.) vibrate, and thus absorb light, at different frequencies in this

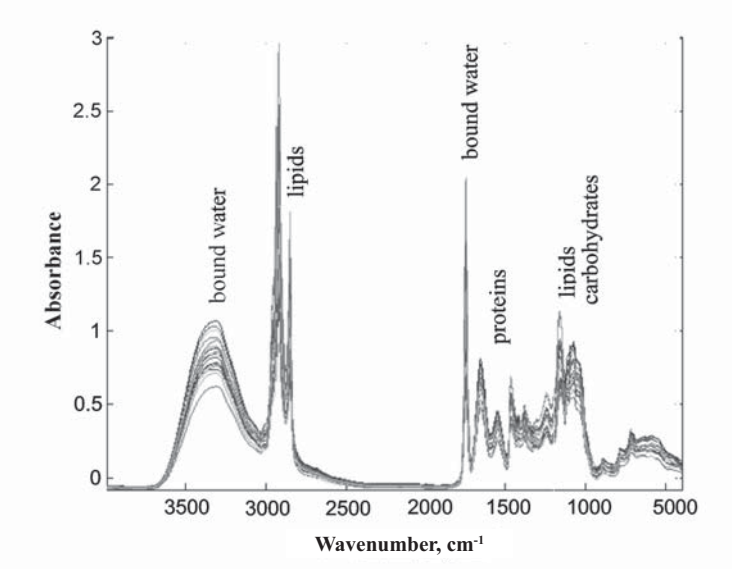


Figure 8. FTIR absorbance spectra of a set of dried bovine milk samples

region of the light spectrum. Hence, milk samples with different levels of these constituents give different absorbance spectra.

Upon closer inspection, some of the spectral differences are found to be due to variations in the physics of the sample - light scattering and sample thickness. These can be estimated and corrected for mathematically, yielding spectra with a wealth of information about the samples' variation in chemical composition,

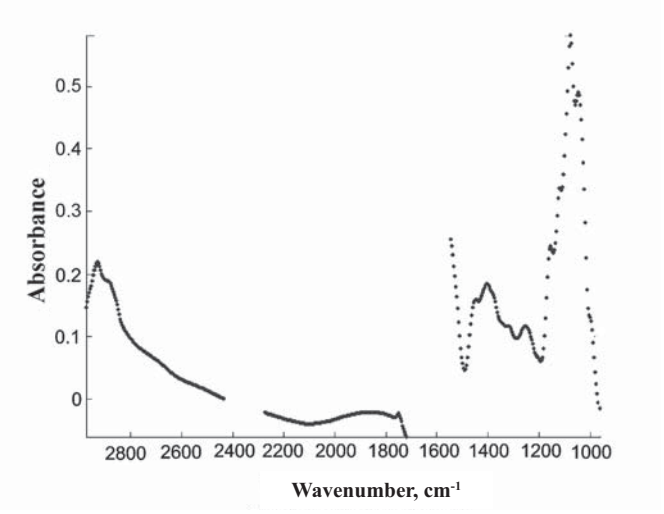


Figure 9. FTIR absorbance of homogenized whole milk

including the main aspects of fatty acid variation and possibly some of the protein variations.

One of the FTIR techniques used routinely for quality assessment of milk (Figure 9) works on intact milk samples - the Foss Electric Milkoscan instrument. Due to the high water content in milk, and the high absorbance of water, this restricts the wavenumber region where precise measurements are possible.

Work is in progress to store millions such bovine milk spectra per year, representing thousands of individual cows several times a year. By multivariate biochemometric analysis, systematic patterns of variation in these spectra will be averaged for the individual sires, and then related to available genetic, genomic and farm management information.

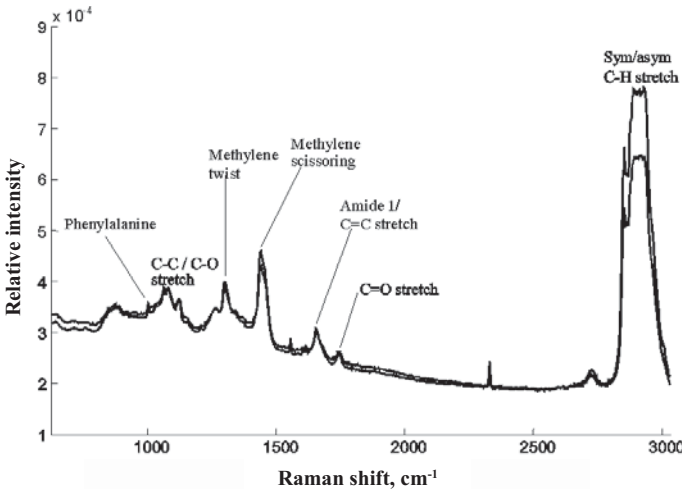


Figure 10. Raman spectra of a milk sample, with band assignment

Figure 10 shows a third biospectroscopic principle: Raman scattering. This type of measurements gives data similar to FTIR and NIR, but is primarily sensitive to symmetrical molecular bonds, such as –C-C– or –C=C–, and is therefore more or less insensitive to the presence of water - in contrast to FTIR. Thus, Raman and infrared spectroscopy complement each other. The figure outlines some of the chemical bond types detectable with this instrumentation.

The molecular vibrations from the infrared region in Figures 8 and 9 can also be observed in the near-infrared (NIR) wavelength region, in terms of their overtones. The absorbance signals are now much weaker, and can therefore be measured easily. Figure 11 shows the NIR spectra a set of natural and modified bovine milk samples, measured by diffuse reflection (top) and diffuse transmission (bottom), using an NIR Systems instrument at our research partners at Copenhagen

University. The data originate from a profiling experiment on the sensory perception of fat, homogenization, etc. in milk (Frøst et al., 2001). Campus Ås has several of these instruments. In both measurement modes the water, lipids, proteins and carbohydrates give rise to broad, somewhat overlapping absorption peaks. In addition, variations in light scattering of these milk samples due to e.g., varying homogenization, cause strong over-all spectral variations. Again, mathematical pre-processing of the spectra can separate the physical scattering variations from the chemical absorbance variations.

Figure 11 also includes the visible wavelength range (400-700 nm), where various pigment molecules display light absorption due to electronic transitions. This wavelength range can be used to predict how consumers will perceive the appearance of milk and milk products.

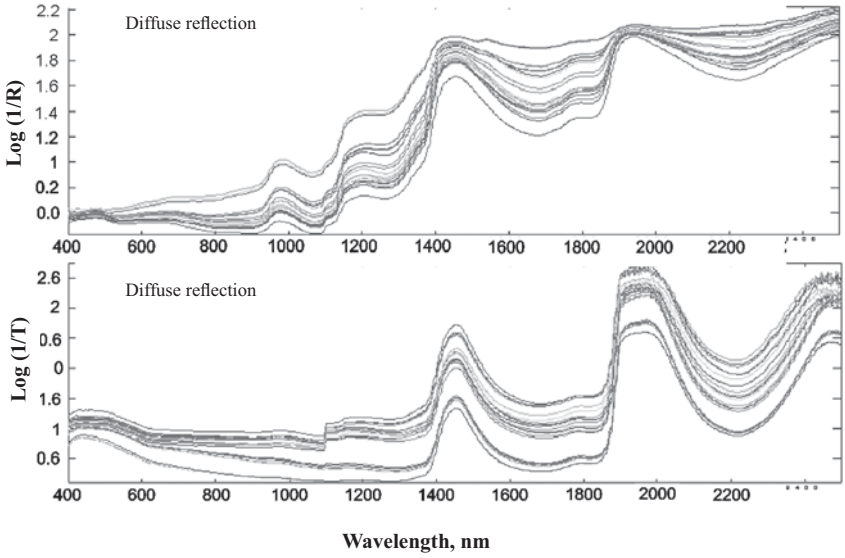


Figure 11. Visible- and near-infrared (NIR) spectra of a set of wet milk samples, obtained by diffuse reflection (top) or diffuse transmission (bottom)

Finally, Figure 12 represents quite a different type of biospectroscopy - namely autofluorescence (Wold et al., 2005). When milk or milk products are illuminated by visible or ultraviolet light, some of the photons are absorbed temporarily by particular milk components, and re-emitted at higher wavelengths. The wavelength shift reflects the type of molecule, while the intensity of the emitted light at a given wavelength reflects the concentration of the corresponding compound(s). The figure illustrates what happens to a given type of cheese when exposed to light from a commercial fluorescent light tube. This experiment showed that different types of ambient light during cheese storage caused different types of

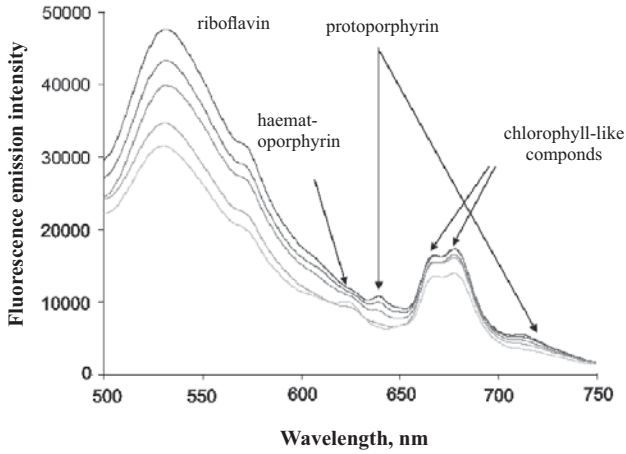


Figure 12. Fluorescence emission spectra from Swiss-like cheese exposed to light from a commercial fluorescent light tube. Light exposure times varied from 0 to 10 h

photo-oxidation and thus different degrees of sensorically perceived rancidity, which can be affect consumer acceptance.

Considering the different nature of the identified fluorescing compounds - some are probably derived from the animal itself, others from the feed - it may well be that fluorescence measurements of series of milk samples can yield phenotypic variations associated with the genome of the animals - or of the feed.

The product quality and production economy

The economic value of the milk, yoghurt, cheese, etc. depends on the willingness of consumers to buy the products. This is, in turn, largely dependent on the sensory properties of the products. In those cases when it has already been clearly established which chemical compounds or physical aspects of the milk that determine variations in its sensory properties, it is sufficient to measure these properties chemically or physically. Otherwise, it is safer to include actual sensory perception studies. Due to developments in the cognitive sciences, etc., modern sensory science is highly informative, reliable and cost-effective. Consumer studies are useful tools for measuring consumer liking - if done professionally (it is not enough just to distribute a simple questionnaire!). Descriptive sensory profiling with a trained assessor panel provides insight into the reasons why the consumers perceive products differently.

Provided that high-throughput methods, like genetic pedigree assessment, genomic profiling or biospectroscopy screening, have been used to identify particularly interesting milk samples, sensory profiling and consumer testing can be used to obtain the commercially relevant assessment of these.

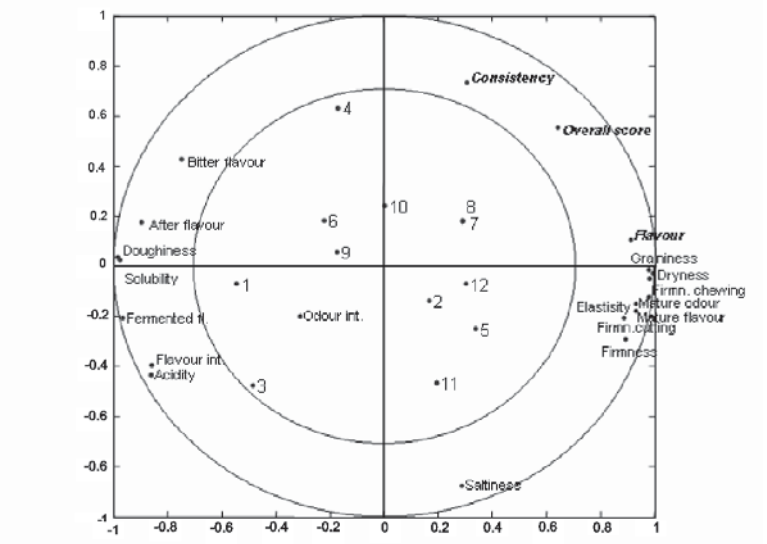


Figure 13. Sensory quality assessment of milk and milk products. Correlation loading plot summarizing a PLSR model of how 17 sensory descriptors (average of 10 trained assessors) predict rating of three quality aspects (bold italics, average of 5 experts) in 12 samples of a given type of cheese, selected to span large quality variation. The same approach can be used for fresh milk samples

Figure 13 shows an example of a sensory study of variations in a milk-based product (Hersleth et al., 2005). Twelve batches of a certain type of cheese were identified as spanning a large range of quality, based on the producer's routine assessment. These samples were submitted to quality ratings by 5 cheese experts as well as descriptive profiling by 10 trained sensory assessors. The figure summarizes how the three over-all quality rating variables (average of five experts) could be explained by the 17 descriptors (average of 10 panelists) by Partial Least Squares Regression, using The Unscrambler software (www.camo.com). The abscissa and ordinate show the correlations between the input variables and the two first latent variables, i.e. the two main patterns of co-variation. The figure shows e.g., that over-all expert score and flavour correlated positively with mature flavour and -odour, graininess, dryness, etc. and negatively with acidity. Consistency reflected minor constrasts in grainness vs firmness, and was negatively associated with saltiness.

The five most extreme cheeses were then selected for subsequent consumer assessment, where 110 consumers assessed their hedonic liking as well as their perception of flavour and texture. Details of the study are given in Hersleth et al. (2005).

The same approach can be used for assessing variations in e.g., fresh milk quality as function of e.g., animal genetics or -feeding.

Integrating the various sources of information

The different blocks of data types in functional genomics outlined in Figure 1 have been illustrated in the subsequent figures. Multivariate data modelling can then be used for integrating the different types of information, in terms of the underlying structures within each of the blocks as well as the relationships between them. Combination of modern bioinformatics, biostatistics and biochemometrics can then bring out the statistically valid information. For instance, the multivariate "soft modelling" approach explained by Martens and Martens (2001) may be used for this purpose - testing hypotheses and discovering unexpected patterns of co-variation within and between various blocks of measured variables.

As Figures 4-12 have demonstrated, milk samples can thus be phenotypic fingerprinted with respect to a number of different properties ranging from proteome details to a variety of metabolomic and quality aspects. By data modelling, these phenotypic data can be related to the gene expression measurements from e.g., udder biopsies (Figure 3), SNP structures (Figure 2) and/or genetic background information, e.g., in terms of estimated haplotype structure. All of these data can in turn be related data-analytically to external environmental or farming management data (Figure 1). Of course, it is not necessary to obtain all types of measurements for all milk samples or all animals. But once the particuarly interesting milk samples have been obtained or particularly interesting animals identified in one research project, we try to secure enough material to allow other types of measurements of these.

This inter-disciplinary research process requires that several different institutions, in different locations and with different financial organizations, have to cooperate. At each stage, professionalism is needed, in order to secure high-quality data. But equally important, distinctly different research cultures will have to meet, ranging from animal science, genetics and genomics *via* analytical chemistry to dairy science and sensory science.

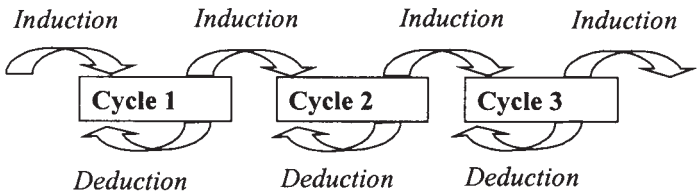
DISCUSSION

One important aspect of this cross-disciplinary effort is to ensure that the research process contains both

– the explorative discovery aspect that stimulates innovation and real-world relevance
– the confirmative testing aspect that satisfies "the hypothetic-deductive method" to guard against false interpretations and wishful thinking
– the mechanistic detailing aspects that can provide causal insight.

One way to overview this work is outlined in Figure 14. The research is considered in more or less formal research cycles. Each of these cycles convert

The cyclic research process:



Data-related elements in one multivariate research cycle:

1. Define purpose, scope and working hypothese from previous cycles.
2. Plan experiment: choice of samples (objects), choice of measurement types (variables)
3. Experimental work: Controlled measuring conditions, sufficient full biological replication, random sample order
4. Handling of raw data: Look for gross errors, average over parallels, preprocess to linearize and to separate e.g., chemical from physical effects and noise, organize data tables with good naming of samples and variables
5. Data modelling: extract main features, test prior hypotheses against errors, look for unexpected clusters and co-variation patterns, look for serious errors and re-analyse without them
6. Demonstrate main results in raw data. Draw conclusions for next selection cycle: identify need for more data, form new hypotheses

Figure 14. Research progress in cross-disciplinary studies: Repeating the research cycle of explorative induction and confirmative deduction

inductively formulated ideas from previous cycles into deductively tested conclusions plus new inductive discoveries. The data-modelling stages within each stage are also outlined in the Figure. In this framework there is a need for all the above-mentioned scientific cultures.

The integrative genetic approach, which includes molecular genomics, high-definition phenotype screening as well end-use quality relevance, is rather generic. For instance, not only the bovine or caprine animal genome and its relationship to milk quality is of interest in this context; the same concepts and methods may also be applied to the feed plants as well as to the intestinal microflora of the animals - to reveal their interactions.

So, why do we try to form this concerted cross-disciplinary environment in milk and milk-related research? It is because we hope to gain more insight and faster research progress by cooperating across scientific traditions. Our campus is large enough to have many types of milk-related expertises, but small enough to foster cooperation synergy. Once an interesting set of samples has been acquired for one given type of measurement, the cost of adding e.g., biospectroscopic fingerprinting is usually small, while the benefit of the resulting added insight can be substantial.

For instance, in animal feeding experiments, unintended genetic differences between animals usually represent a source of unfortunate "biological variation" that creates "random errors". With the present approach they can be characterized as genetic variations that can be identified and compensated for, in the experimental design phase and/or during the subsequent data analysis, thereby increasing the statistical power of the feeding experiments. Genotype x environment interactions may even be revealed. The same goes for dairy science experiments concerning e.g., milk quality. Conversely, in genetic studies, unintended environmental variations in a given phenotype due to e.g., farming practice can be picked up by other phenotypic measurements and corrected for statistically, making the subsequent genetic data modelling more precise - possibly with fewer animals. Finally, the explosive development in available molecular genomics is expected to provide causal insight into both genomic and environmental basis for milk production and milk quality. However, these are early days, and only time will show if the cross-disciplinary platform will be worth while.

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Approximate non-destructive quantification of porphyrins in butter by front face fluorescence spectroscopy*

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ABSTRACT

Dairy products contain natural occurrences of porphyrins, which are active and very effective photosensitizers. The origin and formation of these tetrapyrrols are yet unclear, and the concentration levels are not known. In this study, the approximate concentrations of protoporphyrin IX and haematoporphyrin in butter were determined by the use of non-destructive front face fluorescence spectroscopy. The concentrations for both compounds were in the region 0.02-0.03 ppm.

KEY WORDS: porphyrins, butter, fluorescence spectroscopy, photooxidation

INTRODUCTION

Dairy products are in general susceptible to photooxidation due to natural contents of photosensitizers. To develop storage conditions providing maximal protection, it is necessary to have detailed knowledge of the presence and properties of the photosensitizers in the product (Skibsted, 2000).

Riboflavin has been regarded as the active photosensitizer in dairy products (Borlet et al., 2001). Recent results, however, have demonstrated that dairy products have natural contents of effective photosensitizers acting in the visible spectrum, such as protoporphyrin and chlorophyll-like molecules (Wold et al., 2005). This means that photooxidation in dairy products is caused not only by radiation in the UV - blue region, but also by light in the green-red region.

Presently, we assume that there are at least six different light sensitizers present in dairy products: riboflavin, protoporphyrin, haematoporphyrin, a chlorophyll

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a-like derivative, and two unidentified tetrapyrroles, probably porphyrins (Wold et al., 2006). The origin and formation of these tetrapyrroles are yet unclear, and the concentration levels are not known. We do, however, know that the concentrations vary from product to product and between different production batches of the same product.

The natural occurrence of porphyrins and chlorins in dairy products was discovered by the use of front face fluorescence spectroscopy. This method enables rapid, non-destructive and simultaneous measurement of riboflavin, porphyrins, and chlorophylls. In this study we have used this method to perform an approximate quantification of protoporphyrin and haematoporphyrin in butter.

MATERIAL AND METHODS

Materials

Packages of 500 g from the same batch of dairy butter (Tine Smør) were obtained from TINE BA, Norway. Butter directly from the cooler was placed in glass beakers and weighed to batches of 80 g. Before adding the pure chemicals the butter was tempered to approximately 25°C. At this temperature the butter got a smooth consistence and was easy to stir by hand.

The pure chemicals protoporphyrin IX ($C_{34}H_{34}N_4O_4$, 95%, Sigma CAS 553-12-8) and haematoporphyrin ($C_{34}H_{38}N_4O_6$, 50%, Sigma CAS 14459-29-1) were obtained from Sigma Aldrich Chemie GmbH (Steinheim, Germany).

Design, treatments and measurements

The pure compounds were weighed out in small aluminium vessels by use of a precision balance with readability of 0.0001 g. Two stock butters containing each of the two compounds were made, and held the concentrations 19 ppm and 11 ppm for protoporphyrin IX and haematoporphyrin, respectively. The appropriate amount of stock butter was added to the 80 g butter batches to obtain a reasonable span in the added concentrations, typically from about 0.05 to 0.4 ppm. The stock butter was hand stirred into the butter. Stirring was stopped when no visual particles of the added compound were left. The result was a smooth paste with even colour. Each sample was put in a cuvette, cooled to 4°C and stored in the dark before fluorescence measurements.

Front face fluorescence emission spectra in the range 500-730 nm for excitation wavelength 380 nm, were measured directly on the surface of the butter samples. An optical bench system was used, previously described by Wold et al. (2005). Two parallels of each sample were measured.

Data analysis

A fluorescence spectrum from a butter sample without added compounds were subtracted from the spectra from spiked samples. The peak values for protoporphyrin and haematoporphyrin were then measured at the wavlengths 635 and 620 nm, respectively, for each concentration. A linear regression between these peak values and the known concentrations were made by least squares regression. Based on the regression line, the original intrinsic concentrations could be estimated.

RESULTS

The actual concentrations of added compounds were 0.047, 0.10, 0.21, and 0.41 ppm for protoporphyrin IX, and 0.025, 0.05, 0.10, 0.20, 0.30, and 0.41 for haematoporphyrin.

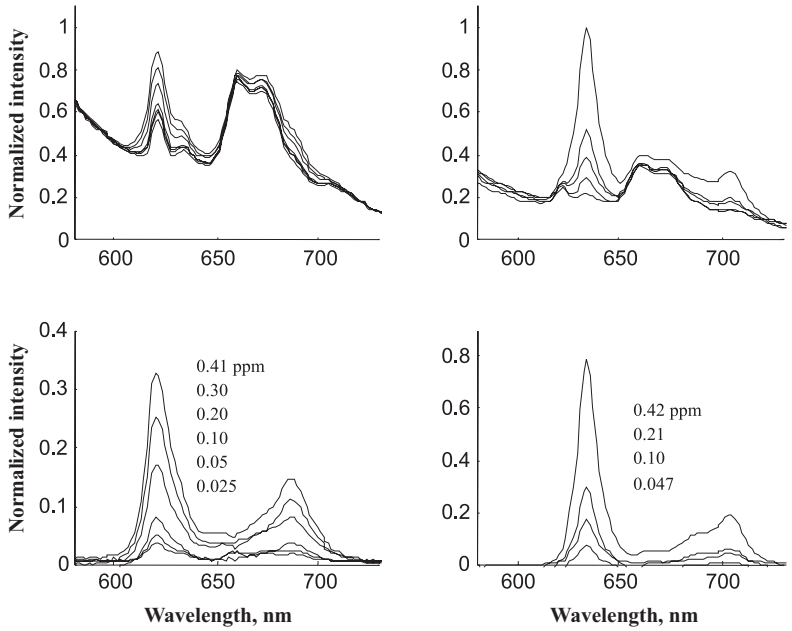

Figure 1. Upper panel: Fluorescence spectra from haematoporphyrin (left) and protoporphyrin (right) spiked samples and butter reference. Under: Spectra after subtraction of butter reference

Figure 1 shows fluorescence spectra of pure butter, and butter spiked with either protoporphyrin IX or haematoporphyrin. Spectra after subtraction of the spectrum from pure butter are also shown, and resemble spectra from the pure compounds.

A systematic increase in peak height was observed with increasing concentration. Note that the original concentrations of the compounds were considerably less than the added concentrations.

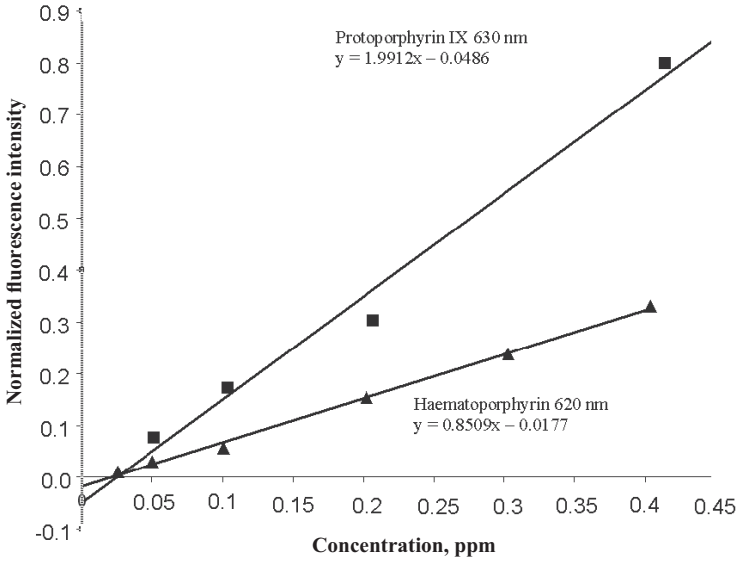

Figure 2. Regression lines for concentrations versus fluorescence intensity

Figure 2 shows regression lines obtained for protoporphyrin IX and haematoporphyrin. These lines intersect the x-axis at 0.024 ppm and at 0.020 ppm for protoporphyrin IX and haematoporphyrin, respectively. These intersections indicate the natural contents of these compounds.

DISCUSSION

The results indicate that the concentrations of protoporphyrin IX and haematoporphyrin in butter are in the 0.02-0.03 ppm region. The exact concentrations in butter vary from batch to batch, and probably from season to season. There may be errors related to the experimental procedures. Thus, the blends were probably not perfectly homogeneous, and some inaccuracies in weighting and measurement may have occurred. Previous fluorescence measurements (Wold et al., 2005) suggest that butter has higher concentrations of porphyrins than other dairy products, such as milk, cheese and sour cream. It is noteworthy that photosensitizers in such low concentrations seem to have a major impact on photooxidation of butter and cheese, as shown by Wold et al. (2005, 2006).

CONCLUSIONS

The concentrations of protoporphyrin IX and haematoporphyrin in butter are in the range of 0.02-0.03 ppm. In spite of these low concentrations, they can be quantified rapidly and non-destructively by front face fluorescence spectroscopy, and may have impact on the photostability of the product.

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Genomic mapping of non-coagulation of milk in the Finnish Ayrshire

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ABSTRACT

The objective of this study was to identify chromosomes affecting non-coagulation of milk in the Finnish Ayrshire. All autosomes were analysed using two pools of DNA samples. Samples from cows milking non-coagulating (NC) and well-coagulating (E) milk were pooled. Pools included 33 NC- and 49 E-animals and they were genotyped using 184 microsatellite markers. Two candidate chromosomes, 24 and 28, were selected based on differences between pools. Chromosomes 24 and 28 were studied using 14 microsatellite markers, individual genotyping and daughter design. Non-parametric interval mapping involved eighteen sire families with a total of 480 daughters. In interval mapping, chromosome 24 associated to NC-milk in one family.

KEY WORDS: milk coagulation traits, non-coagulation of milk, genetics, DNA markers, curd firmness, cheese yield

INTRODUCTION

The average percentage of milk used in cheese production is approximately 50% in the European Union when calculated from production statistics using a 10/L milk to cheese yield ratio (Eurostat, 2006). The high percentage of milk used in cheese production suggests that in many European countries the improvement of raw milk for cheese making should be economically well-motivated. In addition to technological improvements in cheese industry, breeding and management of dairy cattle provide important tools to improve quality of milk for cheese making.

Milk coagulation properties (MCP) are known to play important role in cheese production (Ikonen, 2000; Lucey, 2002). Milk coagulation is dependent on environmental and genetic factors of dairy cows (Ikonen, 2000). In addition,

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technological factors in cheese making affect coagulation (Lucey, 2002; Nájera et al., 2003).

Our studies have shown that in the Finnish Holstein-Friesian and in the Finnish Ayrshire about 12 and 30% of cows produce poorly coagulating milk (Tyrisevä et al., 2004), and about 1% of Holstein-Friesian and 10% of Ayrshire cows produce non-coagulating (NC) milk (Ikonen, 2000; Ikonen et al., 2004; Tyrisevä et al., 2004). Bittante et al. (2002) collected 6909 measurements of MCP from 517 Italian Friesian cows and observed that 19% of measurements were from NC-milk. Four Estonian dairy cattle breeds were studied by Kübarsepp et al. (2005) using 2161 milk samples from 87 cows. From the studied samples 103 samples (4.8%) were NC-milk. A study by Wedholm et al. (2006) analysed coagulation properties of milk collected from 134 cows from the Swedish Red-and-White, the Swedish Holstein and the Danish Holstein-Friesian. More than 30% of cows milked poorly or non-coagulating milk (Wedholm et al., 2006). These studies suggest that the prevalence of poorly and non-coagulating milk should have significant economic outcome in cheese production.

In this study, our main aim was to analyse two candidate chromosomes, 24 and 28, affecting non-coagulation of milk in the Finnish Ayrshire. The genomic mapping in the rest of the chromosomes is in progress and our long-term objective is to characterize chromosomal regions and major genes underlying non-coagulating milk.

MATERIAL AND METHODS

We measured MCP from 4664 cows sired by 91 sires. One milk sample was collected per cow and milk coagulation time (R), curd firming time (K_{20}) and curd firmness (E_{30}) were measured with computerized renneting meter as described by Ikonen et al. (2004).

Eighteen out of 91 sire families were selected for genomic mapping based on phenotypic distribution of MCP among daughters. Selected sires were expected to be heterozygotes for gene(s) underlying NC-milk because each sire had daughters producing both NC-milk and well-coagulating (E) milk. We pooled DNA samples of cows with NC- and E-milk using all families which had DNA extracted from blood samples. Pools included 17 sires and consisted of 33 NC- and 49 E-daughters. Average number of cows per sire was 1.9 and 2.9 daughters for NC- and E-pools, respectively. Using the two pools, all autosomes were analysed with 184 highly polymorphic microsatellite markers. The average number of markers per chromosome was six and the length of intervals between markers varied from 10 to 15 cM.

Based on differences between pools, we selected the two most promising chromosomes for genotyping in individual cows. Chromosomes 24 and 28 were

studied using 14 microsatellite markers in daughter design with 18 sires and 480 daughters. Eighteen families consisted of 193 NC-cows and 287 E-cows (Table 1). Number of daughters in each family varied from 3 to 39 and 8 to 33 in NC- and E-groups (Table 1).

Table 1. The precorrected E_{30} values[1] for all studied sire families grouped by daughters milking non-coagulating and well-coagulating milk

Family	Sire	Non-coagulating (NC)					Well-coagulating (E)				
		n	mean	min	max	Std	n	mean	min	max	Std
1.	Vakio	39	-21.5	-29.8	-14.4	3.8	33	11.9	1.3	21.2	4.4
2.	Vara	2	-25.7	-30.9	-20.5	7.4	27	12.2	1.2	26.8	6.1
3.	Vihtori	17	-22.5	-28.5	-14.3	3.5	16	11.6	1.0	29.7	6.4
4.	Uklaus	13	-24.1	-30.2	-19.7	3.1	17	10.8	0.2	19.3	5.3
5.	Uru	14	-22.2	-31.3	-16.4	4.0	18	12.6	4.8	18.7	3.7
6.	Ugri	5	-24.9	-30.1	-21.1	3.8	16	12.6	3.8	21.7	5.1
7.	Una	11	-21.8	-29.4	-15.2	4.7	16	13.9	5.0	23.3	4.6
8.	Ulaus	8	-21.0	-25.2	-16.1	4.0	9	12.4	6.5	19.6	4.4
9.	Unssi	3	-24.6	-26.1	-21.8	2.4	19	10.0	1.5	19.1	4.4
10.	Upuli	7	-22.8	-27.1	-19.9	2.6	11	7.7	2.5	12.1	3.2
11.	Uuttera	6	-23.7	-30.4	-18.0	4.4	8	12.2	8.0	16.9	3.6
12.	Uisti	10	-23.0	-31.4	-18.6	4.2	17	12.1	5.4	24.6	4.6
13.	Urhea	11	-25.7	-30.8	-0.2	3.3	11	8.0	3.0	15.9	4.9
14.	Uranus	9	-24.0	-28.7	-17.4	3.5	13	10.3	3.1	21.1	4.9
15.	Tunnus	13	-22.8	-30.9	-13.3	4.7	15	8.4	2.1	19.2	4.8
16.	Solttu	4	-20.5	-28.0	-16.7	5.3	12	10.1	1.6	19.7	4.7
17.	Sila	14	-21.3	-28.7	-13.8	4.8	19	11.3	2.6	20.1	4.8
18.	Runsas	7	-22.6	-25.9	-16.4	3.2	10	14.8	8.3	21.2	3.9
Over all families		193	-22.6	-31.4	-13.3	4.0	287	11.4	-0.2	29.7	5.0

[1] E_{30} values are determined as curd firmness 30 min after addition of the clotting enzyme as described by Ikonen et al. (2004)

Curd firmness (E_{30}) was precorrected based on the results from a linear model including parity, lactation stage, measuring unit of a coagulation meter and age of milk sample as fixed effects and herd as a random effect. The precorrected E_{30} values were calculated from the total data set of 4664 cows. The values are given for all studied families in Table 1. Mean and standard deviation (in parenthesis) for precorrected E_{30} values of cows milking NC- and E-milk were -22.6 (4.0) and 11.4 (5.0) over all families in the data set (Table 1). The association between markers and pre-corrected E_{30} values was analysed using nonparametric interval mapping method (Coppieters et al., 1998).

RESULTS

Information contents of microsatellite markers were high (>0.8) along both chromosomes, except on one marker interval on chromosome 28 (0.2-0.8). The most significant association was found in family 4 on chromosome 24 at position 6 cM (Figure 1A). The test statistic (-log(p)=2.23) had the experiment-wise significance level P=0.01 (using Bonferroni correction for two chromosomes). The nearest marker was BM7151. The 90% confidence interval was determined using 1000 bootstraps. According to the confidence interval, the most likely position of the locus (or loci) was from 0 to 14 cM (Figure 1B).

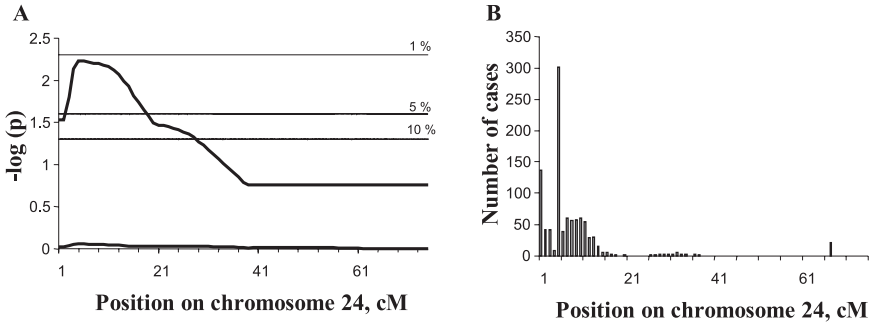

Figure 1. The most significant association between chromomal region and non-coagulation of milk was detected in one family on chromosome 24. A. The test statistic approached 1% significance level in family 4 at position 6 cM (upper curve). The test statistic was nonsignificant in across families analysis (lower curve). B. Based on the 90% confidence interval, the most likely position for a locus (or loci) is from 0 to 14 cM

DISCUSSION

Ikonen (2000) has studied possibilities to genetically improve MCP. Even 40% of the variation in MCP is additive genetic (Ikonen, 2000). This means that it is feasible to reliably estimate breeding values for MCP and improve it through selection of breeding animals. Unfortunately there are technical limitations in MCP measurements (Ikonen, 2000; Lucey, 2002) and currently it is not economically feasible to determine MCP as a part of routine milk recording.

Our previous studies have suggested that poorly coagulating milk and non-coagulating milk have different genetic basis (Ikonen, 2000; Ikonen et al., 2004). However, both poorly coagulating milk and non-coagulating milk have negative effects on cheese making properties when present in bulk milk. Because the incidence of cows with NC-milk is rather high in the Finnish Ayrshire, we expect that elimination of NC-milk from bulk milk could have significant positive effect in cheese production.

In this study we detected the first chromosomal region that affects NC-milk in the Finnish Ayrshire. In our on-going genomic mapping we expect to find several other chromosomal regions and finally major genes that are underlying NC-milk. Characterized variation in major genes is the basis for development of DNA tests for detection of cows milking NC-milk and their male relatives. DNA tests should provide effective means to reduce the occurrence of NC-milk.

CONCLUSIONS

We detected a region on chromosome 24 affecting NC-milk in the Finnish Ayrshire. The region harbouring significant effect on NC-milk was found only in one family out of 18 studied sire families. Our current results from genomic mapping of DNA-pools support the view that there might be several genes affecting NC-milk in the Finnish Ayrshire.

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Proteomic study of regressions between milk yield and whey protein composition

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ABSTRACT

A decrease in casein number (i.e. the content of casein in relation to total protein) in milk during the last 30 years, as reported by the Swedish Dairy Association, may be related to an increasing milk production. The objective of this investigation was to study the relations between whey protein composition and daily milk yield using proteomics combined with multivariate data analysis. The results indicated that several individual minor whey proteins increased with increasing milk yield. Some of these proteins could be identified by mass spectrometry, including: fatty acid binding protein, apolipoprotein A1, lactoferrin and endopin. The technique used proved to be a useful tool for identification of significant proteins extracted from large data sets.

KEY WORDS: milk production, whey proteins, PLS, proteomics, 2D gels, MALDI-TOF

INTRODUCTION

The composition of cows' milk is of great importance for the dairy industry. Surveys carried out by the Swedish Dairy Association (Lindmark-Månsson et al., 2003) have documented variations in the casein number, both at farm-tank level and in dairy silo-tank milk. The Swedish investigation showed that the concentration of casein had decreased from 2.61%, in 1976, to 2.56%, in 1996,

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and the concentration of whey proteins has increased from 0.73 to 0.81% during the same period (Lindmark-Månsson et al., 2003). The results implicate a change in the mean casein number from 78 to 76 during a 30-years period, and indicate a change in the proteins in the milk, either in the overall casein and whey protein content, or in a change in the mutual content of individual proteins. One reason for this change could be the increased milk yield, but the detailed background for the change in protein composition is not known.

Proteomics is a tool for the simultaneous determination of the protein composition of complex samples. One of the tools in proteomics is to combine the separation power of two-dimensional gel electrophoresis (2-DGE) with mass spectrometry (MS) for identification of separated protein spots. This method has been applied for the characterization of composition of milk powder (Galvani et al., 2000). Multivariate statistics may be used to search for systematic variation in large data sets. By partial least squares regression (PLS), the relation between the spot data and other characteristics such as treatments or quality measurements can be analysed (Jessen et al., 2002).

The objective of this study was to investigate the relations between milk production and whey protein composition using these tools.

MATERIAL AND METHODS

Milk samples

Evening whole milk samples (10 L) from 44 Danish Holstein Friesian (SDM) cows were collected at the Research Centre Foulum at the Faculty of Agricultural Sciences as described earlier (Wedholm et al., 2006). Daily milk yield in kg at the day of sampling was recorded for each cow. Protein concentration of milk samples was determined by Milkoscan FT120 (Foss Electric). After two days of cold storage (4°C), individual milk samples were skimmed twice at 3000 × *g* for 10 min. The skimmed milk was preheated at 30°C for 30 min, and fractionated into casein and whey fractions by addition of chymosin (2 ml/L of Chy-Max Plus, 190 International Milk Clotting Units/mL, Christian Hansen A/S). The samples were incubated at 30°C for 30 min, centrifuged at 1000 × *g* for 10 min at 5°C, and the whey fraction was recovered.

Two-dimensional gel electrophoresis and image analysis

A 2-D gel set of 44 gels, one for each individual sample in the experiment, was analysed. For analytical gels subjected to image analysis a volume of the whey protein fractions corresponding to 50 µg was applied, whereas for preparative

gels for MS analysis a volume of sample corresponding to 370 µg was applied. The protein concentrations were based on the Milkoscan determinations and a whey protein:casein ratio of 20:80. Running of the 2-DE analyses was carried out essentially as described by Lametsch and Bendixen (2001). The first dimension of protein separation was carried out in immobilized 11 cm IPG strips (pH 4-7, BioRad), with 8-16% gradient Criterion gels (BioRad) in the second dimension. A Dodeca cell (BioRad) was used, allowing the simultaneous separation of 12 gels. Analytical gels were silver stained according to Lametsch and Bendixen (2001), and the preparative gels were stained according to Shevchenko et al. (1996). The gels were analysed using ImageMaster 2D analyser software (Amersham Pharmacia). After the automatic spot detection carried out by the software, the annotated spots were manually edited. Most protein spots assigned were detected in all gels, i.e. were present in all animals, but some spots were missing in some gels. After manual editing, spots missing in some gels were considered not to be the result of technical error, and were assigned a value of zero (Grove et al., 2006). The relative spot volumes were calculated for each separate gel to overcome gel-to-gel variations in spot intensities and sample loadings.

In gel digestion, desalting and concentration of protein spots

Protein spots of significance were subjected to in-gel digestion by addition of trypsin essentially as described by Jensen et al. (1998), and the peptides were desalted and concentrated as described by Lametsch et al. (2002) by elution in 0.5 µL matrix solution (15-20 g/L of α-cyano-4-hydroxycinnamic acid in 70% acetonitrile) directly onto the MALDI target plate (Bruker Daltonics GmBH).

Partial least squares regression analysis

Partial Least Square regression 1 (PLS1) analysis was carried out by explorative data analysis using the software Unscrambler ver. 9.0 (CAMO ASA) to look for interactions between spot variations and the response Y-variable daily milk production in kg for each cow. The relative spot volumes of the whey proteins comprised the continuous X-variables in the PLS models. Standardized (centred: µ=0, and normalized: 1/SD) variables and full cross validation was used. Protein spots contributing little to the model (i.e. small regression coefficients) were removed through variable selection, and based on the remaining spots (33 contributing factors) significant ($P<0.05$) regression coefficients were identified.

Identification of milk proteins by MALDI-TOF MS

Mass spectra were obtained using a Bruker Ultraflex MALDI-TOF tandem mass spectrometer (Bruker Daltonik GmBH) in reflection mode. The ion accelerating voltage was 25 kV with a delay time of 40 ns. The laser frequency was 50 Hz and 200 laser shots were accumulated for each spectrum. Peptides were identified by mass searches in the database Swiss Prot (Swiss Institute of Bioinformatics) using the MS/MS ion search programme Mascot (Matrix Science).

RESULTS

The daily milk yields of the cows in the experiment varied between 11 and 40 kg, with a mean value of 24.0±7.8 kg.

Two-dimensional gel electrophoresis

A typical gel of the separated whey proteins from one of the samples is shown in Figure 1. By this analysis the majority of the whey proteins were separated. Basic whey proteins with a pI above 7 (like e.g., lactoperoxidase) could, however, not be seen at the selected conditions. The positions in this 2-DGE system of some of the major whey proteins, i.e. α-lactalbumin, β-lactoglobulin, bovine serum albumin and proteose peptone component 3 (PP3)/lactophorin were determined by MALDI-TOF MS, and are shown in Figure 1. Image analysis of the 44 whey protein 2-D gels included annotation of 444 individual protein spots.

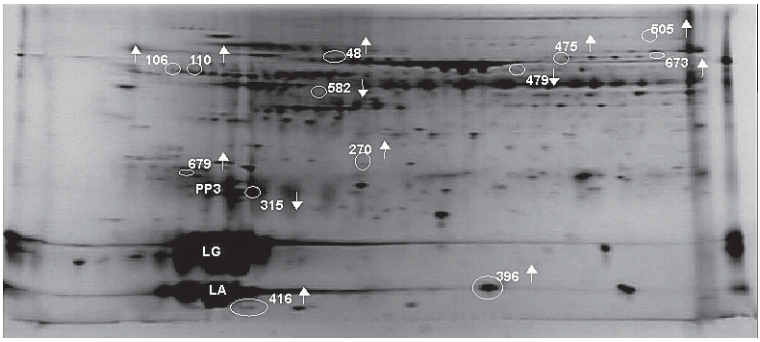

Figure 1. Two-dimensional gel electrophoresis of whey proteins from milk from a single cow in the experiment. The proteins were silver stained. The pH gradient of the first dimension is indicated. The result of the PLS1 analysis is indicated. The ID's of protein spots being positively or negatively associated ($P<0.05$) with daily milk yield are indicated by their number in the image analysis and by a white circle. The positions of the major whey proteins (LA - α-lactalbumin, LG -β-lactoglobulin, PP3 - proteose peptone component 3/lactophorin and BSA: bovine serum albumin) are indicated

PLS1

Result from the PLS1 analysis is presented in Figure 2. It was found that 10 proteins were significantly positively associated with increasing milk yield, whereas 3 proteins were significantly negatively associated with the same trait. These results indicated that more whey proteins were positively associated with high milk production, than negatively associated, and therefore indicate that more whey proteins increased at increasing milk yield. The positions of these spots on the 2D gels are shown in Figure 1. The value of R^2 between measured and predicted Y values in the model was 0.95, indicating a good model.

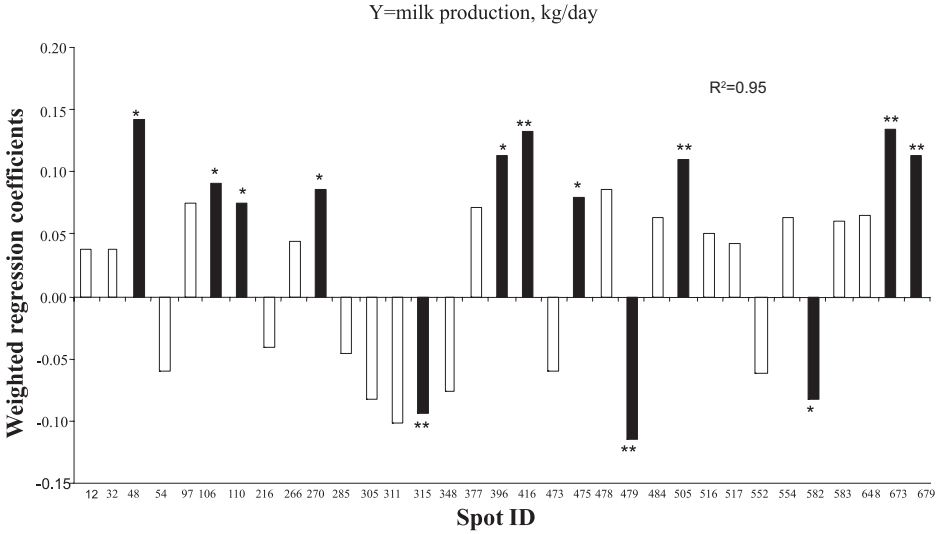

Figure 2. Results from the partial least squares regression analysis presented as the weighted regressions between x-variables of relative spot volumes and the y-variable milk production (kg/day). Black bars represent significant regression coefficients. R^2 is the correlation coefficient between measured and predicted y variables.* P<0.05; ** P<0.01

MALDI-TOF MS identifications

The significant protein spots were excised from preparative gels, and subjected to MALDI-TOF MS. By this analysis the following proteins, which were all positively associated with milk yield, were identified (with their accesskeys in the SwissProt database indicated): spot 48: lactoferrin (P24627); spots 106 and 110: endopin (Q9TTE1), spot 270: apolipoprotein A1 (P15497) and spot 506: fatty acid binding protein (FABP, P10790). It was unfortunately not possible to identify

other significant spots, probably, in part, due to the fact that many of these were minor spots, and therefore not present in substantial amounts sufficient for MS analysis. The three spots negatively associated with milk yield were present in low amounts, and these were not identified.

DISCUSSION

This investigation showed that a large amount of whey proteins could be separated by the gel-based 2-DGE system employed, with a total of 444 individual spots annotated in the image analysis. Of these proteins, many were in a similar position according to molecular mass, but with different pI, lying on a row. This confirms the presence of many post-translational modifications in the whey proteins, e.g., phosporylations and other modification not resulting in significant mass changes.

The results from the PLS1 analysis indicated, that a range of individual whey proteins were positively associated with milk production, i.e. their concentrations were positively associated with milk yield. Some of these proteins were identified by MS, and included lactoferrin, FABP, apolipoprotein A1 and endopin, in addition to other minor whey proteins not identified. FABP participates in the intracellular transport of long-chain fatty acids and their acyl-CoA esters, and in relation to milk in the uptake of preformed fatty acids from feed or body reserves. A possible explanation for the increase in FABP could be linked to an increased output of fatty acids in the milk with increasing milk production. Apolipoprotein A1 is the major protein of high-density lipoprotein (HDL), and participates in cholesterol transport, and therefore also linked to milk fat synthesis. The biological explanation for the increase in lactoferrin and endopin, a proteinase inhibitor, is not known. The increase in concentration of several individual whey proteins, found in this study, could partly explain the decrease in casein number observed along with increasing milk yield, as reported by Lindmark-Månsson et al. (2003).

It should be noted, however, that an experience from this 2-DGE and image analysis, is that the quantification of the large protein spots was difficult to achieve due to the limited dynamic range of the silver staining, and the results therefore should be interpreted according to this caution. A better dynamic range could probably be achieved by the use of fluorescent staining methods for 2-DGE gels, and therefore might result in identification of further major spots associated with milk yield. The investigation demonstrates an application of proteomics integrated with multivariate statistics for the identification of significant protein spots extracted from large datasets.

CONCLUSIONS

The results from the present study showed that several individual whey proteins were positively associated with increasing daily milk yield, whereas a smaller number was negatively associated with the same trait. The proteins that could be identified by MS included two proteins related to milk fat, FABP, apolipoprotein A1, in addition to lactoferrin and endopin.

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