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RESEARCH ARTICLE

# Metabolomic Characterization of Seminal Plasma Associated with Sperm Motility in Indigenous Aceh Bulls

H. Hafizuddin[1,9*], H. Husnurrizal[1], Tongku N. Siregar[1], G. Gholib[2], Arman Sayuti[3], Y. Yusmadi[4,5,] Mitha Kurnia Sari[5], Tulus Maulana[6], Rusli Fidriyanto[6], Muhammad Fajar Amrullah[6], Abdullah Baharun[7] and Mohammed Ahmed Elsharef Abdelbagi[8]

[1]Laboratory of Reproduction, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia; [2]Laboratory of Physiology, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia; [3]Laboratory of Clinics, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh 23111, Indonesia; [4]Departement of Animal Science, Faculty of Agriculture, Universitas Almuslim, Bireuen 24261, Indonesia; [5]Indrapuri Livestock Breeding and Forage Centre (BPTU-HPT), Aceh Besar 23363, Indonesia; [6]Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Cibinong 16911, West Java, Indonesia; [7]Department of Animal Science, Faculty of Agriculture, Djuanda University, Ciawi 16720, West Java, Indonesia; [8]Faculty of Animal Production, University of Khartoum, Khartoum 11115, Sudan; [9]Research Center for Innovation and Feed Technology, Universitas Syiah Kuala, Banda Aceh, Indonesia.
*Corresponding author: hafizuddin_umar@usk.ac.id

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**ABSTRACT**

Seminal plasma contains various metabolites that contribute to sperm function, including energy metabolism, redox homeostasis, and membrane stability. However, information regarding seminal plasma metabolomic profiles associated with sperm motility in tropical indigenous cattle remains limited. This study aimed to characterize the seminal plasma metabolome of Aceh bulls and explore metabolic differences associated with sperm motility. Seminal plasma samples were collected from ten adult Aceh bulls and categorized into high- and low-motility groups based on semen quality evaluation. Metabolomic profiling was performed using gas chromatography–mass spectrometry (GC-MS), followed by multivariate and univariate analyses as well as metabolic pathway enrichment analysis. A total of 63 metabolites were identified, predominantly comprising amino acids, organic acids, carbohydrates, and lipid-related compounds. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) indicated differences in metabolomic profiles between the two motility groups, with partial separation observed between groups. Several metabolites, including phenylpyruvic acid, L-alanine, L-valine, L-aspartic acid, L-glutamic acid, glycerol, and D-fructose, contributed to the observed variation between groups. Pathway analysis indicated the involvement of phenylalanine metabolism, phenylalanine–tyrosine–tryptophan biosynthesis, glycolysis/gluconeogenesis, pyruvate metabolism, and galactose metabolism. These pathways may be associated with cellular energy production, mitochondrial activity, and oxidative stress regulation, which are important for sperm function and motility. Overall, the findings indicate differences in seminal plasma metabolite composition between Aceh bulls with contrasting sperm motility levels. Although exploratory in nature and limited by sample size, this study provides preliminary information that may contribute to future investigations on reproductive physiology and semen quality in tropical local cattle.

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## INTRODUCTION

Artificial insemination (AI) is a cornerstone reproductive biotechnology in modern cattle breeding systems, including in indigenous breeds such as the Aceh bull (Selvaraju *et al*., 2018; Hafizuddin *et al*., 2024). Consequently, bull fertility is a critical determinant of efficient, profitable, and sustainable cattle production (Memili *et al*., 2020). The ability to predict bull fertility in

advance offers substantial economic benefits by improving pregnancy rates and optimizing genetic dissemination. In recent years, omics-based approaches including genomics, transcriptomics, proteomics, and metabolomics have been increasingly applied to identify molecular determinants of bull fertility (Saputra *et al*., 2025). Accumulating evidence indicates that molecular components present in spermatozoa and seminal plasma are significantly associated with fertility outcomes (Velho *et al*., 2018; Al-Gebouri and Eidan, 2024b). However, the application of such molecular assessment tools remains limited in indigenous cattle raised under tropical conditions, and to date, no metabolomic study has been reported in Aceh bulls.

Breeding soundness evaluation in Aceh bulls still relies predominantly on conventional semen quality assessment, primarily focusing on sperm motility, morphology, and sperm concentrations (Hafizuddin *et al*., 2023; Panjaitan *et al*., 2024). Among these parameters, sperm motility is considered a key determinant of fertilization success, as progressive motility enables spermatozoa to traverse the female reproductive tract, undergo capacitation, and penetrate the oocyte membrane (Kumaresan *et al*., 2019; Küçük *et al*., 2020; Amaral, 2022; Ajeesh *et al*., 2026). Although progressive sperm motility is widely recognized as a key predictor of fertilization success (Utt, 2016; Ibanescu *et al*., 2020), the upstream metabolic signatures in spermatozoa and seminal plasma that shape the motility phenotype have not been comprehensively characterized.

Compared with spermatozoa, seminal plasma contains a broader diversity and higher abundance of metabolites. In addition, this biological fluid, seminal plasma contains various bioactive molecules that protect spermatozoa and contribute to sperm quality, motility, and fertility in bulls (Saputra *et al*., 2025). Despite the identification of numerous seminal plasma metabolites, the functional significance of many small molecules in sperm physiology remains insufficiently characterized (Memili *et al*., 2020). Seminal plasma is a complex biological fluid derived from the testis, epididymis, and accessory sex glands (Moura *et al.,* 2018; Jia *et al.,* 2026). Beyond serving as a transport medium, seminal plasma provides energy substrates, antioxidants, osmotic regulators, and signaling mediators that collectively support mitochondrial function, membrane stability, and redox balance in spermatozoa (Egea *et al.,* 2014; Menezes *et al.,* 2019; Ortiz-Rodríguez *et al.,* 2021; Pannu *et al.,* 2022). Previous studies have shown that seminal plasma components play pivotal roles in regulating sperm motility and fertilization capacity (González-Cadavid *et al.,* 2014; Intasqui *et al.,* 2016; Bezerra *et al.,* 2019). Disruptions in these metabolic systems may impair ATP production and compromise flagellar motion stability, ultimately reducing sperm motility (Goodson *et al.,* 2012; Yi *et al.,* 2012; Ferramosca *et al.,* 2016; Gomes *et al.,* 2020; Prieto *et al.,* 2023). Although several studies have investigated metabolic and protein-related markers associated with semen quality and sperm motility, including studies in bulls and buffaloes, the metabolic basis of sperm motility remains incompletely understood, particularly in indigenous cattle breeds (Velho *et al.,* 2018; Musa and Abdulkareem, 2023; Al-Gebouri and Eidan, 2024a; Musa and Abdulkareem, 2024). Nevertheless, comprehensive metabolomic characterization of seminal plasma in tropical indigenous cattle remains scarce.

Aceh cattle, a native Indonesian breed adapted to hot and humid tropical environments, possess unique physiological and adaptive characteristics that may influence reproductive function. Despite their strategic importance in regional breeding programs, molecular indicators that may support evidence-based reproductive selection in this native population remain limited. Gas chromatography–mass spectrometry (GC-MS)-based metabolomics offers a systems-level approach to elucidate the biochemical networks underlying sperm function by capturing small-molecule profiles related to cellular bioenergetics and redox status. To date, no study has comprehensively characterized seminal plasma metabolomic patterns associated with sperm motility phenotypes in Aceh cattle. Therefore, the objectives of this study were: (i) to conduct untargeted GC-MS-based metabolomic profiling of Aceh cattle seminal plasma; (ii) to compare metabolic patterns between high- and low-motility groups; and (iii) to identify discriminant metabolites and enriched metabolic pathways associated with differences in sperm motility. We hypothesized that bulls exhibiting higher progressive motility would show greater abundance of metabolites related to efficient mitochondrial ATP production and redox homeostasis, whereas bulls with lower motility would display metabolic patterns associated with altered glycolytic activity, impaired mitochondrial function, or oxidative imbalance. Collectively, this study aimed to provide preliminary insights into the seminal plasma metabolites associated with sperm motility in native tropical bulls and to establish a basis for future studies exploring metabolite-associated indicators of reproductive performance. Improving reproductive performance in indigenous cattle is essential not only for enhancing breeding outcomes but also for supporting food security, sustainable livestock systems, and the conservation of locally adapted genetic resources in tropical regions.

## MATERIALS AND METHODS

**Animals and Experimental Design:** A total of ten (10) Aceh bulls (4–6 years old) maintained at the Indrapuri Livestock Breeding and Forage Centre (BPTU-HPT), Ministry of Agriculture, Republic of Indonesia, under standardized management conditions were included in the study. Bulls were clinically healthy and routinely used in semen collection programs. All bulls were clinically healthy and routinely used in semen collection programs. The animals were fed a basal diet consisting of forage and a commercial concentrate formulated for breeding beef bulls (Nutrifeed BC 134 Bull; KJUB Puspetasari, Indonesia), with ad libitum access to drinking water. According to the manufacturer's specifications, the concentrate contained a maximum of 14% moisture, 12% ash, 7% crude fat, and 22% crude fiber, with a minimum crude protein content of 12% and a minimum total digestible nutrient (TDN) value of 65% on a dry matter basis. The concentrate also contained 0.60–1.00% calcium, 0.30–0.60% phosphorus, and a maximum urea content of 2%. The concentrate was formulated from rice bran, wheat

bran, molasses, palm kernel meal, copra meal, corn hominy feed, corn gluten feed (CGF), distillers dried grains with solubles (DDGS), vitamins, and mineral supplements. The concentrate was offered at approximately 3% of body weight per animal per day on a dry matter basis, following the manufacturer's recommendations.

**Semen Collection and Evaluation:** Semen was collected using an artificial vagina. Immediately after collection, ejaculates were evaluated for volume, pH (score), mass motility (score), sperm progressive motility, viability and morphology (eosin–nigrosin staining), and sperm concentration. Only ejaculates meeting minimum quality standards were processed further. Progressive sperm motility was assessed by an experienced evaluator under standardized microscopic conditions. Animals were subsequently assigned to either a high-motility group (≥70% progressive motility; n=5) or a low-motility group (<70% progressive motility; n=5) (Indriastuti *et al.,* 2020; Baharun *et al.,* 2023). The selected thresholds were defined a priori to ensure a clear biological distinction between groups and to minimize overlap in seminal plasma metabolic profiles. A progressive motility of ≥70% is widely regarded as indicative of satisfactory semen quality for breeding purposes, as ejaculates meeting this criterion are routinely selected for cryopreservation and use in artificial insemination programs (Odinius *et al.,* 2024). Furthermore, sperm motility is recognized as one of the most important predictors of male fertility and reproductive performance (Utt, 2016). Therefore, ejaculates with progressive motility ≤70% were considered to represent a biologically distinct low-motility phenotype characterized by reduced sperm functional competence and potentially lower fertilizing capacity. Similar threshold-based grouping strategies have been applied in metabolomic studies investigating associations between seminal plasma metabolites and sperm motility (Jia *et al.,* 2026).

**Seminal Plasma Preparation:** Semen samples were centrifuged at 5,000×g for 10min at 4°C to separate spermatozoa. Supernatant (seminal plasma) was carefully collected and centrifuged again t at 5,000×g for 10min at 4°C to remove residual sperm cells and cellular debris. The clarified seminal plasma was then aliquoted and stored at −80°C until metabolomic analysis (Maulana *et al.,* 2025).

**Gas chromatography-mass spectrometry (GC–MS) Metabolomic Analysis:** A 50µL aliquot of seminal plasma was transferred into a 1.5mL microtube. Methanolic heptadecanoic acid (150µL; 1mg/mL; Sigma-Aldrich, St. Louis, MO, USA) was added as an internal standard, followed by 350µL of extraction solution consisting of purified water and methanol (1:4, v/v). The mixture was vortexed for 1min and centrifuged at 13,000rpm for 20min at 4°C. The supernatant was filtered through a syringe filter and transferred into a clean microtube. Solvent removal was carried out at 36°C until dryness (approximately 2h). The dried extract was derivatized by adding 50µL methoxyamine hydrochloride (20mg/mL in pyridine; Sigma-Aldrich, St. Louis, MO, USA), followed by incubation at 30°C for 1h. Thereafter, 100µL N-methyl-N-(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (MSTFA + 1% TMCS; Sigma-Aldrich, St. Louis, MO, USA) was added and the samples were incubated at 70°C for 1h. After centrifugation at 13,000rpm for 10min at 4°C, the derivatized extracts were transferred into 2mL amber GC vials (Agilent Technologies, Santa Clara, CA, USA).

Quality control samples were prepared by pooling equal aliquots from all seminal plasma samples and were analyzed together with the study samples throughout the analytical sequence. Metabolite profiling was performed using a GCMS-QP2010 system (Shimadzu Corporation, Kyoto, Japan) equipped with an Rtx-5MS capillary column (30m×0.25mm i.d.×0.25µm film thickness; Agilent Technologies, Santa Clara, CA, USA). One microliter of each sample was injected into the system. The injector, interface, and ion-source temperatures were maintained at 270°C, 260°C, and 200°C, respectively. Helium (99.9%) was used as the carrier gas at a flow rate of 3mL/min. The oven temperature was programmed from 80°C (held for 2min) to 325°C at 10°C/min and maintained for 6min. Mass spectra were acquired in electron impact mode at 70eV over a mass range of m/z 30–600. Metabolites were identified by comparison with the NIST 20 spectral library and retention index data (https://webbook.nist.gov/chemistry/) (Maulana *et al.,* 2025).

**Data Processing and Statistical Analysis:** Seminal plasma metabolites were identified based on retention time, one target ion, and two qualifier ions by comparison with authentic standards and the NIST mass spectral library. Relative metabolite abundances were calculated by dividing the peak area of each metabolite by the peak area of the internal standard (heptadecanoic acid). Identified metabolites were classified according to the Human Metabolome Database (HMDB version 5.0; https://www.hmdb.ca/) (Wishart *et al.,* 2022). Statistical analyses were performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca), a web-based platform for metabolomics data processing and interpretation (Pang *et al*., 2024). Prior to analysis, metabolite data were subjected to sum normalization, log transformation, and autoscaling. Principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA), Variable Importance in Projection (VIP) analysis, and metabolic pathway enrichment analysis were performed to explore metabolomic patterns associated with sperm motility. Model performance was evaluated using cross-validation, classification accuracy, $R^2$, $Q^2$, and permutation testing. Given the exploratory nature of the study and the limited sample size, interpretation was focused primarily on multivariate and pathway-level analyses.

## RESULTS

**Semen Characteristics of High- and Low-Motility Aceh Bulls:** Semen characteristics of the experimental groups are presented in Table 1. The high-motility group exhibited a mean progressive motility of 82.33±3.51%, whereas the low-motility group showed 62.33±5.86%, confirming a clear distinction between the two experimental groups. Other semen characteristics were generally comparable between groups. Mean semen volume was 4.30±1.79mL in the high-motility group and 4.06±1.80mL in the low-motility group, while semen pH

remained constant at 6.40 in both groups. Mass motility scores averaged 2.40±0.55 and 2.00±0.00, respectively. Sperm viability was similar between groups (77.33±1.53% vs. 76.67±1.26%), whereas the high-motility group tended to show higher sperm concentration (2074±816.26×10⁶/mL) and slightly higher abnormality (15.80±3.75%) than the low-motility group (1414±553.43×10⁶/mL and 10.40±5.87%, respectively).

**Table 1:** Semen characteristics of the high- and low-motility groups

| Parameter | High Motility (*n*=5) | Low Motility (*n*=5) |
|---|---|---|
| Volume (mL) | 4.30±1.79 | 4.06±1.80 |
| pH (score) | 6.40±0.00 | 6.40±0.00 |
| Mass motility (score) | 2.40±0.55 | 2.00±0.00 |
| Progressive motility (%) | 82.33±3.51 | 62.33±5.86 |
| Viability (%) | 77.33±1.53 | 76.67±1.26 |
| Abnormality (%) | 15.80±3.75 | 10.40±5.87 |
| Concentration (×10⁶/mL) | 2074±816.26 | 1414±553.43 |

**Metabolic Signatures Associated with Sperm Motility Using Multivariate Analysis:** Multivariate analysis indicated metabolic differences between seminal plasma samples obtained from Aceh bulls with high and low sperm motility. Plotted on PCA (Fig. 1A), the samples tended to separate along PC1, suggesting that part of the observed variance may be associated with differences in sperm motility. This pattern was further visualized using the PLS-DA model. (Fig. 1B), which showed greater separation between groups (P=0.04, accuracy=0.80, $R^2$=0.75, $Q^2$=0.53), as confirmed by consistent, classifiable metabolic signatures between groups. VIP analysis (Fig. 1C) identified key discriminatory metabolites, primarily associated with carbohydrate metabolism (e.g., lactate, fructose, galactose), energy production intermediates, and lipid-related compounds, indicating that metabolites related to energy metabolism and lipid metabolism differed between groups. Volcano plot analysis identified 19 significantly altered metabolites in seminal plasma between the high- and low-motility sperm groups (P<0.05, fold change >1.5), comprising 10 upregulated and 9 downregulated metabolites. Phenylalanine, D-phenylalanine, phenylpyruvic acid, L-cysteine, methionine, and pyroglutamic acid were markedly elevated in the high-motility group (log2FC >4), whereas N-acetylglucosamine, sorbitol, lactic acid, and the saturated fatty acids stearic and palmitic acid were reduced (Fig. 2).

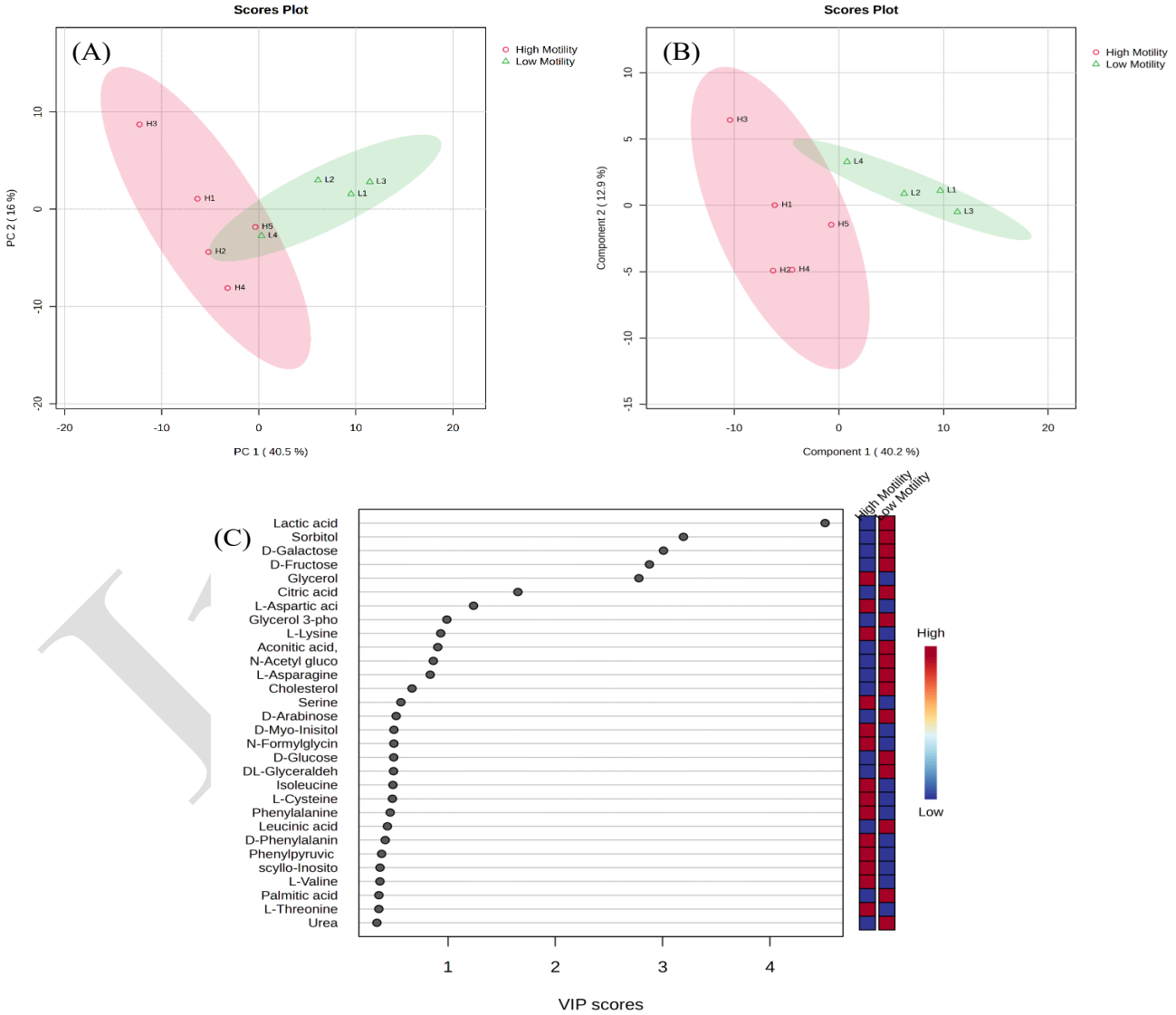


**Fig. 1:** Principal components analysis (PCA) plot (A), partial least square- discriminat analysis (PLS-DA) plot (B), and Variable Importance in Projection (VIP) plot of metabolites associated with high- and low-motility seminal plasma in Indigenous Aceh bulls (C).

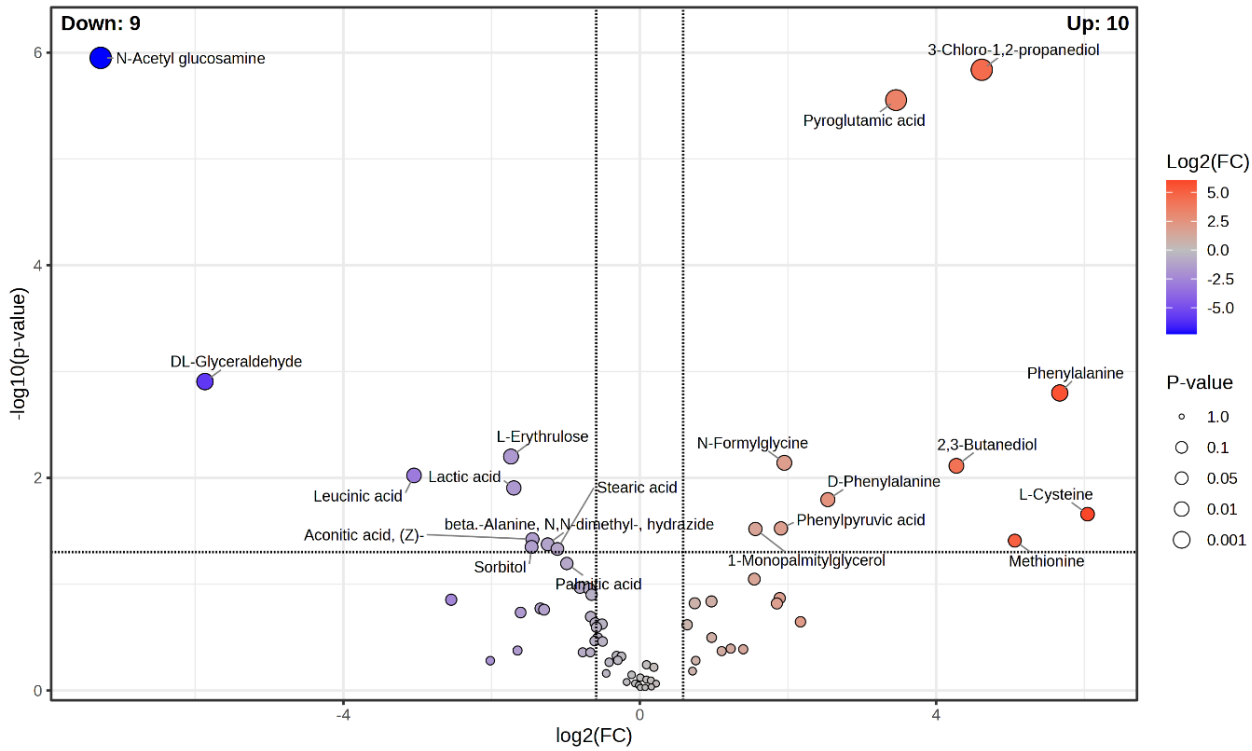


**Fig. 2:** Volcano plot of differentially abundant seminal plasma metabolites between high- and low-sperm-motility groups based on significance criteria of P<0.05 and fold change >1.5.

**Metabolic Signatures Underlying Sperm Motility Differences:** Heatmap analysis revealed a clear metabolic separation between seminal plasma samples of high- and low-motility Aceh bulls (Fig. 3), and hierarchical clustering confirmed distinct and consistent metabolomic profiles in each group. Low-motility samples were characterized by a relative accumulation of metabolites associated with energy metabolism, organic acid production, glycolytic activity, and cellular stress. In contrast, the high-motility seminal plasma group showed increased levels of amino acids and their derivatives, indicating enhanced metabolic adaptation, protein turnover, and cellular maintenance capacity.

**Integrated Metabolic Pathway and Profiling Analysis:** Quantitative pathway enrichment analysis (QPEA) revealed that metabolic differences between the high- and low-motility seminal plasma groups are driven by coordinated changes in pathways for amino acid and energy metabolism (Fig. 4 and Table 2). Pathways associated with aromatic amino acid metabolism, particularly phenylalanine metabolism, and the biosynthesis of phenylalanine, tyrosine, and tryptophan, exhibited the highest combined significance and pathway impact, indicating a central role in modulating sperm functional capacity. In parallel, energy production pathways, including glycolysis/gluconeogenesis, pyruvate metabolism, and galactose metabolism, exhibit moderate to low impact values, suggesting that differences in substrate utilization and bioenergetic efficiency are critical determinants of motility. Additionally, pathways such as taurine and hypotaurine metabolism, as well as the biosynthesis of unsaturated fatty acids, contributed to the overall metabolic landscape, highlighting the involvement of redox regulation and membrane dynamics.

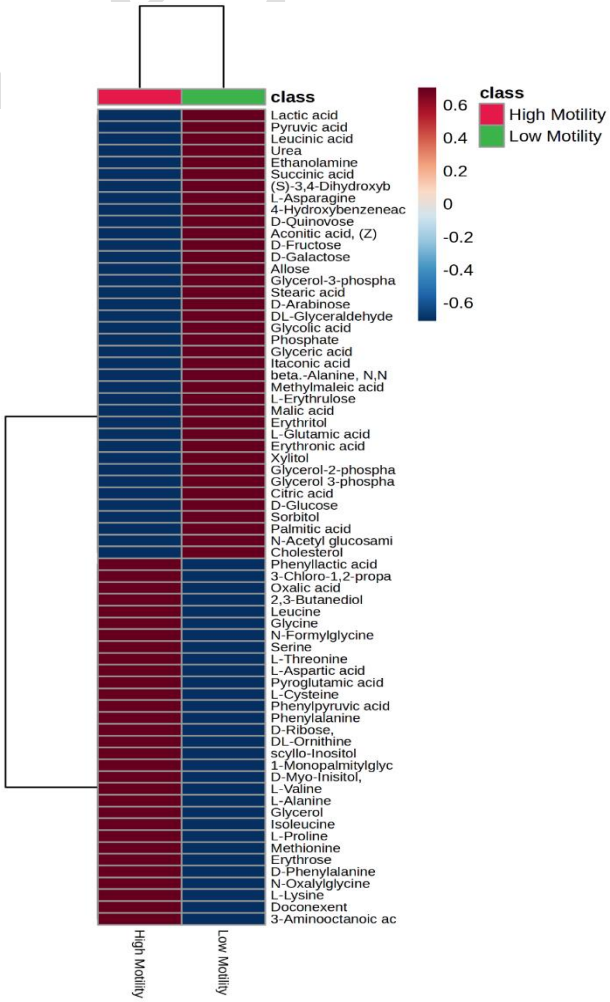


**Fig. 3:** Heatmap analysis of seminal plasma of Indigenous Aceh bulls metabolites with High and Low motility.

**Table 2:** Result from quantitative pathway enrichment analysis (QPEA) of seminal plasma of Indigenous Aceh bulls metabolites with High and Low motility

| No | Metabolism Pathway | Total Cmpd | Hits | Raw p | -LOG10(p) | Impact |
|---|---|---|---|---|---|---|
| 1 | Phenylalanine metabolism | 10 | 2 | 0.002 | 2.699 | 0.619 |
| 2 | Phenylalanine tyrosine and tryptophan biosynthesis | 4 | 2 | 0.002 | 2.699 | 0.500 |
| 3 | Taurine and hypotaurine metabolism | 8 | 1 | 0.020 | 1.706 | 0.000 |
| 4 | Thiamine metabolism | 7 | 1 | 0.020 | 1.706 | 0.000 |
| 5 | Glycolysis or Gluconeogenesis | 26 | 2 | 0.021 | 1.682 | 0.098 |
| 6 | Pyruvate metabolism | 23 | 3 | 0.021 | 1.682 | 0.220 |
| 7 | Biosynthesis of unsaturated fatty acids | 36 | 3 | 0.033 | 1.476 | 0.000 |
| 8 | Galactose metabolism | 27 | 5 | 0.048 | 1.320 | 0.391 |
| 9 | Fructose and mannose metabolism | 20 | 2 | 0.080 | 1.099 | 0.131 |
| 10 | Pentose phosphate pathway | 23 | 2 | 0.100 | 0.998 | 0.000 |
| 11 | Pantothenate and CoA biosynthesis | 20 | 3 | 0.103 | 0.987 | 0.000 |
| 12 | Glycosylphosphatidylinositol (GPI)-anchor biosynthesis | 32 | 1 | 0.109 | 0.965 | 0.039 |
| 13 | Fatty acid biosynthesis | 47 | 1 | 0.109 | 0.965 | 0.015 |
| 14 | Fatty acid elongation | 39 | 1 | 0.109 | 0.965 | 0.000 |
| 15 | Fatty acid degradation | 39 | 2 | 0.109 | 0.962 | 0.000 |
| 16 | beta-Alanine metabolism | 21 | 1 | 0.118 | 0.926 | 0.000 |
| 17 | Nicotinate and nicotinamide metabolism | 15 | 1 | 0.118 | 0.926 | 0.000 |
| 18 | Selenocompound metabolism | 20 | 1 | 0.157 | 0.804 | 0.000 |
| 19 | Glycerolipid metabolism | 16 | 3 | 0.170 | 0.768 | 0.374 |
| 20 | Amino sugar and nucleotide sugar metabolism | 42 | 2 | 0.184 | 0.734 | 0.094 |
| 21 | Lysine degradation | 30 | 1 | 0.207 | 0.685 | 0.000 |
| 22 | Biotin metabolism | 10 | 1 | 0.207 | 0.685 | 0.000 |
| 23 | Starch and sucrose metabolism | 18 | 2 | 0.208 | 0.682 | 0.425 |
| 24 | Steroid biosynthesis | 41 | 1 | 0.212 | 0.674 | 0.028 |
| 25 | Steroid hormone biosynthesis | 78 | 1 | 0.212 | 0.674 | 0.007 |
| 26 | Cysteine and methionine metabolism | 33 | 4 | 0.250 | 0.603 | 0.222 |
| 27 | Tyrosine metabolism | 42 | 2 | 0.285 | 0.546 | 0.000 |
| 28 | One carbon pool by folate | 26 | 4 | 0.286 | 0.544 | 0.107 |
| 29 | Primary bile acid biosynthesis | 46 | 2 | 0.290 | 0.538 | 0.056 |
| 30 | Glycine serine and threonine metabolism | 34 | 6 | 0.325 | 0.488 | 0.536 |
| 31 | Citrate cycle (TCA cycle) | 20 | 4 | 0.347 | 0.459 | 0.214 |
| 32 | D-Amino acid metabolism | 14 | 1 | 0.372 | 0.430 | 0.000 |
| 33 | Sphingolipid metabolism | 32 | 1 | 0.372 | 0.430 | 0.000 |
| 34 | Neomycin kanamycin and gentamicin biosynthesis | 2 | 1 | 0.400 | 0.398 | 0.000 |
| 35 | Valine leucine and isoleucine biosynthesis | 8 | 4 | 0.408 | 0.390 | 0.000 |
| 36 | Valine leucine and isoleucine degradation | 40 | 3 | 0.421 | 0.376 | 0.000 |
| 37 | Purine metabolism | 71 | 1 | 0.425 | 0.372 | 0.000 |
| 38 | Histidine metabolism | 16 | 2 | 0.436 | 0.361 | 0.000 |
| 39 | Alanine aspartate and glutamate metabolism | 28 | 7 | 0.444 | 0.353 | 0.421 |
| 40 | Glycerophospholipid metabolism | 36 | 2 | 0.445 | 0.351 | 0.094 |
| 41 | Arginine biosynthesis | 14 | 4 | 0.454 | 0.343 | 0.186 |
| 42 | Glyoxylate and dicarboxylate metabolism | 32 | 7 | 0.573 | 0.242 | 0.250 |
| 43 | Lipoic acid metabolism | 28 | 2 | 0.635 | 0.197 | 0.002 |
| 44 | Glutathione metabolism | 28 | 5 | 0.797 | 0.098 | 0.119 |
| 45 | Propanoate metabolism | 22 | 1 | 0.870 | 0.061 | 0.000 |
| 46 | Pentose and glucuronate interconversions | 19 | 1 | 0.921 | 0.036 | 0.169 |
| 47 | Arginine and proline metabolism | 36 | 4 | 0.974 | 0.011 | 0.181 |
| 48 | Porphyrin metabolism | 31 | 2 | 0.981 | 0.008 | 0.000 |
| 49 | Nitrogen metabolism | 6 | 1 | 0.998 | 0.001 | 0.000 |
| 50 | Butanoate metabolism | 15 | 2 | 0.998 | 0.001 | 0.000 |

Total cmpd corresponds to total number of compounds in the pathway. Hits correspond to the matched number from the user uploaded data. Raw P correspond to the original P-value calculated from the enrichment analysis. -LOG10(p) correspond to the P-value logarithm. Impact corresponds to the pathway impact value calculated from pathway topology analysis

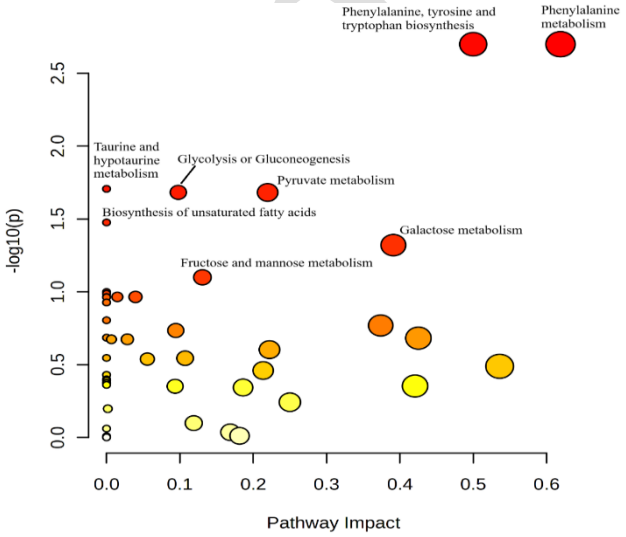


**Fig. 4:** Quantitative pathway enrichment analysis (QPEA) of seminal plasma of Indigenous Aceh bulls metabolites with High and Low motility.

Pathways such as phenylalanine metabolism and the biosynthesis of phenylalanine, tyrosine, and tryptophan showed the highest statistical significance (lowest p-values and highest -log10(p)), suggesting a strong contribution of aromatic amino acid metabolism to sperm functional variation. In contrast, central energy pathways-including glycolysis/gluconeogenesis, pyruvate metabolism, and galactose metabolism- also exhibited enrichment, suggesting that changes in carbohydrate utilization and ATP generation processes contribute to motility status. Furthermore, lipid-related pathways, such as glycerolipid metabolism showed moderate impact values, while steroid biosynthesis showed negligible impact, highlighting the importance of membrane composition and lipid remodeling in sperm function.

Comparative metabolite profiling revealed substantial differences in metabolite abundance between high- and low-motility sperm across amino acid, carbohydrate, and lipid-related compounds (Tables 3–5). Several amino acids and their derivatives, including L-

alanine, leucine, glycine, L-aspartic acid, and L-glutamic acid, were present at higher levels in the high-motility group (Table 3), suggesting enhanced amino acid turnover and potential support for energy metabolism and cellular maintenance. In contrast, major carbohydrates and sugar alcohols, such as D-fructose, D-galactose, sorbitol, and glycerol, exhibited distinct abundance patterns between groups (Table 4), indicating differences in carbohydrate utilization and energy-related metabolic processes associated with motility status. Lipid-related metabolites, including fatty acids (e.g., palmitic acid and stearic acid), glycerophospholipid intermediates, and cholesterol (Table 5), also varied between groups, suggesting differences in membrane composition and lipid remodeling. Notably, metabolites involved in central energy metabolism, including citric acid and lactic acid (Table 3), together with glycerol (Table 4), showed marked differences between groups, further highlighting the importance of bioenergetic regulation in sperm function.

**Table 3:** Amino acid and organic acid-related metabolites identified in seminal plasma of Indigenous Aceh bulls with high and low sperm motility

| Metabolites | RT | RIc | RIf | SI | Low Motility (relative abundance) | High Motility (relative abundance) | SEM |
|---|---|---|---|---|---|---|---|
| Phenyllactic acid | 13.771 | 1596 | 1602 | 92 | 8.722 | 12.452 | 3.333 |
| Phenylpyruvic acid | 13.822 | 1599 | 1575 | 93 | 4.431 | 24.920 | 4.546 |
| L-Alanine | 6.475 | 1105 | 1114 | 97 | 45.204 | 91.761 | 13.129 |
| Leucine | 9.195 | 1277 | 1280 | 96 | 78.681 | s161.076 | 42.886 |
| Glycine | 9.721 | 1312 | 1317 | 96 | 79.855 | 126.228 | 20.979 |
| DL-Ornithine | 16.343 | 1763 | 1756 | 89 | 14.912 | 12.058 | 5.343 |
| Citric acid | 17.559 | 1838 | 1771 | 94 | 1992.096 | 2044.373 | 253.136 |
| L-Lysine | 17.994 | 1864 | 1852 | 96 | 37.959 | 213.174 | 65.385 |
| L-Valine | 8.306 | 1220 | 1221 | 99 | 36.140 | 93.753 | 21.197 |
| Isoleucine | 9.547 | 1300 | 1294 | 95 | 34.713 | 110.426 | 31.987 |
| L-Proline | 9.588 | 1304 | 1309 | 90 | 40.198 | 54.545 | 8.085 |
| N-Formylglycine | 9.937 | 1327 | 1307 | 95 | 7.501 | 39.114 | 6.581 |
| β-Alanine, N,N-dimethyl-, hydrazide | 10.497 | 1365 | 1367 | 84 | 7.684 | 4.978 | 0.843 |
| Serine | 10.534 | 1263 | 1259 | 87 | 78.495 | 240.790 | 58.716 |
| L-Threonine | 10.936 | 1395 | 1390 | 96 | 21.899 | 72.704 | 21.678 |
| Methionine | 11.274 | 1419 | 1412 | 84 | 0.000 | 6.362 | 2.310 |
| L-Aspartic acid | 12.791 | 1429 | 1430 | 96 | 69.914 | 313.699 | 75.101 |
| Pyroglutamic acid | 12.552 | 1511 | 1500 | 75 | 0.000 | 2.229 | 0.410 |
| L-Glutamic acid | 12.897 | 1535 | 1536 | 91 | 277.950 | 501.311 | 140.386 |
| D-Phenylalanine | 13.202 | 1556 | 1547 | 97 | 1.741 | 16.528 | 3.647 |
| L-Cysteine | 13.307 | 1564 | 1568 | 84 | 0.000 | 12.965 | 4.109 |
| L-Asparagine | 13.957 | 1608 | 1582 | 92 | 128.098 | 55.707 | 41.154 |
| N-Oxalylglycine | 14.311 | 1632 | 1628 | 73 | 4.119 | 6.176 | 0.613 |
| Phenylalanine | 14.408 | 1638 | 1644 | 96 | 0.000 | 10.420 | 2.301 |
| Aconitic acid (Z) | 16.203 | 1754 | 1733 | 97 | 127.354 | 72.700 | 16.469 |
| Oxalic acid | 7.142 | 1147 | 1139 | 81 | 45.145 | 69.371 | 10.041 |
| Succinic acid | 9.808 | 1318 | 1320 | 95 | 18.138 | 26.743 | 6.872 |
| Itaconic acid | 10.281 | 1350 | 1359 | 95 | 27.281 | 27.611 | 4.896 |
| Methylmaleic acid | 11.043 | 1402 | 1398 | 82 | 7.096 | 6.405 | 0.642 |
| 3-Aminooctanoic acid | 12.179 | 1484 | 1515 | 73 | 6.916 | 17.839 | 4.521 |
| Lactic acid | 5.834 | 1066 | 1068 | 96 | 2277.368 | 1015.560 | 299.110 |
| Glycolic acid | 6.046 | 1079 | 1080 | 92 | 2.600 | 3.056 | 0.281 |
| Pyruvic acid | 5.633 | 1053 | 1049 | 92 | 13.425 | 12.702 | 2.489 |
| Urea | 8.704 | 1246 | 1246 | 95 | 79.301 | 80.395 | 12.528 |
| Phosphate | 9.249 | 1281 | 1285 | 95 | 254.467 | 337.542 | 58.953 |

RT: Retention time, RIc: Calculated retention index using alkanes standard C7-C40, RIf: retention index from reference (NIST Chemistry WebBook and MS-Search V.3.0 with NIS23 database), SI: Similarity Index

**Table 4:** Carbohydrate and organooxygen metabolites identified in seminal plasma of Indigenous Aceh bulls with high and low sperm motility

| Metabolites | RT | RIc | RIf | SI | Low Motility (relative abundance) | High Motility (relative abundance) | SEM |
|---|---|---|---|---|---|---|---|
| Glyceric acid | 10.128 | 1340 | 1334 | 93 | 5.262 | 4.839 | 0.573 |
| Erythrose | 12.255 | 1489 | 1504 | 72 | 5.398 | 8.094 | 1.116 |
| Erythritol | 12.662 | 1518 | 1501 | 96 | 50.598 | 45.300 | 7.414 |
| Erythronic acid | 13.423 | 1571 | 1577 | 93 | 3.624 | 3.176 | 1.003 |
| D-Ribose | 14.830 | 1666 | 1650 | 92 | 5.338 | 13.293 | 1.913 |
| D-Arabinose | 15.270 | 1696 | 1692 | 87 | 47.895 | 25.040 | 8.697 |
| D-Quinovose | 16.077 | 1746 | 1728 | 85 | 2.864 | 2.729 | 0.370 |
| D-Fructose | 18.574 | 1899 | 1905 | 90 | 1697.248 | 960.648 | 325.710 |
| D-Galactose | 18.807 | 1913 | 1930 | 90 | 992.731 | 277.049 | 261.961 |
| D-Glucose | 19.020 | 1925 | 1932 | 89 | 124.536 | 133.073 | 21.956 |
| Allose | 19.347 | 1944 | 1939 | 89 | 23.604 | 32.425 | 7.475 |
| Sorbitol | 19.536 | 1955 | 1970 | 80 | 1166.191 | 679.998 | 170.034 |
| N-Acetyl glucosamine | 22.313 | 2117 | 2083 | 91 | 21.604 | 0.000 | 3.937 |
| scyllo-Inositol | 22.403 | 2122 | 2090 | 93 | 175.242 | 249.830 | 51.545 |
| 2,3-Butanediol | 5.448 | 1041 | 1040 | 94 | 0.000 | 3.822 | 1.019 |
| L-Erythrulose | 12.124 | 1480 | 1500 | 80 | 11.540 | 5.381 | 1.402 |
| DL-Glyceraldehyde | 8.890 | 1262 | 1267 | 81 | 8.648 | 0.000 | 2.029 |
| Glycerol | 9.223 | 1279 | 1284 | 92 | 296.254 | 1625.719 | 441.768 |
| Xylitol | 15.934 | 1737 | 1743 | 86 | 3.958 | 5.299 | 0.454 |
| D-Myo-Inositol | 29.428 | 2730 | 2742 | 81 | 9.271 | 35.539 | 8.114 |

RT: Retention time, RIc: Calculated retention index using alkanes standard C7-C40, RIf: retention index from reference (NIST Chemistry WebBook and MS-Search V.3.0 with NIS23 database), SI: Similarity Index

**Table 5:** Lipid-related metabolites identified in seminal plasma of Indigenous Aceh bulls with high and low sperm motility

| Metabolites | RT | RIc | RIf | SI | Low Motility (relative abundance) | High Motility (relative abundance) | SEM |
|---|---|---|---|---|---|---|---|
| 1-Monopalmitylglycerol | 27.139 | 2494 | 2480 | 91 | 1.173 | 4.667 | 0.773 |
| Leucinic acid | 8.594 | 1239 | 1245 | 94 | 11.317 | 1.518 | 2.798 |
| Palmitic acid | 21.113 | 2047 | 2050 | 81 | 34.937 | 27.292 | 3.649 |
| Stearic acid | 24.525 | 2254 | 2250 | 96 | 21.689 | 14.954 | 2.213 |
| Doconexent | 27.911 | 2577 | 2566 | 93 | 5.776 | 15.323 | 2.815 |
| Glycerol-2-phosphate | 15.994 | 1742 | 1750 | 93 | 10.146 | 15.340 | 1.867 |
| Glycerol 3-phosphate | 16.601 | 1779 | 1797 | 96 | 665.528 | 738.836 | 116.825 |
| Glycerol-3-phosphate | 24.204 | 2232 | 2228 | 92 | 7.553 | 9.601 | 1.819 |
| Ethanolamine | 9.089 | 1271 | 1269 | 91 | 8.775 | 10.779 | 1.195 |
| 3-Chloro-1,2-propanediol | 7.873 | 1192 | 1185 | 94 | 0.000 | 4.977 | 0.914 |
| 4-Hydroxybenzeneacetic acid | 14.493 | 1644 | 1648 | 95 | 6.320 | 5.311 | 1.031 |
| Cholesterol | 36.612 | 3198 | 3188 | 95 | 102.863 | 68.647 | 17.221 |

RT: Retention time, RIc: Calculated retention index using alkanes standard C7-C40, RIf: retention index from reference (NIST Chemistry WebBook and MS-Search V.3.0 with NIS23 database), SI: Similarity Index.

## DISCUSSION

Progressive sperm motility is widely recognized as one of the most important indicators of semen quality because it reflects the ability of spermatozoa to generate sufficient energy for forward movement and successful fertilization. In Indonesia, fresh semen intended for cryopreservation is required to have a minimum progressive motility of 70% according to the Indonesian National Standard (SNI), ensuring that only ejaculates with adequate functional quality are processed for frozen semen production. Therefore, the 70% threshold was adopted in the present study to establish biologically distinct groups for metabolomic comparison. This grouping strategy is also consistent with previous studies on Indonesian indigenous cattle. Indriastuti *et al.* (2020) evaluated Bali bull ejaculates with more than 70% progressive motility, while Baharun *et al.* (2023) classified Pasundan bull ejaculates into ≥70% and <70% motility groups to investigate seminal plasma and sperm protein biomarkers. Collectively, these studies indicate that the 70% threshold represents a biologically meaningful criterion for distinguishing ejaculates with superior reproductive potential. Furthermore, Suo *et al.* (2023) reported that ejaculates classified as good freezability ejaculates (GFE) exhibited significantly higher sperm motility, progressive motility, viability, acrosome integrity, and mitochondrial activity than poor freezability ejaculates (PFE), highlighting the close relationship between progressive motility and semen freezability. Given this relationship, investigating the metabolic mechanisms associated with sperm motility may provide further insight into the biological processes underlying sperm function.

Sperm motility is a critical determinant of male fertility and is tightly regulated by complex metabolic processes that sustain energy production, membrane integrity, and cellular homeostasis (Patel *et al.,* 2018; Ajeesh *et al.,* 2026). Although conventional semen evaluation relies heavily on kinematic parameters, growing evidence suggests that these metrics do not fully capture the underlying molecular and metabolic determinants of sperm function (de Agostini Losano *et al.,* 2023; Dönmez and İnanç, 2024; de Agostini Losano *et al.,* 2025; Furini *et al.,* 2025). In particular, the metabolic basis of sperm motility in indigenous cattle, such as the Aceh bull, remains poorly characterized, especially at the systems-biology level integrating metabolite profiles and pathway dynamics. Therefore, this study provides preliminary insights suggesting that sperm motility is associated with a distinct metabolic signature, highlighting the contributions of amino acid, carbohydrate, and lipid metabolism to functional sperm quality. Molecular components in seminal plasma are involved in oxidative stress response, spermatogenesis, fertilization, and sperm motility regulation (Saputra *et al.,* 2025). Furthermore, breed-specific molecular profiles in seminal plasma may reflect differences in reproductive physiology among cattle breeds (Saputra *et al.,* 2025). These observations support the importance of investigating metabolomic variation in indigenous tropical cattle such as Aceh bulls.

Recent findings indicate that high-motility samples are associated with metabolic profiles characterized by greater amino acid abundance, whereas low-motility sperm exhibit an accumulation of glycolytic intermediates, suggesting a metabolic bottleneck in downstream mitochondrial respiration and lipid-related processes (Menezes *et al.,* 2019; Pang *et al.,* 2025). From a biological perspective, this pattern indicates that sperm with high motility maintain a more dynamic and adaptive metabolic state, which supports protein turnover, osmotic balance, and cellular repair mechanisms (Wang *et al.,* 2020; Amaral 2022; Miraz *et al.,* 2026). Amino acids such as leucine, glycine, and glutamic acid are known to act not only as substrates for protein synthesis but also as modulators of redox balance and mitochondrial function, which are crucial for maintaining sperm viability and motility (Aitken, 2020; Shrestha *et al.,* 2025). In contrast, the accumulation of glycolytic intermediates and organic acids in sperm with low motility may reflect metabolic inefficiency, impaired mitochondrial respiration, or increased oxidative stress, all of which are generally associated with reduced sperm function.

Results from multivariate and pathway analyses were consistent with this observation by linking statistical separation patterns with underlying biological mechanisms The separation observed in PCA and PLS-DA models suggest that metabolic variation may be associated with differences in sperm motility (Fig. 1), while VIP analysis highlights major metabolites involved in carbohydrate metabolism and energy production. These findings align with the enrichment of glycolysis, pyruvate metabolism, and galactose metabolism pathways, suggesting that differences in substrate utilization and ATP generation are central to sperm functional performance (Prieto *et al.,* 2023). Glycolysis is critical for ATP production in sperm, particularly for motility and hyperactivation (Goodson *et al.,* 2012), whereas pyruvate serves as a key substrate for mitochondrial oxidative phosphorylation to satisfy

bioenergetic demands (Ortiz-Rodríguez *et al.,* 2021; de Agostini Losano *et al.,* 2025). Given that sperm motility is highly dependent on ATP generated through both glycolysis and oxidative phosphorylation, alterations in these pathways likely reflect differences in bioenergetic efficiency and mitochondrial activity between groups (Ortiz-Rodríguez *et al.,* 2021; Amaral, 2022; Prieto *et al.,* 2023). Understanding these bioenergetic mechanisms also contributes to broader insights into reproductive health and cellular function, which are relevant to animal health and well-being.

Consistent with the volcano plot results, elevated phenylalanine, cysteine, and methionine in the high-motility group support a stronger antioxidant amino acid profile, as phenylalanine and related amino acids have been linked to post-thaw sperm viability and antioxidant-related metabolic pathways (Ugur *et al.,* 2020). Conversely, the lower sorbitol and lactic acid levels likely reflect faster glycolytic turnover rather than substrate depletion, since sorbitol and fructose are metabolized through glycolysis to generate the ATP required for sperm motility (Amaral, 2022). Together, these findings suggest that high sperm motility is underpinned by enhanced antioxidant amino acid metabolism and more efficient energy-substrate utilization in seminal plasma.

The important role of aromatic amino acid metabolism, particularly the biosynthesis of phenylalanine, tyrosine, and tryptophan, suggests the existence of an additional layer of metabolic regulation (Table 2). These pathways are closely associated with the production of bioactive molecules involved in redox balance, signaling, and cellular stress response. Recent studies have linked phenylalanine metabolism to the modulation of oxidative stress, while tryptophan derivatives may influence mitochondrial function and apoptosis (Aitken, 2020; Takalani *et al.,* 2023). The increased significance and impact of the pathways observed in this study suggest that these amino acid pathways may help maintain cellular integrity and functional resilience in sperm with high motility (Velho *et al.,* 2018). Furthermore, the involvement of taurine and hypotaurine metabolism further underscores the importance of the antioxidant system, as these metabolites are known to protect sperm membranes from oxidative damage during metabolic stress (Bottrel *et al.,* 2018).

Lipid metabolism also emerges as a significant component of the metabolic landscape that distinguishes sperm motility (Am-in *et al.,* 2011; Serafini and O'Flaherty, 2025). Variations in saturated fatty acids, such as palmitic and stearic acids, imply alterations in the saturated-to-unsaturated lipid ratio, which directly modulates membrane fluidity and lipid remodeling play a critical role in sperm function (Collodel *et al.,* 2020). Sperm membranes are very abundant in polyunsaturated fatty acids, making them highly susceptible to lipid peroxidation (Pannu *et al.,* 2022; Simsek *et al.,* 2025). Therefore, changes in lipid metabolism may reflect differences in membrane stability, fluidity, and resistance to oxidative damage, which are essential for maintaining motility and fertilization capacity (Lancellotti *et al.,* 2025). The variations observed in glycerophospholipid composition further support the notion that membrane dynamics are closely linked to metabolic status and functional performance.

These findings collectively support a conceptual model wherein sperm motility is regulated by an integrated metabolic network involving bioenergetic regulation, amino acid turnover, and lipid remodeling. Sperm with high motility were associated with metabolite profiles indicative of differences in energy and amino acid metabolism, enabling effective ATP production, redox balance, and structural integrity, whereas sperm with low motility exhibit signs of metabolic imbalance and substrate accumulation. From an applied perspective, the identification of specific metabolites and pathways associated with sperm motility may assist future studies investigating potential metabolic biomarkers to enhance semen evaluation and selection strategies in breeding programs. Furthermore, these insights open opportunities for targeted interventions, such as optimizing extender formulations or antioxidant supplementation, aimed at improving sperm metabolic resilience and post-thaw performance. Future studies should validate these candidate metabolic markers in larger populations and integrate metabolomic data with functional fertility outcomes to further elucidate their biological and practical relevance. Importantly, improving the accuracy of semen evaluation and selection strategies in indigenous cattle may enhance breeding efficiency and resource use, thereby supporting sustainable agricultural practices and responsible production systems by contributing to the in situ conservation of locally adapted genetic resources.

Several limitations should be considered when interpreting the present findings. First, the relatively small sample size (n=10) limits statistical power and may reduce the generalizability of the observed metabolic patterns to the broader Aceh bull population. Therefore, the present study should be regarded as exploratory and hypothesis-generating. Second, although the untargeted GC-MS platform enabled the detection of a broad range of metabolites, this approach is inherently semi-quantitative and may not capture all biologically relevant compounds, particularly those present at low concentrations or outside the analytical range of the method. Consequently, some metabolic pathways associated with sperm function may have been incompletely represented. Further evaluation in larger and independent populations will be necessary to determine the consistency and robustness of the observed metabolic patterns, as recommended in previous metabolomics studies (Velho *et al.,* 2018; Menezes *et al.,* 2019; Memili *et al.,* 2020).

Future studies should also employ targeted analytical approaches, such as LC-MS/MS or HPLC-MS/MS, to validate and accurately quantify the candidate metabolites identified in the present study. Although sperm motility is an important indicator of semen quality, fertility is influenced by multiple factors, including sperm function, seminal plasma composition, female reproductive physiology, and post-insemination events within the reproductive tract (Utt 2016). Increasing evidence suggests that seminal plasma components may contribute to the regulation of the uterine environment, endometrial responses, and early embryo development. Therefore, metabolites associated with sperm motility may also have broader implications for reproductive performance. Integrating metabolomic profiles with fertility-related measures, such as conception rate, non-return rate, and

pregnancy outcomes, will be important for determining the practical significance of these metabolites and their potential value as biomarkers of in vivo fertility.

**Conclusions:** The present study demonstrated that seminal plasma metabolomic profiles differed between Aceh bulls with high and low sperm motility. Variations in amino acid, carbohydrate, and lipid metabolism were observed, particularly in pathways related to phenylalanine metabolism, glycolysis/gluconeogenesis, pyruvate metabolism, and galactose metabolism. These pathways may be associated with cellular energy production, oxidative balance, and sperm functional activity. Several metabolites, including amino acids, carbohydrates, and lipid-related compounds, showed differential abundance between motility groups and may provide useful information for understanding sperm physiological status. In summary, the findings provide preliminary metabolomic insights into sperm motility regulation in Aceh bulls and may support future studies aimed at improving semen evaluation and reproductive management in indigenous tropical cattle.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Data Availability:** The data supporting the findings of this study are available from the corresponding author upon reasonable request.

**Ethics Statement:** This study was approved by the Veterinary Ethics Committee, Faculty of Veterinary Medicine, Universitas Syiah Kuala (Ethics Approval No. 527/KEPH/VI/2025). All procedures involving animals complied with institutional ethical guidelines. Semen collection was performed as part of a routine breeding program using standard non-invasive procedures that did not cause additional stress or harm to the animals.

**Authors Contribution:** H. Hafizuddin, G. Gholib, and Tongku N. Siregar conceived and designed the study. The methodology was developed through collaboration among all authors. H. Hafizuddin, H. Husnurrizal, Arman Sayuti, Y. Yusmadi, and Mitha Kurnia Sari were responsible for semen collection and semen quality evaluation. Tulus Maulana, Rusli Fidriyanto, and Muhammad Fajar Amrullah performed sample preparation, metabolomics analysis, and laboratory investigations. Abdullah Baharun and Mohammed Ahmed Elsharef Abdelbagi contributed to data interpretation and scientific discussion. H. Hafizuddin and Tulus Maulana conducted the statistical analyses and prepared the figures and tables. H. Hafizuddin drafted the original manuscript. All authors contributed to the revision of the manuscript, critically reviewed the final version, and approved the manuscript for publication.

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