

# Phenotyping of robustness and milk quality

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*A phenotype describes the outcome of the interacting development between the genotype of an individual and its specific environment throughout life. Animal breeding currently exploits large data sets of phenotypic and pedigree information to estimate the genetic merit of animals. Here we describe rapid, low-cost phenomic tools for dairy cattle. We give particular emphasis to infrared spectroscopy of milk because the necessary spectral data are already routinely available on milk samples from individual cows and herds, and therefore the operational cost of implementing such a phenotyping strategy is minimal. The accuracy of predicting milk quality traits from mid-infrared spectroscopy (MIR) analysis of milk, although dependent on the trait under investigation, is particularly promising for differentiating between good and poor-quality dairy products. Many fatty acid concentrations in milk, and in particular saturated fatty acid content, can be very accurately predicted from milk MIR. These results have been confirmed in many international populations. Albeit from only two studied populations investigated in the ROBUSTMILK project, milk MIR analysis also appears to be a reasonable predictor of cow energy balance, a measure of animal robustness; high accuracy of prediction was not expected as the gold standard method of measuring energy balance in those populations was likely to contain error. Because phenotypes predicted from milk MIR are available routinely from milk testing, longitudinal data analyses could be useful to identify animals of superior genetic merit for milk quality and robustness, as well as for monitoring changes in milk quality and robustness because of management, while simultaneously accounting for the genetic merit of the animals. These sources of information can be very valuable input parameters in decision-support tools for both milk producers and processors.*

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## Introduction

A phenotype can be described as the outcome of the interacting development between the genotype of an individual and its specific environment throughout life (Bowman, 1974). Successful breeding programmes, however, require routine access, preferably at a low cost, to accurate phenotypes on which genetic or genomic evaluations can be performed. The resulting information can be used to make selection decisions, thereby progressing genetic gain. Here we describe the state-of-the-art in phenotyping strategies focusing mainly on animal robustness and milk quality, as breeding goals need to be adopted to accommodate these traits. Milk quality in dairy production systems was traditionally synonymous with the concentrations of the macro constituents of milk fat, protein and lactose, as well as somatic cell count and, in the case of bulk milk samples, bacterial counts. However, fat concentration in its entirety is mainly an accumulation of individual fatty acid concentrations, and similarly protein concentration, as traditionally measured, is mainly an accumulation of casein

and whey fractions, which in turn can further be partitioned into their respective individual components. Similar to milk quality, robustness can be decomposed into its individual components including animal health, fertility and energy balance. Being able to phenotype accurately the individual components of robustness and milk quality not only helps to resolve genetic antagonisms between the sub-components through animal breeding, but also facilitates a greater understanding of the physiological mechanisms underlying these complex phenotypes. Particular emphasis in this review will be on the research results generated by the EU-funded ROBUSTMILK project (<http://www.robustmilk.eu>).

## Phenotyping strategies

Phenotypic information can be collected through a series of mechanisms including (1) farmer-scored (e.g. calving difficulty), (2) professionally scored (e.g. linear type traits) or (3) exploitation of technological advancements (e.g. milk recording). Each strategy has its own advantages and disadvantages, and each strategy is more or less compatible with different phenotypes. Farmer-scored traits, for example, have a low associated

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cost of collection but may be more subjectively recorded, thereby possibly leading to deflated heritability estimates. Professionally scored phenotypes may be more consistently scored, but have a relatively high cost of collection and are generally collected only in selected sub-populations. However, technologically derived phenotypes, although requiring an initial capital cost plus ongoing depreciation and operational costs, are generally more objectively measured and provide access to a plethora of information, some of which has yet to be discovered. Infrared spectroscopy analysis of milk is one such technology that is currently used routinely to determine the concentrations of milk fat, protein, urea and lactose.

Spectroscopy is the study of the interaction between matter and radiated energy. Mid-infrared (MIR) spectroscopy focuses on the mid-infrared region of the electromagnetic spectrum (i.e. 4000 to 400  $\text{cm}^{-1}$ ) and can be used to study fundamental vibrations and the associated rotational vibrational structure of a sample. Mid-infrared spectroscopic analysis of milk samples is the method chosen internationally for the quantification of milk composition, predominantly consisting of fat, protein and lactose concentration. However, MIR could be used to quantify a broader range of chemical moieties in milk.

### Milk quality

Bovine milk represents a unique source of nutrients and bioactive components that act in synergy as well as independently, and that can favourably affect or influence human health (Haug *et al.*, 2007). For example, the average milk fat of a dairy cow contains 70% saturated fatty acids, 25% monounsaturated fatty acids and 5% polyunsaturated fatty acids (Grummer, 1991). Currently, dairy products provide 15% to 25% of the fat consumption in the average human diet, but represent 25% to 35% of the saturated fat intake (Chillard *et al.*, 2001). Hence, tools to help alter the fatty acid profile of bovine milk, for example, would be very beneficial. Furthermore, tools to improve the yield and shelf-life of products as well as other quality measures will also be extremely beneficial.

To date, though, routine measurements of milk quality have generally been confined to fat, protein, urea and lactose concentrations, with some organisations also generating information on casein fractions. Producers in most milk payment systems are financially rewarded to produce milk of better quality. The tools, however, to provide cheap and routine information on more detailed measures of milk quality (e.g. individual fatty acid profile) for incorporation into breeding programmes, payment systems and herd management decisions are not available. Therefore, accurate low-cost methods of quantifying a range of detailed milk quality parameters are required. Because milk samples are routinely available through milk recording and bulk tank sampling, all of which are subjected to MIR analysis, attempting to relate the generated MIR spectrum to various milk quality parameters is one opportunity to generate low-cost phenotypes.

Milk MIR has, to date, been proven to predict accurately some milk fatty acid concentrations, especially groups of fatty

acids (Rutten *et al.*, 2009; Soyeurt *et al.*, 2011; Maurice-Van Eijndhoven *et al.*, 2013). As part of the ROBUSTMILK project, Soyeurt *et al.* (2011), using several different statistical approaches to derive MIR prediction equations for milk fatty acids, reported that 34% to 98% of the variation in individual and groups of fatty acids in an external validation data set ( $n = 250$ ) could be explained by the prediction equations; the average proportion of variation of the individual and groups of fatty acids explained was 0.77. Calibrated using a larger data set ( $n = 1236$ ), including the samples by Soyeurt *et al.* (2011), and chosen based on maximising the MIR spectral variability, Maurice-van Eijndhoven *et al.* (2013) reported that across breed, between 43% and 99% of the variation in individual and groups of fatty acids in milk, could be predicted from the MIR. A similar accuracy of predicting fatty acid composition was reported elsewhere (Rutten *et al.*, 2009). In general, the accuracy of predicting groups of fatty acids was greater than the accuracy of predicting individual fatty acids (Rutten *et al.*, 2009; Soyeurt *et al.*, 2011; Maurice-van Eijndhoven *et al.*, 2013). Saturated fatty acid content in milk could be predicted from MIR, across populations, with accuracy greater than 95% (Rutten *et al.*, 2009; Soyeurt *et al.*, 2011; Maurice-van Eijndhoven *et al.*, 2013).

Lactoferrin, a glycoprotein present naturally in milk, is known to have antibacterial and antifungal effects and also seems to have a role in host defense mechanisms and the modulation of inflammatory processes (Baker, 2005). Increasing lactoferrin content in milk can therefore be potentially useful in host defenses against mastitis and improving the nutritional quality of the milk. Soyeurt *et al.* (2012) documented that 60% of the variation in milk lactoferrin content in an external validation data set ( $n = 274$ ) could be explained by an MIR prediction equation developed using 2499 samples. Predicting lactoferrin content from milk MIR was therefore not accurate as reported for some fatty acids. The ratio of the standard error of prediction to the standard deviation (i.e. RPD) of the ELISA-measured lactoferrin content was 1.86 (Soyeurt *et al.*, 2012). An RPD of  $>3$  is said to be useful for implementation (Williams and Norris, 2001). Therefore, the RPD observed for lactoferrin content was low compared with, for example, 15.7 calculated for saturated fatty acid content, and is currently not deemed suitable for implementation on an individual animal basis (Soyeurt *et al.*, 2011).

Milk MIR analysis has also been documented in other international research to have some, albeit lower, predictive ability for other milk quality attributes including milk coagulation properties (Dal Zotto *et al.*, 2008; De Marchi *et al.*, 2009), mineral content (Soyeurt *et al.*, 2009) and milk protein composition (Bonfatti *et al.*, 2011; Rutten *et al.*, 2011). Dal Zotto *et al.* (2008) using information on milk rennet coagulation time from Holstein-Friesian cows reported RPD values of 1.17 to 1.91; the RPD values for curd firmness varied from 1.19 to 1.33. Soyeurt *et al.* (2009) generated MIR equations to estimate the various mineral contents in milk. They reported RPD values of 2.74 for calcium and 2.74 for phosphorus. Bonfatti *et al.* (2011) reported

RPD values ranging from 1.04 for milk  $\gamma$ -casein percentage in protein to 2.07 for casein content in milk; Rutten *et al.* (2011) obtained RPD values lower than 1.14 for a series of different measures of milk protein composition. Although the RPD values reported for these additional measures of milk quality are all less than the recommended threshold of 3 (Williams and Norris, 2001), expanding the calibration data set to increase the variability present may improve the prediction accuracy sufficiently to justify implementation.

### Robustness

Robustness has previously been defined as the ability of an animal to minimise the extent and duration of energy balance (Berry *et al.*, 2009), where energy balance describes the difference between energy intake and energy expenditure. Dairy cows, like all mammals, are generally in negative energy balance (i.e. energy intake is less than energy expenditure) in early lactation (Berry *et al.*, 2006; Friggens *et al.*, 2007a). Both the extent and the duration of this negative energy balance is known to be associated with animal health and fertility (Beam and Butler, 1999). Associations between energy balance and animal health and fertility also exist at the genetic level (Collard *et al.*, 2000; Veerkamp *et al.*, 2000). Animal health and fertility recorded by individual producers can also provide valuable information on the robustness of the animal. However, producers generally observe only health issues once cows are in the clinical diseased state, and it is difficult to identify animals falling ill sufficiently early before the event to implement remedial action without a compromise in performance. Furthermore, the heritability of animal health (Berry *et al.*, 2011) and fertility (Veerkamp and Beerda, 2007) is low, necessitating a greater quantity of information to generate accurate estimates of genetic merit for these traits within breeding programmes. Therefore, a measurement of energy balance would be appealing as a proxy for general robustness.

Accurate assessment of energy balance can only be achieved in calorimeter chambers, although predictions can be obtained if feed intake measures and measurements of the main energy sinks such as milk production, live weight and body tissue reserves (i.e. proxy for maintenance requirements) are available. However, routine measurement of feed intake on a sufficiently large number of animals necessary for genetic evaluations is prohibitively expensive, and therefore an easy-to-use, low-cost predictor of feed intake or energy status itself would be valuable for day-to-day farm management and animal breeding programmes. Several predictors of energy balance have been suggested including body condition score change (Roche *et al.*, 2009), the ratio of fat to protein in milk (Grieve *et al.*, 1986; Heuer *et al.*, 2000; Friggens *et al.*, 2007b) and the presence of ketone bodies (De Roos *et al.*, 2007). Body condition score change, however, requires assessment by trained assessors. Alternatives such as using computerised image analysis techniques of graphical representations of cow body condition (Roche *et al.*, 2009) or a central unit for human assessment of

images of cow body condition (Ferguson *et al.*, 2006) are one option; however, these options could be expensive. Milk fat to protein ratio, although available routinely from all milk samples, has failed to consistently reflect energy balance at an acceptable level of accuracy for use in individual cow management (Grieve *et al.*, 1986; Heuer *et al.*, 2000; Friggens *et al.*, 2007b). Accurate quantification of ketone bodies can be time-consuming and therefore expensive.

There are several plausible reasons as to why MIR analysis of milk might be able to predict energy balance. Catabolism of adipose fatty acids during body condition score change results in an increase of C18 fatty acids concentration in milk and a consequent inhibition of *de novo* synthesis of fatty acids by the mammary gland (Palmquist *et al.*, 1993; Barber *et al.*, 1997). Previous research (Rutten *et al.*, 2009; Soyeurt *et al.*, 2011; Maurice-van Eijndhoven *et al.*, 2013) clearly documented the ability of MIR analysis of milk to predict the concentration of several milk fatty acids, thereby reflecting body condition score, and subsequently energy balance or robustness provided that the appropriate phenotypic relationships can be established and are reliable enough to be useful. Milk fat and protein concentrations used in the calculation of fat to protein ratio for predicting energy balance are also derived routinely from MIR information. Several studies have attempted to relate infrared spectroscopy analysis in milk to the presence of ketone bodies including acetone, acetoacetate and  $\beta$ -hydroxybutyrate (Hansen, 1999; Heuer *et al.*, 2001; de Roos *et al.*, 2007) as a predictor of ketosis. Milk samples used were either based on raw milk samples from cows (Hansen, 1999; Heuer *et al.*, 2001; de Roos *et al.*, 2007) or samples fortified with acetone (Hansen, 1999). The studies showed that infrared spectroscopy analysis of milk could be used as a screening tool for ketosis, although, on the basis of the current state of the art, Hansen (1999) suggested that the true acetone content of selected samples should be confirmed with a more accurate method than infrared spectroscopy.

These observations provide the basis for presuming that milk MIR may be helpful in providing indirect assessments of energy balance or robustness. This hypothesis was explored as part of the ROBUSTMILK project (McParland *et al.*, 2011 and 2012). Using a data set of up to 3833 test-day records, with measures of both energy status and milk MIR from cows in both confinement and grazing production systems, McParland *et al.* (2012) reported an accuracy of predicting energy status of up to 0.69 using milk MIR plus milk yield. The accuracy of predicting energy intake in the same data set was 0.80. Feed intake was measured using an automated feed intake recording system indoors in Scotland, whereas grass dry matter intake in the Irish grazing cows in that study was predicted using the n-alkane method. The two energy status traits evaluated were energy balance, calculated from dry matter intake, milk energy produced and live weight, and energy content predicted from body condition score and BW (Banos and Coffey, 2010). McParland *et al.* (2011) using a smaller data set documented an inability of milk MIR to predict animal body condition score. However, McParland *et al.* (2011 and 2012)

conceded that a high accuracy of prediction of energy status was not expected because, unlike many gold standard measures of milk quality such as fatty acid composition, errors in recording (e.g. food wastage) as well as the subjectivity of assessment of some traits (e.g. body condition score) is likely to contribute random variation to the estimate of energy balance rendering it itself less accurate.

### Application of phenomics in day-to-day farm management

Phenotypic information can contribute to accurate genetic evaluations on which to make selection decisions and increase genetic gain, and subsequently profitability where traits are economically beneficial. However, phenotypic information also has many uses in day-to-day farm management. Milk recording is used by many producers not only to identify low-producing cows for culling, but also to alert the producer to a possible deterioration in udder health at the individual cow level and also at the herd level. Having access to longitudinal data increases the power of the alert system. Milk processors also use information on milk composition for making payments; in the future, retailers may also incentivise more welfare-friendly production systems where animals are less stressed or under less extreme energy balance. Therefore, milk samples collected routinely on an individual cow or whole-herd basis provide a readily usable potential biological source of information, and because each sample is already routinely subjected to MIR spectroscopy analysis, exploiting the generated spectral information to yield additional information is logical.

To exploit these potential opportunities, the variation in the phenotype being predicted from the MIR must be represented in the calibration data set that is used to derive the prediction equations. Therefore, this involves collection of both MIR and the gold standard phenotype from a diverse range of breeds and production systems, as, for example, was done by Soyeurt *et al.* (2011) in the development of MIR prediction equations for milk fatty acid content within the ROBUSTMILK project. Before the use of a prediction equation derived from MIR (or any other regression model), the similarities between the values of the predictor variables and those in the calibration data set should be assessed (e.g. principal component analysis). Extrapolation beyond the parameters space of the calibration data set should be undertaken with caution, and preferably avoided. More importantly, however, the gold standard measure of the 'outlier' sample should be determined where possible and subsequently added to the calibration data set; this will add statistical power to the prediction equations.

Simply providing raw phenotypic means of predicted traits at a given time point to producers may not be optimal to aid in day-to-day herd management. First, genetic and systematic environmental factors influence most phenotypes including milk quality (Cassandro *et al.*, 2008; Arnould and Soyeurt, 2009; Soyeurt *et al.*, 2010) and robustness (Coffey *et al.*, 2003b; Berry *et al.*, 2007) and these differ

between herds, thereby contributing to phenotypic differences, which may not necessarily reflect herd management. Second, examination of longitudinal data over time can identify trends. As a consequence, producers, through decision-support tools can evaluate the impact of recent management changes. Herd-level solutions from random regression test-day models, which have been adjusted for genetic and systematic environmental effects, can be a useful decision-support tool as well as generating estimates of genetic merit for animals. For example, Bastin *et al.* (2009) applied a test-day model to milk urea content to derive herd-level profiles, which could be subsequently used by producers to aid in feeding management.

### Phenotypes of the future

The methods for measuring phenotypes of the future should ideally be (1) non-destructive and not affect animal performance, (2) low cost, (3) measured in real-time and (4) measurable temporally. Infrared spectroscopy fits most of these requirements; currently, MIR spectroscopy is undertaken in milk laboratories and results are therefore not immediately returned to the producer during the milking process. However, in-line methods of infrared spectroscopy for predicting milk quality, exploiting mainly the visible or near-infrared regions of the electromagnetic spectrum are showing promise (Nguyen *et al.*, 2011; Melfsen *et al.*, 2012), especially for the major milk constituents. Studies on the in-line prediction of animal robustness from infrared analysis of milk are lacking. Nonetheless, other alternative phenotyping strategies for milk quality and, in particular, animal robustness exist. The latter include automatic image analysis (including infrared tomography) of animals for the assessment of body tissue reserves (Coffey *et al.*, 2003a) or udder health (Polat *et al.*, 2010) as well as sensor technology (Ipema *et al.*, 2008). Arguably, one of the greatest challenges of the future will be the derivation of efficient and effective algorithms to exploit the plethora of phenotypes that are being generated for multiple applications. Such approaches could be converted, in conjunction with herd-level information and individual animal genomic or other omic information, into useable and readily understandable information that the producer can use to maximise performance and herd profitability.

### Conclusions

Research undertaken as part of the ROBUSTMILK project, in combination with our interpretation of the literature, signifies that MIR analysis of milk has the potential to enhance phenotyping for milk quality. The current state of knowledge is such that traits such as milk coagulation properties, mineral content and milk protein composition cannot currently be predicted with sufficient accuracy to be useful. Expansion of the calibration data sets to include more variability is required to generate more robust prediction equations. However, estimation of some fatty acid content in milk, especially saturated fatty acid content, is already at a level of accuracy where

consideration might be given to including this approach as part of an overall breeding policy. Collection, storage and translation of MIR data from spectrometers to milk fatty acid is required to generate accurate estimates of breeding values for these milk quality traits. Prediction of animal energy balance from MIR is at a very early stage of research but is nonetheless showing promise. Large data sets with increased variability in energy balance are required, and the measures of energy balance should ideally have minimal error, thereby suggesting energy balance measures from calorimetry studies.

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For more information, please visit the website [www.robustmilk.eu](http://www.robustmilk.eu), read the other papers in these proceedings or contact the project coordinator: Roel F. Veerkamp (E-mail: [Roel.Veerkamp@wur.nl](mailto:Roel.Veerkamp@wur.nl))

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