Quantitative proteomics analysis reveals core and variable tick salivary proteins at the tick-vertebrate host interface

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Abstract
Few studies have examined tick proteomes, how they adapt to their environment, and their roles in the parasite–host interactions that drive tick infestation and pathogen transmission. Here we used a proteomics approach to screen for biologically and immunologically relevant proteins acting at the tick-host interface during tick feeding and, as proof of principle, measured host antibody responses to some of the discovered candidates. We used a label-free quantitative proteomic workflow to study salivary proteomes of (i) wild Ixodes ricinus ticks fed on different hosts, (ii) wild or laboratory ticks fed on the same host, and (iii) adult ticks cofed with nymphs. Our results reveal high and stable expression of several protease inhibitors and other tick-specific proteins under different feeding conditions. Most pathways functionally enriched in sialoproteomes were related to proteolysis, endopeptidase, and amine-binding activities. The generated catalogue of tick salivary proteins enabled the selection of six candidate secreted immunogenic peptides for rabbit immunizations, three of which induced strong and durable antigen-specific antibody responses in rabbits. Furthermore, rabbits exposed to ticks mounted immune responses against the candidate peptides/proteins, confirming their expression at the tick-vertebrate interface. Our approach provides insights into tick adaptation strategies to different feeding conditions and promising candidates for developing antitick vaccines or markers of exposure of vertebrate hosts to tick bites.

KEYWORDS
backbone proteome, humoral recognition, Ixodes ricinus, proteome, saliva, tick-host interface
The hard tick *Ixodes ricinus* is the principal vector of tick-borne encephalitis (TBE) virus and *Borrelia burgdorferi sensu lato*, which causes Lyme borreliosis (Halperin, 2007). *I. ricinus* can also transmit other pathogens of human and veterinary importance, including *Babesia*, *Anaplasma*, and *Rickettsia* species (Burri et al., 2011; Gray et al., 2002; Parola et al., 2005). Hosts can best be protected from these pathogens not only by targeting the pathogens (as achieved by vaccination against TBE or Lyme disease), but also through a comprehensive understanding of their tick vectors, the blood feeding process, and the underlying molecular mechanisms so that these mechanisms can be exploited to disrupt transmission.

Like all hard ticks, *I. ricinus* is an obligate haematophagous ectoparasite and has a wide range of vertebrate hosts (Kazimirová & Štibrániová, 2013). During their long-term blood-feeding, hard ticks must overcome the multifaceted host defence system of haemostasis, inflammation, and immunity through the action of compounds secreted in their saliva (Francischetti et al., 2009). Tick saliva contains many proteins, including protease inhibitors, lipocalins (histamine-binding proteins), disintegrins, enzymes, and tick-specific proteins such as salivary protein 15 (Salp15) and evasins (Déruaz et al., 2008; Wen et al., 2020). Tick saliva also contains nonproteinaceous molecules such as prostaglandins and nucleic acids (Bensaoud et al., 2019; Bowman et al., 1996). To effectively subvert host defences and ensure feeding success on various host species, tick salivary molecules are pluripotent (target more than one host cell population) and functionally redundant (Chmelař et al., 2016). Thus, choosing any single or set of target molecules to disrupt the feeding process is challenging. First, it requires a comprehensive identification of all the molecular determinants of tick blood feeding and then the pathways they participate in.

Over the last three decades, there has been considerable interest in elucidating the interactions between ticks and their ecological surroundings, not least because climate change and human activity have influenced host availability and, consequently, tick distribution and physiology (Alkishe et al., 2017). Among other strategies, ticks have adapted to these changes by modulating gene expression in their salivary glands. Several studies have shown that the analysis of salivary gland gene expression and consequent protein dynamics can provide insights into physiological differences in ticks according to gender, feeding conditions, developmental stage, and environmental adaptability (Chmelař et al., 2016). The tick “sialoverse” describes all tick species’ proteins derived from tick salivary glands that might critically regulate tick-host interactions and host defence mechanisms (Mans, 2020). A complete understanding of the tick sialoverse would provide insights into tick biodiversity and the circulation and evolution of associated pathogens. As such, and taking advantage of modern, high-throughput sequencing techniques, considerable efforts have been made to characterize the entire repertoire of *I. ricinus* salivary gland transcripts. Transcriptomic analyses have now been performed on the whole *I. ricinus* ticks (Charrier et al., 2018), their salivary glands (Schwarz et al., 2013), midguts (Cramaro et al., 2015), and during different stages of their life cycle (Vechtova et al., 2020).

Since the proteome can be regarded as better representing phenotype than the genome or transcriptome, it was anticipated that transcriptomics analyses would subsequently be complemented by proteomic analyses (Ghazalpour et al., 2011). However, in practice, tick proteomics has played a less prominent role in evolutionary and ecological studies than in genomic and transcriptomic studies. There has only been one analysis of the *I. ricinus* proteome from the salivary glands and midguts of ticks during early feeding on animal hosts (Schwarz et al., 2014), despite advances in proteomics techniques enabling a broader and deeper characterization of proteomic landscapes.

Proteomics analyses are particularly useful for validating in silico studies of proteins predicted to regulate tick-host interactions and provide candidate targets for host immunization strategies (e.g., potential epitopes for anti-tick vaccine development) or markers for exposure to tick bites. Several tick antigens involved in host interactions and tick attachment, feeding, reproduction, development, and blood meal digestion have already been identified (Almazán, Fourniol et al., 2020; Almazán, Šimo, et al., 2020). Despite immunization with tick antigens impairing some of these processes and reducing tick viability or engorgement under laboratory conditions, the only marketed antitick vaccine is Gavac (Heber Biotec S.A., Havana, Cuba), which targets a *Rhipicephalus microplus* gut protein Bm86 (Cobon & Hungerford, 1995). Therefore, there is an urgent need to identify other promising target antigens found in tick saliva. Another important parameter for developing antitick vaccines or tick exposure markers is the stable presence of the candidate antigens at the tick-host interface under varying tick feeding conditions, that is, the consistent exposure of tick salivary antigens in vertebrate host humoral response. An integrated and comprehensive *I. ricinus* sialoproteome study addressing different tick feeding conditions would provide unprecedented insights into the tick proteins and epitopes that are consistently found at the tick-host interface and thus candidate antigens for developing anti-tick vaccines or tick exposure biomarkers.

Here, we identified and quantified dynamic changes in salivary proteins secreted at the tick-host interface over time by analysing the *I. ricinus* sialoproteome under different feeding conditions: (i) wild ticks fed on rabbits or guinea pigs to study tick adaptability to different hosts; (ii) wild ticks or ticks reared from the laboratory colonies; and (iii) adult ticks fed with or without nymphs on the same rabbit to examine salivary secretions in the context of competition at the tick-host interface (for the overall experimental workflow, see Figure 1). The generated data enabled us to synthesize six tick-derived antigenic peptides predicted to be highly expressed at the tick-host interface and test the rabbit host humoral response against the candidate peptides. This study sheds light on tick adaptation strategies to ecological threats posed by their molecular
interactions with their parasitizing hosts. It also identifies highly immunogenic peptides/proteins and the pathways within which they are active.

2 | MATERIALS AND METHODS

2.1 | Experimental workflow

The proteomic study of tick saliva under different blood-feeding conditions is summarized in Figure 1.

2.2 | Ticks and sample preparation

We used two sources of ticks: wild ticks collected by flagging in a forest near České Budějovice in the Czech Republic, and inbred ticks from our institutional colony. We used two kinds of animals for feeding, guinea pigs and rabbits from our institutional animal facility. Ticks were induced to salivate by pilocarpine injection. Secreted saliva was collected on the fifth, sixth, or seventh day of feeding. To collect a sufficient quantity of salivary protein for the LC/MS, we used pools of tick saliva from an average of 50 ticks/feeding conditions. The protein concentration was estimated in triplicate using ISOQuant LC–MS/MS. (4) data analysis: Bioinformatic analysis was performed to quantify salivary protein abundance in each feeding condition and for gene ontology analysis. (5) antigenic peptide selection: Six highly expressed peptides were selected based on their sequences and antigenicity as candidates for immunization experiments. (6) peptide synthesis: Peptides were synthesized based on their sequences. (7) rabbit immunizations and exposure to ticks: (7a) a nonimmunized rabbit was exposed repeatedly to ticks; (7b) immunizations of rabbits were performed with three doses of the recombinant peptides with appropriate adjuvant. (8) ELISA: Serum samples collected at different time points from day 0 to day 90 were analysed by ELISA for recognizing the specific tick saliva peptides [Colour figure can be viewed at wileyonlinelibrary.com]
the BCA assay. Pure saliva samples were submitted for quantitative proteomic analysis at the Institute of Immunology, Mainz.

2.3 | Protein digestion

Aliquots (20 μg) of saliva proteins were lyophilized and redissolved in lysis buffer (7 M urea, 2 M thiourea, and 2% CHAPS). Two biological replicates of each experimental sample were prepared. Subsequently, proteins were digested using a modified filter-aided sample preparation (FASP) method (Wiśniewski et al., 2009). Briefly, redissolved proteins were loaded on the filter, and detergents were removed by washing three times with 8 M urea buffer. Proteins were reduced using DTT, alkylated using iodoacetamide, and excess reagent quenched by adding additional DTT and washing through the filters. Buffer was exchanged by washing with 50mM NH₄HCO₃, and the proteins were digested overnight with trypsin (Trypsin Gold, Promega) at an enzyme to protein ratio 1:50. After overnight digestion, peptides were recovered by centrifugation and two additional washes using 50mM NH₄HCO₃. Flowthroughs were combined, lyophilized, and redissolved in 20 μl 0.1% formic acid by sonication. The resulting tryptic digest solutions were diluted with aqueous 0.1% v/v formic acid to a peptide concentration of 200 ng/L and spiked with 25 fmol/μl of enolase 1 (Saccharomyces cerevisiae) tryptic digest standard (Waters Corporation).

2.4 | nanoUPLC–MS configuration

Nanoscale LC separation of tryptic peptides was performed using the nanoAcquity system (Waters Corporation) equipped with a HSS-T3 C18 1.8 μm, 75 μm × 250 mm analytical reversed-phase column (Waters Corporation) in direct injection mode as described previously (Tenzer et al., 2011). 200 ng of digested peptides were injected per technical replicate (three technical replicates performed for each of the two biological replicates of the 11 different experimental conditions; a total of 66 LC–MS experiments). Mobile phase A was water containing 0.1% v/v formic acid, whereas mobile phase B was ACN containing 0.1% v/v formic acid. Peptides were separated with a gradient of 3%–40% mobile phase B over 90 min at a flow rate of 300 nl/min, followed by a 10 min column rinse with 90% mobile phase B. Columns were re-equilibrated at initial conditions for 15 min. The analytical column temperature was maintained at 55°C. The lock mass compound [Glu1]-fibrinopeptide B (100 fmol/l) was delivered by the auxiliary pump of the LC system at 300 nl/min to the reference sprayer of the NanoLockSpray source of the mass spectrometer. Mass spectrometric analysis of tryptic peptides was performed using a Synapt G2-S mass spectrometer (Waters Corporation). For all measurements, the mass spectrometer was operated in v-mode with a typical resolution of at least 25,000 FWHM (full width half maximum). All analyses were performed in positive mode ESI. The time-of-flight analyser of the mass spectrometer was externally calibrated with an NaI mixture from m/z 50 to 1990. Data were post-acquisition lock mass corrected using the doubly charged monoisotopic ions of [Glu1]-fibrinopeptide B. The reference sprayer was sampled at a frequency of 30s. Accurate mass LC–MS data were collected in data-independent analysis modes (Geromanos et al., 2009; Silva et al., 2005) in combination with online ion mobility separations (Giles et al., 2004). For ion mobility separation, a wave height of 40 V was applied. Over the full IMS cycle, travelling wave velocity was ramped from 800 to 500 m/s. The spectral acquisition time in each mode was 0.7 s with a 0.05 s interscan delay. In low energy MS mode, data were collected at constant collision energy of 4 eV. In elevated energy MS mode, the collision energy was ramped from 25 to 55 eV during each 0.7 s integration. One cycle of low and elevated energy data was acquired every 1.5 s. The radiofrequency (RF) amplitude applied to the quadrupole mass analyser was adjusted such that ions from m/z 350 to 2000 were efficiently transmitted, ensuring that any ions observed in the LC–MS data less than m/z 350 were known to arise from dissociations in the collision cell.

2.5 | Label-free quantification using ISOQuant

Label-free quantification was performed using the in-house developed software ISOQuant (25545627). This analysis includes retention time alignment, EMRT (exact mass retention time), and IMS clustering. Only peptides with a minimum PLGS score of 5.5 identified in at least LC–MS runs were considered for cluster annotation, resulting in a peptide-level FDR of 0.2%. The maximum FDR of protein identification was set to 1% (based on decoy hits from the reversed database in the annotated cluster table) after the application of additional data processing steps, including isofrom/homology filtering as described (Distler et al., 2014). Furthermore, ISOQuant was configured to report only proteins identified by at least two peptides with a minimum length of six amino acids and in at least four LC–MS runs. These stringent criteria resulted in a protein-level FDR of 0.7%. Detailed protein and peptide level identification and quantification information, including protein inference, protein sequence coverage, peptide identification scores, and reported posttranslational modifications, are provided in File S1.

2.6 | Data processing and protein identification

LC–MS data were processed and searched using ProteinLynx GlobalSERVER version 3.0.2 (Waters Corporation). Previously, Charrier et al. (2018) assembled a transcriptome based on RNA-seq analysis of whole-body I. ricinus. Coding DNA sequences (CDSs) were predicted, and transcripts were annotated. We aligned this data set with the proteome provided by the UniProt database (Bateman, 2019) and filtered out proteins with no hits. We then eliminated partial and redundant proteins and redundancy based on function, gene ontology, and assigned names of each entry in the UniProt database by merging proteins from UniProt and those predicted by Charrier et al. (2018). The resulting file was clustered using
CD-HIT (Fu et al., 2012), and the obtained clusters were formed by sequences whose alignment had 98% local identity and covered at least 80% of the shorter sequence. Additionally, from these clusters, we included proteins that did not contain UniProt sequences. Through this strategy, we added 17,076 proteins to the 42,337 already in UniProt, resulting in a total of 59,413 proteins. Sequence information of enolase 1 (S. cerevisiae), bovine trypsin, and human keratins were added to the databases to normalize the data sets or for absolute quantification (Patzig et al., 2011). Guideline identification criteria were applied to all searches (Hochberg & Benjamini, 1990). Identified peptides had to meet the following search criteria: (i) trypsin as the digestion enzyme, (ii) up to one missed cleavage allowed, (iii) fixed carboxamidomethylcysteine and variable methionine oxidation set as the modifications, (iv) minimum length of six amino acids, and (v) a minimum of three identified fragment ions. Only proteins identified by at least two peptides were considered. The FDR for peptide and protein identification was determined based on the search of a randomized database generated automatically using PLGS 3.0.2 by reversing the sequence of each entry. The FDR was set to 1% threshold for database searching in PLGS. The experimental data were typically searched with a 3 ppm precursor and 10 ppm product ion tolerance, respectively.

2.7 | Quantification of I. ricinus salivary protein abundance

Proteomic data were measured twice for each sample as parts per million (ppm). For the analysis, the average ppm of both samples was used. If one value was missing, the ppm value of the other sample was used as the average; if both values were missing, the average was set to zero. Peptides quantified in <10 LC-MS runs in at least four samples were filtered out. Data analysis was performed using R version 3.6.2. Log-transformed values were used to perform sample clustering and principal component analysis. To analyse for differentially expressed peptides, limma was used on voom-transformed data. Peptides were graded as significant if the FDR was ≤0.05 and the absolute log2-fold change (logFC) was 1. For each pairwise group comparison, heatmaps were generated showing the top 10, or top 100 (by p-value) differentially expressed peptides.

2.8 | GO terms

To detect significantly enriched functional annotations from gene ontology (GO), we first downloaded the complete annotation file from UniProt ("goa_uniprot_all.gaf.gz" downloaded on 5 February 2020 from ftp://ftp.ebi.ac.uk/pub/databases/GO/goa/UNIPROT/). Using the protein IDs of the generated database (see above), the corresponding GO terms were parsed out of the entire annotation file. 30,613 protein IDs had at least one GO term. The obtained I. ricinus GO annotation file (protein ID - GO terms format) and a list of protein IDs (e.g., differentially expressed proteins) were then used as input of a standalone version of the AnnotationModules algorithm. All protein IDs with at least one functional annotation were used as statistical background.

2.9 | Peptide selection for immunization assays

We synthesized six peptides based on the antigenic regions of six highly abundant proteins in the I. ricinus salivoproteome under all the feeding conditions. These proteins had signal peptides in their complete sequence and were secreted as determined by SignalP. These proteins were already predicted based on existing tick transcriptomes and were TIL domain protein (P1), two thyropin domain protein (P2), microplusin (P3), lipocalin (P4), secreted protein (P5), and vitellogenin-2 (P6). All sequences can be found in the Supporting Information Data.

2.10 | Rabbit immunization

Immunizations and tick infestations were performed at the animal facility of the Institute of Parasitology of the Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic. Three rabbits were immunized intramuscularly with three doses of a combination of P1-P2, P3-P4, and P5-P6 with Freund’s adjuvant (first dose with incomplete adjuvant followed by two doses with complete adjuvant) emulsified with eGFP (control group). A nonimmunized rabbit was exposed twice to ticks and used as a control. Each animal was immunized using 5 ml syringes with 16-gauge needles and boosted twice on days 10 and 19 after receiving the initial dose.

2.11 | Determining antigen-specific antibody titres in sera of immunized animals by enzyme-linked immunosorbent assay (ELISA)

Sera used for ELISA were collected by centrifugation at 2000 g of rabbit blood samples collected after immunization (day 28 post-immunization), the first exposure (day 34 post-immunization), and after the second exposure (day 72 post-immunization). Serum was stored at −20°C. The ELISA plates were coated with 10 μg/mL solutions (500 ng total) of respective peptides diluted in coating buffer (carbonate–bicarbonate buffer at pH 9.6). After overnight coating at 4°C (and covered by seal tape), the wells were blocked using a solution of 5% BSA (blocking buffer) for 4 h at room temperature and then washed using PBS 0.05% Tween 20. Antirabbit IgG-horseradish peroxidase (diluted 1:1000 in blocking buffer) was used as a secondary antibody (Sigma-Aldrich). Finally, after another wash, 50 μl of 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific) were added, and a 2 M solution of H2SO4 stopped the reaction. The optical density (OD) at 450 nm was measured using an Infinite 200 PRO 96-well plate fluorescence reader (Tecan). Antibody titres
were defined as the primary antibody dilution that gave absorbance twice that of the negative control (preimmune serum from the same rabbit).

3 | RESULTS

3.1 | General overview of the *I. ricinus* sialoproteome

An ion mobility-enhanced, data-independent acquisition approach was used to obtain a high-coverage, reproducible quantitative proteomic analysis of tick sialoproteomes under different feeding conditions (Distler et al., 2014). Using a homology-based method and after removing redundancies, 1739 proteins were identified in tick saliva samples by LC–MS/MS, of which 1617 (92.98%) were present in at least four samples (Figure 2a). Relative protein abundance was highly variable under different feeding conditions. A majority of proteins (21.9%) were identified by at least four peptides and 19.9% by nine or more peptides (Figure 2b), indicating high sialoproteome coverage, and there was a >4-fold dynamic range of identified proteins across all fractions (Figure 2c). Based on FASP-based sample preparation and TOP3-based label-free quantification, the acquired data sets were highly reproducible across biological and technical replicates (Figures 2d,e).

Of the total proteins, 1.6% were homologous with host proteins, and 2.0% were other contaminants (Table 1). Putative tick proteins were categorized into 37 groups (Table 1). Most identified proteins were tick-specific and of unknown function (24.4%), followed by proteases (21.0%), protease inhibitors (19.6%), and lipocalins (9.5%). Most of the identified rabbit host proteins were globin, serotransferrin, serum albumin fibrinogen, and peptidoglycan recognition proteins (Table 1, File S1) functionally related to haemoglobin/red blood cell products (13%–58%) followed by cytoskeletal (6%–20%), haem/iron-binding (5%–16%), keratin (2%–30%), and nuclear regulation (2%–20%) proteins (Table 1). Immunity proteins, antimicrobial peptides, protease inhibitors, and proteases were present at ≤8% abundance throughout the feeding.

3.2 | Common and differentially abundant *Ixodes ricinus* sialoproteins and functional analysis

Salivary protein abundance was compared under three different feeding conditions (wild ticks fed on rabbits or guinea pigs; wild or laboratory ticks fed on rabbits; or wild ticks cofed with nymphs on rabbits)
Protein families identified in the sialoproteome

| Protein families                                      | Count |
|------------------------------------------------------|-------|
| TSP of unknown function                              | 395   |
| Protease                                             | 339   |
| Protease inhibitor                                    | 317   |
| Lipocalin                                            | 154   |
| Immunity-related                                     | 71    |
| Metabolism, amino acids                              | 44    |
| Ixostatin                                            | 28    |
| Ixodegrin                                            | 26    |
| Haem/iron binding                                    | 20    |
| Transporter/receptors                                 | 16    |
| Antioxidant/detoxification                            | 15    |
| Cytoskeletal                                         | 14    |
| Extracellular matrix                                  | 14    |
| Cytotoxin-like protein                                | 14    |
| Post-translational modification, protein turnover, chaperones | 13    |
| Glycine rich                                          | 12    |
| Protein modification                                  | 10    |
| Antimicrobial                                         | 9     |
| Nuclear regulation                                    | 8     |
| Metabolism, lipids                                    | 7     |
| IS4 family                                           | 6     |
| Signal transduction                                   | 5     |
| Intracellular trafficking, secretion, and vesicular transport | 3     |
| Metabolism, carbohydrates                             | 2     |
| Metabolism, energy                                    | 2     |
| RNA processing and modification                       | 2     |
| Carbohydrate transport and metabolism                 | 2     |
| Protein synthesis                                     | 2     |
| Metabolism, nucleic acids                             | 2     |
| Transposon                                           | 1     |
| Lipid transport and metabolism                        | 1     |
| Chromatin structure and dynamics                      | 1     |
| Energy production and conversion                      | 1     |
| Mucin                                                | 1     |
| Translation, ribosomal structure and biogenesis       | 1     |

rabbies, where wild ticks are those captured from nature/a forest area) during the late phase of feeding. A total of 249 proteins representing a "backbone proteome" were consistently expressed, independent of the feeding environment (Figure 3a; File S1), and most were metalloprotease and apyrase enzymes, kunitz domain protease inhibitors, and lipocalins (Figure 3b; File S1).

The highest number of differentially abundant proteins (DAPs) was observed in wild ticks fed on different hosts (553 DAPs, 35.49% of the total; 386 proteins increasing and 167 decreasing; Figure 4a). There were 31 DAPs in saliva from ticks fed on rabbits compared with ticks cofed on rabbits with nymphs, of which 20 increased and 11 decreased (Figure 4b). There were 324 DAPs in the saliva of wild ticks compared to laboratory-reared ticks (165 increased and 159 decreased; Figure 4c).

For our detailed analyses of each feeding condition, we focused on DAPs expressed with a log2-fold change (log2FC) > 4 or < -4 (false discovery rate (FDR)<0.05) between conditions and at different feeding time points (5, 6, or 7 days). For all three feeding conditions, most DAPs were proteases, protease inhibitors, lipocalins, and tick-specific proteins of unknown function (Figure 5).

Overall, we observed few time-dependent effects on protein abundance, regardless of feeding condition. The most significant differences were observed when ticks were fed on different animals, and only a few differences were induced by cofeeding. To address tick adaptability towards feeding on different hosts, we first examined DAPs in ticks fed on rabbits or guinea pigs (Figure 6a). There were 290 DAPs, of which 88 (30.2%) were proteases, 62 (21.3%) were tick-specific proteins of unknown function, and 35 (12.0%) were protease inhibitors, with lipocalins (9.2%), metabolism and amino acids (4.8%), and immunity-related proteins (3.4%) present at lesser abundances (File S1). To visualize the abundance of individual secreted proteins under each condition, heatmaps were generated for each DAP for each feeding condition and time point (5, 6, or 7 days; Figures S1–S3). Several proteins were abundant in ticks fed on rabbits on all 3 days of tick feeding, such as vitellogenin 2, is4 protein, alpha-actinin, and Metis2. Relatively low abundance proteins when ticks were fed on guinea pigs were mostly related to 5′-nucleotidase/apyrase and metalloproteases enzymes, lipocalin or kunitz domain protease inhibitors, and tick-specific proteins such as ixoherin, ixostatin, and Salp15.

Transcriptomic studies have shown that tick salivary gland gene expression is influenced by adult ticks competing for the host blood meal with nymphs (Schwarz et al., 2013), so we examined whether this also occurred at the proteome level in adult ticks fed simultaneously with nymphs on the same rabbit. There were only 19 DAPs, mostly proteases, tick-specific proteins of unknown function, and lipocalins (Figures 5 and 6b). Visualization with heatmaps revealed mostly similar expression across the studied conditions, although vitellogenin and lipocalin were more abundant when ticks were not cofed with nymphs or cofed for 7 days compared to those cofed with nymphs after only 5 days of blood uptake.

A significant question in laboratory studies of ticks is how closely tick colonies recapitulate the molecular profiles of wild ticks and how the possible presence of pathogens in ticks can influence gene expression in their salivary glands. We, therefore, compared DAPs in ticks collected from the wild and those reared in the laboratory. A total of 117 proteins were differentially abundant between these groups, of which 19 (33.3%) were proteases, 13 (22.8%) were tick-specific proteins of unknown function, 13 (22.8%) were protease inhibitors, and seven (12.28%) were lipocalins (Figure 6c). Proteins that were overexpressed in wild ticks compared to laboratory ones were mainly vitellogenin 2, tissue factor pathway inhibitor, and proteins involved in post-translational modifications.
3.2.1 | Enrichment analysis and gene ontology

Molecular function gene ontology (GO) analysis of DAPs was performed for proteins with >4 or ≤4 log-fold differences in expression (File S2). DAPs enriched for 78 and 16 pathways in ticks fed on different hosts or those from the wild versus laboratory ticks, respectively. In ticks fed on different hosts, the most enriched molecular functions were endopeptidase inhibitor activity (14.3%) and digestion (7.2%) followed by functions related to the proteolytic activity such as proteolysis and metalloproteinase activity and enzyme activator activity, hydrolase activity acting on ester bonds, nucleotide catabolic processes, and mitigation of host defences by symbionts. Three ontologies were enriched in wild versus laboratory ticks, all of which were related to proteolysis activity: metalloendopeptidase activity (1.5%) followed by metalloproteinase activity (1.1%) and peptidase activity (1%)

There was no significant enrichment in ticks cofed with nymphs versus those fed alone on rabbits

3.3 | Immunogenicity of multiple antigenic peptides in tick saliva

Based on the ppm (part per million) from the LC–MS/MS data, we identified a set of proteins that were highly abundant in all feeding conditions (Table S1). Consequently, we chose six peptides to assess their antigenic potential in immunization experiments. To capture a broad range of functions, peptides were selected from different protein families: til domain protein (P1), two thyropin domains protein (P2), microplusin (P3), lipocalin (P4), secreted protein (P5), and vitellogenin-2 (P6). Enhanced green fluorescent protein (eGFP) was used as a nonspecific control immunogen.
There were no clinical signs nor inflammation at the injection site after immunization in any animal. The presence of humoral responses in the sera from nonimmunized rabbits but exposed to ticks (Figure 7a) provided proof of the concept that these peptides were indeed expressed at the tick-host interface (tick saliva constituents). In addition, the presence of these peptides in tick saliva was sufficient to induce humoral responses in the host organism despite inducing variable antibody titres. On the other hand, immunized rabbits developed specific IgG antibody responses to five out of the six peptides after the third immunization, and antibody titres remained high until at least day 90 after the challenge (Figure 7). P4 (lipocalin) was the most antigenic, with relative antibody titres exceeding 80,000 (reciprocal of the serum dilution), followed by P6 (vitellogenin; 73.467). P1 (TIL) and P3 (microplusin) showed mild responses with antibody titres of around 3000 and 10,000, respectively. P5 was not immunogenic, with measured antibody titres comparable to preimmune rabbit sera. Although antibody titres against all six tested peptides were raised under
repeated infestation to ticks (in the absence of rabbit immunization) (Figure 7a), they were lower than the titres of rabbits immunized with the six peptides (Figures 7b–d).

4 | DISCUSSION

This study represents the first sialoproteome described for I. ricinus ticks, one of the most common tick species in Europe and neighbouring regions, fed under different conditions. Proteomics has become a more accessible and comprehensive methodology, enabling critical research and discoveries in organisms such as ticks that were, until only recently, difficult to study. Here we used label-free LC–MS/MS to provide a high dynamic range of quantification (Bantscheff & Kuster, 2012; Patel et al., 2009) and measure significant changes in protein abundance within the complex mixture of tick saliva as a function of varying tick feeding conditions. To achieve this, biological replicates of tick saliva were analysed from each feeding condition, and sialoproteome variability was studied as a function of different tick feeding conditions: (i) ticks fed on two different vertebrate hosts; (ii) adult ticks cofed with nymphs on the same host; and (iii) ticks collected from the wild or reared in colonies. Secreted saliva was collected on the fifth, sixth, or seventh day of feeding and all protein abundance variations were analysed. As a hard tick, I. ricinus feeds on its vertebrate host for a prolonged period. The slow feeding period starts 1 day postattachment, during which the female ingests about one-third of the total blood meal. The significant portion of the host blood (about two-thirds) is ingested by the mated female during the rapid engorgement phase, taking place during the last 24–48 h before the engorged tick drops off the host. Our study focused on the proteome dynamic of tick saliva, mainly during fast blood feeding. The sequential expression of protein multigenic families in tick saliva throughout feeding results in a continuous antigenic shift while the targeted host process remains blocked. Due to this change in epitope exposure, an effective and timely antigen-specific response cannot be mounted against the tick saliva immunomodulators.

Furthermore, we used the generated I. ricinus sialoproteome catalogue to select six highly abundant candidate proteins for rabbit immunization experiments. We verified experimentally that our proteome analysis indeed described tick proteins found during tick infestation at the tick-host interface, where tick-borne pathogen transmission also occurs. We also demonstrated that these proteins are naturally recognized by the rabbit immune system and that the host humoral response against these tick proteins can be boosted artificially.

Mass spectrometry data revealed a set of proteins expressed across all feeding conditions and over time, which we refer to as the “backbone” sialoproteome. Most of these proteins were proteases, protease inhibitors, and tick-specific proteins of unknown function.
This tick backbone sialoproteome is likely to represent a core, essential group of proteins produced by ticks and contained in their saliva to counteract the vertebrate host immune, inflammatory, and haemostatic defences against ticks. Indeed, these protein families were shown to have relatively stable transcript abundance in previous transcriptomic studies (Perner et al., 2018; Schwarz et al., 2014). Variations or lack of variations in proteome composition, whether between individual ticks, against different hosts, or during feeding, should all be considered when developing antitick vaccines or establishing markers of exposure to ticks. In that sense, the backbone proteome — and especially these salivary proteins predicted to be present in tick saliva — should be ideal candidates for biomedical applications due to their consistent presence at the tick-host interface. Future studies must determine whether the backbone proteome represents proteins essential (cornerstones) for modulating vertebrate host homeostasis during tick infestation. The proteins that make up the backbone sialoproteome are the most generalizable and promising vaccine antigen candidates.

Nonetheless, we should not neglect highly variable proteins because they may inform us about alternate strategies that ensure tick feeding success or may lead to personalized treatments. Since the tick is actively evading the host immune system by varying expression of specific genes and proteins, it is challenging to target these as vaccine antigen candidates and essential to study these complex mechanisms of evasion further. Our proteomic study supports transcriptomic data on the proposed phenomenon of “sialome switching” through which ticks adapt to the host (and stage of blood-feeding) by varying expression of specific gene families. Although we did not focus on this variability, our methodology can certainly help study it. We demonstrated preliminary data showing several proteins exclusive to a host, a feeding condition, or a group of ticks. Ticks have adapted to their ecological environments in the face of sustained pressure from climate change and human activity, with salivary gland genes and proteins evolving over millennia since the Late Cretaceous period (De La Fuente, 2003). To examine the adaptability of ticks to the possible need to feed on different vertebrate hosts, we compared sialoproteomes of ticks fed on rabbits and guinea pigs. Even though the immune system in vertebrates has many similarities to that in ticks, especially innate immunity (external physical barriers, humoral and cellular effector mechanisms, and coagulation [Emson et al., 2011]), we found that ticks vary protein expression in their salivary glands according to their host. The observed variation might be a deliberate adaptation, or stochastic remains to be determined. However, the interaction between tick and host is a dynamic relationship that begins even before the blood meal; ticks secrete different chemicals, known as semiochemicals, into the external environment to mediate essential aspects of tick behaviour (Sonenshine, 2004). Semiochemicals include pheromones (used for conspecific communication), allomones (defence secretions), and kairomones (used for host identification and location), which allow the tick to modulate secretion depending on the host, increase tick blood meal effectiveness, and increase the specificity of secreted salivary molecules depending on the host (Sonenshine, 2006). The blood composition of rabbits and guinea pigs differs in several different ways: some circulatory enzymes such as creatinine kinase are more highly expressed in rabbits, while others such as amylases are more highly expressed in guinea pigs (Evans et al., 2009); rabbits have more blood (160–480ml) than guinea pigs (40–80ml), the mean corpuscular volume and mean corpuscular haemoglobin are different between these species. Therefore, rabbits can afford to lose a higher volume of blood to blood meals (60–160ml vs. 15–30ml, respectively) while sustaining or feeding more individual ticks (60–160 vs. 15–30ml, respectively) (Bousquet, 2015; Cracknell, 2008); and the mean corpuscular volume and mean corpuscular haemoglobin are different between these species. Importantly, rabbits have higher circulating fibrinogen concentrations than guinea pigs (2.4 vs. 1.6 g/L, respectively) and react differently to several snake venoms, with a longer clotting time seen in guinea pigs (Condrey et al., 2020). Furthermore, their immune responses are different: guinea pigs exert more significant antithrombotic responses, highlighting differences in immunological processes and antigen production (Webster & Frank, 1985). These physiological/biochemical differences may explain, at least in part, variable expression of protease inhibitors and proteases as well as different tick adaptation strategies to different animal hosts.

Our results showed high variability in protein abundance, mainly in protease inhibitors and proteases enriched for endopeptidase inhibitor activity. This highlights once again the critical role of proteolysis regulation at the tick-host interface. Our study showed that Kunitz domain protease inhibitors are highly abundant in tick salivary secretions. Kunitz domain protease inhibitors are usually associated with inhibition of trypsin-like serine proteases (Ranasinghe & McManus, 2013), and they are considered to be the most valuable serine protease inhibitors in tick salivary glands as they function as antithrombotic factors by inhibiting various proteases in the coagulation cascade and platelet aggregation. These antihaemostatic Kunitz-type compounds may be helpful to therapeutically treat other cardiovascular or haematological diseases (Chmelar et al., 2012). Our proteomic data showed that cystatins are secreted in the I. ricinus salivary proteome. This protease Inhibitor family is well described for ticks: it is essential to overcome host immune responses, and it may also have promising immunosuppressive activities exploitable as immunotherapeutics (Jmel et al., 2021).

Endopeptidase inhibition is a defensive process used by ticks to inhibit the proteolytic activity of host proteases (Jmel et al., 2021). During host immunological responses to ticks, proteases such as granzymes in cytotoxic lymphocytes; neutrophil elastase, cathepsin G, and proteinase 3 in neutrophils; and chymase and tryptase in mast cells are expressed at the tick-host interface (Heutinck et al., 2010). Most host proteases are monomeric (thrombin is a trypsin-like monomeric protease) or composed of heavy and light chains linked by disulfide dimers (Verespy et al., 2016). These structures are permissive to endopeptidase inhibitor access to active sites to inhibit immunogenic proteolysis. Thus, the variation in protease inhibitor expression under the different experimental conditions may reflect differences in the regulation of proteolytic activity at the sites of tick infestation; these exogenous tick
protease inhibitors are secreted with tick saliva and may interfere with the physiological balance of vertebrate proteases and their endogenous inhibitors upon tick feeding. It is also known that almost all mammals, except rabbits and guinea pigs, express classical chymotryptic enzymes with similarly extended specificities, indicating significant interspecies differences in immunological defences and the need for specific peptidase inhibitors for different animals, including those used in our study (Zhongwei et al., 2019). Endopeptidase inhibitor expression by ticks may also mediate the protection of tick-expressed proteases, which might occur through several mechanisms. We speculate that another possible strategy, as shown in enriched functions of our sialoproteome analysis, may be the use of amine-binding peptides linked to protease N-termini to protect proteases from inhibition and degradation, thereby ensuring delivery to host physiological targets.

As tick saliva secretion are suggested to be variable according to the feeding condition, studies regarding saliva of ticks from laboratory colonies needed to be performed. Our main goal was to investigate the accuracy of data generated from colony ticks which are assumed to be practically pathogen-free and inbred compared to those collected from nature. Apart from the DAP observed, differences in molecular functions were not very drastic. Even though laboratory acclimated ticks might be considered less competitive due to their inbreeding and absence of pathogens, *I. ricinus* maintained effectiveness in vital processes such as blood feeding and, therefore, showed minor changes towards wild species. Nevertheless, some significant differences exist mainly in proteolysis and metalloendopeptidase activities. These differences might be linked to a lesser adaptive response of proteolytic sialome cluster due to the adaptation of proteolytic agents to the colony used to host. A similar loss of proteomic plasticity was observed in a study of human immune response using colony versus wild *Amblyomma americanum* species (Tomás-Cortáz et al., 2017). In fact, colony ticks showed higher immunogenic simulation than wild species, highlighting the loss of proteomic adaptation and, therefore, the production of highly immunogenic proteins (Tomás-Cortáz et al., 2017). In general, tick saliva-related investigations, relied conventionally on the use of samples from laboratory colonies that are considered to be pathogen-free. Thus, the major finding provided here gives great support to proteomic data or any other tick-related experiments that are generated using colony ticks.

We focused on the parasite side of this tick-host interaction, specifically the saliva. However, salivary proteins probably do not act in isolation but through interactions. Currently, the candidates we have proposed only fulfil certain criteria: they are abundantly expressed, their expression is not exclusive to specific conditions that we have tested, and they are certainly introduced into the host via saliva and elicit an antigen-specific host humoral response (antibodies). Including and considering the host proteome in future studies will help generate an interactome, determine the binding partners of candidate antigens at the tick bite site, and support candidate proteins/peptides as vaccine antigens. Our preliminary data suggests that it is possible to isolate specific antibodies and apply them in immunosays to study the candidate proteins: their role(s) in the tick feeding process, their activities, their targets, and the kinetics and specificities of their expression. This will help us choose which candidates to further study, to translate as vaccine antigens, or to target to interrupt the tick life cycle.

5 | CONCLUSIONS

Taken together, our data show for the first time using quantitative proteomics that ticks display a core proteome at the tick-host interface with a variable component in response to ecological adaptation. These proteomic data represent a step towards understanding variations in *I. ricinus* tick salivary antigens at their interface with the vertebrate host and provide a comprehensive characterization of tick salivary protein secretion and their potential to elicit vertebrate humoral responses. This study and the identified protein/peptide candidates offer good starting points for discovering effective targets for antitick vaccine development and markers of tick exposure. It also establishes that the host species must be seriously considered when studying host-ectoparasite interactions at the organismal level to apply research findings from the laboratory to the field successfully.

AUTHOR CONTRIBUTIONS

Stefan Tenzer, Federico Marini, Michael Hackenberg, Michalis Kotsyfakis designed research. Chaïma Bensaoud, Stefan Tenzer, Alicia Poplawski, José María Medina, Mohamed Amine Jmel, Hanne Voet, Imen Mekki, Ernesto Aparicio-Puerta Brent Cuveele, and Ute Distler performed research. All authors analysed the data and wrote at least part of the manuscript.

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CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare that are relevant to the content of this article.

DATA AVAILABILITY STATEMENT

All the data supporting this publication are included in the draft. Proteomic quantification data and .fasta sequence files used for database search are available at Zenodo (10.5281/zenodo.6396487).

BENEFIT-SHARING STATEMENT

Benefits from this research accrue from the sharing of our data and results on public databases as described above. A research collaboration was developed with scientists from the countries listed in the authorship section, all collaborators are included as authors, the results of research have been part of the thesis of two Erasmus Master students from the University of Antwerp, and the research addresses a priority concern, in this case the development of more ecological methods to control ticks and tick-borne diseases. More broadly, our group is committed to international scientific...
Bowman, A. S., Dillwith, J. W., & Sauer, J. R. (1996). Tick salivary proteins irspi and irlip1. *Vaccine*, 8(3), 1–16. https://doi.org/10.1016/0264-410X(96)00049-6

Bensaoud, C., Hackenberg, M., & Kotsyfakis, M. (2019). Noncoding RNAs in parasite–vector–host interactions. *Developmental Biology*, 449(1), 118–128. https://doi.org/10.1016/j.ydbio.2019.02.026

Almazán, C., Fourniol, L., Rakotobe, S., Šimo, L., Bornères, J., Cote, M., Peltier, S., Mayé, J., Versillé, N., Richardson, J., & Bonnet, S. I. (2020). Failed disruption of tick feeding, viability, and molting after immunization of mice and sheep with recombinant *Ixodes ricinus* salivary proteins irspi and irlip1. *Vaccine*, 8(3), 1–16. https://doi.org/10.1016/0264-410X(96)00049-6

Bantscheff, M., & Kuster, B. (2012). Quantitative mass spectrometry in proteomics. *Analytical and Bioanalytical Chemistry*, 404(4), 937–938. https://doi.org/10.1007/s00216-012-6261-7

Bateman, A. (2019). UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Research*, 47(D1), D506–D515. https://doi.org/10.1093/nar/gky1049

Bensaoud, C., Hackenberg, M., & Kotsyfakis, M. (2019). Noncoding RNAs in parasite–vector–host interactions. *Trends in Parasitology*, 35(9), 715–724. https://doi.org/10.1016/j.pt.2019.06.012

Bousquet, T. (2015). Clinical laboratory animal medicine: An Introduction, 4th edition. *The Canadian Veterinary Journal. La Revue Veterinaire Canadienne*, 56(11), 1192.

Bowman, A. S., Dillwith, J. W., & Sauer, J. R. (1996). Tick salivary protaglandins: Presence, origin and significance. *Parasitology Today*, 12(10), 388–396. https://doi.org/10.1016/1089-8961(96)00161-2

Burri, C., Dupasquier, C., Bastic, V., & Kern, L. (2011). Pathogens of emerging tick-borne diseases, *Anaplasmaphagocytophilum*, *Rickettsia* spp., and *Babesia* spp., in *Ixodes* ticks collected from rodents at four sites in Switzerland (Canton of Bern). *Vector-Borne and Zoonotic Diseases*, 11(7), 939–944. https://doi.org/10.1089/vbz.2010.0215

Charrier, N. P., Couton, M., Voordouw, M. J., Rais, O., Durand-Hermouet, A., Hervet, C., Plantard, O., & Rispe, C. (2018). Whole body transcriptomes and new insights into the biology of the tick *Ixodes ricinus*. *Parasites and Vectors*, 11(1), 364. https://doi.org/10.1186/s13071-018-2932-3

Chmelar, J., Calvo, E., Pedra, J. H. F., Francischetti, I. M. B., & Kotsyfakis, M. (2012). Tick salivary secretion as a source of antihemostatics. *Journal of Proteomics*, 75(13), 3842–3854. https://doi.org/10.1016/j.jprot.2012.04.026

Chmelar, J., Kotáš, J., Karim, S., Kopacek, P., Francischetti, I. M. B., Pedra, J. H. F., & Kotsyfakis, M. (2016). Slalomos and Malilomes: A systems-biology view of tick tissues and tick-host interactions. *Trends in Parasitology*, 32, 242–254. https://doi.org/10.1016/j.pt.2015.10.002

Cobon, G. S., & Hungerford, J. (1995). Commercialisation of a recombinant vaccine against *Boophilus microplus*. *Parasitology*, 110(S1), S43–S50. https://doi.org/10.1017/S0031182000001487

Condrey, J. A., Flietstra, T., Nestor, K. M., Schlessor, E. L., Coleman-McCray, J. D., Genzer, S. C., Welch, S. R., & Spengler, J. R. (2020). Prothrombin time, activated partial thromboplastin time, and fibrinogen reference intervals for inbred strain 13/n Guinea pigs (*Cavia porcellus*) and validation of low volume sample analysis. *Microorganisms*, 8(8), 1–11. https://doi.org/10.3390/microorganisms8081127

Cracknell, J. (2008). Anaesthesia of exotic pets. *Veterinary Record*, 162(26), 864. https://doi.org/10.1136/vr.162.26.864

Cramaro, W. J., Revets, D., Hunewald, O. E., Sinner, R., Reye, A. L., & Muller, C. P. (2015). Integration of *Ixodes ricinus* genome sequencing with transcriptome and proteome annotation of the naïve midgut. *BMC Genomics*, 16(1), 871. https://doi.org/10.1186/s12864-015-1981-7

De La Fuente, J. (2003). The fossil record and the origin of ticks (Acari: *Parasitiformes*: *Ixodida*). *Experimental and Applied Acarology*, 29(3–4), 331–344. https://doi.org/10.1023/A:1025824702816

Dérzuaz, M., Frauenschuh, A., Alessandri, A. L., Dias, J. M., Coelho, F. M., Russo, R. C., Ferreira, B. R., Graham, G. J., Shaw, J. P., Wells, T. N., Teixeira, M. M., Power, C. A., & Proudfoot, A. E. (2008). *Journal of Experimental Medicine*, 205(9), 2019–2031. https://doi.org/10.1084/jem.20072689

Distler, U., Kuharev, J., Navarro, P., Levin, Y., Schild, H., & Tenzer, S. (2014). Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. *Nature Methods*, 11(2), 167–170. https://doi.org/10.1038/nmeth.2767

Esmon, C. T., Xu, J., & Lupu, F. (2011). Innate immunity and coagulation. *Journal of Thrombosis and Haemostasis*, 9(S1), 182–188. https://doi.org/10.1111/j.1538-7836.2011.04323.x

Evans, G. O., O’Brien, P., & Watterson, C. L. (2009). *Animal clinical chemistry: A practical guide for toxicologists and biomedical researchers* (2nd ed.). CRC Press.

Francischetti, I. M. B., Sa-Nunes, A., Mans, B. J., Santos, I. M., & Ribeiro, J. M. C. (2009). The role of saliva in tick feeding. *Frontiers in Bioscience (Landmark Edition)*, 14(20), 2051–2088.

Fu, L., Niu, B., Zhu, Z., Wu, S., & Li, W. (2012). CD-HIT: Accelerated for clustering the next-generation sequencing data. *Bioinformatics*, 28(23), 3150–3152. https://doi.org/10.1093/bioinformatics/bts565

Geromonos, S. J., Vissers, J. P., Silva, J. C., Dorschel, C. A., Li, G. Z., Gorenstein, M. V., Bateman, R. H., & Langridge, J. I. (2009). The detection, correlation, and comparison of peptide precursor and product ions from data independent LCMS with data dependant LC-MS/MS. *Proteomics*, 9(6), 1683–1695. https://doi.org/10.1002/pmic.200800562

Ghazalpour, A., Bennett, B., Petyuk, V. A., Orozco, L., Hagopian, R., Mungrue, I. N., Farber, C. R., Sinhjemier, J., Kang, H. M., Furlotte, N., Park, C. C., Wen, P. Z., Brewer, H., Weitz, K., Camp, D. G., 2nd, Pan, C., Yordanova, R., Neuhaus, I., Tilford, C., ... Lusis, A. J. (2011). Comparative analysis of proteome and transcriptome variation in mouse. *PLoS Genetics*, 7(6), 1001393. https://doi.org/10.1371/journal.pgen.1001393

Giles, K., Pringle, S. D., Worthington, K. R., Little, D., Wildgoose, J. L., & Bateman, R. H. (2004). Applications of a travelling wave based radio-frequency-only stacked ring ion guide. *Rapid Communications in Mass Spectrometry*, 18(20), 2401–2414. https://doi.org/10.1002/rcm.1641

Gray, J., Von Stedingk, L. V., Gürtelschmid, M., & Granström, M. (2002). Transmission studies of *babesia microti* in *Ixodes ricinus* ticks and gerbils. *Journal of Clinical Microbiology*, 40(4), 1259–1263. https://doi.org/10.1128/JCM.40.4.1259-1263.2002

Halperin, J. J. (2007). Tick-borne encephalitis. In *Encephalitis: Diagnosis and treatment* (Vol. 34, pp. 157–166). CRC Press, OIE (World Organization for Animal Health). https://doi.org/10.20506/rst.34.2.2371
Heutinck, K. M., ten Berge, I. J. M., Hack, C. E., Hamann, J., & Rowshani, A. T. (2010). Serine proteases of the human immune system in health and disease. *Molecular Immunology*, 47(11–12), 1943–1955. https://doi.org/10.1016/j.molimm.2010.04.020

Hochberg, Y., & Benjamini, Y. (1990). More powerful procedures for multiple significance testing. *Statistics in Medicine*, 9(7), 811–818. https://doi.org/10.1002/sim.4780097010

Jmel, M. A., Aounallah, H., Bensaoud, C., Mekki, I., Chmelar, J., Faría, F., M'hiribi, Y., & Kotsyfakis, M. (2021). Insights into the role of tick salivary protease inhibitors during ectoparasite-host crossstalk. *International Journal of Molecular Sciences*, 22(2), 892. https://doi.org/10.3390/ijms22020892

Kazimírová, M., & Štibrániová, I. (2013). Tick salivary compounds: Their role in modulation of host defences and pathogen transmission. *Frontiers in Cellular and Infection Microbiology*, 3, 43. https://doi.org/10.3389/fcimb.2013.00043

Mans, B. J. (2020). Quantitative visions of reality at the tick-host Interface: Biochemistry, genomics, proteomics, and transcriptomics as measures of complete inventories of the tick SialoVerse. *Frontiers in Cellular and Infection Microbiology*, 10, 574405. https://doi.org/10.3389/fcimb.2020.574405

Parola, P., Paddock, C. D., & Raoult, D. (2005). Tick-borne rickettsioses around the world: Emerging diseases challenging old concepts. *Clinical Microbiology Reviews*, 18(4), 719–756. https://doi.org/10.1128/CMR.18.4.719-756.2005

Patel, V. J., Thalassinos, K., Slade, S. E., Connolly, J. B., Crombie, A., Murrell, J. C., & Scrivens, J. H. (2009). A comparison of labeling and label-free mass spectrometry-based proteomics approaches. *Journal of Proteome Research*, 8(7), 3752–3759. https://doi.org/10.1021/pr90080y

Patzig, J., Jahn, O., Tenzer, S., Wichert, S. P., de Monasterio-Schrader, P., Rosfa, S., Kuharev, J., Yan, K., Bormuth, I., Bremer, J., Aguzzi, A., Orfaniotou, F., Hesse, D., Schwab, M. H., Mobiús, W., Nave, K. A., & Werner, H. B. (2011). Quantitative and integrative proteome analysis of peripheral nerve myelin identifies novel myelin proteins and candidate neuropathy loci. *Journal of Neuroscience*, 31(45), 16369–16386. https://doi.org/10.1523/JNEUROSCI.4016-11.2011

Perner, J., Kropáčková, S., Tenzer, S., & Ribeiro, J. M. C. (2018). Sialome BENSAOUD et al.

Sonenshine, D. E. (2004). Pheromones and other semiochemicals of ticks and their use in tick control. *Parasitology*, 129(SUPPL), S405–S425. https://doi.org/10.1017/S003118200400486X

Sonenshine, D. E. (2006). Tick pheromones and their use in tick control. *Annual Review of Entomology*, 51, 557–580. https://doi.org/10.1146/annurev.ento.51.111010.151150

Tenzer, S., Docter, D., Rosfa, S., Wlodarski, A., Kuharev, J., Rekik, A., Kneuer, S. K., Bantz, C., Nawroth, T., Bier, C., Sirirattanapan, J., Mann, W., Trequel, L., Zellner, R., Maskos, M., Schild, H., & Stauber, R. H. (2011). Nanoparticle size is a critical physicochemical determine of the human blood plasma corona: A comprehensive quantitative proteomic analysis. *ACS Nano*, 5(9), 7155–7167. https://doi.org/10.1021/nn201950e

Tomás-Cortázár, J., Martín-Ruiz, I., Barriales, D., Pascual-Itoiz, M. Á., de Juan, V. G., Caro-Maldonado, A., Merino, N., Marina, A., Blanco, F. J., Flores, J. M., Sutherland, J. D., Barrio, R., Arjas, A., Martínez-Chantar, M. L., Carracedo, A., Simó, C., García-Cañas, V., Abecia, L., Lavin, J. L., ... Anguita, J. (2017). The immunosuppressive effect of the tick protein, Salp15, is long-lasting and persists in a murine model of hematopoietic transplant. *Scientific Reports*, 7, 10740. https://doi.org/10.1038/s41598-017-11354-2

Vechtova, P., Fussy, Z., Cegan, R., Sterba, J., Erhart, J., Benes, V., & Grubhoffer, L. (2020). Catalogue of stage-specific transcripts in *Ixodes ricinus* and their potential functions during the tick life-cycle. *Parasites & Vectors*, 13, 311. https://doi.org/10.1186/s13071-020-04173-4

Verespy, S., Mehta, A. Y., Afsoah, D., Al-Horani, R. A., & Desai, U. R. (2016). Allosteric partial inhibition of monomeric proteases. Sulfated coumarins induce regulation, not just inhibition, of thrombin. *Scientific Reports*, 6(1), 1–13. https://doi.org/10.1038/srep24043

Webster, A. C., & Frank, C. L. (1985). Comparison of immune response stimulated in sheep, rabbits and Guinea pigs by the administration of multi-component clostridial vaccines. *Australian Veterinary Journal*, 62(4), 112–114. https://doi.org/10.1111/j.1751-0813.1985.tb07254.x

Wen, S., Wang, F., Ji, Z., Pan, Y., Jian, M., Bi, Y., Zhou, G., Luo, L., Chen, T., Li, D., Ding, Z., Abi, M. E., Liu, A., & Bao, F. (2020). Salp15, a multifunctional protein from tick saliva with potential pharmaceutical effects. *Frontiers in Immunology*, 10, 3067. https://doi.org/10.3389/fimmu.2019.03067

Wiśniewski, J. R., Zougman, A., Nagaraj, N., & Mann, M. (2009). Universal sample preparation method for proteome analysis. *Nature Methods*, 6(5), 359–362. https://doi.org/10.1038/nmeth.1322

Zhou, X., Akula, R., Li, L., Ding, Z., Abi, M. E., Liu, A., & Bao, F. (2020). Salp15, a multifunctional protein from tick saliva with potential pharmaceutical effects. *Frontiers in Immunology*, 10, 3067. https://doi.org/10.3389/fimmu.2019.03067

SUPPORTING INFORMATION

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