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Evaluation of The Efficacy of Three Extenders on Boar Semen Quality after Liquid Storage

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ABSTRACT

This study aimed to evaluate the efficacy of three commercial semen extenders, Modena, Zorlesco, and Vim on boar sperm quality during liquid storage in tropical Tanzania. Semen was collected from six healthy boars, representing three breeds (Large White, Duroc, and Dupi), and analyzed for motility, viability, acrosome integrity, morphology, and bacterial contamination. Ejaculates with a concentration of ≥40 million sperm/mL and ≥80% motility were selected for the study. Each ejaculate was split and extended with the three extenders, then stored at 17°C, with sperm quality assessed every 24 hours for up to 7 days. Modena demonstrated superior performance across all parameters, particularly in large white boars, maintaining sperm motility at 95.00±3.50% initially and 40.00± 5.67 % by 120 hours. It also preserved viability at 97.00± 0.65 % at 0 hours, decreasing to 37.50±8.67 % by 120 hours. Additionally, Modena was free from bacterial contamination across all breeds, significantly outperforming both Zorlesco and Vim. In contrast, Zorlesco exhibited the poorest performance, with sperm motility dropping from 92.00±4.30 % at 0 hours to 31.50±5.87 % by 120 hours in Large White. It also had the highest bacterial contamination, particularly with Staphylococcus spp. and Pseudomonas spp., and affected all breeds, with Dupi being the most contaminated. Vim showed moderate performance, particularly for Duroc and Dupi, where motility started at 85.00±13.67 % and 80.00±10.00 % but dropped to 20.00±2.67 % and 25.00±1.30 % respectively, by 120 hours. Microbial analysis revealed that Staphylococcus spp. is the predominant contaminant (42.86%), followed by Pseudomonas spp. (33.33%). These findings highlight the importance of selecting semen extenders tailored to breed-specific needs and challenging environmental conditions, particularly in tropical climates where bacterial contamination and temperature fluctuations pose significant challenges to semen preservation.

Keywords: Bacterial contamination, Liquid storage, Modena, VIM, Zorlesco.

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INTRODUCTION

Artificial insemination (AI) has become an integral part of modern swine production, providing a practical method for rapidly disseminating superior genetic material from select boars to large populations of sows. This technique has significantly contributed to genetic progress and enhanced overall herd performance **(Gadea, 2003).** However, the success of AI is heavily dependent on the quality of semen used, which in turn is influenced by the semen extenders employed during storage **(Althouse and Lu, 2005).** These extenders are essential for maintaining key sperm parameters such as viability, motility, morphology, and acrosome integrity, which are crucial for successful fertilization (Knox, 2006). Worldwide, more than 99% of the estimated 19 million AIs performed each year in commercial swine operations utilize extended semen that can be transported and/or stored in a liquid state at 15-20°C for up to 5 days **(Gadea, 2003).**

In tropical climates, the selection of appropriate semen extenders is critical. Extenders must not only provide the necessary nutrients and stabilize the environment for the sperm but also possess antimicrobial properties to inhibit bacterial growth **(Luther *et al.*, 2021; Mapeka *et al.*, 2012).** Various commercial extenders, such as Beltsville Thawing Solution (BTS) and Androhep, have been widely used; however, their efficacy can vary depending on the environmental conditions and the specific breeds of boars **(Gadea, 2003).**

Breed-specific responses to semen extenders are another critical factor that can influence AI outcomes **(Tremoen., 2018).** Identification of sperm

parameters and gene variants influencing boar fertility. Different boar breeds may exhibit varying levels of resilience to environmental stressors, including temperature fluctuations and bacterial contamination **(Flowers, 2015).** For instance, Large White and Duroc boars have been reported to show robust sperm quality even under suboptimal storage conditions, while other breeds may be more susceptible to environmental stressors **(Kondracki *et al*., 2012).** These breed-specific differences highlight the need for tailored approaches in selecting the ideal semen extenders that cater to the unique requirements of each breed **(Bonet *et al*., 2012).**

In addition to chemical composition and breed-specific considerations, the physical and environmental factors of semen storage, such as temperature and humidity, play a significant role in determining sperm quality **(Malmgren and Larsson, 1984).** Heat stress, in particular, has been identified as a major factor that can adversely affect sperm motility and viability, leading to reduced fertility rates **(Boni, 2019).** The impact of heat stress is further compounded in tropical regions, where maintaining a consistent cold chain during semen storage can be challenging **(Flowers, 2022).** Studies have shown that fluctuations in storage temperature can result in cold shock, causing irreversible damage to spermatozoa **(Johnson *et al*., 2000).**

Bacterial contamination in boar semen is common, as many sources are listed, including animal faeces, preputial cavity fluids, skin and hair, water, feed, ventilation systems, collection areas, and laboratory semen processing systems **(Althouse *et al*., 2000).** The presence of contaminants in semen has a damaging effect on the sperm quality since bacteria compete with sperm for nutrients in the semen extender **(Kuster and Althouse, 2016).** Also, the bacterial metabolic byproducts, lipopolysaccharides and endotoxins, damage the sperm membrane, reduce motility, and decrease overall sperm viability **(Frydrychova *et al*., 2010).** There are a variety of bacterial contaminants in boar ejaculates reported in many countries, such as *Staphylococcus, Streptococcus, Pseudomonas, Enterobacter, Bacillus, Proteus* and *Escherichia coli* in Polish boar semen **(Gączarzewicz *et al*., 2016);** *Escherichia coli*, Proteus, Serratia, Enterobacter, Klebsiella, Staphylococcus, Streptococcus and Pseudomonas in Cuba **(Martín *et al*., 2010);** as well as Enterobacteria, *Pseudomonas spp*., Pasteurella, *Globicatella sanguinis*, *Delftia acidovorans* and *Micrococcus spp.* in Thailand **(Ngo *et al*., 2023).**

Since it was stated that bacterial contamination decreased the sperm viability by 6.4% for every extra log10 of the total bacteria count **(Ngo *et al*., 2023),** a comprehensive study on bacteriospermia in boar semen used for AI is of paramount importance for success in AI services in the pig industry using liquid semen. The present study hypothesized that the effectiveness of semen extenders in preserving boar semen quality varies across extenders and breeds. Therefore, the objectives of the present study were to evaluate the effectiveness of three commercial semen extenders: Modena, Zorlesco, and Vim across three boar breeds (Large White, Duroc, and DUupi) and to identify the bacterial contaminants affecting boar semen used for AI services in the tropical conditions of Tanzania. The findings aim to provide practical recommendations for optimizing artificial insemination protocols in similar climates.

MATERIALS AND METHODS

1. Ethical Clearance

The research was carried out per laws and regulations for conducting experiments on live animals in Tanzania. Animals were handled carefully throughout the experiment to avoid unnecessary stress while experiments were performed according to laboratory guidelines and regulations. The study was conducted after getting ethical clearance (DPRTC/R/186/22) from the Directorate of Research, Technology Transfer, and Consultancy of the Sokoine University of Agriculture.

2. Animals

This study was conducted at the College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture (SUA) . In total, six healthy purebred boars (two boars from each breedline of Duroc, Large White, and Dupi) were used in this study. The boars aged between 18 to 24 months were housed in individual, well-ventilated pens and maintained under the same environmental and sanitary conditions throughout the study, which was carried out from January to April 2024. The animals were fed twice a day (an average of 3 kg/day) with a homemade balanced concentrate containing 15-16% crude protein and 3,000 Kcal/kg metabolizable energy. The feed ingredients and their respective proportions in a 100 kg batch consist of 60 kg maize, 25 kg soybean meal, 5 mixed minerals (calcium and phosphorus), 2 kg iodized salt, 1 kg lysine (Evonik Animal Nutrition), and 7 kg vitamin and mineral premix (Kemin Industries). Clean drinking water was provided ad libitum via water nipples.

3. Experimental design

The experimental layout was a 3×3 factorial arrangement of treatment in a complete design with three types of boars and three types of commercially available extenders. The ejaculate with a high concentration of spermatozoa (40 x 10^6 sperm/mL) and more than 80% of sperm viability, motility, morphological normalcy and acrosomal integrity was thrice split and diluted in three extenders; Modena and Zorlesco **(Gadea, 2003)**, and Vim (Viddavet Ph), and also supplemented with antibiotics (1 mg/ml penicillin and 1mg/ml streptomycin). Each extended semen was divided and pipetted into 8 sterile cryovials, stored at 17°C and evaluated for sperm viability and motility

under a light microscope at 400x magnification every 24 hours.

4. Semen Collection

Semen collection was done weekly in the mornings between 08:00 and 09:00 hours using a gloved-hand method **(Frangež *et al*., 2005).** Each boar provided one semen sample per week, resulting in a total of 70 semen samples. During semen collection, the semen was filtered through gauze and only the sperm-rich fraction was collected into graduated funnel tubes and macroscopically evaluated for volume, colour and pH. Qualified ejaculates were placed in a water bath maintained at 37ºC then sent directly to the laboratory for quality analysis

5. Semen quality analysis

Semen quality assessment was carried out on 70 samples from boars of the three breeds (Large white-24, Duroc -24, and Dupi-22 samples). Each semen sample was analyzed separately to ensure that the data reflected individual performance. Quality assessment analysis included microscopic analysis of sperm concentration, morphology, motility, viability, acrosomal integrity, and bacteriology for contamination.

5.1. Spermatozoa concentration

Spermatozoa concentration was measured using a spectrophotometer assay **(Hansen *et al.*, 2006).** The sperm count was calculated based on the optical density using the formula; C = (11,170 × Absorbance) – 90. Where C = spermatozoa concentration.

5.2. Spermatozoa motility

Progressive sperm motility was assessed using a phase contrast microscope (Olympus, Tokyo, Japan) following established methods **(Kondracki *et al.*, 2017)** with a bio-thermal stage facility maintained at 37ºC. Briefly, 25 µL of 1:10 PBS-diluted semen was spread on a clean, grease-free glass slide, covered with a clean coverslip, and examined under 400× magnification of a phase contrast microscope with a bio-thermal stage facility maintained at 37ºC. 200 spermatozoa were examined during each motility assessment, and each sample was examined at three different microscopic fields. Duplicate counts were done, and the average value was recorded as percentage motility. Motility results were expressed as a percentage of cells displaying forward motility. Motility assessment was made by the same person to minimize differences during interpretation.

5.3. Spermatozoa viability and morphology assessment (Plasma Membrane Integrity)

The eosin-nigrosin staining technique was used for the assessment of spermatozoa viability **(Kondracki *et al.*, 2017).** When assessing viability for each sample, a drop (about 50 µl aliquot) of semen was mixed in a prewarmed ceramic well with the same amount (50 µl) of Eosin-Nigrosin dyes [0.6% Eosin and 5% Nigrosin dissolved in distilled water]. The suspension was incubated for 30 s at room temperature (20°C). Then a small drop (5 µL) of the mixture was spread on a clean, grease-free glass slide. Two smears were made from each sample. The smears were air-dried and examined directly for the vitality of spermatozoa using phase-contrast microscopy (1000x). The viable spermatozoa with intact plasma membranes did not get stained by the eosin dye (spermatozoa were white), whereas the dye penetrated the membrane-damaged spermatozoa and presented as dark pink or red spermatozoa heads. In each sample, the percentage of viable spermatozoa was calculated based on 200 total spermatozoa counted. In morphology assessment, at least two hundred spermatozoa from each sample were examined for normalcy and abnormalities such as proximal cytoplasmic droplet, distal cytoplasmic droplet, folded tail, coiled tail, head defects, and midpiece defects.

5.4. Acrosome integrity

Acrosome integrity was determined using a Giemsa stain procedure as described by **Bedair *et al.*, (2020).** A drop of semen was thinly smeared on a pre-warmed, grease-free slide and air-dried. The smears were fixed by immersion in 10% buffered formal saline for 15 minutes and then washed in running tap water and air-dried. The slide smear was stained using 3 ml of absolute Giemsa solution (Sigma Aldrich, Germany) prepared according to the manufacturer's instructions. Thereafter, the stained smear was rinsed briefly in distilled water, air dried, and finally examined under a light microscope (Olympus, Japan) at 100X using oil immersion. The acrosome was considered normal when the sperm head showed evenly distributed purple colour starting from the sperm anterior to the equatorial segment, while the sperm with damaged acrosome was characterized by a pale lavender head. The percentage of normal/abnormal acrosomes was then calculated.

5.5. Bacterial isolation and identification

Standard microbiological procedures to grow and characterize bacterial contaminants from both raw and extended semen were performed at the College of Veterinary Medicine and Biomedical Science Microbiology laboratory, Sokoine University of Agriculture. Initially, an equal volume (1 mL) of buffered peptone water and semen samples was thoroughly mixed. From each dilution, 1.0 mL was pipetted onto three agar plates (0.5 mL/plate): Columbia blood agar, Mac-Conkey agar (Oxoid, Hampshire, UK), and EMBL (Eosin-Methylene Blue) agar (Oxoid, Hampshire, UK), then incubated at 37°C for 24 hours. Isolated bacteria were identified using standard microbiological procedures: growth and colonial morphologic characteristics, gram staining, and biochemical characteristics following IMViC (Indole, Methyl red, Voges-Proskauer, and Citrate) tests, Triple Sugar Iron (TSI) tests, and urease tests.

6. Statistical Analysis

Data were analyzed using SPSS **(SPSS 29.0.0).** The semen quality parameters were presented as mean ± SEM across breed groups and compared by two-way variance analysis (ANOVA). Multiple analysis of variance was carried out to determine the effect of breed and extenders on the semen quality traits. The variations in parameters were regarded as significant at the level $P<0.05$.

RESULTS

Table 1 showed the average values of volume, pH, progressive motility, sperm concentration, viability, acrosomal integrity, and normalcy in the fresh semen before extension; of Large white, and Duroc and Dupi boar breeds.

Table 1: Fresh semen parameters of Large white, Duroc and Dupi boars' breeds (Mean ± SE)

Parameters	Large White	Duroc	Dupi	p-value
Volume (mL)	157.50 ± 27.57^{a}	191.15 ± 10.68^{b}	154.70 ± 54.81^{c}	0.021
pH	7.7 ± 0.25	7.2 ± 0.50	6.9 ± 0.62	0.065
Progressive Motility (%)	96.00 ± 0.71^{a}	93.75 ± 1.25^{b}	85.00 ± 2.04^{c}	0.010
Concentration (spermatozoa/mL)	$295.00 \pm 8.08 \times 10^{7a}$	$223.00 \pm 3.72 \times 10^{7b}$	$245.00 \pm 7.18 \times 10^{7b}$	0.015
Viability (%)	96.00 ± 3.81^{a}	93.00 ± 3.81^{b}	86.75 ± 1.20^{c}	0.015
Acrosomal integrity (%)	93.25 ± 1.74^{a}	90.00 ± 1.25^{a}	86.88 ± 3.07^{b}	0.018
Morphological normal spermatozoa (%)	94.90 ± 1.64	94.49 ± 0.94	92.14 ± 0.42	0.071

^{ab}Different letters indicate significant differences within rows ($P < 0.05$).

The effects of boar breeds (Large White, Duroc, and Dupi), extenders (Modena, Zorlesco, and Vim), and storage periods (1 to 7 days) on sperm viability, motility, morphology, and acrosome integrity are presented in Tables 1–4. The comparative analysis of the three extenders revealed significant differences in semen quality parameters across storage periods. From the start (0 hours) to 72 hours, there was a gradual decline in sperm motility **(Table 2)**, viability **(Table 3)** and acrosome integrity **(Table 4)** across all extenders and breeds. However, after 72 hours, a sharp decline was observed for most semen parameters. Modena maintained semen quality better than the other extenders, although at reduced levels, up to 168 hours of storage **(Table 5)**. In contrast, Zorlesco and Vim preserved semen quality up to 120 hours but showed significant reductions thereafter **(Table 2-4).**

Table 2: Effect of boar breeds, extenders and storage time on spermatozoa motility (%) after dilution with extenders.

Storage time (hr)	Breed	Extender type			p-value
		Modena	VIM	Zorlesco	
0	Large white	95.00 ± 3.50	95.00 ± 5.00	90.00 ± 5.00	0.021
	Duroc	90.00 ± 0.00	85.00 ± 13.67	85.00 ± 4.33	0.032
	Dupi	85.00 ± 5.00	80.00 ± 10.00	70.00 ± 13.67	0.018
24	Large white	85.00 ± 5.00	80.00 ± 8.67	70.00 ± 0.00	0.007
	Duroc	80.00 ± 5.00	75.00 ± 5.33	60.00 ± 5.00	0.005
	Dupi	70.00 ± 5.00	65.00 ± 4.67	55.00 ± 5.00	0.036
48	Large white	75.00 ± 5.00	70.00 ± 5.00	60.00 ± 5.00	0.008
	Duroc	70.00 ± 5.67	65.00 ± 0.00	55.00 ± 0.00	0.012
	Dupi	60.00 ± 0.00	55.00 ± 8.67	45.00 ± 5.00	0.042
72	Large white	60.00 ± 0.00	60.00 ± 5.00	50.00 ± 3.33	0.001
	Duroc	55.00 ± 7.33	50.00 ± 5.00	45.00 ± 6.77	0.001
	Dupi	50.00 ± 2.33	45.00 ± 0.00	40.00 ± 1.67	0.035
96	Large white	50.00 ± 3.33	45.00 ± 5.00	40.00 ± 4.67	0.021
	Duroc	45.00 ± 5.67	40.00 ± 0.00	35.00 ± 5.67	0.049
	Dupi	40.00 ± 9.33	35.00 ± 8.67	30.00 ± 0.00	0.021
120	Large white	40.00 ± 5.67	35.00 ± 0.00	30.00 ± 0.00	0.004
	Duroc	35.00 ± 5.00	20.00 ± 2. 67	20.00 ± 6.67	0.022
	Dupi	30.00 ±3.33	25.00 ± 1.33	10.00 ±4.33	0.008
144	Large white	25.00 ± 5.00	20.00 ± 3.33	0.00	0.045
	Duroc	20.00 ± 5.00	0.00	0.00	0.047
	Dupi	15.00 ± 5.00	0.00	0.00	0.029
168	Large white	15.00 ± 1.67	10.00 ± 3.33	0.00	0.041
	Duroc	10.00 ± 5.00	0.00	0.00	0,049
	Dupi	0.00	0.00	0.00	1.00

Table 3: Effect of boar breeds, extenders and storage time on spermatozoa viability

Storage time (hr)	Breed	Extender		
		Modena	VIM	Zorlesco
0	Large white	97.00 ± 0.65	95.00 ± 1.45	92.00 ± 4.30
	Duroc	92.00 ± 4.67	89.50 ± 3.99	82.70 ± 4.30
	Dupi	8086.00 ± 1.55	777.00 ± 8.01	70.00 ± 5.35
24	Large white	87.50 ± 3.67	83.10 ± 2.86	72.70 ± 5.33
	Duroc	78.80 ± 3.27	74.10 ± 1.99	65.90 ± 8.33
	Dupi	70.00 ± 5.33	65.60 ± 6.34	57.20 ± 3.67
48	Large white	75.00 ± 7.76	71.30 ±2.00	62.40 ± 3.40
	Duroc	67.50 ±3.00	62.80 ± 8.11	55.00 ± 5.00
	Dupi	60.00 ±4.50	56.30 ± 7.09	49.50 ± 4.53
72	Large white	62.50 ± 5.00	59.40 ± 3.37	52.10 ± 1.55
	Duroc	56.30 ± 6.67	51.40 ± 4.32	45.20 ± 4.56
	Dupi	50.00 ± 5.05	47.00 ± 7.67	41.70 ± 8.94
96	Large white	50.00 ± 4.32	47.50 ± 1.00	41.80 ± 2.34
	Duroc	45.00 ± 3.85	40.10 ± 3.65	35.40 ± 4.87
	Dupi	40.00 ± 4.21	37.50 ± 9.76	33.90 ±5.76
120	Large white	37.50 ± 8.67	35.60 ± 0.85	31.50 ± 5.87
	Duroc	33.80 ± 4.09	28.80 ± 5.00	21.30 ± 0.90
	Dupi	30.00 ± 6.99	28.20 ± 5.55	13.50± 3.22
144	Large white	25.00 ± 5.00	23.80 ± 6.02	21.20 ± 2.50
	Duroc	22.00 ± 2.02	0.00	0.00
	Dupi	19.80 ± 1.76	0.00	0.00
168	Large white	12.50 ± 4.10	11.90 ± 1.10	10.90 ± 3.45
	Duroc	11.30 ± 6.02	0.00	0.00
	Dupi	0.00	0.00	0.00

Table 4: Effect of boar breeds, extenders and storage time on spermatozoa acrosomal integrity

Storage time (hr)	Breed	Extender type		
		Modena	VIM	Zoresco
0	Large white	97.60 ± 1.00	95.20 ± 2.10	81.00 ± 4.90
	Duroc	90.50 ± 4.51	85.00 ± 1.50	73.70 ± 3.25
	Dupi	80.50 ± 2.80	75.00 ± 5.00	65.30 ± 2.34
24	Large white	87.10 ± 6.67	83.10 ± 5.08	70.80 ± 3.75
	Duroc	79.10 ± 3.65	74.10 ±2.09	64.40 ± 3.60
	Dupi	70.10 ± 3.21	65.60 ± 3.54	55.90 ± 2.58
48	Large white	74.60 ± 3.74	71.30 ± 7.10	60.60 ± 5.45
	Duroc	67.60 ± 5.49	62.80 ± 3.33	55.90± 3.21
	Dupi	59.60 ± 6.00	56.30 ± 2.33	47.60 ± 4.32
72	Large white	62.10 ± 2.30	59.40 ± 6.37	50.30 ± 2.98
	Duroc	56.20 ± 3.33	51.40 ± 3.45	47.30 ± 2.25
	Dupi	49.10 ± 1.50	47.00 ± 6.34	39.30 ±4.45
96	Large white	49.60 ± 2.34	47.50 ± 5.98	40.10 ± 3.67
	Duroc	44.80 ± 4.04	40.10 ± 4.36	36.60 ± 2.78
	Dupi	38.60 ± 2.98	37.50 ± 2.98	31.00 ± 4.50
120	Large white	37.10 ± 5.76	35.60 ± 2.37	29.80 ± 9.88
	Duroc	33.40 ± 4.44	28.80 ± 4.87	13.60 ± 3.65
	Dupi	28.10 ± 4.87	28.20 ± 2.67	5.30 ± 3.05
144	Large white	24.60 ±2.54	23.80 ± 5.08	19.60 ± 3.08
	Duroc	22.10 ± 2.77	0.00	0.00
	Dupi	17.60 ± 6.00	0.00	0.00
168	Large white	12.10 ± 1.15	12.00 ± 2.55	10.30 ± 2.34
	Duroc	11.00 ± 3.45	0.00	0.00
	Dupi	7.10 ± 3.30	0.00	0.00

Table 5: Effect of different commercial semen extenders and storage time on three boar seminal parameters.

Storage time (hr)	Parameter	Extender		
		Modena	VIM	Zorlesco
0	Sperm Motility (%)	90.00 ± 2.89	86.67 ± 4.41	81.67 ± 6.01
	Sperm Viability (%)	95.00 ± 1.45	90.00 ± 4.30	100.00 ± 0.00
	Acrosomal Integrity (%)	97.60 ± 1.00	95.20 ± 2.10	81.00 ± 4.90
24	Sperm Motility (%)	78.33 ± 4.41	73.33 ± 4.41	61.67 ± 4.41
	Sperm Viability (%)	83.10 ± 2.86	72.70 ± 5.33	87.50 ± 3.67
	Acrosomal Integrity (%)	83.10 ± 5.08	70.80 ± 3.75	87.10 ± 6.67
48	Sperm Motility (%)	68.33 ± 4.41	63.33 ± 4.41	53.33 ± 4.41
	Sperm Viability (%)	71.30 ± 2.00	62.40 ± 3.40	75.00 ± 7.76
	Acrosomal Integrity (%)	71.30 ± 7.10	60.60 ± 5.45	74.60 ± 3.74
72	Sperm Motility (%)	55.00 ± 2.89	51.67 ± 4.41	45.00 ± 2.89
	Sperm Viability (%)	59.40 ± 3.37	52.10 ± 1.55	62.50 ± 5.00
	Acrosomal Integrity (%)	59.40 ± 6.37	50.30 ± 2.98	62.10 ± 2.30
96	Sperm Motility (%)	45.00 ± 2.89	40.00 ± 2.89	35.00 ± 2.89
	Sperm Viability (%)	47.50 ± 1.00	41.80 ± 2.34	50.00 ± 4.32
	Acrosomal Integrity (%)	47.50 ± 5.98	40.10 ± 3.67	49.60 ± 2.34
120	Sperm Motility (%)	35.00 ± 2.89	26.67 ± 4.41	20.00 ± 5.77
	Sperm Viability (%)	35.60 ± 0.85	31.50 ± 5.87	37.50 ± 8.67
	Acrosomal Integrity (%)	35.60 ± 2.37	29.80 ± 9.88	37.10 ± 5.76
144	Sperm Motility (%)	20.00 ± 2.89	6.67 ± 6.67	0.00 ± 0.00
	Sperm Viability (%)	23.80 ± 6.02	21.20 ± 2.50	25.00 ± 5.00
	Acrosomal Integrity (%)	23.80 ± 5.08	19.60 ± 3.08	24.60 ± 2.54
168	Sperm Motility (%)	8.33 ± 4.41	3.33 ± 3.33	0.00 ± 0.00
	Sperm Viability (%)	11.90 ± 1.10	10.90 ± 3.45	12.50 ± 4.10
	Acrosomal Integrity (%)	12.00 ± 2.55	10.30 ± 2.34	12.10 ± 1.15

Microbial contamination was observed in 35.71% (25 out of 70) of extended semen samples examined, with different contamination levels depending on the extender used and the boar breed. The most prevalently isolated bacteria were *Staphylococcus* (42.86%) and *Pseudomonas* (33.33%). The least isolated bacteria were *Proteus mirabilis, Enterobacter spp.,* and *Escherichia coli* **(Table 6).** From the bacteriologically positive samples, nine samples (42.86%) had mixed contamination, whereas twelve samples (57.14%) presented single bacterial contamination. In the semen samples collected from the Large White boars, the semen extended with VIM was mainly contaminated with *Staphylococcus spp.* and *Pseudomonas spp.,* while the sample extended with Zorlesco only showed the presence of *Staphylococcus* spp. and *E. coli.* However, no bacterial contamination was detected in the large white semen sample extended with Modena. For the Duroc semen, contamination was found in the samples extended with both Zorlesco and VIM, which harbored *Staphylococcus spp.* and *Pseudomonas spp.,* while the sample extended with Modena remained free from contamination. Similarly, in the Dupi boar semen, Zorlesco was associated with contamination by *Pseudomonas* spp. and *Proteus*, as was VIM, but the sample extended with Modena did not show any bacterial presence. Modena was the only extender that consistently prevented bacterial contamination in all semen samples, while Zorlesco and VIM were associated with varying levels of bacterial presence.

Table 6. Summary of bacteria from doses of extended boar semen samples (n=70) isolated in pure or mixed cultures.

Bacteria	Positive semen samples	
	Number	percentage
Staphylococcus spp.	9	42.86
Pseudomonas spp.	7	33.33
Escherichia coli	3	14.29
Proteus mirabilis	1	4.76
Enterobacter spp.	1	4.76

DISCUSSION

This study assessed the effects of long-term storage of boar semen using three extenders VIM, Modena, and Zorlesco on boar semen quality parameters, including viability, motility, morphology, acrosome integrity, and bacterial contamination. The current study's findings revealed significant differences between extenders and boar breeds. Viability is one of the most critical parameters for successful AI, as it directly influences the fertilizing potential of sperm **(Knox, 2016).**

The higher viability rates observed with Modena are likely due to its antioxidant-rich formulation, which effectively combats oxidative stress, a known cause of sperm damage during storage **(Rienprayoon *et al*., 2012).** The observed differences in boar semen motility at 0 hours between fresh semen and semen diluted with extenders highlight the immediate impact of extender dilution on sperm functionality. These changes are likely due to osmotic stress, biochemical alterations, or interactions between extender components and sperm membranes **(Watson, 2000).** Such findings underscore the need to optimize extender formulations to minimize adverse effects on sperm motility immediately after dilution.

In this study, Modena consistently demonstrated superior performance in maintaining sperm viability compared to VIM and Zorlesco across all boar semen tested. Studies have shown that antioxidants like those present in Modena can reduce reactive oxygen species (ROS), thereby preserving sperm membrane integrity **(Henning *et al*., 2012; Karageorgiou *et al*., 2016).** Zorlesco, on the other hand, exhibited a more pronounced decline in viability, particularly in the Dupi breed, suggesting that its composition may be less effective in mitigating oxidative stress **(Vyt *et al*., 2004).**

Sperm motility, which is critical for fertilization, declined over time in all extenders, but Modena again outperformed the others. Although differences in motility were not statistically significant, the trends indicate that Modena may provide better protection against motility loss during storage, particularly in the Duroc breed **(Gadea, 2003).** The protective effects of antioxidants on sperm membrane stability may explain Modena's performance. Zorlesco's lower motility preservation in this study aligns with findings from previous research, which suggested that its extender composition may not be as conducive to preserving membrane fluidity and motility under storage conditions **(Buhr, 1990).** The analysis of sperm morphology revealed significant differences between extenders, with Modena again preserving normal sperm morphology more effectively than VIM or Zorlesco.

The importance of normal sperm morphology cannot be bypassed, as abnormal sperm shapes are associated with reduced motility and fertility potential **(Bryła and Trzcińska, 2015).** The use of extenders containing protective agents like bovine serum albumin and cryoprotectants may have contributed to Modena's superior performance in this parameter. Morphological abnormalities observed with Zorlesco, particularly in the Dupi breed, likely stem from the extender's inability to prevent oxidative damage effectively **(Kommisrud *et al*., 2002).** Acrosome integrity is crucial for sperm's fertilizing capability, as it plays a pivotal role in the sperm's ability to penetrate the oocyte **(Tello-Mora *et al*., 2018).** The study found significant differences in acrosome integrity among the extenders, where the Modena extender maintained higher integrity levels than Zorlesco, particularly in the Large White breed. These findings are in harmony with previous studies that emphasized that antioxidants and membrane-stabilizing agents in extenders are vital for preserving acrosome integrity during storage **(Lopez Rodriguez *et al*., 2013).** Zorlesco's poor performance may be due to its inability to preserve the acrosomal membrane effectively, leading to premature capacitation-like changes **(Watson and Holt, 2001).**

The results also demonstrated breed-specific responses to extenders, emphasizing the need for tailored approaches in AI programs. The large white breed consistently exhibited the highest sperm quality across all extenders, suggesting genetic resilience to oxidative stress and sperm membrane damage. In contrast, the Dupi breed showed a marked decline in sperm quality, particularly with Zorlesco, indicating a higher sensitivity to environmental stressors during storage **(Menegat *et al*., 2017).** These findings underscore the importance of selecting extenders that cater to breed-specific needs, as genetic factors can influence the resilience of spermatozoa to storage conditions **(Llavanera, 2024).**

Bacterial contamination is a major challenge in semen preservation, as it can significantly impact the sperm quality and consequently the success of artificial insemination (AI). The results of the current study highlight clear differences in the bacterial loads of the three extenders, VIM, Modena, and Zorlesco, used to preserve boar semen. The current findings showed semen samples extended with Zorlesco extender had the highest level of bacterial contamination, while Modena demonstrated the lowest levels of contamination. These differences could be attributed to the formulation of the extenders, where Zorlesco extender had lower levels of antimicrobial agents, allowing for greater bacterial growth, while Modena extender contained more effective antimicrobial compounds. The current results emphasized, like the previous research, the importance of antimicrobial agents in semen extenders to mitigate

bacterial contamination **(Luther *et al*., 2023).** The identification of *Staphylococcus spp. and Pseudomonas spp.* as the predominant bacteria in the semen samples is particularly concerning. These bacteria are known to produce endotoxins, which can damage the sperm plasma membrane, decrease motility, and reduce overall sperm viability **(Pinart and Morrell, 2023).**

The presence of these bacteria in extenders like Zorlesco and VIM, and their absence in Modena, highlights the effectiveness of Modena in preventing bacterial contamination. The findings of this study are consistent with those of **Tvrdá *et al*., (2021)** who observed that extenders with specific antimicrobial agents, such as Modena, can significantly reduce the bacterial loads and improve semen quality during preservation. Breed-specific responses to bacterial contamination were also evident in this study. For instance, Large White semen samples showed relatively good motility and viability even in the presence of contamination, suggesting that this breed may possess a genetic predisposition to resist bacterial damage. This observation aligns with the research by **Roca *et al*., (2004)** who suggested that some breeds may have inherent resistance to bacterial infections, particularly in tropical regions where bacterial contamination is more rampant. These breed-related differences further emphasize the need to consider breed characteristics when selecting semen extenders for AI, particularly in regions prone to higher bacterial loads.

CONCLUSIONS

In tropical regions like Tanzania, where high ambient temperatures and unreliable refrigeration can compromise semen storage, selecting the right extender is critical for AI success. The formulation of extenders that provide enhanced protection against oxidative stress, heat damage, and bacterial contamination will be crucial for improving the efficiency of AI programs in regions with challenging environmental conditions. The results of this study suggest that Modena is the most suitable extender for use in tropical climates, given its superior performance in maintaining sperm quality and its ability to minimize bacterial contamination. Bacterial contamination remains a significant challenge in AI programs, but extenders with strong antimicrobial properties, like Modena, can mitigate these risks. The findings of this study have important implications for improving the success of AI programs, particularly in tropical climates where maintaining optimal storage conditions is difficult.

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Conflict of interest

The authors declare they have no competing interest.

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