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Biomarker and proteome analysis of milk from dairy cows with clinical mastitis: Determining the effect of different bacterial pathogens on the response to infection

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ABSTRACT

Antimicrobial usage (AMU) could be reduced by differentiating the causative bacteria in cases of clinical mastitis (CM) as either Gram-positive or Gram-negative bacteria or identifying whether the case is culture-negative (no growth, NG) mastitis. Immunoassays for biomarker analysis and a Tandem Mass Tag (TMT) proteomic investigation were employed to identify differences between samples of milk from cows with CM caused by different bacteria. A total of 94 milk samples were collected from cows diagnosed with CM across seven farms in Scotland, categorized by severity as mild (score 1), moderate (score 2), or severe (score 3). Bovine haptoglobin (Hp), milk amyloid A (MAA), C-reactive protein (CRP), lactoferrin (LF), α-lactalbumin (LA) and cathelicidin (CATHL) were significantly higher in milk from cows with CM, regardless of culture results, than in milk from healthy cows (all *P*-values <0.001). Milk cathelicidin (CATHL) was evaluated using a novel ELISA technique that utilises an antibody to a peptide sequence of SSEANLYRLLELD (aa49–61) common to CATHL 1–7 isoforms. A classification tree was fitted on the six biomarkers to predict Gram-positive bacteria within mastitis severity scores 1 or 2, revealing that compared to the rest of the samples, Gram-positive samples were associated with CRP < 9.5 μg/ml and LF ≥ 325 μg/ml and MAA < 16 μg/ml. Sensitivity of the tree model was 64%, the specificity was 91%, and the overall misclassification rate was 18%. The area under the ROC curve for this tree model was 0.836 (95% bootstrap confidence interval: 0.742; 0.917). TMT proteomic analysis revealed little difference between the groups in protein abundance when the three groups (Gram-positive, Gram-negative and no growth) were compared, however when each group was compared against the entirety of the remaining samples, 28 differentially abundant protein were identified including β-lactoglobulin and ribonuclease. Whilst further research is required to draw together and refine a suitable biomarker panel and diagnostic algorithm for differentiating

Abbreviation: AMU, Antimicrobial usage; AMR, Antimicrobial resistance; SCM, Sub-clinical mastitis; CM, Clinical mastitis; No growth, NG, Culture-negative mastitis; LA, α-lactalbumin; Hp, Haptoglobin; MAA, Mammary amyloid A; CATHL, Cathelicidin; NMC, National Mastitis Council; HuCAL, Human Combinatorial Antibody Library; CV, coefficient of variation; ROC, receiver operating characteristic curve; BSA, Bovine serum albumin; DAP, differentially abundant protein; kDa, Kilo Daltons; Mw, Molecular weight; ELISA, Enzyme linked immunoassay; TMT, Tandem Mass Tag.

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Gram- positive/negative and NG CM, these results have highlighted a potential panel and diagnostic decision tree. Host-derived milk biomarkers offer significant potential to refine and reduce AMU and circumvent the many challenges associated with microbiological culture, both within the lab and on the farm, while providing the added benefit of reducing turnaround time from 14 to 16 h of microbiological culture to just 15 min with a lateral flow device (LFD).

1. Introduction

Mastitis, one of the most common and economically impactful health problems of dairy cattle, is caused mainly by intramammary bacterial infection. Its control and management are the main reasons for antimicrobial usage (AMU) in dairy cattle with the potential for the development of antimicrobial resistance (AMR) (Addis et al., 2016; Naranjo-Lucena and Slowey, 2023; Selim et al., 2022; Titouche et al., 2022). Mastitis can occur as sub-clinical mastitis (SCM) or can be clinically evident with observable changes visible to the naked eye, which may include swelling, tenderness, raised temperature, and redness of the affected udder quarter, as well as the presence of clots in the milk. Depending on the severity of clinical mastitis (CM), three forms have been recognised based on clinical signs: mild (abnormal milk), moderate (abnormal milk and abnormal udder) and severe (abnormal milk, abnormal udder and abnormal cow) (Pinzon-Sanchez et al., 2011; Roberson, 2012).

In cases of CM, there is considerable value in distinguishing between the causative bacteria, whether they are Gram-positive or Gram-negative, or identifying culture-negative (no growth, NG) mastitis. The use of antimicrobial therapy has been shown to have limited benefits in cases of mild to moderate clinical mastitis caused by *Escherichia (E.) coli* or other Gram-negative species (Roberson, 2012; Suojala et al., 2013). While there are numerous compounds available to treat Gram-negative bacteria, the indiscriminate and untargeted use of antimicrobials may lead to the selection of resistant bacteria. Selective treatment protocols used for CM have shown no difference in cure rate, recurrence rate, or long-term production output when antimicrobial treatment was withheld from cases where either Gram-negative bacteria, no bacteria, or yeasts were cultured; there was however an overall reduction in the use of antimicrobials (Bates et al., 2020; Griffioen et al., 2021; Lago et al., 2011).

Differentially diagnosing CM by Gram classification or as NG mastitis is only achievable following microbiological culture and, where present, bacterial identification; this may require the sample to be dispatched to a laboratory, with inherent delay in the result being provided before a clinical decision can be made. On-farm culture procedures are gaining popularity, but these may also take up to 24 h prior to result generation (Malcata et al., 2020; Malcata et al., 2021).

To overcome these limitations there has been an increased emphasis on identifying and measuring host-derived biomarkers that are present in milk (Giagu et al., 2022). Differentiating clinically mastitic milk samples may offer significant potential to refine and reduce AMU and circumvent the many challenges associated with microbiological culture, both within the lab and on the farm. Focusing on host-derived biomarker proteins in milk increases the diagnostic potential with increased availability of platforms with improved sensitivity, rapid on farm point of care usage and the ability to multiplex, digitalise and expediate results. In light of the proteomic and peptidomic development over the last 10 years a wide number of inflammatory (and not so inflammatory) biomarkers have been identified, beyond the "traditional APPs". There are also those whose concentration changes in the absence of overt inflammation and/or infection. Furthermore, acute phase proteins are regarded as serum proteins that are synthesised and secreted from the liver, whereas the biomarkers of mastitis and even those that are also present in serum (Hp, MAA/SAA) are produced locally in the mammary gland.

Highly abundant milk proteins such as α-lactalbumin (LA) and serum albumin in milk have been long established as milk biomarkers from classical investigations of the quantifiable changes in these proteins in response to intramammary infection and the resulting inflammation (Gronlund et al., 2003; Hogarth et al., 2004). Further mastitis biomarker identification and more recently, increased diagnostic utility of existing mastitis biomarkers, has been enabled by the combined use of proteomic, peptidomic and immunodiagnostic platforms (Bilic et al., 2018; Mudaliar et al., 2016; Turk et al., 2021).

These combined advancements have enabled very low abundant milk proteins, such as haptoglobin (Hp) and mammary amyloid A (MAA), to be identified and confirmed as key mastitis biomarkers enabling sensitive immunoassays to be developed and deployed across varied mastitis research programmes. Cathelicidin (CATHL), a neutrophil derived anti-microbial peptide, has also been identified as a biomarker of mastitis (Mudaliar et al., 2016) with measurement via immunoassay established (Addis et al., 2016). Whilst the increased availability of immunoassays for some mastitis biomarkers has enabled a range of studies and investigations to be undertaken, most of these have investigated and compared the difference in biomarker concentration between milk from healthy dairy cows to milk from those with SCM or CM with little regard to the bacteria, if present, that may be causing disease (Wollowski et al., 2021; Turk et al., 2021; Boehmer, 2011).

This study aimed to investigate the diagnostic utility and application of a range of established mastitis biomarkers to determine if these, either individually or in combination could differentiate a diagnosis of CM into cases caused by Gram-positive or Gram-negative bacteria and identify CM cases with no bacterial growth. Data could then be used to develop a diagnostic decision tree to reveal the optimal biomarkers for differentiating these groups.

Furthermore, a quantitative proteomic approach was also undertaken with a view of identifying additional biomarkers that could be utilised to differentiate CM diagnosis between these groups utilising recent developments in the proteomic analysis milk proteins (Das et al., 2022). The aims of the study are therefore to determine the value of previously identified milk biomarkers in differential diagnosis of Gram-positive, Gram-negative or NG mastitis and to identify by quantitative proteomics additional biomarkers that could be utilised in such differential diagnosis.

2. Material and methods

This research was approved by the Ethics and Welfare Committee, School of Biodiversity, One Health and Veterinary Medicine, University of Glasgow, UK (Ref EA24/19).

2.1. Milk sample collection from dairy cows with clinical mastitis

Seven dairy farms in Scotland were selected for participation based on location and willingness to collaborate in the study. The study recruited farm personnel, such as milkers and herd managers, who were then trained to recognize cases of CM and categorize them as either mild (presence of abnormal milk such as milk with clots or flakes or serous milk), moderate (presence of signs of udder tissue inflammation: hardness, swelling, redness, heat or pain) or severe (animals with additional systemic signs of disease, such as fever, tachycardia, tachypnoea, dehydration, anorexia and decreased ruminal function) (Pinzon-Sanchez et al., 2011). The National Mastitis Council (NMC)'s guidelines for aseptic milk sample collection were used to train the farm personnel on

how to collect milk samples in a sterile manner (NMC, 2017). The trained staff cleaned, pre-dipped and dried the teat, fore-stripped 2 to 3 times, scrubbed the teat using swabs and surgical spirit and collected milk in 30 ml sterile universal containers (Henry Schein, Melville, United States of America) held in a diagonal position and without touching the cap to avoid contamination. Universal containers are the most used milk sampling vessels in mastitis studies. No preservative was added to the milk samples.

If multiple quarters of a cow were affected simultaneously, all affected quarters were sampled. Any CM episode in the same quarter occurring >14 days after the previous episode was considered a new CM case, regardless of the pathogen isolated. If the second CM episode occurred in the same quarter within 14 days with a different etiologic agent than that of the first episode, it was also considered a different CM case (Hertl et al., 2014). Animals were eligible for inclusion in the first week after calving but no animals included were within 14 days of administration of antimicrobial products. Samples were collected in two periods from January to May 2018 and from September to November 2020, stored on-farm at −20°C and transported to the Veterinary Diagnostic Services laboratory of the University of Glasgow where they were stored at −20°C until processing. Samples were then thawed at ambient temperature for up to 8 h and split into two aliquots. One aliquot underwent standard bacteriology culture (Veterinary Diagnostic Services, School of Biodiversity, One Health and Veterinary Medicine, University of Glasgow) and one aliquot was used for biomarker analysis. A total of 94 mastitic milk samples were collected from 94 Holstein Friesian cows (*Bos taurus*) and cultured for bacteriology within 4 weeks from CM detection. Milk samples ($n = 29$) from clinically healthy dairy cows were also collected and stored under similar conditions.

2.2. Standard bacteriology culture

Standard laboratory techniques were used to process the milk samples for microbiological analysis (NMC, 2017). To prepare the samples, a sterile cotton swab was soaked in the milk and then used to create lawn cultures in the corners of MacConkey and 5% sheep blood agar plates (E & O Laboratories). The plates were then streaked with a sterile loop and incubated aerobically at 37 °C for 24–48 h before being examined. Samples that did not show visible colonies were classified as negative for mastitis-associated pathogens. Samples that displayed three or more colony types were regarded as contaminated and therefore excluded from data analysis following NMC guidelines.

Standard laboratory methods were employed to identify all organisms, including assessments of colony morphology, Gram stain, pattern of haemolysis, and biochemical profile (Hogan et al., 1999). In cases where speciation was necessary, API® (bioMérieux Ltd., Basingstoke, UK) were used, namely API®20E, API®32 Staph, API®20 Strep, API® 32STREP, API® Coryne.

2.3. Biomarker analysis

2.3.1. Biomarker immunoassay

The concentrations of a number of established milk biomarkers of mastitis were determined, with bovine haptoglobin (Hp), milk amyloid A (MAA), C-reactive protein (CRP) and lactoferrin (LF) being determined by Spatial Proximity Analyte Reagent Capture Luminescence (SPARCL™) immunoassays (Life Diagnostic Inc., West Chester, USA) according to the manufacturer's instruction and described in Turk et al. (2021). Milk concentrations of α-lactalbumin (LA) were determined by enzyme linked immunoassay (Bethyl Laboratories, USA) according to the manufacturer's instructions.

2.3.1.1. CATHL antibody production. The milk concentration of CATHL was determined by ELISA based on antibodies raised against a peptide sequence common to bovine CATHL 1–7 and prepared in collaboration with Abingdon Health Ltd., (Birmingham, UK). The CATHL antibodies were produced using HuCAL (Human Combinatorial Antibody Library), technology (Bio-Rad, Hemel Hempstead, UK). A peptide sequence, SSEANLYRLLELD (aa49–61), that was conserved between the seven isoforms of bovine CATHL was identified on the Uniprot website (www.uniprot.org) and this peptide was synthesised and used as the target in antibody production. A total of 14 CATHL antibodies were generated and were initially assessed by Western blot for cross reactivity to bovine CATHL.

2.3.1.2. CATHL antibody assessment by western blot. To test each of the 14 CATHL antibodies, four CM and one healthy milk sample were separated by SDS-PAGE. Protein concentrations of milk samples were determined by the bicinchoninic acid (BCA) assay (ThermoFisher, Paisley, UK), with protein concentrations being determined using a bovine serum albumin (BSA; Sigma-Aldrich, Poole, UK) protein standard. Samples were diluted in water and mixed with 2× Laemmli sample buffer (Bio-Rad, Hemel Hempstead, UK), containing 5% beta-mercaptoethanol (Sigma-Aldrich, Poole, UK), to give a final protein loading of 10 μg per sample. Diluted samples were heated on a heating block for 4 min at 95 °C and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4–15% Criterion™ TGX™ precast protein gels (Bio-Rad, Hemel Hempstead, UK). Gels were run at 300 V in 1× Tris/Glycine/SDS running buffer (Bio-Rad, Hemel Hempstead, UK), for 20 min. Proteins were subsequently transferred to nitrocellulose membrane (Bio-Rad, Hemel Hempstead, UK), at 70 V for 60 min using a Criterion™ blotter system (Bio-Rad, Hemel Hempstead, UK).

For Western blot, nitrocellulose membrane was blocked overnight at 4 °C in 5% *w/v* powdered skimmed milk dissolved in TTBS buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween®20). The membrane was washed with TTBS three times and incubated with the CATHL antibody (1 μg/ml diluted in TTBS containing 1% *w/v* powdered skimmed milk), for one hour at room temperature. The membrane was washed as before and incubated with HRP-linked goat polyclonal secondary antibody to human IgG (Bio-Rad, Hemel Hempstead, UK), at a 1:5000 dilution in TTBS containing 1% *w/v* powdered skimmed milk, for one hour at room temperature. The membrane was washed and incubated with enhanced chemiluminescence (ECL) Western Blotting Substrate (ThermoFisher, Paisley, UK). It was then exposed to Hyperfilm™ photographic film (GE Healthcare, Buckinghamshire, UK), and the film was developed with an X-ray film developer. Exposure times were optimised, and films scanned (Umax PowerLook III, Umax, Taiwan).

2.3.1.3. CATHL immunoassay. A sandwich ELISA was developed for measuring CATHL in milk. Two CATHL antibodies (#15480 and #15484), were selected from those tested by western blotting. CATHL antibody (#15484) was labelled with HRP using the Lightning-Link® kit (Abcam, Cambridge, UK), as per the manufacturer's protocol and was used as the detection antibody. Briefly, modifier reagent was mixed with antibody (1.2 mg/ml). This was then added to HRP conjugation mix and incubated in the dark for three hours at room temperature. Quencher reagent was added, and the HRP-conjugated antibody was ready to use 30 min later and stored at 4°C for subsequent use.

MaxiSorp 96-well plates (ThermoFisher, Paisley, UK), were coated with 100 μl/well of CATHL antibody (#15480), diluted to 1 μg/ml in coating buffer (0.06 M carbonate buffer, pH 9.6), and incubated overnight at 4 °C. Plates were washed with PBST (0.01 M PBS, pH 7.4 containing 0.05% Tween®20), and blocked with 250 μl/well of PBST containing 2% w/v BSA for 30 min at room temperature. Plates were washed and 100 μl/well of diluted standard or sample were added. Following a one hour incubation at 37°C, plates were washed and 100 μl/well of HRP-labelled CATHL antibody was added (1:10,000 dilution in PBST containing 1% w/v BSA). Plates were incubated for one hour at 37°C and then washed. 100 μl/well of TMB solution was added (Pierce™

TMB Substrate Kit; Thermo Fisher, Paisley, UK), and plates were incubated for 30 min at room temperature. The reaction was stopped by adding 50 µl/well of stop solution (2 M H_2SO_4), and the optical density was measured at 450 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, Aylesbury, UK). The concentration of CATHL in samples was determined against the standard curve using a 4-parameter fit model.

A milk sample with a high concentration of CATHL was calibrated against standards of the bovine CATHL 1–7 peptide, used as immunogen and following serial dilution in PBS buffer, was used to form a standard curve from 0.78 to 50 µg/ml of CATHL. Samples were diluted 1:20 in the same PBS buffer. During assay development it was determined that for optimal immunoassay the standard and samples required heating to 90°C for 10 min, after they had been diluted. The repeatability of the assay was determined by calculation of the inter assay coefficient of variance (CV) of control samples included in every assay ($n = 9$) with the intra assay CV determined by the root mean square method using duplicate samples in the assay. Sensitivity was assessed by determination of the limit of the blank as the value of CATHL determined at 3 standard deviations (SD) above the mean of blank samples of buffer alone. Specificity was demonstrated by the Western blots of milk samples from udder quarters with CM using the anti-bovine CATHL as first antibody.

2.3.2. Bovine serum albumin detection by Western blot

The presence of BSA in the milk samples was detected by Western blot with a rabbit polyclonal anti-BSA-HRP (Antibody Online, GmbH, Germany ABIN6190479A) that did not require the use of a second antibody as peroxidase was already conjugated to the antibody. The procedure for SDS-PAGE and blotting to cellulose nitrate was as described above for CATHL western blots except that the blot was incubated overnight in anti-bovine BSA-HRP at a dilution of 1:5000 in TBST before washing and visualisation with ECL. A standard of BSA at 100 µg/ml was included in each gel and the relative intensity of the BSA band in samples compared to standard using ImageJ 1.44 (https://imagej.nih.gov/ij/) providing a semi-quantitative measure of the BSA concentration in each milk sample.

2.3.3. Statistical analysis of biomarkers

Biomarker results (Hp, CRP, LF, MAA, LA and CATHL) were used to compare the Gram-positive, Gram-negative, NG, Healthy groups, and to fit classification tree models. Percentile bootstrap confidence intervals (Efron and Tibshirani, 1993) for the mean biomarker values per group were calculated using 1000 bootstrap replications. Kruskall-Wallis tests (Hollander et al., 2014) were used to compare the distributions of each biomarker in the groups (NG, Gram-positive, Gram-negative, and Healthy) simultaneously. For biomarkers in which the Kruskal-Wallis test showed that at least one of the groups differs from the others, pairwise Wilcoxon rank sum tests were performed to determine which group pairs differ from each other. Bonferroni corrections were applied to control the family-wise Type I error rates in each set of pairwise comparisons.

A classification tree model (Breiman et al., 1984) was fitted on the six biomarkers to predict Gram-positive bacteria (that is, Gram-positive vs. the rest). Leave-one-out cross-validation was used to avoid over-fitting, and the final tree model was pruned back to minimise the cross-validation misclassification error. To evaluate goodness of fit for classification tree model, the area under the receiver operating curve (ROC) was calculated. Sensitivity, specificity, and overall classification accuracy were calculated for the final biomarker tree model.

Fisher's exact test for count data was used to compare the semi-quantitative albumin concentrations of the NG, Gram-negative and Gram-positive groups, with Bonferroni corrections for the multiple pairwise tests.

2.4. Proteomics of milk protein in clinical mastitis

2.4.1. Sample preparation for tandem mass tag labelled mass spectrometry

A proportional subset of 49 milk samples were selected for quantitative proteomics, which consisted of 14 with NG, 15 with Gram-negative bacterial and 20 with Gram-positive bacterial infections utilising the criteria of (i) only using samples with mastitis severity 1 or 2 and excluding severity score 3; (ii) equal numbers of samples from the bacterial infection with the largest number of samples in the Gram-negative and Gram-positive groups, being *E. coli* ($n = 10$) and *Streptococcus (S.) uberis* (n = 10) respectively; (iii) inclusion of samples with other Gram-negative infections, *Pasteurella multocida* ($n = 2$) and *Klebsiella oxytoca* ($n = 3$) and with other Gram-positive infection, *S. uberis* (n = 10) *S. aureus* ($n = 4$), *S. dysgalactiae* ($n = 6$); (iv) NG samples were selected from samples derived from udder quarters of severity scores 1 (n = 4) or 2 (n = 4) and from those were severity was not recorded (n = 6); (v) samples collected in the first period of January to May 2018.

The samples were prepared for quantitative proteomics with an ultra-centrifugation step (150,000 x*g*) for 60 min at 4 °C to reduce the casein content of milk whey samples (Mudaliar et al., 2016) modified by the use of 0.1 M triethyl ammonium bicarbonate (TEAB) buffer replacing PBS of the original procedure. The whey samples were subsequently prepared for tandem mass tag (TMT) proteomics using a ten-plex TMT kit (Thermo Fisher Scientific, Bremen, Germany) with conjugation of tryptic peptides within each samples to isobaric tags and analysed by LC-MS/MS using the Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) connected to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as previously described (Turk et al., 2021).

2.4.2. Proteomic statistics

The statistical analysis of the TMT proteomic results were processed as described previously (Turk et al., 2021). Briefly, Wilcoxon rank sum tests (Hollander et al., 2014) were used to compare the proteomic distribution locations of the Gram-positive samples against the rest of the samples. We evaluated both the unadjusted *p*-values and the p-values adjusted with false discovery rate (FDR) to control the family-wise Type I error rate in the ensemble of tests. The sample size was relatively small, with a large number of candidate proteins, many of which the accession names indicated that they are similar. FDR adjustment would have been too conservative in this context, possibly leading to promising candidate proteins being overlooked for further studies.

3. Results

3.1. Bacteriology results

A total of 94 milk samples from udder quarters where CM was observed were collected with a summary of the bacteria identified and the severity of the condition detailed in Table 1. The bacterial determination yielded samples with NG ($n = 21$), Gram-negative ($n = 44$) and Gram-positive ($n = 29$) results. The majority of the Gram-negative infections were caused by *E. coli* ($n = 31$), while there was a spread of Gram-positive infections with *Streptococcus uberis* being the largest group ($n = 12$) followed by *Streptococcus dysgalactiae* ($n = 6$) and *Staphylococcus aureus* ($n = 5$). The severity score was recorded in 87% (82/94) of the samples. Of the ones recorded, the score was the highest in the Gram-negative group of which 10 samples were classified as severe while only 1 sample with Gram-positive growth originated from a severe case and no cases of the NG group had this degree of severity. The severity score 3 samples were excluded from the subsequent analysis, to avoid confounding between the Gram-negative pathogens and disease severity. In addition, targeted treatment of Gram-negative CM is only recommended for non-severe cases.

Table 1
Bacteriology results for 94 bovine milk samples from quarters with clinical mastitis of varying severity. Proportion of samples that were no growth (NG), gram positive or negative is indicated in second column.

	Number of samples (% of total sample number)	Severity score				Pathogen detected (73)
		Mild (1)	Moderate (2)	Severe (3)	Not recorded	
No growth	21 (22%)	7	8	0	6	Not Applicable
Gram negative	44 (47%)	13	21	10	0	*Escherichia coli* (31) *Klebsiella pneumoniae* (7) *Klebsiella oxytoca* (4) *Pasteurella multocida* (2) *Streptococcus uberis* (12) *Streptococcus dysgalactiae* (6)
Gram positive	29 (31%)	8	14	1	6	*Staphylococcus aureus* (5) *Staphylococcus equorum* (2) *Staphylococcus epidermis* (1) *Staphylococcus sciuri* (1) *Staphylococcus haemolyticus* (1) *Trueperella pyogenes* (1)

3.2. Cathelicidin antibodies and ELISA

Two of the HuCAL antibodies to bovine CATHL, #15480 and #15484 were selected on the basis of western blots to be used in development of the CATHL ELISA and an example of a western blot with anti-bovine CATHL #15480 is shown in Supplementary Fig. S2 showing the reaction of antibody #15480 to 16 samples of milk from clinical mastitis cases and a sample of healthy milk, antibody #15484 gave a similar pattern of reactions on western blotting with milk from cases of CM. Both antibodies exhibited (Fig. S1, supplementary information) cross reactions with protein of 16 kDa and 30 kDa likely to be monomer and dimers of the precursor of bovine CATHL. Using these antibodies to quantify CATHL in milk, produced an ELISA which with control samples in multiple assays ($n = 9$) giving inter assay CVs of 15% and 34% at CATHL concentrations of 49 μg/ml and 2.3 μg/ml respectively an intra assay CV of 13% based on 35 duplicate samples and a limit of blank of 0.53 μg/ml.

3.3. Biomarker analysis

3.3.1. Biomarker concentration in clinical mastitis milk

Biomarker concentrations from cases of CM are detailed, alongside results from healthy milk samples (determined as no CM, SCC $<200 \times 10^3$ cells per ml and CMT = 0, in Table 2 and Fig. 1). Box plots show the median, interquartile range, and range for results for Hp, CRP, LF, MAA, LA and CATHL. Biomarker concentrations were significantly higher in milk from cows with CM, regardless of culture results, than in milk from healthy cows (all *P*-values <0.001, after Bonferroni corrections for multiple tests). For the CM cases, some of the pairwise differences were significant for Hp, CRP, and LA; compared to Gram-negative samples, NG samples had lower Hp ($P = 0.01$) and higher LA concentrations ($P = 0.01$), and Gram-negative samples had higher CRP concentrations than Gram-positive samples ($P = 0.03$). No significant differences were found for LF ($P = 0.07$), MAA ($P = 0.54$), or CATHL ($P = 0.45$) between any of the groups of samples from clinical mastitis. The Spearman rank correlations (Table 3) between the biomarkers showed that the strongest correlation was between Hp and CATHL at 0.88, while most of the other pairwise correlations were moderately to strongly positive. LA was negatively correlated with all other biomarkers, ranging from −0.46 with LF, to a correlation of −0.82 with CRP.

The Western blot for BSA (Fig. 2) included milk samples from all groups of the CM samples. Within the 21 samples of NG bacterial test result, 4/21 (19%) samples were found with high concentrations of albumin estimated as being >1000 mg/ml, while in the Gram-negative and Gram-positive groups, 31/44 (70%) and 14/29 (48%) had this high concentration of albumin respectively (Table 4). Pairwise Fisher's exact tests showed that albumin concentrations of NG differed significantly from Gram-negative (Bonferroni corrected $P = 0.0004$) but not from Gram- positive (corrected P = 0.07) samples. Albumin concentrations in Gram-negative and Gram-positive samples did not differ (corrected P = 0.5).

3.3.2. Combination of biomarkers by classification tree in differential diagnosis of mastitis pathogen

The classification tree model (Fig. 3), based on the set of CM samples with disease severity = 1, 2, or NA, with NG ($n = 21$), Gram-negative ($n = 34$), Gram-positive ($n = 28$), showed that compared to the rest of the samples (Gram-negative and NG together), Gram-positive samples were associated with the following combination of biomarker concentrations: CRP < 9.5 μg/ml and LF ≥ 325 μg/ml and MAA < 16 μg/ml. A total of 64% (18/28) of the Gram-positive samples had this combination of biomarker concentrations, compared to 9% (5/55) of the other samples (Gram-negative and NG). Sensitivity of the tree model was thus 64%, the specificity was 91%, and the overall misclassification rate was 18%. The area under the ROC curve for this tree model is 0.836 (95% bootstrap confidence interval: 0.742; 0.917).

Table 2
Mean milk biomarker concentrations in clinical mastitis samples (non-severe cases only; n = 83 samples) and healthy controls (SCC < 200,000 cells/ml; $n = 29$). 95% Bootstrap percentile confidence intervals are in brackets.

	Hp (μg/ml)	CRP (μg/ml)	LF (μg/ml)	MAA (μg/ml)	LA (mg/ml)	CATHL (μg/ml)
No growth	369.76	10.60	880.13	264.30	0.50	169.19
(n = 21)	(198.89; 610)	(4.35; 18.16)	(603.05; 1209.67)	(36.79; 646.08)	(0.39; 0.60)	(108.29; 237.98)
Gram negative	787.67	14.92	683.83	258.32	0.29	239.75
(n = 34)	(535.62; 1119)	(10.57; 19.99)	(505.90; 892.39)	(49.51; 606.72)	(0.23; 0.35)	(174.02; 311.08)
Gram positive	685.56	7.82	895.61	74.33	0.39	201.11
(n = 28)	(388.73; 1018)	(3.77; 12.65)	(720.19; 1086.23)	(24.25; 142.74)	(0.32; 0.46)	(130.44; 277.55)
Healthy	0.17	0.09	298.88	0.59	0.84	<0.53
(n = 29)	(0.11; 0.24)	(0.08; 0.12)	(253.31; 355.64)	(0.32; 0.93)	(0.72; 0.96)	

Haptoglobin (Hp), C-reactive protein (CRP), Lactoferrin (LF), Milk amyloid A (MAA), α-lactalbumin (LA), Cathelicidin (CATHL).

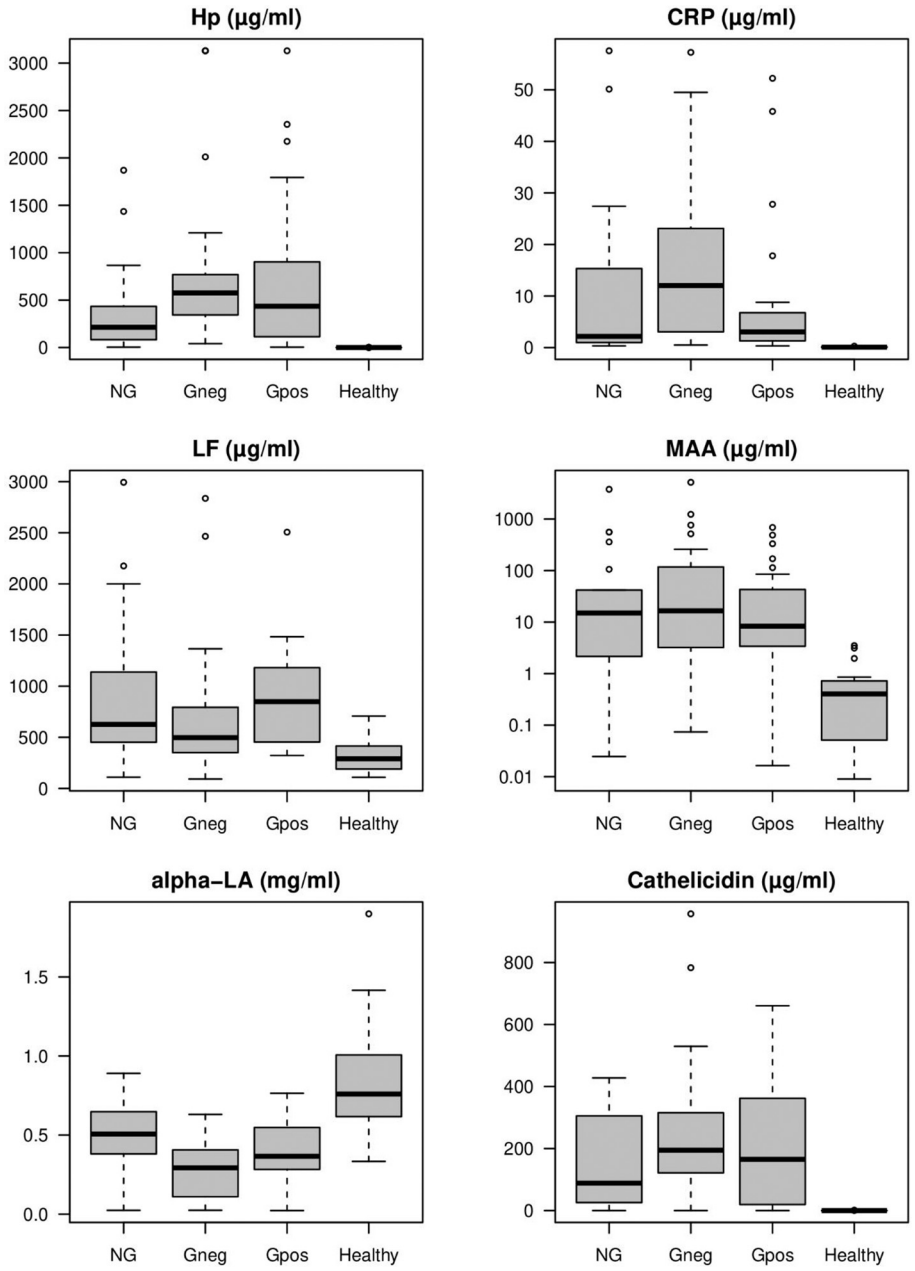

Fig. 1. Biomarker concentrations, haptoglobin (Hp), C-reactive protein (CRP), lactoferrin (LF), milk amyloid A (MAA), α-lactalbumin (LA), cathelicidin (CATHL), in all milk samples (n = 112) from udder quarters with clinical mastitis with no growth (NG); gram negative (Gneg), gram positive (Gpos) and healthy controls (Healthy).

Table 3
Spearman rank correlations between the biomarkers (non-severe clinical mastitis cases, n = 83; healthy controls, n = 29).

	Hp	CRP	LF	MAA	LA	CATHL
Hp	1.00	0.79	0.64	0.64	−0.81	0.88
CRP	0.79	1.00	0.47	0.53	−0.82	0.73
LF	0.64	0.47	1.00	0.51	−0.46	0.60
MAA	0.64	0.53	0.51	1.00	−0.54	0.66
LA	−0.81	−0.82	−0.46	−0.54	1.00	−0.71
CATHL	0.88	0.73	0.60	0.66	−0.71	1.00

Haptoglobin (Hp), C-reactive protein (CRP), Lactoferrin (LF), Milk amyloid A (MAA), α-lactalbumin (LA), Cathelicidin (CATHL).

3.4. Proteomic comparison of bacterial groups

Comparison of the distribution of bacterial cause of CM and the descriptive statistics for biomarker concentration in the samples (n = 49) selected for proteomics (Supplementary Tables S1 and S2 respectively) and the equivalent data for the whole set (Tables 1 and 2 respectively) demonstrate that the selected samples are representatives of the groups.

Overall, in the groups of milk samples from udder quarters with CM examined by TMT proteomic analysis, there were 931 proteins and 510 master proteins identified (Supplementary Excel Table S3). When the three groups of samples (Gram-negative (n = 15) or Gram-positive (n = 20) or no bacterial growth (n = 14)) analysed by proteomics, there was little difference between the groups in protein abundance. The comparisons that yielded the most informative results occurred when each

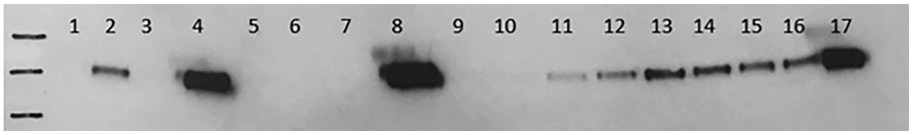

Fig. 2. Example of western blot analysis of milk from cows with varying health status and bacteria culture results with antibody to bovine serum albumin. Samples 1: healthy milk; 2: mastitis control on every gel; 3–10: no growth; 11–13: gram negative; 14–16: gram positive; 17: bovine serum albumin control on every gel. Samples in lanes 4 and 8 had visible signs of contamination from serum.

Table 4
Semi-quantitative Western blot analysis for serum albumin for 94 bovine milk samples from quarters with clinical mastitis divided based on their culture results (No growth, gram negative and gram positive). Number and proportion (in brackets) of samples based on their culture results (No growth, gram positive or gram negative).

Clinical mastitis group	Number of samples	Undetectable <30 μg/ml	30–1000 μg/ml	>1000 μg/ml
No growth	21	14 (67%)	3 (14%)	4 (19%)
Gram negative	44	8 (18%)	5(12%)	31 (70%)
Gram positive	29	8 (28%)	7 (24%)	14 (48%)

individual group was compared against the entirety of the remaining samples, e.g. Gram-positive vs Gram-negative + NG; Gram-negative vs Gram-positive + NG and NG vs Gram-positive + Gram- negative as shown in Supplementary Excel Table S4. In this case, there were 28 differentially abundant protein (DAP)s when each group was compared to the remaining samples (Table 5). Only one protein, NPC intracellular cholesterol transporter 2 was a DAP in the Gram-negative samples compared to all other samples. There were 23 proteins that were DAPs in comparing Gram-positive samples to all other samples, of which 13 were decreased in Gram-positive and 10 were increased. The former included alpha S1 casein, butyrophilin and β-lactoglobulin while the latter included ribonuclease A and related isoforms. In the comparison of samples showing NG of bacteria to all others, there were 9 DAPs, with increased abundance in 4 proteins, for example of alpha s1 casein, and 5 that were lower in NG such as chain X of β-lactoglobulin. The biomarkers, Hp, CRP, LF, MAA, LA and CATHL, measured by immunoassay (Table 2, Fig. 1) were detected by TMT proteomics, in some cases with multiple isoforms (Table 6 and Supplementary Excel Table S3) but were not differentially abundant between the CM groups.

4. Discussion

The first and main aim of the investigation was to identify a biomarker or combination of biomarkers, within clinical mastitic milk samples, that could differentiate the bacteria causing the mastitis, where present, as Gram-positive or Gram-negative. In line with the overarching aim of this research, which is to enable a more strategic approach to the provision of antimicrobial treatment, our study focused on leveraging biomarkers capable of distinguishing between Gram-positive from Gram-negative infections and/or Gram-positive from the rest of the samples (Gram-negative and NG); this could enable a reduction in overall AMU mitigate the emergence of AMR. While this aim was not achieved with respect to a single biomarker being able to provide such differentiation, the combination of results from multiple biomarker analysis may have this potential, and with it, the ability to multiplex a single diagnostic test for on farm use.

Whilst there had been a notable growth in interest for on farm diagnostic tests for mastitis and the emergence of a new diagnostic market for on-farm and point of care tests for mastitis, the majority of these are based on identification of a bacterial cause of the mastitis. Undertaken on farm these may remove the transport associated delay in receiving results, but they still take over 12 h and the inherent issues associated with bacterial contamination remain. Focusing on

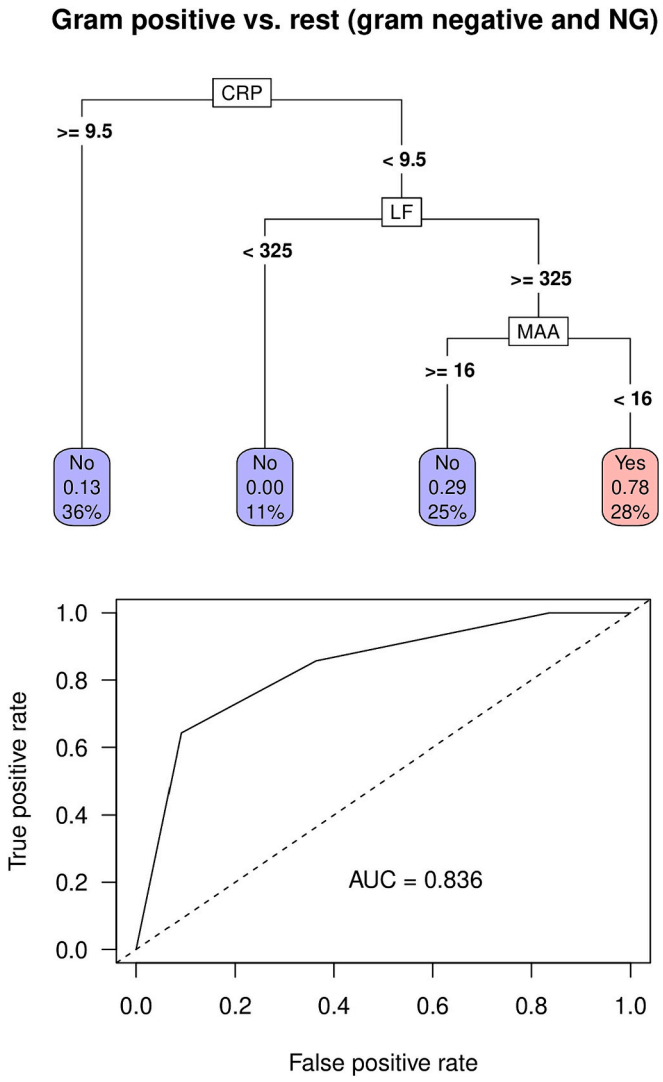

Fig. 3. Biomarker classification tree for clinical mastitis samples (n = 83), to predict gram positive pathogens vs. Rest (gram negative and no growth together). A blue box indicates that the majority of samples in the node are not gram positive (gram positive = No), while a red box indicates a majority of gram-positive samples (gram-positive = Yes). For example, the tree model predicts that samples in the left-most node are not gram-positive, as only 13% of these samples in our data are gram positive; 36% of all clinical mastitis samples ended up in this node. The area under the receiving operating characteristic curve (ROC) is 0.836 (95% bootstrap confidence interval: 0.742; 0.917). Sensitivity of the model is 64%, specificity is 91%, and overall misclassification error rate in our sample data is 18%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

endogenous protein biomarkers as this study has, addresses many of these challenges as their measurement can be undertaken within minutes utilising for instance, lateral flow immunochromatographic

Table 5
Summary of proteins that were differentially abundant ($p < 0.05$) in milk from cases of bovine CM when comparing $\log_2$ fold change (log2FC) between groups (red indicating increase and blue decrease) with gram negative, gram positive bacteria and NG following bacteriological culture. Full proteome results in Supplementary Excel Table S3.

Accession number	Description	log2FC GramNeg vs Gram Pos +NG	log2FC GramPos vs Gram Neg + NG	log2FC NG v Gram Pos +2 + Gram Neg
296482166	78 kDa glucose-regulated protein precursor		-0.182	
146386603	actin, cytoplasmic 1		-0.310	
385251867	actin, cytoplasmic 2		-0.310	
159793189	alpha S1 casein		-0.733	0.809
1387271778	alpha-S1-casein-3			0.748
741951254	apolipoprotein A-IV			-0.245
312893	apolipoprotein E		-0.239	
163126	beta-1-4 galactosyltransferase		0.244	
22655316	beta-actin		-0.338	
520	beta-lactoglobulin		-0.482	0.566
49259423	beta-lactoglobulin (chain X)			-0.617
12647311	butyrophilin		-0.383	
557804859	butyrophilin subfamily 1 member A1		-0.419	
146345391	complement factor B		-0.337	0.466
122144501	endoplasmic reticulum chaperone BiP		-0.182	
296482932	epididymal secretory protein E1			-0.293
257096531	heat shock 70 kDa protein 1-like		-0.182	
148887197	heat shock-related 70 kDa protein 2		-0.182	
1387193716	NPC intracellular cholesterol transporter 2	0.251		-0.298
674651038	osteopontin		0.415	-0.467
528941645	protein CREG1		0.260	
20663516	ribonuclease A		0.405	
573014712	ribonuclease A C2		0.352	
194320100	ribonuclease pancreatic		0.405	
194320101	ribonuclease pancreatic A		0.405	
809297	ribonuclease S (S-PROTEIN)		0.352	
157833719	RNASE S		0.352	
345101069	xanthine dehydrogenase/oxidase		-0.358	

platforms, which would reduce the waiting time for a result significantly below the 8 h that many dairy farmers have described as desirable (Griffioen et al., 2016). Whilst further research is required to draw together and refine a suitable biomarker panel and diagnostic algorithm, the results herein have highlighted a potential panel and diagnostic decision tree.

Of the six biomarkers measured in milk from dairy cows with CM, five (Hp, MAA, CRP, LF and CATHL) were all increased by a considerable amount, while LA was decreased in the presence of CM. These reactions to intramammary infection have been previously described (Eckersall et al., 2001; Pyörälä et al., 2011) although their relative increase in CM cases has not been explored in detail. Hp had the highest correlation to CATHL among the other biomarkers, which indicates a similarity in the reaction to the infecting bacteria while MAA, with the lowest correlations to the other biomarkers, would be indicative of a difference in the inflammatory mechanisms. While these biomarkers were significantly different from their concentrations in healthy milk, individual biomarkers were not significantly associated with the three categories of bacteriology results. Furthermore CATHL, a more recent addition to the mastitis biomarker panel, also did not show any individual benefit over the more established biomarkers in differential diagnosis in CM. The CATHL ELISA used in this study utilised antibody to a peptide sequence of SSEANLYRLLELD (aa49–61) common to CATHL 1–7 isoforms, but on the Western blot the antibodies react with protein in the region of 14 and 28 kDa. These are likely to be monomer and dimer of the precursor of bovine CATHL with a predicted Mw of 13,039. It is likely that the requirement of heating of diluted milk sample to enable the ELISA is related to the formation of such dimers and the location of the peptide sequence in an internal location in the precursor protein. It would also mean that immunoassays based on these antibodies would not likely be usable in on-farm testing if a heating step would be necessary.

Each biomarker showed large variation in concentration, for instance with Hp varying from 3.5 to 3130 μg/ml and MAA from 0.016 to 5161 μg/ml across all the samples with CM. Turk et al. (2021) reported the concentrations of Hp and MAA (SAA-1) in subclinical and clinical mastitis and the concentrations found in our investigation were of the same order for clinical mastitis. It is possible that the dynamics of biomarker concentration changes are more aligned with the severity of the host response to infection rather than the causative agent. Given that severity can be observed clinically, biomarkers are not of significant diagnostic benefit in that regard.

As no single biomarker was appropriate for differential identification of the bacterial class involved in an individual mastitis case, a classification tree was fitted to determine if a combination of biomarker results could enhance the bacterial class identification. The classification tree model allows fitting of non-linear effects and complex interactions between the biomarkers, with fewer assumptions on the population distributions of the biomarkers than, for example, a logistic regression model. The final tree model showed that Gram-positive mastitis was associated with a combination of lower CRP, higher LF, and lower MAA concentrations than Gram-negative or NG cases. Moreover, the estimated biomarker thresholds for the tree splits can serve as cut-off points for diagnostic test devices with binary outcomes, such as lateral flow

Table 6
Known biomarkers of mastitis identified in proteomics but not differentially abundant when comparing samples from clinically affected quarters showing NG, gram negative or gram positive growth on bacterial culture.

Accession	Description	Sum PEP score*	Coverage [%]	Peptides	Unique peptides
296,486,410	TPA: serum albumin precursor [*Bos taurus*]	1054	82	56	14
229,552	albumin	815	72	46	4
529,482,051	Chain A, Serum albumin	1054	85	56	14
11,610,591	alpha lactalbumin [*Bos taurus*]	298	75	12	12
134,104,332	Chain B, Alpha-lactalbumin	298	87	12	12
296,474,766	TPA: cathelicidin-1 precursor [*Bos taurus*]	66	50	7	7
296,491,742	TPA: cathelicidin-4 precursor [*Bos taurus*]	32	31	3	3
1,168,625	RecName: Full = Cathelicidin-3;	13	18	3	2
1,708,945	RecName: Full = Cathelicidin-6;	12	25	3	1
183,240,984	cathelicidin 7 [*Bos taurus*]	16	21	3	1
296,491,743	TPA: Cathelicidin 1-like [*Bos taurus*]	66	50	7	7
183,240,946	cathelicidin 2, partial [*Bos taurus*]	31	33	5	3
183,240,986	cathelicidin 7 [*Bos taurus*]	16	21	3	1
208,969,128	cathelicidin 6 [*Bos taurus*]	12	25	3	1
408,928	lactoferrin [*Bos taurus*]	631	62	41	1
193,299,659	lactoferrin [*Bos taurus*]	647	62	41	1
255,762,013	lactoferrin precursor, partial [*Bos taurus*]	647	63	41	1
157,830,374	Chain A, Lactoferrin	647	64	41	1
122,137,096	RecName: Full = Haptoglobin; Contains: RecName:	2	2	1	1
296,477,882	TPA: haptoglobin precursor [*Bos taurus*]	2	2	1	1
218,963,155	serum amyloid A 3, partial [*Bos taurus*]	24	29	4	4
79,158,764	Serum amyloid A 3 [*Bos taurus*]	24	24	4	4

* Data for PEP Score, Coverage [%], Peptides, Unique Peptides are from the group with highest abundance.

devices.

Classification tree models are however prone to over-fitting. Cross-validation avoids over-fitting by balancing goodness-of-fit to the sample data set with ability of the model to correctly classify future data sets. Another issue, which is more difficult to address, is that strongly correlated biomarkers contain similar information about the inflammatory processes and may substitute one another in the tree model for different subsets of the data. Therefore, while the final tree is representative of our sample data, a different sample from the same study population may lead to a tree model with splits on slightly different thresholds, on perhaps a different combination of the biomarkers. Larger, confirmatory studies are needed to validate these classification tree results. The classification tree model also attaches equal cost to a false positive classification (i.e. predicting Gram-positive when the sample is Gram-negative or culture negative) and a false negative classification (i.e. predicting not Gram-positive when the sample contains Gram-positive pathogens). Economic and welfare cost-benefit analyses are required to determine the relative costs of these two types of classification errors, to refine the biomarker threshold values used in the tree model splits. As discussed by (Malcata et al., 2021), the weighting of false-positive and false-negative implications may differ between societies, whereby some would prefer to minimise AMU at the risk of withholding treatment from infected cows whereas others would prefer to minimise the possibility of leaving cows untreated at the risk of contributing to AMU and concerns about public health impacts of AMR.

Although not analysed by an established biomarker immunoassay, the presence or absence of bovine serum albumin in milk from dairy cows with CM may provide information on differential diagnosis. Western blotting of the milk samples from CM cases revealed two distinct patterns of reaction with specific antibody to albumin. It was noticeable that the NG group had significantly more samples (14/21 = 67%) in which albumin was not detectable than the Gram-positive (28%) or the Gram-negative (18%) groups. In a few samples ($n = 4$) in the NG group there was visible discolouration associated with major influx of serum into the mammary gland and if these were excluded from the observed pattern then the proportion of NG samples with negligible albumin was even higher (14/17 = 83%). As a step towards limiting mastitis treatment to Gram-positive cases and narrow-spectrum antimicrobials, which would be desirable from an antimicrobial stewardship perspective, the identification of NG samples might help to reduce AMU in areas where broad-spectrum products are used routinely, or where people are hesitant to leave any bacterial infection untreated.

To identify additional biomarkers, a subset of milk samples was submitted for proteomic analysis. Importantly, the composition of the subset was comparable to the full sample set, in terms of the bacterial cause of disease and the distribution of biomarker concentrations (as shown in the supplementary information Tables S1 and S2). The majority of the biomarkers measured by immunoassay and discussed above were detected in the proteomic analysis but they were not present in the list of DAP when the bacterial groups were compared. This was to be expected considering that the immunoassays did not reveal statistically significant differences between groups.

Of the 28 DAP that were suggestive of some difference between CM groups, this was reduced to 17 when isoforms were eliminated and the majority of these were in the group of Gram-positive against the rest. Attempts were made to identify an antibody that would react with these bovine proteins (β-lactoglobulin and RNAse) but neither could be validated as the antibodies either showed no reaction or gave multiple non-specific bands following Western blotting. Of these, RNAse may be a protein worthy of further investigation as six different isoforms were apparent in the proteomics investigation that were suggestive as DAP. Furthermore, bovine RNAse, as a secretory protein involved in host defences (Rosenberg, 2011), has anti-bacterial activity (Cho and Zhang, 2007) that may support its role in the host response to mastitis. There were other proteins identified in the proteomic investigation that showed large variation in all the sample groups, including forms of the biomarkers described above but also further proteins which are expressed in response to mastitis. Although the biomarker assays showed significant differences between CM groups for Hp, CRP and LA these were not detected by the TMT proteomics probably due to the lower sensitivity of the TMT methodology using relative abundance to a pooled control, compared to the use of immunoassay for specific proteins. As with the measured biomarkers, it could be that these show the

different stages of the infectious process and these are likely to be variable given that the samples derive from naturally occurring CM cases from dairy herds, a population reflective of one for which a diagnostic mastitis biomarker panel would be applied, rather than an experimental study. This highlights both the necessity to investigate biomarkers in 'real-time' and the complexities of the overlapping of time-course of infection and bacterial cause. Price, labour, and reliability of this proteomics approach are not yet suitable for practical use. As a research paper, the exploration of convenience characteristics and reliability was not within the scope of this study series at this stage. Once a biomarker panel and a suitable diagnostic method, such as a lateral flow assay, have been developed, it would be appropriate to investigate these aspects. Further considerations on this topic have been discussed more broadly in a publication by Malcata et al. (2020).

The increased momentum towards investigating endogenous biomarkers, differentially abundant between clinically mastitic milk of differing bacterial aetiologies, is notable with other early work highlighting protein kinase C-binding protein NELL2, thrombospondin-1, and complement factor I as possibly diagnostic targets for differentiating staphylococci and streptococci intramammary natural infection and inflammation (Maslov et al., 2023). It is notable that like the current study, natural infection was used, and whilst there are differences in biomarkers highlighted, further work is indicated to characterise the mastitomic changes across different bacterial infection types and crucially, where mastitis is present but where no bacterial growth is cultured. This may provide more accurate estimates of the capacity of host responses to eliminate invading bacteria and provide information on the appropriate timing of initiation and discontinuation of antimicrobial treatment or supportive treatment such as anti-inflammatory treatment.

5. Conclusion

Although the investigation of six biomarkers of bovine mastitis did not identify a single biomarker that can differentiate between the bacterial causes of clinical mastitis as Gram-positive, Gram-negative or culture negative, the combination of biomarker results identified in a classification tree model indicated that use of multiple markers such as a combination of CRP, LF and MAA may have the potential for such differentiation. While this will need more extensive assessment with more sample results included in the classification trees and additional biomarkers included, it should be possible to use multiplex analysis to provide an indication of the bacterial cause of the intramammary infection, thus enabling more directed antimicrobial therapy to reduce their use and limit resistance being developed.

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CRediT authorship contribution statement

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Declaration of competing interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

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