Eurasia Specialized Veterinary Publication

International Journal of Veterinary Research and Allied Science

ISSN:3062-357X

2023, Volume 3, Issue 2, Page No: 94-111

Available online at: www.esvpub.com/

Spermadhesin-1 Dominates Seminal Plasma Proteome of Young Nellore Bulls and Correlates with Breeding Soundness

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ABSTRACT

The objective of this work was to investigate the protein composition of seminal plasma in young Nellore bulls. Twenty animals, aged between 19.8 and 22.7 months, were selected and separated into two categories based on their Breeding Soundness Evaluation (BSE) outcomes: approved (FIT; n = 10) and not approved (UNFIT; n = 10). Scrotal circumference was recorded, and semen samples were collected via electroejaculation. Sperm movement, collective motility, and vigor were assessed by optical microscopy, while sperm defects were evaluated through phase-contrast microscopy. Seminal plasma was separated from sperm cells by centrifugation and analyzed using LC-MS/MS proteomics. Protein identification utilized MASCOT Daemon (v2.4.0), and relative quantification followed the Exponentially Modified Protein Abundance Index (emPAI) via SCAFFOLD Q+ (v4.0). Protein functions were annotated using the KOG ontology, and clustering analyses were carried out in DAVID. No significant differences were found in scrotal size or basic semen traits between groups, but the UNFIT bulls exhibited a higher rate of sperm defects ($p < 0.05$). Across all samples, 297 proteins were detected, with 11 showing distinct abundances ($p < 0.05$): two elevated in FIT bulls (Spermadhesin-1 and Ig gamma-1 chain C region) and nine in UNFIT bulls (Vasoactive intestinal peptide, Metalloproteinase inhibitor 2, Ig lambda-1 chain C region, Protein FAM3C, Hemoglobin beta, Seminal ribonuclease, Spermadhesin 2, BSP-30kDa, and Spermadhesin Z13). Spermadhesin-1 represented the most abundant protein overall (36.7%), corresponding to 47.7% in FIT and 25.7% in UNFIT animals. The most represented biological functions included post-translational modification, protein degradation, and chaperone activity. Functionally enriched clusters were linked to phospholipid transport, ATP-binding mechanisms, and chaperonin complexes. Proteins increased in FIT bulls were related to sperm capacitation and antioxidant defense, while those overrepresented in UNFIT bulls were linked to impaired motility, membrane cholesterol loss, and oxidative damage. In summary, seminal plasma from FIT bulls contained proteins that support fertilization processes, whereas the UNFIT group showed patterns associated with reduced fertility potential. The relative concentration of Spermadhesin-1 could serve as a biomarker for reproductive assessment in young Nellore bulls.

Keywords: Proteome, Fertility, Semen, Bovine, Breeding

Received: 09 September 2023
Revised: 28 November 2023
Accepted: 04 December 2023

How to Cite This Article: Ribeiro G, Castro G, Duarte M. Spermadhesin-1 Dominates Seminal Plasma Proteome of Young Nellore Bulls and Correlates with Breeding Soundness. Int J Vet Res Allied Sci. 2023;3(2):94-111. https://doi.org/10.51847/MMSB8cxE3N

Introduction

Brazil is among the world's foremost beef producers, ranking second globally in meat exports [1]. This achievement is driven by extensive pasturelands, efficient use of natural and cultivated grass, and a predominance

of Nellore cattle and their crossbreeds in the national herd [2]. Such conditions provide Brazil with notable advantages in beef production efficiency.

Nellore cattle are particularly valued for their adaptability, hardiness, and resistance to parasitic infections. Ongoing genetic selection for traits like early maturity and rapid weight gain has further improved productivity, shortening production cycles and enhancing overall output [3, 4].

The implementation of reproductive biotechnologies has accelerated genetic progress in recent decades [5]. Still, many beef producers depend primarily on natural mating to reproduce their herds [6]. Since a single bull may mate with numerous cows, its reproductive soundness has a direct influence on herd fertility and farm profitability [7].

To ensure reproductive potential, Breeding Soundness Evaluation (BSE) is routinely applied. This assessment includes clinical and reproductive examinations, semen quality analysis, and sperm morphology [8]. Bulls that meet established parameters are considered FIT for breeding, while those failing to meet criteria are labeled UNFIT; the latter may undergo re-examination after about 60 days if the cause of disqualification is reversible.

Even with comprehensive testing, subtle biochemical factors within the seminal fluid can still impair sperm functionality and fertility, escaping detection in conventional evaluations [9]. The seminal plasma (SP)—the liquid fraction of semen—plays a vital role in supporting, nourishing, and protecting spermatozoa [10].

SP is formed by secretions from the testes, epididymis, and mainly the accessory sex glands, containing diverse molecules such as sugars, ions, and proteins [11]. Because sperm cells lack active transcription, many of their essential functions depend on proteins present in SP [12]. These proteins regulate key reproductive processes, including motility, maturation, energy metabolism, membrane stability, capacitation, and fertilization [13–15].

Proteomic profiling of SP provides insight into sperm health, the physiological state of the reproductive tract, and male fertility. It also enables the identification of potential molecular markers linked to semen quality, contributing to more reliable sire selection [16, 17].

Hence, the present research sought to determine the proteomic landscape of seminal plasma in young Nellore bulls classified as FIT and UNFIT, and to identify differential protein expression associated with sperm morphology traits.

Materials and Methods

Ethical approval

The experimental protocol was reviewed and authorized by the Committee on Animal Use of the Federal University of Viçosa (approval number 28/2017). All experimental stages complied with national and institutional regulations for animal welfare and were conducted following the ARRIVE recommendations (https://arriveguidelines.org, accessed 17 July 2017).

Animal selection

A total of twenty Nellore bulls, ranging in age from 19.8 to 22.7 months, were included in the study. These animals belonged to a genetic selection program aimed at improving body weight, early sexual development, and carcass traits. The bulls were raised in Aquidauana, Mato Grosso do Sul, Brazil (19°48′53.3124″ S, 55°40′8.2704″ W), on open pasture with Brachiaria brizantha grass and unlimited access to water.

Two equal groups were formed based on reproductive performance: FIT (Group 1; n = 10) and UNFIT (Group 2; n = 10). Classification followed the standards set by the Brazilian College of Animal Reproduction (CBRA) [18], which stipulate a minimum of 50% sperm motility and 70% morphologically normal cells for approval as breeding stock.

Each bull underwent a Breeding Soundness Evaluation (BSE) that included both internal and external genital examinations. Internal organs (seminal vesicles and prostate) were assessed via transrectal palpation, while the penis, prepuce, testes, and epididymides were examined for symmetry, shape, firmness, and size. Scrotal circumference was measured with a flexible tape at the widest point. Semen was then obtained by electroejaculation, and its physical and morphological characteristics were analyzed [19].

Semen evaluation was performed subjectively by the same operator. Mass motility was rated from 0 (no movement) to 5 (strong collective motion). Individual motility was estimated as the percentage of motile sperm from 0% to 100%. Vigor was scored from 0 (no motility) to 5 (rapid and strong progression). Analyses were conducted under light microscopy (CX31, Olympus, Tokyo, Japan) at 100× magnification, examining four

microscopic fields per sample, and the average value was recorded, following Okano *et al*. [20]. The volume (mL) and appearance of ejaculates were also scored as 1 = watery, 2 = slightly turbid, 3 = milky, and 4 = creamy.
For morphological evaluation, a semen aliquot was mixed with 1 mL of formaldehyde-buffered saline [21]. The preparations were examined under phase-contrast microscopy (BX41, Olympus, Tokyo, Japan) at 1000× magnification using immersion oil. In each sample, 400 sperm cells were analyzed to identify abnormalities in the acrosome, head, midpiece, and tail regions. Defects were classified as major (likely to affect fertility), minor (less critical forms), and total (sum of all defects), following Blom's classification [22] and CBRA guidelines [18].

Seminal plasma processing for proteomic study
Seminal plasma (SP) was separated from sperm cells by centrifugation at 10,000× g for 10 minutes using a Heraeus Multifuge X1R centrifuge (Thermo Scientific, Waltham, MA, USA). The SP fraction was collected, transferred into 0.5 mL straws, and stored in liquid nitrogen (-196 °C). For analysis, samples were thawed, filtered through 0.22 µm membranes, and used for subsequent proteomic procedures [23].

Extraction and quantification of soluble proteins
Each 100 µL portion of filtered SP was mixed with 600 µL of an ice-cold acetone: ether mixture (1:1, v/v) containing 5% (w/v) trichloroacetic acid and 1 mM dithiothreitol (DTT; Sigma-Aldrich, Burlington, NJ, USA). The resulting protein pellets were dissolved in 250 µL of solubilization buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 40 mM DTT). Protein concentration was determined using the Bradford colorimetric method, calibrated with a bovine serum albumin standard curve [24].

SDS-PAGE electrophoresis
For verification of extraction quality, 25 µg of protein from each sample was resolved by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolving gel was prepared with 14% T and 3.0% C, and the stacking gel with 5.1% T and 2.6% C. Electrophoresis was conducted at 80 V for 15 minutes, followed by 60 V for 4 hours using Mini-PROTEAN Tetra Cell equipment (Bio-Rad, Hercules, CA, USA). Broad-range molecular weight standards (Bio-Rad) were included. Gels were stained with Coomassie Brilliant Blue R-250 for 2 hours and destained for 10 hours in 25% methanol and 7.5% acetic acid (v/v) [25].

Pooling of samples
After confirming protein profiles, 60 µg of protein from each individual sample was pooled to create two representative samples: one for the FIT and another for the UNFIT group. Each pooled sample was divided into five replicates containing 50 µg of protein. The replicates were electrophoresed approximately 1 cm into 12.5% polyacrylamide gels (resolving gel: 12.5% T, 3.3% C; stacking gel: 5.1% T, 2.6% C). Gels were fixed in 40% methanol and 5% acetic acid for 2 hours [26]. One portion was stained with Coomassie Brilliant Blue R-250 for visualization, and the remaining portion was kept in 5% acetic acid for enzymatic digestion.

Enzymatic digestion of gel proteins
Protein bands from all five gel replicates were individually excised, pooled together, and minced into fine fragments before being transferred to clean microcentrifuge tubes. To eliminate residual Coomassie dye, the gel pieces were subjected to three consecutive washes, each with 200 µL of a 1:1 (v/v) mixture of acetonitrile (ACN; Sigma-Aldrich, Burlington, NJ, USA) and 25 mM ammonium bicarbonate (AMBIC; pH 8.0; Sigma-Aldrich, Burlington, NJ, USA).
Following this, the fragments were dehydrated twice with 200 µL of pure ACN for 5 minutes per cycle and subsequently dried using a vacuum concentrator (Concentrator Plus AG–22331, Eppendorf, Hamburg, Germany). Proteins embedded within the gel matrix were reduced in 100 µL of 65 mM dithiothreitol (DTT) prepared in 100 mM AMBIC at 56 °C for 30 minutes. Reduction was followed by alkylation using 100 µL of a 200 mM iodoacetamide solution in 100 mM AMBIC, performed in darkness at room temperature for 30 minutes. The treated fragments were then rinsed twice sequentially with AMBIC and ACN to ensure removal of residual reagents, rehydrated, dehydrated again, and finally vacuum-dried.
For digestion, the dried gels were reconstituted in a trypsin working solution (Promega, Madison, WI, USA) at a concentration of 25 ng/µL in activation buffer composed of 40 mM AMBIC and 10% (v/v) ACN. After allowing

45 minutes of rehydration on ice, 100 µL of activation buffer without trypsin was added, and enzymatic digestion proceeded for 22 hours at 37 °C. Post-digestion, the tubes were sonicated for 10 minutes, vortexed for 20 seconds, and the resulting supernatants were transferred into new microtubes.

To recover residual peptides, 200 µL of extraction buffer containing 5% (v/v) formic acid and 50% (v/v) ACN was added to the remaining gel fragments. Each tube was vortexed for 20 seconds, incubated for 15 minutes at room temperature, and sonicated for 2 minutes. The supernatant was collected and pooled with the initial digest. This extraction was repeated twice more, and the combined peptide extracts were dried in a vacuum centrifuge [27].

The concentrated peptides were redissolved in 10 µL of 0.1% (v/v) trifluoroacetic acid (TFA; Sigma-Aldrich, Burlington, NJ, USA) in ultrapure water. For desalting, samples were processed using C18 reversed-phase microcolumns (Millipore, Burlington, NJ, USA) and eluted in 50% ACN containing 0.1% (v/v) TFA.

LC–MS/MS analysis

Each tryptic digest replicate was dissolved in 20 µL of 0.1% (v/v) aqueous formic acid (LC–MS grade, Sigma-Aldrich, Burlington, NJ, USA) and transferred to vials for nano LC–MS/MS analysis. A 1 µL aliquot of each sample was analyzed using a nanoAcquity UPLC system (Waters, Milford, MA, USA) equipped with a Symmetry® C18 trap column (2G-V/MTrap, 5 µm, 180 µm × 20 mm), operated at a flow rate of 7 µL/min for 3 minutes. Peptides were then separated on a BEH130 column (1.7 µm, 100 µm × 100 mm) at 0.3 µL/min.

The mobile phase consisted of solvent A (ultrapure water containing 0.1% (v/v) formic acid) and solvent B (acetonitrile with 0.1% (v/v) formic acid). The chromatographic gradient was as follows: 2% B for 1 minute; 2–30% B over 299 minutes; 30–85% B for 5 minutes; held at 85% B for 5 minutes; then decreased to 2% B over 5 minutes, and maintained at 2% B for an additional 5 minutes, resulting in a total runtime of 320 minutes.

Eluting peptides were analyzed using a MAXIS 3G mass spectrometer (Bruker Daltonics, Billerica, MA, USA) coupled to a CaptiveSpray ion source and operated online with the chromatographic system. Data were acquired using the IE_GCF_01-02-2017 method, under the following conditions: drying gas flow of 3 L/min, ion source temperature of 150 °C, and a capillary voltage of 2 kV. The resulting raw spectra were converted into Mascot Generic Format (MGF) files for database comparison.

Protein identification

Protein identification was carried out using Mascot Daemon software version 2.4.0 (Matrix Science, London, UK), searching the canonical Bovidae protein database (Taxonomy ID 9895) from UniProt Knowledgebase (UniProtKB; https://www.uniprot.org/; accessed 28 March 2018). Search parameters included trypsin as the proteolytic enzyme with one permitted missed cleavage, carbamidomethylation of cysteine as a fixed modification, and methionine oxidation as a variable modification. The allowed mass tolerance was set to 30 ppm for precursor ions and 0.6 Da for fragment ions, with charge states ranging from +2 to +4.

Mascot output files were validated using Scaffold Q+ software version 4.0 (Proteome Software Inc., Portland, OR, USA). Peptide identification confidence was assessed with the Peptide Prophet algorithm [28], while protein-level validation employed the Protein Prophet algorithm [29]. Acceptance thresholds were set at a minimum probability of 0.95, a false discovery rate (FDR) ≤ 1%, and identification supported by at least two distinct peptides. Only proteins detected in a minimum of two replicates were retained for subsequent analysis.

Functional protein classification

The biological functions of the identified proteins were assigned using Gene Ontology (GO) annotations retrieved from the UniProtKB database. Enrichment of these functional categories was analyzed through the Database for Annotation, Visualization, and Integrated Discovery (DAVID) platform (https://david.ncifcrf.gov/home.jsp; accessed on 11 November 2022) [30].

For orthology-based grouping, proteins were aligned against the EuKaryotic Orthologous Groups (KOG) database via the RPSBLAST tool included in BLAST version 2.13.0, employing an E-value threshold of 1×10^{-10} to retain statistically significant matches [31, 32].

Quantification of identified proteins

Quantitative assessment included only proteins detected in at least three independent technical replicates per treatment. Relative protein abundance was determined using the Exponentially Modified Protein Abundance

Index (emPAI) implemented in Scaffold Q+, version 4.0 [33]. Proteins with missing quantitative values were excluded from further statistical testing.

Differentially abundant proteins (DAPs) were identified using the msmsTests package within R software, version 4.2.2 (https://cran.r-project.org/, accessed on 29 March 2018) [34]. Two statistical models were employed: a complete model representing the alternative hypothesis (UNFIT group) and a nested model corresponding to the null hypothesis (FIT group). Significance values were corrected using the Benjamini–Hochberg procedure, and proteins were classified as differentially abundant when the false discovery rate (FDR) was below 0.05.

Protein interaction network construction

Proteins exhibiting differential expression between the experimental groups were analyzed through the STRING database, version 11.5 (http://www.string-db.org, accessed on 11 November 2022) [35]. This approach integrates interaction data derived from multiple evidence sources, including physical associations, functional correlations, genomic neighborhood information, co-expression patterns, experimental datasets, and published literature.

Evaluation of phenotypic variables

Semen trait differences among experimental groups were examined using Analysis of Variance (ANOVA) through SAEG software, version 9.1, applying a 5% significance level [36]. To determine the degree of association among the evaluated traits, Pearson correlation coefficients were calculated, also considering a 5% probability threshold for statistical significance.

Results and Discussion

Semen characteristics of FIT and UNFIT bulls

There were no significant distinctions ($p < 0.05$) between the FIT and UNFIT bulls in terms of mass motility (MM), sperm motility (Mot), or vigor (Vig). Nevertheless, the percentage of sperm abnormalities (Morp) was considerably higher in UNFIT bulls compared to the FIT group **(Table 1)**. Additionally, scrotal perimeter (PE) averages showed no statistical difference between groups.

Table 1. Physical and morphological semen traits of Nellore bulls categorized as FIT or UNFIT for reproduction, as evaluated by microscopy following electroejaculation.

Variable	FIT Bulls	UNFIT Bulls	Overall Mean	Coefficient of Variation (%)	Minimum Value	Maximum Value
PE	34.91 ± 1.22	34.51 ± 1.52	34.61 ± 1.39	3.99	31	36.6
Vol	3.40 ± 0.69	4.70 ± 1.33	4.05 ± 1.23	26.35	2	6
Asp	2.70 ± 0.67	2.60 ± 0.51	2.65 ± 0.58	22.68	2	4
MM	1.65 ± 1.49	0.70 ± 0.82	1.17 ± 1.27	102.53	0	4
Mot	70.00 ± 11.05	61.00 ± 23.66	65.00 ± 18.56	28.2	0	90
Vig	3.15 ± 0.34	2.80 ± 1.01	2.97 ± 0.75	-	0	4
Morp	12.30 ± 3.27^{b}	32.90 ± 9.48^{a}	22.60 ± 12.62	31.37	9	50

Abbreviations: CV – Coefficient of variation; PE – scrotal perimeter (cm); Vol – ejaculate volume (mL); Asp – aspect; MM – mass motility (0–5); Mot – sperm motility (%); Vig – vigor (0–5); Morp – morphology (%). Mean values followed by different lowercase letters denote statistical differences ($p < 0.05$).

Seminal plasma proteomic overview

Using LC–MS analysis, a total of 297 seminal plasma proteins were identified across both FIT and UNFIT bulls. According to KOG-based categorization, the most represented groups were: post-translational modification, protein turnover, and chaperones [O]; carbohydrate transport and metabolism [G]; and general functional prediction [R] **(Figure 1)**.

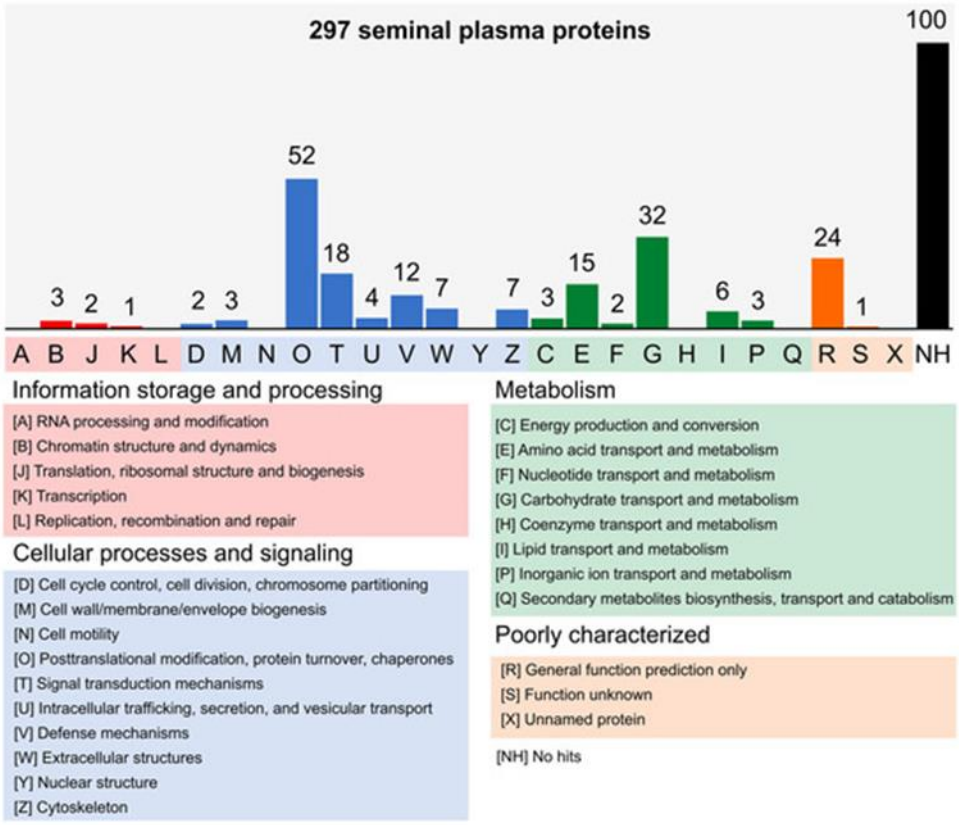


Figure 1. Functional categorization of seminal plasma proteins from Nellore bulls. From the total identified, 197 proteins were assigned to four major KOG categories, whereas 100 proteins did not yield significant hits (NH) and thus remained unclassified. The letters A–Z correspond to the functional KOG classification system.

Other functionally relevant protein groups

Additional protein functions of note for sperm biology included signal transduction [T], amino acid metabolism and transport [E], and defense-related pathways [V] **(Figure 1)**. These categories were represented in both FIT and UNFIT bulls, though their expression levels varied. Exceptions were observed for the following proteins: epididymal sperm-binding protein 1 (UniProtKB E1B9P4), SERPIN-domain protein (UniProtKB L8I2G8), ARSG protein (UniProtKB A6QLR7), protocadherin fat 2 (UniProtKB L8IHR1), insulin-like growth factor II (UniProtKB P07456), leucine-rich alpha-2-glycoprotein 1 (UniProtKB Q2KIF2), vitamin D–binding protein (UniProtKB Q3MHN5), GDP-fucose O-fucosyltransferase 2 (UniProtKB Q7YRE5), and gastrin-releasing peptide (UniProtKB Q863C3).

Among these, E1B9P4 and L8I2G8 were exclusive to FIT bulls, while the remaining proteins appeared only in UNFIT animals.

In FIT bulls, spermadhesin-1 accounted for 47.7% of the total protein signal, compared with 25.7% in the UNFIT group. The next most prevalent were seminal plasma protein PDC-109 (2.3% FIT; 8.2% UNFIT) and serine protease inhibitor clade E member 2 (3.9% in both). Together, 19 proteins represented 70.5% of total abundance in FIT samples and 72.1% in UNFIT ones **(Table 2)**. The remaining 278 proteins occurred in much lower amounts.

Table 2. Nineteen most abundant seminal plasma proteins detected in FIT and UNFIT Nellore bulls. Collectively, these account for 70.5% (FIT) and 72.1% (UNFIT) of the full set of 297 proteins. The remaining 278 proteins were classified as low-abundance.

UniProtKB ID	Protein Name	Average in FIT Bulls	% of Total in FIT	Average in UNFIT Bulls	% of Total in UNFIT
P29392	Spermadhesin-1	428.58	47.7%	240.36	25.7%

Q8HZY1	Serine protease inhibitor clade E member 2	34.75	3.9%	36.74	3.9%
P02784	Seminal plasma protein PDC-109	20.32	2.3%	76.44	8.2%
A0A4W2BP54	Jacalin-type lectin domain-containing protein	14.70	1.6%	22.09	2.4%
G3MWX7	Lipocalin cytosolic fatty-acid-binding domain	13.69	1.5%	18.87	2.0%
P17697	Clusterin	13.23	1.5%	6.43	0.7%
P80311	Peptidyl-prolyl cis-trans isomerase B	12.12	1.3%	18.63	2.0%
A0A0E3UT00	Metalloproteinase inhibitor 2	11.79	1.3%	37.80	4.0%
L8HPF5	Ig gamma-1 chain C region	11.54	1.3%	3.31	0.4%
P81019	Seminal plasma protein BSP-30 kDa	11.05	1.2%	32.77	3.5%
P79345	NPC intracellular cholesterol transporter 2	10.87	1.2%	20.18	2.2%
F1MCF5	Glutathione peroxidase	10.69	1.2%	9.20	1.0%
D4QBC5	Hemoglobin beta	9.02	1.0%	4.86	0.5%
P82292	Spermadhesin Z13	7.80	0.9%	50.14	5.4%
F1MGQ1	Deoxyribonuclease	7.15	0.8%	17.13	1.8%
D4QBD1	Hemoglobin beta	6.93	0.8%	18.93	2.0%
W5P198	Vasoactive intestinal peptide	4.46	0.5%	28.53	3.0%
Q4R0H2	Spermadhesin 2	3.46	0.4%	21.71	2.3%
P00669	Seminal ribonuclease	1.95	0.2%	10.68	1.1%
Other proteins			**29.5%**		**27.9%**

AVG: mean spectral count.

Functional enrichment of seminal plasma proteins

The enrichment study grouped the detected proteins into 48 clusters, reflecting links to major biological pathways, molecular roles, and cellular structures. Clusters with the highest enrichment values were associated with biological processes (BP) such as sperm capacitation, molecular functions (MF) involving protein maintenance and stabilization, and cellular components (CC) related to reproductive protein complexes **(Table 3)**.

Table 3. Principal cluster annotations for seminal plasma proteins in Nellore bulls. Proteins from FIT and UNFIT groups were assigned Gene Ontology (GO) terms under BP, MF, and CC categories.

GO Term	Functional Annotation Cluster	Number of Proteins	Enrichment Score
BP	Phospholipid efflux	4	3.12
	Sperm capacitation	4	
	Positive regulation of sperm capacitation	3	
MF	ATP binding	16	4.42
	Unfolded protein binding	11	
	ATPase activity	10	
	Protein binding involved in protein folding	8	
CC	Chaperonin-containing T-complex	4	2.04
	Cell body	4	
	Microtubule	4	

Enrichment Score: indicates how strongly a category or GO term is represented in comparison to a reference dataset.

Quantitative proteomic comparison between FIT and UNFIT bulls

Statistical evaluation of the label-free quantification data by principal component analysis (PCA) and hierarchical clustering demonstrated a distinct separation between the FIT and UNFIT bull groups **(Figure 2)**. From these data, 11 proteins were determined to be differentially abundant (DAP; FDR < 0.05), with two showing greater abundance in FIT bulls (spermadhesin-1 and Ig gamma-1 chain C region) and nine elevated in UNFIT individuals **(Figure 3)**.

Pearson's correlation results revealed that the two high-abundance proteins in FIT bulls were negatively correlated with the proportion of both major and minor sperm defects. Conversely, three proteins—FAM3C, tissue inhibitor of metalloproteinase-2 (TIMP-2), and vasoactive intestinal peptide (VIP)—exhibited positive associations with the rate of major morphological defects among UNFIT bulls. No meaningful correlation was noted between Ig lambda-1 chain C and sperm abnormality levels. Although the mean protein abundance was greater in the UNFIT group, high variance among replicates was observed for both groups **(Figure 4)**.

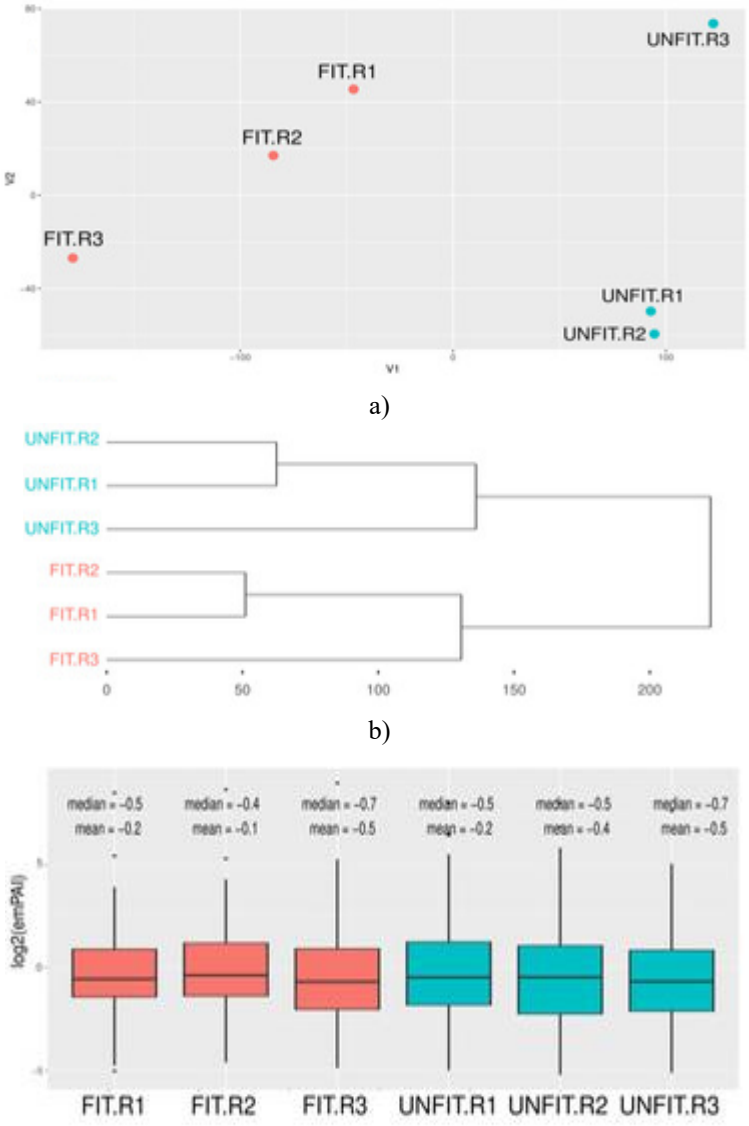
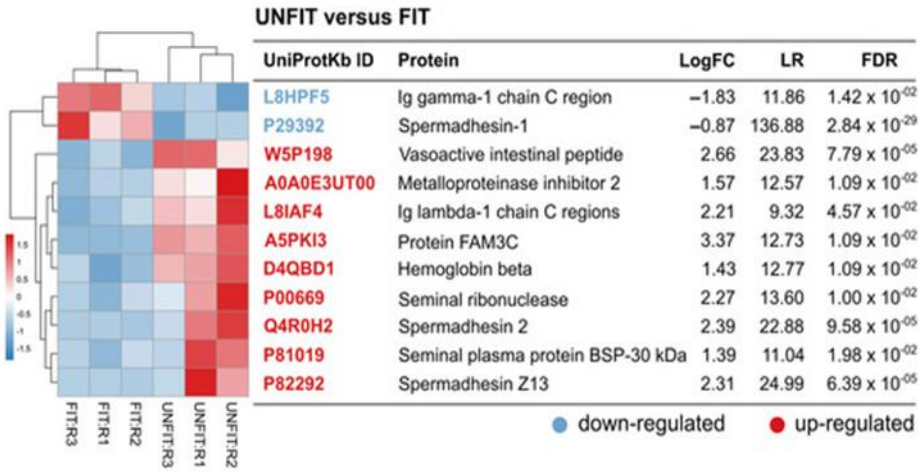


Figure 2. PCA and clustering plots for label-free protein quantification.
(a) PCA clearly separates the technical replicates of FIT and UNFIT groups. (b) Cluster analysis using UPGMA (unweighted pair group method with arithmetic mean) shows comparable group division. (c) Boxplots display replicate dispersion along with corresponding mean and median values.



UniProtKb ID	Protein	LogFC	LR	FDR
L8HPF5	Ig gamma-1 chain C region	−1.83	11.86	1.42×10^{-02}
P29392	Spermadhesin-1	−0.87	136.88	2.84×10^{-29}
W5P198	Vasoactive intestinal peptide	2.66	23.83	7.79×10^{-05}
A0A0E3UT00	Metalloproteinase inhibitor 2	1.57	12.57	1.09×10^{-02}
L8IAF4	Ig lambda-1 chain C regions	2.21	9.32	4.57×10^{-02}
A5PKI3	Protein FAM3C	3.37	12.73	1.09×10^{-02}
D4QBD1	Hemoglobin beta	1.43	12.77	1.09×10^{-02}
P00669	Seminal ribonuclease	2.27	13.60	1.00×10^{-02}
Q4R0H2	Spermadhesin 2	2.39	22.88	9.58×10^{-05}
P81019	Seminal plasma protein BSP-30 kDa	1.39	11.04	1.98×10^{-02}
P82292	Spermadhesin Z13	2.31	24.99	6.39×10^{-05}

Figure 3. Differentially abundant seminal plasma proteins identified in FIT and UNFIT Nellore bulls. The label-free comparison revealed 11 DAPs. The heatmap represents the replicate-specific distribution of emPAI values; red indicates higher, blue lower abundance.
LogFC: log fold-change; LR: likelihood ratio; FDR: false discovery rate adjusted by the Benjamini–Hochberg method.

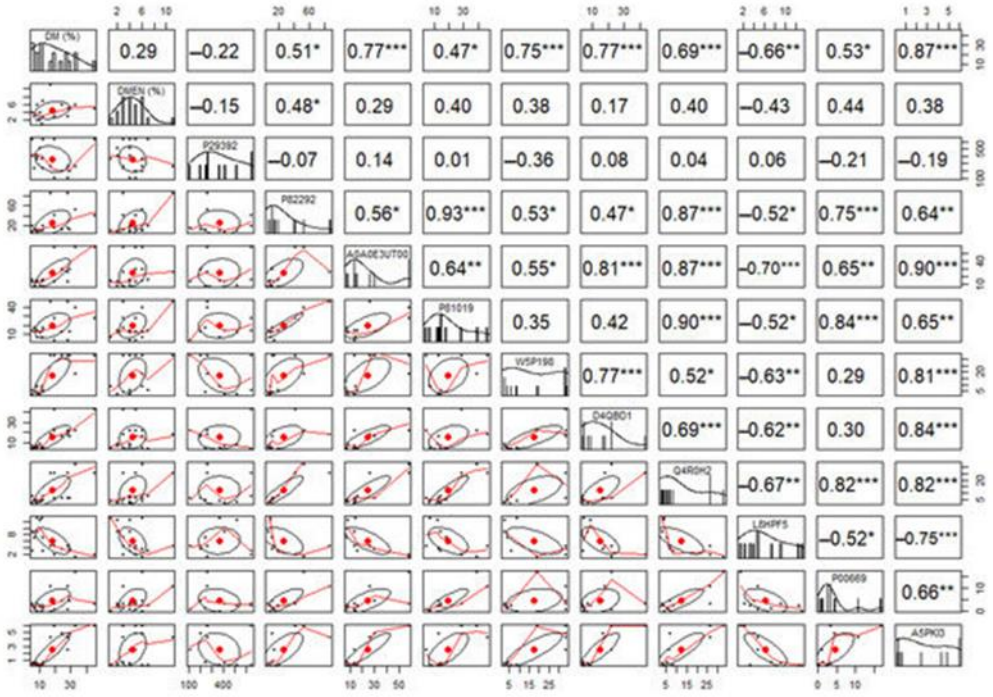


Figure 4. Pearson's correlation assessment between the proportions of major (DM) and minor (DMEN) sperm abnormalities and the differentially abundant proteins (DAPs) identified in the seminal plasma (SP) of Nellore bulls classified as FIT or UNFIT for reproductive performance.
DAPs: P29392 – Spermadhesin 1; P82292 – Spermadhesin Z13; A0A0E3UT00 – Tissue inhibitor of metalloproteinase 2; P81019 – Seminal plasma protein BSP–30 kDa; W5P198 – Vasoactive intestinal peptide; D4QBD1 – Hemoglobin beta; Q4R0H2 – Spermadhesin 2; L8HPF5 – Immunoglobulin gamma–1 chain C region; P00669 – Seminal ribonuclease; A5PKI3 – Protein FAM3C.
Significance thresholds: $p < 0.05$ (), $p < 0.01$ (), $p < 0.001$ ().

Computational modeling of protein–protein interaction (PPI) networks among DAPs revealed that Spermadhesin Z13 (SPADH2) interacts with Clusterin (CLU) and several Binder of Sperm Proteins (BSP1, BSP3, BSP5). Conversely, the seminal plasma BSP–30 kDa protein (BSP5) exhibited connections with Spermadhesin–1 (SPADH1) and Tissue Inhibitor of Metalloproteinase–2 (TIMP2). The latter, TIMP2, was further linked to

multiple metalloproteinase enzymes. In parallel, protein FAM3C demonstrated interactions with transmembrane and ubiquitin-like domain–containing protein 1 (TMUB1), Bos taurus transmembrane protein 126A (TMEM126A), Pyridoxine-5-phosphate oxidase (PNPO), and a cadherin-like protein (CPED1) **(Figure 5)**.

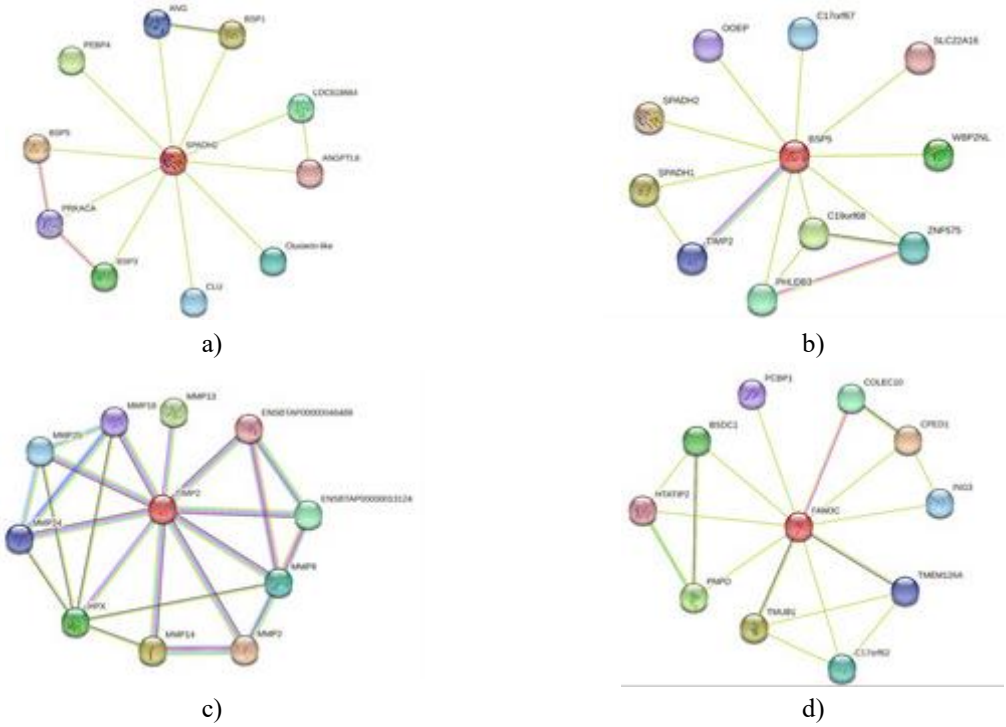


Figure 5. Interaction maps depicting seminal plasma protein networks identified in Nellore bulls, constructed using STRING v11.5 (https://string-db.org/, accessed 11 November 2022). Only DAPs identified in UNFIT bulls were included. (a) SPADH2 – Spermadhesin Z13; CLU – Clusterin; BSP1 – Binder of sperm protein 1; BSP3 – Binder of sperm protein 3. (b) BSP5 – Seminal plasma protein BSP–30 kDa. (c) TIMP2 – Tissue inhibitor of metalloproteinase–2. (d) FAM3C – Protein FAM3C; TMUB1 – Transmembrane and ubiquitin-like domain–containing protein 1; TMEM126A – Bos taurus transmembrane protein 126A; PNPO – Pyridoxine-5-phosphate oxidase; CPED1 – Cadherin-like protein.

This investigation utilized young Nellore bulls, categorized through Breeding Soundness Evaluation (BSE) as either reproductively FIT or UNFIT, to explore proteomic differences in seminal plasma (SP) between animals exhibiting low versus high levels of sperm abnormalities. No significant variation ($p < 0.05$) was detected between groups in traits such as mass motility, total motility, vigor, or average scrotal circumference, likely reflecting the genetic selection protocols applied within the herd improvement program. However, the frequency of sperm morphological defects (Morp) was notably reduced in the FIT bulls compared with the UNFIT group, confirming the suitability of these animals for detecting proteomic distinctions associated with sperm pathology rates. Broadly, these bulls displayed genetic merit superior to the mean of contemporary young Bos indicus populations. All bulls originated from a genetically selected Nellore breeding herd maintained under optimal management and animal welfare standards. Procedures for semen collection via electroejaculation and the assessment of phenotypic semen parameters were strictly followed by the standardized farm protocol and were executed uniformly for every individual.

The advent of molecular and proteomic methodologies has revolutionized the study of andrological biology, uncovering molecular details beyond the reach of conventional semen evaluations. Among these, proteomic profiling provides an exceptional window into reproductive physiology, identifying proteins that differ between animals with shared phenotypic patterns. Although much prior work has centered on adult bulls, the economic impact of maintaining elite genetic stock has driven efforts to develop early, precise assessments for identifying UNFIT young bulls. Expanding such research to the younger age class supports more informed breeding decisions and improves efficiency within assisted reproductive programs.

This study employed LC–MS/MS analysis, a highly sensitive and discriminative technique that permits quantitative detection of low-abundance proteins. The dataset showed that 278 proteins, representing only about 28–30% of the total signal from 297 identified proteins, fall into this low-abundance category. Such proteins, though scarce, often perform essential regulatory and signaling roles, influencing gene expression, response mechanisms, and cellular communication.

The functional classification of proteins from FIT and UNFIT semen underscores the critical contribution of seminal plasma to sperm maintenance, quality, and physiological readiness. The SP is indispensable in processes such as maturation, capacitation, motility, and acrosome reaction [37]. This was reflected in the functional cluster annotations, which showed elevated enrichment scores for these categories. The KOG classification "Post-translational modification, protein turnover, and chaperones" contained the greatest number of protein entries — a finding expected since the SP constitutes the extracellular environment of mature spermatozoa, whose own translational and transcriptional activity ceases post-spermatogenesis. Consequently, sperm depend heavily on SP-derived proteins and their post-translational modifications for executing reproductive functions [38, 39].

In the present investigation, which focused on the seminal plasma (SP) proteome of young Nellore bulls, Spermadhesin-1 emerged as the most prevalent protein identified. This contrasts with reports for adult bulls, where Binder of Sperm Proteins (BSPs) typically account for approximately 60% of the total SP protein content [40]. Spermadhesin-1, secreted by seminal gland epithelial cells in Nellore bulls, fulfills multiple essential reproductive roles [41]. It is capable of binding to the sperm plasma membrane, thereby enhancing motility and providing protection against oxidative stress. In addition, this protein interacts with cervical mucus, promoting the smoother passage of spermatozoa through the female reproductive tract. Another significant role involves the modulation of local immune responses, fostering a microenvironment favorable to sperm viability and fertilization by binding to glycoproteins on immune cell surfaces and inhibiting their recognition of spermatozoa [42].

Moreover, Spermadhesin-1 associates with membrane phospholipids of spermatozoa stored within the ampullae of the vas deferens before ejaculation, resulting in reduced mitochondrial metabolism. This mechanism prevents lipid peroxidation damage to the sperm membrane and temporarily suppresses motility, effectively extending sperm lifespan. Some investigations further indicate that residual Spermadhesin-1 on the sperm surface after capacitation may serve as a primary zona pellucida receptor, contributing to gamete recognition during fertilization [43–46]. High concentrations of this protein in SP have also been correlated with improved cryotolerance and post-thaw fertility of semen samples [47, 48]. Conversely, the lower Spermadhesin-1 abundance detected in UNFIT bulls may explain their reduced fertilizing ability.

Although the underlying cause for Spermadhesin-1 dominance in the SP of young bulls remains uncertain, its multifunctional properties and correlation results suggest that elevated levels of this protein indicate superior sperm quality. Quantitative analysis revealed that FIT bulls exhibited 78.3% higher Spermadhesin-1 abundance compared to UNFIT bulls, with standard deviations below 20% across three replicates. Given its notably high overall representation, Spermadhesin-1 may serve as a potential early biomarker, differentiating reproductively approved (FIT) bulls from non-approved (UNFIT) individuals. Implementing such early identification could streamline herd management and lower breeding costs by enabling the prompt removal of low-fertility young bulls.

Among the 297 SP proteins identified in young Nellore bulls, Spermadhesin-1 ranks among the 19 proteins exceeding 1% abundance in both FIT and UNFIT groups. This finding is particularly noteworthy since, despite the clear proteomic distinction between groups evidenced by PCA and clustering, 11 of these abundant proteins also qualified as differentially abundant proteins (DAPs). According to Kovac *et al*. [49], suitable non-invasive biomarkers for diagnosing male infertility through proteomics should be early-detectable, reproducible, economical, and quantifiable. The highly expressed DAPs identified herein align with these criteria and represent promising molecular marker candidates, as most are directly engaged in key reproductive mechanisms.

In contrast to Spermadhesin-1, both Spermadhesin-2 and Spermadhesin Z13 displayed higher relative abundances in the UNFIT bulls. Although they belong to the same protein family, Spermadhesin-2 and Spermadhesin Z13 are phylogenetically and sequentially distinct from Spermadhesin-1, which likely accounts for the observed differences in abundance profiles. These proteins are implicated in fertilization events, especially binding to the oviductal epithelium and regulating sperm function. Nevertheless, studies indicate that elevated levels of Spermadhesin-2 and Z13 may correlate with reduced fertility, owing to their inhibitory impact on motility through interference with Dynein ATPase activation—an enzyme essential for ATP hydrolysis within the axonemal

microtubules [50, 51]. Although a direct mechanistic link between Spermadhesin Z13 abundance and the high proportion of sperm defects in UNFIT bulls could not be conclusively established, in silico PPI analyses confirmed an interaction between Spermadhesin Z13 (SPADH2) and Clusterin (CLU).

Clusterin functions as a cell-protective glycoprotein that mitigates oxidative stress by neutralizing reactive oxygen species, thereby minimizing oxidative injury to spermatozoa. It is also associated with other seminal plasma components, maintaining the stability of lipid and protein structures. Nonetheless, this protein has been negatively correlated with the proportion of morphologically normal sperm, suggesting a link to inferior semen quality in bulls [52].

The seminal plasma protein BSP-30 kDa (BSP5) and seminal ribonuclease (SRN) have been extensively investigated regarding their roles in SP composition and bovine fertility [52]. Both have previously been described as positive phenotypic indicators associated with enhanced fertility and greater semen cryoresistance in bulls [53, 54]. However, the outcomes of the current research contradict these earlier reports, as BSP5 and SRN were detected in higher concentrations among bulls classified as UNFIT for reproduction, and both exhibited strong positive correlations with the percentage of major sperm abnormalities, implying a potential contribution to defective sperm formation. When spermatozoa are exposed to elevated levels of BSP5 for prolonged periods, damage to the plasma membrane may occur through excessive depletion of cholesterol and phospholipids within the bilayer [55].

Under physiological conditions, BSP5 partially associates with cholesterol and phosphatidylcholine of the sperm membrane during ejaculation [56]. Through this interaction, it participates in critical reproductive events such as oviductal binding, capacitation, and oocyte–sperm recognition [57]. Nevertheless, Moura *et al*. [51] demonstrated a nonlinear (quadratic) relationship between BSP5 concentration and fertility, indicating that optimal levels of BSP5 are beneficial, whereas excessive amounts impair fertility—confirming the dose-dependent effect of this protein.

Seminal ribonuclease contributes to sperm capacitation and exerts cytoprotective effects through its anti-spermatogenic, antitumor, and immunosuppressive properties. Despite these functions, Westfalewicz *et al*. [58] observed an elevated abundance of this enzyme during warmer seasons, coinciding with lower semen quality parameters. They proposed that high ambient temperatures promote ribonuclease oligomerization, leading to enhanced enzymatic and cytotoxic activity, which in turn generates a deleterious seminal environment for sperm cells [59]. The Aquidauana region in Mato Grosso do Sul, Brazil, where this study was carried out, experiences higher average temperatures than Olecko, Poland, where Westfalewicz *et al*. [58] performed their work. Thus, the thermal influence on seminal ribonuclease may have been more pronounced in our samples, though additional experiments are needed to confirm this effect.

Another protein showing increased abundance among UNFIT bulls and displaying strong positive correlations with major sperm defects was the Tissue Inhibitor of Metalloproteinases 2 (TIMP-2). This protein acts as a regulatory component of semen, modulating proteolytic enzyme activity, particularly matrix metalloproteinases (MMPs), by forming inhibitory complexes [60]. Both MMPs and TIMPs are crucial for extracellular matrix remodeling and tissue restructuring, participating in processes such as seminal liquefaction in the female reproductive tract, trophoblast invasion of the endometrium, and angiogenesis during embryo implantation [61].

Although prior investigations have detected TIMP-2 in the SP of high-fertility bulls and in human sperm exhibiting low DNA fragmentation, excessive TIMP-2 levels can also serve as a marker of testicular degeneration [53, 62, 63]. This is attributed to its involvement in inflammatory reactions, structural alterations in Sertoli cells, and blood–testis barrier dysfunction, as reported by Pereira *et al*. [64] in bulls subjected to thermal stress. Similarly, Newton *et al*. [65] documented that TIMP-2 expression rises with increasing testicular temperature, while Boe-Hansen *et al*. [10] observed a positive relationship between high TIMP-2 abundance and a greater proportion of sperm defects in young Brahman bulls.

In this study, TIMP-2 was also positively correlated with seminal ribonuclease abundance. Elevated levels of both proteins may reflect deteriorated sperm quality caused by thermal elevation, as previously noted. An increase in scrotal temperature can alter the biochemical composition of SP, either directly through testicular or epididymal changes, or indirectly via hormonal and neural mechanisms affecting accessory gland secretions [66]. It is well established that many major sperm pathologies originate from testicular dysfunction and disruptions in spermatogenesis induced by heat stress [67]. Excessive testicular temperature interferes with spermatogenesis across all stages, leading to higher rates of morphological defects, decreased motility, and lower sperm counts due to cellular apoptosis [68, 69].

The Vasoactive Intestinal Peptide (VIP), a neuropeptide distributed throughout various tissues, including the testes, accessory glands, and nervous system, acts both as a neurotransmitter and a neuroendocrine modulator [70, 71]. In mammalian and avian species, VIP also functions as a prolactin-releasing factor, influencing multiple reproductive and physiological pathways [72].

In males, prolactin suppresses hypothalamic GnRH release, consequently reducing FSH and LH secretion from the pituitary, which diminishes testicular testosterone production. These hormonal disruptions can impair spermatogenesis, resulting in poorer sperm quality and potential infertility [73]. Studies on roosters have demonstrated that VIP can negatively affect reproductive performance and semen traits in young males, primarily due to its role in prolactin stimulation [74]. These findings may partly explain our observation of higher VIP abundance in the UNFIT group, which also exhibited a positive correlation with major sperm defects and with increased levels of FAM3C and Hemoglobin beta.

The proteins FAM3C and Hemoglobin beta are known to participate in several biological and metabolic processes, such as bone formation (osteogenesis), energy regulation, and oxygen transport [75, 76]. Within bull seminal plasma, both are believed to contribute to sperm protection mechanisms that counteract lipid peroxidation induced by reactive oxygen species, thereby maintaining cellular integrity and fertility potential [57, 77].

The precise defense mechanisms exerted by FAM3C and Hemoglobin β remain uncertain; however, evidence suggests they are linked to their metal-binding properties, particularly with iron and copper ions. When present at elevated concentrations, these ions can initiate free-radical generation through cholesterol peroxidation of the cellular membrane [78]. Additionally, an excess of unbound iron in the seminal plasma (SP) can indirectly promote oxidative imbalance, since many pathogenic microbes use iron as a metabolic cofactor. This process triggers immune activation, the recruitment of macrophages, and neutrophil stimulation, all of which intensify the production of reactive oxygen species (ROS) [79-81].

The participation of immunoglobulin G (IgG) in SP is still debated. Some research indicates that IgG may assist in gamete fusion and modulation of local immune tolerance in the male tract, while other studies associate its presence with reduced fertility. In the latter case, IgG molecules bind to antigens on spermatozoa, promoting agglutination and loss of motility, which obstructs cervical mucus penetration and compromises the acrosomal reaction, resulting in fertilization failure [82, 83].

In this work, immunoglobulins appeared as differentially abundant proteins (DAPs) in both FIT and UNFIT bulls, reinforcing the complex and dual nature of their function. The Ig gamma-1 chain C region, representing the constant segment of the IgG1 heavy chain, was up-regulated in FIT bulls. This fragment is critical to antibody effector activity once antigen binding occurs. Significant negative correlations were observed between this protein and major sperm defects, accompanied by elevated levels of FAM3C, Hemoglobin β, and TIMP-2.

By contrast, the Ig lambda-1 chain C region exhibited higher expression in UNFIT bulls. This segment corresponds to the light-chain region of lambda-1 immunoglobulins, which identify and neutralize foreign molecules. The appearance of antibodies in semen is frequently related to disruption of the blood–testis barrier and to other pathological changes in the reproductive tract [49].

Accordingly, the enhanced concentrations of FAM3C, Hemoglobin β, and Ig lambda-1 chain C region observed in the UNFIT group may reflect an oxidative stress condition that negatively impacts sperm functional parameters. Both FAM3C and Hemoglobin β showed strong positive associations with major sperm abnormalities, supporting this interpretation.

Overall, the DAP pattern in UNFIT bulls indicates a cellular response to oxidative damage. The structural alterations within the testicular parenchyma appear consistent with degenerative events leading to oxidative imbalance **(Figure 6)**. Such changes likely contribute to the higher incidence of sperm pathologies identified in this group.

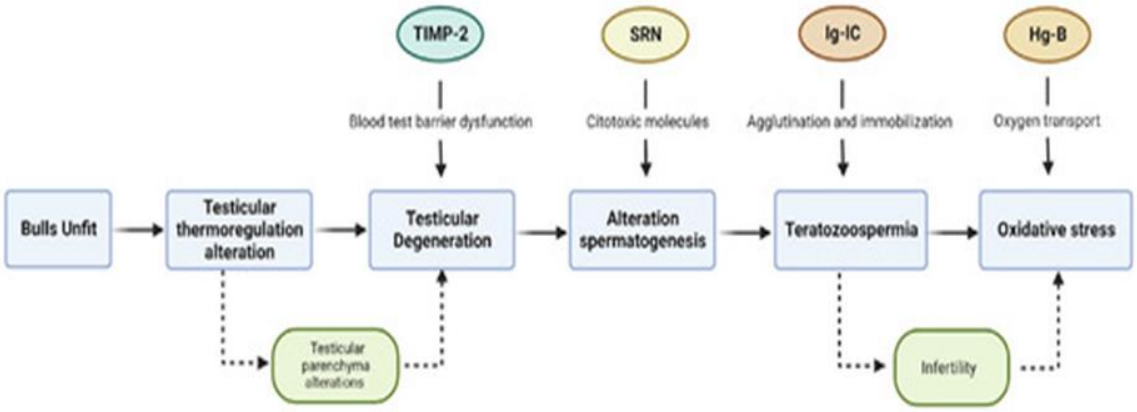


Figure 6. Functional representation of differentially abundant proteins (DAPs) linked to sperm defect formation. Young Nellore bulls designated as UNFIT displayed nine up-regulated seminal plasma proteins. Among them, TIMP-2 (A0A0E3UT00), SRN (P00669), Ig-C (L8IAF4), and Hg-B (D4QBD1) are involved in molecular pathways that contribute to sperm impairment.

Conclusion

Through LC–MS/MS proteomic profiling, 297 seminal plasma proteins were detected in young Nellore bulls categorized as FIT or UNFIT for reproduction. Of these, 93.6 % exhibited abundances below 1 %, classified as low-abundance proteins. The data revealed a clear proteomic distinction between the two groups, despite 11 highly abundant proteins being common to both. These 11 proteins, identified as DAPs, meet the requirements for reproductive molecular biomarkers, being functionally tied to fertility, detectable in early evaluation stages, and quantifiable with precision.

Among all identified proteins, Spermadhesin-1 displayed the greatest mean abundance across both groups, marking it as a promising biomarker for reproductively sound (FIT) bulls. Quantitative analysis showed up-regulation of Spermadhesin-1 and Ig gamma-1 chain C region in the FIT group, whereas Spermadhesin Z13, TIMP-2, BSP-30 kDa, VIP, Hemoglobin β, Spermadhesin-2, Seminal Ribonuclease, FAM3C, and Ig lambda-1 chain C region were up-regulated in UNFIT bulls.

The proteins overexpressed in the UNFIT cohort are primarily associated with oxidative-stress pathways, which likely explain the increased sperm deformities found in these animals. As oxidative stress interferes with spermatogenesis at multiple stages, it can induce structural and morphological abnormalities in developing spermatozoa, ultimately reducing fertility.

Acknowledgments: None

Conflict of Interest: None

Financial Support: None

Ethics Statement: None

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