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

S.E.C. van Altena[a,*], B. de Klerk[b], K.A. Hettinga[c], R.J.J. van Neerven[a,d], S. Boeren[e], H.F.J. Savelkoul[a], E.J. Tijhaar[a]

[a] Cell Biology and Immunology Group, Wageningen University, P.O. Box 338, Wageningen, The Netherlands
[b] Animal Breeding and Genomics Centre, Wageningen University, P.O. Box 338, Wageningen, The Netherlands
[c] Dairy Science and Technology Group, Wageningen University, 6700 EV Wageningen, The Netherlands
[d] FrieslandCampina, Stationsplein 4, 3818 LE Amersfoort, The Netherlands
[e] Laboratory of Biochemistry, Wageningen University, 6700 EV Wageningen, The Netherlands

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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.

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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,

* Corresponding author at: De Elst 1, 6708 WD Wageningen, The Netherlands.
E-mail address: christine.vanaltena@wur.nl (S.E.C. van Altena).

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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

mode in the linear trap (MSMS threshold = 5.000, 45s exclusion duration for the selected m/z +/−25 ppm).

LC–MS runs with all MSMS spectra obtained were analysed with MaxQuant 1.3.0.5 (Cox and Mann, 2008) using default settings for the Andromeda search engine (Cox et al., 2011) except that extra variable modifications were set for de-amidation of N and Q.

A bovine database downloaded from Uniprot (http://www.uniprot.org) was used together with a contaminants database that contains sequences of common contaminants as for instance: BSA (P02769, bovine serum albumin precursor), Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human) and Keratin K1CI (P35527, human).

In the MaxQuant analysis, only peptides and proteins with a false discovery rate (FDR) of less than 1% were accepted. Reversed hits were deleted from the MaxQuant protein groups result table. The protein groups result was filtered further to keep only proteins with at least 2 identified peptides of which at least one should be unique and at least one should be unmodified.

2.3. Lactoferrin ELISA

The bovine lactoferrin ELISA was performed according to manufacturer instructions (Bovine Lactoferrin ELISA Quantitation Set, Bethyl Laboratories Inc., Montgomery USA). Milk samples were diluted 500, 1000 or 4000 times in Blocking Reagent (Roche Applied Science, Mannheim, Germany). All samples and standards were prepared and measured in duplicate at 450 nm using a Filtermax F5 Plate Reader (Molecular Devices, Sunnyvale, California). The within assay coefficient of variation is <5% (Soyeurt et al., 2012).

2.4. Gamma glutamyltransferase 1 ELISA

Levels of gGT1 were determined using the bovine gamma glutamyltransferase 1 ELISA kit according to the manufacturers' instructions (NovaTeinBio, Woburn, USA).

2.5. Statistical analysis

Groups of high- and low-resistant cows were matched for age, parity, milk production, SCC, fat percentage, protein percentage and DIM. The association of disease status or condition with LF levels were analysed with PROC MIXED in SAS 9.3 (SAS Inst. Inc. Cary, NC). To approximate normality, the natural logarithm (ln) of LF levels were calculated and analysed. Preliminary analyses indicated that the fixed continuous effect of a 305 days milk production in kg, age of the cows in years, DIM, the interaction between age and DIM, and the random class effect of farms (farm 1–9) significantly affected LF levels. These effects were, therefore, maintained as correction factors within the models (base model). Inspection of the distribution of the residuals and the Q-Q plot indicated 2 possible outliers which were LF levels below the detection limit. These observations were, therefore, removed from the dataset, resulting in 43 high-resistant and 34 low-resistant cows.

To assess whether disease status or condition status was associated with LF levels, disease and condition variables were categorized into a limited set of different classes as some categories only had 1 observation. For mastitis, lameness, metabolic diseases and retained placenta the data were converted to a binary trait consisting of either having a condition (1) or not having a condition (0). For metritis the data was categorized in three classes consisting of having metritis once (1), having metritis more than once (2) or not having metritis (0). The fixed class effect of disease status or condition status was then individually added to the base model in separate analyses. To assess whether significant differences exist between cows that are not ill and cows that are ill with or without a certain condition, cows were categorized for each individual condition as either not ill, ill with a condition, or ill without a condition. These categories were individually added to the base model in separate analyses. Results are displayed in vertical scatter plots as the original individual observation (uncorrected LF level in μg/ml) for each disease status and/or condition.

3. Results

3.1. Comparison of milk serum samples of high- and low-resistant cows by proteomics

To characterize potential biomarkers for disease resistance in dairy cows, differences in milk serum proteins between 4 high- and 4 low-resistant cows were determined by NanoLC–MS/MS. In total, 78 proteins were identified and quantified in these samples (Supplementary Table 1). Each protein is represented in Fig. 1 as a $\log_2$ ratio of the protein abundance in high-resistant versus low-resistant cows. $\log_2$ protein ratios between −1 and 1 were considered as natural variation, which may be caused by, for example, differences in cow genetics and individual farms. The proteins outside these limits may indicate true variation between high- and low-resistant cows. The expression of 10 proteins was significantly ($p < 0.05$) different between the high- and low-resistant cows (open squares in Fig. 1). All 10 proteins were 2.3–6 times more abundant in milk serum of low-resistant than high-resistant cows (Table 1). To reduce the chance of missing potential biomarkers, while testing only a small number of cows, initially also proteins with $0.05 < p < 0.07$ were included, which resulted in the inclusion of 3 additional proteins (open triangles in Fig. 1) and a total of 13 proteins. The cut-off at $p < 0.07$ was chosen to include potential biomarkers, but still outside the natural variation between cows ($\log_2$ protein ratios between −1 and 1). The proteins that were selected were detected in at least 3 out of 4 cows per group.

3.2. Selection potential biomarkers

The 13 proteins that differed between high- and low-resistant cows ($p < 0.07$) were judged for their potential as a biomarker for bovine health. Most proteins were membrane-bound and were therefore less suitable as a biomarker since sample preparation and protein quantification with a high-throughput technique like ELISA is more difficult (Table 1). We therefore selected two soluble proteins for further investigation: gamma-glutamyl transpeptidase 1 (gGT1) and lactoferrin (LF). Gamma-GT1 is an enzyme involved in the Meister glutamyl cycle and is responsible for the transfer of glutathione across the cell membrane to maintain homeostasis (Meister and Anderson, 1983). Furthermore, gGT1 levels in blood are related to liver performance and intoxication (Pompella et al., 2007). Gamma GT1 levels measured in urine can be an indicator for renal damage (Ferguson et al., 2008). The association with disease and damage makes gGT1 in milk an interesting candidate marker for disease resistance. Lactoferrin is a glycoprotein produced by glandular epithelial cells and neutrophils and is present in all body fluids. It is part of the innate immune system and has antimicrobial, antiviral, antifungal, anti-inflammatory and anti-oxidative properties, among others by iron sequestering (García-Montoya et al., 2012; Kanwar et al., 2015) and can also act as an acute phase protein (Tothova et al., 2014).

3.3. LF levels in milk of high- and low-resistant cows, selected on disease registration data

New, full fat milk samples were selected from the "Resilient cattle" biobank to determine if LF and gGT1 were suitable markers for health-related problems in cows. Milk samples of 43 high-resistant

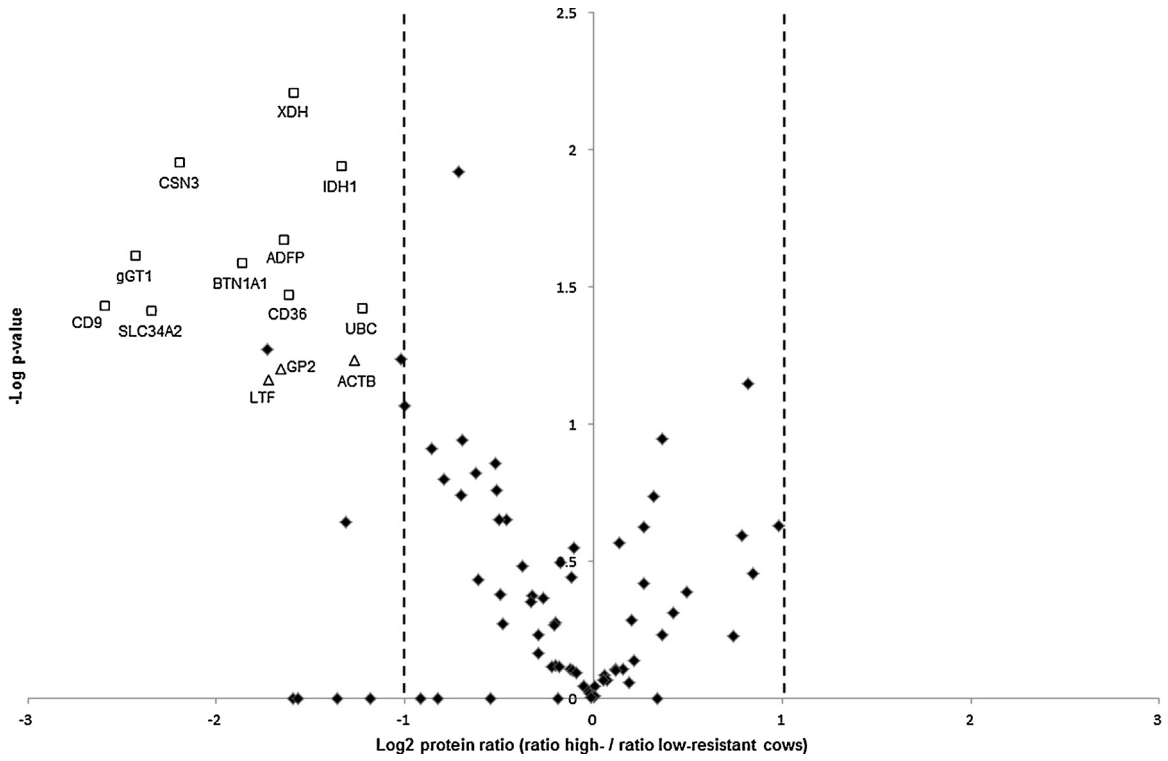

Fig. 1. Ratio of milk serum proteins of high- to low-resistant cows.
The diamonds, triangles and squares indicate the proteins found in milk serum samples from 4 high- and 4 low-resistant cows (n = 78). Proteins are represented as a $\log_2$ ratio of (ratio protein expression in high-resistant cows/protein expression in pooled milk sample) divided by (ratio protein expression in low-resistant cows/protein expression in pooled milk sample). $\log_2$ protein ratios between −1 and 1 are considered as natural variation. Open squares: proteins that differ between high-and low-resistant cows ($p < 0.05$). Open triangles: proteins that differ between high- and low-resistant cows ($0.05 < p < 0.07$). The abbreviations correspond to the proteins listed in Table 1.

Table 1
Milk serum proteins that differ between high- and low-resistant cows.

Protein	Full name	Uniprot ID	Subcellular location	Fold change	p-value
CD9	CD9 antigen	P30932	M	6.0	0.037
GGT1	Uncharacterised protein; Gamma-glutamyl transpeptidase 1, gamma-glutamyltransferase 1	G3N2D8	M, S	5.4	0.024
SLC34A2	Solute carrier family 34 (sodium-dependent phosphate transport protein 2B) member 2	F1N6D4	M	5.1	0.039
CSN3	Casein kappa	P02668	E	4.6	0.011
BTN1A1	Butyrophilin subfamily 1 member A1	P18892	M	3.6	0.026
LTF	Lactotransferrin	P24627	E, S	3.3	0.069
GP2	Uncharacterised protein; Glycoprotein 2, zymogen granule membrane, pancreatic secretory granule membrane major glycoprotein	F1N726	E, S	3.1	0.063
PLIN2/ADFP	Perilipin 2, adipophilin, adipose differentiation-related protein	F1N1N6	M	3.1	0.021
CD36	CD36 molecule, platelet glycoprotein IV, thrombospondin receptor	P26201	M	3.1	0.034
XDH	Xanthine dehydrogenase/oxidase	F1MUT3	C, E	3.0	0.006
IDH1	Isocitrate dehydrogenase 1 (NADP+)	Q9XSG3	C	2.5	0.011
ACTB	Actin, cytoplasmic 1	P60712	C	2.4	0.060
UBC	Polyubiquitin	P0CH28	C	2.3	0.037

C = cytosol, E = extra-cellular space, M = membrane, S = secreted.

cows and 34 low-resistant cows were collected based on comprehensive disease registration data and milk recording data from January 2010 until July 2015. These cows did not have annotations the disease registration data in the month before and after sampling. LF and gGT1 levels were determined using commercially available ELISAs. The gGT1 levels in full fat milk samples were below the detection limit (data not shown) and were therefore not further investigated. Lactoferrin levels were detectable using ELISA and were expressed in μg per ml (Fig. 2). For statistical analysis, the LF levels were ln transformed and corrected for milk production, days in milk (DIM), age, farm and the interaction between DIM and age as these variables significantly influenced LF levels in milk. No association was found for LF levels and somatic cell counts (data not shown). The least squares means (corrected mean) for LF levels in full fat milk were 244 μg/ml and 311 μg/ml for high- and low-resistant cows, respectively, and were significantly higher in low-resistant cows ($p = 0.047$) (Fig. 2). This is consistent with the previous results obtained by NanoLC–MS/MS for the 8 milk serum

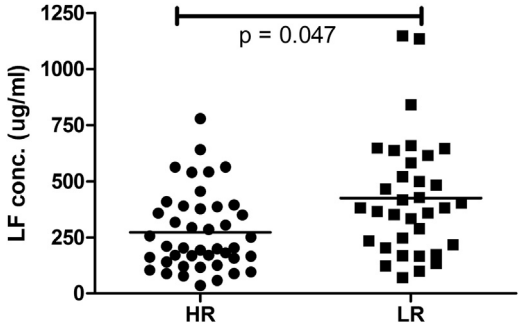

Fig. 2. LF levels in full fat milk samples of high- versus low-resistant cows.
LF levels were measured in full fat milk samples from 43 high- and 34 low-resistant cows using ELISA. LF levels were expressed as the original LF concentration in μg/ml. The horizontal line indicates the mean. Statistics were performed on the ln transformed and corrected data. (Correction for farm, 305 days production, age, days in milk (DIM) and the interaction between age and DIM.) LF levels were significantly higher in low-resistant cows than high-resistant cows ($p = 0.047$).

samples. This implies that being high- or low-resistant is significantly associated with LF levels in full fat milk, even after correction for milk production, DIM, age, farm and the interaction between DIM and age.

3.4. Association of LF levels in milk and specific diseases

Next, we determined if the LF levels in full fat milk were associated with specific classes of diseases, independent of the resistance status of the cows. Cow performance was monitored by disease registration data and milk recording data. Diseases were categorised in: mastitis, other udder problems, lameness, retained placenta, metritis, respiratory diseases, metabolic diseases and "other" (diseases different than the previous categories or e.g. trauma). Cows with high disease resistance had none of the aforementioned health problems, while the low-resistant cows had at least two times an annotation in the disease registration data. Based on disease incidence, the association with LF levels in milk was determined for the following health-related problems in cows: mastitis, metabolic diseases, lameness, metritis and retained placenta (Supplementary Fig. 1).

We did not observe a significant difference in LF levels in milk between cows that had mastitis, metabolic diseases, metritis or a retained placenta in the past and cows without these particular diseases (Supplementary Fig. 1a–d). Nevertheless, cows that had suffered from lameness ($n = 14$) were significantly different from cows without lameness ($p = 0.024$) as cows with lameness had higher LF levels in their milk (Supplementary Fig. 1e).

3.5. Association of LF levels in milk with mastitis and lameness in high- and low-Resistant cows

Independent of being high- or low-resistant, LF levels were determined with respect to the different diseases. When the high- and low-resistance status was taken into account, the low-resistant cows that had suffered from mastitis showed similar LF levels in milk as the low-resistant cows without mastitis (Fig. 3a). Low-resistant cows with lameness had significantly higher LF levels in milk compared to high-resistant cows without lameness ($p = 0.014$) (Fig. 3b). LF levels appeared to be higher in low-resistant cows that had suffered from lameness compared to low-resistant cows without lameness, although, this difference was not significant ($p = 0.13$) (Fig. 3b).

3.6. Prognostic value lactoferrin

The LF levels were measured in milk samples collected during the summer of 2014. One year later, summer 2015, we investigated which of the high-and low-resistant cows were culled or not as an indicator for cow performance. Fig. 4 shows the LF levels in full fat milk of high- and low-resistant cows that were still alive and those that were culled within one year. The low-resistant cows that were culled within one year after sampling had on average higher LF levels (least squares means = 394.0 μg/ml) than high-resistant cows that were alive (265.2 μg/ml), high-resistant cows that were culled (166.1 μg/ml) and low-resistant cows that were still alive (288.6 μg/ml). This difference was significant between high-resistant cows that were still on the farm and low-resistant cows that were culled within one year ($p = 0.035$) and between high- and low-resistant cows that were culled ($p = 0.0017$) (Fig. 4). A similar trend was observed for low-resistant cows that were still alive and those that were culled ($p = 0.10$). In addition, high-resistant cows that were culled had lower LF levels in milk compared to high-resistant cows that were still alive ($p = 0.041$) and low-resistant cows that were still alive ($p = 0.017$). Taken together, these results indicate that low-resistant cows with higher LF levels had a higher chance of being culled.

4. Discussion

In this study we identified LF as a putative marker for lameness with prognostic value for early culling. Potential biomarkers were characterised in milk to indicate cows that are at risk to develop disease using a proteomics-based approach. The initial comparison of milk serum samples from 4 high- versus 4 low-disease-resistant cows revealed 13 proteins that were differentially expressed between both groups (ten $p < 0.05$ and three $p < 0.07$). These proteins were all more abundantly detected by proteomics in milk serum of low-resistant cows compared to high-resistant cows. Using ELISA we confirmed in a much larger set of full fat milk samples that the levels of one of these proteins, LF, were significantly increased in low-resistant cows. A positive association was observed between LF levels in milk and cows that had suffered from lameness. No significant associations were found for LF levels and metabolic disease, metritis, retained placenta or mastitis. The LF levels were also associated with culling and had a prognostic value for culling as low-resistant cows that were culled within one year had higher LF levels in milk.

The two potential biomarkers, gGT1 and LF, were selected from the proteins that were significantly different between high- and low-resistant cows, because these were soluble proteins which were related to disease in practice and literature. Elevated gGT1 levels in blood is an indicator for cholestasis and liver failure and is already used in practice. In literature, enhanced gGT1 activity in serum of *Rathi* cattle is a marker for stress and metabolic dysfunction (Kataria and Kataria, 2012). Increased gGT1 levels in urine were related with renal injury (Ferguson et al., 2008). Additionally, gGT1 levels were used as an indicator for colostrum uptake in young calves and lambs (Maden et al., 2003). Unfortunately, the gGT1 levels in milk were below the detection limit of the available capture ELISA, therefore the relevance of gGT1 levels in milk for bovine health could not be determined in this study. LF levels were shown to be significantly higher in low-resistant cows. Lactoferrin has different functions (García-Montoya et al., 2012; Kanwar et al., 2015; Tothova et al., 2014) and plays e.g. an important role in the induction of innate immunity by sequestering iron and thereby limiting the availability of free iron, which is essential for bacterial growth. Therefore, it might appear counterintuitive that a protective agent like LF is increased in low-resistant cows, since higher LF

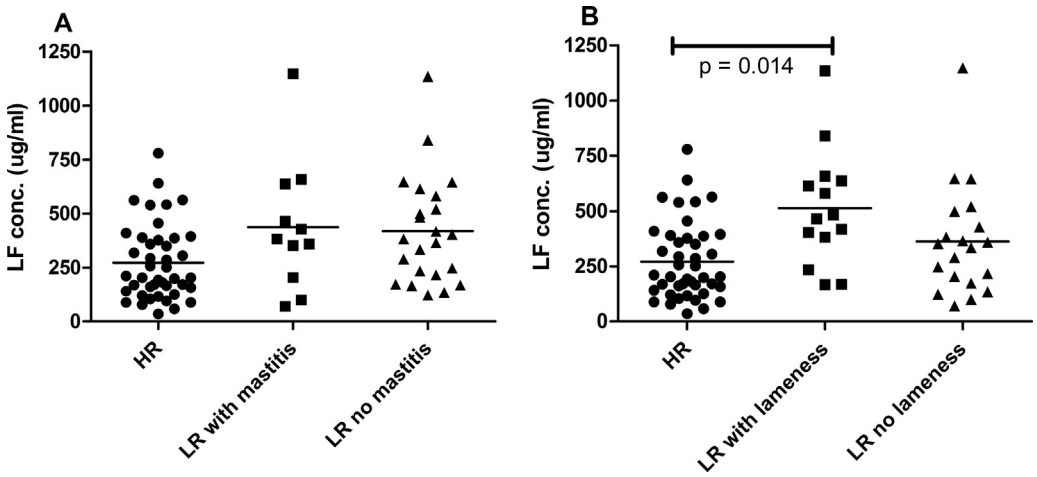

Fig. 3. LF levels in full fat milk related to mastitis and lameness.
LF levels were determined in full fat milk samples and related to disease thereby comparing high-resistant cows, low-resistant cows which had suffered from a specific disease and low-resistant cows without that particular disease. Individual LF levels were expressed in μg/ml. Statistics were performed on the ln transformed and corrected data. (3A) High resistant cows, low-resistant cows which had suffered from mastitis and low-resistant cows which had suffered from other diseases than mastitis had similar LF levels in milk. (3B) Low-resistant cows which had suffered from lameness had significantly higher LF levels in milk compared to high-resistant cows (p = 0.014).

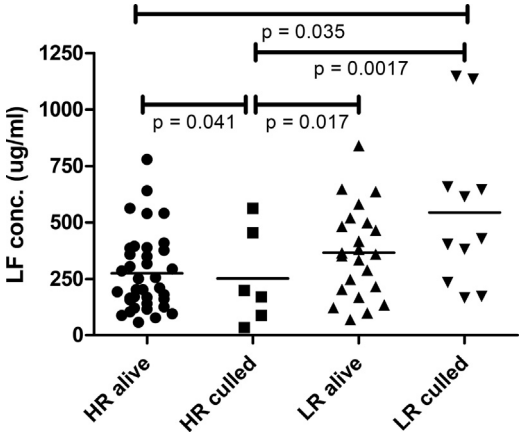

Fig. 4. LF levels in full fat milk and culling.
To determine the prognostic role of LF, LF levels in milk were related to culling. LF levels in milk were determined in high- and low-resistant cows that were culled or alive one year after sampling. Individual LF levels were expressed in μg/ml. Statistics were performed on the ln transformed and corrected data. LF levels were lower in high-resistant cows which were culled compared to high-resistant cows that were still alive (p = 0.041) or low-resistant cows that were still alive (p = 0.017). Low-resistant cows that were culled had higher LF levels in milk than high-resistant cows which were still alive (p = 0.035) or culled (p = 0.0017).

levels imply a better protection against disease. However, a similar situation is seen for SCC. A higher SCC is usually caused by an influx of neutrophils in the udder to fight a bacterial infection (Paape et al., 2002). Similar to LF, the higher SCC level helps to control the infection, which is favourable for the cow. At the same time the higher SCC level is an indication of a recent or ongoing infection, which will occur more often in low-resistant cows.

Lactoferrin levels were significantly associated with lameness i.e. cows that had suffered from lameness in the past had higher LF levels in milk. Other studies showed relations between lameness and elevated levels of the acute phase proteins serum amyloid A, haptoglobin and fibrinogen in serum (Kujala et al., 2010; Tóthová et al., 2011). However, in our milk samples these proteins were not significantly different in high- and low-resistant cows or were not detected at all by NanoLC–MS/MS (Supplementary data 1), suggesting that LF was not produced due to an acute phase response. As we excluded cows with annotations in the months before and after sampling, this implies that LF could indicate lameness on a long term in contrast to e.g. serum amyloid A which indicates an ongoing infection.

Lactoferrin in milk is released by neutrophils and epithelial cells and its production is positively related with the influx of neutrophils and SCC (Lindmark-Månsson et al., 2006). Since SCC were on average equal between high- and low-resistant cows (data not shown), it is less likely that LF levels were increased due to neutrophil infiltration in the udder. Lactoferrin and haptoglobin levels in milk were also associated with the energy status of cows (Hiss et al., 2009). Cows with higher serum NEFAs levels around parturition, an indicator for increased fat mobilisation and a negative energy balance (NEB), had significantly higher LF and haptoglobin levels in milk for several weeks afterwards. Moreover, cows with more metabolic stress after calving had more health disorders (Gross et al., 2011), like lameness (Collard et al., 2000). In line with this, the low-resistant cows in our study had higher LF levels in milk, suffered at least two times from periparturient diseases, and showed a significant association between LF levels and lameness. Unfortunately, data on the cows' energy status was lacking in our study and a significant association between LF levels in milk and periparturient diseases was not observed (data not shown).

In contrast to other studies, no association was observed between LF levels in milk and SCC (Chaneton et al., 2013; Cheng et al., 2008; Liu et al., 2010). However, we selected for cows with SCC below 250.000 cells/ml and excluded cows with annotations in the disease registration data in the month before and after sampling, thereby excluding an association with mastitis. In addition, we found an increase in CD36, BTN1A1, IDH1 and kappa-casein in milk of low-resistant cows, while others showed these proteins to be decreased in milk from cows with ongoing mastitis (Huang et al., 2014; Moyes et al., 2009a). This supports the notion that the increased LF levels observed in this study were not caused by mastitis.

Initially, we found an association between LF levels and disease-resistance in cows, but a prognostic marker for disease-resistance would be more valuable. The prognostic value of LF was studied based on culling rates one year after our analysis. LF levels were significantly higher in low-resistant cows that were culled within one year compared to high-resistant cows. A similar trend was observed comparing low-resistant cows which were culled or still alive. This suggest that LF is an indicator for culling risk and might be a result of repeated health problems. High-resistant cows that were culled

within one year surprisingly had significantly lower LF levels compared to the high-resistant cows that were still alive, suggesting that these cows were culled for other reasons than low-resistant cows. Although, it should be taken into account that the number of high-resistant that were culled were low (n = 6). Despite the variability in basal LF levels between cows (Stelwagen et al., 2009) and the short half-life of LF in milk (Kutila et al., 2002), we did show an association between LF levels in milk and lameness and culling. Based on our data, a clear cut-off value for LF to indicate "at risk" cows cannot yet be chosen, but they warrant a more extensive study to determine the full potential of LF as a biomarker to indicate cows with a higher risk to develop health problems.

5. Conclusion

In conclusion, using a proteomics approach, we have established a potential biomarker for cows with health problems. Low-resistant cows have higher lactoferrin levels in milk even though these cows were clinically healthy at the moment of sampling. The LF levels were positively associated with lameness and may be a long term indicator for this disease. Moreover, cows with high LF levels were more likely to have been culled one year after sampling, indicating that LF has potential as a prognostic biomarker for premature culling.

Conflict of interest

RJJvN is an employee of FrieslandCampina. FrieslandCampina was not involved in this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2016.04.005.

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