Final report

Biomarkers for reducing non-compliance in beef carcases

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Abstract

Beef carcase pH non-compliance is a complex meat industry problem leading to economic penalties for producers that may be avoided through early detection. This project was undertaken to help identify candidate protein biomarkers in the saliva of cattle that could be used as prognostic markers of pH non-compliance before slaughter or along the supply chain. Across three separate trials, saliva was collected from cattle four weeks before slaughter, then both saliva swabs and ultimate pH readings were taken from the same animals post-slaughter. Mass spectrometry-based proteomics was conducted on a subset of on-farm and post-slaughter saliva samples to identify and quantify differentially expressed proteins between pH compliant and non-compliant animals. A suite of 40 candidate biomarkers of pH non-compliance were identified. The proteins identified in this project can be used in protein biomarker assays or form part of a multidimensional prediction model that incorporates other physiological measurements. Application of these results may help identify animals at risk of pH non-compliance, providing industry the opportunity to select alternative management pathways pre-slaughter for susceptible animals.

Executive summary

Background

Beef that is classified as pH non-compliant is typically inferior in quality and represents a significant financial burden to the Australian beef industry. Diagnostic tests to identify animals at risk of pH non-compliance could be used by suppliers or processors to help reduce the level of non-compliance. Studies have investigated the relationship between pH non-compliance and factors such as animal physiological and psychological stress, feeding practices and environmental conditions. This project differs to other investigations, as it explores the potential of 'omic' technologies on-farm and pre-slaughter for early prediction of non-compliance in live cattle. Specifically, it sought to identify protein biomarkers that can be used on farm or during pre-processing to classify animals at risk of non-compliance, allowing alternative management of these animals and avoidance of penalties due to non-compliance.

Objectives

This project aimed to:

1. Identify biomarkers of non-compliance in the saliva of cattle prior to slaughter and how they can be used along the supply chain (including development of a prediction model).
2. Learn from producers and meat processors about the practicalities of implementing and willingness to pay for an early detection method.
3. Provide the requisite knowledge base and foundations for a non-invasive, targeted on farm detection biosensor (not actually develop the biosensor).

Methodology

A stakeholder engagement process was initiated to explore the experience of non-compliance in the industry and the desire and qualities of a tool for the detection of non-compliant animals. A systematic review was also conducted on reported candidate biomarkers, options for their detection and the current state of biosensor capability. This data helped inform the design of the subsequent biomarker trials in cattle. Saliva was chosen as a sampling method due to its non-invasive nature, grass-fed animals were targeted due to the higher incidence of non-compliance, and sampling was performed on-farm and post-slaughter to observe animals at different points in the production chain.

Three sperate field trials were conducted and saliva samples were collected from grass-fed animals at beef producer's properties four weeks before slaughter. Saliva was then collected from the same animals at different abattoirs immediately post-slaughter. In addition, ultimate pH was measured by MSA accredited chiller assessors. For the second and third trials, CSIRO personnel also took pH readings. Mass spectrometry-based proteomics was used to identify differentially abundant proteins in the saliva of non-compliant animals compared to compliant animals. Using different statistical analyses a panel of candidate biomarkers was developed. A schematic overview of the project is provided in **Fig. 1.**

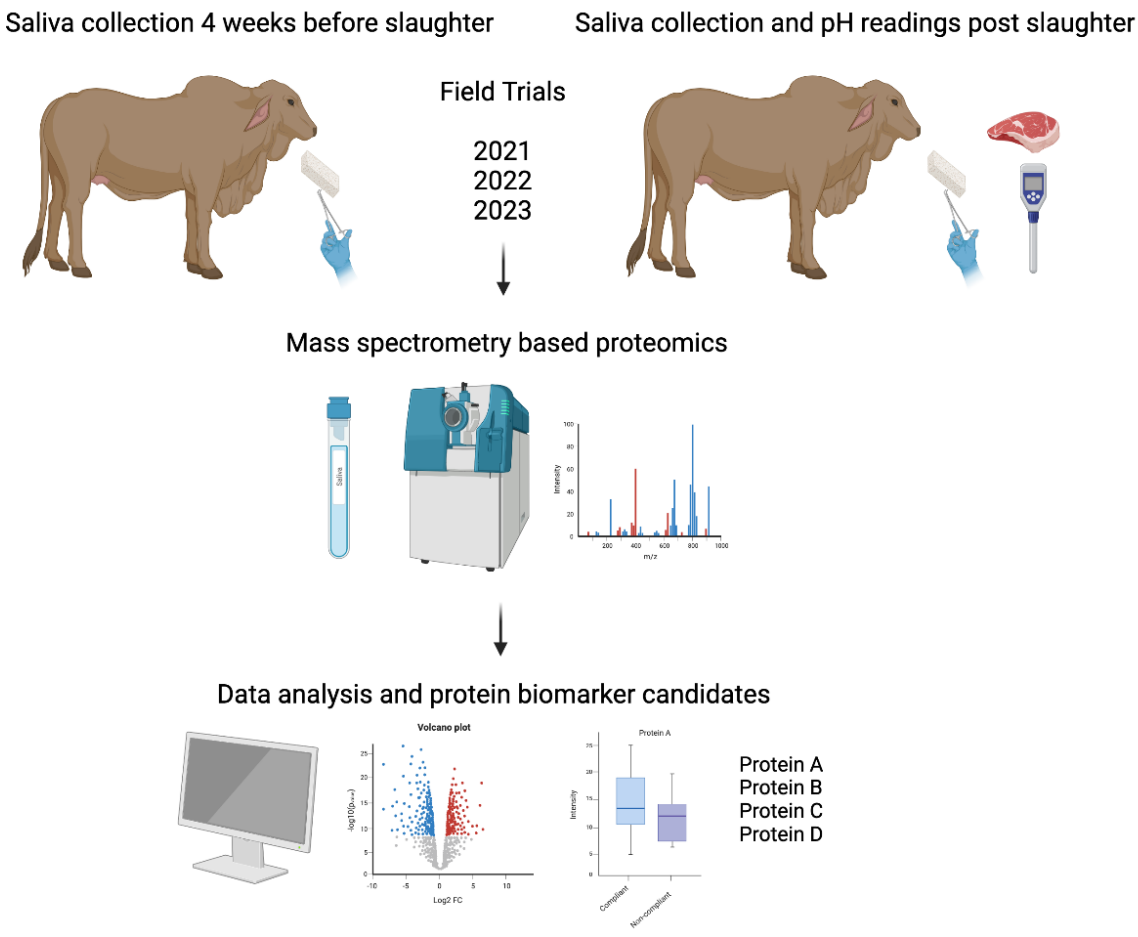

Figure 1. Overview of the project design.

Results/key findings

Forty protein biomarker candidates were identified across the three field trials. These proteins represent promising targets that could be combined as a panel for the prediction of risk of non-compliance in cattle.

Benefits to industry

The candidate proteins identified in this project may identify animals pre-slaughter that are predisposed to pH non-compliance. Identification of such animals will help reduce the incidence of dark cutting and associated economic penalties.

Future research and recommendations

- Engage with industry to determine acceptable cut-off points for the prediction of pH non-compliance. In particular, desired cut-offs for the ratio of non-compliant animals correctly classified (true positives) and the ratio of compliant animals that may be incorrectly classified (false positives).

- It is likely that more than one biomarker will be required to accurately predict non-compliance, in particular for applications requiring the identification of individual animals at risk.
- Pooled sample analysis could be investigated as a potential approach to examine the risk of non-compliance for cohorts.
- Access to samples from cohorts with high prevalence of clearly elevated ultimate pH will be required in the future to establish a higher degree of certainty.
- Functional assays may help identify underlying molecular mechanisms driving susceptibility of pH non-compliance in animals.

Table of contents

1. Background

Beef carcase pH non-compliance occurs when a carcase returns a high ultimate pH (pH_u) reading at grading (>5.70). Meat that is pH non-compliant is also known as "dark cutting" beef as it is typically darker in colour and poorer in quality. It is perceived negatively by consumers due to appearance, shelf life, cooking inconsistencies and results in significant income loss for beef producers and processors.

Non-compliance can fluctuate by producer based on many different reasons, e.g. feed type, age, location, breed, climate, management practices etc. In the 2019/20 financial year the average national non-compliance for pH was 4% of non-grain fed animals (although this number may be down weighted due to under-reporting). Critically, 25% of producers observed non-compliance greater than 11.7%, whilst 10% were greater than 27.3%. This indicates that while the rate of non-compliant carcases appears satisfactory at a national level, there are a significant number of producers (2,841) consigning approximately 8.2% of the total national slaughter (307,445 carcases) that receive non-compliance levels over 11.7%. Over the 2019/20 financial year, using an average $50/head deduction, this equates to $7.5M in lost income to producers.

It is generally accepted that low muscle glycogen levels predispose an animal to dark cutting, however the underlying triggers for perturbed glycogenesis and glycogenolysis pathways are complex and multifactorial. Meat quality can be influenced by pre-slaughter stress (Steel et al., 2021), nutrition status and feed composition (Steel at al., 2022), water supply and other physical and environmental conditions (McGilchrist et al., 2014). Despite this knowledge there is currently no pre-slaughter predictive or diagnostic measure of pH non-compliance in cattle. Our survey of the literature reveals preliminary evidence that suggests that dark, high pH meat contains specific proteins or small molecule biomarkers that differ from compliant meat (Nelis et al., 2022). Prognostic biomarkers would help industry predict which animals are likely to present with high ultimate pH readings. This would allow suppliers or processors to implement measures such as rest periods for at-risk animals to reduce animal stress and improve meat quality (Loudon et al., 2019).

This project aimed to assess if the proteins present in the saliva of cattle could be used to predict carcase pH non-compliance pre-slaughter or along the supply chain. Saliva sampling is minimally invasive and has been shown to be a valuable source of biomarkers in a range of disease states (Song et al., 2023). The protein biomarkers of interest identified in this project could be used in a protein biomarker assay (individually or in combination), or as part of a multidimensional prediction model that incorporates other physiological measurements with the overall aim of reducing the incidence of non-compliance in cattle.

2. Objectives

The project objectives are defined below and were met successfully:

1. Identify biomarkers of non-compliance in the saliva of cattle prior to slaughter and how they can be used along the supply chain (including development of a prediction model).
2. Learn from producers and meat processors about the practicalities of implementing and willingness to pay for an early detection method.
3. Providing the requisite knowledge base and foundations for a non-invasive, targeted on farm detection biosensor (not actually develop the biosensor).

3. Methodology

3.1 Producer and processor survey

A small number of processors and producers were interviewed for the initial stakeholder engagement. The interview process was reviewed by the CSIRO Social and Interdisciplinary Science Human Research Ethics Committee (CSSHREC) and ethics clearance was granted.

The initial consultation process used a qualitative methodology. Quantitative data analysis was not aimed for at this stage. The questionnaire used in the interview was developed by the research team based on prior experience with stakeholder consultation gained in other projects, including the ONPrime process, a guided customer engagement process in sprint format. The interviews were conducted in person or over the telephone after initial contact was made and agreement to participate in the interview process had been confirmed. Interviewees were identified from existing contacts through previous projects and from contacts through membership of the Southern Australian Livestock Research Council.

Interviews were transcribed into notes and summarised qualitatively.

3.2 Literature review of biosensor applications and biomarkers of stress

A literature search was conducted in Web of Science, using a range of keywords such as 'meat quality', 'dark cutting', 'non-compliance', 'biomarkers', 'stress markers' and related search terms. Relevant publications were filtered by iterative scanning.

3.3 Sample collection

After consultation with producers and meat processors a minimally invasive and robust sample collection and processing protocol was developed. In 2021, 2022 and 2023 saliva samples were collected from cattle on beef producer's properties and processors in Central QLD. In total, saliva samples were collected from 837 animals at different farms approximately four weeks before slaughter. Saliva was then collected from 827 of these animals at different processors immediately post-slaughter. In addition, pH_u readings were collected for all carcases.

3.3.1 On-farm sample collection

Animals were herded into a race and restrained in a crush with head bail. While each animal was restrained, saliva samples were collected. A sponge held between forceps was placed inside the animal's cheek, for at least 10–15s. The sponges containing saliva were placed into 60mL syringes and saliva samples were expressed directly into 5mL tubes and placed immediately into dry ice. The forceps holding the sponges were cleaned with 100% ethanol between each animal. All samples were stored at ~-80°C until proteomic analysis.

3.3.2 Post-slaughter sample collection

All abattoirs practiced Halal slaughter. Saliva samples were collected from each animal on the cradle after stunning and before the throat was cut. Saliva was collected with sponges as per the on-farm sampling protocol.

3.3.3 pH measurement

Carcases were graded by MSA-accredited Graders. For the second and third trials, CSIRO personnel also conducted independent pH measurements using a TPS-WP80M pH meter and electrodes (an MSA approved pH meter). The pH meter was calibrated for both pH (buffers at pH 7.00 and 4.00) and temperature.

3.4 Proteomic sample preparation

3.4.1 Protein quantification and extraction from study cohort samples

The protein concentration of saliva samples was determined prior to extraction using the colorimetric Bradford protein assay. Proteins (25µg) were then extracted using 200µL of buffer (8M urea, 2M thiourea, 4% CHAPS and 50mM dithiothreitol in 100mM Tris, pH 8.0). The sample was vortexed, sonicated for 5min in a sonic water bath, and shaken at 1000rpm for 30min at RT in a thermomixer. The supernatant was harvested following centrifugation (20,800 x *g*, 15 min).

3.4.2 Protein digestion

Protein (25µg) was subjected to filter-assisted sample preparation using 30kDa molecular weight cut-off filters (Millipore, Australia). Proteins were washed twice with 200µL of UA buffer (8M urea in 0.1M Tris-HCl, pH8) and buffers were removed using centrifugation (20,800 x *g*, 15min). Cysteine residues were alkylated with 50mM iodoacetamide for 30min at room temperature in the dark before washing with 200µL of UA buffer and removal through centrifugation. The buffer was exchanged using 50mM ammonium bicarbonate (pH 8.0) by two consecutive wash/centrifugation steps. Sequencing grade porcine trypsin (Promega, Alexandria, Australia) was added (protein-trypsin ratio 1:50) and incubated for 16h at 37°C. The filters were transferred to fresh centrifuge tubes and the filtrate (digested peptides) was collected following centrifugation (20,800 x *g*, 10min). The filters were washed twice with 200µL of 50mM ammonium bicarbonate and the filtrates were combined and lyophilised in a SpeedVac. The resultant peptides were re-suspended in 25µL of 0.1% formic acid (i.e. 1 µg/µL). Pooled samples were used as biological quality controls (PBQC) to monitor data acquisition performance and assess data structure and quality.

3.5 LC-MS data acquisition and analysis

3.5.1 Data acquisition

Peptides resulting from proteolytically digested saliva samples were analysed using a data-independent acquisition method with chromatographic separation on an Ekspert nanoLC415 (Eksigent, Dublin, CA, U.S.A.) directly coupled to the OptiFlow ion source of a TripleTOF 6600 LC-MS/MS (SCIEX, Redwood City, CA, USA). The peptides were desalted for 3min on a Trajan ProteCol C18 (3µm, 120Å, 10mm × 0.3mm) trap column at a flow rate of 10 µL/min 0.1% FA, and separated on a ChromXP C18 (3µm, 120Å, 150mm × 0.3mm) column at a flow rate of 5 µL/min at 30˚C. A linear gradient from 3-25% solvent B over 68min was employed followed by: 5min from 25% B to 35% B; 2min 35% B to 80% B; 3min at 80% B, 80-3% B, 1min; and 8min re-equilibration. The solvents were: (A) 5% DMSO, 0.1% FA, 94.9% water; (B) 5% DMSO, 0.1% FA, 90% acetonitrile, 4.9% water. The instrument parameters were: ion spray voltage 4500V, curtain gas 30psi, GS1 30psi and GS2 30psi, heated interface 150°C. MS accumulation time was set to 50ms, while MSMS spectra (100-1400 m/z) were acquired using 100 variable windows, where a set of optimised m/z windows was stepped across the mass range (400-1250 m/z), with a 1Th overlap, such that on average an equivalent ion current is distributed across each window. Each window was acquired for 25ms. PBQC samples were interspersed throughout each sample type to monitor acquisition quality.

3.5.2 Protein identification and quantification

Protein identification and quantitation were carried out using Neural Networks (DIA-NN) software v1.8.1 (https://github.com/vdemichev/DiaNN) using standard library-free settings. DIA-NN both creates and applies spectral libraries from sequence information in the process of extracting quantitative data from samples. The sequences provided to DIA-NN software included the contemporary Bos taurus proteome from uniport.org and common contaminant proteins from thegpm.org.

3.5.3 Statistical analyses

Protein abundance values from DIA-NN were $\log_{10}$ transformed and Pareto scaled. Multivariate unsupervised principal component analysis (PCA) was performed to assess data acquisition quality whereby quality is characterised by all PBQC samples showing low variance. T-test (≤ 0.05) and log fold change (≥ 1.5) were used to identify candidate biomarkers supported by p-values and measures of abundance perturbation. Multivariate orthogonal partial least squares discriminant analysis (oPLS-DA) was performed to identify robust proteins responsible for separating the pH compliant and pH non-compliant animals. The 25 most important features that were identified by oPLS-DA were reported as potential protein markers of pH non-compliance. The spectral library from DIA-NN was processed in PeakView (SCIEX) to quantify proteins before differences in protein abundances were determined with a linear mixed model (≤ 10^{-5}) using MSstats in R (Kerr et al., 2019). Receiver operating characteristic (ROC) curves were calculated in Metaboanalyst. GraphPad Prism v9.3.2. was used to perform statistical analyses and visualise the data.

4. Results

4.1 Producer and processor survey

Four processors were interviewed on the incidence and impact of pH non-compliance in beef carcases at their plants. One primary producer was formally interviewed, and another five had been previously involved in preliminary discussions on the topic. An overview of key findings is provided below:

- An early detection method for identifying pH non-compliant animals was of interest to all four processors.
- All processors indicated a decreased value for pH non-compliant carcases, which was passed back to the producer. The main financial benefit was identified by producers for grass-fed cattle directly sold to processors which would be MSA graded.
- Several of the processors work with their producer supply chain to try to minimise pH non-compliant carcases at processing. This includes management of stock between the farm, feedlot and processor. Interventions include nutrition, travel conditions, ensuring health and welfare throughout the journey, and some aimed to identify animals susceptible to stress.
- All processors highlighted ‘the earlier the detection, the better’.
- Producers showed interest in the ability to identify animals with an increased risk of non-compliance and identified the opportunity to hold at-risk animals over to mature further and go to slaughter with following consignments.
- In terms of sample type, most processors said it was ‘pretty easy to do a mouth swab’. With suggestions from on-farm to post-slaughter for sample collection.
- It was noted that the test needed to be cost-effective and relatively quick. One processor said they would expect to pay around $10 per test.
- One processor said that understanding more about the mechanism of pH non-compliance was of interest to them. This processor also indicated that a cost-benefit analysis would be

important to develop as an incentive for the 7,000 producers that supply to them to take on board an early detection test.
- Another processor said they are trialling cameras to see how animals act when they arrive at the facility and they can see this linking nicely with the biomarker test.

4.2 Literature review of biosensor applications and biomarkers of stress

A comprehensive literature review, on existing and emerging biosensor detection methods for potential biomarkers, was conducted. The review focussed on methodological aspects of providing pen-side processing of biosensor data and hence considered a wide range of potential biomarkers, including small molecules such as lactate and glucose which have already been investigated by other teams. The literature review was published in Comprehensive Reviews in Food Science and Food Safety (Nelis et al., 2022). An overview of key findings is provided below:

- Lactate, glucose and β-hydroxybutyrate concentration can be detected directly on-site during sampling from tail bleeds with cost-efficient and rapid commercially available devices that have been validated in cattle.
- For all other small molecule there is clear indications that these compounds can be present in various fluids, but for most compounds research is limited to the presence in blood and biological relevance in other fluids should be investigated.
- Diagnostics biomarkers of disease and stress that are observed haematologically are often found in the saliva, albeit at lower concentrations.
- The biomarkers of interest in this project would act in a prognostic fashion, in that they would be used for the prediction of high pH meat, as a forecasting tool.
- Some stressors facilitate biological and physiological changes in the saliva that are detectable post-stress. These stressors may be of a behavioural exposure (e.g. sheep shearing), artificial biochemically simulated (e.g. cattle adrenocorticotropic hormone or ACTH administration) or immune functionality (e.g. porcine virus exposure) nature and are likely to have different biomarker profiles based on the type of stress in which the animal is faced.

4.3 Sample collection and measurement

4.3.1 Sample size, animal characteristics and associated measurements

Collection of saliva samples was successfully performed in 2021, 2022 and 2023 from cattle on beef producer's properties and processors in Central QLD. Saliva samples were collected from a total of 837 animals four weeks before slaughter then collected from 827 of the same animals immediately post-slaughter. In addition, pHu was measured as per MLA standards. **Table 1** provides an overview of the three trials performed and a range of on-farm and post-slaughter animal information and measurements.

Table 1. Details of the animals sampled in this project and associated measurements.

	Trial 1 Aug/Sept 2021	**Trial 2** Sept/Oct 2022	**Trial 3** Jun/July 2023
On Farm			
Total animals sampled	161	245	431
Sex			
Steers	161	245	306
Heifers	0	0	125
After slaughter			
Total animals sampled	160	240	427
Compliance			
Compliant	147	211	410
Non-compliant	13	29	17
Non-compliance (%)	8%	12%	4%
Ultimate pH (pHu)			
Median pHu of total compliant	5.56	5.55	5.55
Median pHu of total non-compliant	5.82	5.83	5.76
Range pHu total compliant	5.39-5.70	5.43-5.70	5.39-5.70
Range pHu total non-compliant	5.72-5.95	5.72-6.69	5.71-5.86
Mass spectrometry-based proteomics			
Number of animals (on-farm and post-slaughter saliva samples were analysed for each animal)	26	58	57
Compliance			
Compliant	13	29	40
Non-compliant	13	29	17
Ultimate pH (pHu)			
Median pHu of compliant selected for proteomics	5.49	5.54	5.45
Median pHu of non-compliant selected for proteomics	5.82	5.83	5.76
Range pHu of compliant selected for proteomics	5.43-5.55	5.47-5.65	5.40-5.50
Range pHu of non-compliant selected for proteomics	5.72-5.95	5.72-6.69	5.71-5.86

4.3.2 Ultimate pH measurements for animals selected for proteomic studies

Post-slaughter pH_u reading were recorded for each animal. Comparative analyses were performed to assess the measured pH differences between the non-compliant and compliant sample groups used in the proteomic studies. As expected, the pH_u was found to be significantly ($P<0.0001$) different between the two experimental classes, with non-compliant animals presenting higher pH_u than compliant animals. The median pH_u in Trials 1, 2, and 3 for non-compliant animals was 5.82, 5.83 and 5.76, respectively. The median pH_u in Trials 1, 2, and 3 for compliant animals was 5.49, 5.54 and 5.45, respectively.

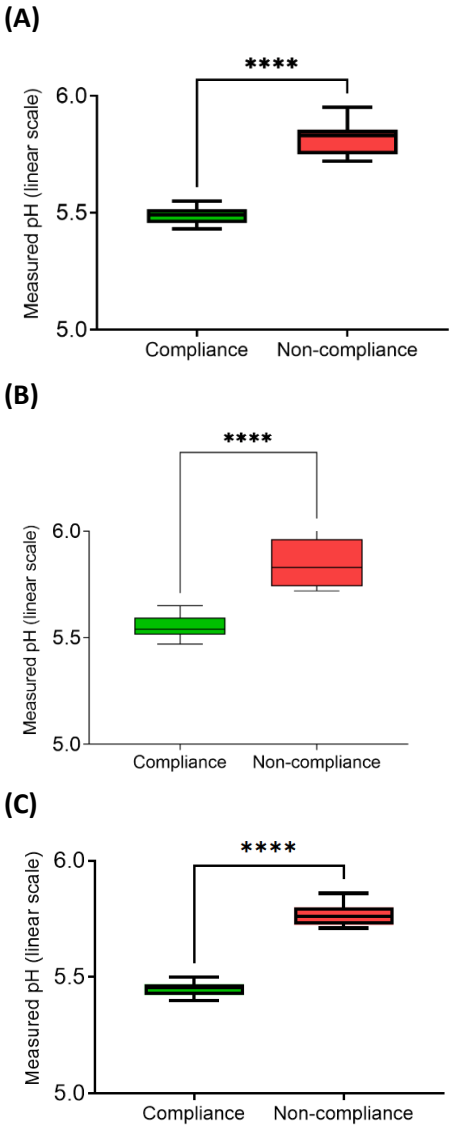

Figure 2. Comparison of measured pH value at grading between compliant and non-compliant animal samples using a Mann-Whitney test (two-tailed) (**** = p<0.0001). **(A)** Trial 1, n = 13 per group. **(B)** Trial 2, n=29 per group. **(C)** Trial 3, n= 40 compliant and 17 non-compliant.

4.4 Proteome measurements and comparisons

The proteomes of a subset of saliva collected on-farm and post-slaughter from each of the three trials were characterised by mass spectrometry based-proteomics using a data-independent acquisition method. This enabled relative quantification of proteins in each saliva sample. To assess instrument performance, PBQCs were prepared by aliquoting equal amounts of each digested sample into a single tube. The PBQC samples were analysed throughout the mass spectrometry acquisitions and were processed simultaneously with the experimental samples. In all analyses the results show excellent correlation (R^2=0.99) between a PBQC acquired at the beginning and at the end of the batch. An average of 2,566 bovine proteins were identified in the saliva samples across all three trials (**Table 2**) which is in line with other recent proteomic analyses of bovine saliva (Franco-Martinez et al., 2021).

Table 2. Sample number and confident protein identifications in each trial.

	Trial 1	Trial 2	Trial 3
Saliva samples analysed	26	58	114
Protein passing a false discovery rate cut-off of 1%	2,272	2,465	2,961

Proteomic data quality for all sample sets acquired was further evaluated by PCA (typical PCA provided in **Fig. 3A**). Tight clustering of PBQC samples was observed; however, the experimental samples showed substantial variance and limited clustering within compliance class. To facilitate the identification of biomarkers a supervised multivariate analysis method (oPLS-DA) was implemented (typical oPLS-DA provided in **Fig. 3B**). This method stratifies the compliant and non-compliant animals and reveals which variables (proteins) are responsible for class discrimination. While oPLS-DA can provide indications of variable importance, statistical testing permits the identification of candidate biomarkers supported by p-values. Both t-tests (≤ 0.05) and a linear mixed model (≤ 10^{-5}) were used in addition to log fold change calculations (≥ 1.5) to measure significant changes in protein abundance. As an example, proteins Protein 4 and Protein 18 were significantly different in non-compliant animals, exhibiting higher and lower abundance, respectively (**Fig. 4**).

(A) **(B)**

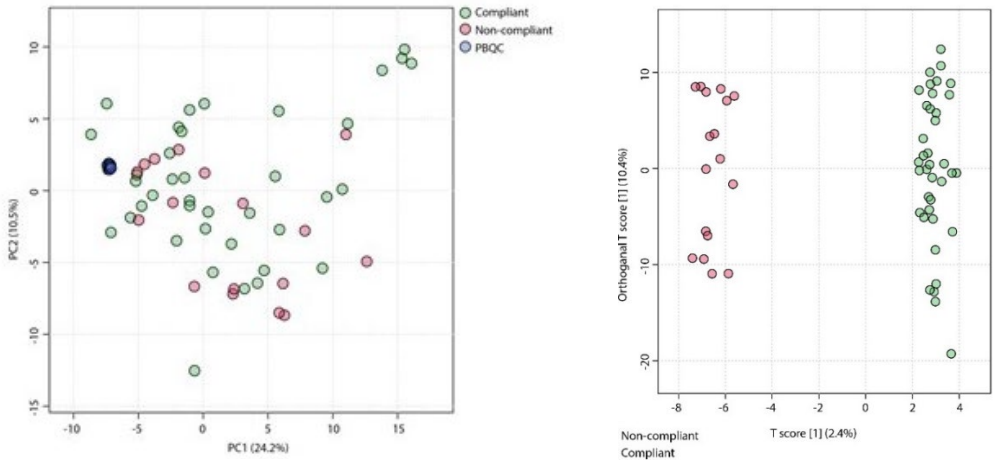

Figure 3. Proteome data quality assessment and predictive profiling of saliva samples from Trial 3 on-farm. **(A)** PCA shows clustering of PBQCs (Blue), but no clear separation of non-compliant and compliant samples. **(B)** oPLS-DA chart showing distinct separation of the experimental groups in the t-dimension (predictive component versus orthogonal component). Pink – non-compliant; green – compliant.

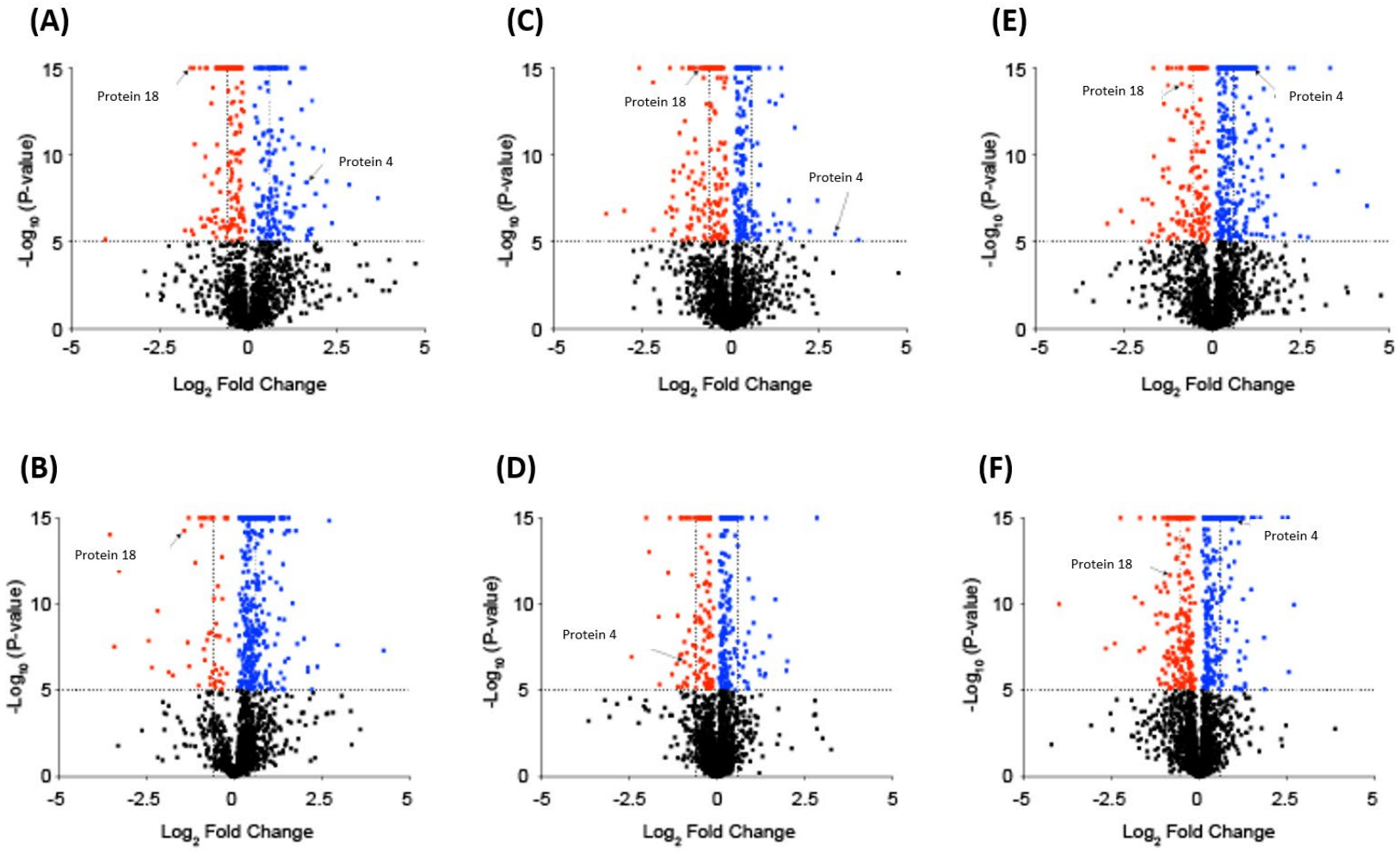

Figure 4. Volcano plots representing differential protein abundance between compliant and non-compliant samples. **(A)** Trial 1 on-farm, **(B)** Trial 1 post-slaughter, **(C)** Trial 2 on-farm, **(D)** Trial 2 post-slaughter, **(E)** Trial 3 on-farm, **(F)** Trial 3 post-slaughter. Blue = higher in non-compliant; Red = lower in non-compliant and Black = non-significant proteins. The horizontal dotted represents significance ($-\log_{10}$ p-value; ($\leq 10^{-5}$). The vertical dotted lines show the cut-off for fold change ($\log_2$ fold change; ≥ 1.5).

4.5 Protein candidate performance

Proteins that were identified using oPLSA-DA or that were differentially abundant and met both statistical and fold change thresholds were combined into a list of candidate proteins (934 proteins). To identify targets of high quality and performance a threshold was applied where the differentially abundant proteins must have been identified in at least two trials at the same time, on-farm or post-slaughter, with a fold change in the same direction. In this final list of 40 proteins (**Table 3**), 25 proteins were increased in abundance and 15 proteins were decreased in abundance in non-compliant samples. Proteins 4 and 5 were increased in abundance in saliva from non-compliant animals on-farm in all three trials. Protein 18 was decreased in abundance in saliva from non-compliant animals on-farm in all three trials. These three proteins therefore have high potential as biomarkers as they were identified in three independent experimental cohorts. In the post-slaughter samples, no proteins were observed to be consistently differentially abundant across all three trials. However, Protein 4, 5 and 18 were all identified at least once in post-slaughter samples further supporting their potential as prognostic biomarkers.

ROC curves (non-parametric) were generated for the 40 candidate proteins to determine biomarker performance. The area under the ROC curve (AUROC) was also calculated for all proteins and the values are represented in a heatmap in **Fig. 5**. Proteins 2, 5 and 9 performed well across trials on-farm with AUROC-values >59. In the post-slaughter samples, no proteins were observed to have consistently high AUROC values. To visualize performance the three well-performing proteins on-farm, the ROC curves for these three proteins are presented in **Fig. 6.** A ROC curve plot illustrates the ratio of true positives (*y*-axis) to false positives (*x*-axis) for each cutoff point. Curves closer to the top-left corner indicate better performance, as the true positive is closer to one and the false positive is closer to zero. For all three proteins, the true positive rate ranged between approximately 0.6-0.8 meaning 60-80% of animals will be correctly allocated as non-compliant. For all three proteins, the false positive rate ranged between approximately 0.2-0.4, meaning up to 40% of animals could be incorrectly assigned as non-compliant. These values represent an "optimal cutoff" and can be adjusted based on the preferences of beef producers, suppliers or processors. For example, if the strategy is to provide rest periods for at-risk animals a higher true positive rate might be preferable at the expense of more false positives as the animals will simply spend extra time feeding before processing. However, if the strategy is to pivot at risk animals into processing pathways for lower quality meats, then it may be preferable to keep the number of incorrectly classified compliant animals to a minimum.

The discovery of the candidate biomarkers herein is highly encouraging. The experimental design used industry samples rather than a highly controlled study animals and hence the samples represent within-cohort variation from a range of sources that are inherent to industry populations and processing including: diet, genetics, saliva composition and grading time post-slaughter. Nonetheless, a range of proteins have still been identified herein with significant differences and multiple lines of evidence that supports their function as biomarkers. This suggests that these proteins likely have substantial perturbation to have been identified. In summary, 40 candidate protein biomarkers were identified in this study. Five of these have high potential as they were consistently identified on-farm across the three trials or performed well as determined by ROC analyses. These proteins have varying functions, including cellular transport, energy production, and regulation of insulin metabolic pathways. Their functionality further supports the relevance of the candidates for the identification of animals at-risk of pH non-compliance.

Table 3. Protein candidates identified as increased (UP) or decreased (DOWN) in non-compliant animals.

	On-farm			Post-slaughter		
Protein	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 2
Protein 1	UP		UP	UP		
Protein 2	UP		UP			
Protein 3		UP	UP			
Protein 4	**UP**	**UP**	**UP**		**DOWN**	**UP**
Protein 5	**UP**	**UP**	**UP**	**UP**		
Protein 6		UP	UP	UP		UP
Protein 7	UP		UP	UP		
Protein 8	UP		UP			
Protein 9	UP		UP	UP		
Protein 10	UP		UP		UP	
Protein 11	UP		UP			
Protein 12	UP		UP			
Protein 13	DOWN		DOWN	UP		DOWN
Protein 14	DOWN	DOWN		DOWN	DOWN	
Protein 15		DOWN	DOWN			UP
Protein 16		DOWN	DOWN			
Protein 17	DOWN	DOWN		UP		
Protein 18	**DOWN**	**DOWN**	**DOWN**	**DOWN**		**DOWN**
Protein 19		DOWN	DOWN			
Protein 20		DOWN	DOWN	DOWN		DOWN
Protein 21	DOWN		DOWN			
Protein 22		DOWN	DOWN	UP		
Protein 23	DOWN	DOWN				
Protein 24		UP		UP	UP	
Protein 25	DOWN			UP		UP
Protein 26				UP		UP
Protein 27				UP		UP
Protein 28			UP	UP	UP	
Protein 29		UP			UP	UP
Protein 30				UP		UP
Protein 31				UP		UP
Protein 32				UP	UP	
Protein 33				UP		UP
Protein 34				UP		UP
Protein 35			DOWN	UP		UP
Protein 36		UP		UP	UP	
Protein 37				DOWN		DOWN
Protein 38				DOWN		DOWN
Protein 39				DOWN	DOWN	
Protein 40					DOWN	DOWN

	On-farm			Post-slaughter		
Protein	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 2
Protein 1	0.73	0.51	0.71	0.64	0.51	0.57
Protein 2	**0.68**	**0.60**	**0.75**	**0.51**	**0.52**	**0.56**
Protein 3	0.56		0.53			0.59
Protein 4	0.55			0.65		0.58
Protein 5	**0.72**	**0.63**	**0.71**	**0.77**	**0.56**	**0.53**
Protein 6	0.60			0.61		0.62
Protein 7	0.65	0.52	0.52		0.67	0.62
Protein 8	0.66	0.54		0.56	0.57	0.52
Protein 9	**0.69**	**0.59**	**0.78**	**0.72**	**0.55**	**0.53**
Protein 10	0.62			0.70	0.58	0.60
Protein 11	0.53	0.51	0.78	0.62	0.56	0.51
Protein 12	0.66	0.53	0.58	0.65	0.57	0.55
Protein 13	0.56	0.51	0.56			0.60
Protein 14	0.59	0.68	0.51	0.62	0.65	0.55
Protein 15	0.52					0.52
Protein 16						0.61
Protein 17	0.65			0.61	0.54	0.58
Protein 18	0.67	0.73	0.54	0.67	0.55	0.64
Protein 19	0.53					0.56
Protein 20	0.63	0.60	0.63	0.64	0.51	0.72
Protein 21	0.60	0.63	0.76	0.61	0.52	0.53
Protein 22	0.67					0.62
Protein 23	0.56	0.61	0.54	0.59	0.54	0.56
Protein 24	0.51			0.65		
Protein 25	0.65					0.54
Protein 26	0.66	0.55	0.64	0.53	0.58	0.68
Protein 27	0.56		0.51			0.57
Protein 28	0.54	0.61	0.51			0.60
Protein 29			0.70		0.63	0.56
Protein 30	0.56	0.53	0.57	0.62	0.61	0.57
Protein 31	0.61		0.55	0.57	0.50	0.51
Protein 32	0.67					0.55
Protein 33	0.65				0.62	0.58
Protein 34	0.54	0.50	0.56	0.57	0.64	0.55
Protein 35	0.61					0.56
Protein 36	0.51	0.66	0.51	0.53	0.60	0.57
Protein 37	0.62	0.62	0.62	0.72	0.52	0.70
Protein 38	0.58		0.70	0.72		0.62
Protein 39	0.53					0.53
Protein 40						0.54

Figure 5. Heatmap of area under the ROC curve (AUROC) calculations for all 40 candidate biomarkers. Higher AUROC-values (blue) have better predictive value and AUROC-values under 0.5 were not reported and are therefore blank.

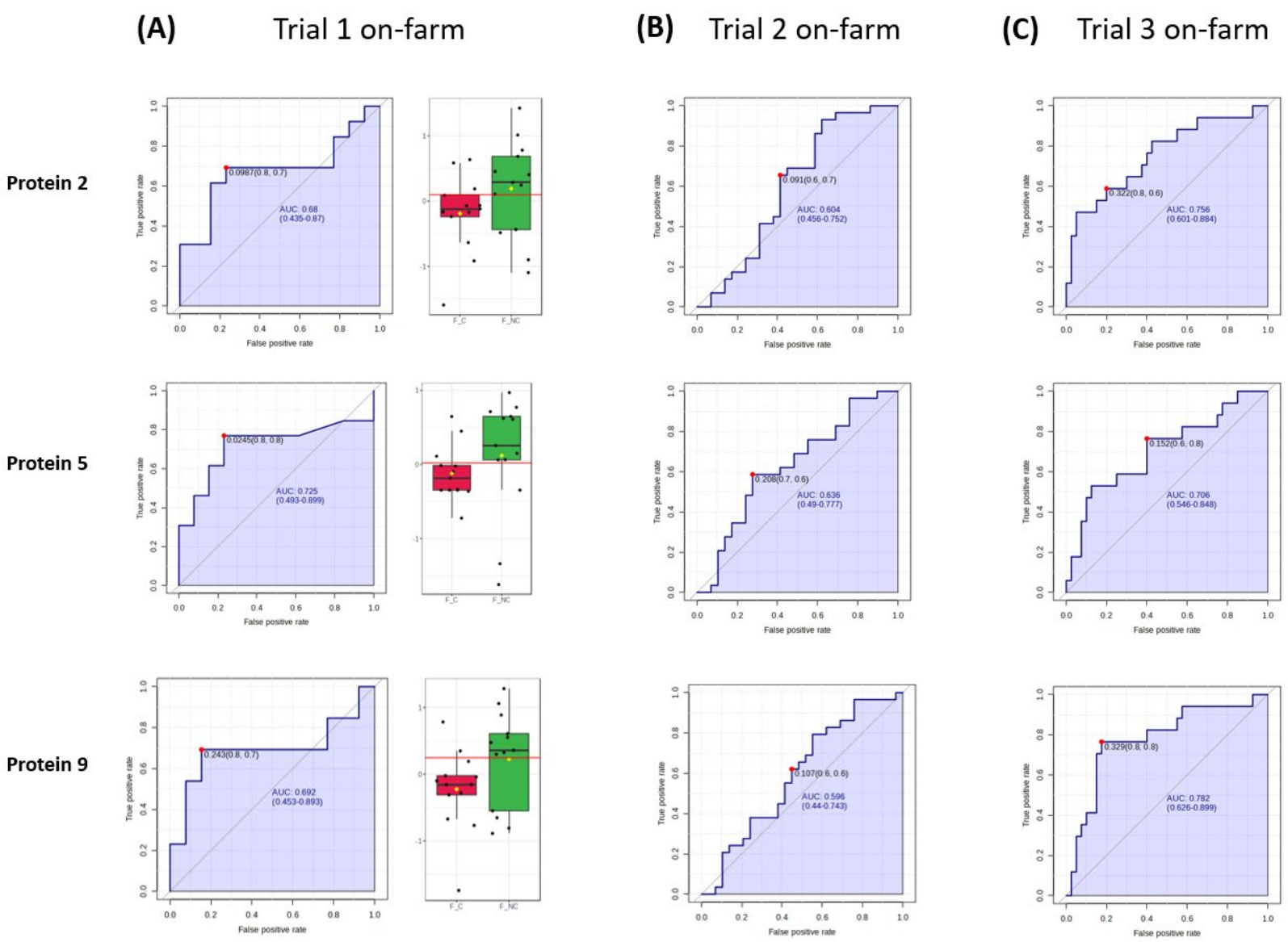

Figure 6. ROC curves for three candidate biomarkers. The ratio of true positives (*y*-axis) to false positives (*x*-axis) are represented and the red dot indicates an "optimal" cutoff point. **(A)** Trial 1 on-farm, **(B)** Trial 2 on-farm, **(C)** Trial 3 on-farm. A box plot (green non-compliant) is presented in (A) to visualize the cut-off point (red) and the abundance of the protein for individual animals (black

5. Conclusion

The overall results of the project are encouraging. After consultation with producers and meat processors a minimally invasive and robust sample collection and processing protocol were established. In addition, the project team has published a comprehensive review of the scientific literature summarising published efforts to identify pH non-compliance/dark cutting biomarkers. The review confirmed that the analysis of saliva as a sample as well as the advanced prediction of non-compliance are highly novel approaches in this area. Collectively, the proteome analyses identified a panel of 40 candidate biomarkers. These were deemed to be of high quality and performance as they were identified in at least two independent experimental cohorts. Five of these have been ranked as having the highest potential based on presence and performance as a biomarker and may be used to predict pH non-compliance.

5.1 Key findings

1. Identification of 40 candidate proteins that can be included in prognostic tools for the identification of animals at-risk animals of pH non-compliance.
2. Saliva samples can be collected readily at pen-side and be used to produce high-quality measurements of animal proteins.
3. Informative biomarkers for non-compliance can be identified from saliva four weeks before slaughter.
4. Several biomarkers for non-compliance in saliva appear both at four weeks pre-slaughter and at slaughter.

5.2 Benefits to industry

The candidate proteins identified in this project may identify animals pre-slaughter that are predisposed to pH non-compliance. Identification of such animals will help reduce the incidence of dark cutting and associated economic penalties.

Possible use cases identified with industry partners over the duration of the project, and supported by the obtained key findings, include

- Identification of individual animals at high risk of non-compliance before or after transport to the processor, with the option for intervention, e.g. rest on feed before slaughter.
- Identification of cohorts with a high risk of non-compliance on average across the cohort, with the option for intervention, e.g. longer period on high quality feed before transport.

6. Future research and recommendations

Moving forward it is important industry is engaged to further determine preferences for an early detection method, and to assess willingness to pay. In addition, acceptable cut-off points for the prediction of pH non-compliance. These include desired cut-offs for the ratio of non-compliant animals correctly classified (true positives) and the ratio of compliant animals that may be incorrectly classified (false positives).

It is likely that more than one biomarker will be required to accurately predict non-compliance, in particular for applications requiring the identification of individual animals at risk. Pooled sample analysis could be investigated as a potential approach to examine the risk of non-compliance for cohorts. Access to samples from cohorts with high prevalence of clearly elevated ultimate pH will be required in the future to establish a higher degree of certainty and the application of these biomarkers for grain-fed animals will need to be assessed. Functional assays may help elucidate underlying molecular mechanisms driving susceptibility of pH non-compliance in animals.

7. References

Franco-Martínez, L., Muñoz-Prieto, A., Contreras-Aguilar, M. D., Želvytė, R., Monkevičienė, I., Horvatić, A., Kuleš, J., Mrljak, V., Cerón, J. J., & Escribano, D. Changes in saliva proteins in cows with mastitis: A proteomic approach. *Research in veterinary science* **2021**, *140*, 91–99. https://doi.org/10.1016/j.rvsc.2021.08.008

Kerr, E. D., Caboche, C. H., & Schulz, B. L. Posttranslational Modifications Drive Protein Stability to Control the Dynamic Beer Brewing Proteome. *Molecular & cellular proteomics* **2019**, *18*(9), 1721–1731. https://doi.org/10.1074/mcp.RA119.001526

Loudon, K.M.W.; Tarr, G.; Pethick, D.W.; Lean, I.J.; Polkinghorne, R.; Mason, M.; Dunshea, F.R.; Gardner, G.E.; McGilchrist, P. The Use of Biochemical Measurements to Identify Pre-Slaughter Stress in Pasture Finished Beef Cattle. *Animals* **2019**, *9*, 503. https://doi.org/10.3390/ani9080503

McGilchrist P., Perovic J. L., Gardner G. E., Pethick D. W., Jose C. G. The incidence of dark cutting in southern Australian beef production systems fluctuates between months. *Animal Production Science* **2014**, 54, 1765-1769. https://doi.org/10.1071/AN14356

Nelis, J. L. D., Bose, U., Broadbent, J. A., Hughes, J., Sikes, A., Anderson, A., Caron, K., Schmoelzl, S., & Colgrave, M. L. Biomarkers and biosensors for diagnosis of pH noncompliant, dark cutting beef predisposition, and welfare in cattle. *Comprehensive Reviews in Food Science and Food Safety*, **2022**, 21, 2391–2432. https://doi.org/10.1111/1541-4337.12935

Song, M., Bai, H., Zhang, P. *et al.* Promising applications of human-derived saliva biomarker testing in clinical diagnostics. *Int J Oral Sci* 2023, 15, 2. https://doi.org/10.1038/s41368-022-00209-w

Steel, C.C.; Lees, A.M.; Bowler, D.; Gonzalez-Rivas, P.A.; Tarr, G.; Warner, R.D.; Dunshea, F.R.; Cowley, F.C.; McGilchrist, P. Abattoir Factors Influencing the Incidence of Dark Cutting in Australian Grain-Fed Beef. *Animals* **2021**, *11*, 474. https://doi.org/10.3390/ani11020474

Steel, C.C.; Lees, A.M.; Tarr, G.; Dunshea, F.R.; Bowler, D.; Cowley, F.; Warner, R.D.; McGilchrist, P. Feedlot Factors Influencing the Incidence of Dark Cutting in Australian Grain-Fed Beef. *Animals* **2022**, *12*, 1989. https://doi.org/10.3390/ani12151989

8. Abbreviations

Table 4. Abbreviation List

Abbreviation	Description
µg	Microgram
µL	Microlitre
AUROC	The area under the ROC curve
DIA	Data-independent Acquisition
FASP	Filter-assisted sample preparation
oPLS-DA	Multivariate orthogonal partial least squares analysis discriminate analysis
PBQC	Pooled biological quality controls
PCA	Principal component analysis
pH_u	Ultimate pH
ROC	Receiver operating characteristic (ROC) curves
rpm	Revolutions per minute
RT	Room Temperature
SEM	Standard error of the mean
SWATH-MS	Sequential window acquisition of all theoretical fragment ion spectra mass spectroscopy
TRIS	Tris(hydroxymethyl)aminomethane
UA buffer	8M urea in 0.1M Tris-HCl, pH8