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A proteomics-based identification of putative biomarkers for disease in bovine milk

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ABSTRACT

The objective of this study was to identify and characterize potential biomarkers for disease resistance in bovine milk that can be used to indicate dairy cows at risk to develop future health problems. We selected high- and low-resistant cows i.e. cows that were less or more prone to develop diseases according to farmers' experience and notifications in the disease registration data. The protein composition of milk serum samples of these high- and low-resistant cows were compared using NanoLC–MS/MS. In total 78 proteins were identified and quantified of which 13 were significantly more abundant in low-resistant cows than high-resistant cows. Quantification of one of these proteins, lactoferrin (LF), by ELISA in a new and much larger set of full fat milk samples confirmed higher LF levels in low- *versus* high-resistant cows. These high- and low-resistant cows were selected based on comprehensive disease registration and milk recording data, and absence of disease for at least 4 weeks. Relating the experienced diseases to LF levels in milk showed that lameness was associated with higher LF levels in milk. Analysis of the prognostic value of LF showed that low-resistant cows with higher LF levels in milk had a higher risk of being culled within one year after testing than high-resistant cows. In conclusion, LF in milk are higher in low-resistant cows, are associated with lameness and may be a prognostic marker for risk of premature culling.

1. Introduction

The objective of this study was to identify biomarkers for disease resistance in bovine milk, thereby providing a prognostic tool to indicate dairy cows at risk to develop future health problems. The last decades dairy farming in the Netherlands has changed enormously and the number of cows per farm increased with 40% during the last 10 years (CRV, 2015). Clinical mastitis, one of the major health problems in dairy farming, has an incidence of about 33 cases per 100 cows annually (Santman-Berends et al., 2015) with associated annual costs of approximately €61 to €97 per cow based on worldwide estimations (Hogeveen et al., 2011). Also fertility problems and lameness are important issues in dairy farming (Huxley, 2013; Weaveret al., 2007). About 75% of the diseases in dairy cows occur in the first month after calving (LeBlanc et al., 2006). Around parturition, the immune system is compromised and the feed intake does not meet the energy requirements of the cow resulting in a negative energy balance (NEB), which makes the cow susceptible for diseases (Ingvartsen and Moyes, 2013; LeBlanc et al., 2006; van Knegsel et al., 2007).

To monitor the health status of cows, several studies were performed to obtain specific biomarkers. For example the energy balance, and thereby the risk of developing disease, can be measured by the levels of not-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA) in blood (Ospina et al., 2010). Pre-partum NEFA serum levels were shown to be positively correlated with the risk of mastitis after parturition (Holtenius et al., 2004; Moyes et al., 2009b). High post-partum NEFA levels are also a predictor for clinical ketosis, retained placenta, metritis and displaced abomasum (Ospina et al., 2010). Acute phase proteins (APP) in cows, like haptoglobin and serum amyloid, are common markers for infection and inflammation (Ceciliani et al., 2012; Eckersall and Bell,

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2010; Eckersall et al., 2006). Haptoglobin and mammary-associated serum amyloid A (M-SAA3) were consistently increased in milk and subsequently in blood after a *Staphylococcus aureus*-induced subclinical mastitis (Eckersall et al., 2001; Eckersall et al., 2006). In milk, an increase in somatic cell counts (SCC) or lactate dehydrogenase (LDH) are markers for mastitis (Åkerstedt et al., 2011; Hiss et al., 2007) and are now routinely tested. Furthermore, ketosis can also be determined in milk by the rise in BHBA levels.

The risk for development of important diseases in dairy cattle can thus be monitored by the levels of some of these markers, which are used for regular screening in dairy farming already. A regularly used marker like SCC is specifically related to detection of mastitis, but does not indicate other diseases. Therefore, we are aiming for prognostic markers in bovine milk that are related to diseases different than mastitis. Markers in milk are preferred since milk samples are already collected regularly for routine screening, in contrast to blood samples. Nowadays, hundreds of unique proteins can be identified in different fractions of bovine milk by mass spectrometry (Hettinga et al., 2011; Nissen et al., 2013). This makes a proteomics approach a valuable tool for discovery of novel biomarkers (Boehmer et al., 2010; Ferreira et al., 2013). Here, we use shotgun proteomics (NanoLC–MS/MS) to compare milk samples of cows with a good health history (high-resistant cows) to milk samples of cows with a poorer health history (low-resistant cows). In this study, we consider high-resistant cows as having a low susceptibility to the development of disease. Likewise low-resistant cows have a high susceptibility for disease development. To exclude the detection of acute disease related markers, all samples were taken from cows that had not experienced health problems in the preceding 4 weeks. With this approach, we aimed to identify novel candidate biomarkers in milk for disease incidence in dairy cows, which were then evaluated in a larger number of milk samples from high- and low-resistant cows, selected on basis of comprehensive disease registration data collected during this study.

2. Materials and methods

2.1. Samples

Milk samples were obtained from the Resilient Cattle ("Weerbaar Vee") biobank established in the Netherlands from 2010 until 2015. Cows from 29 conventional Dutch dairy farms were sampled multiple times during this period with the highest sampling frequency in 2014. In 2014, all full fat milk samples tested in the general milk recording and monitoring program were also stored in Resilient Cattle biobank at −80° C (5–14 samples per cow). The average number of dairy cows per farm was 114 with a range of 63–266 cows. From 2010 until 2015 comprehensive disease registration data of these cows were collected. The disease registration data were carefully documented as instructed and supervised by one veterinarian and contained information about the diseases, applied treatments and medications the cows received including data about the duration of disease and treatment, vaccinations and hoof trimming. Diseases were categorised by the same veterinarian into: mastitis, other udder problems, lameness, retained placenta, metritis (uterus-related problems), respiratory diseases, metabolic diseases (e.g. ketosis) and "other" (diseases different than the previous categories for example trauma due to accidents).

First, milk serum (whey) samples used for proteomics analysis were selected based on the farmer's opinion on perceived disease resistance of the cows in combination with disease registration data. At that moment, the average number of dairy cows per farm was 108 with a range of 59–230 cows. In consultation with the veterinarian, farmers were asked to identify their five highest and five lowest performing cows in terms of health problems, which are henceforward called high- and low-resistant cows. These cows were checked for health problems using the recorded disease registration data and milk recording data. Cows with somatic cell counts above 250,000 cells/ml were excluded to reduce the chance on including cows with an ongoing mammary infection (Sampimon et al., 2010). In addition, cows were excluded with annotations in the disease registration data within one month before or after the moment of sampling. High-resistant cows had no or only minor health problems, while low-resistant cows had recurrent health problems. Four high-resistant and four low-resistant cows were selected for proteomics analysis. These two group of cows were matched for age, parity, milk production, somatic cell counts (SCC), fat percentage, protein percentage and days in milk (DIM). At the moment of milk sampling all cows in both groups were clinically healthy based on disease registration and milk recording data. The individual milk serum samples were compared to a pooled of milk serum sample derived from 26 cows. This randomly chosen "average group" is matched to both groups of low-resistant and high-resistant individual samples in terms of age, parity, milk production, SCC, fat percentage, protein percentage and DIM.

The second and larger group of 43 high- and 36 low-resistant cows were selected based on the disease registration data obtained from the beginning of 2010 until summer 2014. Cows in the high- and low-resistant groups were matched for farm (n = 9), age, parity, milk production, SCC, fat percentage, protein percentage and DIM. Other inclusion criteria for the cows were: raised on the selected farms, born between 2008 and 2011, more than 30 days in lactation, production above the average production per farm and somatic cell count at sampling below 250,000 cells/ml. High-resistant cows had no annotations in the comprehensive disease registration data except for vaccinations. Farmers were carefully instructed and coached by the same veterinarian in keeping the disease registration accurate and up to date. Low-resistant cows had at least two annotations in the disease registration data (excepting regular vaccinations).

2.2. NanoLC–MS/MS

Milk serum samples were prepared by centrifugation at 1500*g* for 10 min at 10° C. The supernatant was collected (without fat layer) and diluted 1:1 in 0.05 M ammonium bicarbonate buffer pH = 8.0 (ABC buffer, NH_4HCO_3 in water), then ultra-centrifuged at 100,000*g* for 90 min at 30° C. The clear supernatant (milk serum) was collected and prepared for proteomics analysis as described by (Zhang et al., 2015b). Milk serum samples were treated using the filter-aided sample preparation (FASP) method (Wisniewski et al., 2009) to clean the samples and perform trypsin digestion. After trypsin digestion, the resulting peptides were labelled by dimethyl labelling (Lu et al., 2011). The amine-group of each peptide reacts with formaldehyde (for light label) or deuterated formaldehyde (for heavy label) forming a so called Schiff base, which is subsequently reduced by cyanoborohydride resulting in a light or heavy label attached to each peptide (Boersema et al., 2009). The milk serum samples from high- and low-resistant cows were individually labelled with a light label and compared to a pool of milk serum from 26 cows containing a heavy label. Protein quantity is expressed as a $\log_2$ ratio of the individual milk serum samples to the pooled milk serum sample. All eight individual samples can be compared with each other due to this labelling approach.

NanoLC–MS/MS analysis was performed as described by (Zhang et al., 2015a). Full scan positive mode FTMS spectra were measured between *m*/*z* 380 and 1400 on a LTQ-Orbitrap XL (Thermo electron, San Jose, CA, USA) in the Orbitrap at high resolution (60,000). CID fragmented MSMS scans of the four most abundant 2+ and 3+ charged peaks in the FTMS scan were recorded in data dependent