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SEMINAL PLASMA PROTEOMES OF INDIGENOUS INDONESIAN BULLS: INSIGHTS INTO REPRODUCTIVE BIOLOGY AND FUNCTIONAL PATHWAYS

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Abstract. This study aimed to characterize the seminal plasma proteome of three indigenous Indonesian bull breeds - Bali, Pasundan and Donggala - to identify reproductive-associated proteins and provide molecular insights into breed-specific fertility traits in tropical livestock systems. Seminal plasma was collected from 15 bulls (five from each breed) and analyzed using 1D SDS-PAGE followed by LC-MS/MS, with functional annotation conducted via Gene Ontology and STRING databases. A total of 364 unique proteins were identified, with Bali bulls exhibiting the highest number of breed-specific proteins (195), followed by Donggala (172) and Pasundan (130). No protein was shared across all three breeds, indicating distinct proteomic profiles. Functional enrichment analyses revealed that Bali bulls had proteins related to motility and metabolism, Pasundan bulls to structural and acrosomal functions and Donggala bulls to transport and cytoskeletal stability. Key reproductive-associated proteins such as BSP1, TEX101, IZUMO1 and HSPA1L were identified and interaction networks highlighted specialized molecular strategies related to sperm function. These findings demonstrate significant inter-breed variation in seminal plasma composition, reflecting adaptations to tropical environments and provide a molecular foundation for fertility biomarker discovery and precision breeding in underrepresented bovine populations.

Keywords: *Seminal plasma proteomics, indigenous Indonesian bulls, fertility biomarkers, protein profiling, LC-MS/MS.*

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1. Introduction

Male fertility is central to reproductive efficiency, genetic progress and overall livestock productivity. In breeding programs - particularly those using artificial insemination - the fertilizing capacity of bulls determines herd-wide reproductive success.

While semen evaluation traditionally focuses on traits like motility, viability and morphology, recent studies have emphasized the crucial role of seminal plasma as a dynamic biochemical medium influencing sperm function beyond structure (Kasimanickam *et al*., 2019; Kumaresan *et al*., 2020).

Seminal plasma contains a complex mixture of proteins, enzymes and signaling molecules that support key sperm functions critical for successful fertilization, including capacitation and immunological interaction (Diansyah *et al*., 2021, 2022). In taurine breeds such as Holstein, Angus and Simmental, seminal plasma proteomic profiling has enabled the identification of reproductive-associated proteins linked to fertility variation (Westfalewicz *et al*., 2017; Zoca *et al*., 2022; Baharun *et al*., 2023). These discoveries have facilitated the development of molecular markers to predict fertility performance and improve sire selection in high-production systems.

Despite these advances, proteomic studies on bulls in tropical environments remain scarce. Most available data come from Bos taurus breeds raised in temperate climates, with minimal representation of indicine or indigenous bulls, particularly those kept in smallholder or low-input systems (Utsunomiya *et al*., 2019). Indonesia, for instance, is home to genetically distinct breeds such as Bali, Pasundan and Donggala, which are well adapted to tropical conditions and play a vital role in rural livestock systems (Tawaf *et al*., 2017; Mangun *et al*., 2024; Hariyono *et al*., 2025). However, molecular-level research on their reproductive physiology remains sparse and fragmented.

This gap in the literature highlights a lack of understanding of reproductive biology in indigenous bulls. Although some initial studies have reported basic semen characteristics in these breeds (Tawaf *et al*., 2017), no comprehensive proteomic profiling of their seminal plasma exists to date. Without such insights, identifying breed-specific fertility markers and designing diagnostics or management tools tailored to tropical breeding programs remains a challenge (Yaro *et al*., 2017; Ayantoye *et al*., 2025).

This study aims to characterize the seminal plasma proteome of three indigenous Indonesian bull breeds - Bali, Pasundan and Donggala - with a focus on identifying reproductive-related proteins potentially linked to male fertility. By addressing this gap, the study seeks to provide foundational insights into breed-specific reproductive physiology and support biomarker-based approaches for fertility evaluation and sustainable bull selection in tropical systems.

2. Materials and Methods

Ethical Approval

All animal procedures in this study were conducted in compliance with ethical guidelines for animal welfare and were approved by the Animal Ethics Commission of the National Research and Innovation Agency (Certificate No. 050/KE.02/SK/03/2023). The protocol for frozen semen production followed the Indonesian National Standard (SNI 4869-2:2021) for artificial insemination.

Animal Selection and Semen Collection

A total of fifteen reproductively mature and clinically healthy bulls comprising five individuals each from the Bali, Pasundan and Donggala breeds were selected from certified government breeding centers in Indonesia. All bulls were aged between 5 and 8 years, confirmed to be free of reproductive disorders and had documented records of acceptable semen quality based on previous breeding performance. Semen collection was

performed by artificial vaginal method referring to the protocol Nirmala et al. (2025), was collected biweekly over a two-month period using an artificial vagina, with each bull contributing three independent ejaculates. Immediately after collection, the semen samples were placed into pre-warmed sterile tubes and transported in a thermal container maintained at 37 °C to the laboratory for further analysis.

Seminal Plasma Protein Extraction

Seminal Plasma Protein Extraction following the protocol of Diansyah et al. (2022). Seminal plasma was separated from freshly collected semen samples by centrifugation at 6,500 rpm for 30 minutes at 4 °C using a refrigerated centrifuge. Approximately 1 mL of semen per sample was transferred into sterile 1.5 mL microcentrifuge tubes. The supernatant was carefully collected and stored at -20 °C until further analysis. Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, USA), with bovine serum albumin (BSA; Sigma-Aldrich, USA) as the standard. Each reaction contained 5 µL of sample and 250 µL of Bradford reagent, incubated at room temperature for 10 minutes. Absorbance was measured at 595 nm using a microplate reader. Protein concentrations were calculated from a standard curve and expressed as milligrams per milliliter (mg/mL). All measurements were performed in triplicate.

SDS-PAGE Protein Fractionation and In-Gel Digestion

Seminal plasma proteins were separated by one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1D SDS-PAGE) (Baharun *et al*., 2021) using a 12% resolving gel and a 4% stacking gel. Electrophoresis was performed at a constant voltage of 60 V and a current of 20 mA for 3.5 hours. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, USA) and destained using 50 mM ammonium bicarbonate in a 1:1 (v/v) mixture of acetonitrile and ultrapure water at 37 °C for 30 minutes. Distinct protein bands were manually excised and transferred to sterile 1.5 mL microcentrifuge tubes.

The gel slices were washed and equilibrated with 50 mM ammonium bicarbonate, followed by reduction with 10 mM Tris(2-carboxyethyl)phosphine (TCEP; Sigma-Aldrich, USA) at 37 °C for 30 minutes. Alkylation was then carried out using 55 mM iodoacetamide (IAA; Sigma-Aldrich, USA) at room temperature in the dark for 30 minutes (Diansyah *et al*., 2023). After rinsing, in-gel digestion was performed using sequencing-grade modified trypsin (Promega, USA) at a concentration of 12.5 ng/µL in 50 mM ammonium bicarbonate. The samples were incubated overnight at 37 °C. Resulting peptides were collected and stored for subsequent purification and LC-MS/MS analysis (Diansyah *et al*., 2023).

Peptide Purification and LC-MS/MS Analysis

Tryptic peptides were desalted and purified using Thermo Scientific™ Pierce™ C18 Spin Columns following the manufacturer's protocol. Elution was performed with 70% acetonitrile containing 0.1% formic acid. The eluates were dried under vacuum using a SpeedVac concentrator and reconstituted in 20 µL of 0.1% formic acid for subsequent LC-MS/MS analysis (Davidovics *et al*., 2022).

Peptide separation and analysis were carried out using a high-resolution Orbitrap mass spectrometer (Q Exactive™ HF, Thermo Fisher Scientific, USA) coupled with an EASY-nLC 1200 nano-flow liquid chromatography system. Peptides were loaded onto a PepMap™ RSLC C18 analytical column (75 µm × 50 cm, 2 µm particle size, 100 Å pore

size) and separated at a flow rate of 300 nL/min using a linear gradient of solvent B (80% acetonitrile with 0.1% formic acid) from 5% to 35% over 90 minutes. Solvent A consisted of 0.1% formic acid in water.

The mass spectrometer was operated in data-dependent acquisition (DDA) mode. Full MS scans were acquired at a resolution of 60,000, followed by MS/MS fragmentation of the top 20 most intense ions using higher-energy collisional dissociation (HCD). Dynamic exclusion was set to 30 seconds.

Raw spectral data were processed using MaxQuant software (version 2.0.3.0). Spectra were searched against the Bos taurus UniProtKB reference proteome (used as a proxy for Bos javanicus) using the Andromeda search engine. Carbamidomethylation of cysteine was set as a fixed modification, while oxidation of methionine was set as a variable modification. The false discovery rate (FDR) was controlled at <1% at both the peptide and protein levels (Iskandar *et al*., 2023).

Bioinformatics and Functional Annotation

Functional annotation of the identified proteins was performed using the Gene Ontology (GO) framework and protein-protein interaction analysis via the STRING database version 12.0 (https://string-db.org). Protein identifiers obtained from LC-MS/MS analysis were matched to entries in the UniProt database (https://www.uniprot.org), which served as the primary reference for determining protein function. UniProt provides comprehensive annotations, including molecular roles, associated biological processes and subcellular localization, enabling the functional interpretation of each identified protein (Iskandar *et al*., 2023).

Enrichment analysis was conducted to identify significantly overrepresented GO terms among the detected proteins. To examine interaction patterns, protein-protein interaction networks were generated in STRING with a minimum required confidence score of 0.7. Both experimental and predicted associations were included to construct high-confidence functional clusters and regulatory modules. The resulting interaction maps were used to explore molecular relationships involved in sperm motility, capacitation and other reproductive-associated functions (Shen *et al*., 2021).

3. Result

Comparative Proteomic Profiling of Seminal Plasma Proteins in Indigenous Indonesian Bulls

Proteomic analysis of seminal plasma from Bali, Pasundan and Donggala bulls revealed a total of 364 unique proteins across all samples. As depicted in the Venn diagram (Figure 1), Bali bulls showed the highest number of breed-specific proteins, totaling 195 proteins (37.5%), followed by Donggala bulls with 172 proteins (35.5%) and Pasundan bulls with 130 proteins (25.3%). Shared proteins between two breeds were also observed: 12 proteins between Bali and Donggala, 6 between Pasundan and Donggala and 1 between Bali and Pasundan. Notably, no protein was found to be shared across all three breeds. This distribution indicates clear proteomic divergence among the three indigenous cattle breeds studied.

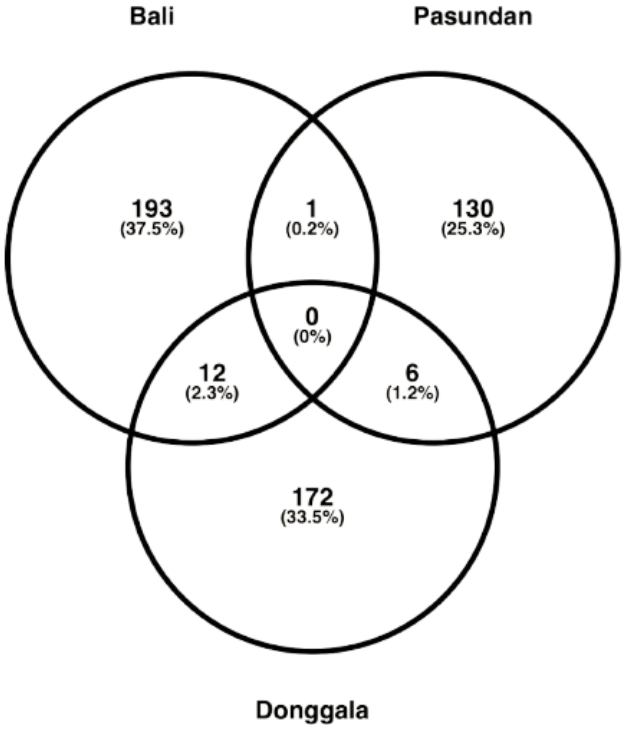

Figure 1. Distribution and overlap of identified seminal plasma proteins among Bali, Pasundan and Donggala bulls

Functional Annotation of Identified Proteins in Indigenous Indonesian Bulls

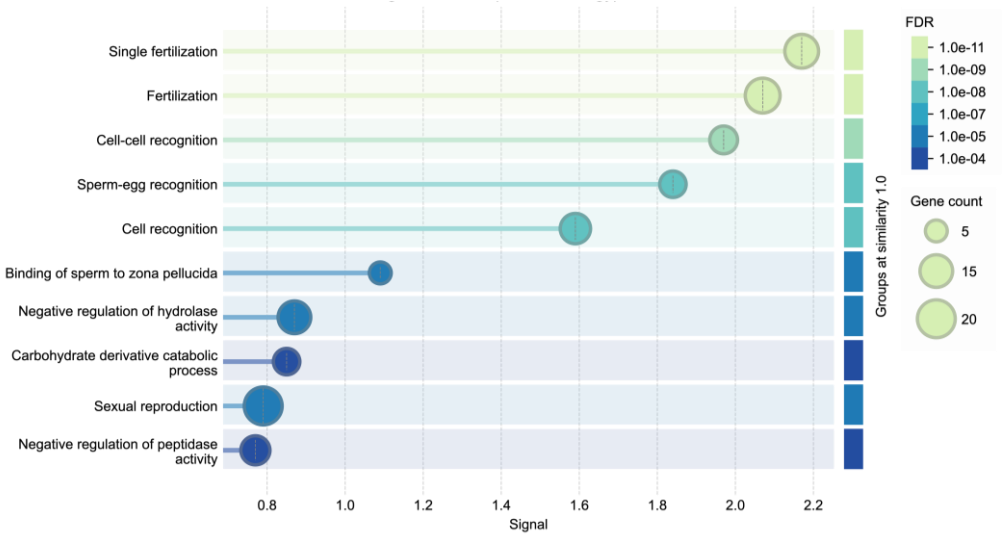

Figure 2. Gene Ontology (GO) enrichment analysis of seminal plasma proteins from Bali bulls

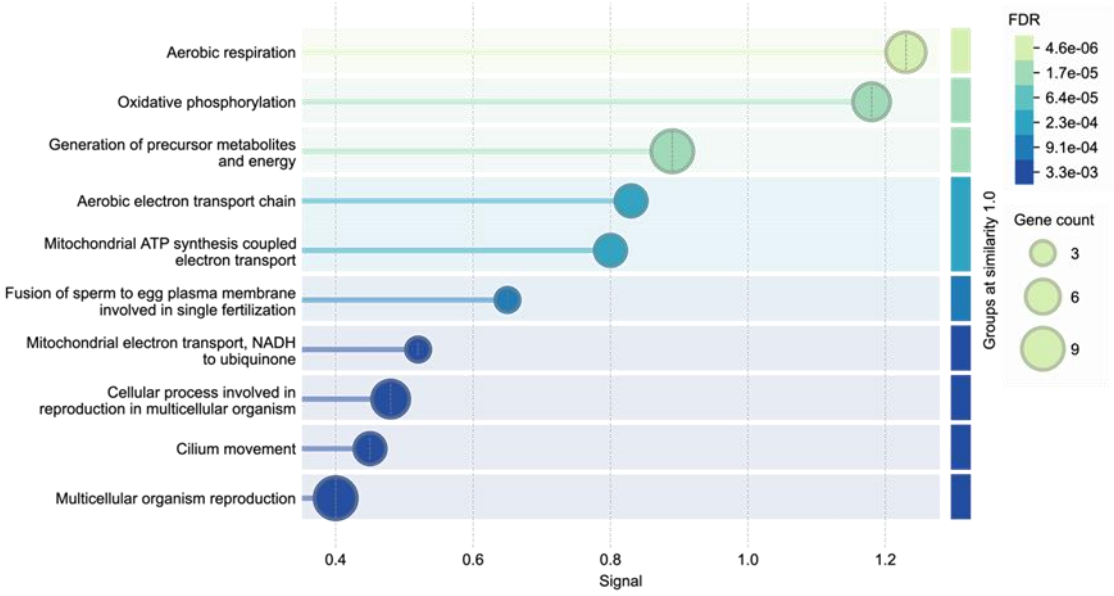

Figure 3. Gene Ontology (GO) enrichment analysis of seminal plasma proteins from Pasundan bulls

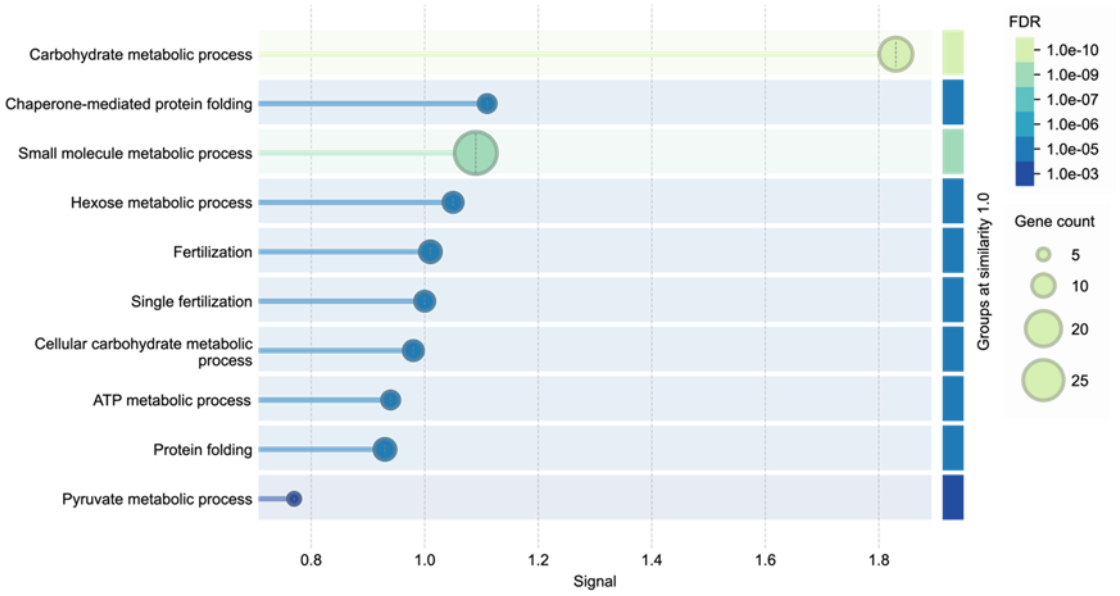

Figure 4. Gene Ontology (GO) enrichment analysis of seminal plasma proteins from Donggala bulls

Functional classification based on Gene Ontology analysis revealed distinct enrichment patterns for each breed. In Bali bulls (Figure 2), enriched terms were related to cellular motility, metabolic regulation and reproductive activity. Several annotations demonstrated high statistical confidence (FDR < $1e^{-10}$) with considerable gene counts, indicating a strong representation of functionally significant proteins in this breed. For Pasundan bulls (Figure 3), enriched terms included pathways associated with structural development, intracellular signaling and gamete interaction. Although the number of contributing proteins was relatively lower, the annotations remained statistically significant, suggesting relevant biological functions specific to this breed. In Donggala bulls (Figure 4), the enrichment profile highlighted functions associated with molecular transport, cellular structure and energy-related processes. Several terms showed both high

statistical significance (FDR < $1e^{-09}$) and a broader spectrum of protein involvement, indicating a diverse functional proteome in Donggala seminal plasma. These annotation results provide insight into the molecular landscape of seminal plasma across breeds and serve as a foundation for identifying fertility-associated proteins in indigenous Indonesian bulls.

Comparative Profile of Reproductive-Associated Proteins in Indigenous Indonesian Bulls

Table 1. Comparative List of Reproductive-Related Proteins of Seminal Plasma in Indigenous Indonesian Bulls

Breed	ID Gene	Accession
Bali	ZPBP	F1N369
	ELSPBP1	E1B9P4
	CRISP1	E1BC47
	ROPN1	F1N058
	FOLR1	P02702
	TEX101	A6QPE3
	BSP1	P02784
	BSP3	P04557
	BSP5	P81019
Pasundan	RSPH9	A0A4W2BZV4
	ZPBP	F1N369
	ROPN1	A0A4W2CZC0
	CIMIP2A	A0A4W2DPP6
	TEKT3	A0A4W2DWK5
	CABYR	A0A4W2BU18
	IZUMO1	A0A4W2ILL7
	SPACA1	A0A4W2D0I6
Donggala	CCT5	A0A4W2IMM7
	HSPA1L	A0A4W2I2Q2
	CCT7	A0A4W2FDX9
	ARSA	A0A4W2DSL6
	ATP1A4	A0A4W2G6J0
	LYZL4	A0A4W2BQ23

Interaction Networks of Reproductive-Associated Proteins in the Seminal Plasma of Indigenous Bulls

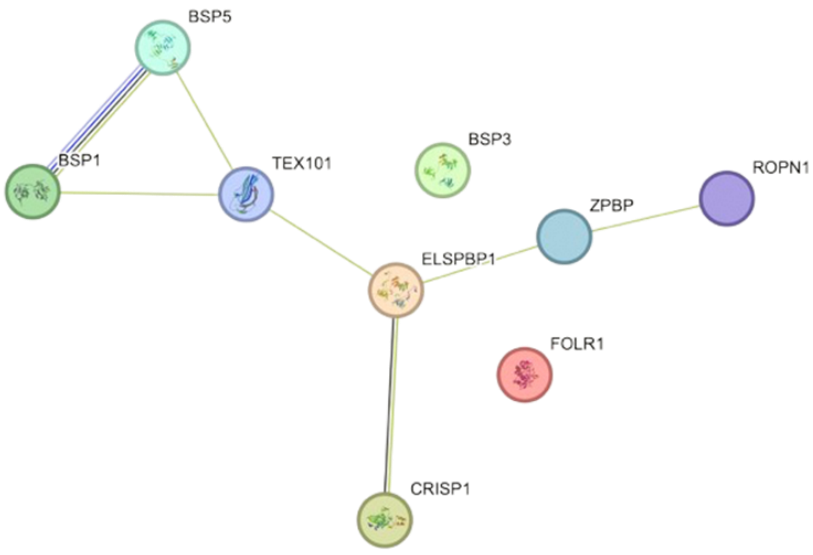

Figure 5. Protein Reproductive Function Interaction of seminal plasma Bali bulls

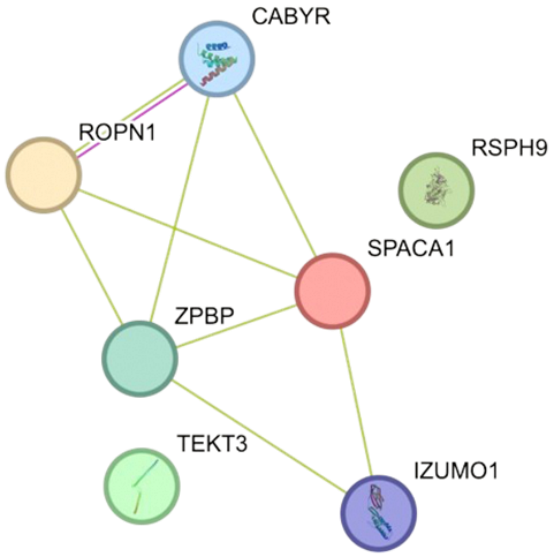

Figure 6. Protein Reproductive Function Interaction of seminal plasma Pasundan bulls

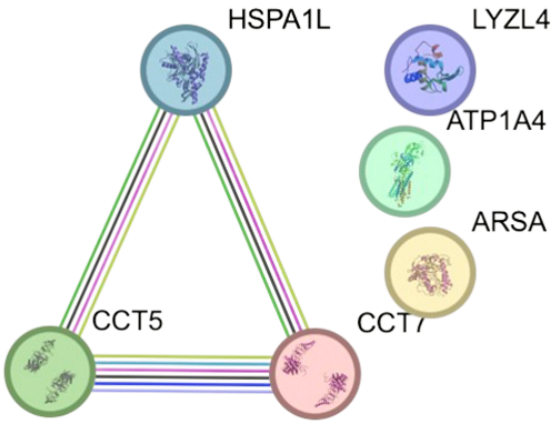

Figure 7. Protein Reproductive Function Interaction of seminal plasma Donggala bulls

4. Discussion

The Proteomic analysis of seminal plasma from three indigenous Indonesian bull breeds revealed marked molecular divergence, with minimal protein overlap (Figure 1). This observation suggests that the biochemical environment of spermatozoa is shaped not only by individual genetics but also by breed-specific evolutionary pressures. The high number of unique proteins, especially in Bali bulls, suggests co-evolution of seminal plasma composition with traits like heat tolerance, metabolic efficiency and reproductive investment under tropical conditions (Baharun *et al*., 2023). Similar patterns of proteomic divergence have been reported in Bos indicus populations exposed to varying environmental pressures (Hsu *et al*., 2013), highlighting the role of local adaptation in shaping reproductive molecular profiles.

The Gene Ontology enrichment results (Figures 2-4) underscore this hypothesis. Bali bulls showed a concentration of proteins involved in energy metabolism and motility regulation, possibly reflecting a metabolic strategy favoring rapid sperm activation. This aligns with findings by Gacem et al. (2023), who demonstrated that ATP-regulated

proteins are positively correlated with motility indices in fertile bulls. In Pasundan bulls, the prominence of acrosome assembly and sperm-egg interaction pathways suggests a molecular emphasis on fertilization precision rather than pre-fertilization competition, which could be a response to specific breeding system dynamics. Donggala bulls showed enrichment in proteins for transport and cytoskeletal stability - traits linked to sperm longevity and resilience (Frezarim *et al*., 2025). These proteomic patterns may indicate differing reproductive strategies, where Bali and Pasundan bulls optimize fertilization efficiency, while Donggala bulls prioritize sperm preservation under variable environmental stressors.

The reproductive-associated proteins listed in Table 1 offer mechanistic insights into these functional themes. For example, the consistent identification of BSP proteins (BSP1, BSP3, BSP5) in Bali bulls suggests a key role in sperm activation, particularly through membrane remodeling mechanisms (Freitas *et al*., 2021) - may explain their enrichment in motility-related GO terms. The co-detection of TEX101 and ELSPBP1 further supports a role in sperm-epithelial interaction and zona binding, pathways associated with higher fertilizing capacity. In contrast, the presence of IZUMO1 and SPACA1 in Pasundan bulls aligns with observations by Hernández-Falcó et al. (2022), who showed that these proteins are essential for sperm-oocyte fusion and acrosomal targeting. The detection of TEKT3 and CABYR - proteins involved in flagellar stiffness and calcium signalling - suggests a tightly controlled activation mechanism during fertilization.

In Donggala bulls, the abundance of molecular chaperones such as HSPA1L and CCT5 indicates a stress-adaptive proteomic signature. These proteins have been shown to stabilize protein structure and function during thermal or oxidative stress (Özbek *et al*., 2021; Malheiros *et al*., 2021), a relevant trait for bulls maintained in high-humidity, low-input systems. The presence of ATP1A4, which regulates ionic gradients in sperm tails, may support sperm longevity under environmental stress (McDermott *et al*., 2021).

The STRING-based interaction networks (Figures 5-7) highlight functional specialization across breeds. In Bali bulls, a tightly connected cluster of sperm activation proteins indicates coordinated molecular regulation of membrane dynamics. Pasundan bulls showed clustered interactions around flagellar and acrosomal proteins, which may contribute to a rapid and synchronized acrosome reaction. Donggala bulls, meanwhile, displayed a chaperone-dominated network, implying a systemic emphasis on sperm preservation and structural integrity. These observations are consistent with studies in tropical cattle indicating adaptive selection for heat tolerance and regulated sperm activation timing (Naranjo-Gómez *et al*., 2021) and may reflect evolutionarily optimized sperm strategies in challenging environments.

Taken together, the observed proteomic divergence likely reflects functional adaptations rather than mere taxonomic differences. These findings emphasize that fertility is not a uniform trait, but a complex phenotype shaped by molecular, physiological and environmental factors (Jiménez *et al*., 2023). From a translational perspective, the presence of breed-specific reproductive proteins presents valuable opportunities to develop targeted fertility diagnostics and breeding tools (Mogielnicka-Brzozowska & Cichowska, 2024). Nonetheless, the limited sample size (five bulls per breed) constrains generalizability. Future studies involving larger cohorts are needed to validate and expand upon these findings.

5. Conclusion

This study presents a comprehensive proteomic characterization of seminal plasma in Bali, Pasundan and Donggala bulls, revealing substantial inter-breed divergence in protein composition, functional pathways and molecular interactions related to male fertility. The identification of breed-specific reproductive-associated proteins underscores the existence of distinct physiological strategies likely shaped by genetic and environmental factors. These findings not only enhance the molecular understanding of bull reproduction in tropical systems but also provide a foundation for developing fertility biomarkers, precision breeding strategies and AI-based prediction tools for reproductive success. Such molecular insights could support the creation of diagnostic kits, inform sire selection protocols and optimize breeding outcomes in indigenous and low-input cattle systems. Despite the need for further phenotypic validation, the results offer a valuable reference for advancing reproductive management, conservation efforts and molecular breeding strategies in underrepresented bovine populations.

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Data Availability

The raw mass spectrometry data generated in this study are co-owned by collaborating institutions and are not publicly available at this time. However, the data may be made available from the corresponding author upon reasonable request and with permission from all data contributors.

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