Asian Journal of Agriculture and Biology
https://doi.org/10.35495/ajab.2025.199

Original Article

Integrated proteomic and metabolomic analysis identifies candidate biomarkers defining the dichotomy of bovine seminal plasma and spermatozoa

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Received: 12 September 2025 / Revised: 04 November 2025 / Accepted: 11 November 2025 / Published Online: 21 November 2025

Abstract

Semen quality is one of important factor that impacts cattle reproduction. However, the seminal plasma and spermatozoa separation is still unclear. We attempted proteomic and metabolomic techniques on fresh bovine semen and its compartments to molecularly characterise and cross these two compartments. The proteomic profile of the semen plasma showed pronounced accessory proteins enhancing lipid binding, ion homeostasis, and membrane dynamics, particularly PDC-109, enolases, VDAC2, and SP-10, while the spermatozoa comprised scaffolding, anchoring, and mitochondrial enzymatic proteins such as AKAP3, tektins, cylicins, and COX5B. The dichotomy was reinforced by complementary metabolomic analysis, with seminal plasma containing antioxidants and lipids such as taurine, ergothioneine, palmitoylglycine, and stearamide, while spermatozoa were enriched in metabolites associated with energy, including citrate, inosine, succinic semialdehyde, and pantothenic acid. Pathway analysis reinforced plasma specialisation about antioxidants and lipids rather than spermatozoa on glycolysis and amino acid metabolism with oxidative phosphorylation. Collectively, these results illustrate the presence of interconnected but non-interchangeable molecular domains in which seminal plasma provides protective and regulatory buffering, while spermatozoa are specialised in structural and energetic components for fertilisation. The candidate biomarkers identified from this study such as PDC-109, AKAP3, cylicins, taurine, and citrate illustrate molecular outputs corresponding to the quality of semen and provide a systematised context for enriched understanding of bovine reproductive biology.

Keywords: Bovine semen, Metabolomics, Seminal plasma, Spermatozoa, Proteomics

How to cite this article:

Sahiruddin S, Yusuf M, Diansyah AM, Masturi M, Herdis H, Maulana T and Wibowo S. Integrated proteomic and metabolomic analysis identifies candidate biomarkers defining the dichotomy of bovine seminal plasma and spermatozoa. Asian J. Agric. Biol. 2026: e2025199. DOI: https://doi.org/10.35495/ajab.2025.199

Introduction

Bovine reproduction is central to livestock productivity and food security, as cattle represent a major source of both meat and milk. In Indonesia and many other regions, rising demand for beef and dairy continues to outpace domestic production, creating pressure to improve reproductive efficiency. Artificial insemination (AI) has become a cornerstone of breeding programs, yet its success remains variable, with semen quality identified as a major determinant of fertilization outcomes. Despite the widespread use of semen extenders to support sperm function during handling, the molecular basis of semen quality and the complementary roles of seminal plasma and spermatozoa remain poorly defined (Said, 2020; Yusuf and Sahiruddin, 2020; Hasrin et al., 2020; Surahman et al., 2021).

Recent advances in omics technologies provide new opportunities to address this gap. Proteomics enables the systematic identification of seminal plasma and spermatozoal proteins that regulate motility, capacitation, and membrane stability, while metabolomics offers complementary insights into small molecules acting as energy substrates, antioxidants, and signaling mediators. Together, these approaches allow a systems-level view of semen composition, capable of revealing how seminal plasma functions as an extracellular buffer and regulatory medium, while spermatozoa concentrate structural and energetic machinery optimized for fertilization (Sahiruddin et al., 2016; Masturi et al., 2021; Tethool et al., 2022; Sahiruddin et al., 2020; Costes et al., 2024; Maulana et al., 2024).

Although proteomic and metabolomic approaches have been applied in livestock reproduction, their use in cattle semen has remained limited (Khan et al., 2021; Poudyal et al., 2021; Lombó et al., 2021; Diansyah et al., 2022; Talluri et al., 2022). By integrating analyses of seminal plasma and spermatozoa, it is possible to uncover discriminant proteins and metabolites that reflect their distinct but interdependent functions. The present study applies such a multi-omics strategy to define the molecular dichotomy of bovine semen and to identify candidate biomarkers that provide mechanistic insights into semen quality and fertility potential.

Material and Methods

Sample collection and protein preparation

Fresh semen samples were collected from healthy Bali (Balinese) bulls (age 4–6 years; body condition score [BCS] 4–6) and immediately processed to separate seminal plasma from spermatozoa by centrifugation. A total of five (n = 5) bulls were enrolled; each bull contributed five ejaculates collected once weekly across five separate sessions (≈7-day intervals). For proteomic and metabolomic analyses, ejaculates from the same bull were pooled at equal volumes to generate one composite sample per fraction (seminal plasma and spermatozoa) per bull; thus, the biological unit for inference was the bull (n = 5). Cross-contamination between fractions was minimized through double-wash steps and visual inspection of pellet clarity before plasma decanting. All procedures were approved by the Ethics Committee of the Faculty of Animal Science, Universitas Hasanuddin (Approval No. 011/UN4.12/EC/V/2025; 21 May 2025).

Protein identification and quantification

The extraction of seminal plasma proteins followed the procedure of Diansyah et al. (2022) using a protein lysis buffer. Soluble proteins were resolved using one-dimensional SDS-PAGE (12% polyacrylamide gels). Protein bands were excised, washed with ammonium bicarbonate–acetonitrile, reduced with tris(2-carboxyethyl) phosphine (TCEP), and alkylated with iodoacetamide (IAA). Digestion was performed with sequencing-grade trypsin at 37 °C for 4 hours. The resulting peptides were purified using C18 spin columns. Peptide fractionation and mass spectrometric analysis were carried out using a Nano LC-Q Exactive Plus Orbitrap HRMS system (Shimadzu, Japan), enabling the identification of proteins associated with semen quality. Raw MS/MS spectra were searched against the UniProt Reference Proteome for *Bos taurus* (downloaded and accessed 30 July 2025) including canonical and isoform sequences; a reversed decoy database was appended for FDR estimation. Protein identifications were filtered using a 1% false discovery rate (FDR) at both the peptide and protein levels. Quantitative information was obtained from normalized spectral intensity values, which were subsequently used for downstream statistical analyses (Diansyah et al., 2022).

https://doi.org/10.35495/ajab.2025.199

Metabolomic analysis

Metabolomic profiling was performed on bovine seminal plasma to identify metabolites associated with semen quality. The metabolomics workflow used pooled, equal-volume composites per bull (5 bulls × 1 composite per fraction), matched to the proteomics design. Metabolite extraction was carried out following the procedure of Nurlatifah et al. (2023) with minor modifications. Briefly, 150 µL of methoxamine hydrochloride in methanol was added as an internal standard, together with 350 µL of extraction solvent (1:4 ultrapure water:methanol) to 50 µL of seminal plasma. The mixture was vortexed for 1 minute, centrifuged at 13,000 rpm for 20 minutes at 4 °C, and the supernatant was collected and filtered through a syringe filter.

The filtrate was evaporated to dryness in an evaporator at 36 °C for approximately 2 hours and stored at −20 °C until derivatization. For derivatization, 100 µL of N-trimethylsilyl-N-methyl trifluoroacetamide (MSTFA) containing 1% trimethylchlorosilane (TMCS) was added to the dried extracts, and the mixture was incubated at 70 °C for 1 hour. After derivatization, samples were centrifuged at 13,000 rpm for 10 minutes at 4 °C, and the supernatant was transferred into amber glass vials (GC vials) for analysis. Metabolite identification was performed using gas chromatography–mass spectrometry (GC-MS; Shimadzu, Japan). The resulting chromatograms were processed for peak detection, alignment, and annotation against standard libraries, generating metabolite profiles associated with osmotic stability, redox buffering, and protection of spermatozoa within bovine seminal plasma (Nurlatifah et al., 2023).

Statistical analysis

All statistical analyses were conducted in MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/; accessed 30 July 2025). For proteomic data, raw protein intensity values were $\log_2$-transformed, Pareto-scaled, and for metabolomic data captured through GC–MS, raw values were similarly normalised prior to analysis. To minimize bias from sparse features, missing values were imputed minimally using k-nearest neighbors after transformation. Because ejaculates were pooled per bull, the biological unit for inference was the bull (n = 5 per fraction).

Each dataset was first examined with Principal Component Analysis (PCA) to visualize overall structure and within-cohort reproducibility. We then applied Partial Least Squares Discriminant Analysis (PLS-DA) to summarize group separation. Model reliability was evaluated using leave-one-out cross-validation (LOOCV), reporting accuracy, R^2, and Q^2, and further contextualized by 1,000 permutation tests that preserved class sizes (5 vs 5). Given the limited sample size, multivariate results are interpreted cautiously and primarily as visualization rather than definitive classification. (Nurlatifah et al., 2023).

Beyond multivariate modeling, we performed paired two-sample t-tests comparing seminal plasma and spermatozoa within bull. P-values were adjusted using the Benjamini–Hochberg procedure with an FDR threshold of 0.05 as the criterion for statistical significance. We reported effect sizes (Cohen's *d* for paired designs) and 95% confidence intervals and expressed directional changes as $\log_2$ fold-changes. VIP scores (threshold > 1.0), together with PLS-DA regression coefficients, were used only for feature ranking and not as stand-alone evidence of significance. For the metabolomics subset, pathway contextualization was performed in MetaboAnalyst using KEGG; pathway impact plots combine effect and adjusted p-values and are presented as exploratory summaries consistent with the pooled, bull-level design.

Network analysis

Protein–protein interaction (PPI) topologies were delineated via STRING version 12.0 (https://string-db.org). Discriminative entities emerging from multivariate analyses were uploaded through their UniProt accession identifiers, constrained to the Bos taurus taxon. Complete interaction modalities were leveraged, encompassing experimental assays, manually curated repositories, co-expression data, and textual annotations. Graph-theoretical descriptors encompassed node and edge counts, mean node degree, and the global clustering coefficient. Network significance was gauged through STRING-provided enrichment probabilities, thus determining whether the degree of interconnectivity appreciably surpassed stochastic baselines. Independent subnetworks were computed for seminal plasma- and spermatozoa-purified proteomes. Nodes classified as centrality hubs or as structural bottlenecks were cross-referenced against their statistical prominence (assessed via variableimportance in projection (VIP) scores, coefficients, and false-discovery rates) facilitating the synthesis of systems-level interconnectivity with empirical statistical thresholds.

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Results

Proteomic analysis

Principal component analysis (PCA)

A data-driven approach employing unsupervised principal component analysis (PCA) (Figure 1) was applied to delineate the overarching architecture of the proteomic corpus. At the level of the scores plot, a decisive partition was manifest between plasma and sperm fractions. Principal component one (PC1) captured the largest share of variance (≈99%), whereas PC2 explained <1%. The scores plot shows clear fraction-level separation between seminal plasma and spermatozoa, with plasma localized toward negative PC1 and sperm toward positive PC1. This indicates that the fraction-specific molecular predominantly with PC1(Fu et al., 2019).

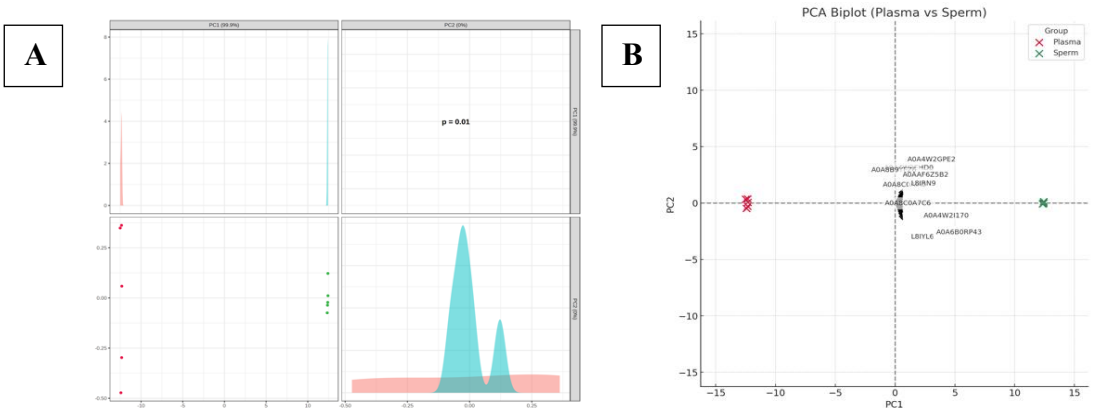

Figure-1. Principal Component Analysis (PCA) of seminal plasma and spermatozoa proteomes. (A) Score plot shows clear separation between seminal plasma (red) and spermatozoa (green) along PC1. (B) Biplot displays the top proteins contributing to this separation.

Partial least squares discriminant analysis (PLS-DA)

A detailed partial least squares discriminant analysis (PLS-DA) model was constructed to enhance visualisation and to identify the most informative variables (refer to Figure 2). The analysis corroborated the earlier principal component analysis (PCA) findings: component 1 (C1) explained 99.9% of the total variance, thereby completely differentiating seminal plasma from spermatozoa. The subsequent component (C2) explained merely 0.1% of the variance, confirming its negligible role in discriminant capability. Classification was unequivocal; all experimental replicates were clearly allocated to their respective groups with no observed overlap. Examination of the loading plots revealed extensive dispersion of relative weights for a limited subset of proteins along C1, identifying these proteins as the principal determinants of the observed separation (Fu et al., 2019).

The integrity of the PLS-DA model was established via complementary methodologies: cross-validation and permutation testing. Cross-validation yielded Accuracy = 1.0, $R^2 = 1.0$ and $Q^2 = 1.0$, metrics indicative of both near-ideal model fit and substantial predictive validity. These metrics remained consistent across higher-order components, thereby demonstrating that the classification achieved in Component 1 did not arise from model overfitting. Concurrently, a permutation procedure involving 1,000 iterations was executed, producing a p-value of 0.01; this statistic situated the observed metric well beyond the empirical 95th percentile of the null distribution, thereby substantiating the conclusion that the observed distinction between seminal plasma and spermatozoa is statistically robust and improbable to stem from random fluctuation (Fu et al., 2019).

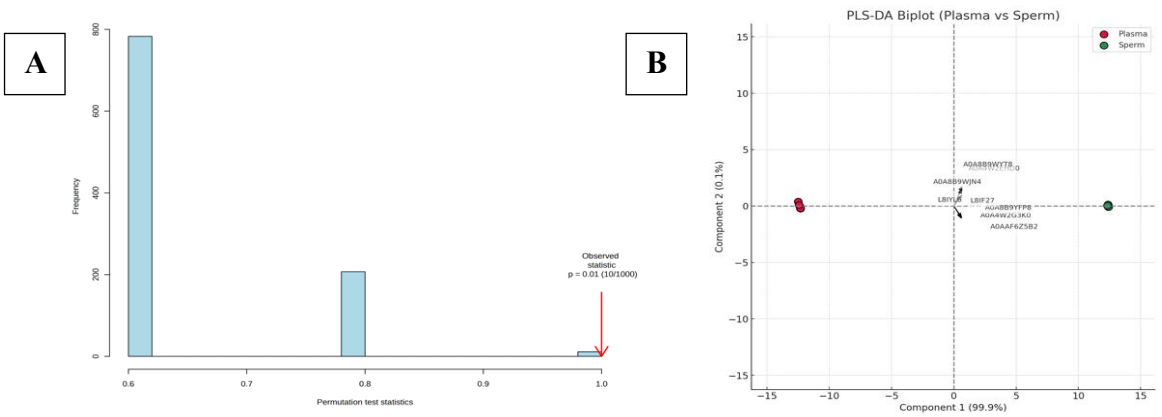

Figure-2. Partial Least Squares–Discriminant Analysis (PLS-DA) of seminal plasma and spermatozoa proteomes. (A) Permutation testing confirmed the robustness of the PLS-DA model (p = 0.01). (B) The PLS-DA biplot shows clear separation between plasma (red) and spermatozoa (green), with the main discriminant proteins indicated.

Univariate differential testing

Independent univariate testing accompanied the multivariate models, employing t-tests subject to false discovery rate (FDR) adjustment on each analyzed protein. Observed effects were noted to be exceedingly pronounced across the protein panel, with absolute t-statistics routinely surpassing the threshold of 100, associated p-values consistently well below 1×10^{-13}, and corrected FDR estimates staying consistently below 1×10^{-12}. The observed effects were observed to be very strong with corrected FDR consistently remaining below 1×10^{-12}, and associated p-values consistently well below 1×10^{-13}, with t-statistics routinely above the threshold of 100. These results confirm that the primary element of the group difference is found in significant protein-level signals, supporting the pronounced clustering shown in both principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA). A simultaneous analysis of univariate significance and multivariate importance metrics showed that highly congruent profiles, supporting the observed biological differentiation across the cohorts, and confirming that the discriminatory proteins found in the models have both statistical and putative biological significance (Fu et al., 2019; Li et al., 2023).

Variable importance in projection (VIP) and coefficients

Integration of variable importance in projection (VIP) scores with partial least squares-discriminant analysis (PLS-DA) coefficients (Figure 3) yielded refined rankings of discriminatory proteins. Proteins exhibiting VIP scores exceeding 1.0 together with pronounced absolute coefficient magnitudes were categorically regarded as highly discriminant, with coefficient polarity elucidating functional enrichment. Negative coefficients designated seminal plasma enrichment, whereas positive coefficients indicated enrichment in spermatozoa. Among seminal plasma proteins, several repeatedly occupied the apex of discriminant rankings. Pre-eminent amongst these was seminal plasma protein PDC-109 (P02784), manifesting markedly elevated VIP scores, pronounced negative coefficients, and t-test p-values achieving false discovery rate (FDR) thresholds below 1×10^{-13}. These concordant metrics unequivocally identify PDC-109 as the definitive plasma fraction biomarker.

Supplementary plasma markers were the tubulin alpha chain (A0AAF6Z5B2), the EF-hand domain structural protein (A0A8B9WYT8), and two isoforms of phosphopyruvate hydratase (enolase: A0A4W2EHD0 and A0A4W2CSG1), each exhibiting negative fold-change ratios, elevated VIP scores, and t-test-derived p-values within the same stringent FDR range. Additionally, the voltage-dependent anion channel isoform 2 (VDAC2; A0A4W2EB14) evidenced plasma enrichment, as did two isoforms of cytochrome c oxidase subunit 5A, mitochondrial (L8IRN9 and A0A8B9WJN4). These later proteomic annotations emphasize the mitochondrial and metabolic context of seminal plasma proteomes, corroborating the penetration of organellar constituents into the extracellular fluid. Although putatively associated with the sperm acrosome, the SP-10 isoform X2

(A0A6P5AZX4) demonstrates enrichment on the plasma membrane exterior, suggesting a mechanism for its shed release into the surrounding extracellular milieu (Pessoa et al., 2023).

Spermatozoa analyses revealed pronounced enrichment of structural and mitochondrial proteins. The most conspicuous discriminants were the non-crystallin proteins, specifically cylicin 1 (A0A8C0AC17) and cylicin 2 (F1MI94). These proteins, both residing within the perinuclear theca, displayed markedly high positive loadings, VIP scores exceeding 1.5, and stringent FDR thresholds of $< 10^{-12}$, confirming their pivotal roles in stabilising sperm head architecture. The mitochondrial fraction was largely indexed by cytochrome c oxidase subunit 5B (L8IV97) and the glycolytic regulatory protein, triosephosphate isomerase (A0A8B9YFZ6). Each of these proteins not only exhibited enrichment within the spermatozoa but also attained significance thresholds indicative of profound biological effect, reinforcing the notion of energetically demanding structure maintenance prior to oocyte interaction (Pessoa et al., 2023).

Spermatozoa presented a noteworthy enrichment of proteins directly associated with flagellar architecture and rhythmic activity. Among these, A-kinase anchoring protein 3 (AKAP3, L8IF27) emerged as a principal sperm-specific marker, underlining its established duty of approximating protein kinase A to the length of the flagellum. A concomitant enrichment was detected for tektins (L8IYL6 and A0A6P5BPQ1), fundamental to the conformation of the axoneme, alongside outer dense fiber protein 1 (A0A8C0A7C6), a protein endowing lateral rigidity to the flagellar canvas. A more expansive set of discriminating entities comprised sperm equatorial segment protein 1 (A0A8C0AIA2), directly implicated in mediating sperm–oocyte interaction, a smaller ribonuclease A-domain–containing peptide (A0A4W2I1Z1), alongside a representative of nuclear constituents, MENT (A0A6B0S904), each exhibiting pronounced and statistically significant spermatozoa-centric accumulation (Pessoa et al., 2023).

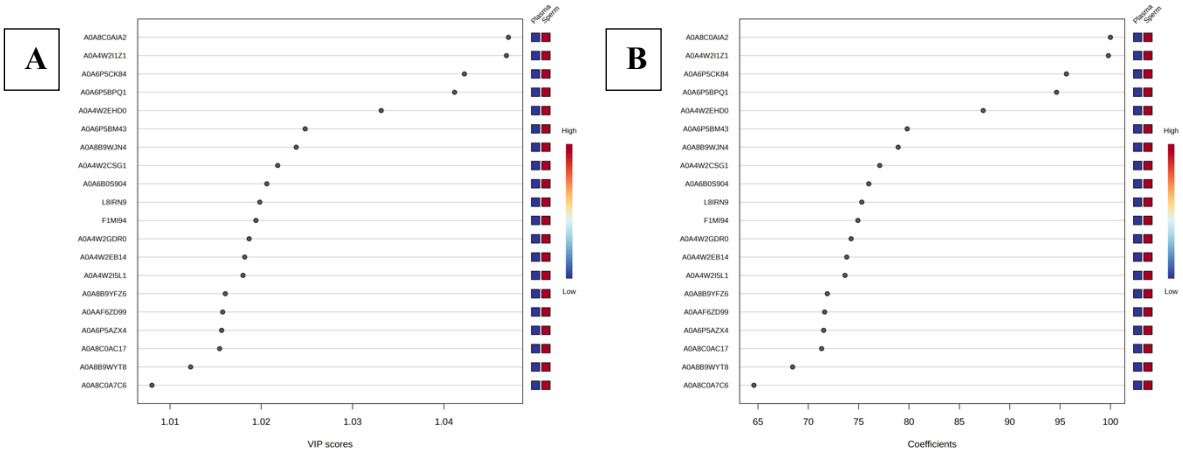

Figure-3. Variable Importance in Projection (VIP) scores and PLS-DA coefficients for discriminant proteins. VIP scores (A) and PLS-DA coefficients (B) highlight the proteins most responsible for the separation between seminal plasma and spermatozoa. Color gradients indicate relative abundance across groups, with red representing higher levels and blue representing lower levels.

Comparative STRING analysis

Protein–protein interaction (PPI) networks (Figure 4) were created for seminal plasma–enriched and spermatozoa-enriched proteins through STRING. For the seminal plasma network, STRING provided 7 nodes and 7 edges (two less than expected by chance). The mean node degree was 2.0 and the mean local clustering coefficient was 0.619, p = 0.0119 (PPI enrichment). This suggests that seminal plasma proteins are interconnected to a greater extent than expected, creating a moderately clustered network. The central network was formed by VDAC2, COX5A and ENO2, connecting mitochondrial respiration, glycolysis and transport of metabolites. Leaf/branch tips were tubilin (TUBA1B) as well as EFHD1, and

BSP1 (PDC-109) and ACRV1 (SP-10) were more distal to effect pathways (Tirpák et al., 2022).

STRING found 9 nodes and 5 edges for the spermatozoa network, and no edges would be expected by chance. Average node degree was 1.11, average clustering coefficient was 0.593, and the PPI enrichment was $p = 2.95 \times 10^{-5}$. This greater enrichment emphasizes a biochemically meaningful module. The network centered on AKAP3, which partnered with ODF1, TEKT1, and SPESP1 to form a signaling–structural axis. Cylicin 1 and cylicin 2, as well as ODF1 and TEKT1, composite metabolic proteins (COX5B, TPI1), and RNASE1, and energy and RNA regulation, respectively, were tightly coupled (Tirpák et al., 2022).

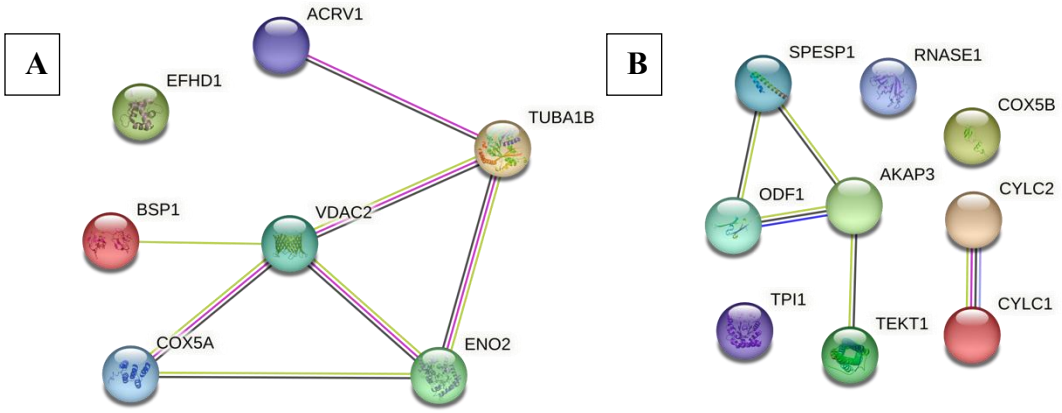

Figure-4. Protein–protein interaction (PPI) networks of discriminant proteins identified in seminal plasma and spermatozoa. (A) Network of seminal plasma–enriched proteins (B) Network of spermatozoa–enriched proteins.

Metabolomic analysis

Principal component analysis (PCA)

The PCA shown in Figure 5 revealed that the metabolomic dataset is highly reproducible and dramatically separated seminal plasma from spermatozoa. The first principal component explained 88.5% of the variance, whereas PC2 represented only 1.7% of the variance. This suggests that a single axis of variation conveys approximately all of the metabolic differences across these compartments. Furthermore, the scores plot indicates that spermatozoa are distributed on the positive side of PC1, whereas plasma samples are categorised on the negative side. These metabolic fingerprints are accurate, demonstrated by the distinct difference between the two groups and the little distribution of biological replicates within each group. The significance of this classification was verified by a statistical test based on permutations ($p = 0.014$) (Fu et al., 2019).

The loading graph demonstrated exactly how several metabolites contributed to the separation. Choline, the major electrolyte metabolism osmolyte; S-methylthioglycolate, a sulphurous thiol redox metabolite; and 5-[(phenylsulfonyl)methyl]-2-furoic acid, a sulfide-containing compound with the potential antioxidant properties, were among the distinguishing characteristics in plasma. Those compounds confirm the claim that plasma functions as a stabilising and protective extracellular matrix. 1-[3-azido-5-O-(12-azidododecanoyl)-2,3-dideoxy-D-glucopyranosyl], a potential glycolipid indicative of extracellular lipid signalling, is one of the atypical derivatives found in plasma (Li et al., 2023).

On the contrary, compounds directly related to energy metabolism and structural maintenance were correlated with sperm cells. Pantothenic acid, known as Vitamin B5, was a discriminant metabolite, supporting the importance of mitochondrial activity in sperm, as it is an essential precursor for coenzyme A. Mitochondrial respiration was additionally underlined by succinic semialdehyde, a GABA-shunt intermediary which contributes to the TCA cycle. Whereas the detection of a tetrapeptide (L-valyl-L-isoleucyl-L-alanyl-L-lysine) revealed a continuing proteolytic turnover process inside these cell types, enrichment of PHHdiA-PE (a phosphatidylethanolamine derivative) indicated membrane remodelling in processes that might rupture sperm cells. Subsequently, 6-amino-2,4-diimino-5-

nitro-1,3-pyrimidine, a pyrimidine derivative, indicated nucleotide metabolism that may be directly or indirectly related to DNA stability and the acrosome response (Tirpák et al., 2022).

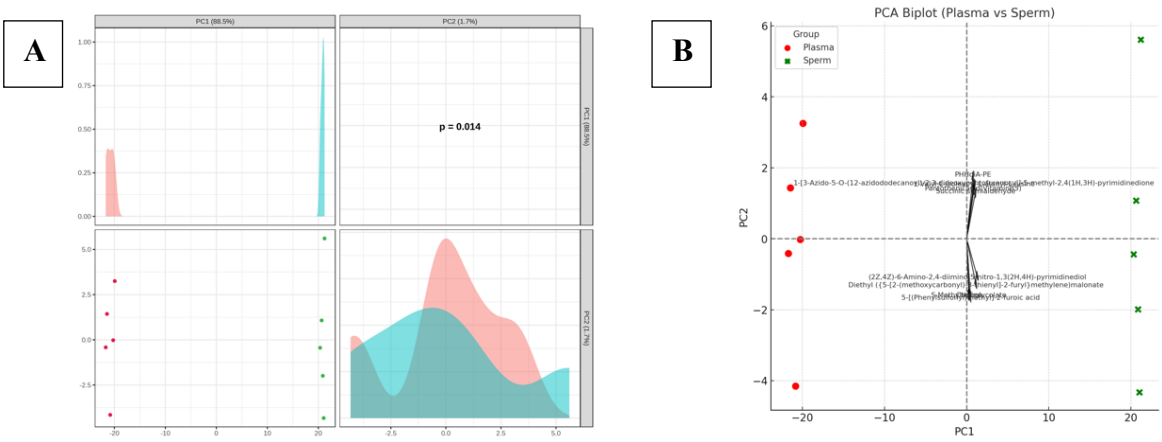

Figure-5. Principal Component Analysis (PCA) of metabolomic profiles in seminal plasma and spermatozoa. (A) Pairwise score plot and density distribution showing clear separation between seminal plasma (red) and spermatozoa (green) along PC1, which explains the majority of variance ($p < 0.05$). (B) PCA biplot illustrating group clustering and the top discriminant metabolites driving separation between seminal plasma and spermatozoa.

Partial least squares discriminant analysis (PLS-DA)

Principal Components Analysis (PCA) dimensional decomposition provided an initial unsupervised view of dataset variance, but supervised Projection to Latent Structures-Discriminant Analysis (PLS-DA) (Figure 6) significantly improved group characterisation by plasma and spermatozoa. A single latent component (Component 1) was adequate to yield complete discrimination of the two sample cohorts, with leave-one-out cross-validation demonstrating unity across metrics of accuracy, variance explained (R^2), and predictive capability (Q^2; all equal to 1.0). The stability of the resulting predictive model was affirmed by permuted evaluation, with 1,000 iterations returning a statistical threshold ($p = 0.007$), thereby excluding hypotheses of spurious fitting.

The partial least squares discriminant analysis (PLS-DA) loadings corroborated the discriminative metabolites previously identified by principal component analysis (PCA) while reordering them according to their capacity to enhance inter-class separation. Within plasma samples, metabolites determined to exert the greatest influence included choline, S-methylthioglycolate, and variants of antioxidant-like aromatic compounds. Analysis of sperm-derived samples revealed a differing phenotype, wherein the strongest loadings were attributable to pantothenic acid, succinic semialdehyde, and various molecular forms of phosphatidylethanolamine, coupled with contributory signals from short tetrapeptides and pyrimidine derivatives, all of minor magnitude in comparison.

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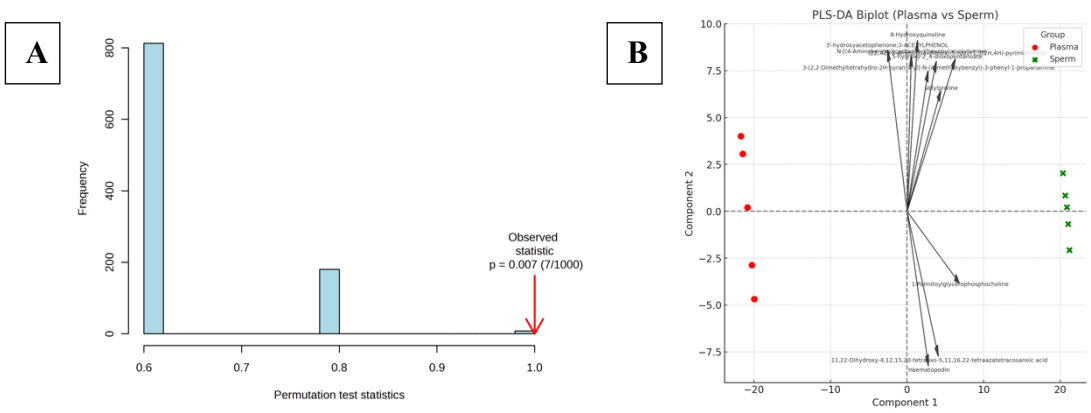

Figure-6. Partial Least Squares Discriminant Analysis (PLS-DA) of metabolomic profiles in seminal plasma and spermatozoa. (A) Permutation test (B) PLS-DA biplot showing clear separation between seminal plasma (red) and spermatozoa (green).

Variable importance in projection (VIP) and coefficient analysis

Variable Importance in Projection (VIP) scores and coefficient weighting from the Projection to Latent Structures Discriminant Analysis (PLS-DA) model afforded an additional layer of refinement in the identification of differentially abundant metabolites distinguishing seminal plasma from spermatozoa (Figure 7). Within the plasma compartment, metabolites exhibiting consistently elevated VIP scores and pronounced positive regression coefficients were identified. A prominent component, 1-palmitoylglycine, emerged as the foremost contributor; its identification reinforces the notion that seminal plasma participates in lipid signaling and regulates fatty acid-derived bioactive pools. Concomitantly, a (2Z,4Z)-6-amino-2,4-hexadienoate analogue, structurally associated with the pyrimidine biosynthetic pathway, together with N-acetylanthranilate demonstrated significant enrichment in the plasma compartment. Both metabolites are previously documented to serve antioxidant and metabolic buffering functions, thereby providing additional corroboration that seminal plasma operates as a physicochemically stable extracellular microenvironment, buffering against oxidative and metabolic perturbation (Tirpák et al., 2022; Pessoa et al., 2023).

The plasma analyzed here was additionally enriched in L-ergothioneine, a thiol-antioxidant acquired exclusively through diet, whose role in safeguarding cellular structures from oxidative insult is well documented. Co-occurring in significant concentrations were stearamide, a long-chain fatty acyl amine implicated in membrane stabilization and intercellular signaling, and deoxyloganin, an iridoid glycoside noted for attenuating pro-inflammatory pathways. The three compounds collectively accentuate the seminal plasma's capacity to furnish an oxidative, modulatory, and homeostatic milieu, thereby contributing to the preservation and optimal functioning of gametes, seminal vesicle-derived structures, and adjacent somatic tissues (Tirpák et al., 2022).

In contrast, spermatozoa exhibited a marked enrichment profile, primarily comprising metabolites associated with energetic, nucleotide, and membrane pathways. The nucleotide metabolite inosine surfaced as a dominant element, serving as an indicator of ATP hydrolysis and simultaneously exerting a cytoprotective role through scavenging of reactive oxygen species. The observation of succinic semialdehyde, an intermediate of the gamma-aminobutyric acid (GABA) shunt funneling into the tricarboxylic acid (TCA) cycle, substantively reinforced the hypothesis that mitochondrial respiration critically supports spermatozoal mechanical performance. The parallel accumulation of citramalic acid, another TCA-borne derivative, substantiates the metabolic reprogramming that equips the sperm for extended progressive motility. Phosphatidylcholine derivatives [PC(15:1GZ)/0:0)] were recovered as lipid species indicative of membrane restructuring, underscoring their role in regulating fluidity and viscoelastic response through the capacitation and acrosome reaction phases. Conclusive discrimination was achieved with the

identification of 1-(2-methoxy-5H-pyrrol-2-yl) nucleoside surrogates and diethyl-substituted dicarboxylic acids, metabolites that likely modulate oxidative equilibria and contribute to the intricate balancing of sperm nitrogen metabolism (Tirpák et al., 2022).

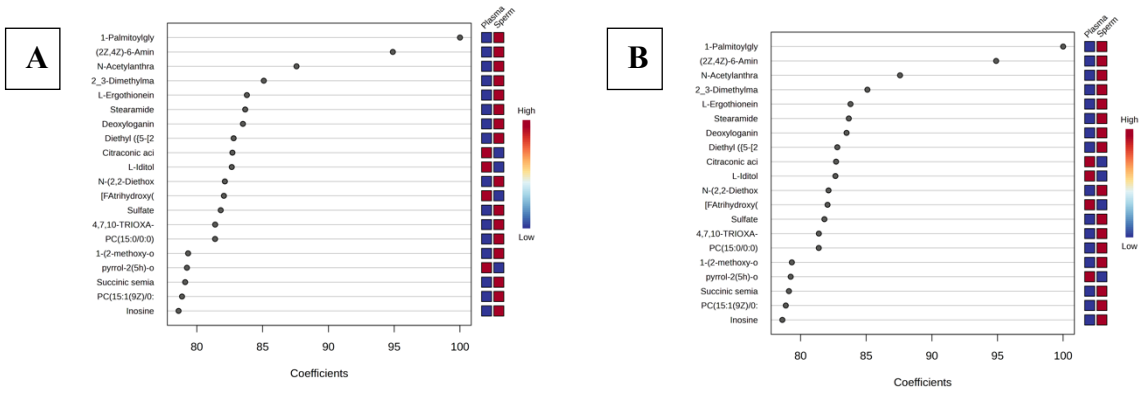

Figure-7. Variable Importance in Projection (VIP) scores and regression coefficients from PLS-DA of seminal plasma and spermatozoa metabolomes. (A) VIP score ranking of the top discriminant metabolites, highlighting those with the greatest contribution to group separation (VIP > 1.0). (B) PLS-DA regression coefficients indicating the direction and magnitude of metabolite enrichment between seminal plasma (red) and spermatozoa (blue).

Univariate analysis (t-test)

To provide further validation of the multivariate outcomes, independent univariate t-tests were performed, quantifying the abundance of discriminant metabolites between seminal plasma and spermatozoa. The metabolites identified as discriminants in the PCA and PLS-DA analyses displayed consistently low p-values in the t-tests, reinforcing their status as biologically relevant, rather than spurious, findings. Within seminal plasma, 1-palmitoylglycine, N-acetylanthranilate, and L-ergothioneine showed significantly higher concentrations than in spermatozoa ($p < 0.05$, after FDR adjustment), thereby substantiating the earlier multivariate interpretations regarding an enrichment of lipid-derived conjugates and endogenous antioxidants. Moreover, the exact magnitude of the t-test differences validated the discriminative ability of stearamide and deoxyloganin, which were already identified in the VIP plots as potential plasma biomarkers (Tirpák et al., 2022; Pessoa et al., 2023).

The measured levels of inosine, succinic semialdehyde, and citramalic acid in the sperm metabolites observed to be significantly higher than those in plasma. The presence of these metabolites, in particular as concentrated energy and nucleotide metabolic metabolites in spermatozoa, is strongly supported by their validation using t-test statistical analysis, and they appeared as significant features in both PCA and PLS-DA. In addition, derivatives of phosphatidylcholine, including PC (15:1GZ)/0:0 and others, also achieved statistical significance, confirming their function in the remodelling and specialisation of sperm membranes.

Pathway enrichment analysis

We highlighted the biochemical disparities between seminal plasma and spermatozoa at the systems level by combining PCA, PLS-DA, VIP, univariate analysis, and pathway enrichment studies. Metabolites such 1-palmitoylglycine, N-acetylanthranilate, L-ergothioneine, and stearamide, which have strong positive coefficients and a discriminant power (> 4.0 VIP in the PLS-DA model), are linked to and fall within the lipid biosynthesis and redox balancing pathways in seminal plasma. Enrichment analysis identified bile acid biosynthesis ($p = 0.0119$, impact = 0.045), taurine and hypotaurine metabolism ($p = 0.0306$, impact = 0.429), and GPI-anchored protein biosynthesis ($p = 0.1179$, impact = 0.040) as the most representative pathways. These findings are in line with the PCA biplot that demonstrated the predominance of certain metabolites associated with plasma samples, which strongly separated along PC1 and PC2 (Tirpák et al., 2022).

Furthermore, the observed enrichment of glycerophospholipid metabolism coupled with unsaturated fatty acid biosynthesis corroborates the

prominent role of the identified fatty acyl derivatives (palmitoylglycine and stearamide) within the variable importance in projection (VIP) analysis. Within the spermatozoa, the metabolites that exerted the greatest influence on partial least squares discriminant analysis (PLS-DA) spatial separation comprised inosine, succinic semialdehyde, citraconic acid, and derivatives of butanoate. These metabolites corroborate subsequent pathway enrichment analysis (Table 1) via the tricarboxylic acid (TCA) cycle (p = 2.1E-04, false discovery rate [FDR] = 0.0128, impact = 0.132), pyruvate metabolism (p = 3.2E-04, FDR = 0.0128, impact = 0.028), and metabolism of alanine, aspartate, and glutamate (p = 5.8E-04, FDR = 0.0156, impact = 0.099). complementarily, additional pathways were implicated, notably arginine biosynthesis, butanoate metabolism, glycolysis/gluconeogenesis, and glycerolipid metabolism. These pathways exhibit direct biochemical connectivity with the observed proteomic enrichment of specific subunits of cytochrome c oxidase (COX5A, COX5B), triosephosphate isomerase (TPI1), and A-kinase anchor protein 3 (AKAP3), which coalesce to form the energetic and structural scaffolding requisite for flagellar motility. The pronounced uniform clustering manifested in both principal component analysis (PCA) and PLS-DA score plots substantiate this biochemical convergence focused on energy generation, corroborative of the spermatozoa's acknowledged role in fertilization and subsequent intracellular dynamics (Tirpák et al., 2022).

If we look at the data as a whole, this pathway analysis has a pattern similar to that found by the multivariate model. The presence of the PDC-109 protein, which is an EF-hand calcium-binding protein, and the same extracellular form of enolase corresponds to the main pathway in plasma, which is related to maintaining lipid stability, protecting against oxidative stress, and sending signals outside the cell. Then, the discovery of more cyclicin, tektin, and outer dense fibers is apparently related to processes related to sperm that focus on several pathways: energy production through the TCA cycle, glycolysis, and pyruvate metabolism, then the management of amino acids such as arginine and glutamate, and changes in cell membrane structure through glycerolipid metabolism. We can conclude that based on proteomic and metabolomic results from seminal plasma and sperm indicate that they are metabolically complementary (Tirpák et al., 2022).

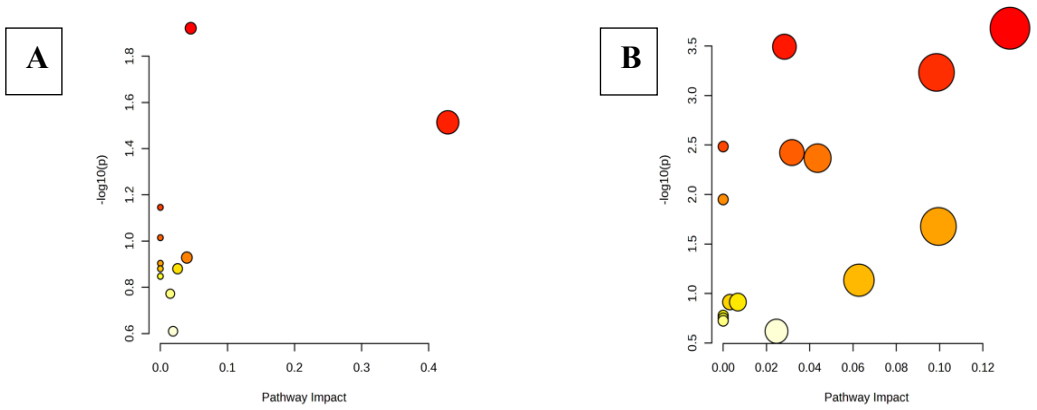

Figure-8. Pathway enrichment analysis of metabolites in seminal plasma and spermatozoa. (A) Significantly enriched pathways in seminal plasma, with bubble size representing pathway impact and color intensity indicating –log10(p) significance. (B) Significantly enriched pathways in spermatozoa, showing stronger clustering of energy metabolism, amino acid metabolism, and lipid remodeling pathways.

Table-1. Summary of significantly enriched metabolic pathways.

Compartment	Pathway	p-value	–log10(p)	FDR	Pathway Impact
Plasma	Primary bile acid biosynthesis	0.0119	1.9209	0.9598	0.0452
Plasma	Taurine and hypotaurine metabolism	0.0306	1.5137	1.0000	0.4286
Plasma	Ubiquinone and other terpenoid-quinone biosynthesis	0.0715	1.1458	1.0000	0.0000
Plasma	One carbon pool by folate	0.0967	1.0145	1.0000	0.0000
Plasma	Glycosylphosphatidylinositol (GPI)-anchor biosynthesis	0.1179	0.9285	1.0000	0.0395
Plasma	Glycine, serine and threonine metabolism	0.1249	0.9035	1.0000	0.0000
Plasma	Biosynthesis of unsaturated fatty acids	0.1318	0.8801	1.0000	0.0000
Plasma	Glycerophospholipid metabolism	0.1318	0.8801	1.0000	0.0257
Plasma	Fatty acid elongation	0.1421	0.8475	1.0000	0.0000
Plasma	Fatty acid degradation	0.1421	0.8475	1.0000	0.0000
Plasma	Fatty acid biosynthesis	0.1690	0.7720	1.0000	0.0147
Plasma	Purine metabolism	0.2457	0.6096	1.0000	0.0189
Sperm	Citrate cycle (TCA cycle)	2.09E-4	3.6802	0.0128	0.1325
Sperm	Pyruvate metabolism	3.21×10^{4}	3.4934	0.0128	0.0283
Sperm	Alanine, aspartate and glutamate metabolism	5.84×10^{4}	3.2337	0.0156	0.0986
Sperm	Arginine biosynthesis	0.0033	2.4845	0.0577	0.0000
Sperm	Butanoate metabolism	0.0037	2.4237	0.0577	0.0318
Sperm	Glycerolipid metabolism	0.0043	2.3674	0.0577	0.0436
Sperm	Glycolysis or Gluconeogenesis	0.0112	1.9497	0.1283	0.0000
Sperm	Glycerophospholipid metabolism	0.0210	1.6773	1.0000	0.0994
Sperm	Purine metabolism	0.0735	1.1339	1.0000	0.0627
Sperm	Fructose and mannose metabolism	0.1222	0.9129	1.0000	0.0031
Sperm	Pantothenate and CoA biosynthesis	0.1222	0.9129	1.0000	0.0068
Sperm	Lipoic acid metabolism	0.1672	0.7768	1.0000	0.0000
Sperm	Inositol phosphate metabolism	0.1781	0.7493	1.0000	0.0000
Sperm	Glyoxylate and dicarboxylate metabolism	0.1889	0.7238	1.0000	0.0000
Sperm	Tyrosine metabolism	0.2409	0.6181	1.0000	0.0246

Disucussion

Our findings indicate a clear division of labor between bovine seminal plasma and spermatozoa that directly supports fertilization. Seminal plasma provides the nourishing and stabilizing extracellular milieu that helps time capacitation, whereas spermatozoa concentrate the structural and enzymatic machinery for movement, efficient energy delivery, and a properly executed acrosome reaction (Swamy, 2004). Taken together, the integrated proteomic–metabolomic signal suggests these compartments are not merely different but complementary along the pathway to fertilization.

Proteomic evidence shows that seminal plasma is an active regulatory compartment that contributes to energy balance, redox control, and ionic buffering while carrying and modulating enzymes and channels. PDC-109 (BSP1) stands out as a membrane-binding protein that promotes controlled efflux of cholesterol and specific phospholipids, initiating membrane remodeling that tunes the timing of capacitation stabilizing membranes during early transit in the female tract and priming responsiveness to zona pellucida cues before the acrosome reaction. Additional factors such as VDAC2, SP-10, and enolase align with redox and membrane-dynamics control, and pathway maps support a modular organization integrating metabolic and fertility regulators to support sperm maturation. On the sperm side, proteomes emphasize integration and specialization: axonemal scaffolds (e.g., tektins, outer dense fibers) and

energy enzymes (COX5B, TPI1) form a coupled locomotion and ATP-supply system, while AKAP3 anchors PKA signaling along the principal piece to coordinate phosphorylation-driven hyperactivation with local ATP availability from glycolysis and mitochondrial OXPHOS. STRING visualizations illustrate these modules (Alghamdi et al., 2009; Iskandar et al., 2022).

Metabolomics corroborates this division of labor. In seminal plasma, taurine acts as an osmolyte and small-molecule antioxidant that quenches reactive oxygen species and helps preserve membrane fluidity; together with ergothioneine, it creates a "redox buffer zone" that protects sperm lipids during storage and transport and supports acrosomal integrity under osmotic/thermal stress. In spermatozoa, enrichment of the TCA cycle, glycolysis, and amino acid metabolism is accompanied by metabolites such as citrate, inosine, and phosphatidylcholine species. Citrate functions both as a pH buffer and a TCA-cycle hub maintaining a microenvironmental pH conducive to capacitation signaling while indicating ATP-generating capacity and flexible substrate switching among β-oxidation, glycolysis, and OXPHOS. Inosine supports purine salvage and can provide rapid energy with cytoprotective effects under oxidative stress, consistent with the high ATP demand of hyperactivated motility. Together, the data support a two-compartment working model: seminal plasma maintains sperm in a protected standby state until entry into the female tract, after which the sperm's internal architecture anchored signaling (AKAP3), axonemal/cytoskeletal elements, and energy enzymes drives efficient motility, on-time capacitation, and acrosome readiness.

These biology-first interpretations suggest a practical path forward. A composite panel that combines functionally coherent markers higher PDC-109 and AKAP3 together with protective-range taurine and citrate, alongside inosine may yield a semen-quality score that outperforms single markers for bull selection, process monitoring (e.g., extenders and handling), and cryopreservation strategies emphasizing redox–membrane stability. Nonetheless, our effective sample size at the bull level is modest (n = 5 per fraction) and we used pooled ejaculates, which reduces statistical power and increases the risk of overfitting in supervised models despite cross-validation and permutation tests; generalizability is therefore uncertain. Larger cohorts with non-pooled samples and an independent validation set tied to prospective fertility outcomes will be essential to establish robustness and clinical utility (Ashwitha et al., 2022; Pessoa et al., 2023).

Conclusion

This study successfully described a comprehensive multiomic profile of bovine semen, providing new insights into the molecular segregation between seminal plasma and spermatozoa. Proteomic analysis showed that seminal plasma contains extracellular proteins directly involved in membrane remodeling, ion regulation, and antioxidant defense. Furthermore, spermatozoa are filled with structural components, signaling anchors, and mitochondrial enzymes, which are crucial for motility and fertilization. Furthermore, the results of the metabolomic analysis corroborate the segregation findings from previous proteomic concepts, where lipid conjugates and antioxidant metabolites were most abundant in seminal plasma. Meanwhile, spermatozoa exhibited TCA cycle intermediates and phospholipids associated with membrane dynamics. Our research demonstrates that seminal plasma and spermatozoa function as two interdependent compartments within the fertility system. Plasma can act as a protective and regulatory buffer, while spermatozoa can form units optimized for fertilization.

Acknowledgments

The authors would like to thank the Ministry of Research, Technology, and Higher Education (Kemristekdikti), Republic of Indonesia, through the BIMA Program for funding support under contract number 02209/UN4.22/PT.01.03/2025.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: This research was financially suppported by Ministry of Research, Technology and Higher Education (Kemristekdikti), Republic of Indonesia, through the BIMA Program for funding support under contract number 02209/UN4.22/PT.01.03/2025.

Contribution of Authors

Sahiruddin S, Yusuf M, Wibowo S & Diansyah AM: Conceived idea, designed the experiment and interpreted data.

Sahiruddin S, Yusuf M, Masturi M & Diansyah AM: Performed the experimental procedures, collected and analyzed data.

Herdis H, Maulana T & Wibowo S: Supervised and coordinated the research, provided clinical data and edited the manuscript.

Diansyah AM, Maulana T & Wibowo S: Conducted statistical analysis and data interpretation.
Sahiruddin S, Yusuf M & Diansyah AM: Prepared the initial draft of the manuscript and interpreted data.

All authors critically reviewed and approved the final draft of the manuscript.

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