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RESEARCH ARTICLE

# Investigations on the Role of the 22-24 kDa Seminal Plasma Protein in Bull Breed Fertility and Semen Quality through Proteomics

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**ABSTRACT**

Seminal plasma contains proteins that serve as a protective shield for sperm. These proteins potentially play an important role both in sperm quality and fertility. This study aimed to identify the proteins in seminal plasma that can significantly improve the quality of semen and enhance reproductive performance of bulls of different breeds. Semen samples were collected weekly for five weeks from each of five bulls of six breeds (Limousin, Friesian Holstein, Wagyu, Angus, Ongole, and Brahman). The quality of the semen was assessed, and seminal plasma proteins were identified using a proteomic approach through SDS-PAGE and LC-MS/MS analysis. The semen quality parameters were analyzed using two-way ANOVA, and protein identification was conducted using UniProt. Results showed that sperm quality was significantly different ($P<0.05$) among the breeds, whereas there was no difference among weeks of semen collection. The LC-MS/MS analysis revealed that Ongole bulls had the highest number (18) of seminal plasma proteins, while the Wagyu had the lowest number of only five proteins. In summary, the ejaculate of six bull breeds contained a wide range of 22-24kDa proteins, with a total of 36 different proteins. Four identical proteins were detected in all breeds, which were seminal ribonuclease (RNASE1-2), acrosin-binding protein (ACRBP), T-cell surface glycoprotein CD3 zeta chain (CD247) and renin receptor (ATP6AP2). These seminal plasma proteins were assessed to show strong correlation with sperm fertility. Therefore, they have the potential to be used as biomarkers for the evaluation of bull fertility.

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## INTRODUCTION

Bovine fertility plays a pivotal role in maintaining sustainable cattle farming, given that infertile or sub-fertile bulls can lead to substantial financial ramifications (Özbek *et al.*, 2021). Commonly employed semen quality assessment techniques, such as assessing sperm morphology and the physical characteristics of semen, do not universally serve as reliable indicators of fertility and reproductive efficiency of bulls (Bollwein and Malama, 2023). Furthermore, assessing the outcomes of artificial insemination (AI) once from one cow is susceptible to biases, and the record of a large number of successful inseminations is required to achieve a reliable fertility parameter. Moreover, the process of gathering data on AI outcomes is time-consuming (Willforss *et al.*, 2021). The identification and understanding of the role of proteins involved in bull breed fertility and semen quality have become crucial in the field of reproductive biology. Earlier studies have posited the role of seminal plasma proteins in

various aspects of the reproductive performance of a bull. Baharun *et al.* (2023) documented the presence of numerous seminal plasma protein expressions in high-quality semen of Simmental bulls, attributing roles in sperm capacitation, motility, and fertility to these proteins. Moreover, findings from a meta-analysis study indicated that seminal plasma harbours numerous proteins associated with the seminal binding of sperm proteins, contributing to the enhancement of motility and quality of *Bos taurus* spermatozoa (Viana *et al.*, 2022).

Seminal plasma, a naturally occurring component, comprises proteins that serve to protect sperm against different physical and chemical insults. Certain proteins within bovine seminal plasma undergo adsorption onto the sperm surface upon ejaculation, contributing to the preservation of sperm quality. However, investigations in humans have identified several proteins in seminal plasma that exhibit overexpression or under-expression in cases of poor sperm quality (Selvam and Agarwal, 2019). In Bali cattle, 52% of the protein fraction of seminal plasma was detected at a molecular weight of 25kDa (Sarsaifi *et al.*, 2015). The proteins with a molecular weight of 23-24kDa play multiple roles within the reproductive system, serving as necessary surface proteins for sperm cells during fertilization and functioning as sperm-binding proteins (Somashekar *et al.*, 2017). Additionally, a protein weighing 24kDa has been identified which is implicated in the apoptosis pathway and hypothesized to affect fertility in *Bubalus bubalis* (Almadaly *et al.*, 2023).

Proteomic methodology has been applied to determine the identity and to investigate the seminal plasma proteins linked to bull breed fertility. The 22-24kDa seminal plasma proteins have emerged as potential candidates for further investigation. Previous studies have suggested that these proteins may play a crucial role in reproductive processes. However, the specific function and mechanism of action of these proteins remain unclear. By analyzing the protein composition of bull semen using proteomic techniques, the present study aimed to determine the presence and abundance of the 22-24kDa seminal plasma proteins and explore their potential interactions with other proteins involved in reproduction. Therefore, identifying the role and function of these proteins could potentially enhance the quality of frozen semen and improve reproductive outcomes in bull breeding programs.

## MATERIALS AND METHODS

**Ethics and animals:** This research protocol received ethical clearance from the Ethics Committee of the National Research and Innovation Agency, Central Jakarta, Indonesia, with Decree number 018/KE.02/SK/02/2023. This study utilized bulls of six breeds (Limousine, Frisian Holstein-FH, Wagyu, Angus, Ongole and Brahman) kept at the Artificial Insemination Center of Lembang, Indonesia. The criteria of selection were based on age (4-6 years), body weight (>400kg) and body condition score (3-4). The farm is situated at elevations ranging from 1,312 to 2,084 meters above sea level, with coordinates of 6°54'53.0784"S latitude and 107°36'35.3160"E longitude. The ambient temperatures in the region fluctuated between 17 to 24°C with relatively low humidity. Semen collection was undertaken in the morning using an artificial vagina method. Semen collection and quality assessment were performed weekly for five weeks, involving five bulls from each breed.

**Semen quality assessment:** Immediately after collection, semen quality parameters (ejaculatory volume, colour, consistency, pH, wave motion, sperm motility, and sperm concentration) were evaluated following the protocol of Azizah *et al.* (2023), with some modification. Briefly, macroscopic assessments of ejaculatory volume, colour, consistency, and pH were directly conducted within 15mL tubes; semen volume was determined by reading the scale printed on the tube, while pH was measured using a digital pH meter. Wave motions were recorded using a fresh semen slide observed under 100X magnification of a light microscope, with mass wave thickness and speed were assessed. Assessment criteria were categorized as 3(+++), 2(++), and 1(+). Sperm motility was evaluated by observing the semen slide under 400X magnification of a light microscope. Sperm concentration was quantified using a spectrophotometer (Photometer SDM 6 Minitube) calibrated at the wavelength of 535nm used for bovines (Saif-ur-Rehman *et al.*, 2019).

**Extraction and isolation of proteins:** For Extraction and isolation of proteins, ejaculates were transferred to 10mL conical tubes and supplemented with a protease inhibitor cocktail (Promega, USA). Seminal plasma was isolated from the ejaculates by centrifugation at 6,000rpm for 30min at 4°C. SDS-PAGE analysis was conducted to identify proteins of 22-24kDa, following the method described by He (2011) with slight modification. The proteins were mixed with Novex™ Tris-Glycine SDS Sample Buffer (2X) (Invitrogen, USA) and heated at 85°C for 5min. The proteins were separated using a Mini-Protean Tetra Cell. Electrophoresis was conducted at 80volts. The gel was stained with colloidal Coomassie G-250 GelCode™ Blue Stain Reagent (Thermo Fisher Scientific, USA), followed by overnight washing with ultrapure water (Invitrogen, USA).

**Proteins preparation, reduction, alkylation, and digestion:** The proteins underwent digestion using the PierceTM In-Gel Tryptic Digestion Kit (Thermo Fisher Scientific, USA). The targeted protein (22-24kDa) was excised into pieces using a scalpel (Fig. 1). These gel fragments were subjected to twice washing with 200µL of the destaining solution, followed by incubation at 37°C for 30min. Then 30µL of reduction buffer was added and incubated at 60°C for 10min. The gels were treated with 30µL of alkylation buffer and incubated in the dark at room temperature (1h). The gels underwent twice washing and incubated at 37°C for 15min. The gels were then dehydrated by adding 50µL of acetonitrile and incubating at room temperature for 15min. The gels were then rehydrated with 20µL of activated trypsin solution and incubated at room temperature for 15min. Then 25µL of digestion buffer was added to each tube, and the samples were incubated overnight at room temperature. Proteins were transferred to a clean tube for liquid chromatography (LC-MS/MS) analysis.

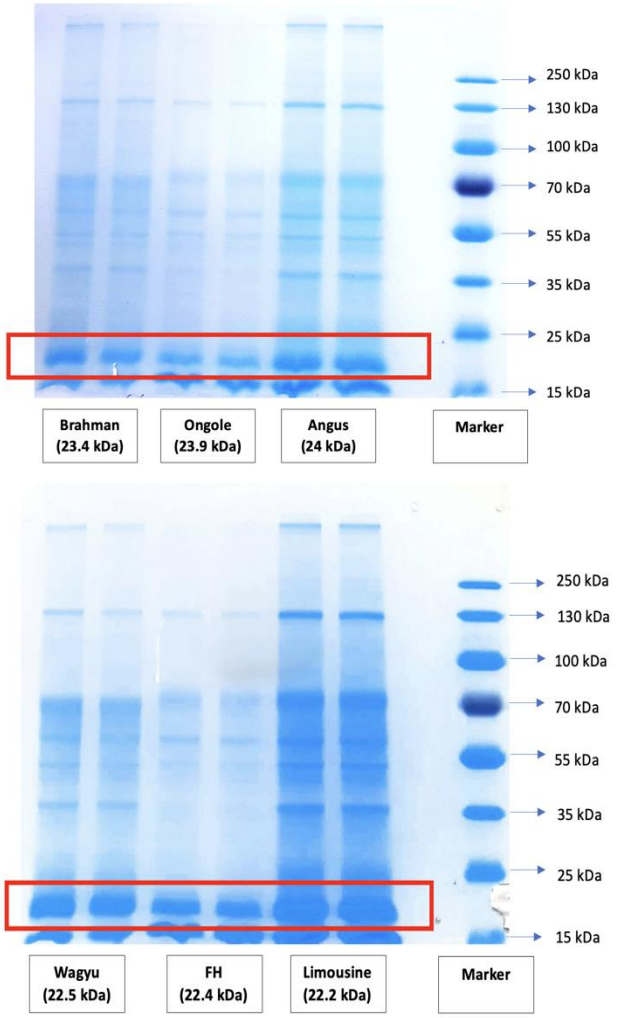


**Fig. 1:** Separation of 22-24kDa seminal plasma proteins with 1D-SDS-PAGE.

**Liquid chromatography with tandem mass spectrometry (LC-MS/MS):** All experimental procedures were conducted using the NanoLC Ultimate 3000 Series System coupled with the Tandem Q Exactive Plus Orbitrap HRMS (Thermo Fisher Scientific). Proteins were loaded onto a trap column (30µm diameter and 5mm length) (Thermo Fisher Scientific, USA). Protein separation was performed on a capillary column (PepMap RSLC C18; Thermo Fisher Scientific, USA) at a flow rate of 300nL/min. The mass spectrometer was operated within a mass range of 200-2000m/z.

**Statistical analysis:** The semen quality parameters were analyzed, using the two-way ANOVA, while significant groups were compared using Duncan's multiple range test (SPSS software version 25). Data implementation was subjected to principal component analysis (PCA; JMP Pro 14). Protein identification was conducted using resources from www.uniprot.org and subjected to descriptive analysis. Interaction prediction between the most abundant proteins and fertility was assessed using STRING version 12.0 (https://string-db.org). Gene Ontology (GO) analysis was performed with the PANTHER Classification System version 18.0 (www.pantherdb.org).

## RESULTS

**The quality of semen of bulls of six breeds:** The semen quality assessment conducted on six bull breeds revealed adherence to established norms within the bovine species. A comprehensive analysis of semen quality parameters based on breeds and weeks of semen collection is shown in Table 1. Principal Component Analysis (PCA) elucidated that Component 1 and Component 2 respectively explained 38.2 and 23.3% of this observed variance (Fig. 2). PCA also explained the grouping of cattle breeds based on semen quality parameters. Sperm concentration and consistency had a positive correlation because they were in the same quadrant, which was found in Angus breed. Wave motion, sperm motility, and ejaculatory volume showed a positive correlation and were found in Ongole cattle. Traits such as ejaculatory volume, pH, mass motion, and sperm motility exhibited relatively consistent characteristics across multiple breeds. The particular significance of fertility assessment was the wave motion and sperm motility parameters derived from semen characteristics. Table 1 shows that sperm quality was significantly different ($P<0.05$) among bulls of six breeds, whereas there was non-significant difference among weeks of semen collection. Weeks x breeds interaction was also non-significant. Significant differences ($P<0.05$) in wave motion and sperm motility were observed in Brahman, Ongole, Angus, Wagyu, and Friesian Holstein (FH) bulls when compared to Limousine. The sperm motility of these breeds remained consistently above 60%, whereas Limousine exhibited significantly lower ($P<0.05$) sperm motility of 57.50±19.60%. Limousine also displayed lower wave motion (1.53±0.56) and sperm concentration (679.59±281.45x$10^6$/mL) than other breeds.

**Identification of proteins in six bull breeds:** Numerous seminal plasma proteins within the 22–24kDa range were effectively characterized across six distinct breeds (Ongole, Limousine, Wagyu, Brahman, Angus, and FH). Table 2 presents a comprehensive summary of these proteins, including protein names, gene symbols, protein accession, proximity labelling, and peptide coverage. The proximity labelling technique employed here entails the robust interaction of small peptides derived from proteins enriched via trypsin digestion, subsequently subjected to ionization for LC-MS/MS analysis. The percentage of peptide coverage serves as a critical metric facilitating the identification of peptide sequences within the database. It is noteworthy that the Ongole displayed the highest number of proteins (22-24kDa), with a total of 18 proteins identified through LC-MS/MS analysis; Wagyu exhibited the lowest number, identifying only five proteins within this size range. Proteins such as seminal ribonuclease (RNASE1-2), Acrosin-binding protein (ACRBP), T-cell surface glycoprotein CD3 zeta chain (CD247), and renin receptor (ATP6AP2) were consistently detected across all breeds. Conversely, the presence of the Fibronectin type-II domain-containing protein was notably absent in the Wagyu. IF rod domain-containing protein was exclusively identified in Brahman, Angus, and FH, highlighting breed-specific protein variations in seminal plasma composition.

**Table 1:** Mean and standard deviation of semen quality parameters of six bull breeds based on breeds and week

| Parameter | Breeds of bulls | | | | | | P Value | |
|---|---|---|---|---|---|---|---|---|
| | Ongole | Brahman | Limousin | Angus | Wagyu | Friesian Holstein | Breeds | Breed x Week |
| Volume (mL) | 6.8±2.2$^{b}$ | 6.8±1.8$^{b}$ | 6±2.3$^{ab}$ | 6.7±2.2$^{b}$ | 5.2±1.3$^{a}$ | 8.4±1.9$^{c}$ | 0.01 | 0.12 |
| Color* | 1.8±0.4$^{abc}$ | 1.5±0.5$^{a}$ | 2.1±0.3$^{c}$ | 1.6±0.6$^{ab}$ | 1.7±0.5$^{ab}$ | 1.8±0.4$^{bc}$ | 0.01 | 0.93 |
| Consistency* | 1.7±0.5$^{bc}$ | 1.7±0.5$^{c}$ | 1.2±0.4$^{a}$ | 1.9±0.4$^{c}$ | 1.7±0.5$^{bc}$ | 1.4±0.5$^{ab}$ | 0.01 | 0.37 |
| pH | 6.6±0.1$^{a}$ | 6.6±0.1$^{ab}$ | 6.7±0.1$^{c}$ | 6.6±0.1$^{ab}$ | 6.7±0.1$^{bc}$ | 6.6±0.1$^{ab}$ | 0.01 | 0.25 |
| Wave motion | 1.9±0.4$^{b}$ | 2±0.0$^{b}$ | 1.5±0.6$^{a}$ | 1.8±0.4$^{b}$ | 1.9±0.3$^{b}$ | 1.9±0.3$^{b}$ | 0.01 | 0.87 |
| Motility (%) | 68.3±13.0$^{b}$ | 71.1±2.1$^{b}$ | 57.5±19.6$^{a}$ | 68.6±7.6$^{b}$ | 70±6.7$^{b}$ | 70±3.7$^{b}$ | 0.01 | 0.97 |
| Conc. (x10$^{6}$ /mL) | 947.2±379.4$^{b}$ | 1043.6±290.5$^{bc}$ | 679.6±281.5$^{a}$ | 1175.7±151.1$^{c}$ | 999.3±303.3$^{bc}$ | 937.9±354.4$^{b}$ | 0.01 | 0.59 |
| | Weeks of semen collection | | | | | | | |
| | I | II | III | IV | V | | Weeks | Breed x Week |
| Volume (mL) | 6.6±2.1 | 6.6±1.8 | 6.5±1.8 | 6.5±2.2 | 6.3±2.8 | | 0.99 | 0.12 |
| Color* | 1.7±0.4 | 1.8±0.4 | 1.7±0.5 | 1.7±0.5 | 1.86±0.3 | | 0.80 | 0.93 |
| Consistency* | 1.5±0.5 | 1.5±0.5 | 1.5±0.5 | 1.6±0.5 | 1.4±0.5 | | 0.89 | 0.37 |
| pH | 6.6±0.1 | 6.6±0.1 | 6.6±0.1 | 6.6±0.1 | 6.6±0.1 | | 0.61 | 0.25 |
| Wave motion | 2.1±0.3 | 2.1±0.2 | 2.1±0.3 | 2.1±0.3 | 2.1±0.3 | | 0.87 | 0.87 |
| Motility (%) | 68.2±10.7 | 65±17.1 | 65.4±10.9 | 62.3±19.1 | 68.8±7.1 | | 0.50 | 0.97 |
| Conc. (x10$^{6}$ /mL) | 901.5±349.2 | 918.77±6=363.9 | 947.1±335.9 | 936.1±329.8 | 902.4±369.7 | | 0.98 | 0.59 |

Mean values with different superscripts in the same row show a significant difference (P<0.05). Color*: 1=cream; 2=white; Consistency*: 1=thin; 2=thick.

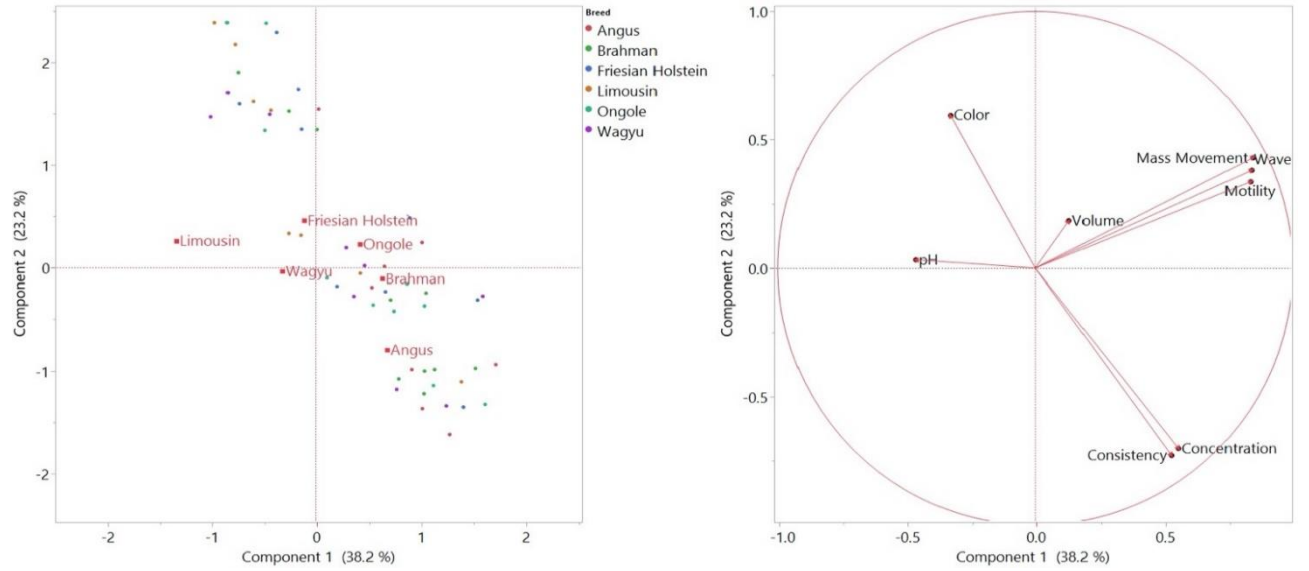


**Fig. 2:** Principal Component Analysis of semen quality of each bull breed.

**String analysis of the most abundant seminal plasma protein related to fertility:** The STRING analysis of bulls of six breeds defined the relationships between protein clusters based on their functional roles (Fig. 3). Multiple protein clusters were identified, which had a complex connection to the reproductive system. In Brahman, four protein clusters were identified (Fig. 3D), with two clusters specifically associated with fertility (ACRBP and the Peptidase C14A family). Limousine displayed three protein clusters with distinct roles in the reproductive system (Fig. 3B). Ongole exhibited two notable protein clusters in the reproductive system (Fig. 3A). Despite the lower number of proteins identified in Wagyu (Fig. 3C), the clusters formed through STRING analysis are involved in reproductive processes, with ACRBP and RNASE1-2 clusters showing homology to those in the Limousine, and the E1BDS9_BOVIN cluster being homologous to the Angus (Fig. 3E). The STRING analysis in FH revealed more complex protein clusters within the reproductive system Fig. 3F). ACRBP, RNASE1-2, and E1BLI4_BOVIN were interconnected, forming a large cluster that interacted with several proteins, and the CCDP2 cluster, which belonged to the sperm-adhesin superfamily.

**Classification of proteome based on gene ontology (GO):** Plasma proteins in *Bos indicus* and *Bos taurus* exhibited similar proportions across various functional categories, including molecular function, biological process, cellular component, protein class, and pathway (Fig. 4). Meanwhile, breed-specific gene ontology results demonstrated considerable variation (Fig. 5). The cellular anatomical entity function was the most prevalent across all breeds. Specifically, hydrolase activity was predominant in Ongole (63%), structural molecule activity was prominent in Limousine (14%) and Brahman (10%), binding function was notable in Angus (33%) and Limousine (14%), structural molecule activity was prominent in FH (17%), and catalytic activity was substantial in Brahman (10%). The biological processes of Wagyu seminal plasma proteins displayed a balanced distribution, with each function comprising 14%. Ongole (27%) and FH (17%) class proteins primarily acted as metabolite interconversion enzymes. Wagyu exhibited a similar function at 25%, with an equivalent proportion functioning as defense/immunity proteins and transmembrane signal receptors. Brahman's primary protein classes included both metabolite interconversion enzymes and protein modifying enzymes. Seminal plasma

**Table 2:** Identification of 22-24kDa seminal plasma proteins in six bull breeds

| Breed | Protein Name | Gene symbol | Protein Accession | Proximity labelling | Peptides (% coverage) |
|---|---|---|---|---|---|
| Ongole | Seminal ribonuclease | RNASE1-2 | A0A4W2ENM6 | 9.1 | 24 |
| | Secretoglobin family 1D member | SCGB1D2 | A0A4W2FHX1 | 8.18 | 20 |
| | AP complex mu/sigma subunit domain-containing protein | AP2M1/AP2S1 | A0A6B0S911 | 9.19 | 13 |
| | Fibronectin type-II domain-containing protein | FN2 | A0A4W2CIX0 | 6.54 | 9 |
| | Acrosin-binding protein | ACRBP | A0A4W2DY29 | 5.4 | 8 |
| | Mitochondrial peptide methionine sulfoxide reductase | MSRA | Q9UJ68 | 8.09 | 7 |
| | Renin receptor | ATP6AP2 | A0A3Q1MUT5 | 5.87 | 5 |
| | T-cell surface glycoprotein CD3 zeta chain | CD247 | A0A6B0S2X7 | 7.34 | 4 |
| | Dual specificity phosphatase 15 | DUSP15 | A0A4W2CYC8 | 9.74 | 4 |
| | Phospholipase A2 domain-containing protein | PLA2G2A | A0A6B0RXI6 | 9.11 | 3 |
| | GPRIN family member 3 | GPRIN3 | A0A4W2DYH9 | 6.96 | 3 |
| | Microtubule-associated protein | MAP1B | A0A4W2CDT2 | 6.34 | 3 |
| | Glycosyl hydrolase | HGNC | D0MUI5 | 6.44 | 2 |
| | F-actin monooxygenase | MICAL1 | A0A4W2ID41 | 8.84 | 2 |
| | Major capsid protein | MCP | Q2VSL5 | 6.42 | 2 |
| | WD domain-containing protein, putative | WDR44 | D0N9R4 | 6.15 | 2 |
| | Centrosomal protein of 162kDa | CEP162 | A0A4W2E595 | 5.54 | 1 |
| | YLP motif-containing protein 1 | YLPM1 | L8I8T7 | 6.44 | 1 |
| Limousin | Fibronectin type-II domain-containing protein | FN2 | A0A4W2CIX0 | 6.54 | 21 |
| | Seminal ribonuclease | RNASE1-2 | A0A4W2ENM6 | 9.1 | 13 |
| | Homeobox domain-containing protein | HOXC5 | A0A8B9WKR5 | 8.66 | 9 |
| | Acrosin-binding protein | ACRBP | A0A4W2DY29 | 5.4 | 8 |
| | Endonuclease/exonuclease/phosphatase domain-containing protein | EEPD1 | A0A6B0R6P1 | 9.19 | 5 |
| | Fibronectin type-II domain-containing protein | FN2 | A0A4W2ECX5 | 9.52 | 5 |
| | Renin receptor | ATP6AP2 | A0A3Q1MUT5 | 5.87 | 5 |
| | Transforming growth factor beta induced | TGFBI | A0A4W2EY86 | 7.25 | 5 |
| | T-cell surface glycoprotein CD3 zeta chain | CD247 | A0A6B0S2X7 | 7.34 | 4 |
| Wagyu | Seminal ribonuclease | RNASE1-2 | A0A4W2ENM6 | 9.1 | 13 |
| | Acrosin-binding protein | ACRBP | A0A4W2DY29 | 5.4 | 13 |
| | 14-3-3 domain-containing protein | YWHAQ | A0A6B0RXV9 | 4.79 | 8 |
| | Renin receptor | ATP6AP2 | A0A3Q1MUT5 | 5.87 | 5 |
| | T-cell surface glycoprotein CD3 zeta chain | CD247 | A0A6B0S2X7 | 7.34 | 4 |
| Brahman | Phosphoglycerate mutase | PGAM1 | L8I0N8 | 8.9 | 23 |
| | CUB domain-containing protein | CDCP1 | A0A8C0AK87 | 8.16 | 14 |
| | Seminal ribonuclease | RNASE1-2 | A0A4W2ENM6 | 9.1 | 13 |
| | Renin receptor | ATP6AP2 | A0A3Q1MUT5 | 5.87 | 12 |
| | Fibronectin type-II domain-containing protein | FN2 | A0A4W2CIX0 | 6.54 | 11 |
| | Acrosin-binding protein | ACRBP | A0A4W2DY29 | 5.4 | 8 |
| | Malate dehydrogenase | MDH1 | L8ISK9 | 8.54 | 6 |
| | Elafin-like | LOC113902985 | A0A4W2FYE5 | 9.36 | 6 |
| | IF rod domain-containing protein | IFC-2 | A0A6B0RD99 | 8.51 | 5 |
| | Fibronectin type-II domain-containing protein | FN2 | A0A4W2ECX5 | 9.52 | 5 |
| | T-cell surface glycoprotein CD3 zeta chain | CD247 | A0A6B0S2X7 | 7.34 | 4 |
| | Peptidase S1 domain-containing protein | LOC786126 | A0A4W2DSL1 | 9.58 | 4 |
| Angus | Fibronectin type-II domain-containing protein | FN2 | A0A4W2CIX0 | 6.54 | 20 |
| | Phosphoglycerate mutase | PGAM1 | L8I0N8 | 8.9 | 13 |
| | Seminal ribonuclease | RNASE1-2 | A0A4W2ENM6 | 9.1 | 13 |
| | Acrosin-binding protein | ACRBP | A0A4W2DY29 | 5.4 | 13 |
| | 14-3-3 domain-containing protein | YWHAQ | A0A6B0RXV9 | 4.79 | 8 |
| | Renin receptor | ATP6AP2 | A0A3Q1MUT5 | 5.87 | 5 |
| | T-cell surface glycoprotein CD3 zeta chain | CD247 | A0A6B0S2X7 | 7.34 | 4 |
| | IF rod domain-containing protein | IFC-2 | A0A6B0RD99 | 8.51 | 3 |
| | IF rod domain-containing protein | IFC-2 | A0A6B0RAY2 | 8.31 | 2 |
| FH | Seminal ribonuclease | RNASE1-2 | A0A4W2ENM6 | 9.1 | 24 |
| | Fibronectin type-II domain-containing protein | FN2 | A0A4W2CIX0 | 6.54 | 21 |
| | Acrosin-binding protein | ACRBP | A0A4W2DY29 | 5.4 | 12 |
| | Fibronectin type-II domain-containing protein | FN2 | A0A4W2ECX5 | 9.52 | 7 |
| | Renin receptor | ATP6AP2 | A0A3Q1MUT5 | 5.87 | 11 |
| | Phosphoglycerate mutase | PGAM1 | L8I0N8 | 8.9 | 12 |
| | CUB domain-containing protein | CDCP1-2 | A0A4W2C5E4 | 5.74 | 10 |
| | T-cell surface glycoprotein CD3 zeta chain | CD247 | A0A6B0S2X7 | 7.34 | 4 |
| | IF rod domain-containing protein | IFC-2 | A0A6B0RD99 | 8.51 | 3 |

proteins in Ongole, Limousine, Wagyu, Brahman, and FH were also involved in the T cell activation pathway. Additionally, these proteins played a role in the glycolysis pathway in Wagyu, Brahman, and FH. In contrast, the predominant protein classes and pathways in Angus were adapter proteins (33%) and pathways associated with Parkinson's disease, epidermal growth factor (EGF), and fibroblast growth factor (FGF) receptor signaling, each constituting 20%.

## DISCUSSION

Seminal plasma comprises highly complex proteins across various species; these proteins are essential for numerous functions associated with the fertilization process (Andrade *et al.*, 2022). Proteins of the 22-24kDa range are associated with the sperm capacitation process and the acrosome reaction (Frolikova *et al.*, 2018).

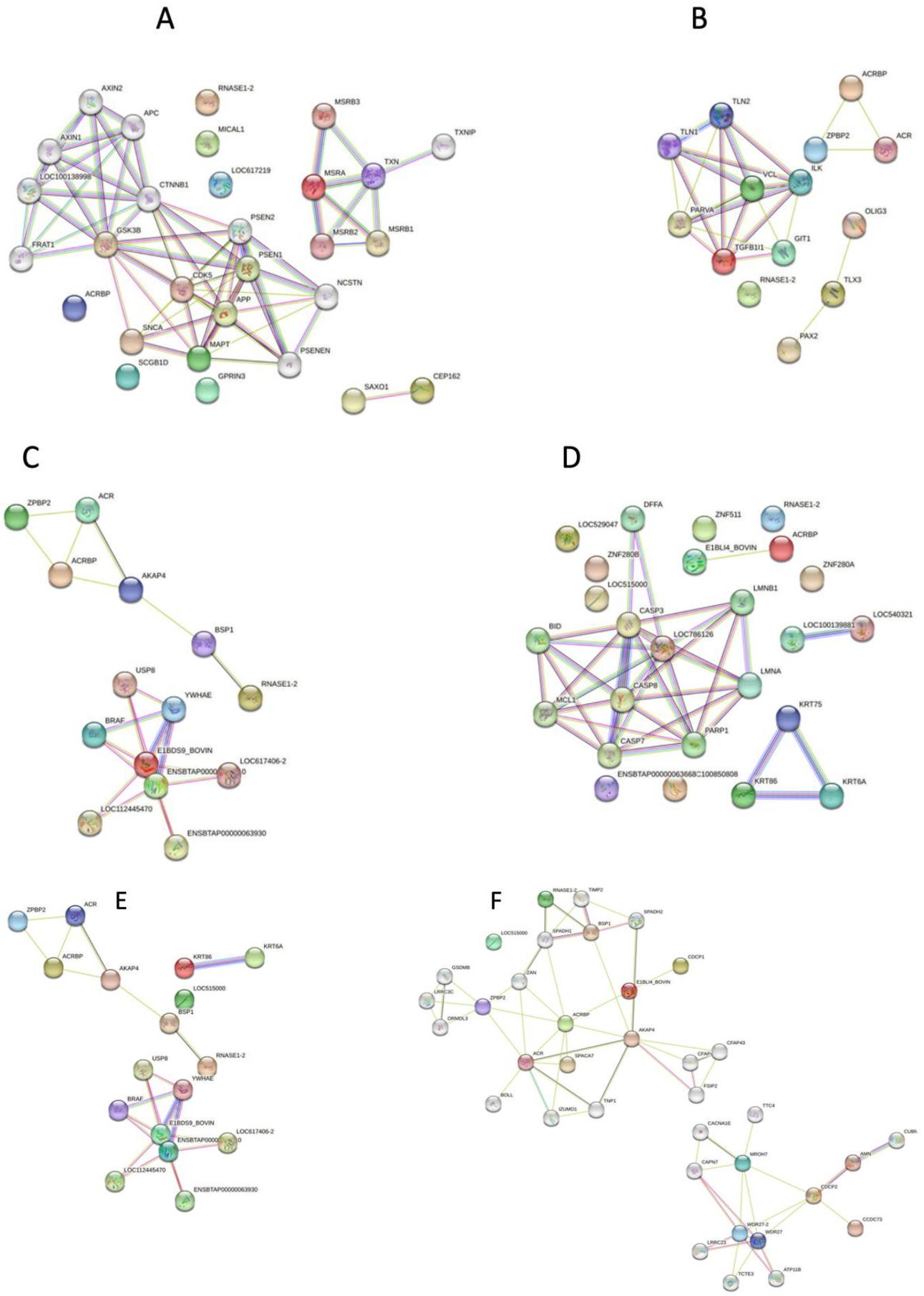


**Fig. 3:** Proteomic strings of 22-24kDa seminal plasma proteins among six breeds: (A) Ongole, (B) Limousine, (C) Wagyu, (D) Brahman, (E) Angus, (F) Friesian Holstein.


**Fig. 4:** Gene Ontology of seminal plasma proteins between *Bos indicus* and *Bos taurus*.

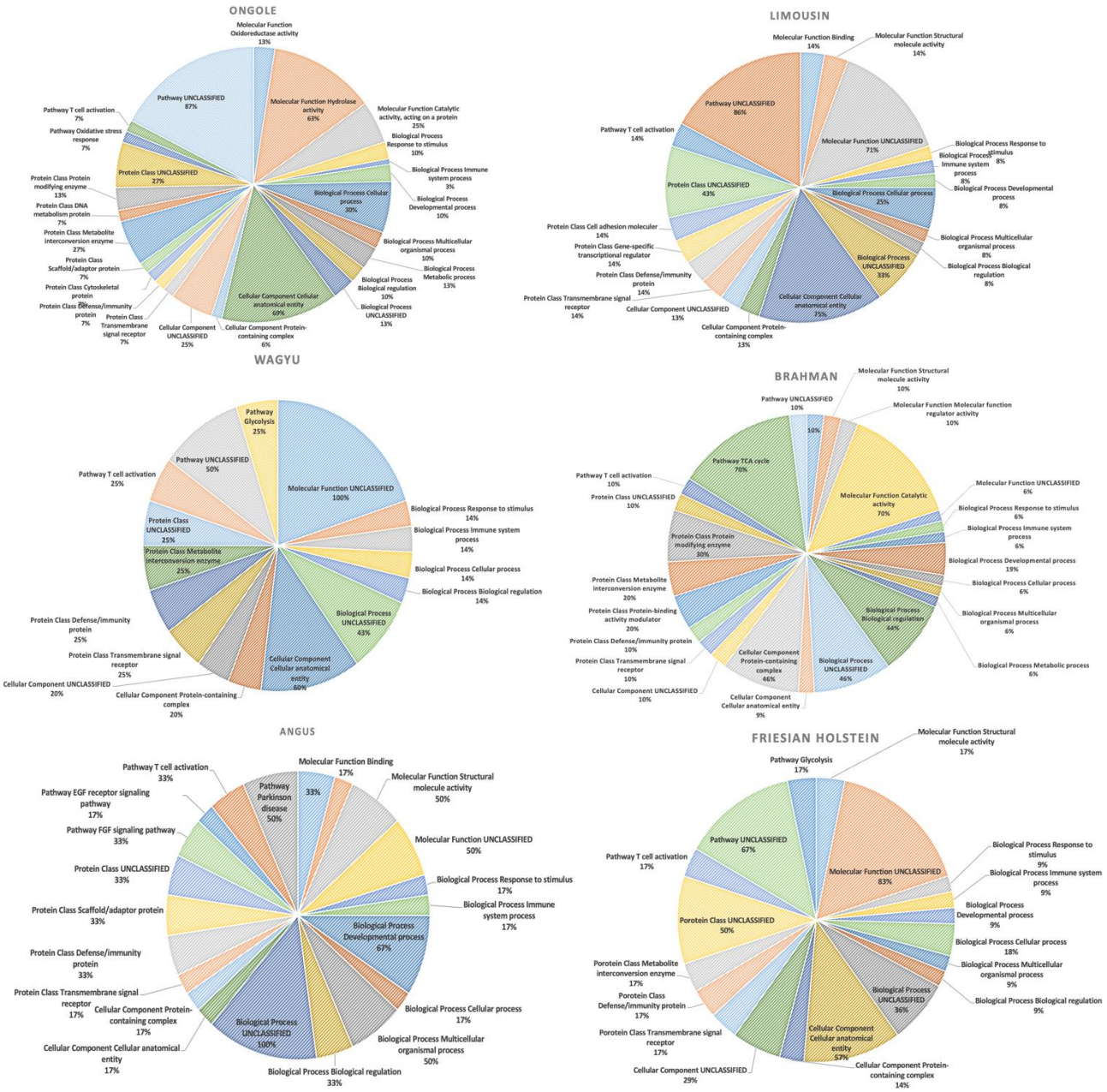


**Fig. 5:** Gene ontology of protein seminal plasma from all bull breeds.

Additionally, the 22kDa protein band is linked to male fertility through its role in protecting sperm from heat-induced damage (Miller *et al.*, 2024). In the present study, RNASE1-2, ACRBP, and CD247 detected in the seminal plasma of bulls of six breeds have been reported to show a strong correlation with fertility, sperm motility, stress response, and spermatogenesis (Xu *et al.*, 2018; Kato *et al.*, 2021; Ma *et al.*, 2023). The Renin Receptor (ATP6AP2) was also detected in all breeds; however, its role in the reproductive system remains unclear. ATP6AP2 is crucial in the renin-angiotensin system (RAS) and is present in seminal plasma, suggesting its role in regulating seminal plasma electrolytes (Gianzo and Subirán, 2020). FN2 protein was detected in nearly all breeds, except Wagyu, and is associated with reproduction. This protein is expressed along the male reproductive tract and binds to the sperm surface during maturation and capacitation through the PC-binding pathway (Şahin *et al.*, 2009; Baharun *et al.*, 2023).

In our study, Wagyu bulls exhibited good sperm motility despite the fewer identified proteins than other breeds. Sperm motility is critical for the successful fertilization of the ovum (Satrio *et al.*, 2022) and for the quality of frozen semen (Andila *et al.*, 2023). The motility of Wagyu sperm could be related to findings from String analysis. The protein interrelationships detected in Wagyu formed clusters with other proteins that play important role in the reproductive process. ACRBP and RNASE1-2 are indirectly associated with forming a linear cluster that binds to AKAP4 and BSP1. AKAP4, derived from the maturation of proAKAP4, is essential for coordinating major transduction signals regulating sperm motility and fertility (Sergeant *et al.*, 2019). BSP1, a family of proteins found in the seminal plasma of several mammalian species, plays a role in forming male fertility markers (Wariata *et al.*, 2022). BSP1 enhances the fertilizing capacity of bull spermatozoa through interaction with heparin-like glycosaminoglycans in the female genital tract (Hung and Suarez, 2012). Within the same cluster, ACRBP and AKAP4 also interact with Acrosin (ACR), contributing to the acrosome reaction. In a separate cluster, E1BDS9_BOVIN is a 14-3-3 domain-containing protein that primarily functions in cell cycle arrest and recovery (Gardino and Yaffe, 2011). E1BDS9_BOVIN also binds to SERPIN domain-containing protein family. Physiologically, the serpin family proteins inhibit excessive inflammatory responses to prevent further tissue damage (Kelly-Robinson *et al.*, 2021). These findings suggest that the protein clusters formed synergistically control tissue remodeling and repair in the reproductive system. According to Gene Ontology (GO) results, the proteins in Wagyu seminal plasma are involved in immune system processes, defense/immunity proteins, and T-cell activation, which may relate to the role of seminal plasma proteins in protecting sperm cells from oxidative stress. Considering the role of each binding protein, it appears that these two protein clusters play a strong role in bull fertility. The clusters formed in Wagyu are similar to those in Angus, as most of the proteins detected were the same, likely due to both being *Bos taurus*.

Although Limousin and FH bulls are also *Bos taurus* and share some identical proteins, the complexity of their protein clusters differs. TLX3, a homeobox domain-containing protein, binds to PAX2 and OLIG3, influencing sperm motility dynamics. Homeobox genes are expressed in testicular germ cells in neonates and somatic cells in the testes and epididymis in adults (Lindsey and Wilkinson, 1996). Spermatozoa undergo significant physiological and biochemical changes to gain fertilizing capability during transit in the epididymis, a tract rich in seminal plasma fluid (Dcunha *et al.*, 2022). The association between motility and homeobox proteins likely arises because spermatozoa acquire motility potential in the epididymis. However, Limousin sperm motility was lower compared to other breeds, possibly due to OLIG3 binding to TLX3, which inhibits sperm development. OLIG3, known for determining the fate of motor neurons, also acts as a gene repressor (Zhang *et al.*, 2022). Despite the low motility, Limousin seminal plasma contained the TGFB1/1 protein, forming a complex cluster, which functions as an androgen receptor and plays a crucial role in fertilization. TGFB1/1 exerts an immune-regulatory effect on the female reproductive tract, contributing to spermatozoa survival and fertilization (Pierucci-Alves *et al.*, 2012). Protein function annotation based on GO reveals that Limousin seminal plasma proteins are involved in cellular processes, developmental processes, and immune functions, indicating that Limousin fertility can still be enhanced through sperm cell regeneration during spermatogenesis.

In FH, proteins ACRBP, RNASE1-2, and E1BLI4_BOVIN are interconnected, forming a large cluster that interacts with several other proteins. The interplay among these three proteins significantly contributes to essential reproductive processes such as acrosome assembly (Kato *et al.*, 2021), spermatogenesis (Kim *et al.*, 2015) and fertilization (Eisa *et al.*, 2021). Another distinctive cluster in FH cattle involves CDCP2, which belongs to the same superfamily as spermadhesin. Spermadhesin plays a crucial role in protecting the sperm membrane from damage due to lipid peroxidation and in deactivating sperm motility in the vas deferens, thereby extending sperm lifespan (Ramírez-López *et al.*, 2023). This protein relationship likely contributes to FH sperm motility being comparable to that of Wagyu. Cellular processes and structural molecule activity enhance the structural integrity of complexes or their assembly inside or outside sperm cells, resulting in high motility.

In contrast, *Bos indicus* seminal plasma proteins form distinct protein complexes in both Ongole and Brahman. MAPT, a member of the microtubule-associated protein superfamily, functions in cellular processes involving microtubules in seminiferous tubules (Sigala *et al.*, 2014; Nishida *et al.*, 2023) Seminiferous tubules contain Leydig cells and Sertoli cells, both crucial for spermatogenesis. The second cluster comprises MSRA which serves as a repair enzyme for oxidized proteins, activating oxidation reactions (Bhattacharya *et al.*, 2020). These methionine sulfoxide reductase family bonds in seminal plasma likely contribute to protecting sperm from oxidative stress. Despite the high molecular function in terms of hydrolase activity, the presence of proteins involved in oxidoreductase activity via the oxidative stress response pathway and high cellular processes suggests that sperm quality can thrive in seminal plasma.

ACRBP in Brahman forms a direct binding with E1BL14_BOVIN, contributing to various aspects of

reproductive system function, similar to the FH bulls. Peptides from the peptidase S1 family, represented by LOC786126, exhibited interconnections within a cluster. Previous studies have shown that the S1 family peptidase plays a role in reproduction through the process of apoptosis (Page and Di Cera, 2008; Zupanič *et al.*, 2023). LOC786126, along with Caspase, BID, and PARP1 proteins, participates in the apoptosis process (Said *et al.*, 2004; Celik-Ozenci and Tasatargil, 2013; Asadi *et al.*, 2021; D'Orsi *et al.*, 2021), indicating potential disturbances in sperm cells. Despite the involvement of numerous proteins in the cell death process, Brahman sperm motility remained unaffected. MCL1 plays an anti-apoptotic role, contributing to cell survival and inhibiting the action of proteins involved in apoptosis (Sancho *et al.*, 2022). This is corroborated by the function of Brahman seminal plasma proteins as a molecular function regulator of activity and the TCA cycle pathway, ensuring undamaged sperm cells with high motility.

**Conclusions:** The 22-24kDa proteins detected in the ejaculates of bulls of six breeds exhibited considerable diversity, encompassing a total of 36 distinct proteins, with four proteins consistently identified across all breeds. These seminal plasma proteins within the 22-24kDa range demonstrate a significant association with sperm fertility, indicating their potential utility as biomarkers for fertility evaluation of bulls. However, the protein profiles of each breed exhibit unique roles and functions within the reproductive system. Furthermore, these proteins are implicated in critical reproductive processes, including sperm motility, oxidative stress response, apoptosis, spermatogenesis, fertilization and acrosome reaction.

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**Authors contribution:** FS, NA, and DAK conceptualized the design of the study, and the methodology was designed in cooperation with all authors. ZM, AFA, and DAK were responsible for semen collection and assessment of the quality of semen. NA, SAA, and IM conducted the extraction and isolation of proteins. NP, AS, and TH digested the proteins and analyzed using LC-MS/MS. The data obtained was statistically analyzed by FS and TPP, and visualized and validated by IM, TS, TK, and LA. The original manuscript was written by FS and NA, revised and edited by IM, TK, TS, and LA. All authors have read and approved the final manuscript.

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