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Differential seminal plasma proteome signatures of boars with high and low resistance to hypothermic semen preservation at 5°C

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Abstract

Background: Hypothermic storage at 5°C has been investigated as an alternative to promote the prudent use of antibiotics for boar artificial insemination doses. However, this temperature is challenging for some ejaculates or boars.

Objective: The present study aimed to identify putative biomarkers for semen resistance to hypothermic storage at 5°C by comparing the seminal plasma proteomes of boars with high and low seminal resistance to preservation at 5°C.

Materials and methods: From an initial group of 34 boars, 15 were selected based on the following criteria: ejaculate with ≤20% abnormal spermatozoa and at least 70% progressive motility at 120 hours of storage at 17°C. Then, based on the response to semen hypothermic storage at 5°C, boars were classified into two categories: high resistance—progressive motility of >75% in the three collections (n = 3); and low resistance—progressive motility of <75% in the three collections (n = 3). Seminal plasma proteins were analyzed in pools, and differential proteomics was performed using Multidimensional Protein Identification Technology.

Results: Progressive motility was lower at 120 hours of storage in low resistance, compared to high resistance boars ($P < .05$). Acrosome and plasma membrane integrity were not affected by the boar category, storage time, or their interaction ($P \geq .104$). Sixty-five proteins were considered for differential proteomics. Among the differentially expressed and exclusive proteins, the identification of proteins such cathepsin B, legumain, and cystatin B suggests significant changes in key enzymes (eg, metalloproteinases) involved in spermatogenesis, sperm integrity, and fertilizing potential.

Discussion and Conclusion: Differences in the seminal plasma suggest that proteins involved in the proteolytic activation of metalloproteinases and proteins related to immune response modulation could disrupt key cellular pathways during spermatogenesis and epididymal maturation, resulting in altered resistance to chilling injury.

Further in vivo studies focusing on the immunological crosstalk between epithelial cells and gametes might explain how the immune regulators influence sperm resistance to hipothermic storage.

KEYWORDS
extended semen, liquid preservation, proteomic, storage temperature

1 | INTRODUCTION

Artificial insemination (AI) is the most common biotechnology used in the pig industry being the liquid preservation almost exclusively used. To avoid cold shock, extended boar semen is traditionally liquid-preserved at 15-20°C for up to 5 days.[1] However, in this storage temperature range, and due to media composition, bacteria can grow easily. Bacteriospermia negatively affects the quality of semen doses due to the higher incidence of sperm-to-sperm agglutination, damaged acrosomes, poor sperm motility, and reduced sperm longevity.[2,3]

Aiming to promote the prudent use of antibiotics, a recent commercial antibiotic-free boar semen extender has been tested under hypothermic storage.[4,5] Liquid-extended boar semen without antibiotics preserved at 5°C or 10°C reduced bacterial load and maintained satisfactory sperm motility (>75%) and plasma membrane integrity (>80%) during 120 hours of storage.[4] Moreover, high fertilizing capacity was also evidenced, with a farrowing rate of 92% after the insemination of sows and gilts with antibiotic-free doses stored at 5°C.[5] These promising results indicate that the use of temperatures as low as 5°C for boar semen preservation is a feasible alternative to mitigate semen dose bacterial contamination.

Individual resistance of boar to chilling injury in semen cryopreservation protocols has already been evidenced[6] and the same assumption may be applied to liquid-preserved semen. Indeed, boars can perform differently at 17°C[7,8] and also at 5°C.[9] At the moment of semen collection, it is not possible to predict whether a given ejaculate will respond to liquid preservation at cold temperatures, since raw semen often presents acceptable seminal trait values. Therefore, the use of seminal biomarkers associated with seminal resistance to cooling could bring several benefits to boar stud farms. Recently, the association of individual boar sperm resistance to storage at 17°C for up to 144 hours with proteins of the seminal plasma has been already established.[10] However, it remains unclear whether seminal plasma proteins would modulate the resistance to chilling injury at 5°C. Thus, the present study aimed to compare seminal plasma proteomes of boars with high and low resistance to hypothermic preservation at 5°C, identifying putative biomarkers for seminal resistance to cooling.

2 | MATERIALS AND METHODS

All procedures involving animals were approved by the Research Committee of the Universidade Federal do Rio Grande do Sul (project number 32493).

2.1 | Animals and facilities

Boars (AGPIC 337®; Agroceres PIC, and Traxx®; Topigs Norsvin) from a commercial boar stud farm located in the south of Brazil were collected weekly for routine AI dose production. The average age was 13.3 ± 3.8 (±SD) months, and boars were housed individually in crates (0.70 × 2.4 m) with slatted floor, in a temperature-controlled barn (16-20°C) under a 16-hour light/d regime. Water access was ad libitum, and all boars were fed 2.1-2.4 kg/d of the same nutritional corn and soybean meal diet (3.02 Mcal metabolizable energy/kg, 19.68% crude protein, and 1.07% total lysine).

2.2 | Experimental design

One normospermic ejaculate from each boar (n = 34) was used (Figure 1). Boars were selected for the study if they presented satisfactory libido, if the ejaculate presented >80% normal spermatozoa, and if the extended semen reached a minimal 70% threshold of progressive motility at 120 hours of storage at conventional preservation (17°C). Selected boars (n = 15) had three consecutive ejaculates analyzed to verify the resistance to liquid preservation at 5°C for up to 120 hours, totaling 45 ejaculates.

After the three semen collections, boars were classified, according to the progressive motility of the extended semen after 120 hours of storage under hypothermic preservation (5°C), into two categories: high resistance (HR)—progressive motility of >75% in the three collections (n = 3); and low resistance (LR)—progressive motility of <75% in the three collections (n = 3). Animals with doses of progressive motility of <75% in one and/or two collections (n = 6) were not included in the analyses. In addition, two selected boars were removed due to low progressive motility at 17°C and one boar did not complete the third semen collection. Thus, nine ejaculates from each category were considered for the proteomic analyses.

2.3 | Semen collection, processing, and sperm analyses

Ejaculates were collected using a semi-automatic collection system (BoarMatic; Minitüb GmbH). Normospermic ejaculates[11] were extended isothermically (34°C) diluted in one step with Androstar® Premium extender (Minitüb GmbH) to produce doses with 1.5×10^9 spermatozoa and stored in 60-mL tubes (QuickTip Flexitube®; Minitüb GmbH) with a total volume of 45 mL. The extended semen doses were placed in

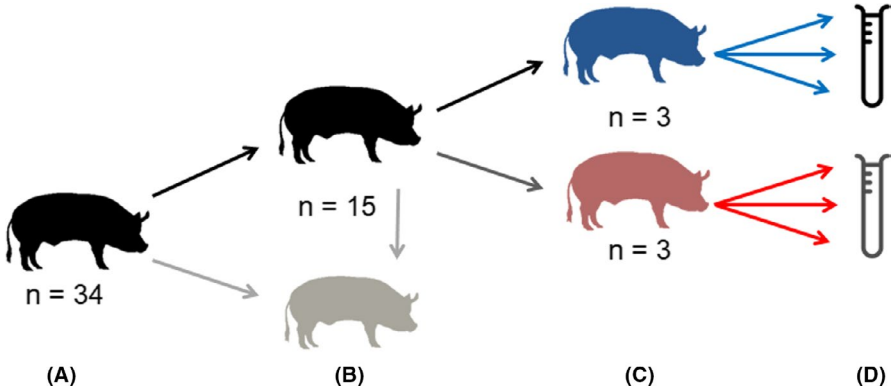

FIGURE 1 Schematic view of the experimental design. A, One normospermic ejaculate from each 34 boar was evaluated during 120 h of 17°C storage. Boars with ejaculates presenting >80% normal spermatozoa and progressive motility >70% at 120 h of storage were selected. B, Three semen collections were performed for selected boars (n = 15 boars). Boars were classified, according to the progressive motility of the extended semen at 120 h of storage, under hypothermic preservation (5°C), into two categories: high resistance (HR)—progressive motility >75% in the three collections (n = 3); and low resistance (LR)—progressive motility <75% in the three collections (n = 3). C, Three ejaculates of each boar were used for the proteomic analyses and D, nine ejaculates were pooled, according to the boar category

a temperature-controlled box (22-24°C) until arrival at the laboratory for storage and further analysis (maximum 4 hours, including processing and transport time). Once in the laboratory, the extended semen doses were placed into temperature-controlled cabinets at either 17°C or, after 6-hour stabilization period at room temperature, at 5°C.[4,5]

For analyses performed during storage, a different semen dose tube was used at each time of evaluation (24, 72, and 120 hours of storage) to avoid the influence of manipulation. Total and progressive motility were assed using a CASA system (AndroVison® version 1.1.4; Minitüb GmbH) under phase contrast microscopy (200× magnification, Axio Scope.A1, Zeiss®).[4,5]

Samples of raw and extended semen were fixed in a formalin-citrate solution for sperm morphology and acrosome integrity analyses,[4,5] respectively. All samples were analyzed under a phase contrast microscope at 1000× magnification, and 200 spermatozoa were assessed for sperm morphology and classified as normal or abnormal.[12] Plasma membrane integrity was assessed at arrival in the laboratory, and at 24, 72, and 120 hours of storage using a double-staining method with the fluorescent probes SYBR-14 and propidium iodide, PI (LIVE/DEAD® Sperm Viability kit; Thermo Fisher Scientific).[11,13] Analyses were performed with the CASA system equipped with a viability module and fluorescence microscope (Axio Scope.A1 HAL 100-FL, Filter Set 09, BP 450-490; Zeiss®).[11,13] Upon arrival at the laboratory, sperm membrane integrity was 87.4 ± 1.3%.

2.4 | Statistical analyses

All analyses were performed using the Statistical Analysis System software (SAS, version 9.4, Institute Inc). The sperm motilities, acrosome integrity, and plasma membrane integrity were analyzed as repeated measures, considering binomial distribution, using the GLIMMIX procedure. The category of boars, storage time, and their interaction were included as fixed effects, whereas the boar was included as a random effect. Tukey's test was used as a post hoc test for pairwise comparisons of means at significance level of 5%, and results are expressed as LSmeans ± standard error of the mean.

2.5 | Preparation of seminal plasma protein extracts

Aliquots of 5 mL of ejaculates were centrifuged at 600 *g* for 15 min to separate the seminal plasma from spermatozoa. A further clarification of the seminal plasma was obtained with another centrifugation at 12 000 *g* for 1 hour at 4°C in order to remove cell debris and other particles. After addition of protease inhibitors (P8340; Sigma-Aldrich), total protein contents were quantified in all samples using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) and stored at −80°C. Only samples from boars classified as HR or LR resistant boars were then used for the subsequent steps.

In order to minimize the individual variation among the ejaculates of each boar, LC-MS/MS analyses were performed with a pool composed of three subsequent ejaculates of each boar. For the preparation of the pool, a sample of 300 μg of protein was extracted from the seminal plasma from each of the three ejaculates. Then, a second pool was prepared using the pool of seminal plasma protein extracts from two different boars from the same category. Taken together, three pools of seminal plasma protein extracts were evaluated in the HR group and another three pools were evaluated in the LR group.

2.6 | Protein digestion and MudPIT analyses

For Multidimensional Protein Identification Technology (MudPIT), proteins were precipitated by a chloroform/methanol method.[14] Pellets were resuspended in 100 mmol/L Tris-HCl pH 7.0, containing 8 mol/L urea, and submitted to a tryptic digestion. Briefly, disulfide bonds were reduced in 5 mmol/L dithiothreitol (DTT) for 20 minutes at 37°C, and

then cysteines were alkylated in 25 mmol/L iodoacetamide (IAM) for 20 minutes at room temperature in the dark. Urea was diluted to 2 mol/L with 100 mmol/L Tris-HCl pH 7.0, trypsin was added at mass ratio of 1:100 enzyme/protein with 1 mmol/L $CaCl_2$ and the sample incubated overnight at 37°C; 5% v/v of formic acid was added to stop the reaction. Peptide separation was performed on an in-house-made 20 cm reverse-phase column (5 μm ODSAQ C18; Yamamura Chemical Lab) using a nanoUPLC (nanoLC Ultra 1 D plus; Eksigent). Mobile phase A consisted of water/acetonitrile/formic acid (95:5:0.1) and mobile phase B consisted of water/acetonitrile/formic acid (10:90:0.1). Approximately 2 μg were injected in technical triplicates through the autosampler. The flow rate was set to 300 nL and a 120-minute reversed-phase gradient was used: 0%-5% B for 5 minutes; 5%-25% B for 60 minutes; 25%-50% B for 20 minutes; 50%-80% B for 15 minutes; 80% B for 5 minutes; 80%-5% B for 1 minute; and 5% B for 14 minutes. The LC system was coupled to a hybrid mass spectrometer (LTQ-XL and LTQ Orbitrap Discovery, Thermo) by a nano-electrospray ion source (Thermo Fischer Scientific, EUA). The mass spectrometer was operated in a data-dependent mode and data collected with one MS1 full scan in the Orbitrap (400-1600 m/z range; 30 000 resolution). The eight most abundant ions per scan were selected to CID MS2 in the ion trap. Three technical replicates were performed per pool.

2.7 | Data analysis

The MS/MS spectra acquired from precursor ions were searched with comet[15] in the PatternLab for Proteomics platform[16] against a database contained a non-redundant *Sus scrofa* proteome (ID UP000008227, www.uniprot.org) and reverse sequences. The validity of the peptide-spectra matches (PSMs) generated was assessed using PatternLab's Search Engine Processor (SEPro) with a false discovery rate of 1% based on the number of decoys.

Venn diagrams were generated using PatternLab for Proteomics platform module. Proteins were grouped by maximum parsimony, and the presence of proteins in at least seven replicates was required for each condition. Volcano plots were generated by comparison between two semen sample groups using TFold module in PatternLab for Proteomics platform. Proteins were grouped by maximum parsimony, and normalized spectral abundance factor (NSAF) was used to normalize spectral count data. Benjamin–Hochberg (BH) *q*-value was set at 0.02. A variable fold-change cutoff for each individual protein was calculated according to the *t* test *P*-value using an F-stringency automatically optimized by the TFold software. Low-abundance proteins were removed using an L-stringency value of 0.4.

2.8 | Bioinformatics

Identified proteins were categorized by Gene Ontology (GO) annotation[17] according to biological process, molecular function and cellular component, using Blast2GO 5.2.[18] Also, protein-protein interactions were evaluated using the STRING v.11 server (http://string-db.org/) database, indicating physical and functional associations and generating an interaction network.[19]

2.9 | In silico protein structure prediction

The structure modeling of the porcine cathepsin B (A0A287BF94), cystatin E/M (F1RU34), and legumain (I3LKM9) was performed using Phyre2 server[20] in intensive modes. The stereochemical quality and structural integrity of the model were validated by RAMPAGE[21] (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php), QMEAN[22] (https://swissmodel.expasy.org/qmean/), MolProbity 4.4[23] (https://github.com/rlabduke/MolProbity), and Verify 3D[24] (https://servicesn.mbi.ucla.edu/Verify3D/). Also, predicted models were analyzed using ProSA-web server[25,26] (https://prosa.services.came.sbg.ac.at/prosa.php) to validate protein structure and to calculate a *Z*-score for each model and, thus, to determine the overall model quality. Generated PDB files were uploaded to ProFunc (https://www.ebi.ac.uk/thornton-srv/databases/ProFunc/),[27,28] a metaserver that combines sequence-, global structure-, and local structure-based methods to obtain a set of function predictions. Structures were visualized using PyMol 2.0.

2.10 | Protein-protein docking

The validated predicted structure models for cathepsin B, legumain, and cystatin E/M were subjected to protein-protein docking in silico. Three different servers were used to validate the docking of cystatin E/M to cathepsin B and legumain: CoDockPP,[29] a multistage framework for both ab initio protein-protein docking and site-specific docking that applies the efficient FFT-based method to systematically evaluate shape complementarity and focuses on the ligand protein poses with good surface complementarity (http://codockpp.schanglab.org.cn/); HawkDOCK,[30] where protein-protein interactions were predicted by combining ATTRACT docking and HawkRank re-scoring and the key residues for PPIs highlighted by the MM/GBSA free energy decomposition (http://cadd.zju.edu.cn/hawkdock/); and HDOCK,[31] a pipeline based on a hybrid docking algorithm of template-based modeling and free docking (http://hdock.phys.hust.edu.cn). The best docking models were refined using GalaxyWeb RefineComplex server[32] (http://galaxy.seoklab.org/index.html). Validations of interaction interfaces between proteins were accessed using the servers PDBePISA[33] (https://www.ebi.ac.uk/pdbe/pisa/) and EPPIC[34] (http://www.eppic-web.org/). Finally, binding interfaces were evaluated using LigPlot+.[35]

3 | RESULTS

3.1 | Extended semen quality between categories of boars during 120 hours storage at 5°C

There was a significant interaction between category and storage time for total ($P = .006$) and progressive ($P = .002$) motilities (Figure 2).

Only at 120 hours of storage the sperm motilities were higher in the HR category compared to LR ($P < .05$). Furthermore, HR boars did not show reductions in motilities during storage at 5°C, while the LR boars showed a reduction in total motility at 120 hours of storage, and in progressive motility at 72 and 120 hours of storage. Integrities of acrosome and membrane are shown in Table 1. Membrane ($P \geq .104$) and acrosome integrity ($P \geq .126$) were not affected by the category of boars, storage time at 5°C, or interaction of category × storage time.

3.2 | Differences between seminal plasma proteomes

In the present study, 65 proteins were considered for the comparison between HR and LR boars for hypothermic preservation at 5°C. Fifty-four proteins were identified in both phenotypes, and only four and seven proteins were exclusive in the HR and LR groups, respectively (Figure 3). Five proteins were differentially expressed between groups, being highly expressed in the seminal plasma of LR boars. The proteins cystatin E/M, cathepsin B, complement regulator factor H, and lipocalin 5 were found in the UniProt database. One protein (A0A286ZTC9) was a non-characterized protein (entry submitted based on predicted data from genomic studies). Based on sequence homology, it might be an isoform of Ig-like domain-containing protein (A0A287B626). Table 2 summarizes relevant information regarding the exclusive and differentially expressed proteins. The complete list of identified proteins is available as supporting information.

The comparison of the GO analyses of both proteomes revealed few differences, with an average increase of 5% in the number of hits of each term (Figure 4). Regarding biological processes, the higher hits accounted for cellular process, biological regulation, development, reproductive process, and reproduction. The most prevalent molecular functions were ion binding, protein binding, carbohydrate binding, and hydrolase activity. Since the proteins were found in the seminal plasma, most of them are expected to be secreted. Indeed, more than 70% of the proteins were associated with the extracellular region and extracellular space. However, several proteins were also identified as membrane or endomembrane proteins as well of cytoplasmic origin. This is an expected result, since fragments of cell lysis from reproductive tissues are frequently present in the semen.

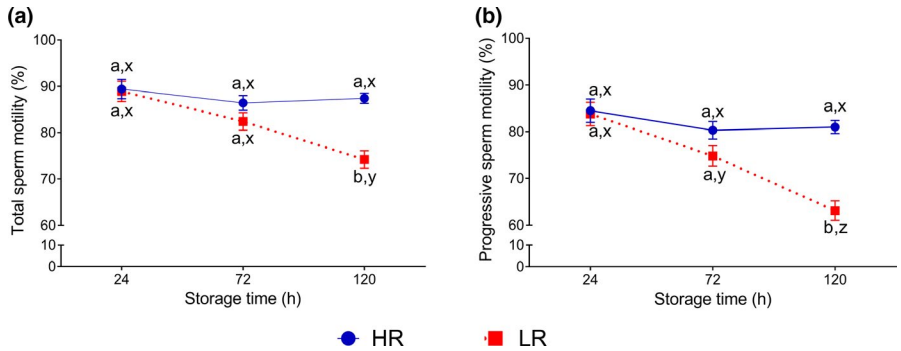

FIGURE 2 Effect of the interaction between boar category × storage time for total ($P = .006$) and progressive motility ($P = .002$) in extended semen of HR (n = 3) and LR boars (n = 3) during 120 h of hypothermic storage (5°C). a and b indicate significant differences between boars of the HR and LR categories within the storage time ($P < .05$). *x*, *y*, and *z* denote significant differences between storage time within the category of boars ($P < .05$). HR: progressive motility of >75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections. LR: progressive motility of <75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections (n = 9 ejaculates per category of boar)

TABLE 1 Acrosome integrity and plasma membrane integrity according to the category of boars and storage time of AI doses stored at 5°C for up to 120 h (n = 9 ejaculates per category of boar)

	Category of boars[a]			Storage time, h			*P*-values		
	HR	LR	SEM[b]	24	72	120	Category of boars	Storage time	Category × Storage time
Acrosome integrity	94.0	95.5	0.8	95.2	95.2	93.8	.186	.104	.719
Plasma membrane integrity	84.9	82.6	1.7	82.2	83.9	85.1	.331	.213	.126

Note: All selected boars presented progressive motility of >80% at 120 h of storage at conventional preservation (17°C). Values expressed as LSmeans.

[a]HR: included boars (n = 3) that produced AI doses with progressive motility of >75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections. LR: included boars (n = 3) that produced AI doses with progressive motility of <75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections.

[b]The highest value of the standard error of mean was used.

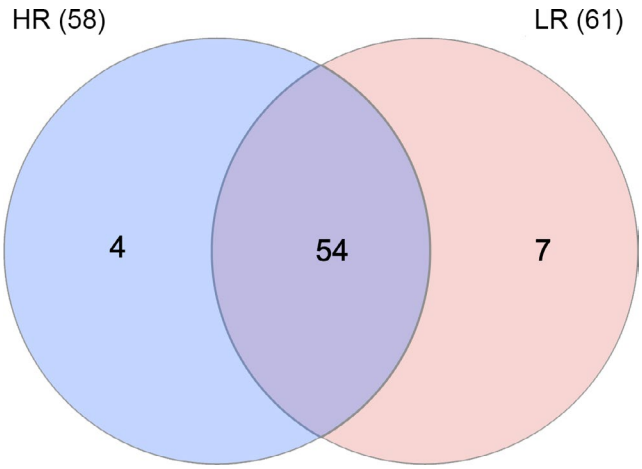

FIGURE 3 Venn diagram depicting differences in the proteomes of seminal plasma of HR (n = 3) and LR boars (n = 3). HR: progressive motility of >75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections. LR: progressive motility of <75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections (n = 9 ejaculates per category of boar)

The analysis of protein-protein interaction (Figure 5) revealed a close association of cystatin E/M with legumain and cathepsin B, cysteine proteases with several roles in tissue metabolism and remodeling. Also, interactions between clusterin and complement regulator factor H, prostaglandin-H2 D-isomerase, and lipocalin 5 are evidence of immune modulation by the seminal plasma proteins.

3.3 | Cathepsin, legumain, and cystatin E/M in silico structural modeling

Phyre2 server was used to generate structural models of cathepsin (Figure 6), legumain (Figure 7), and cystatin E/M (Figure 8). The best-ranked models were verified by Ramachandran plot analysis, qualitative model energy analysis (Verify 3D, MolProbity, QMEAN) and ProSA-web, and presented *Z*-scores and values within the range of scores typically found for native proteins of similar size (Table 3). Also, functional and GO evaluation by ProFUNC confirmed that the generated 3D models were closely associated with their homologs.

TABLE 2 List of differentially expressed and exclusive proteins found in seminal plasma of high-resistance (HR) and low-resistance (LR) boars

Differentially expressed proteins				
UniProtKB	**Protein name**	**Gene name**	**Log_2FC**	***P*-value**
A0A287BD18	Complement regulator factor H	*CFH*	−1.4583	.0463
F1RU34	Cystatin E/M	*CST6*	−1.6129	.0162
A0A287BF94	Cathepsin B	*CTSB*	−1.5717	.0170
A0A287AC59	Lipocalin 5 (cytosolic fragment)	*LCN5*	−1.1977	.0228
A0A286ZTC9	Uncharacterized protein	–	−1.3750	.0298
Proteins found exclusively in seminal plasma of HR boars				
UniProtKB	**Protein name**	**Gene name**		
I3LKM9	Legumain	*LGMN*		
A0A286ZW70	Peptidyl-prolyl cis-trans isomerase	*PPIB*		
K7GND8	Clusterin	*CLU*		
A0A287AUN3	Ribonuclease A family member 9	*RNASE9*		
Proteins found exclusively in seminal plasma of LR boars				
UniProtKB	**Protein name**	**Gene name**		
Q7YR83	Epididymal sperm-binding protein 1	*ELSPBP1*		
F1SKB1	Ceruloplasmin	*CP*		
I3LVD5	Actin gamma 1	*ACTG1*		
F1RNP2	Ig-like domain-containing protein	*AZGP1*		
A0A287BJ64	MHC_I-like_Ag-recog domain-containing protein	*AZGP1*		
A0A287APX1	Prostaglandin-H2 D-isomerase	*PTGDS*		
A0A287ALA0	Brain abundant membrane attached signal protein 1	*BASP1*		

Note: HR: included boars that produced AI doses with progressive motility of >75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections. LR: included boars that produced AI doses with progressive motility of <75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections.

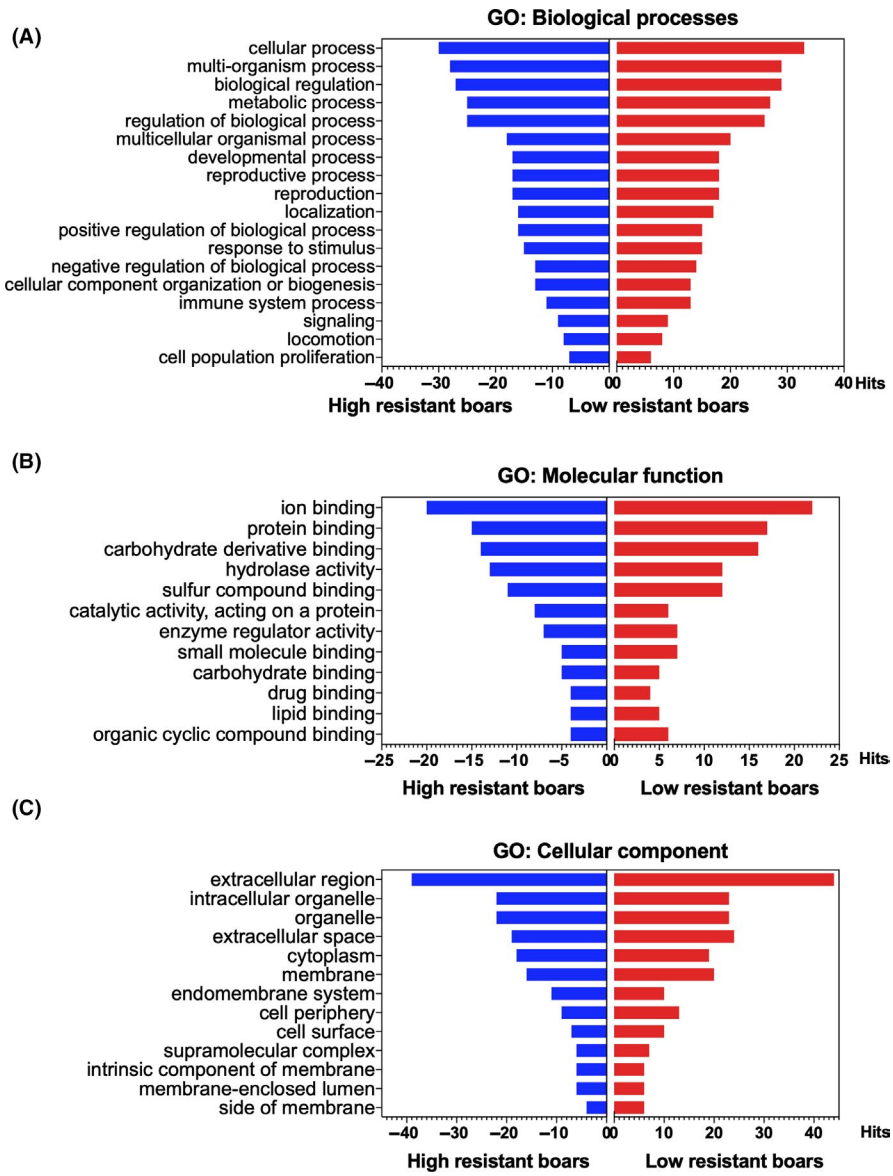

FIGURE 4 Gene Ontology analysis of the proteomes of seminal plasma collected from HR and LR boars. HR: progressive motility >75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections. LR: progressive motility <75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections

3.4 | Validation of cystatin E/M docking in predicted cathepsin B and legumain models

To verify the protein-protein interaction between cathepsin B and cystatin E/M and between legumain and cystatin E/M, four different protein-protein docking servers were used. The summary of the results of the top five predictions generated by the servers and the results of docking complex refinement are presented as Supporting Information. Protein-protein interactions provided by the different docking servers were accessed by PDBePISA and EPPIC servers (Tables S1-S9), showing that CoDOCKPP generated best docking results for cathepsin B and cystatin E/M binding (Figure 9) and HawkDOCK for legumain and cystatin E/M binding (Figure 10).The residues involved in the binding between cystatin E/M and cathepsin B and legumain are presented in Figures 8C and 9C, respectively.

4 | DISCUSSION

Boar spermatozoa are highly sensitive to lower temperature, as demonstrated by the lipid phase transition and fluidity decrease of plasma head membranes occurring in the temperature zone from 30°C to 10°C.[36,37] Losses in sperm motility and membrane integrity caused by the cooling become apparent in the conventional semen storage at 17°C[38] and might be even more pronounced at 5°C.[39,40] Previous studies have provided evidence that cooling from 30°C to 5°C is a viable strategy for boar semen preservation when using a protective extender for hypothermic storage.[4,5] In the present study, we investigated the differences in the sensitivity of spermatozoa from different boars to the liquid 5°C preservation. To clarify, the 75% progressive motility was established to identify boars that would be safely selected for the hipothermic storage technology for boar studs. Boars with progressive motility close to the minimal

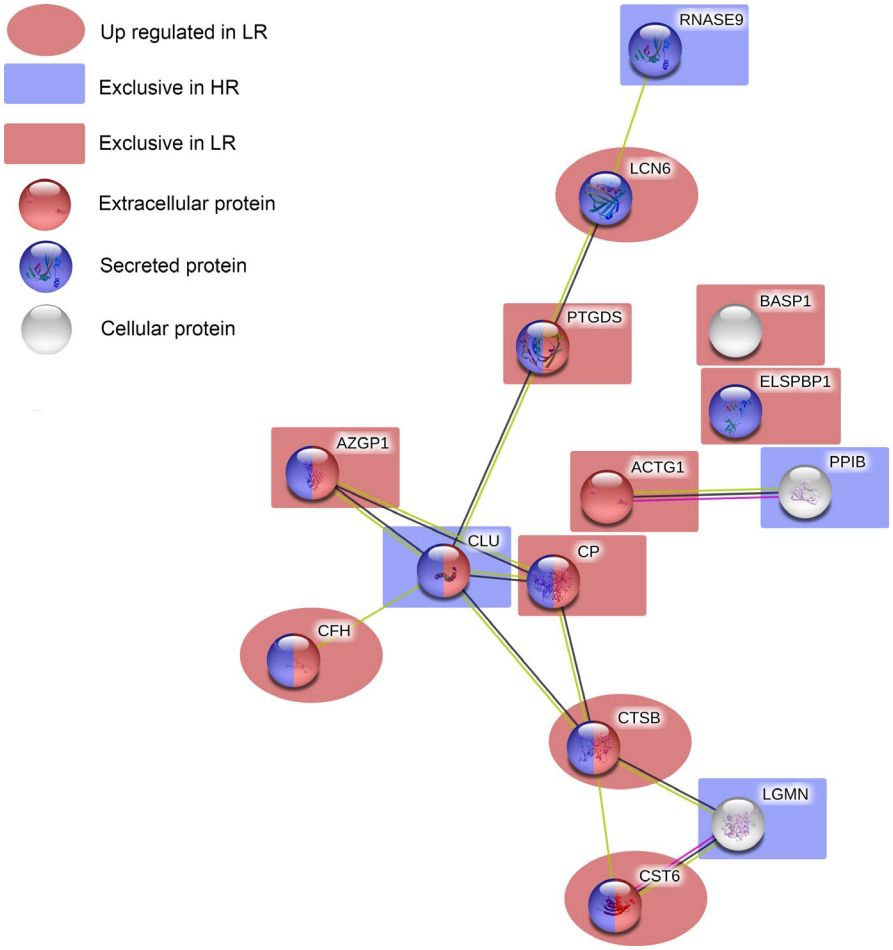

FIGURE 5 Protein-protein interaction network of the differentially expressed (light red ellipse) and exclusive proteins found in the seminal plasma of HR and LR boars. Red nodes indicate proteins associated with GO term extracellular space (GO: 0005615); purple nodes indicate proteins identified as secreted (KW-0964) in the UniProt database. Gray nodes indicate cellular proteins. Network generated by STRING 5.0 server. HR: progressive motility of >75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections. LR: progressive motility of <75% at 120 h of hypothermic storage (5°C) in three subsequent semen collections

threshold for AI would represent a risk for acceptable reproductive performance.

The percentage of motile spermatozoa is an indicator of chilling and storage damage in spermatozoa and is considered as the most important indicator used in boar AI centers, with a minimum threshold of 60%-80% for usable semen.[41] Although the high levels of acrosome and plasma membrane integrity observed during 120 hours of storage, it seems that boar effect exists when considering the hypothermic storage. Boars from both groups had >78% progressive motility at 17°C (data not shown) and at 24 hours at 5°C storage (Figure 1), but low resistant group showed a steady reduction up to 120 hours of 5°C storage. However, chilling injury can occur to a sublethal level that does not cause loss of motility but may impair other parameters such as changes in membrane lipid architecture.[42,43]

It is important to consider that the satisfactory in vivo results previously obtained[5] were from sows inseminated with heterospermic doses from pools of a total of 23 boars. Thus, the boar effect was masked to some extent. In a recent study, a significant individual effect was found on the ability to maintain high sperm quality at 5°C preservation.[9] For this reason, the identification of proteins in seminal plasma of boars with high or low resistance to hypothermic preservation can bring more clarity to this topic. In our study, five proteins were highly abundant in the seminal plasma of boars showing lower resistance to seminal cooling at 5°C. Functional analyses of these proteins provide new information on hypothermic preservation of boar semen.

The overall comparison between the seminal plasma proteomes of HR and LR boars under preservation at 5°C showed mild differences regarding the number of proteins in both proteomes and differentially expressed proteins. Previous works comparing seminal plasma proteomes from boars with different semen qualities[44,45] and resistance to the conventional storage at 17°C[10] also observed few quantitative differences in the protein profiles. In the present work, from a total of 34 boars evaluated for sperm resistance to hypothermic storage at 5°C, six males were used for the proteomic analysis of the seminal plasma. Despite the reduce number of boars per group, we used three ejaculates per individual in order to improve biological significance of the experimental groups. Sample pooling was used to avoid individual variability, since our main goal was to find putative biomarkers between groups. However, this strategy brings some limitations such as the identification of outlier proteins and possible masking of one individual with significantly different protein content. In order to minimize the experimental design limitations, a higher stringency was set in the data analysis. Nevertheless, a closer look in the roles of the differentially expressed proteins in metabolic pathways allows a more precise

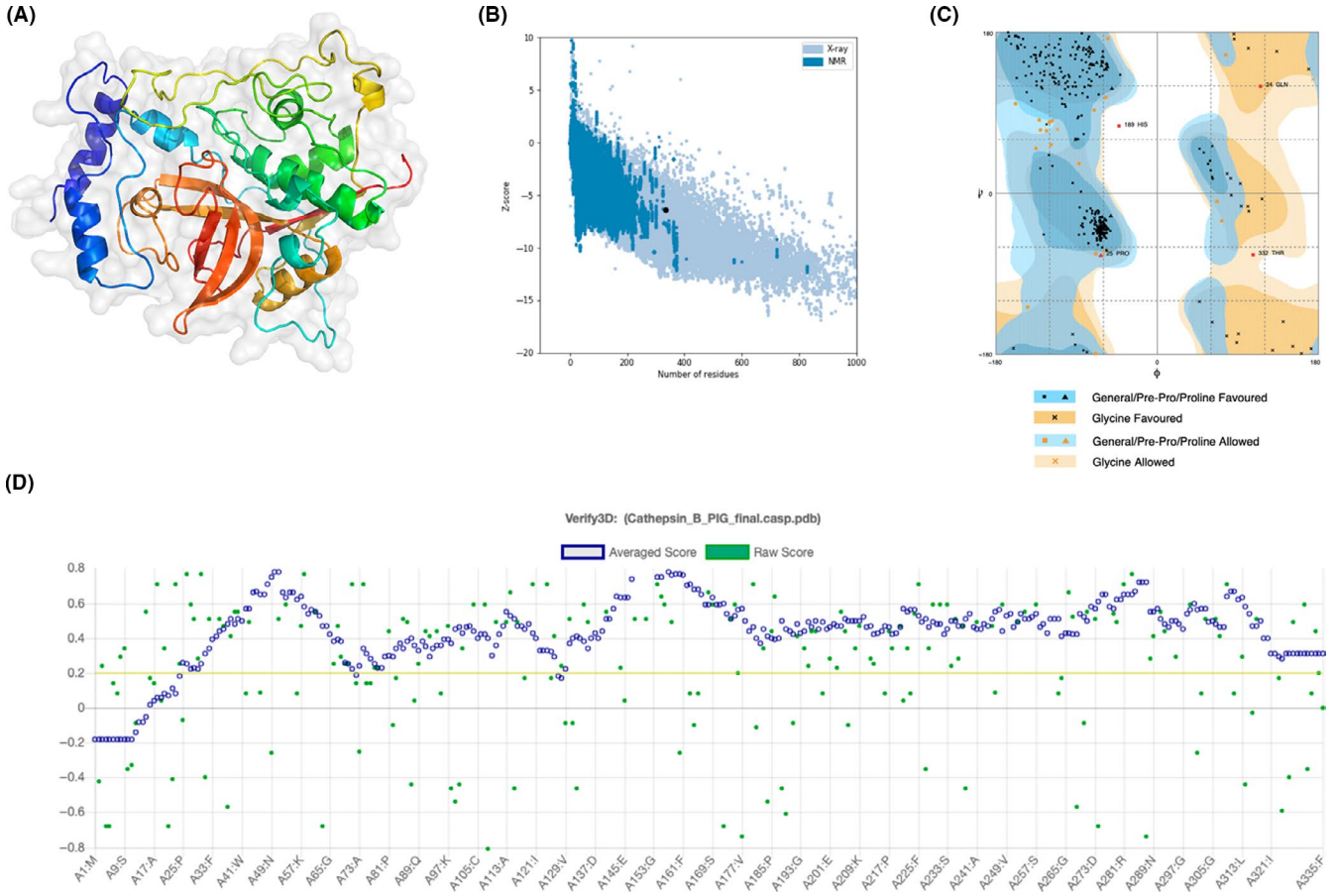

FIGURE 6 In silico prediction of porcine cathepsin B based on its sequence (335 amino acids; UniProtKB A0A287BF94). A, Cartoon representation of the tertiary structure of porcine cathepsin B as predicted by Phyre2. B, ProSA-web results of the predicted model, with a *Z*-score of −6.4 (overall model quality). C, Ramachandran plot analysis of the model generated by RAMPAGE. D, Validation of porcine cathepsin B predicted model by Verify 3D showing that 91.94% of the residues have average 3D-1D score of ≥0.2

understanding of the possible impacts of the increase or reduction in specific proteins on the resistance of boar spermatozoa submitted to hypothermic storage at 5°C. In the following paragraphs, proteins with possible involvement in sperm resistance will be discussed based on their main biochemical properties, participation in testicular and epididymal signaling pathways and known roles in sperm physiology.

4.1 | Possible roles of proteolytic pathways on sperm resistance to storage at 5°C

The present findings suggest a role of serine proteases and metalloproteases in the modulation of sperm resistance against the cold damage from hypothermic preservation at 5°C. The protein-protein interaction network analysis indicated a close relationship among cathepsin B, cystatin E/M, and legumain, proteins involved in important proteolytic events in several tissues, including semen. In fact, cystatin E/M is a well-known inhibitor of cathepsin B and legumain activities. Therefore, the increased abundance of these proteins in the seminal plasma of boars with low resistance to storage at 5°C might be associated with changes in the proteolytic pathways triggered by legumain and cathepsin B.

Cathepsin B is a member of the papain-like family of cysteine proteases, synthesized as a preproenzyme[46] with endopeptidase and exopeptidase activity. Cathepsin B is an important participant within cellular proteolytic networks; its overexpression is frequently associated with altered trafficking of the enzyme to the plasma membrane and secretion into the extracellular milieu.[47] Cathepsin B degrades and remodels the extracellular matrix (ECM), thus promoting migration and invasion of tumor cells. Also, it contributes to proteolytic networks as an activator of downstream serine proteases and metalloproteases frequently involved tumorigenesis and invasion.[48,49] Recent studies have demonstrated that cathepsin B is overexpressed in the seminal plasma of boars with low seminal quality[45] and boars with high resistance to storage at 17°C.[10] Two possible events could explain these contrasting findings: (a) the intrinsic enzymatic activity of cathepsin B, dependent on maintenance of its native state, and (b) the presence of cathepsin B inhibitors. Recently, cathepsin B activity was associated with oocyte quality, being associated with insulin signaling pathways[50] and control of autophagy.[51] Future studies should determine

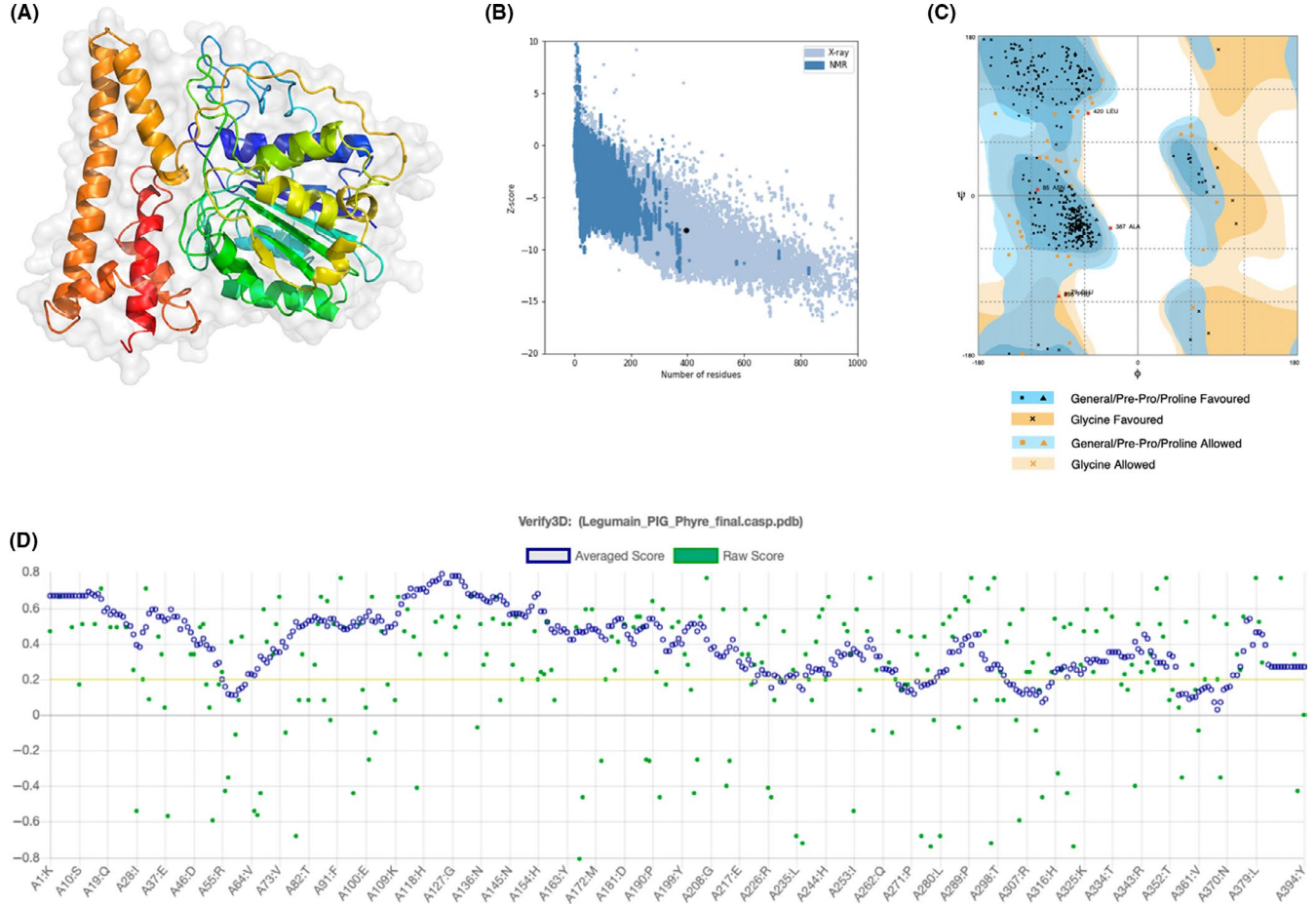

FIGURE 7 In silico prediction of porcine legumain based on its sequence (394 amino acids; UniProtKB I3LKM9). A, Cartoon representation of the tertiary structure of porcine legumain as predicted by Phyre2. B, ProSA-web results of the predicted model, with a *Z*-score of −8.18 (overall model quality). C, Ramachandran plot analysis of the model generated by RAMPAGE. D, Validation of porcine legumain predicted model by Verify 3D showing that 85.53% of the residues have average 3D-1D score of ≥0.2

whether different levels of cathepsin B activity are involved in boar spermatozoa functional and structural preservation.

Legumain is a member of the C13 family of cysteine proteases and cleaves protein substrates at the C-terminus of asparagine.[52] Legumain was first observed to be located in the endosome/lysosome,[53] in the nucleus,[54,55] on the surface of cells,[56] and in the ECM.[57] In reproductive tissues, legumain can be found in the testis,[53] epididymal fluid,[58] and seminal plasma[59,60] in different species. Similar to cathepsin B, legumain is involved in many physiological and pathological processes,[61] such as antigen processing,[62] cell migration,[56] and proliferation.[55] The upregulation of legumain expression has been reported in various solid tumors, positively correlating with their invasive and metastatic potential.[56,63] This observation might be directly associated with the activation of matrix metalloproteins (MMPs) by legumain,[64] in a similar pattern as cathepsin B.

Legumain has been previously related to male fertility. Increased legumain is found in human semen with oxidative stress,[65] and has been correlated with acrosome intact live bull spermatozoa.[60] Legumain expression is regulated by JD-1, a coactivator to various transcription factors, including the androgen receptor and p53.[66] DJ-1 is associated with infertility of rats and mice and participates in fertilization during the interaction of the spermatozoa with the zona pellucida.[67] Studies of DJ-1 in a knockout mouse model revealed a significant increase in legumain expression and activity in the epididymis[68]; however, the consequences for sperm quality and fertility were not investigated. Nonetheless, DJ-1 levels are significantly lower in seminal plasma from asthenozoospermic men compared to that from healthy donors.[59]

Cystatins form a superfamily of cysteine protease inhibitors that is subdivided into four subfamilies: stefins, cystatins, kininogens, and non-inhibitory cystatins.[69] Cystatins are mainly localized intracellularly. However, extracellular locations have also been described, including the secretions of seminal plasma.[70-72] They are potent inhibitors of papain and papain-like enzymes such as cathepsin B. Also, cystatin E/M is the most potent legumain inhibitor.[73,74] Primarily associated with prostasomes,[70] together with cathepsin B, cystatins are negatively correlated with the freezing resistance of ram spermatozoa.[72]

Given the physiological importance of cathepsin B, legumain, and cystatin E/M and their putative role in the resistance of boar sperm to hypothermic storage at 5°C, several in silico tools have been used to investigate the interaction between porcine

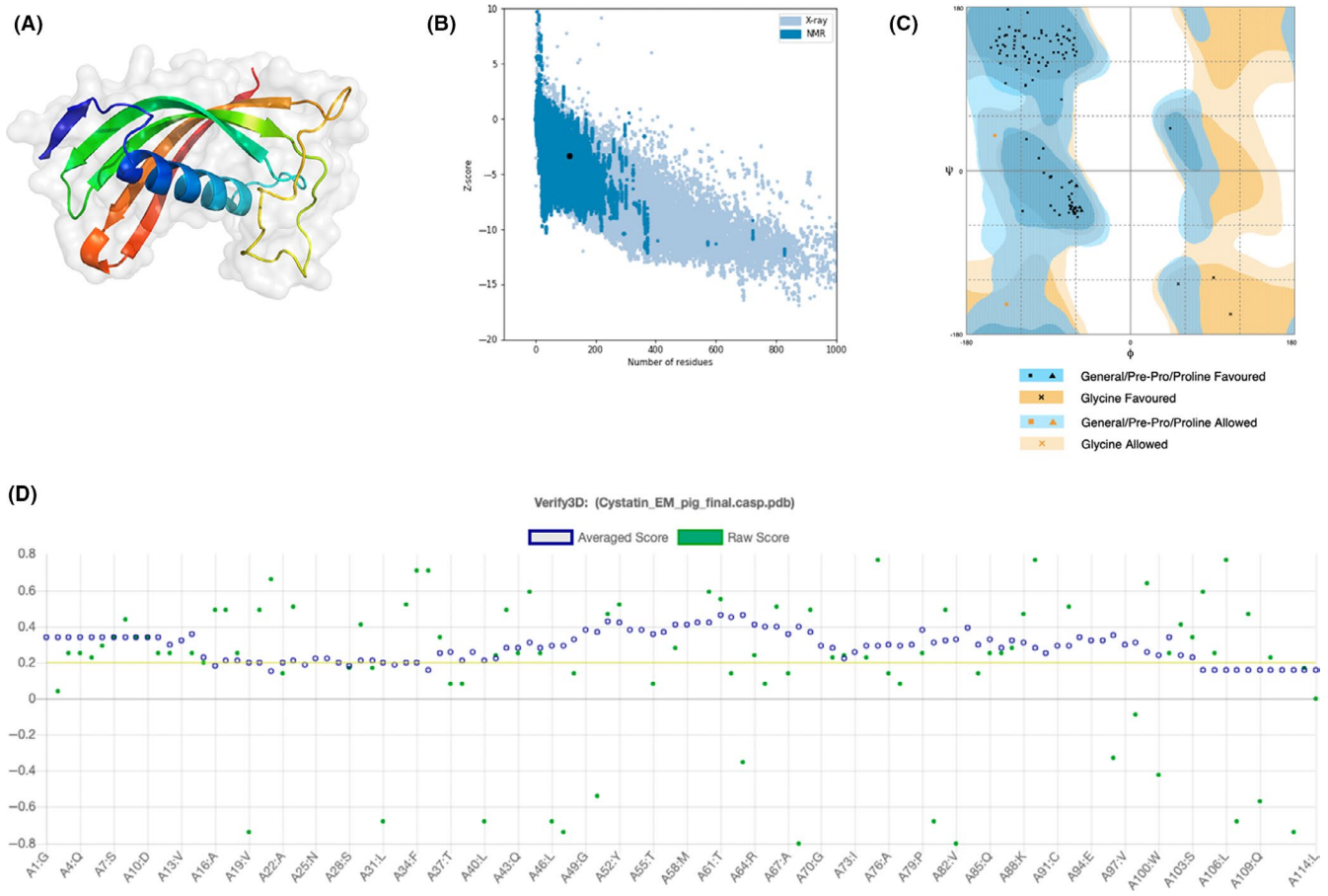

FIGURE 8 In silico prediction of the porcine cystatin E/M based on its sequence (114 amino acids; UniProtKB I3LKM9). A, Cartoon representation of the tertiary structure of porcine cystatin E/M as predicted by Phyre2. B, ProSA-web results of the predicted model, with a Z-score of −3.34 (overall model quality). C, Ramachandran plot analysis of the model generated by RAMPAGE. D, Validation of porcine cystatin E/M predicted model by Verify 3D showing that 85.09% of the residues have average 3D-1D score of ≥0.2

TABLE 3 Model validation of protein structures predicted using Phyre2 server

	Cathepsin B	Legumain	Cystatin E/M
QMEANDisCo global score	0.81 ± 0.05	0.80 ± 0.05	0.84 ± 0.08
ProSA-web Z-score	−6.4	−8.18	−3.34
MolProbity	2.63	3.2	1.6
Verify 3D	91.94%	85.53%	85.09%
Ramachandran plot			
Residues in favored region	92.5%	90.3%	98.2%
Residues in allowed region	6.3%	8.4%	1.8%
Residues in outlier region	4%	1.3%	0%

cystatin E/M and cathepsin B and legumain. Since no protein structures were available for in silico studies, structure modeling was performed after obtaining acceptable predicted models for the three proteins. After validation, the interaction of the generated structures of porcine cystatin E/M with cathepsin B and legumain was confirmed using different protein-protein docking servers. In silico binding prediction was necessary since, to date, there is no information available on these interactions based on X-ray crystallography or NMR. The docking results were verified using the servers PDBePISA and EPPIC, demonstrating better predictions of the interaction of legumain and cystatin E/M, using HawkDOCK and CoDOCKPP for cathepsin B and cystatin E/M. Since the validation depends on previously described structures, and that porcine homologs are not yet available, the dockings results were considered acceptable. Therefore, the in silico approach strengthens the explanation of possible mechanisms involved in proteolysis-dependent events during the hypothermic preservation of boar semen at 5°C.

As previously mentioned, proteases such as cathepsins and legumain are key proteins in a myriad of physiological pathways involving the activation of MMPs, important constituents of epididymal[58] and ejaculated semen.[75] These proteins belong to a group of proteolytic zinc-dependent enzymes (endopeptidases), which, alongside their inhibitors (tissue inhibitors of metalloproteinases, TIMPs), have a role in remodeling the extracellular matrix. Matrix

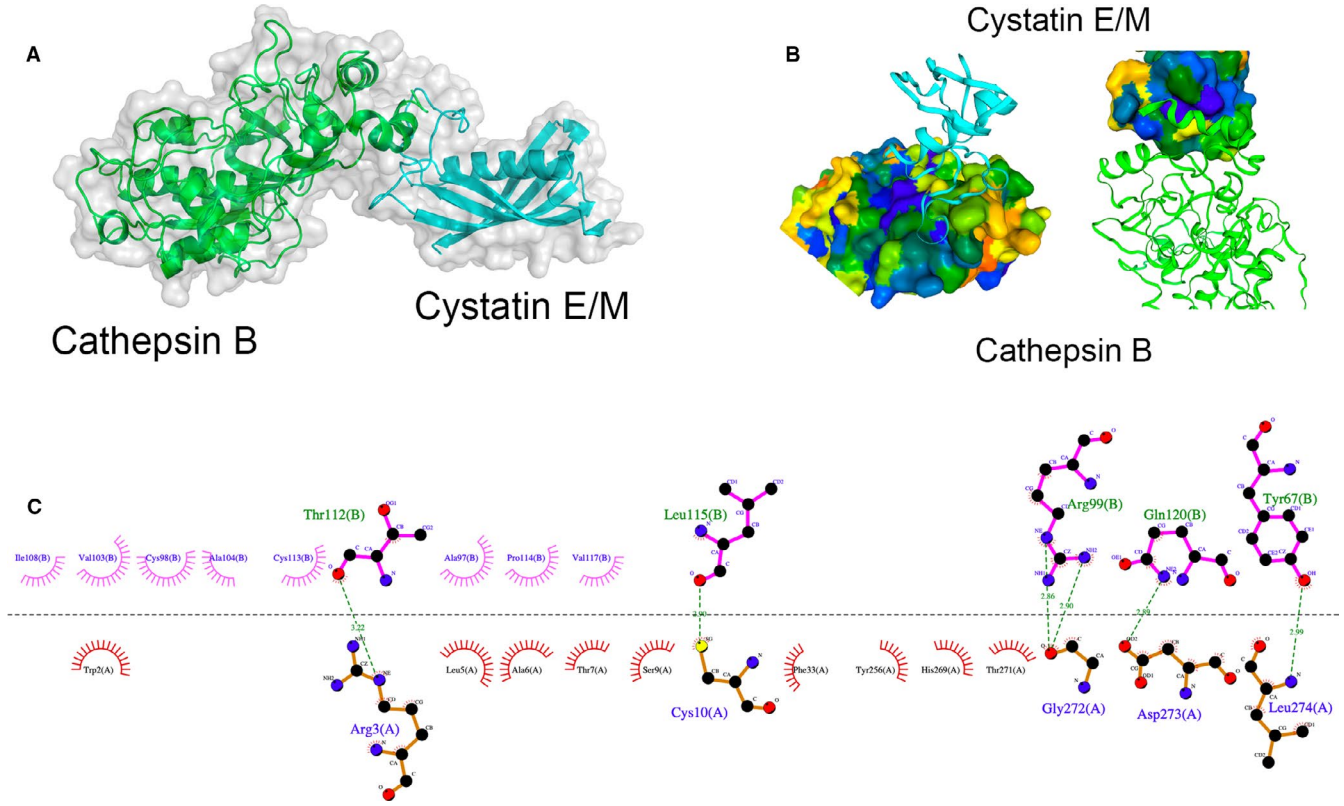

FIGURE 9 In silico docking prediction of porcine models of cathepsin B with cystatin E/M. A, Generated structure as predicted by the CoDOCKPP server. B, Surface and ribbon representations of the validations of the docking results by EPPIC server. Colored bar indicates values from the multiple sequence alignment mapped: Blue colors indicate low entropies (high conservation), and colors toward yellow correspond to increasingly higher entropies (lower conservation). C, LigPlot+ scheme showing the interaction between cathepsin B (chain A) and cystatin E/M (chain B) residues. Green lines indicate hydrogen-bond formation, and red arches indicate hydrophobic interactions

metalloproteinases have also been shown to affect sperm differentiation and morphological modifications, as they have important roles in the reconstruction of sperm cellular morphology during spermatogenesis.[76] Also, the interaction of MMPs with sperm proteins has been associated with sperm viability, capacitation, and fertilization, and is modulated by the presence of different hormones, such as gonadotrophins.[77] Based on our findings, one may consider the association of changes in sperm MMP activities with the resistance of spermatozoa to preservation at 5°C. Although specific proteolytic activities were not measured, differences in cathepsin B and legumain abundances suggest a disruption in the role of MMPs in spermatozoa, contributing to the variable resistance to hypothermic storage.

In addition, it is worth mentioning that clusterin, found only in the seminal plasma of HR boars, also modulates the activity of MMPs and TIMPs.[78] This protein is present in the seminal plasma of several domestic and wild animals in moderate-to-high concentrations.[79] Clusterin has been shown to have a powerful effect against stress-induced apoptosis in the testis,[80] and it has been positively correlated with men with disrupted spermatogenesis presenting non-obstructive azoospermia.[81] Future studies addressing the activities of specific porcine MMPs and sperm resistance to liquid storage at 17°C and 5°C must be performed to confirm our findings.

4.2 | Possible implications of immune regulatory and inflammatory molecules in sperm resistance

Interestingly, immune regulatory molecules are associated with the resistance of boar spermatozoa to storage at 5°C. Seminal plasma contains many immune and inflammation interacting biomolecules, but their physiological roles are generally unknown. Cytokines such as prostaglandin E (PTGE) have many functions in physiological and pathological processes in the testis and seminal plasma that are mediated in a coordinated manner.[82] During infections, an increase in PTGE might inhibit the cell-mediated response to spermatozoa, while the overall cytokine production during the humoral response is enhanced in the fight against infection.[83] Prostaglandin-H2 D-isomerase, found exclusively in the seminal plasma of LR boars, is responsible for the synthesis of precursors of PTGE. Its presence might indicate an inflammatory condition in the male reproductive tract. It would be important to determine the origin of the prostaglandin-H2 D-isomerase found in the seminal plasma and to evaluate whether an inflammatory process is present and how inflammatory and anti-inflammatory agents influence sperm resistance.[84-86]

Three other proteins were upregulated or found exclusively in LR boars (complement regulator factor H;

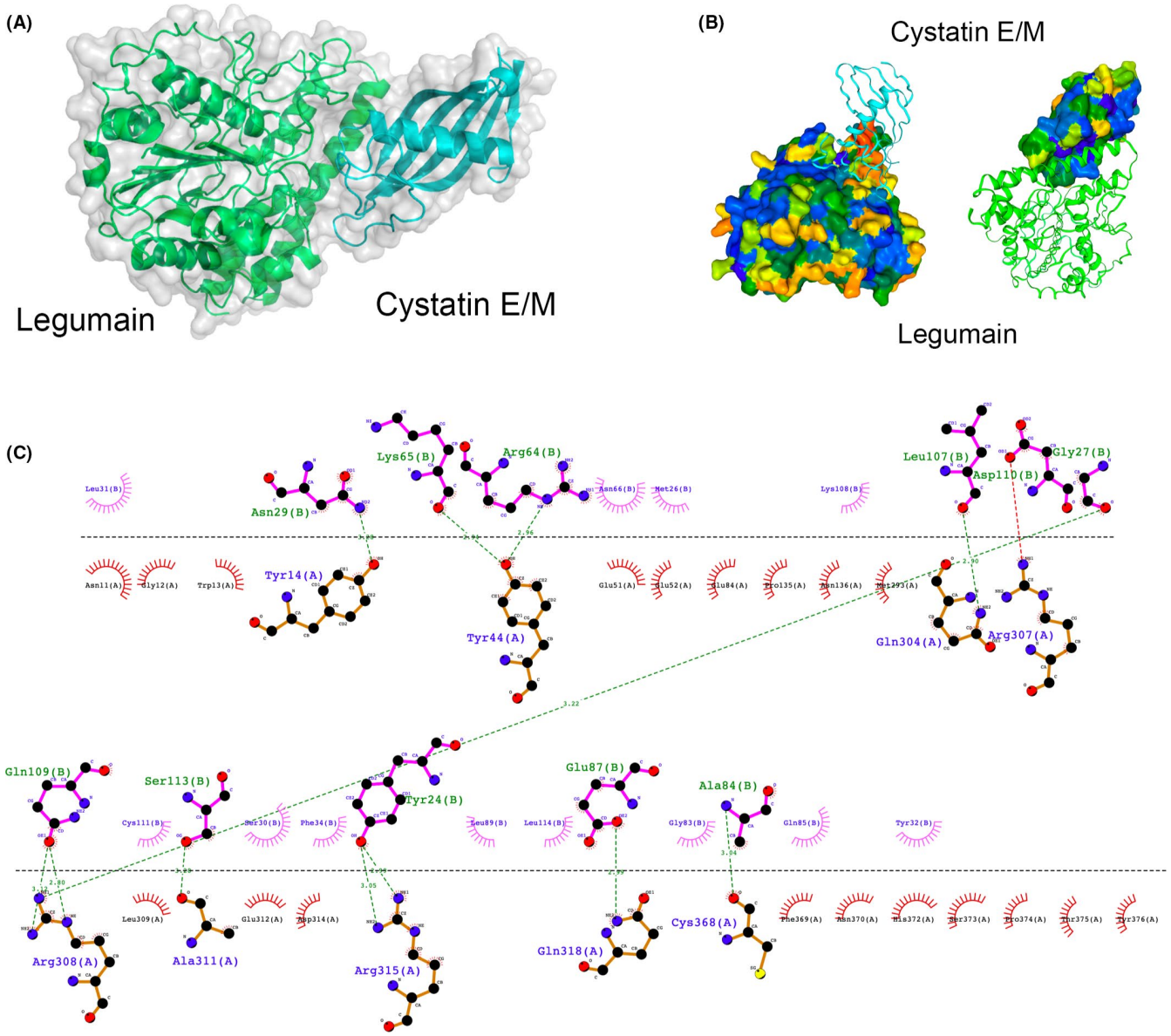

FIGURE 10 In silico docking prediction of porcine models of legumain with cystatin E/M. A, Generated structure as predicted by the HawkDOCK server. B, Surface and ribbon representations of the validations of the docking results by EPPIC server. Colored bar indicates values from the multiple sequence alignment mapped: Blue colors indicate low entropies (high conservation), and colors toward yellow correspond to increasingly higher entropies (lower conservation). C, LigPlot+ scheme showing the interaction between legumain (chain A) and cystatin E/M (chain B) residues. Green lines indicate hydrogen-bond formation, and red arches indicate hydrophobic interactions

Ig-like domain-containing protein and MHC_I-like_Ag-recog domain-containing protein). Also, the uncharacterized protein (A0A286ZTC9) has sequence homology to immunoglobulin mu heavy chain of several mammal species. The presence of immunoglobulins in boar epididymal fluid[58] and seminal plasma has been previously described,[45,87] and they have an important role in sperm protection in male and female reproductive tracts. However, there are findings suggesting that immunoglobulins, particularly IgG, can be expressed by sperm cells and may play a role in fertilization and infertility.[88]

Ribonuclease A family member 9 (RNASE9) and peptidyl-prolyl cis-trans isomerase (PPIB) were found exclusively in the seminal plasma of boars resistant to preservation at 5°C. RNASE9 is a member of the ribonuclease A superfamily that is expressed only in the epididymis and lacks ribonuclease activity. Data from *Rnase*$^{-/-}$ mice have shown that RNASE9 is dispensable for fertility, but its absence during epididymal transit results in impaired sperm maturation.[89] Due to its antibacterial activity, RNASE9 is associated with host defense of the male reproductive tract.[90] PPIB catalyzes the cis/trans isomerization of peptidyl-prolyl peptide bonds, preceded by a phosphorylated serine or threonine residue. It is expressed intensively in Sertoli cells, and results from the knockout model for PPIB show a disruption in the blood-testis barrier.[91] Also, germ cells in post-natal mouse *Pin1*$^{-/-}$ testis are able to initiate and complete spermatogenesis, producing mature spermatozoa. However, a progressive and age-dependent degeneration of

the spermatogenic cells in *Pin1*$^{-/-}$ testis was observed, which led to a complete germ cell loss by 14 months of age.[92] The present results show that the roles of RNASE9 and PPIB in sperm physiology deserve a closer attention to validate them as biomarkers for sperm resistance.

Despite our present understanding of the functions and roles of these proteins in spermatogenesis, epididymal maturation, interaction with the female tract, and fertilization, the precise mechanisms that promote a better or worse resistance of spermatozoa to the challenge of hypothermic preservation at 5°C are yet unknown. Further investigations, including in vivo studies, should focus on the immunological crosstalk between epithelial cells and gametes in order to provide information about how the immune regulators influence the resistance of boar spermatozoa to chilling injury. The sample pooling strategy did not allow for identifying differences between individual males. Thus, the development of new experiments to confirm the differentially expressed proteins as biomarkers for boar semen resistance to preservation at 5°C in different production systems and breeds as well possible variations between ejaculates and individuals are needed.

5 | CONCLUSIONS

To the best of our knowledge, this is the first report on putative molecular markers of the resistance of boar semen to hypothermic preservation at 5°C. Important differences in the seminal plasma suggest that proteins involved in the proteolytic activation of metalloproteinases (cathepsin B, legumain, cystatin E/M and clusterin) and proteins related to immune and inflammatory modulation (prostaglandin-H2 D-isomerase, complement regulator factor H; Ig-like domain-containing protein and MHC_I-like_Ag-recog domain-containing protein; ribonuclease A family member 9 and peptidyl-prolyl cis-trans isomerase) could disrupt key cellular pathways during spermatogenesis and epididymal maturation, resulting in altered sperm resistance to chilling injury. Further studies must be performed to validate the differentially expressed proteins as biomarkers for semen resistance to hypothermic storage at 5°C considering production systems and breeds.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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