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Sperm characteristics in frozen semen using microscopy and flow cytometry in breeding bulls from Cajamarca, Peru

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ABSTRACT

Evaluating bulls is crucial for selecting sires and ensuring the sustainability of livestock herds. This study aimed to assess sperm quality in frozen semen from breeding bulls in Cajamarca, Peru. Three bulls from the Holstein, Brown Swiss, and Fleckvieh breeds were selected, and three 0.5 mL straws from each bull were analyzed. Optical microscopy revealed an overall motility of 40.37±18.29%, a concentration of 89.07±39.51 million spermatozoa/mL, and normal morphology of 81±5.95%, surpassing the minimum international quality control standards for commercialization. Flow cytometry analysis showed that plasma membrane integrity (PMI), assessed using SYBR/PI, reached 17.5±7.79%; mitochondrial membrane potential (MMP), evaluated with MitoTracker Deep Red FM, was 22.67±8.96%; and acrosomal membrane integrity (AMI), assessed with FITC-PSA/PI, was 19.24±8.15%. PMI varied among breeds, being lowest in Holstein bulls (11.50±5.32%), followed by Brown Swiss (20.49±9.57%) and Fleckvieh (20.52±4.05%). A similar trend was observed for MMP, with Holstein exhibiting the lowest values (16.01±8.25%), followed by Brown Swiss (23.80±8.9%) and Fleckvieh (28.19±5.28%) ($P<0.05$). No differences in AMI were found among breeds ($P>0.05$). A direct correlation was observed between PMI and MMP ($r= 0.763$; $P=0.000$), PMI and AMI ($r= 0.850$; $P=0.000$), and MMP and AMI ($r= 0.635$; $P=0.000$). Normal sperm morphology was correlated with PMI ($r= 0.4$; $P=0.039$) and inversely correlated with AMI ($r=-0.534$; $P=0.004$). In summary, sperm characteristics assessed via microscopy in breeding bulls from Cajamarca showed acceptable values. However, flow cytometry evaluations revealed lower values, particularly in Holstein bulls, which exhibited lower percentages of PMI and MMP compared to Brown Swiss and Fleckvieh breeds.

Introduction

Livestock farming contributes to global food security by providing direct access to animal-derived food products, generating income through livestock sales, and supplying other animal-derived goods (Herrero *et al*., 2013). However, maintaining productivity and achieving sustainability require proper reproductive management of livestock herds. Therefore, bull selection is crucial for optimizing genetics and facilitating artificial insemination. This selection is primarily based on genetic indices, andrological characteristics, and seminal traits (Sakase and Fukushima, 2020; Hutchins *et al*., 2023). Nevertheless, these characteristics vary across regions due to environmental conditions and breed differences (Fuerst-Waltl *et al*., 2006; Filipčík *et al*., 2023).

It has been noted that the role of livestock varies across different regions. Whether these roles are beneficial or detrimental, they must be acknowledged by the scientific community. Research agendas should capitalize on the challenges associated with livestock as opportunities for improvement, while continuing to highlight their positive contributions. These factors are essential for enabling society to make well-informed decisions regarding the future role of animals in sustainable food production, economic growth, and poverty alleviation (Herrero *et al*., 2013). Therefore, targeted studies are needed to assess the genetics of breeding bulls and identify the most suitable individuals for each specific region.

Artificial insemination remains one of the most widely used reproductive techniques worldwide, playing a key role in disseminating high-quality animal genetics. Following semen collection, analyses are conducted to assess its quality, with microscopy being one of the most accessible techniques due to its low cost and ease of use. However, this conventional method involves a degree of subjectivity, underscoring the need to complement it with more objective techniques such as flow cytometry (Silva and Gadella, 2006; DeJarnette *et al*., 2022). The combination of these methods has proven effective in evaluating the sperm characteristics of bulls (Varela *et al*., 2020).

Cajamarca is a livestock-producing region where the cryopreservation of semen from breeding bulls of various breeds is practiced, although its reach is limited. Semen quality assessment is traditionally performed using microscopy, analyzing seminal and sperm variables subjectively. Since the cryopreservation process induces cellular damage in spermatozoa, more objective, precise, and reproducible methods are required to evaluate sperm quality before their use in assisted reproductive techniques (Tanga *et al*., 2021; Hernández-Avilés, 2024). This study aimed to assess sperm quality in commercial doses of frozen semen from breeding bulls in Cajamarca, Peru, using optical microscopy and flow cytometry.

Materials and methods

Ethical approval

Semen collection in breeding bulls is a common practice in livestock management. The present study complied with the Animal Protection Law of the Peruvian State (Law No. 30407).

Bull selection

Semen samples were collected from three sexually mature bull breeds (Holstein, Brown Swiss, and Fleckvieh) aged between 1.5 and 8 years, free from infectious diseases, and exhibiting typical libido. Three bulls per breed were selected, and semen was collected artificially. These bulls were located in the Cajamarca valley, Peru, and reared under an extensive grazing system, fed on Cajamarca ecotype Ryegrass (Lolium multiflorum) and white clover (*Trifolium repens*), with *ad libitum* access to water.

Semen collection and preservation

Each bull was subjected to weekly semen collection using an artificial

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vagina (38–40°C) with a cow serving as a mount dummy. Initially, two false mounts were performed, followed by the collection of two ejaculates.

Only ejaculates that met the volume ≥2 mL criteria, concentration ≥500×10^6 sperm/mL, and motility ≥70% criteria were processed and cryopreserved. The samples were diluted in two commercial extenders (Andromed® and Triladyl®), packaged in 0.5 mL PVC straws via mechanical aspiration, and sealed with polyvinyl alcohol. Subsequently, they were equilibrated at 5°C for 12 hours in a water bath. The straws were then placed horizontally on a freezing rack, which was exposed to liquid nitrogen vapor at 6 cm and 4 cm above the nitrogen level for 10 and 5 minutes, respectively. Finally, the straws were submerged in liquid nitrogen and stored in a cryogenic tank.

Microscopy analysis

Three straws per bull were retrieved from the cryogenic tank to evaluate motility, morphology, and sperm concentration via optical microscopy. Assessments were conducted at 400× magnification using microscope slides with a glass coverslip placed on a stage heated to 37.5°C. Sperm concentration was determined by counting spermatozoa in the central and peripheral quadrants of a Neubauer chamber. The interval between semen cryopreservation and evaluation was 10 years for Holstein and Brown Swiss bulls, and 2 years for Fleckvieh bulls.

Flow cytometry analysis

Similarly, three straws per bull were retrieved from the cryogenic tank and analyzed using a flow cytometer (Amnis, Seattle, WA, USA) to assess sperm viability, acrosomal membrane integrity, and mitochondrial membrane potential (Silva and Gadella, 2006). These analyses were conducted at the Animal Reproduction Laboratory of the Faculty of Veterinary Medicine at the National University of San Marcos in Lima, Peru.

Plasma Membrane Integrity (PMI) – Sperm viability

Each sample was washed twice by centrifugation with PBS at 600 g for 8 minutes, followed by removal of the supernatant and resuspension of the pellet in 100 µL of PBS. The LIVE/DEAD® Sperm Viability Kit (L-7011, Molecular Probes) was employed, containing SYBR-14 fluorochrome, which emits green fluorescence as an indicator of viable sperm with an intact plasma membrane, and propidium iodide (PI), which emits red fluorescence as an indicator of non-viable sperm with a damaged plasma membrane.

A 100 µL sample was incubated with 0.5 µL of SYBR-14 stock solution (20 µM) to achieve a final concentration of 100 nM. Subsequently, 0.5 µL of PI stock solution (2.4 mM) was added, resulting in a final concentration of 12 µM, and the mixture was incubated at 38°C for 10 minutes.

The solution was analyzed using a flow cytometer, with 5,000 sperm-compatible events per sample considered. A 488 nm laser at 15 mW was used for excitation, with fluorescence emissions read at 505–560 nm (Ch02) for SYBR-14 and 642–740 nm (Ch05) for PI (Figure 1). The flow cytometer's imaging system captured live sperm stained with SYBR-14 and dead sperm stained with PI (Juárez and Santiani, 2019).

Acrosomal membrane integrity (AMI)

Acrosomal status was evaluated using *Pisum sativum* agglutinin conjugated with fluorescein isothiocyanate (FITC-PSA). A 100 µL sample was incubated at 38°C for 8 minutes with 2.5 µL of FITC-PSA stock solution (100 µg/mL), yielding a final concentration of 2.5 µg/mL. Additionally, 0.5 µL of PI stock solution (1 mg/mL) was added, resulting in a final concentration of 5 µg/mL.

Samples were analyzed using a flow cytometer equipped with INSPIRE® v.100.3.218.0 (Amnis, Seattle, USA), and the data were processed using IDEAS® v.6.2 (Amnis, Seattle, USA). Ten thousand sperm-compatible events were evaluated based on their size and the length-to-width ratio. Excitation was performed using a 488 nm laser at a power of 20 mW. FITC-PSA fluorescence was detected at 505–560 nm (Ch02), while PI fluorescence was detected at 642–740 nm (Ch05) (Figure 2).

Additionally, images were recorded to confirm lectin binding to the acrosomal region. Sperm were classified into four categories: (a) live sperm with intact acrosomes (FITC-negative, PI-negative), (b) live sperm with damaged acrosomes (FITC-positive, PI-negative), (c) dead sperm with intact acrosomes (FITC-negative, PI-positive), and (d) dead sperm with damaged acrosomes (FITC-positive, PI-positive) (Ugarelli *et al*., 2017).

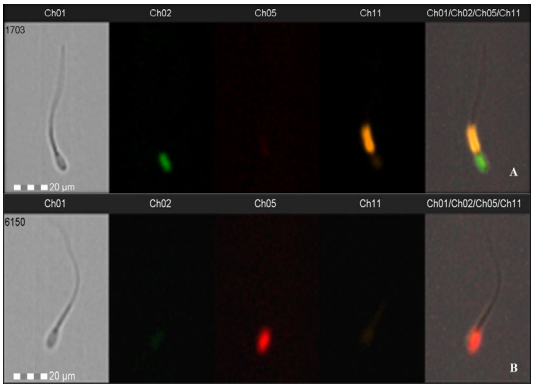

Figure 1. Spermatozoa from breeding bulls in Cajamarca analyzed by imaging flow cytometry using the fluorochromes SYBR-14 and propidium iodide (PI), observed under bright field (Ch01), fluorescence (Ch02/Ch05/Ch11), and a combination of bright field and fluorescence (Ch01/Ch02/Ch05/Ch11). (A) Live spermatozoon emitting green fluorescence due to SYBR-14 staining; (B) Dead spermatozoon emitting red fluorescence due to PI staining.

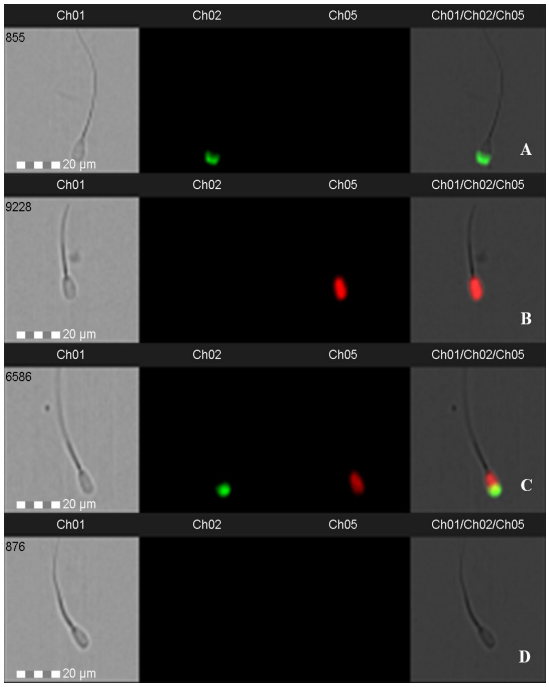

Figure 2. Sperm patterns of breeding bulls in Cajamarca incubated with fluorescein isothiocyanate–peanut agglutinin (FITC-PSA) and propidium iodide (PI), observed under bright field (Ch01), a combination of bright field and fluorescence (Ch01/Ch02/Ch05), and fluorescence (Ch02/Ch05). (A) Live spermatozoon with an acrosomal reaction (PSA+ and PI−); (B) Dead spermatozoon without an acrosomal reaction (PSA− and PI+); (C) Dead spermatozoon with an acrosomal reaction (PSA+ and PI+); (D) Live spermatozoon without an acrosomal reaction (PSA− and PI−).

Mitochondrial Membrane Potential (MMP)

A stock solution of 50 µg Mitotracker Deep Red in 92 µL of DMSO was prepared, yielding a concentration of 1 mM. The solution was homogenized, aliquoted into 2 µL portions, and frozen at 0°C for 60 minutes. A working solution was prepared by diluting 2 µL of stock Mitotracker

solution in 100 µL of PBS, achieving a final concentration of 20 µM. A 100 µL washed sperm sample was incubated with 0.5 µL of the 20 µM Mitotracker working solution, resulting in a final concentration of 100 nM. The sample was then incubated for 10 minutes at 38°C in the dark.

Samples were analyzed using a flow cytometer equipped with an image analyzer. A total of 10,000 sperm were evaluated per sample. Mitotracker excitation was performed with a 642 nm laser, and fluorescence emission was detected at 642–740 nm in channel 11 (Ch-11). Sperm exhibiting red fluorescence were considered to have mitochondrial activity, and results were expressed as the percentage of sperm with mitochondrial activity (Allauca *et al*., 2019).

Statistical analysis

Results were processed using SPSS v.27. Sperm characteristics were analyzed using descriptive statistics. Data normality for each variable within each breed was assessed using the Shapiro-Wilk test. Based on significance, means were compared using one-way ANOVA, and medians were compared using the Kruskal-Wallis test with post hoc Tukey HSD and Dunn tests, respectively. Additionally, correlations between sperm characteristics evaluated by microscopy and flow cytometry were analyzed using Spearman's rho for non-parametric data and Pearson's correlation coefficient for parametric variables. A significant level of $P<0.05$ was considered.

Results

A high sperm concentration ($\times10^6$) was observed in the three evaluated breeds, with the highest average recorded in the Brown Swiss bull straws. However, these concentrations did not show statistically significant differences ($P>0.05$). Similarly, no significant differences were found among breeds in terms of motility, normal sperm morphology, and the IMA ($P>0.05$). In contrast, significant differences were identified in PMI and MMP, with lower values observed in the Holstein breed ($P<0.05$). The Brown Swiss and Fleckvieh breeds did not exhibit statistically significant differences between them (Table 1).

Bivariate analyses revealed a direct positive correlation between normal sperm morphology, as assessed by microscopy, and PMI ($P<0.05$). Stronger correlations were observed between normal sperm morphology and AMI, PMI, and MMP, as well as AMI and MMP ($P<0.01$) (Table 2).

Discussion

The concentration of spermatozoa in the ejaculates, normal morphology, and sperm motility of bulls from the three breeds were within acceptable ranges. However, cryopreservation and thawing induced considerable structural and molecular changes in the spermatozoa, as most PMI, MMP, and AMI percentages were below 50%. Although it is expected that freezing results in a loss of sperm vitality, it has been indicated that at least 40% of spermatozoa with intact PMI are required (Bollwein and Malama, 2023).

Sperm motility exhibited a high coefficient of variation in Holstein and Brown Swiss bull samples. The highest motility percentage was observed in the Fleckvieh breed (47.58±9.72%). However, most values were above 30%, the minimum acceptable threshold (Bollwein and Malama, 2023). Nevertheless, other studies have reported more promising val-

Table 1. Sperm characteristics of frozen semen from breeding bulls in Cajamarca (Peru), assessed by microscopy and flow cytometry

Breed	Statistical	Optical microscopy			Flow cytometry		
		Motility (%)	Concentration ($\times10^6$)	Normal morphology (%)	PMI (%)	MMP (%)	AMI (%)
Holstein	Mean±SE	36.67±6.67[a]	83.78±11.68	82.44±1.34[a]	11.50±1.77[a]	16.01±2.75	15.44±2.76
	SD	20	35.05	4.03	5.32	8.25	8.27
	Median (IQR)	40 (15-55)	82 (58-103)[a]	81 (79-87)	12.00 (7.25-15.40)	13.40 (10.66-19.55)[a]	14.60 (7.49-23.75)[a]
	Min.-Max.	10-60	32-152	77-87	3.41-20.90	8.22-35.30	4.31-25.60
	CV%	54.55	41.83	4.89	46.3	51.52	53.57
	Normality	0.22	0.82	0.09	0.98	0.03	0.30
Brown Swiss	Mean±SE	36.67±7.45[a]	110.33±15.77	80.78±2.34[a]	20.49±3.19[b]	23.80±2.97	21.24±3.51
	SD	22.36	47.3	7.01	9.57	8.9	10.53
	Median (IQR)	30 (15-60)	114 (64-114)[a]	79 (79-86)	20.00 (15.35-24.15)	22.90 (15.15-31.80)[ab]	19.50 (17.45-23.00)[a]
	Min.-Max.	10-70	39-188	66-90	6-41.20	11.70-37.10	6.07-45.90
	CV%	60.98	42.87	8.68	46.69	37.39	49.56
	Normality	0.33	0.77	0.14	0.36	0.75	0.02
Fleckvieh	Mean±SE	47.78±3.24[a]	73.11±9.31	79.78±2.25[a]	20.52±1.35[b]	28.19±1.76	21.02±1.17
	SD	9.72	27.93	6.74	4.05	5.28	3.51
	Median (IQR)	50 (40-55)	88 (39-92)[a]	79 (74-85)	18.20 (17.60-23.80)	30.60 (21.65-32.45)[b]	21.30 (18.20-23.70)[a]
	Min.-Max.	30-60	34-106	72-92	15.70-27.90	20.80-33.70	15.60-27.00
	CV%	20.34	38.21	8.45	19.74	18.72	16.68
	Normality	0.27	0.03	0.60	0.29	0.03	0.10
Total	Mean±SE	40.37±3.52	89.07±7.60	81.00±1.14	17.50±1.50	22.67±1.72	19.24±1.57
	SD	18.29	39.51	5.95	7.79	8.96	8.15
	Median (IQR)	40 (30-60)	88 (57-114)	80 (78-86)	17.50 (12.30-22.10)	21.80 (13.40-31.10)	19.50 (15.60-22.90)
	Min.-Max.	10-70	32-188	66-92	3.41-41.20	8.22-37.10	4.31-45.90
	CV%	45.3	44.35	7.34	44.5	39.51	42.37
	Normality	0.03	0.30	0.62	0.2	0.07	0.01

SE: Standard error; SD: Standard deviation; IQR: Interquartile range; CV: Coefficient of variation.
PMI: Plasma membrane integrity; MMP: Mitochondrial membrane potential; AMI: Acrosomal membrane integrity.
a,bDifferent letters in the same column indicate a statistically significant difference between breeds ($P<0.05$). Means were compared using one-way ANOVA with Tukey's HSD post hoc test. Medians were compared using the Kruskal-Wallis test with Dunn post hoc analysis.

Table 2. Cross-correlations between sperm characteristics assessed by microscopy and flow cytometry in frozen semen from breeding bulls in Cajamarca, Peru.

Correlation		Motility	Concentration	Morphology	PMI	MMP	AMI
Motility	ρ	1	-0.01	-0.01	0.11	-0.07	0.12
	P-value		0.95	0.97	0.58	0.74	0.44
Concentration	r		1	0.13	0.24	-0.12	0.05
	P-value			0.53	0.23	0.55	0.81
Morphology	r			1	0.40	-0.34	-0.53
	P-value				0.039*	0.08	0.004**
PMI	r				1	0.76	0.85
	P-value					0.000**	0.000**
MMP	r					1	0.64
	P-value						0.000**
AMI	r						1
	P-value						

*Significant at 95% (P<0.05); ** Significant at 99% (P<0.01).
PMI: Plasma membrane integrity; MMP: Mitochondrial membrane potential; AMI: Acrosomal membrane integrity.
Sperm concentration, morphology, PMI, and MMP were analyzed using Pearson's correlation coefficient (r) due to their parametric nature. Motility was correlated using Spearman's rho (ρ), as its data did not follow a normal distribution.

ues, with an average of 50.8±2.7% in Friesian bulls (Khalil *et al*., 2018), 64.0±14.0% in beef bulls, and 59.0±14.0% in dairy bulls (Morrell *et al*., 2018). Similarly, findings comparable to those of the present study have been reported, with a motility of 43.6±16.3% in Angus and Holstein bulls (Varela *et al*., 2020). The lower sperm motility values observed in Holstein and Brown Swiss bulls may be influenced by the longer storage duration (eight years) compared to Fleckvieh bulls, whose semen was stored for only two years. Prolonged storage has been shown to negatively affect sperm metabolism, reducing motility and viability due to oxidative and osmotic stress (Malik *et al*., 2015).

The mean post-thaw sperm concentration was 89.07±39.51×10^6/mL. This result is similar to a previous study reporting a concentration of 88.0±20.0 million spermatozoa in beef bull semen and 55.0±19.0 million spermatozoa in dairy bull semen (Morrell *et al*., 2018). It is also encouraging compared to another study that reported a sperm concentration of 21.9±0.012 (10^6/mL) in beef and dairy bull semen (DeJarnette *et al*., 2022). A high sperm concentration significantly contributes to the efficiency of artificial insemination programs, as it increases fertilization probabilities by maintaining the protective components of seminal plasma, intracellular components, and the sperm surface. This compensates for spermatozoa damage, particularly at the plasma membrane level, enhancing their competitive advantage (Saacke, 2008; Patil *et al*., 2020).

On the other hand, a high sperm concentration can increase oxidative stress levels, negatively affecting cell viability and motility (Murphy *et al*., 2013). Even at low concentrations, where membrane integrity and mitochondrial membrane potential remain high, results can be satisfactory as long as acrosome integrity is not compromised (Kastelic, 2013). This is because acrosomal membrane integrity is a non-compensable characteristic, regardless of the number of spermatozoa reaching the fertilization site.

The overall percentage of spermatozoa with normal morphology was 81.0±5.95%, exceeding the 70% minimum threshold for a satisfactory breeding bull (Thundathil *et al*., 2016). Similar values have been reported in other studies, with 76±8% in beef bulls and 87.0±6.0% in dairy bulls (Morrell *et al*., 2018). A combined analysis of Angus and Holstein bulls reported 81.0±22.2% of spermatozoa with normal morphology (Varela *et al*., 2020). Sperm morphology is a crucial indicator of fertilization capacity, as spermatozoa with a higher proportion of normal morphology have a greater likelihood of traversing the cow's reproductive tract and fertilizing the oocyte.

Among all semen samples, only 17.5±7.79% of spermatozoa exhibited an intact PMI. In contrast, other reports have found significantly higher values, ranging from 40.0±11.0% in beef bulls to 46.0±8.0% in dairy bulls (Morrell *et al*., 2018). Similarly, another study reported a considerably higher PMI than the present work, with an average of 44.6±15.2% in beef and dairy bulls (Varela *et al*., 2020). A highly significant difference (P<0.01) in sperm viability was observed among breeds, with the lowest values in Holstein (11.50±5.32%), followed by Brown Swiss (20.49±9.57%) and Fleckvieh (20.52±4.05%). While these results are lower than those reported elsewhere, they highlight the potential influence of genetic, metabolic, and environmental factors on semen quality, which can vary significantly among breeds due to their distinct production purposes and physiological adaptations, as well as factors such as oxidative stress, age of the breeding bulls, and storage duration.

Regarding spermatozoa with MMP (16.01±8.25%) and AMI (15.44±8.27%), the results of this study were markedly lower than those reported in other studies, which recorded values exceeding 50% (Morrell *et al*., 2018). However, the total MMP (22.67±8.96%) was slightly higher than the 18.3±15.5% described by Varela *et al*. (2020). Significant differences (P<0.01) were found in MMP among breeds, with the lowest values in Holstein bulls (16.01±8.25%), followed by Brown Swiss (23.8±8.9%) and Fleckvieh (28.19±5.28%). Viability, MMP, and AMI are key markers of sperm functional capacity, relying on energy metabolism, which is essential for motility, capacitation, and successful fertilization of the oocyte.

The correlation coefficient indicated significant (P<0.05) and directly proportional correlations between morphology and PMI (r= 0.40), but a negative correlation between morphology and AMI (r=-0.53). Stronger correlations were also observed between PMI and MMP (r= 0.763), PMI and AMI (r= 0.850), and MMP and AMI (r= 0.635). These correlations align with the findings of Varela *et al*. (2020), who determined that PMI and MMP assessed via flow cytometry are related to sperm characteristics evaluated through conventional methods.

The main limitations of this study were the limited sample size, which may affect the representativeness of the results, and the semen storage duration, which exceeded 10 years for Holstein and Brown Swiss bulls compared to only 2 years for Fleckvieh bull straws. This factor may have influenced post-thaw sperm quality. However, a strength of this study is the incorporation of complementary analyses using flow cytometry, allowing for a more detailed and objective assessment of sperm quality. Additionally, the findings are relevant for optimizing artificial insemination programs in the livestock industry of Cajamarca. Future research should expand the sample size, include more bulls, and consider the im-

pact of environmental factors on semen quality.

Conclusion

The sperm characteristics of frozen semen from breeding bulls in Cajamarca, evaluated via microscopy, showed satisfactory averages. However, parameters assessed using flow cytometry were low, particularly in Holstein bulls, which exhibited lower viability and MMP percentages compared to Brown Swiss and Fleckvieh breeds.

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Conflict of interest

The authors have no conflict of interest to declare.

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