Serum proteomes of Santa Gertrudis cattle before and after infestation with *Rhipicephalus australis* ticks

Running title "Host proteomics and cattle tick resistance"

Ali Raza[1#], Benjamin L. Schulz[2], Amanda Nouwens[2], Lousie A. Jackson[3], Emily K. Piper[4], Peter James[1], Nicholas N. Jonsson[5], Ala E. Tabor[1,2#]

[1]Centre for Animal Science, Queensland Alliance for Agriculture & Food Innovation, University of Queensland, St Lucia 4072 and Dutton Park 4102, Australia

[2]School of Chemistry and Molecular Bioscience, University of Queensland, Brisbane 4072, Queensland, Australia

[3]Biosecurity Sciences Laboratory, Department of Agriculture and Fisheries, Brisbane 4108, Queensland, Australia

[4]Global Genetics Laboratory Operations and Customer Support, Zoetis, 333 Portage St., KZO300-210SE, Kalamazoo, MI 49007

[5]The University of Glasgow, Institute of Biodiversity, Animal Health and Comparative Medicine, Glasgow, G61 1QH UK

[#]*Corresponding authors:* a.raza@uq.edu.au; a.tabor@uq.edu.au

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Author contributions

AET and PJ conceived the research grants; AR undertook proteomics and analyses; AR, AET and NNJ drafted the manuscript; BLS supervised the SWATH analyses; AN conducted the mass spectrometry analysis; NNJ, EKP, and LAJ designed and conducted the Santa Gertrudis trial and sampling; EKP and LAJ undertook the immune analysis.

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Abstract

Aims

Previous studies have applied genomics and transcriptomics to identify immune and genetic markers as key indicator traits for cattle tick susceptibility/resistance, however, results differed between breeds, and there is lack of information on the use of host proteomics.

Methods and Results

Serum samples from Santa Gertrudis cattle (naïve and phenotyped over 105-days as tick-resistant (TR) or -susceptible (TS)) were used to conduct differential abundance analyses of protein profiles. Serum proteins were digested into peptides followed by identification and quantification using sequential window acquisition of all instances of theoretical fragment ion mass spectrometry. Before tick infestation, abundance of 28 proteins differed significantly (adjusted $P<10^{-5}$) between TR and TS. These differences were also observed following tick infestation (TR vs TS) with a further eight differentially abundant proteins in TR cattle, suggesting possible roles in adaptive responses. The intragroup comparisons (TS-0 vs TS and TR-0 vs TR) showed that tick infestation elicited quite similar responses in both groups of cattle, but with relatively stronger responses in TR cattle.

Conclusion

Many of the significantly differentially abundant proteins in TR Santa Gertrudis cattle (before and after tick infestation) were associated with immune responses including complement factors, chemotaxis for immune cells, and acute phase responses.

Keywords: Cattle, Santa Gertrudis, *Rhipicephalus australis,* Host resistance, Proteomics, Biomarker discovery

1. Introduction

Rhipicephalus microplus, commonly referred to as the cattle tick, is a species complex with five recognised clades (clades A, B and C, *Rhipicephalus annulatus,* and *Rhipicephalus australis* "the Australian cattle tick"). [1,2] Cattle ticks can cause direct effects on cattle through their feeding behaviour, including discomfort, skin damage, loss of milk and meat production, and anemia, as well as indirect effects via the transmission of tick fever pathogens including *Babesia* spp. and *Anaplasma marginale* reviewed by Hurtado, Giraldo-Ríos [3]. These pathogens cause serious illnesses in bovines, thereby reducing farm profitability and increasing costs

63 associated with livestock products. A recent estimate suggests that approximately 80% of the
64 world's cattle populations are at risk of ticks and tick-borne diseases, causing economic losses
65 of US$ 22-30 billion per year. [4] There are no recent estimates available for the economic losses
66 to the Australian cattle industry (dairy and beef), however, in 2015, it was reported that ticks
67 and tick-borne diseases cause annual economic losses of ~$AUD 161 million due to reduced
68 income and increased expenses. [5] Traditionally, acaricides have most widely been used to
69 control ticks across the world with considerable success. However, widespread acaricide
70 resistance, environmental contamination, increasing demand for drug-residue free animal
71 products, and the cost related to developing new acaricides limit the use of acaricides reviewed
72 by Rodriguez-Vivas et al [6]. In addition, implementation of biological and immunological
73 control strategies have had limited success. [7]

74 *Bos indicus* cattle carry 10-20% as many ticks as *Bos taurus* cattle, given the same challenge.
75 [8] Genetically controlled variation in tick numbers has also been shown within breeds, the trait
76 having a heritability of greater than 40%. [9,10] Tick-host interaction is a complex phenomenon
77 and tick resistance in hosts is a composite trait, involving many components including non-
78 immune components such as skin color and thickness, [11] and grooming behaviour. [12] The
79 adaptive immune components include variation in hypersensitivity reaction, [13] humoral, [8,14]
80 and cellular responses to tick attachment. [15,16] However, the role of host physical barriers and
81 immunological parameters in tick resistance is still poorly understood, as these responses differ
82 between susceptible and resistant breeds as well as within the same breed, as reviewed by Tabor
83 et al [17]. Several studies have attempted to identify genetic markers for the resistance of cattle
84 to tick burden, for example, protein-based analyses, [18] immunological methods, [19,20] genome-
85 wide analysis studies, [21,22] and quantitative trait analysis in tropically adapted genotypes. [23]

86 Comparative proteomics allows the investigation of the differences and similarities in health
87 and disease conditions between individuals, groups, breeds, and species, reviewed by Bilić et
88 al [24]. Previously, a comparative proteomics analysis indicated that five proteins including
89 epidermal structural proteins (keratin-5 and keratin-14), hair (keratin-33B), and chromatin
90 (H2A histone) structural proteins and lipocalin-9 were up-regulated in the skin of highly tick
91 resistant Belmont Red cattle. [25] This study used isobaric tags for relative and absolute
92 quantification (iTRAQ) analysis and detected very few differentially abundant proteins. The
93 authors concluded that protein concentration and degree of expression changes might be the
94 limiting factors for adequate quantification by this approach. A recent report suggested that
95 although iTRAQ is faster than sequential window acquisition of all theoretical ions mass

spectrometry (SWATH-MS), it is less sensitive, reliable and robust. [26] Unlike iTRAQ, SWATH-MS is a label-free technique and therefore does not limit the number of experimental groups. In addition, SWATH is a data-independent acquisition method, in which permanent records of the fragment ion spectra of a sample are measured independently and can be reexamined if the library used in the downstream application is updated. [27] Therefore, with limited numbers of clinical samples collected at multiple time points, SWATH analysis can identify significant proteins associated with a disease condition. In this study, we explored the potential of SWATH-MS to measure and quantify the relative abundance of serum proteins in cattle before and after exposure to ticks.

2. Methods

2.1. Serum Samples

Serum samples used in this study were collected from Santa Gertrudis (SG) heifers phenotyped as tick susceptible (TS) or tick resistant (TR) as reported by Piper et al [19]. Briefly, 35 SG heifers aged 12 months were acquired from a tick-free region of Australia. The cattle had no previous tick exposure and were vaccinated against tick fever pathogens (*Babesia bovis*, *Babesia bigemina* and *Anaplasma marginale*) four weeks before commencing the trial. For this trial, 30 animals were kept at the Pinjarra Hills facility which were divided into resistance status groups following an intensive tick infestation trial over 105 days, in which each animal was infested with 10,000 (0.5g) larvae of Non-Field Resistant Strain of *R. australis* weekly for 13 weeks in addition to the natural infestation in the tick-infested pastures. Six animals (tag IDs: 501, 679, 783, 809, 821, 825) with the consistently lowest tick counts were classified as "tick resistant (TR)", whereas the six animals (tag IDs: 607, 615, 629, 639, 797 and 907) with the highest tick counts were classified as "tick susceptible (TS)", and the remaining (18 animals) were classified as "middle". Blood samples were collected into 2×9 mL Vacuette® Z clot activator tubes at each time point and serum was harvested and stored at -20^{0}C for further use. The serum samples collected at day-0 (before tick infestation, referred to as tick-susceptible naïve (TS-0) and tick-resistant naïve (TR-0)) and at the end of the tick infestation trial (105 days post first infestation) when the animals were fully phenotyped, (referred to as fully phenotyped, tick-susceptible (TS) and tick-resistant (TR)) were used in this study.

2.2. Filter-aided sample preparation

Serum samples were denatured, reduced, and alkylated using Pierce concentrator 10K molecular weight cutoff (MWCO) columns (Thermo Fisher Scientific®, USA) as described

previously. [28] For each sample, protein concentration was measured by Nanodrop spectrophotometer (Thermo Fisher Scientific®, USA), 150 µg of total protein was denatured by adding 100 µL of 8*M* urea, 50 mM ammonium bicarbonate (ABC) at 45°C, and 600 rpm for 10 min using thermomixer (Eppendorf Thermomixer® C, Hamburg, Germany). The denatured sample was transferred to the top of 10K MWCO columns followed by centrifugation at 14,000 × g at room temperature (RT) until solution passed through the membrane (approximately 40 minutes) and 20 µL remain in the top of the MWCO column. Proteins were washed by adding 500 µL of the wash solution (8*M* urea and 50 mM ABC) followed by repeating the centrifugation step, after which the filtrate was discarded. Proteins were reduced by adding 200 µL of wash solution with 5 mM DL-Dithiothreitol (DTT) (Sigma-Aldrich®) and incubated at 56°C for 30 min. Cysteines were alkylated by adding iodoacetamide (IAA) to a final concentration of 25 m*M* and incubating for 30 min at RT in dark. The excess IAA was quenched by adding DTT to a final concentration of 5 m*M* followed by recommended centrifugation, the filtrate was discarded. Proteins were dissolved in 100 µL of 50 mM ABC and digested by the addition of 6 µg trypsin (Proteomics grade, Sigma-Aldrich®) and overnight incubation at 37°C in thermomixer with 400 rpm. The digested peptides were collected by centrifugation and the filter membrane was rinsed with 50 µL of 0.5 M NaCl and centrifuged. The two filtrates were combined, and trypsin digested peptides were desalted with C18 ZipTips (Millipore®, USA) following the manufacturer instructions. A pooled sample generated by taking 5 µL from each sample (before desalting), totalling approximately 120 µg peptides was subjected to fractionation using Pierce High pH Reversed-phase Peptide Fractionation kit (Thermo Fisher Scientific®, USA). Peptides were applied to a 20 mg of resins in a 1:1 water/ DMSO slurry, washed with 500 µL of LC-MS Grade water (Thermo Fisher Scientific®, USA) followed by elution in eight separate fractions of acetonitrile (500 µL for each 5%, 7.5%, 10%, 12.5% 15%, 17.5%, 20% and 50%) in triethylamine (0.1%). These eluted peptides were lyophilized and resuspended in 0.1% trifluoroacetic acid.

2.3. Mass Spectrometry

Peptides were measured by LC-MS/MS using a Shimadzu® Prominence nanoLC system with a TripleTOF 5600 mass spectrometer with a Nanospray III interface (SCIEX®) as described previously. [29] Approximately 2 µg (as estimated by ZipTip binding capacity) of peptides were desalted on an Agilent C18 trap (pore size 300 Å, particle size 5 µm, 0.3 mm i.d. × 5 mm) at a flow rate of 30 µL/ min for 3 min, followed by separation on a Vydac EVEREST reverse-phased C18 HPLC column (pore size 300 Å, particle size 5 µm, 150 µm i.d. × 150 mm) at a

flow rate of 1 µL/ min. Peptides were separated with buffer A (1 % acetonitrile / 0.1% formic acid) and buffer B (80% acetonitrile / 0.1% formic acid) with a gradient of 10-60% buffer B over 45 min. Gas and voltage were adjusted as required. MS-TOF scan across 350-1800 *m/z* was performed for 0.5 sec for data-dependent acquisition (DDA), followed by DDA MS/MS with an automated selection of top 20 peptides with intensity greater than 100, across 40-1800 *m/z* (0.05 sec per spectrum) using a collision energy of 40 ± 15 V. For data-independent acquisition (DIA) SWATH analyses, MS scans across 350-1800 *m/z* were performed (0.05 sec), followed by high sensitivity DIA mode using 26 *m/z* isolation windows for 0.1 sec, across 400-1250 *m/z*. Collision energy values for SWATH samples were automatically assigned by Analyst software based on *m/z* mass windows (SCIEX®).

2.4. Data analysis

Proteins from DDA data were identified using ProteinPilot software (SCIEX®5.02), searching against all bovine proteins in UniProtKB (downloaded 11 May 2020; 46754 total entries). The ID search settings were as follows: sample type = identification, cysteine alkylation = iodoacetamide, instrument = TripleTOF5600, species = *Bos taurus*, ID focus = biological modifications, digestion = trypsin, search effort = thorough ID. False discovery rate (FDR) was analyzed with limits of 99% confidence and 1% local FDR. Peptides with a confidence >99% were included in further analysis. An ion library from proteins identified with ProteinPilot was used to measure peptide abundance in each sample using PeakView 2.1 (SCIEX®), with settings: shared peptides = allowed, peptide confidence threshold = 99%, FDR = 1%, XIC extraction widow = 6 min, XIC width = 75 ppm. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [30] partner repository with the dataset identifier PXD020518. Statistical analyses were performed as described by Kerr et al [31] using ReformatMS and MSstats (2.4) in R [32], with Benjamini and Hochberg corrections to adjust for multiple comparisons and a significance threshold of $P < 10^{-5}$. Those proteins with a $\log_2$FC cut-off value of > 0.3 were included in further analyses. Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) was used to identify protein-protein interaction and enrichment analysis for gene ontology (GO) terms and biological pathways using Uniprot accession identifiers (IDs) of significantly differentially abundant (DA) proteins as a target list [33]. *Bos taurus* genome was used as background in the STRING analysis with the following basic settings: meaning of network edges as evidence; active interaction sources included were experiments, databases, co-expression, neighbourhood, gene expression and co-occurrence;

highest confidence (0.900) for the minimum required interaction score, and *k*-means clustering with the number of clusters set at 3.

3. Results

3.1. Protein identification

Proteins were identified from DDA LC-MS/MS of high pH fractionated pooled samples and unfractionated individual samples of trypsin digested proteins from TS and TR cattle before (tick naïve: day 0) and after (fully phenotyped) exposure to cattle ticks. A total of 223 unique proteins were identified by ProteinPilot software (SCIEX®5.02) (Table S1). SWATH-MS was used to measure the relative abundance of each protein within each individual, unpooled sample,, quantifying 106 proteins by PeakView 2.1 (SCIEX®) with an FDR cutoff of 1% (Table S2). The serum proteomes of the two groups of cattle (TS & TR) were compared at two time points (before and after exposure to ticks) to explore the differences in serum proteomes before infestation (intergroup comparison TS-0 vs TR-0); determine the effect of tick exposure on cattle serum proteomes (intragroup comparisons TS-0 vs TS and TR-0 vs TR), and identify the differences in proteomes of TS and TR cattle in response to exposure to cattle tick (intergroup comparison TS vs TR).

3.2. Serum proteomes before tick infestation

The comparison of constitutive serum proteomes of TS-0 and TR-0 cattle before exposure to cattle ticks (samples collected at day 0) identified a set of 28 proteins which were significantly different in abundance between the two groups of cattle ($\log_2FC > 0.3$) with 21 proteins having significantly higher, and 7 proteins significantly lower abundance in TR-0 cattle than TS-0 group of cattle (Table 1; Figure 1). Based on the $\log_2FC$ values, the top three significantly abundant proteins in TR-0 cattle included SERPIN-A10 (A5PJ69), alpha-1-acid glycoprotein (Q5GN72) and coagulation factor V (F1N0I3). Three complement proteins (complement 5a anaphylatoxin (F1MY85), complement 8 beta chain (F1N102) and complement 9 (Q3MHN2)) were also among the significantly differentially abundant proteins. In TS-0 cattle, gelsolin (F1N1I6) and two uncharacterized proteins (immunoglobulin V-set domain (G3MZH0) and transferrin domain (E1BI82)) were among the top three highly abundant proteins.

The STRING interaction map of highly abundant proteins in TR cattle showed strong connectivity for the proteins involved in immune response and lipid metabolism, which were grouped using *k*-mean clustering analysis (Figure 2). Based on the *k*-mean clustering, three

complement proteins (C5a, C9 and C8) and vitronectin showed strong interaction and were grouped in one cluster. Similarly, the second *k*-mean cluster contained apolipoproteins (APOA-I, APOA-II, APOB), SERPIN A-10, coagulation factor V (F5) and an uncharacterized protein (belonging to the alpha 2 macroglobulin domain). Also, two acute-phase response proteins leucine-rich alpha-2-glycoprotein-1 (*LRG-1*) and alpha-1-acid glycoprotein (*ORM-1*) were strongly connected in a separate cluster. The seven highly abundant proteins in the TS-0 group did not show any predicted functional associations. GO analysis of the TR-0 group DA proteins showed that C5a, C9, apolipoproteins (APOA-I and APOA-IV) and alpha-1 acid glycoprotein (ORM1) were associated with host immune response by contributing to complement activation (C5, C9), regulation of immune response (APOA1, C5, C9), inflammatory response (C5, ORM1) and response to stimuli (APOA1, APOA4, C5, C9, ORM1) (see Table S3 for a full list of biological process (BP) GO terms and KEGG pathways). On the other hand, no enrichments were observed for BP GO terms and KEGG pathways for TS-0 group DA proteins.

3.3. Serum proteomes after tick infestation

To understand the impact of tick infestation on host biology, changes in proteomes of each group (TS and TR) of cattle in response to tick infestation were observed by comparing the serum proteomes of tick naïve susceptible and resistant cattle (TS-0 & TR-0) with the same group of cattle following tick infestation and full characterisation of the phenotype (TS & TR). The intragroup group comparison of susceptible cattle between timepoints (TS-0 vs TS) showed 46 significantly differentially abundant proteins, 30 of which at higher abundance and 16 at lower abundance in TS cattle (Figure 3). Of these DA proteins, eight highly abundant and two lowly abundant proteins had a $|\log_2 FC| > 1$ (adjusted P-value $< 10^{-5}$). The most highly abundant proteins in TS cattle included conglutinin (P23805), kinesin family member 12 (F1MMK9), kininogen-1 (A0A140T8C8), apolipoprotein C-III (V6F9A3), uncharacterized protein (F1MLW8), C8 beta chain (F1N102), clusterin (P17697), and complement factor I (Q32PI4), while the two most highly abundant proteins in TS-0 (before infestation) were transthyretin (O46375) and serotransferrin (Q29443) (Table S4).

Tick-resistant cattle responded to tick infestation similarly to tick-susceptible cattle. The between timepoint comparison of TR cattle with the tick resistant naïve (TR-0) group identified 58 proteins as DA, of which 35 proteins were higher and 23 proteins were lower in abundance in TR than TR-0 cattle (Figure 4; Table S5). Of these, 12 proteins (six upregulated and six downregulated) had $|\log_2 FC| > 1$ (adjusted P-value $< 10^{-5}$). The DA proteins with higher

abundance were conglutinin, apolipoprotein C-III, kinesin family member 12, gelsolin (F1N1I6), kininogen-1, adiponectin (Q3Y5Z3), hemopexin (Q3SZV7), and inter-alpha-trypsin inhibitor heavy chain H1 (F1MMP5). The proteins with the lowest abundance included coagulation factor V (F1N0I3) and angiotensinogen (serpin peptidase inhibitor A8) (Q3SZH5). Tick infestation elicited a quite similar response in TR and TS cattle, which was evident from the identification of 34 proteins common to both intragroup group comparisons (TR vs TR-0 and TS vs TS-0). Most of these proteins were upregulated in both groups following tick infestation. Gelsolin, antithrombin-III (F1MSZ6), and apolipoproteins A-I and A-II were some of the proteins which showed higher abundance in TR cattle only following tick infestation.

The STRING analysis also suggested similar protein-protein interactions for the DA proteins in both groups of cattle (TS and TR) following infestation when compared to the respective naïve groups (Supplementary figures S1A and S2A). In both groups, the proteins with higher abundance in tick-exposed cattle were mainly grouped into two *k*-mean clusters, for example, five complement factors showed strong connectivity and were grouped in one cluster. In addition, in TS cattle, apolipoproteins (APOA-IV, APOB and APOC-III) were grouped into a separate cluster along with plasminogen, kininogen-1 and fibronectin as well as the copper transport protein ceruloplasmin. In TR cattle, the apolipoproteins (APOA-I, APOA-II, APOA-IV and APOC-III) showed strong connectivity and grouped in one k-mean cluster with kininogen-1, ceruloplasmin and the SERPINs (C-1 and D-1). Whereas the highly abundant proteins in naïve cattle of both groups (TS-0 and TR-0) showed strong connectivity for four immunoglobulin-like proteins which were clustered together, all other proteins showed weaker or no interactions within the query proteins (Supplementary figures S1B and S2B).

The DA proteins upregulated in TS cattle were assigned to 103 enriched BP GO terms and 12 KEGG pathways (a full list of GO terms and KEGG pathways with observed gene counts is given in Table S6). Similarly, GO analysis identified 164 BP GO terms for DA proteins in TR cattle (Table S7), of which 95 GO terms were the same as identified in the TS group following exposure to cattle tick (TS vs TS-0). Some of these BP GO terms suggested that proteins were involved in complement activation (classical and alternate pathways), immune response, regulation of the biological process, cytokine-mediated signalling, intracellular signal transduction, response to stimuli and inflammatory response. Also, DA proteins in the TR group of cattle were enriched for some specific GO terms including regulation of Cdc42 protein signal transduction, interleukin-8 production and regulation of production of molecular mediators of the immune response which were not present in GO term analysis of TS cattle.

3.4. Comparison of induced serum proteomes after infestation

The induced serum proteomes of TR cattle following tick infestation showed overlapping of proteomic profile with TS cattle when compared to their respective naïve samples, therefore, the induced serum proteomes of TR and TS cattle were compared to identify DA proteins among the two groups. This analysis detected 22 DA proteins (adjusted P-value < 10^{-5}) with 16 proteins having significantly higher and six proteins significantly lower abundance in TR cattle (Figure 5; Table 2). The two most abundant proteins in TR cattle with $|\log_2 FC| > 1$ (adjusted P-value < 10^{-5}) were serotransferrin and transthyretin. Most of the proteins with significantly higher abundance in TR cattle following tick exposure were also observed as highly abundant proteins in TR naïve cattle when compared to the TS naïve cattle (TR-0 vs TS-0, for example, C9, APOA-I preprotein, APOA-IV, leucine-alpha rich glycoprotein-1 and SERPINA-10. Of six highly abundant proteins in TS cattle, four were identified as uncharacterized in the Protein Pilot search, three of which belonged to the immunoglobulin domain. The protein-protein interaction showed that the apolipoproteins (APOA-1, APOA-2 and APOC-3) clustered with SERPINA-10 and ITIH-2, similar to the comparison of naïve samples (TR-0 vs TS-0) (Figure 6). Similarly, the acute-phase response proteins (alpha-1 acid glycoprotein and leucine-rich alpha-2-glycoprotein-1) were clustered with C-X-C chemokine motif (*PPBP*), an inflammatory mediator. The highly abundant proteins in TR cattle were associated with 111 BP GO terms and four KEGG pathways. The KEGG pathways responsible for host immune response were complement and coagulation cascade, cholesterol metabolism, and peroxisome proliferator-activated receptor (PPAR) signalling pathways. Additional BP GO terms and KEGG pathways identified can be found in Supplementary Table 8. The DA proteins with higher abundance in TS cattle showed no significant GO term (BP) enrichment, but were associated with two KEGG pathways including *Staphylococcus aureus* infection and systemic lupus erythematosus pathway.

4. Discussion

In this study, SWATH-MS identified differentially abundant proteins in serum samples of tick susceptible and resistant Santa Gertrudis cattle in the naïve state and after infestation with the cattle tick, *R. australis.* Tick infestation elicited quite similar responses in both TS and TR cattle, with a relatively higher abundance in TR cattle of proteins involved in immune responses, for example, acute-phase response (APR) proteins, complement factors, proteins involved in lipid metabolism, and chemokines, as compared to the proteins upregulated in TS cattle. In addition, following tick infestation, the TR group exhibited persistent levels of some

proteins associated directly or indirectly with the immune response (similar to the pre-infestation proteomics profile), potentially impairing tick attachment and feeding success. These findings suggest that proteomics could be applied as a potential tool to determine the candidate protein(s) associated with tick-resistance phenotype, for example, apolipoproteins (APOA-I and APOA-II), complement factors (C3, C5a, and C9), APR proteins, clusterin and plasminogen in different cattle breeds and validate the association of these proteins as a potential biomarker(s) for tick resistance/ susceptibility in cattle.

The intergroup comparison of naïve samples (TS-0 vs TR-0 and TR vs TS) showed that most of the DA proteins with significantly higher abundance in TR cattle were involved in the regulation of immune responses, complement activation, inflammatory responses, responses to stimulus and stress, lipid metabolism, and haemostasis. Many of these mechanisms have already been associated with variability in host resistance to ticks, for example, blood coagulation, angiogenesis, inflammation, iron transport, and lipid metabolism. [34-36] Moré et al [36] recently proposed that modulation of such protective mechanisms could help tick-naïve hosts to achieve a better initial response against tick antigens when tick feeding starts. The current findings support the idea that the initial protective response against tick challenge could be achieved more effectively in naïve TR cattle, for example, the highly abundant proteins included three complement factors (C5a, C8b and C9), two uncharacterised proteins (associated with complement activation and inflammatory response) and APR proteins that may enable these cattle to promptly react to tick infestation. The complement system plays an important role in adaptive as well as innate immunity, thus likely contributes to host protection. It is known that saliva of *R. microplus* can inhibit the activation of classical and alternative pathways of the host complement system can which ultimately prevent the formation of the membrane attack complex and production of inflammatory mediators, including C5a. [37] Abundance of complement proteins such as C9, C8b and C5a at the time of tick feeding could enable a better protective cellular response in naïve TR cattle as compared to naïve susceptible cattle. In TR cattle (TR vs TS), only C9 showed higher abundance among the complement proteins. STRING analysis showed that these complement proteins were clustered closely along with vitronectin, a protein indirectly involved in immune response. Also, *k*-mean clustering showed a strong interaction between apolipoproteins (APOA-1, APOA-4, APOB) SERPIN A10, coagulation factor V (F5) and an uncharacterised protein (associated with complement activation and inflammatory responses), through which the apolipoprotein cluster was connected with the complement protein cluster. In addition, two APR proteins (*ORM-1*

and *LRG-1*) were linked with the apolipoprotein-SERPIN cluster. Highly abundant proteins in TR cattle, when compared to the TS group (TR vs TS) also showed apolipoprotein-SERPIN A-10 and ARP protein clusters (*LRG-1*, *ORM-1* and transthyretin). However, the highly abundant apolipoproteins in TR cattle were APOA-1, APOA-2 and APOC-3, and the cluster also contained serotransferrin. The *LRG-1*(leucine-rich alpha-2-glycoprotein-1) and *ORM-1* (alpha-1-acid glycoprotein) are positive APRs, whose concentrations increase in response to inflammation, whereas serotransferrin and transthyretin are negative APRs whose concentrations decrease in response to inflammation. [38] Carvalho et al [18] reported decreased levels of *ORM-1* and serotransferrin in *Bos indicus* and *Bos taurus* cattle, respectively, in response to tick infestation, whereas, level of haptoglobin was increased in *Bos taurus* following heavy tick infestation. [18] These findings suggest that TR cattle may develop a more controlled or directed inflammatory response to tick challenge which can be protective instead of facilitating tick feeding. This association of high resistance with dampened inflammation is consistent with gene expression studies in taurine vs indicine cattle [39] and with histological and immunohistochemical observations in taurine vs indicine [40] and high vs low resistant taurine × indicine cattle. [41] In addition, as with other proteins, the effect of APR protein levels can involve many other processes apart from inflammation. For example, transthyretin is also involved in retinol metabolic process (vitamin A1 alcohol), retinol binds to retinoic acid receptors (*RAR*) and peroxisome proliferator-activated receptor (*PPAR*) and plays a role in immunity, thus transthyretin may have an indirect role in immune response. [42,43] Moré et al [36] has recently reported that retinol-binding protein-1 coding gene was upregulated in the skin of tick resistant Braford cattle following tick exposure as compared to susceptible cattle, while this gene was downregulated in TS cattle following tick exposure.

The C-X-C motif chemokine (platelet factor 4 precursor) was significantly more abundant in TR than TS cattle and was downregulated in TS cattle following tick infestation (TS-0 vs TS). The C-X-C motif chemokine or PF4 attracts leukocytes and neutrophils to the site of inflammation, which suggests a role in protective responses to tick challenge. Domingues et al [44] previously reported significant downregulation of *CXCL8* in blood samples of tick susceptible crossbred (Gir × Holstein) cattle 24 and 48 h after infestation compared to uninfected samples, suggesting that downregulation of C-X-C chemokine in response to tick infestation may contribute to a limited immune response to tick challenge. Previous studies have obtained contrasting results with respect to the direction of the association between resistance to tick infestation and the scale of the inflammatory response. In some studies

reduced local inflammation at the site of attachment was associated with high resistance [40,41,45] and in other studies, the opposite was observed. [34]

In naïve susceptible cattle (TS-0) compared with TR-0 cattle, gelsolin (GSN) was significantly more abundant, which as a part of free actin (released into extracellular space due to cellular death and lysis) scavenging system, helps in controlling acute inflammatory responses and wound healing in humans and animals reviewed by Piktel et al [46]. Due to the observation of increased GSN abundance in plasma in a variety of disorders, and its ability to predict clinical outcomes in a variety of health conditions, it has been suggested that extracellular GSN should be considered as a universal predictor of general health rather than a specific biomarker for any given disease. [46]

Tick infestation elicited quite similar responses in tick-susceptible and tick-resistant animals, as they shared 34 DA proteins following tick challenge, 22 being up-regulated and 12 down-regulated. The proteins that increased in abundance following infestation in both TR and TS cattle were divided into two major clusters: an apolipoproteins-SERPINs cluster, and a complement factors cluster. The proteins in the apolipoprotein cluster in TS cattle (APOA-IV, APOB and APOC-III) were mainly associated with lipid metabolism, whereas the proteins in the apolipoprotein cluster in TR cattle (APOA-I, APOA-II, APOA-IV and APOC-III) were also involved in immune mechanisms in addition to lipid metabolism, for example, APOA-I and APOA-II. This is broadly consistent with the immunological analysis of the blood samples from the same herd of Santa Gertrudis animals, in which immune cell subsets were similar in both TR and TS groups following tick infestation when compared to the unexposed control animals with an unknown resistance or susceptibility profile. [19] The only real differences in immunological profiles were in the immunoglobulin activities in serum; the TS cattle developed significantly higher tick-specific IgG1 antibody titres compared to the TR animals. In the proteomic investigation, half of the proteins at higher abundance in TS relative to TR cattle (3 of 6) had immunoglobulin domains or were identified as uncharacterised. One of the uncharacterised proteins (G3MXG6) was identified from protein sequence in NCBI BLAST protein database as bovine immunoglobulin gamma (IgG), with 69.70% identity and 93% query cover. Hence, the proteomic findings are consistent with the direct immunological findings from these animals, as previously reported by Piper et al [19]. One potential non-equivalence between the immunological assays described previously with the present proteomic study is that in the immunological assays, the IgG1 and IgG2 responses were differentiated from each other. The IgG1 response after infestation was significantly higher in

TS cattle compared to TR cattle vs control, while the level of IgG2 response varied considerably. SWATH-MS analysis measures IgG as a whole molecule/ protein, and may not differentiate between IgG1 and IgG2; this may also be due to the lack of distinct sequences for IgG1 and IgG2 proteins in the database. Another point of potential non-equivalence between the two approaches is that the immunological assays were specific to tick antigen, whereas the proteomic approach does not differentiate according to IgG specificity. Taken together, the proteomic and immunological analyses suggest that (at least in the group of Santa Gertrudis cattle under study) increased antibody response either does not play a role in resistance, or might contribute to increased susceptibility to infestation.

Some proteins showed differentially higher abundance specific to each group in response to tick infestation. For example, plasminogen, beta-2 glycoprotein-1 (APOH), and clusterin were upregulated only in TS cattle following tick exposure. Plasminogen, an inactive precursor of plasmin, which catalyzes fibrinolysis, helps in tick feeding activity, [47] plasminogen activator-tissue gene has been previously reported as a highly upregulated gene in the skin of susceptible cattle following tick infestation. [48] Moré et al [36] reported that genes involved in plasminogen activator pathway were upregulated in both resistant and susceptible Braford cattle skin following tick infestation which differs to this study showing up-regulation in TS cattle only. Beta-2 glycoprotein-1 exhibits anticoagulant and/ or pro-coagulant, and complement inhibition activities depending on the surrounding environment (health and disease), reviewed by McDonnell et al [49]; for example, it participates in plasminogen activation and also inhibits activation of protein C, and disrupts the anticoagulant annexin V shield. Although beta-2 glycoprotein-1 also showed higher abundance in TR naïve and TR cattle in intergroup comparisons (TR-0 vs TS-0 and TR vs TS), it can be predicted that in the presence of higher levels of plasminogen, beta-2 glycoprotein-1 may act as an anticoagulant and facilitate tick feeding. Similarly, clusterin is a multifunctional glycoprotein which contributes to a variety of physiological and pathological processes including lipid transport, apoptosis, cell to cell and cell to matrix interactions as well as inhibition of complement systems. [50] In humans, circulating clusterin has been implicated in the development of colorectal cancer, as it may limit the host response to pathogenic bacteria, thus allowing damage to the mucoid intestinal barrier and favouring inflammation and cancer. [51] This suggests that the presence of proteins that facilitate tick feeding by either stimulating fibrinolysis or inhibiting immune response may contribute to host susceptibility to tick infestation. It is consistent with the observation that bovine hosts that are susceptible to tick infestations exhibited an increased clotting time for

blood collected from the immediate vicinity of haemorrhagic feeding pools in skin infested with different developmental stages of *R. microplus.* [52]

Tick-resistant cattle specifically showed higher expression of some proteins involved in lipid metabolism (apolipoprotein A-I and A-II, and paraoxonase-1). Apolipoproteins (A-I and A-II) are involved in a variety of immune-related functions such as positive regulation of phagocytosis, regulation of Cdc42 protein signal transduction, and IL-8 biosynthetic process. In addition, serum samples of TR cattle also exhibited a higher abundance of antithrombin III, a vertebrate serpin that functions as an endogenous inhibitor of thrombin, coagulation factors IX and X, which makes it the major regulatory protein of vertebrate coagulation under physiological conditions. [53] This higher abundance of antithrombin III might be a physiological response to maintain haemostasis and flow of immune cells in the presence of proteins that enhance blood coagulation. These mechanisms may further enhance the potential anti-tick response that contributes to differentiating hosts regarding resistance, for example, the Cdc42 protein and cytoskeleton organization are upregulated in response to an injury and play important roles in wound healing in animals. [36,54]

The findings of this study support the proposal that modulation of immune response including cytokines, acute-phase response proteins, cell adhesion molecules, and chemokines with the ability to attract T and B lymphocytes and granulocytes could be associated with tick resistance. [40,55] The findings suggest that the proteins with higher abundance in TR cattle before tick exposure including complement factors (C5a, C9 and C8B), APR proteins (leucine-rich alpha-2-glycoprotein-1and alpha-1-acid glycoprotein) and apolipoproteins (APOA-I, APOA-II and APOB) may be used as potential biomarkers for tick resistance. However, further studies are required to validate these findings across different cattle breeds and challenge conditions. It is also important to design future experiments to include the skin tissue to better understand the systemic and local response to tick challenge and identify variations in proteomic profiles of different cattle with varying phenotype to the cattle tick.

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Figure legends

Figure 1. Volcano plot illustrating the DA proteins between tick-susceptible and tick-resistant groups of cattle before exposure to cattle tick (TS-0 vs TR-0). Red, significantly different in abundance ($P < 10^{-5}$). Blue, not significantly different in abundance ($P > 10^{-5}$).

Figure 2. STRING protein interaction map based on biological process GO terms of differentially abundant proteins in tick-resistant naïve cattle (before exposure to cattle ticks) when compared with tick-susceptible naïve cattle (TS-0 vs TR-0). Each node represents an individual protein. *k*-mean clusters showing strong interactions are highlighted: green = apolipoprotein-SERPINA-10 cluster with coagulation factor V; red = Complement factors with vitronectin cluster.

Figure 3. Volcano plots illustrating the DA proteins between naïve tick-susceptible and tick-susceptible (TS-0 vs TS) groups of cattle. Red, significantly different in abundance ($P < 10^{-5}$). Blue, not significantly different in abundance ($P > 10^{-5}$).

Figure 4. Volcano plots illustrating the DA proteins between naïve tick-resistant and tick-resistant (TR-0 vs TR) groups of cattle. Red, significantly different in abundance ($P < 10^{-5}$). Blue, not significantly different in abundance ($P > 10^{-5}$).

Figure 5. Volcano plot illustrating the DA proteins between tick-susceptible and tick-resistant groups of cattle (TS vs TR) following exposure to cattle tick. Red, significantly different in abundance ($P < 10^{-5}$). Blue, not significantly different in abundance ($P > 10^{-5}$).

Figure 6. STRING protein interaction map based on biological process GO terms of differentially abundant proteins in tick-resistant cattle when compared to tick-susceptible cattle (TR vs TS). Each node represents an individual protein. *k*-mean clusters showing strong interactions are highlighted: green = apolipoprotein-SERPINA-10 cluster with serotransferrin; red = acute-phase response proteins with C-X-C motif chemokine cluster.

686 **Table 1.** Significantly abundant proteins between tick susceptible and resistant cattle before tick exposure (TS-0 vs TR-0). Negative and positive
687 values indicate proteins with higher and lower abundance in serum from tick-resistant cattle

UniProt Accession ID	Protein names*	log2FC	Biological Process
A5PJ69	SERPINA10 protein	0.9	Negative regulation of endopeptidase activity
Q5GN72	Alpha-1-acid glycoprotein	0.8	Acute phase protein, Neutrophil degranulation
F1N0I3	Coagulation factor V	0.7	Blood coagulation
E1B805	Uncharacterized protein	0.7	Haemolytic complement-like
E1BNR0	Apolipoprotein B	0.6	Lipid metabolism, Regulation of gene expression, Response to virus
F1MVK1	Uncharacterized protein	0.6	Complement activation; Inflammatory response
A0A140T843	Beta-2-glycoprotein 1	0.6	Blood coagulation
Q2KIU3	Protein HP-25 homolog 2	0.6	Collagen like protein
Q3MHN2	Complement component C9	0.6	Cell killing, complement activation (Classical and alternate pathways)
Q5EA67	Inter-alpha inhibitor H4 (Plasma Kallikrein-sensitive glycoprotein)	0.5	Hyaluronan metabolic process
D4QBB4	Globin A1 (Haemoglobin beta)	0.5	Oxygen transport
F1N102	Complement C8 beta chain	0.5	Complement activation, Immune response
F1N3Q7	Apolipoprotein A-IV	0.4	Lipid metabolism, removal of superoxide radicals
Q6T182	Sex hormone-binding globulin	0.4	
V6F9A2	Apolipoprotein A-I preproprotein	0.4	Lipid metabolism, Regulation of Cdc42 protein signal transduction, Negative regulation of cytokine secretion involved in immune response, Positive regulation of phagocytosis
G8JKW7	Serpin A3-7	0.4	Negative regulation of endopeptidase activity
Q2KIF2	Leucine-rich alpha-2-glycoprotein 1	0.4	Regulation of angiogenesis and endothelial cell proliferation
F1MY85	Complement C5a anaphylatoxin	0.4	Complement activation; Inflammatory response; Negative regulation of macrophage chemotaxis; Positive regulation of chemokine secretion

A5D7R6	Inter-alpha-trypsin inhibitor heavy chain H2	0.3	Hyaluronan metabolic process
Q3ZCH5	Zinc-alpha-2-glycoprotein	0.3	Lipid metabolism, Antigen processing and presentation of endogenous peptide antigen via MHC class Ib; positive regulation of T cell mediated cytotoxicity
Q3ZBS7	Vitronectin	0.3	Cell adhesion mediated by integrin; Cell migration; Endodermal cell differentiation; Immune response
Q9BGU1	Histidine-rich glycoprotein	-0.3	Regulation of gene expression, Regulation of platelet activation and blood coagulation, Regulation of actin cytoskeleton organization,
F1MMP5	Inter-alpha-trypsin inhibitor heavy chain H1	-0.3	Hyaluronan metabolic process
Q1RMN8	Immunoglobulin light chain, lambda gene cluster	-0.3	Immunoglobulin like
Q3SYR8	Immunoglobulin J chain	-0.5	Adaptive immune response; Antibacterial humoral response; Innate immune response; Positive regulation of respiratory burst
E1BI82	Uncharacterized protein	-0.6	Belongs to the transferrin family
G3MZH0	Uncharacterized protein	-0.7	
F1N1I6	Gelsolin	-1.0	Actin filament polymerization; Actin filament reorganization, Phagocytosis, Positive regulation of protein processing in phagocytic vesicle

688 *All proteins are significantly different between naïve tick-susceptible and naïve tick-resistant cattle with $P < 10^{-5}$

689

690 **Table 2:** Significantly abundant proteins between tick susceptible and resistant cattle following tick exposure (TS vs TR). Negative and positive
691 values indicate proteins with higher and lower abundance in serum from tick-resistant cattle

UniProt Accession ID	Protein names*	log2FC	Function
Q29443	Serotransferrin (Transferrin)	2.0	Cellular iron ion homeostasis; Iron ion transport
O46375	Transthyretin (Prealbumin)	1.7	purine nucleobase metabolic process; Retinol metabolic process; Thyroid hormone transport
F1MVK1	Uncharacterized protein	0.7	Complement activation, Inflammatory response
V6F9A2	Apolipoprotein A-I preproprotein	0.6	Lipid metabolism, Regulation of Cdc42 protein signal transduction, Negative regulation of cytokine secretion involved in immune response, Positive regulation of phagocytosis
Q5GN72	Alpha-1-acid glycoprotein	0.6	Regulation of immune system process
P81644	Apolipoprotein A-II	0.6	Lipid metabolism, Positive regulation of phagocytosis, Negative regulation of cytokine secretion involved in immune response; Positive regulation of IL8 biosynthetic process
G8JKW7	Serpin A3-7	0.5	Negative regulation of endopeptidase activity
A5PJ69	SERPINA10 protein	0.4	Negative regulation of endopeptidase activity
V6F9A3	Apolipoprotein C-III	0.4	Lipid metabolism, G-protein-coupled receptor signalling pathway; Regulation of Cdc42 protein signal transduction
A5D7R6	Inter-alpha-trypsin inhibitor heavy chain H2	0.4	Hyaluronan metabolic process
Q2KIW1	Paraoxonase 1	0.4	Lipid metabolism, Response to toxins, Positive regulation of transporter activity
Q2KIF2	Leucine-rich alpha-2-glycoprotein 1	0.3	Regulation of angiogenesis and endothelial cell proliferation
Q3SZV7	Hemopexin	0.3	Cellular iron ion homeostasis, Positive regulation of immunoglobulin production, Positive regulation of humoral immune response mediated by circulating immunoglobulin
F1MD83	C-X-C motif chemokine	0.3	Chemokine-mediated signalling pathway; Immune response; Inflammatory response; Leukocyte chemotaxis; Neutrophil chemotaxis

A0A140T843	Beta-2-glycoprotein 1	0.3	Blood coagulation
Q3MHN2	Complement component C9	0.3	Cell killing, Complement activation (classical and alternate pathways)
Q5E9E3	Complement C1q subcomponent subunit A	-0.3	Complement activation (classical pathway); Innate immune response; Microglial cell activation; Neuron remodelling
G3MXG6	Uncharacterized protein	-0.4	Innate immune response, B cell receptor signalling pathway, Phagocytosis
A0A1K0FUD3	Globin C1	-0.4	Oxygen transport
E1BI82	Uncharacterized protein	-0.5	
F1MZ96	Uncharacterized protein	-0.5	Immune response
F1MLW8	Uncharacterized protein	-0.9	Immune response, Immunoglobin production

692 **All proteins are significantly different between tick-susceptible and tick-resistant cattle with $P < 10^{-5}$

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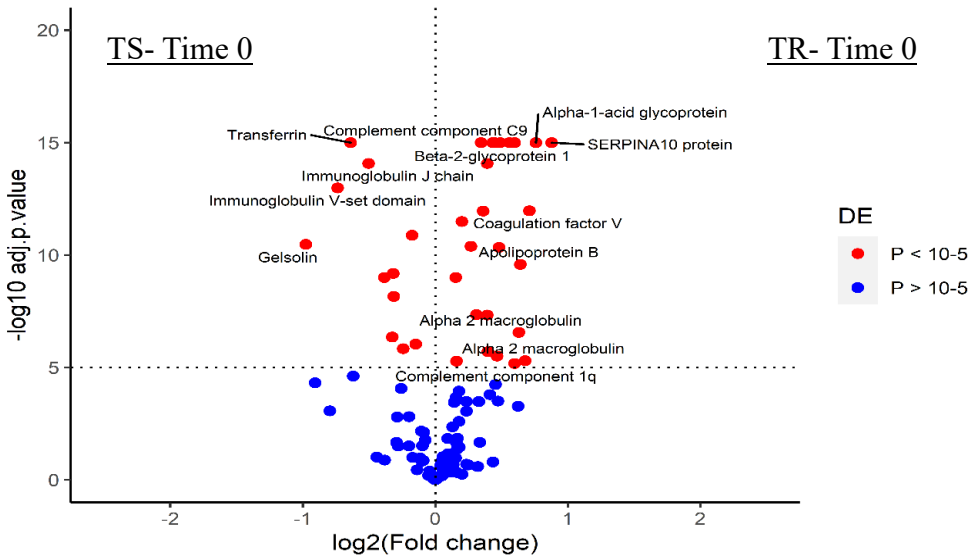

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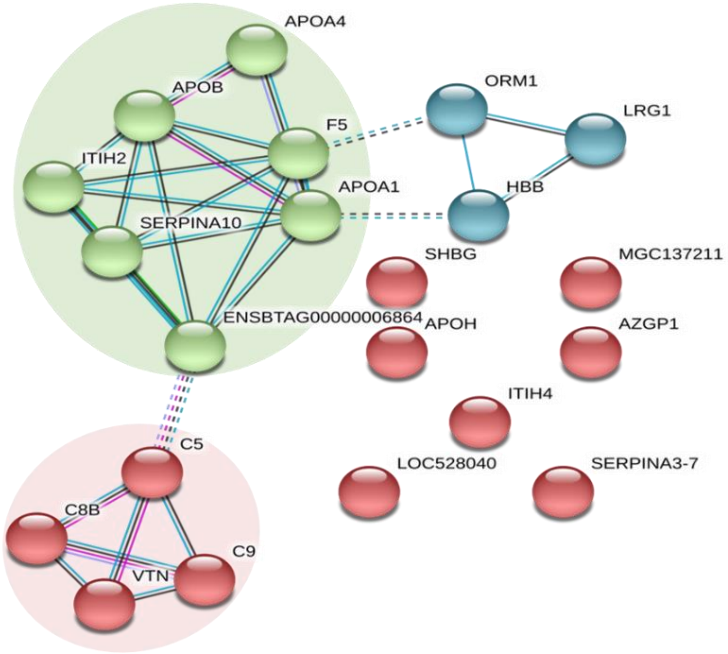
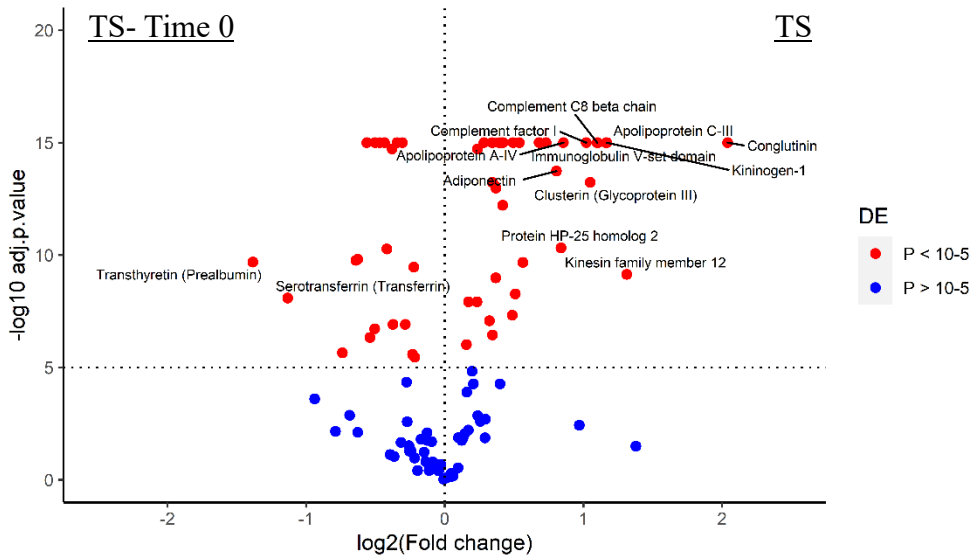


Figure 2.



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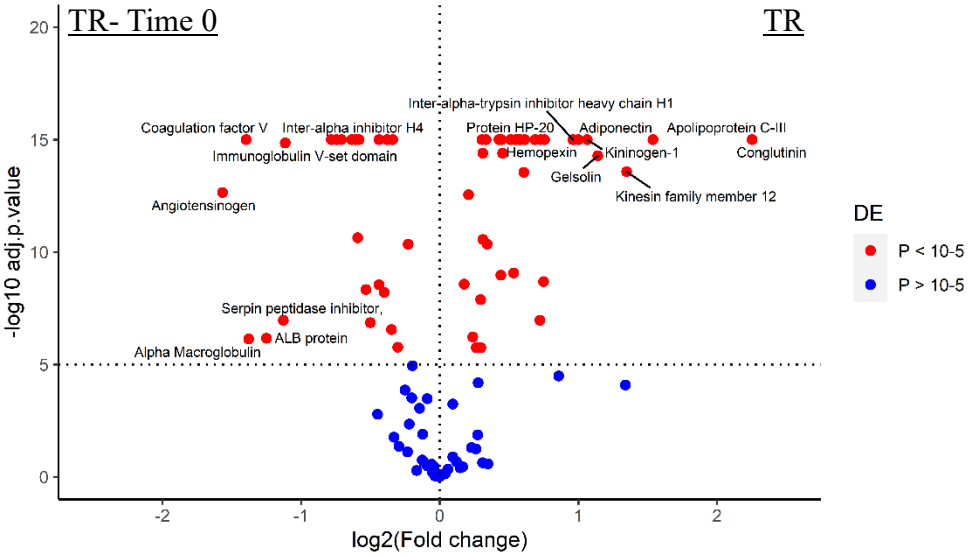

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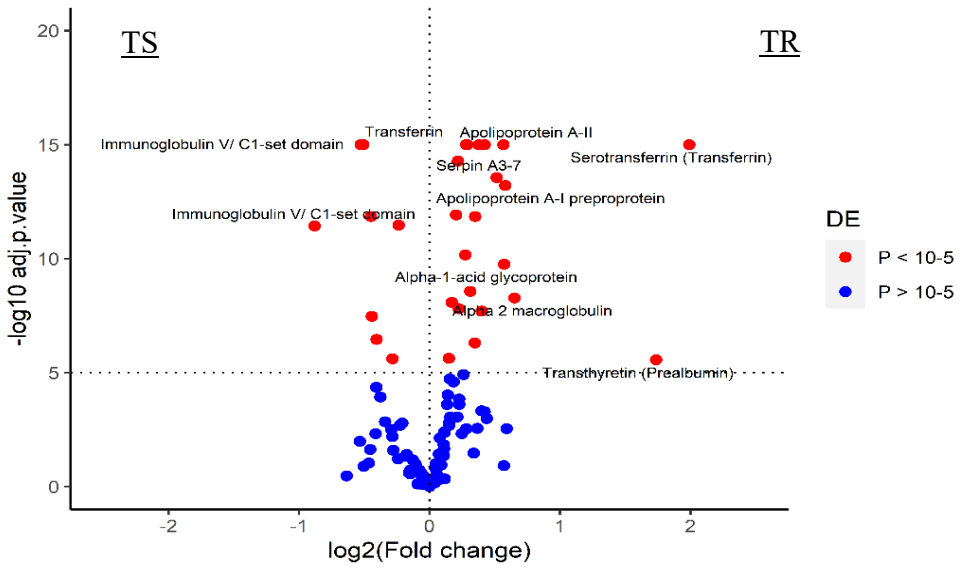


Figure 5.

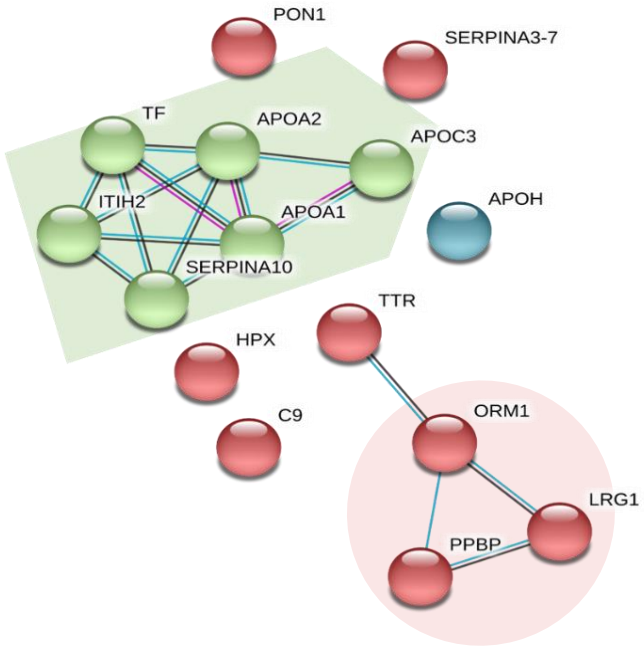
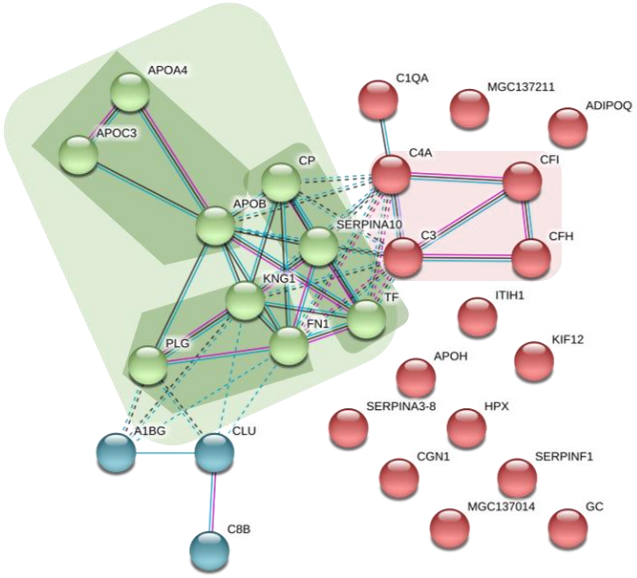


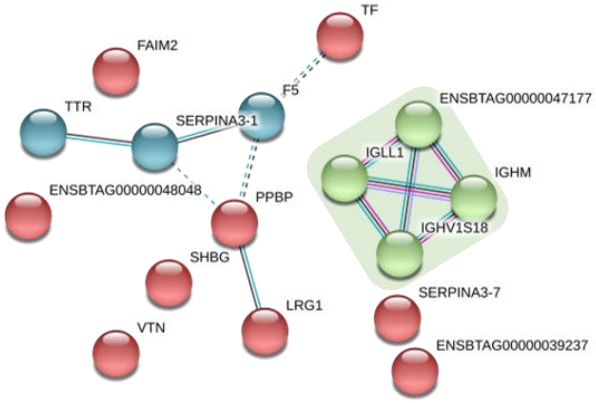
Figure 6.

Supporting Information

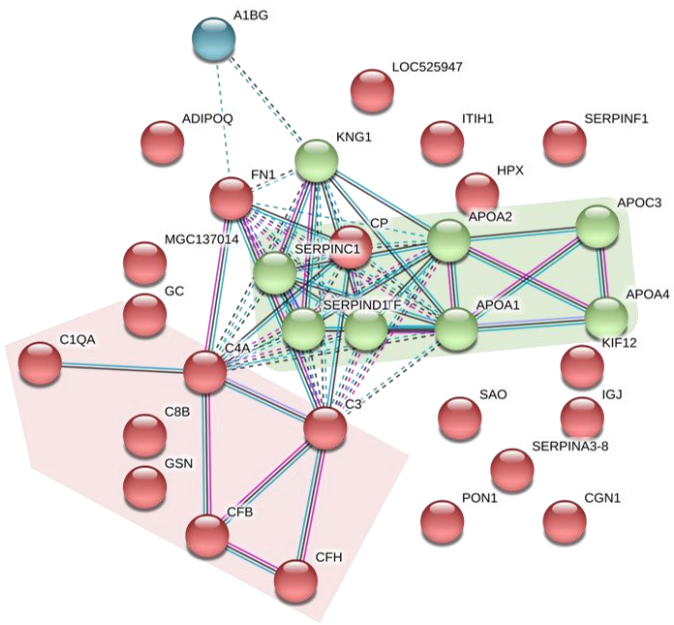
Serum proteomes of Santa Gertrudis cattle before and after infestation with *Rhipicephalus australis* ticks



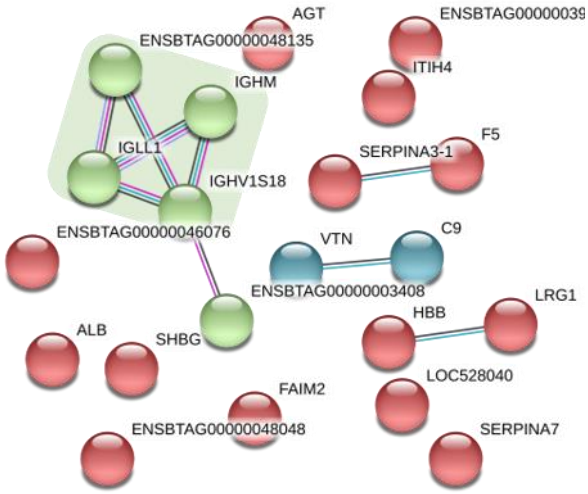
Supplementary Fig. 1A. STRING protein interaction map based on enrichment of proteins with significantly higher abundance in tick-susceptible cattle (TS) compared to tick-susceptible naïve cattle (TS-0). Each node represents an individual protein. *k*-mean clusters are highlighted: green = apolipoprotein-SERPINA-10 cluster with plasminogen and fibronectin-1; red = Complement factors cluster



Supplementary Fig. 1B. STRING protein interaction map based on enrichment of proteins with significantly higher abundance in tick-susceptible naïve cattle (TS-0) compared to tick-susceptible cattle (TS). Each node represents an individual protein. *k*-mean clusters are highlighted: green = Immunoglobulin like proteins



Supplementary Figure 2A: STRING protein interaction map based on enrichment of proteins with significantly higher abundance in tick-resistant cattle (TR) compared to tick-resistant naïve cattle (TR vs TR-0). Each node represents an individual protein. *k*-mean clusters are highlighted: green = apolipoprotein-SERPINs (C1 & D1) cluster with fibronectin-1 and serotransferrin; red = Complement factors cluster



Supplementary Figure 2B: STRING protein interaction map based on enrichment of proteins with significantly higher abundance in tick-resistant naïve cattle (TR-0) compared to tick-resistant cattle (TR). Each node represents an individual protein. *k*-mean cluster is highlighted: green = Immunoglobulin like proteins