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PREFACE

In this edition, Volume 29 No 2, we proudly present articles from animal and veterinary sciences including genetic, reproduction; animal physiology; and veterinary from scientist all over the world. The articles published in this edition are:
"Optimal and Tolerant Conditions for Alginate and Calcium Chloride for the Semen Encapsulation of Pasundan Bull"; "The Effect of Different Ovarian Storage Times and Oocyte Collection Methods on the Quantity, Quality, and Maturation of Buffalo Oocytes *In Vitro*"; "Effects of Different Levels of Fish Meal in the Diet on Carcass Traits and Meat Quality of Bali Cattle"; "Utilization of Sago Waste Fermented by *Neurospora* sp. as Alternative Corn Substitution on Laying Duck Production"; "Gastroprotective Effects of *Curcuma zedoaria* in Ethanol-Induced Gastric Ulcers of Experimental Rats"; "Improving Storage Stability and Kinetics of Pasundan Bull Sperm Encapsulation Using Alginate Solid Beads"; and "Estimating the Genetic Situation of Native Upper Egypt Subpopulations of Rabbits Using Microsatellite Markers".

We extent high appreciation to all peer reviewers who make this journal academically high value. Hopefully, these articles would offer any benefit to readers and the end-users of technological innovation, and attract interests from scientists to contribute their papers to the Indonesian Journal of Animal and Veterinary Sciences.

Chief Editor

Bogor, June 2024

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LIST OF CONTENT

Optimal and Tolerant Conditions for Alginate and Calcium Chloride for the Semen Encapsulation of Pasundan Bull

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ABSTRAK

Khan A, Manan MA, Samsudewa D, Pamungkas FA, Zulfiqar H, Irfan S, Haidari K, Wulandari V, Hadi DN. 2024. Kondisi alginat dan kalsium klorida yang optimal dan toleran untuk enkapsulasi semen sapi Pasundan. JITV 29(2):56-66. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3430.

Penelitian ini bertujuan untuk menguji toleransi spermatozoa sapi pasundan dengan konsentrasi alginat dan kalsium klorida ($CaCl_2$) yang berbeda untuk mengidentifikasi kondisi optimal enkapsulasi spermatozoa. Sampel semen sapi pasundan dikoleksi setiap minggu dengan vagina buatan. Semen yang dikoleksi dibagi menjadi 9 volume yang sama. Sampel pertama diencerkan dengan tris kuning telur (kontrol), 4 sampel diencerkan dengan tris kuning telur dengan konsentrasi alginat yang berbeda (0,25; 0,5; 0,75, dan 1%), dan 4 sampel lainnya $CaCl_2$ yang berbeda (2,5; 5; 10, dan 20 mM). Evaluasi semen ditentukan setelah inkubasi 5 menit pada suhu ruang dan setelah penyimpanan selama 24 jam pada suhu 5 °C. Hasil penelitian menunjukkan setelah penyimpanan 24 jam, nilai motilitas total dan progresif pada kelompok $CaCl_2$ 2,5 mM dan alginat 0,25% tidak menunjukkan perbedaan dengan kelompok kontrol, perbedaan yang signifikan ($P<0,05$) diperlihatkan oleh kelompok $CaCl_2$ dan alginat lainnya. Nilai motilitas progresif pada kelompok dengan konsentrasi alginat lebih besar dari 0,25% menurun secara signifikan ($P<0,05$). Tidak ada perbedaan antara kelompok alginat dan $CaCl_2$ dalam nilai viabilitas dan integritas membran plasma spermatozoa. Kesimpulan dari penelitian ini adalah spermatozoa sapi pasundan dalam pengencer tris kuning telur ditambah dengan 2,5 mM kalsium klorida atau 0,25% alginat lebih toleran terhadap paparan yang lebih lama dan proses enkapsulasi spermatozoa.

Kata Kunci: Alginat, Kalsium Klorida, Toleransi Sperma, Enkapsulasi, Semen Sapi Pasundan

ABSTRACT

Khan A, Manan MA, Samsudewa D, Pamungkas FA, Zulfiqar H, Irfan S, Haidari K, Wulandari V, Hadi DN. 2024. Optimal and tolerant conditions for alginate and calcium chloride for the semen encapsulation of Pasundan bull. JITV 29(2):56-66. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3430.

The study aimed to determine the tolerance of Pasundan bull's sperm with different alginate and calcium chloride (CaCl2) concentrations to identify optimal conditions for sperm encapsulation. Semen samples were collected weekly with artificial vaginas from pasundan bulls. Pooled semen was divided into 9 equal volumes. The first sample was diluted with tris egg yolk extender (control), 4 samples were diluted with tris egg yolk extender supplemented with different concentrations of alginate (0.25, 0.5, 0.75, and 1%), and 4 other samples with CaCl2 (2.5, 5, 10, and 20 mM). Evaluation of semen was determined after 5 min incubation at room temperature and after overnight storage at 5 °C. Results showed that after 24h of refrigerated storage, the values for total motility and progressive motility in the groups with 2.5 mM CaCl2 and 0.25% alginate showed no difference compared to the control group. In contrast, a significant difference ($P<0.05$) was found between the other groups with CaCl2 and alginate. The progressive motility value in the group with alginate concentration greater than 0.25% decreased significantly ($P<0.05$). There was no difference between the groups (both alginate and CaCl2) in the spermatozoa viability and plasma membrane integrity variable. In conclusion, sperm with 2.5 mM calcium chloride and 0.25 % alginate was more tolerant of appropriate prolonged exposure and the sperm encapsulation process.

Key Words: Alginate, Calcium Chloride, Sperm Tolerance, Encapsulation, Pasundan Bull Semen

INTRODUCTION

Pasundan cattle are local Indonesian livestock recognized as a genetic resource based on the Decree of the Minister of Agriculture of the Republic of Indonesia Number 1051/Kpts/SR.120/10/2014 dated 13 October 2014. The production of frozen pasundan bull semen and its distribution through artificial insemination (AI) technology was used to preserve and maintain this local Indonesian genetic heritage (Sutarno & Setyawan 2015).

The success of the application of AI technology is determined by the high percentage of pregnancies achieved, which are influenced by the reproductive health of females, the quality of the inseminated semen, and the practical and timely application of AI techniques (Abdel Aziz et al. 2023). The low probability of a pregnancy resulting from incorrect timing of AI can be due to either a decrease in sperm viability while waiting for the ovum to be ready for fertilization or loss of the sperm due to contraction and phagocytosis processes in the female reproductive tract (Anchordoquy et al. 2022).

Sperm encapsulation technology can potentially solve the problems and challenges mentioned above. From a male perspective, it increases the viability and stability of the sperm membrane over a more extended period. The sperm are gradually released from the microcapsule to remain available for a more extended period, waiting to be released from the encapsulation membrane when the egg is ready for ovulation (Kang et al. 2014). From a female perspective, on the other hand, sperm reflux is reduced during AI, and the process of phagocytosis in the female reproductive tract is prevented, allowing the technology to remain available for longer and potentially solving problems related to the duration of artificial insemination (Sánchez-Sánchez et al. 2022).

Biomaterials, both natural and synthetic polymers, have been used as matrices in the sperm encapsulation process. Alginate is an example of an organic anionic polysaccharide derived from brown seaweed and is known for its general properties of biocompatibility, non-immunogenicity, and non-toxicity (Ahmad et al. 2021). Numerous studies have shown that alginate has a rich reservoir of antioxidants (Król et al. 2017), antibacterial (Karbassi et al. 2014), antiviral (Ahmadi et al. 2015), and fungicidal (Tøndervik et al. 2014; Hernández-Figueroa et al. 2024) properties. Calcium chloride ($CaCl_2$) is a versatile chemical known for its use in de-icing highways in the winter, as it can lower the freezing point of water so that ice cannot form, making it the best choice for encapsulating sperm at low temperatures (Nutile & Solan 2019). Some studies use a combination of alginate and $CaCl_2$ in sperm encapsulation, including in goat (Silva et al. 2015), dairy cattle (Kusumaningrum et al. 2015), sheep (Thiangthientham et al. 2020), mice (Veisi et al. 2022), and horses (Pruß et al. 2022).

However, some of these studies still use very different concentrations of alginate and $CaCl_2$, although too high concentrations would impair sperm viability or motility. In contrast, an insufficient concentration would not provide sufficient protection against oxidative stress during storage (Pruß et al. 2022). Therefore, in order to maintain sperm in animal breeding over a long period, it is necessary to perform tolerance tests to facilitate the creation of a stable environment that balances structural integrity in the encapsulation matrix while preserving sperm function (Kunkitti et al. 2016). The research aims to evaluate the optimal and tolerant conditions of pasundan bull semen at different concentrations of alginate and $CaCl_2$ for the sperm encapsulation approach.

MATERIALS AND METHODS

Animals

The study is conducted at the Center for Artificial Insemination Breeding and Development of Beef Cattle, Ciamis, West Java, Indonesia. A total of 3 Pasundan bulls aged 3-6 years with body weights range of 380-430 kg were used in this study. The bulls were fed similar diets consisting of 10% Pennisetum purpureum grass and 1% commercial concentrate with 16% crude protein. Fresh drinking water is given ad libitum, and feed is administered twice daily. Approval (Protocol No. 011/KE.02/SK/01/2023) was obtained from the Ethical Clearance and Foreign Research Permit, Directorate of Management for Research and Innovation Permit, and the Scientific Authorities, National Research and Innovation Agency before the start of the experiments. Animal care and experimental protocols followed the Manual for the Care and Use of Laboratory Animals.

Diluents preparation

The primary diluent used for this study is Tris egg yolk. Tris buffer consisted of 3.03 g Tris (hydroxymethyl) aminomethane, 1.78 g citric acid, and 1.25 g fructose in 100 ml aquabidest, as Darussalam et al. (2020) used. Tris egg yolk consisted of 85% Tris buffer, 15% egg yolk, 1000 IU/mL penicillin, and 1 mg/mL streptomycin, which were thoroughly mixed and used as a diluent as done by Kusumaningrum et al. (2015) for the preservation of dairy bull semen.

Semen collection

By the Standard Procedure of the Center for Artificial Insemination, Breeding and Development of Beef Cattle, semen samples were collected from each pasundan bull once a week for one month using an artificial vagina in the morning between 8:00 to 10:30 a.m. Before pooling, the Computer Assisted Sperm Analyzed (CASA) instrument Androvision® (Minitube-Germany) was used to measure the motility of the sperm at 100X magnification using a heating plate. In this study, semen samples with less than 75% motility values were not used. Nine equal volumes of pooled semen from each bull were obtained. One sample was diluted with tris egg yolk extender (control); four samples were diluted with tris egg yolk extender supplemented with various concentrations of sodium alginate or sodium salt from brown algae (Sigma, A2033 CAS Number. 9005-38-3),

0.25, 0.5, 0.75, and 1%. The remaining four samples were diluted with tris egg yolk extender supplemented with different concentrations of Calcium chloride or $CaCl_2$ (Sigma SA C4901), 2.5, 5, 10, and 20 mM. The final concentration of sperm was around 100×10^6 sperm ml^{-1}. After that, the diluted semen was placed into a 15 ml corning tube. Then, the samples were placed in a glass beaker with a water jacket. Sperm microscopic analysis was assessed during a 5-minute incubation period at room temperature and following an overnight storage period at 5°C.

Evaluation of the tolerance of sperm to alginate and calcium chloride

Sperm motility was assessed using the CASA instrument Androvision® (Minitube-Germany). An 8 µl semen sample was mixed with 4 ml of 0.9% NaCl; the sample was dropped on a slide with a coverslip, observed using a microscope with objective magnification of 10x10 connected to a computer and installed with CASA, observing five fields of view, and scoring from 0% to 100 %. The motility value variables of CASA are total motility (TM), progressive motility (PM), fast motility (FM), slow motility (SM), local motility (LM), immotile (IM), the curvilinear velocity (VCL), straight-line velocity (VSL), average pathway velocity (VAP), linearity (LIN), straightness (STR), wobbles (WOB), the amplitude of lateral displacement (ALH), and beat cross frequency (BCF), as shown in Table 1.

Sperm viability was determined using eosin-nigrosin staining (each 100 mL contains 1.1 g eosin Y, 0.5 g Na citrate, 6.67 g nigrosin, and distilled water to 100 mL) according to Singh et al. (2022) with modifications. A semen sample of 2 µL and 16 µL eosin-nigrosin dye were homogenized. A test was made on a glass object, dried on a hot plate, and viewed with a microscope at 10×40 magnification. The preparation is tested in at least five fields of view or >200 sperm. Alive (viable) sperm are marked with an unstained (transparent) head, and dead sperm are marked with a purple head.

Plasma membrane integrity was assayed with hypoosmotic swelling solution (HOS test) (each 100 ml has 0.736 g Na citrate, 1.352 g fructose, and 100 ml distilled water) according to Hufana-Duran et al. (2015). Three hundred (300) µl of the HOS test solution was placed in microtubes, and 3 µl of the semen sample was dropped and incubated in a water bath at 37 °C for 30 min. Then, 15 µL of the mixture was removed and dropped onto a microscope slide, covered with a cover glass, and observed with a microscope at 400× magnification. Observations were made in at least two hundred cells. Sperm with intact plasma membrane show a reaction, namely a circular on the end of the tail.

Statistical analysis

The data collected from the different groups were analyzed using statistics according to various criteria. A One-way ANOVA was explicitly used to calculate each parameter's mean values and group differences, supported by the SPSS software when there was only one independent variable (such as the control) and several dependent variables. The results of the ANOVA test indicated significant differences between at least two

Table 1. Sperm kinematic descriptors and their corresponding definition (Barbas et al. 2018)

Kinematic Descriptor	Measurement Unit	Descriptor Definition
Curvilinear velocity (VCL)	(µm/s)	The average path velocity of the sperm head along its true
Straight-line velocity (VSL)	(µm/s)	The average path velocity of the sperm head along its true trajectory per unit time
Average path velocity (VAP)	(µm/s)	The average velocity of the sperm head along its average trajectory per unit time
Linearity index (LIN)	(%)	The ratio between VSL and VCL (x 100)
Straightness index (STR)	(%)	The ratio between VSL and VAP (x 100)
Wobble coefficient index (WOB)	(%)	The ratio between VAP and VCL (x 100)
Amplitude of lateral head displacement (ALH)	(µm)	The average value of the extreme side-to-side movement of the sperm head in each beat cycle
Beat cross-frequency (BCF)	(Hz)	The frequency with which the actual sperm trajectory crosses the average path trajectory

groups if the p-value was below a predetermined significance level (P≤0.05). Post-hoc tests, such as the Duncan test, were used to detect group differences in significant cases (Chen et al. 2019).

RESULTS AND DISCUSSION

The results of the analysis of total motility, progressive motility, and kinematics of sperm (fast, slow, circle, local, and immotile) after incubation in a tris egg yolk diluent containing different concentrations of alginate and calcium chloride are shown in Figure 1 and Table 2-5. In the $CaCl_2$ groups, there were no differences in the total and progressive motility characteristics that were assessed at 0 hours. However, after 24h of cold storage, the total motility value was nsignificant in the 2.5 mM $CaCl_2$ compared with a control group. In contrast, a significant difference was found between the other groups (P<0.05). At the same time, the values of progressive motility in the 2.5 and 5 mM $CaCl_2$ groups were almost identical to those of the control group.

As for fast and slow motility in the $CaCl_2$ groups, after 24h of cold storage, the numerically highest rate was found in the 2.5 mM $CaCl_2$ group, and a significant statistical difference was found compared with the 20 mM $CaCl_2$ groups (P<0.05). The local and immotile values were higher in the 20 mM group than in the other groups (P<0.05). In addition, there were no differences between groups in all kinematic sperm characteristics assessed at 0 hours in the $CaCl_2$ groups. For kinematic sperm such as VSL, VAP, LIN, and ALH, values found a significant difference between the other groups (P<0.05) after 24 hours of cold storage. However, the values in the 2.5 mM $CaCl_2$ groups were almost identical to the control.

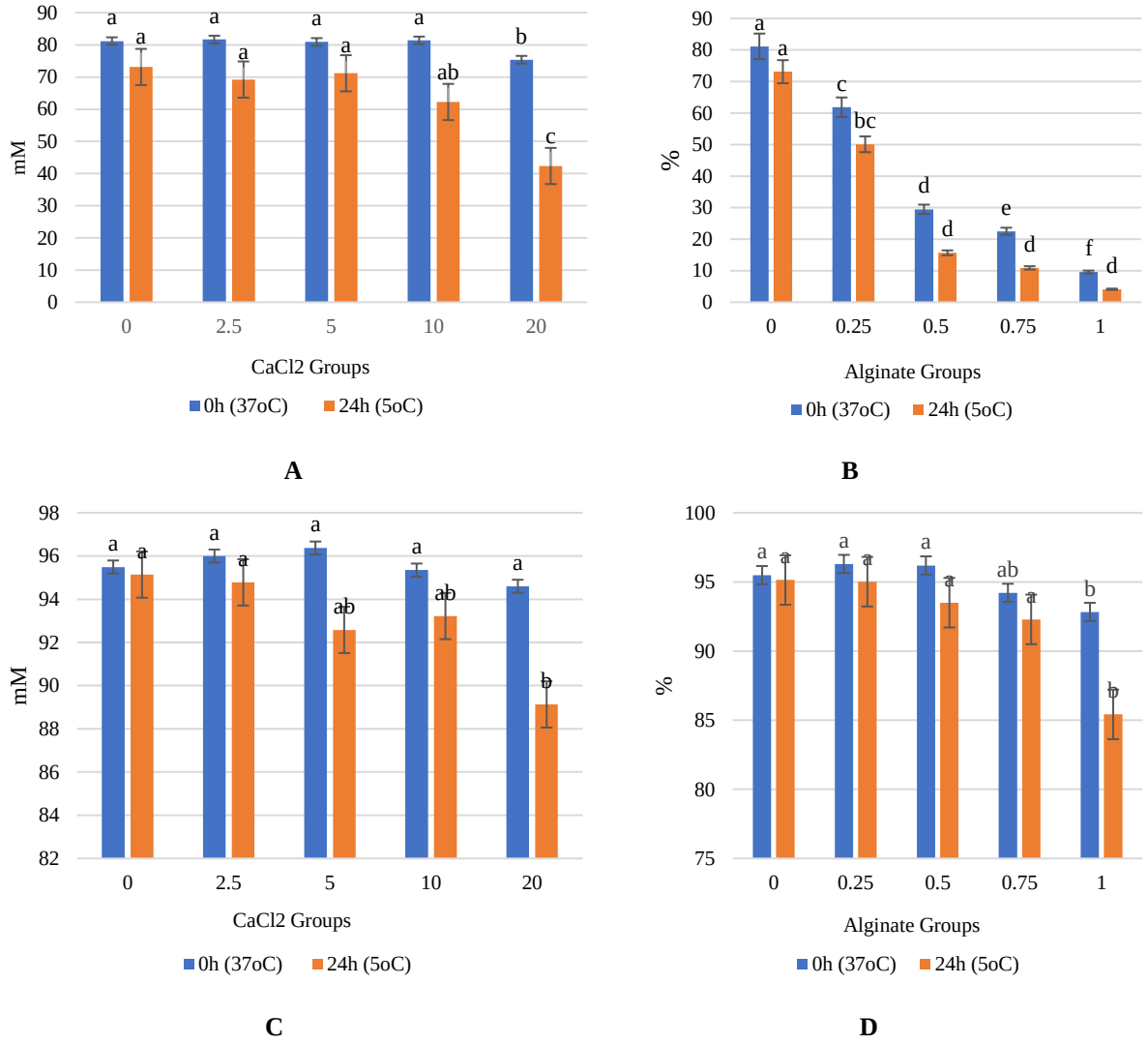

Figure 1. Evaluated progressive motility (A and B) and total motility (C and D) of sperm after incubation in a tris egg yolk diluent containing different amounts of alginate and calcium chloride, and the data were examined at 0hours (37ºC) and 24hours (5ºC). Statistically significant differences (P≤0.05) between the analysis times are labeled with different letters

Table 2. Assessment of sperm motility after incubation in a tris egg yolk diluent containing different amounts of calcium chloride and the data were examined at 0 hours (37ºC) and 24 hours (5ºC).

Calcium Chloride Group (mM)	Sperms (%)									
	Fast Motility		Slow Motility		Circle Motility		Local Motility		Immotile	
	0hour	24hours	0hour	24hours	0hour	24hours	0hour	24hours	0hour	24hours
0	11.33±2.48^{ab}	10.47±1.99^{a}	67.53±4.05^{a}	56.67±3.92^{a}	1.14±0.94^{a}	0.26±0.07^{a}	14.33±0.92^{b}	27.72±5.08^{b}	4.50±0.74^{b}	4.85±0.86^{c}
2.5	12.07±1.95^{ab}	11.18±1.61^{a}	69.14±1.46^{a}	57.58±3.17^{a}	0.46±0.19^{b}	0.45±0.15^{a}	14.32±1.37^{b}	25.56±4.01^{c}	3.99±0.60^{b}	5.21±0.74^{c}
5	15.09±1.82^{a}	10.44±2.23^{a}	66.59±1.58^{a}	50.76±6.99^{ab}	0.65±0.16^{b}	0.25±0.07^{a}	15.45±2.88^{b}	31.12±7.43^{a} b	3.62±0.29^{b}	7.42±1.90^{ab}
10	13.61±1.65^{ab}	9.77±2.10^{a}	65.47±2.71^{a}	52.15±3.97^{a}	0.46±0.16^{b}	0.31±0.19^{a}	13.95±1.72^{b}	30.96±4.99^{b} c	4.65±0.69^{b}	6.78±0.92^{bc}
20	10.05±1.86^{b}	5.11±1.79^{b}	64.87±1.81^{a}	38.18±8.77^{b}	0.46±0.26^{b}	0.04±0.01^{b}	20.05±2.76^{a}	45.78±7.73^{a}	5.39±0.99^{a}	10.87±2.68^{a}

Statistical results are given as Mean ± SE; the One-Way ANOVA analysis revealed significant differences (P≤0.05) between the alphabetically separated groups

Table 3. Assessment of sperm motility after incubation in a tris egg yolk diluent containing different amounts of alginate and the data were examined at 0 hours (37ºC) and 24 hours (5ºC)

Alginate Group (%)	Sperms (%)									
	Fast Motility		Slow Motility		Circle Motility		Local Motility		Immotile	
	0h	24h	0hour	24hours	0hour	24hours	0hour	24hours	0hour	24hours
0	11.33±2.48^{a}	10.47±1.9^{a}	67.53±4.05^{a}	56.67±3.92^{a}	1.14±0.94^{a}	0.26±0.07^{a}	14.33±0.92^{a}	27.72±5.08^{a}	4.50±0.74^{a}	4.85±0.86^{a}
0.25	1.69±0.49^{b}	0.12±0.02^{b}	60.17±2.07^{a}	48.93±2.45^{a}	0.00±0.00^{b}	0.00±0.00^{b}	36.01±3.74^{b}	44.91±3.12^{b}	3.69±0.68^{a}	4.97±1.04^{a}
0.5	0.22±0.73^{b}	0.12±0.02^{b}	29.26±0.46^{b}	15.53±1.90^{b}	0.00±0.00^{b}	0.00±0.00^{b}	66.70±0.66^{b}	77.83±1.47^{c}	3.81±0.40^{a}	6.50±0.78^{a}
0.75	0.24±0.03^{b}	0.12±0.05^{b}	22.26±1.45^{c}	10.76±1.73^{b}	0.00±0.00^{b}	0.00±0.00^{b}	71.70±1.59^{c}	81.41±1.67^{c}	5.78±0.76^{a}	7.70±0.98^{a}
1	0.05±0.02^{b}	0.07±0.03^{b}	9.52±1.67^{d}	4.03±0.48^{b}	0.00±0.00^{b}	0.00±0.00^{b}	83.26±1.29^{c}	81.32±1.86^{c}	7.16±0.77^{a}	14.57±1.58^{b}

Statistical results are shown as Mean ± SE; the One-Way ANOVA analysis revealed significant differences (P≤0.05) between the alphabetically distinct groups

Calcium chloride is an essential component in extenders because it prevents the formation of ice crystals from water and thus protects the sperm from potential damage (Nutile and Solan 2019). The biocompatibility results of $CaCl_2$ at a concentration of 2.5 mM and 5 mM against sperm of pasundan bulls show no significant difference in terms of the tested factors after exposure at 0 hours at room temperature and after 24 hours of cooling at 5 °C. According to Ashizawa et al. (2013), $CaCl_2$ does an excellent job restoring sperm motility. The results show that sperm has a good tolerance to these $CaCl_2$ concentrations at room temperature, but prolonged exposure to high concentrations (20Mm $CaCl_2$) significantly decreases these parameters. Faustini (2011) reported that CaCl2 concentrations of 5-25 mM were used for sperm tolerance in other species. The loss of sperm motility of pasundan semen from 81.39% to 62.25% after 24 hours of cold storage was attributed to calcium chloride and previously reported in numerous studies for other species (Fernandez-Novo et al. 2021).

The increase in the internal calcium ion concentration stimulates the cyclic adenosine monophosphate (cAMP) signaling pathway, activating sperm motility patterns in different species. Calcium chloride is a prerequisite for regulating sperm motility in different species (Bondarenko et al. 2017). Ionic calcium concentration not only plays a role in triggering sperm motility but is also related to the flagellar beating pattern (Bondarenko et al. 2017; Dzyuba et al. 2017). Consequently, increased extracellular calcium concentration promotes asymmetrical movement and curvilinear velocity (VCL) of sperm (Sandoval-Vargas et al. 2021). However, sperm activated with the calcium-free solution tended to reduce VCL and VAP values. Pérez et al. (2016) also found a moderate reduction in VCL and VAP values and the VSL and BCF of sperm activated with a calcium-free solution.

In the alginate groups, the total motility values in the 0.25 and 0.5 % alginate groups were numerically almost the same in the control, with no statistical difference. However, after 24h of cold storage, the total motility value in the 0.25% alginate group was numerically almost the same. It did not differ from the control group, while a statistical difference was found in the 1% alginate group ($P<0.05$). On the other hand, the value of progressive motility in the alginate groups was found to decrease with increasing alginate concentration. The control group had the highest value of progressive motility, which gradually decreased, followed by the 0.25, 0.5, 0.75, and 1% alginate groups. However, after the 0.25% alginate group, the rate of progressive sperm motility decreased dramatically, and as Fig. 1 shows, there was a significant difference ($P<0.05$) between the other groups. In addition, in the alginate groups, after 24 hours of cold storage, the highest rate of fast and slow motility was found in the control group, and a statistical difference was found between the other groups ($P<0.05$). Fast and slow motility was highest in the control group, followed by the 0.25, 0.5, 0.75, and 1 % alginate groups. VCL, VSL, VAP, and ALH values decreased with increasing alginate concentration in the alginate groups. The control group had the highest value, which gradually decreased, followed by the groups with 0.25, 0.5, 0.75, and 1% alginate.

Alginate is commonly employed as a gelling agent. Therefore, it was expected to increase the viscosity of extenders and cause the sperm velocities in samples containing the alginate-fortified extender to be lower (Kumar et al. 2019). Fertilization requires the female reproductive tract to maintain sperm motility for the whole course of the reproductive cycle. As demonstrated by the outcomes of the in vitro incubation test, the current study's findings suggest that supplementing semen extenders with alginate can result in sperm motility being sustained for extended durations of time. Additionally, a spermatozoon needs a functioning and undamaged plasma membrane for progressive motility to be sustained for the cell to have the ability to fertilize. The current study suggests that adding alginate to egg yolk extender reduced membrane damage during preservation. This discovery supports the outcomes of an earlier investigation wherein the incorporation of alginate showed efficacy in preserving the plasma membrane integrity of salmonid fish sperm following brief preservation at 4°C (Merino et al. 2017). Alginate is expected to increase the viscosity of the extender as it is widely used for gelling agents. Due to the high viscosity, sperm velocity decreases, resulting in sperm energy conservation. The result showed that alginate is also used as a food and helps in the controlled release of nutrients; in this way, it increases sperm lifespan and helps in storage (Gheorghita Puscaselu et al. 2020). An increase in alginate content causes a decrease in progressive motility and an increase in local sperm motility. Possibly due to the higher viscosity of the medium, higher alginate levels were associated with decreased progressive motility and increased local sperm. As in Feyzmanesh et al. (2022), sperm motility decreases due to alginate, but the other parameters, like viability and membrane integrity, remain the same as the control. Pruß et al. (2022) also reported similar findings while working on the alginate encapsulation of stallion sperm to increase its storage stability. These concentrations show that sperm have an excellent tolerance to alginate hydrogel. Besides that, Veisi et al. (2022) conclude that alginate hydrogel enhances the proliferation of spermatogonial stem cells.

The results of viability and membrane integrity analysis after incubation in a tris egg yolk diluent with different concentrations of alginate and calcium chloride are shown in Figure 2. In the $CaCl_2$ groups, the viability

Table 4. Kinematic characteristic of Pasundan bull sperm in extender with different concentrations of $CaCl_2$ during storage

Kinematic	Hour	Calcium chloride group (mM)				
		0	2.5	5	10	20
VCL (µm/s)	0	76.54±1.89^{a}	72.05±3.20^{a}	73.21±4.05^{a}	76.89±4.12^{a}	70.83±4.10^{a}
	24	55.70±8.88^{a}	62.26±4.48^{a}	54.37±9.62^{a}	50.22±7.02^{a}	37.79±11.11^{a}
VSL (µm/s)	0	42.55±3.39^{a}	41.74±8.71^{a}	41.08±1.73^{a}	46.24±4.72^{a}	40.19±1.98^{a}
	24	35.41±4.14^{ab}	39.96±1.92^{a}	29.84±5.51^{ab}	30.03±3.16^{ab}	19.56±6.24^{b}
VAP (µm/s)	0	46.46±3.23^{a}	42.64±2.00^{a}	43.85±1.81^{a}	48.89±4.89^{a}	42.63±2.01^{a}
	24	35.43±3.83^{ab}	36.78±2.38^{a}	31.73±5.71^{ab}	32.05±3.29^{ab}	21.06±6.52^{b}
LIN (%)	0	0.57±0.02^{a}	0.55±0.01^{a}	0.56±0.01^{a}	0.59±0.03^{a}	0.56±0.01^{a}
	24	0.53±0.01^{ab}	0.55±0.01^{a}	0.54±0.01^{ab}	0.54±0.01^{ab}	0.50±0.01^{b}
STR (%)	0	0.94±0.00^{a}	0.94±0.00^{a}	0.93±0.00^{a}	0.94±0.00^{a}	0.94±0.00^{a}
	24	0.93±0.00^{a}	0.93±0.00^{a}	0.93±0.00^{a}	0.93±0.00^{a}	0.91±0.01^{a}
WOB (%)	0	0.60±0.02^{a}	0.60±0.01^{a}	0.60±0.01^{a}	0.64±0.02^{a}	0.59±0.01^{a}
	24	0.57±0.01^{a}	0.58±0.00^{a}	0.58±0.01^{a}	0.57±0.01^{a}	0.54±0.01^{a}
ALH (µm)	0	1.94±0.02^{a}	1.95±0.06^{a}	1.95±0.09^{a}	1.96±0.09^{a}	1.82±0.09^{a}
	24	1.66±0.12^{a}	1.66±0.10^{a}	1.42±0.20^{ab}	1.49±0.13^{ab}	1.06±0.24^{b}
BCF (Hz)	0	1.16±0.05^{a}	1.34±0.14^{a}	1.27±0.09^{a}	1.20±0.07^{a}	1.20±0.12^{a}
	24	1.58±0.17^{a}	1.54±0.08^{a}	1.41±0.14^{a}	1.55±0.11^{a}	1.40±0.04^{a}

VCL= Curvilinear velocity, VSL= Straight-line velocity, VAP= Average path velocity, LIN= Linearity index, STR= Straightness index, WOB= Wobble coefficient index, ALH= Amplitude of lateral head displacement, BCF= Beat cross-frequency. The One-Way ANOVA analysis showed significant differences (P<0.05) between the alphabetically separate groups; the statistical data are shown as Mean±S.E

Table 5. Kinematic performance of Pasundan bull sperm diluted in extender with varied concentrations of alginate during different storage durations

Kinematic	Observation time (hour)	Alginate group (%)				
		0	2.5	5	0.75	1
VCL (µm/s)	0	76.54±1.81^{a}	46.66±2.00^{b}	29.03±0.66^{c}	25.60±1.33^{c}	17.71±2.04^{d}
	24	55.70±8.88^{a}	40.64±1.81^{b}	21.87±0.95^{c}	18.44±1.20^{c}	12.52±0.75^{c}
VSL (µm/s)	0	42.55±3.39^{a}	27.18±1.35^{b}	16.45±0.76^{c}	16.37±0.69^{c}	11.97±1.37^{c}
	24	35.41±4.14^{a}	23.28±1.03^{b}	12.97±0.42^{c}	11.16±0.68^{c}	8.57±1.06^{c}
VAP (µm/s)	0	46.46±3.23^{a}	28.01±1.11^{b}	18.68±0.43^{c}	16.88±0.67^{cd}	11.98±1.36^{d}
	24	35.43±3.83^{a}	23.55±1.07^{b}	13.85±0.52^{c}	12.05±0.69^{c}	8.47±0.58^{c}
LIN (%)	0	0.57±0.02^{bc}	0.56±0.00^{c}	0.60±0.00^{abc}	0.61±0.01^{b}	0.62±0.00^{a}
	24	0.53±0.01^{b}	0.54±0.00^{b}	0.58±0.00^{a}	0.60±0.01^{a}	0.61±0.02^{a}
STR (%)	0	0.94±0.00^{a}	0.94±0.00^{a}	0.94±0.00^{a}	0.94±0.00^{a}	0.93±0.00^{a}
	24	0.93±0.00^{a}	0.93±0.00^{a}	0.92±0.00^{ab}	0.92±0.00^{ab}	0.91±0.00^{b}
WOB (%)	0	0.60±0.02^{bc}	0.60±0.00^{c}	0.64±0.00^{ab}	0.66±0.01a	0.67±0.00^{a}
	24	0.57±0.01^{c}	0.57±0.00^{c}	0.63±0.00^{b}	0.65±0.01^{ab}	0.67±0.01^{a}
ALH (µm)	0	1.94±0.02^{a}	1.29±0.04^{b}	0.90±0.02^{c}	0.87±0.02^{c}	0.62±0.04^{d}
	24	1.66±0.12^{a}	1.21±0.03^{b}	0.77±0.04^{c}	0.66±0.02^{cd}	0.49±0.02^{d}
BCF (Hz)	0	1.16±0.05^{b}	1.59±0.11^{a}	1.63±0.06^{a}	1.57±0.04^{a}	1.54±0.14^{a}
	24	1.58±0.17^{a}	1.62±0.18^{a}	1.40±0.17^{a}	1.25±0.19^{a}	1.16±0.11^{a}

VCL= Curvilinear velocity, VSL= Straight-line velocity, VAP= Average path velocity, LIN= Linearity index, STR= Straightness index, WOB= Wobble coefficient index, ALH= Amplitude of lateral head displacement, BCF= Beat cross-frequency. The One-Way ANOVA analysis showed significant differences (P<0.05) between the alphabetically separate groups; the statistical data are shown as Mean±S.E

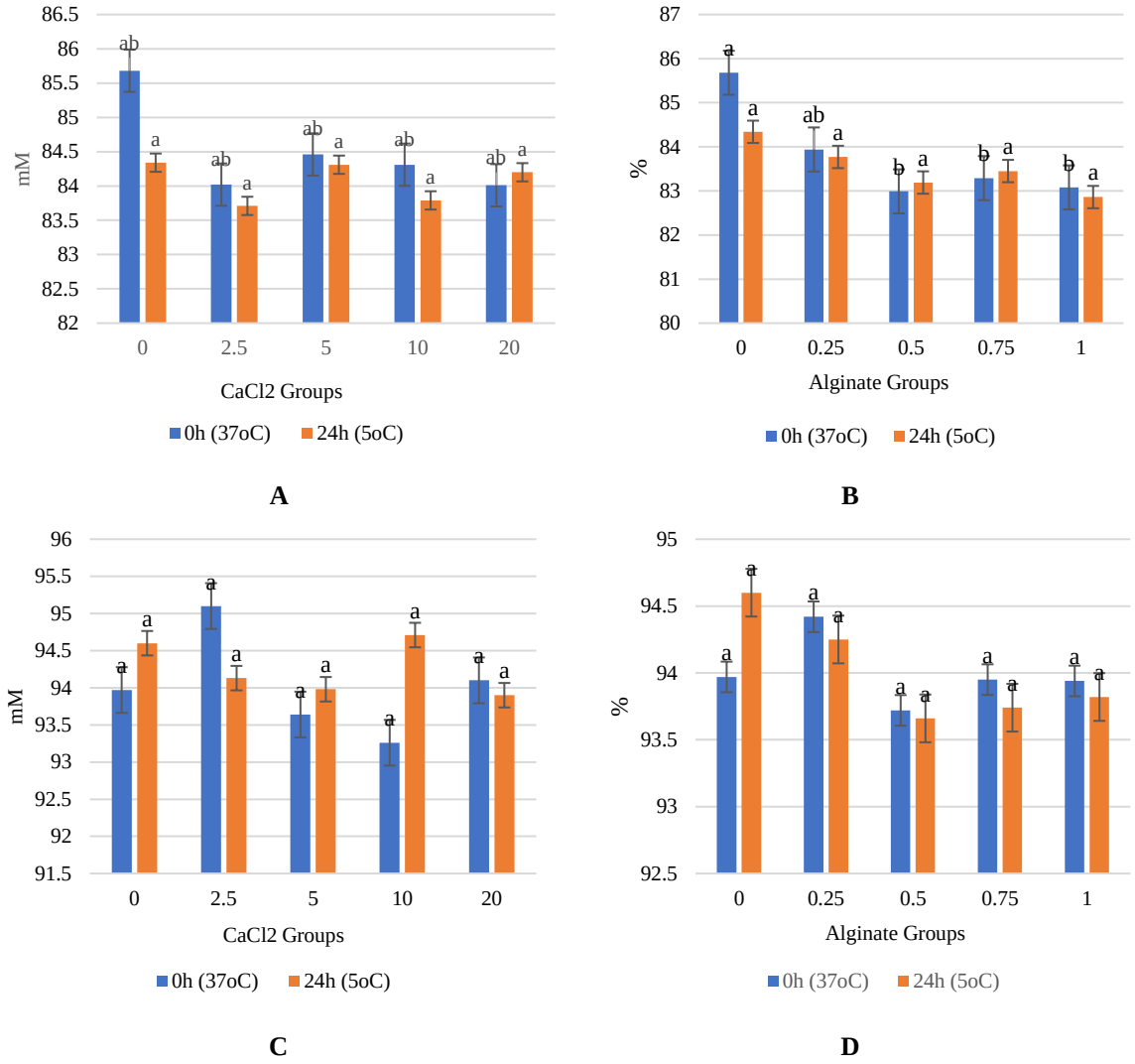

Figure 2. Evaluation of viability (A and B) and membrane integrity (C and D) of sperm after incubation in a tris egg yolk diluent containing different concentrations of alginate and calcium chloride and the data were examined at 0 hours (37ºC) and 24 hours (5ºC). Statistically significant differences (P≤0.05) between the analysis times are indicated with different letters

values of the 2.5 mM $CaCl_2$ groups were numerically the same as the control group, with a statistical difference between the other groups (P<0.05) when tested at 0 hours. However, after 24 hours of cold storage, there was no difference between the groups regarding viability parameters. Viability assessment provides information about the overall health of sperm under different experimental or clinical conditions (Eckel et al. 2017). The interaction of different elements, such as the presence of chemicals like calcium chloride and alginate, in combination with physical properties like temperature and nutrition availability, controls cell survival and metabolic activity (Yuan et al. 2022).

However, in the alginate groups, there was no difference in viability parameters after incubation in a tris egg yolk diluent containing different alginate groups, which were examined at 0 and 24 hours. In addition, the results of many studies indicate that a slight increase in the viscosity of alginate leads to an improvement in the semen quality in sheep (Yániz et al. 2005), rabbits (Rosato & Iaffaldano 2011) and boars (Gil et al. 2014). The results of the present study indicate that supplementations of semen extenders with alginate may destabilize the plasma membrane components when temperatures decrease during preservation (Swami et al. 2017). The results of the present study suggest that supplementation of alginate in the extender minimized the membrane damage during preservation. This finding confirms the results of a previous study in which adding alginate to boar semen improved the integrity of the plasma membrane after thawing (Hu et al. 2014).

In addition, the statistical analysis results show no difference between the groups in terms of plasma membrane integrity parameters after incubation in a tris

egg yolk diluent containing different alginate and calcium chloride concentrations at 0 and 24 hours. Since the plasma membrane serves as a barrier that regulates the flux of ions and molecules, maintaining its integrity is essential for cell activity and overall survival (Dias & Nylandsted 2021); this means that, within the range studied, neither the different concentrations of alginate nor calcium chloride had a significant effect on the plasma membrane integrity. The stability of the plasma membrane integrity under these different experimental conditions demonstrates that the different concentrations of calcium chloride and alginate did not affect the structural integrity of the cell's plasma membrane.

CONCLUSION

In this study, we demonstrate the sperm tolerance of Pasundan bulls to calcium chloride and alginate concentrations to select conditions for sperm encapsulation. Sperm with 2.5 mM calcium chloride and 0.25 % alginate were more tolerant to corresponding prolonged cold storage and can be used as a concentration for sperm encapsulation.

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The Effect of Different Ovarian Storage Times and Oocyte Collection Methods on the Quantity, Quality, and Maturation of Buffalo Oocytes *In Vitro*

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ABSTRAK

Hendri, Udin Z, Masrizal, Jaswandi, Nanda S. 2024. Pengaruh berbagai waktu penyimpanan dan metode koleksi oosit terhadap kuantital, kualitas dan maturasi *in vitro* kerbau. JITV 29(2):67-78. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3192.

Penelitian ini bertujuan untuk mengetahui pengaruh metode koleksi oosit dan lama penyimpanan terhadap jumlah, kualitas, dan kecepatan maturasi oosit kerbau secara *in vitro*. Ovarium dikoleksi dari rumah potong hewan pada empat waktu penyimpanan yang berbeda (0, 3, 6, 9 dan 12 jam) sebelum diproses dengan tiga teknik: slicing, aspirasi, serta gabungan slicing dan aspirasi. Menurut morfologi lapisan sel kumulus yang melekat erat pada zona pelusida oosit dan penampakan sitoplasma oosit, oosit dibagi menjadi 4 kelompok: A, B, C, dan D. Kompleks kumulus-oosit (COCs) dipindahkan ke media maturasi *in vitro* (IVM) TCM-199 (Sigma) selama 24 jam. Tahapan pematangan inti diperiksa dengan pewarnaan COC yang diperbesar dari masing-masing kelompok. Hasil penelitian menunjukkan bahwa rata-rata jumlah oosit per ovarium berkisar antara 8,08±1,28 hingga 9,83 ±1,89 dan tidak berpengaruh nyata (P>0,05) terhadap jumlah oosit per ovarium. Rata-rata kualitas oosit per ovarium yang berkisar antara 0,2 hingga 5,00 merupakan pengaruh yang sangat nyata antara lama penyimpanan terhadap kualitas oosit (P<0,01). Tingkat pematangan pada stadium M-II berkisar antara 1,33-3,92 per ovarium, dan lama penyimpanan berpengaruh nyata (P<0,05) terhadap tingkat pematangan kerbau lokal. Cara pengambilan yang dilakukan mempunyai pengaruh yang sangat nyata (P<0,01) terhadap jumlah oosit antara pemotongan dan aspirasi, juga antara aspirasi plus pemotongan, dan tidak berbeda nyata (P>0,05) antara pemotongan dan aspirasi plus pemotongan. Rata-rata kualitas oosit berkisar antara 0,10-4,95 per ovarium dan tertinggi pada metode slicing plus aspirasi (P>0,05). Rata-rata tingkat pematangan pada stadium M-II berkisar antara 1,00 hingga 3,85 oosit per ovarium, dan metode pengumpulan oosit berpengaruh signifikan (P<0,05) terhadap tingkat pematangan kerbau lokal. Disimpulkan bahwa waktu penyimpanan hingga 6 jam dan aspirasi plus pengirisan cocok untuk pematangan in vitro kerbau lokal.

Kata Kunci: Kerbau, Oosit, Waktu Penyimpanan Oavrium, Teknis Koleksi, Maturasi *In Vitro*

ABSTRACT

Hendri, Udin Z, Masrizal, Jaswandi, Nanda S. 2024. The effect of different ovarian storage times and oocyte collection methods on the quantity, quality, and maturation of buffalo oocytes *in vitro*. JITV 29(2):67-78. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3192.

This study aimed to ascertain the impact of oocyte harvest methods and storage times on the amount, quality, and rate of buffalo oocyte maturation in vitro. Ovaries were collected from slaughterhouse at four different time storages (0, 3, 6, 9 and 12 h) before processing with three collection techniques slicing, aspiration, and slicing plus the aspiration of oocytes. According to the morphology of cumulus cell layers closely attached to the zona pellucida of the oocytes and the cytoplasmic appearance of the oocytes, the oocytes were divided into 4 groups (A, B, C, and D). Cumulus-oocyte-complexes (COCs) were transferred into in vitro maturation (IVM) medium of TCM- 199 (Sigma) for 24 h. The stages of nuclear maturation were examined by staining the enlarged COCs from each group. The result found that the average quantity of oocytes per ovary ranged from 8.08±1.28 to 9.83 ±1.89 and no significant effect (P>0.05) on the quantity of oocytes per ovary. The average of oocytes quality per ovary ranges from 0.2 to 5.00 was a highly significant effect of storage time on quality oocytes (P<0.01). The maturation rate in the M-II stage ranges from 1.33 to 3.92 per ovary, and storage time is a significant effect (P<0.05) on the maturation rate of local buffalo. The collection methods were highly significant (P<0.01) on the quantity of oocytes between slicing and aspiration, also between aspiration plus slicing, and no significant (P>0.05) between slicing and aspiration plus slicing. The Average oocyte quality ranged from 0.10 to 4.95 per ovary and the highest in slicing plus aspiration methods (P>0.05). The average maturation rate in M-II stage was ranged from 1.00 to 3.85 oocytes per ovary, and the methods of oocytes collection were significant (P<0.05) on the maturation rate of local buffalo. In conclusion that the storage time of up to 6 h and aspiration plus slicing is suitable on in vitro maturation of local buffalo.

Key Words: Buffalo, Oocyte, Ovary Storage Time, Collection Techniques, *In Vitro* Maturation

INTRODUCTION

The population of buffalo in west Sumatra consists of indigenous swamp buffalo or local buffalo, and almost all these buffalo are reared by smallholder farmers. The Buffalos had poor reproductive performance, silent estrus symptoms, seasonal anestrus, delayed adolescence, delayed first calving, delayed postpartum conception, a long calving gap, and a low total number of follicles in the ovary, among other reproductive issues (Fitriani et al. 2023). The main issue with buffalo is low reproductive efficiency, indicated by low conception rate and high mortality. Countries have prioritized improve the genetic potential of swamp buffalo for meat production. The research on buffalo has been focused on assisted reproductive technologies (ARTs), including in vitro fertilization (IVF) and in vitro maturation (IVM). ATR might be useful for enhancing the state of cattle reproductive at the moment (Srirattana et al. 2022).

Buffalo embryo in vitro development has proved unsuccessful due to a number of issues, including insufficient follicles on the ovaries and a low rate of oocyte recovery. Oocytes are matured in vitro (IVM), sperm are activated, matured oocytes are fertilized in vitro (IVF), and fertilized oocytes are then cultured in vitro (IVC) until the blastocyst stage. Events that take place during oocyte maturation, fertilization, and the subsequent growth of the fertilized oocytes have a significant impact on in vitro embryo development. In vitro maturation (IVM) is used in assisted reproductive technology to prepare oocytes for in vitro fertilization. The first and most crucial step in developing quality and quantity-conscious embryos in vitro is the careful recovery of oocytes. The time between collection and delivery to the laboratory within 4 hours of the slaughterhouse is not always easily managed. Only high-quality oocytes were selected for in vitro maturation because a higher proportion of high-quality oocytes promotes a faster rate of cumulus growth and maturation (Rajesh et al. 2018). It is feasible to increase the tolerance time for ovary collection by up to 6 hours and increase the effectiveness of the IVEP by ensuring that the temperature of the ovarium is kept below 30°C throughout transit (Francesco et al. 2007). In contrast to other preservation periods of up to 2 hours, (Abdel-Khalek et al. 2010)found that preserving ovaries at 25°C for 5–6 hours successfully sustained the oocytes' quality and recovery rate. For the generation of blastocysts on day 7 of IVC, shipping bovine oocytes from slaughterhouses for either 18 or 24 hours was preferable to the standard maturation approach (Barceló-Fimbres et al. 2015).

Domestic animals have been used to extract oocytes utilizing a variety of techniques, including ovary slicing and post-aspiration slicing of ovarian follicles. However, the effectiveness of these techniques varied (Udin et al. 2020; Rajendar et al. 2024). The oocytes from the abattoir ovaries are harvested using a variety of procedures. The aspiration, slicing, and penetration methods are among them (Saleh 2017). That may suggest the effectiveness of the slicing approach as a collecting strategy for the quantity and quality of buffalo oocytes (Gabr et al. 2014). Buffalo oocyte in vitro maturation was also enhanced in TCM-199 with hormone additions. The method of oocyte harvest for IVM is also the first and most important step toward effective in vitro embryo formation (Segers et al. 2015). The slicing approach, in accordance with (Habeeb and Hussain 2018), produced a much higher quantity of buffalo oocytes 220 distribution grade A (90), B (50), and C. (80). The better procedure for recovering an oocyte for additional (*in vitro*) research is aspiration (Singh et al. 2018). The goal of the current study was to establish an appropriate window of time between the slaughterhouse and the laboratory's ovarian recovery method and technique for collecting local buffalo oocytes.

MATERIALS AND METHODS

Chemical and media

Ovaries from Slaughterhouse buffalo in saline solution for transportation ovaries PBS (phosphate buffer solution) for handling, washing, and evaluation oocytes. Medium maturation TCM-199 + BSA (bovine serum albumin) + hormones from Sigma chemical C0, USA. Gentamicin (50 µg/ml) was added to the medium used for transportation, washing of the ovaries, collecting the oocytes, and maturing the oocytes before use. Before use, handling, collection, and maturation media were equilibrated with 5% CO_2 in a humidified atmosphere at 38°C for at least 2 hours. The maturation grade was then determined using the fixation solution of ethanol and acetic acid.

Experiment-1. Effect of ovaries stored for a period of time on the amount, quality, and maturation of oocytes from local buffalo

Total of 60 ovaries from a local buffalo slaughterhouse were used in this investigation for five groups at different times. Ovarian storage times were divided into five groups in the lab, including 0 hours, 3 hours, 6 hours, 9 hours, and 12 hours. In each group, there were 12 ovaries.

Experiment-2: Effect of collecting method on local buffalo oocyte quantity, quality, and maturation rate.

Oocytes were removed from 60 ovaries using three different techniques: aspiration, slicing, and slicing with

aspiration. Twenty ovaries were used in each oocyte collection technique.

Collection of ovaries

As soon as the buffalo was slaughtered, the ovaries were taken from the nearby slaughterhouse in Padang, West Sumatra. The ovaries were transported to the lab in a thermos filled with normal saline (0.9%) containing gentamicin and maintained between 27 – 30°C. Ovaries from slaughterhouses were split into five groups for storage time: 0 hours, 3 hours, 6 hours, 9 hours, and 12 hours. These groups were processed in the lab. There were 60 ovaries overall, divided into 12 groups.

Collection methods of oocytes

Oocytes were removed from the ovaries using three techniques: aspiration and slicing-aspiration. The ovaries were put in a clean glass petri dish with 2 cc of PBS. Aspiration and slicing-aspiration were the methods employed to collect oocytes. Each collection technique consisted of 20 ovaries, and the total was 60 ovaries Aspiration technique: The ovaries aspiration from individual ovaries after carefully washing to remove extraneous tissue and placed in a Petri dish containing 1 ml of PBS. Oocytes were aspiration from the visible follicle on the ovarian surface with a 22gauge needle fixed to a 5 ml of disposable syringe. Slicing procedure: Using forceps, the ovaries were held firmly in a sterile glass Petri dish with 2 cc of PBS. The blade was attached to the artery forceps and used to cut the ovaries into potentially tiny parts. The PBS medium-containing oocyte was put in a Petri dish and inspected using a stereomicroscope. Combining aspiration and slicing: The aspirated ovaries underwent additional slicing to count the remaining oocytes (post-aspiration slicing), and the number of oocytes obtained using each approach was then counted. The oocytes were microscopically examined, after which they were moved to a grading plate with PBS. The PBS medium was made, and 2 ml of BSA was added to each ml of PBS. A 0.22 µm Millipore filter was used to filter the medium after the pH medium was adjusted to 7.2 to 7.4 and the osmolarity to 280 to 300 ml Osmol kg^{-1}.

Evaluation of oocytes

Five hundred twenty-one oocytes were collected using three different procedures, and 522 oocytes were stored in five different groups of storage duration. According to (Ebrahimi et al. 2010), oocytes were examined under stereomicroscope and divided into four categories: (1) cumulus oocytes-complexes (COCs) with compact cumulus cell (3 layers and homogenous ooplasm, (2) expanded cumulus cell oocytes, (3) denuded oocytes with completely devoid cumulus cells and heterogeneity ooplasm, and (4) partial.

***In vitro* maturation**

The oocytes were matured in TCM-199 maturation media supplemented with 0.3% BSA, 100 IU penicillin G, 100 µg streptomycin/ml, and the hormone FSH at a concentration of 0.04 ml. The 0.22 µm Millipore filter was used to filter the medium. 500 µl of the ready-made maturation media were put into a three-well dish for storage, and three different methods of oocyte collection using sterile mineral oil. The medium was incubated in a CO_2 incubator (5% CO_2, at 38°C with saturated humidity) for at least 60 min to achieve temperature and gas equilibrium before the oocytes were placed on culture dishes. Before being cultured in the intended maturation medium, the COCs were washed three times in the washing medium and twice in it. The maturation period was 24 hours and 10-20 oocytes were placed in maturation media, liquid paraffin oil was added on top, and the Petri dishes were then moved into a CO_2 incubator. In experiment-1, there were 247 oocytes (grade A and B), and in experiment-2, there were 247 oocytes (grade A and B) collected using three different methods.

Fixation, staining, and examination of oocytes

Oocytes were washed in PBS containing 1 mg/ml of hyaluronidase to get rid of the cumulus cells after the 24-hour maturation phase. Oocytes were then placed onto a clean slide after being washed twice in PBS treated with 2% BSA. Overnight, slides were soaked in a fixation solution (three ethanol to one glacial acetic acid). Then, using phase-contrast microscopy, oocytes were stained with 1% orcein in 45% acetic acid to detect different stages of maturation, such as the germinal vesicle (GV), which indicates that chromosomes are enclosed within a nuclear membrane, the germinal vesicle break down (GVBD), which shows the absence of a visible nuclear membrane and chromatin condensation. Metaphase-1 (M1): Chromosomes are condensed in pairs without a polar body being visible; Metaphase-2 (M2): Chromosomes have formed an equatorial plate, and the remaining chromosomes are either substantially condensed or have the first polar body or mature oocytes, extruded from them; Degenerated (Deg) is an example of vacuolated oocytes with constricted cytoplasm or chromatin.

Statistical analysis

The obtained data were statistically evaluated using SPSS 16 and the chi-square test to compare two treatments at each experiment stage.

RESULT AND DISCUSSION

Experiment-1: Effect of ovaries stored for a period of time on the amount, quality, and maturation of oocytes from local buffalo

The average number of oocytes per ovary was 9.833±1.89, 8.03±1.28, 8.75±1.72, 8.33±2.46, and 8.41±1.92 in storage periods of 0 hours, 3 hours, 6 hours, 9 hours, and 12 hours, respectively (Fig. 1). The amount of buffalo oocytes was not significantly affected by storage time ($P > 0.05$). At 0 and 12 hours, grade A oocyte quality ranged from 1.7% to 5.9% on average. The highest-quality oocytes were grade B for all oocyte collection methods, and the average oocyte quality was 28.0% at 9 hours, 32.7% at 12 hours, 46.4% at 3 hours, 50.8% at 0 and 6 hours, and 57.1% at 6 hours (Fig. 2). The average grade C oocyte quality ranged from 10.5% at 6 hours to 25.8% at 12 hours. For grade D, the average oocyte quality increased after 9 hours and decreased after 3 hours of ovarium preservation. The quality of the local buffalo's oocytes is significantly affected by storage duration ($P<0.05$). The percentage of oocytes that matured was lower in GV; it ranged from 8.20% at 3 hours of storage time to 28% at 12 hours, whereas it ranged from 0.1% at 0 hours of storage time to 25.60% at 12 hours. The average M-1 storage time ranged from 0.00% at 6 hours to 6.10% at 0 hours. The in vitro maturation percentage for M2 grade was 74.20% at 0 h, 73.50% at 3 h, 72.50% at 6 h, 50.00% at 9 h, and 46.20 at 12 h storage time. In vitro maturation was not affected by storage time between 0 h, 3 h, and 6 h ($P>0.05$) but was affected by storage time between 9 h and 12 h ($P<0.05$). This finding demonstrated that while oocyte number and in vitro maturation % were not significantly impacted by storage time, the quality of native buffalo oocytes was.

Effect of storage time on oocytes quantity of local buffalo

This result found that the quantity of oocytes tends to decrease by prolonging the time storage of ovaries before being processed in the laboratory. This finding, supported by Kandil et al. (2023), indicates that buffalo oocyte developmental ability is not negatively impacted by delaying ovary collection and processing by up to 6 h. The number of oocytes in this study was higher than (Rajesh et al. 2018), who found that the recovery rate for oocytes from ovaries with CL was 1.35%, while that for ovaries without CL was 1.97%, and (Pitroda et al. 2021), who found that the recovery for oocytes from Riverine buffalo ovaries was 2.74 ± 0.194. The oocyte recovery rate was discovered to be 3.12 per ovary by Ruhil and Purohit (2015) , but the presence result was less favorable than that of Abdel-Khalek et al. (2010) found that the average number of oocytes per ovary ranged from 9.9 to 12.4 and that the oocyte recovery rate decreased barely over time, from 68.1% at 5 hours to 78.6% at 9 hours. If the specimen is kept in a cool box between 4 and 8 degrees Celsius after the animal has been slaughtered, it is preferable to transport it there immediately (Saleh 2017). Additionally, it was hypothesized that photoperiod influences buffalo heifer ovarian function independently of dietary and metabolic conditions and that slaughterhouse specimens can be used to acquire more oocytes (Salzano et al. 2019) . In addition, the quantity and quality of oocytes were unaffected by the calorie content of the diet (Sales et al. 2015). The developmental potential of oocytes was considerably ($P<0.05$) decreased after ovaries were kept at 4°C for 12 or 24 hours (Ravindranatha et al. 2003). The geographic location, the amount of processed ovaries, the season of recovery and the recovery technique used, the reproductive condition of the donor

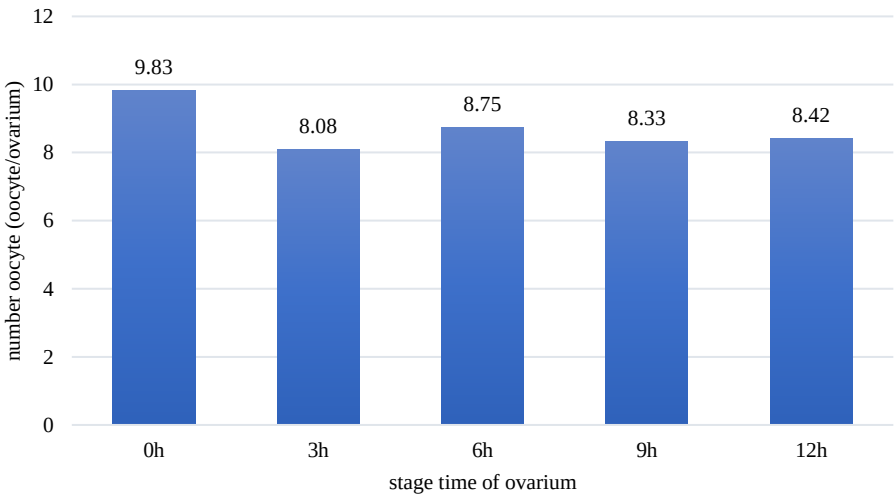

Figure 1. Effect of storage time on quantity of oocytes of local buffalo

animal from which the oocytes were retrieved, and the method used to select ovaries from the slaughterhouse could all contribute to the discrepancies (Al-Shammari and Almeeni 2022). The size of the follicles and other functional features of the ovaries, such as CLs, have been discovered to impact the rate at which oocytes recover in buffalo (Shabankareh et al. 2015) . This study indicated that extending the ovarian storage period to 12 hours was appropriate for the amount of local buffalo oocytes.

Effect of storage time on oocytes quality of local buffalo

The findings that storage time had a significant impact on the quality of buffalo oocytes were confirmed by studies by Saleh (2017), Francesco et al. (2007), and Ravindranatha et al. (2003) that found that storing ovaries at 4°C for 12 or 24 hours significantly (P<0.05) decreased the developmental potential of oocytes. This result's percentage of high-quality oocytes was lower than those in (Saleh 2017): 55%–75% range, Jamil et al. (2008): 50–56% range in autumn and summer, and Abdel-Khalek et al. (2010): range of 42–65.7%. This result demonstrated that prolonged storage times directly impacted oocyte quality. (Saleh 2017) and (Lonergan and Fair 2016) concurred that while several factors directly affect oocyte quality, the time of slaughter has the greatest impact. That element might prevent in vitro oocyte maturation, resulting in low-quality embryos (Aguila et al. 2020). Sonowal et al. (2017) state that good-quality oocytes have more than 4-5 layers of cumulus cells around the zona pellucida and uniform cytoplasmic appearance. When evaluating the in vitro development competence of oocytes, the oocytes quality is crucial (Lemseffer et al. 2022).

This present study was similar to (Sonowal et al. 2017) in that the superior quality grade A +B COCs were 0.2±0.1 to 1.1±0.2, caused the ovarian function in buffalo heifer is fluencies by photoperiod independent of nutritional status and metabolic status. It has been speculated that buffalo abattoir-derived oocytes are particularly affected by cellular damage due to autolytic processes, especially when they reside in excised ovaries for prolonged periods (Kumar et al. 2023). Furthermore, the reproductive status, estrous cycle stage, existence of a CL, and mode of oocyte retrieval all impacted the number of ovarian follicles, yield, and quality of oocytes (Ahmed et al. 2015). Additionally, the frequency for grade I oocytes was significantly higher in the winter and spring (8.31.29 and 9.411.32, respectively) (P<0.05). In the spring (10.52.08), grade II oocyte frequency was greater (P<0.05). In the summer and fall, grades III and IV were slightly higher (P<0.05) (Mansor 2019) .

Effect of storage time of ovary on maturation rate of local buffalo

The maturation rate of local buffalo in this present study was similar with Yousaf and Chohan (2003) was reached the M2 67.1 % and 79.1 % from follicles 4-<6 mm and 6-<8mm, respectively, and Deb et al. 2016) was 74.16 ±5.49 %. The result in the storage time of ovaries up to 6 h was significantly higher in vitro maturation in M2 of local buffalo than Elbaz et al. (2019) was 42.0 % (1.68±0.3) in ovaries with CL and 55.6 % (5.9±1.3) with ovaries without CL, and (Habeeb and Hussain 2018) founded 33.33 % in TCM-199 medium and 13.9% in SOF. (Mohammed et al. 2019) claim that variations in follicular fluid compositions are caused by variances in follicle size, nutrition level, and animal species, which either promote or impede oocyte maturation and subsequent embryonic development. Additionally, the buffalo oocyte maturation rate in the TCM-199 medium was 74.82% (Aquino et al. 2015).

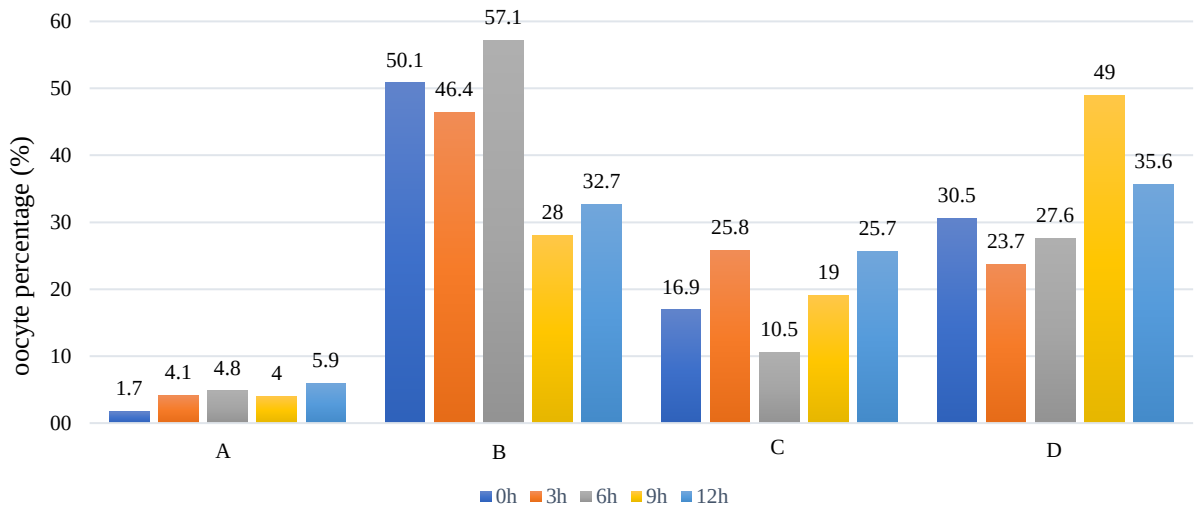

Figure 2. Effect of storage time on percentage of oocytes quality of local buffalo

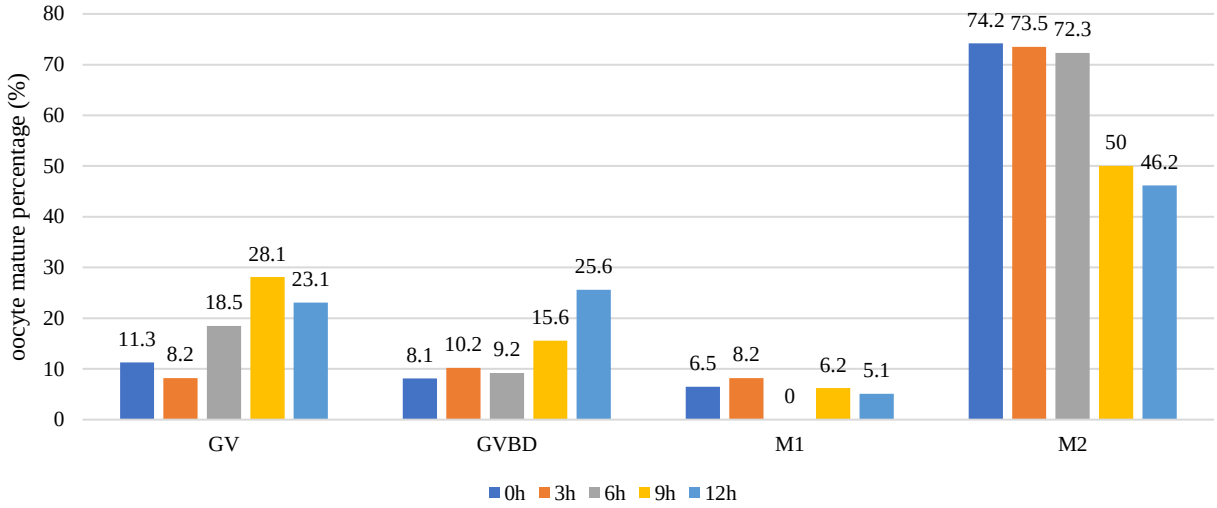

Figure 3. Effect of storage time on *in vitro* maturation percentage of local buffalo

The results of the current study demonstrated that the maturation rate of local buffalo was significantly affected by the length of time between the slaughterhouse and laboratory processing, which was linked to the quality of oocytes recovered from ovaries. In the creation of IVM, the amount and quality of recovered oocytes from each ovary are crucial factors. An oocyte's outward appearance and the amount of cumulus cells it contains have been used to estimate or evaluate its developmental potential, or its capacity to go through typical maturation, fertilization, and development to the blastocyst stage. FBS was superior to EBS for IVM of buffalo oocytes (Puri et al. 2015). EBS may be employed as a serum substitute for oocyte maturation, although the pace was slower than FBS. Therefore, in buffalo, it is possible that ALC influences mitochondrial function, controls paracrine factors produced from oocytes, and boosts steroid hormone production, improving in vitro embryonic development and the quality of developed oocytes (Xu et al. 2018). The results imply that buffalo ovary storage duration is longer in 6 h and there is no tendency to reduce the quantity up to 9 h or 12 h ($P > 0.05$) but the considerable impact on oocyte quality, higher grade at COCs, and maturation rate up to 6 hours of storage.

Experiment-2: Effect of collection technique on quantity, quality, and maturate rate of local buffalo oocytes

The average number of oocytes per ovary using the slicing, aspiration, and slicing plus aspiration procedures was 11.31.63, 4.152.39, and 10.61.2, respectively (Fig.4). Oocyte number per ovary between slicing and aspiration had a highly significant effect ($P<0.01$), whereas oocyte number per ovary between slicing and aspiration plus slicing had no significant effect ($P> 0.05$). Aspiration and aspiration combined with slicing had a substantial ($P<0.05$) impact on the quantity of oocytes in each ovary. According to slicing, aspiration, and aspiration plus slicing, the percentage of oocytes with grade A quality was 3.15%, 2.40%, and 5.70%, respectively. In the three categories of slicing, aspiration, and aspiration plus slicing, the proportion of grade B was 42.5, 37.30, and 46.705, respectively. In the slicing, aspiration, and aspiration plus slicing processes, the percentage of oocytes with grade C quality was 20.4%, 34.10%, and 16.10%, respectively. Slicing, aspiration, and aspiration with slicing each produced 34.10%, 36.10%, and 31.10% of quality oocytes in grade D, respectively (Fig.5). The quality of the buffalo oocytes was not significantly affected ($P>0.05$) by the collection method. In slicing, aspiration, and aspiration plus slicing, respectively, there were 17.50%, 28.20%, and 15.30% of GVs that had undergone in vitro maturation. Sliced, aspirated, and aspirated plus sliced GVBD rates were 10.7%, 18.20%, and 12.60%, respectively. Slicing, aspiration, and aspiration with slicing in M1 were 7.8%, 3.00%, and 2.70%, respectively. According to slicing, aspiration, and aspiration plus slicing, the maturity percentage of M2 was 64.2%, 60.60%, and 69.40%, respectively (Figure.6). The percentage of local buffalo that matured in vitro was not significantly affected by the collection technique ($P>0.05$).

Effect of collection technique on oocyte quantity of local buffalo

This finding supported Gabr et al. (2014) that the slicing technique yielded significantly more oocytes per ovary than follicle by aspiration technique, and Habeeb and Hussain (2018) that the oocyte recovery rate was

better with slicing than the aspiration in buffalo. The slicing methods were higher than aspiration plus slicing and aspiration methods on the quantity of local buffalo. Saleh 2017) asserts that aspiration procedures produce a reasonable number of oocytes with good quality and good embryo production, whereas slicing methods produce more oocytes with intermediate quality. The outcome of the presence research is superior to that of Hammad et al. (2014) found that the number of oocytes per ovary of buffalo was 4.1±0.30, 3.1±0.10, and 3.67±0.27 in slicing, aspiration, and aspiration plus slicing respectively. Puncture was 3.46±0.31, and aspiration methods were 2.38±0.19. Slicing yielded a significant ($P<0.01$) number of oocytes per ovary that was 7.98±0.70. Additionally, it was shown that an ovary without CL was better suited to collecting a greater quantity and higher caliber of COCs (Rajesh et al. 2018). In comparison to the dissection, aspiration, and aspiration plus slicing techniques, the recovery of oocytes utilizing the slicing approach enhanced the oocyte recovery rate. It produced a larger percentage of good-grade oocytes (COCs and enlarged oocytes) (Gabr et al. 2014). By slicing, the mean number of oocytes recovered per ovary was significantly larger (7.88) than it had been using the follicular puncture (3.59) or aspiration (2.50) techniques (P 0.05) (Mahesh et al. 2014). Because some follicles are present that are deeply lodged in the cortex and are freed by slicing the ovary, the study's finding that fewer oocytes were aspirated may be explained by the presence of these follicles. Some oocytes may even be lost when aspirating a follicle, which is not feasible when using the slicing methods. The aspiration approach is the better strategy for recovering oocytes for use in subsequent (in vitro) research, according to a separate (Singh et al. 2018) study.

In the current study, the buffalo ovaries' post-aspiration slicing resulted in an extra 39.15% of oocytes per ovary. This variation in oocyte collection may be caused by the release of cells from the deeper cortex and both surface follicles in slicing techniques (Rose 2014). The fact that certain follicles are deeply entrenched inside the cortexes and are liberated by slicing the ovary may be the cause of the aspiration method's decreased recovery of oocytes. While aspirating follicles, is not possible when employing the slicing procedure, some of the oocytes may even be lost (Gabr et al. 2014). Furthermore, (Saleh 2017) argued that it was ethical to keep the ovarian samples that were aspirated because they may still produce new oocytes by slicing those ovaries. On the other hand, the recovery of buffalo oocytes was unaffected by cutting, aspiration, or a combination of approaches (da-Silva et al. 2021). The reproductive state may be the cause of the various outcomes. This finding established that the method of collecting local buffalo oocytes had a substantial impact on the quantity and that the way of slicing them produced more oocytes from the local buffalo.

Effect collection technique on oocytes quality of local buffalo

The oocytes quality of buffalo in the presence study found that the aspiration plus slicing technique was higher than slicing and aspiration and supported Singh et al. (2018) and (Saleh (2017) that the slicing technique could be considered as the best technique for harvesting the quality of the bovine oocyte. The percentage and quality number per ovary were higher in grade B was 42.5%, 37.30%, to 46.30% in slicing, aspiration, and aspiration plus slicing, respectively (Fig.5). This finding was lower than reported by Gabr et al. (2014) in buffalo was 51.24%, 63.17%, 51.34%, and 42.02% in aspiration plus slicing, slicing, aspiration, and dissection, respectively and Saleh (2017) was 80%, 55%, 45%, and 40% in aspiration, slicing, slicing plus aspiration and slicing, respectively and also the recovery of A+B grade oocyte was 86.01% and 81.76 % by aspiration and slicing technique, respectively (Singh et al. 2018).

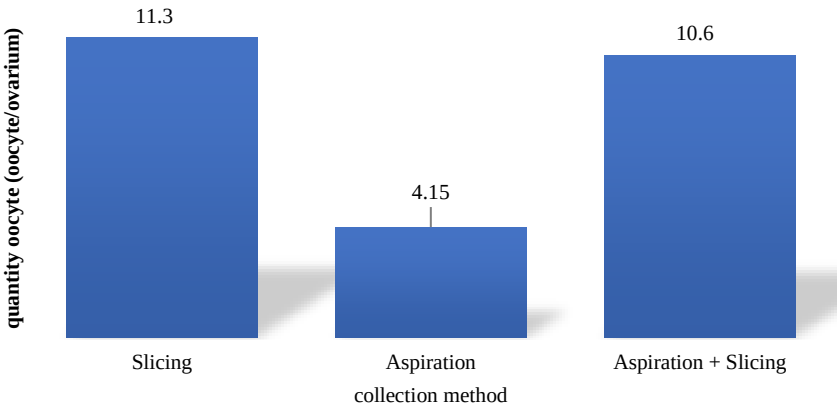

Figure 4. Effect of collection methods on oocytes quantity of local buffalo

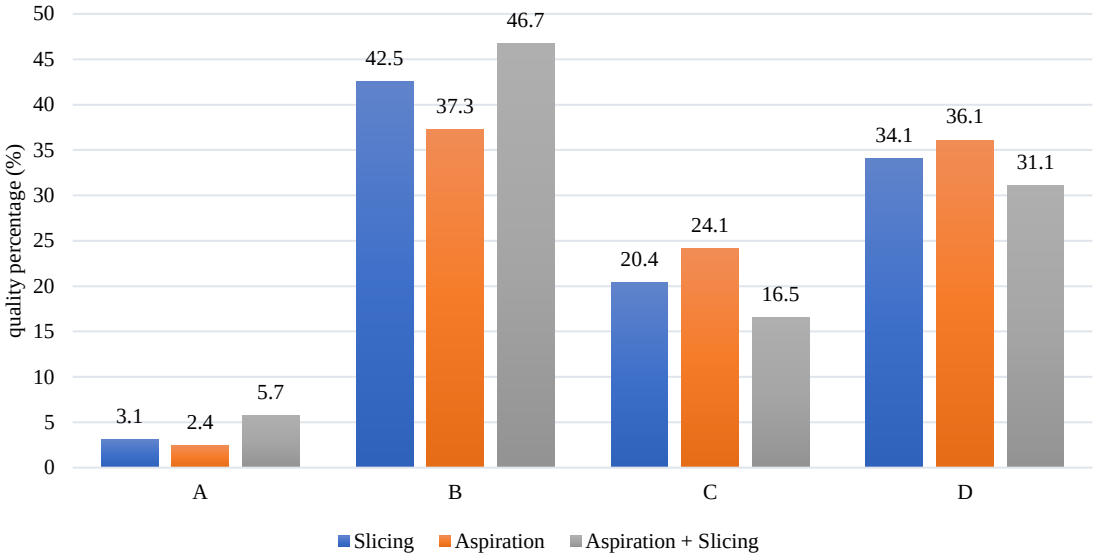

Figure 5. Effect of collection technique on oocytes quality of local buffalo

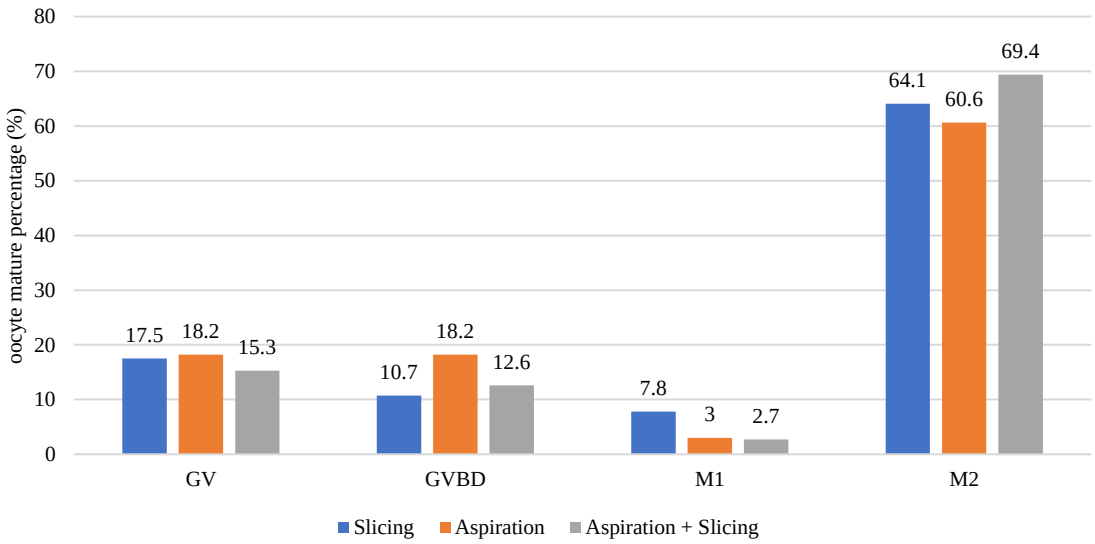

Figure 6. Effect of collection methods of oocytes on maturation percentage of local buffalo

According to Hammad et al. (2014) and (Mahesh et al. 2014), the COCs category was 51.34% in aspiration, 63.17% in slicing, and 51.24% in aspiration plus slicing. The average culture grade A and B COCs recovery was 65.79%. This result was higher than that reported by (Mehmood et al. 2011), who found that the COCs recovery per ovary was 0.9 and 2.2 in aspiration and slicing, respectively, and 0.4 -3,85 good COCs/ovary in buffalo (Nandi et al. 2002), as well as by Jamil et al. (2008), who found that of the three oocyte recovery methods used, dissection produced the highest percentage (36.7). The efficiency of the slicing approach as a collecting method on the amount and quality of buffalo oocytes was also demonstrated byHammad et al. (2014).

The percentage of oocyte quality in the three collection techniques was A grade lower than B, C, and D grade. Aspiration plus slicing technique was higher in A and B grades and least in C and D grades. This finding was supported by Singh et al. (2018), that was no significant difference in the overall recovery rate between the technique, and Ahmed et al. (2015) reported lower recovery rates grade A (38.77%) and B (27.02%). According to Elbaz et al. (2019) that oocytes aspirated from medium-sized follicles (3-6mm) were of higher quality and maturation rate than large (>6 mm) and small-size follicles (≤3 mm). For grade I oocytes. Meanwhile, the slicing method yielded a significantly higher number of oocytes in grades A, B, and C was 40.90 %, 22.7 %, and 36.4%, respectively. While the

aspiration method in grades A, B, and C produced 32%, 28%, and 40% of COCs per ovary, respectively, the slicing approach produced more COCs per ovary (Habeeb and Hussain 2018). The result is different with Mahesh et al. 2014) that the percentage of COCs collected by slicing was higher (P<0.05) at 63.17%, was higher than (P<0.05) than aspiration at 51.34%, aspiration plus slicing was 51.24 %.

Effect of collection technique of oocytes on maturation rate of local buffalo

The maturation rate of COCs at the M2 stage per (A+B) is comparable to that reported by Mehmood et al. (2011), which was 65% in the aspiration technique and 40.0% in slicing technique, and to that reported by Gabr et al. 2014), which was 67% in TCM-199 medium and 58.6 in HamsF10 (P0.05), but higher than that reported by (Saleh 2017), which was 45% in Mendes et al. (2018), findings that the average nuclear maturation in cattle was 58.2% confirm this finding. The result obtained via extrusion of the first polar body is less than that of (Deb et al. 2016), which was 74.16%. Despite a marginally higher percentage of M2 oocytes being found in A-grade COCs obtained by slicing and puncture (84.13.1% and 82.94.7%, respectively) than those obtained by aspiration I and II (78.61.9 and 81.72.5%, respectively), there was no statistically significant difference in the proportion of M2 oocytes after IVM of A grade and B COCs harvested by 4 methods (Vuong et al. 2020). The percentage of M2 oocytes in A grade COCs was not significantly different between the slicing and puncture methods, as well as between the aspiration I and aspiration II methods. The proportion of first polar body extrusion matured in vitro at 56.5°C was 79.5% and 63% (A and B), and at 38,5°C was 85.41% and 74.1%, according to Sen & Kuran (2018) (A and B). In FBS and EBS, the percentage maturation rate was 83.80% and 77.45%, respectively (Puri et al. 2015). Season, oocyte quality, cumulus expansion, maturation, and developmental competence of buffalo oocytes' in vitro maturation all had a significant relationship with one another (Hasbi et al. 2022). According to Gad et al. (2018) , polar body rates (95.8 and 45.5%, respectively) and expansion rates (P<0.05) demonstrated higher maturation rates. Furthermore, the buffalo oocytes' recovery rate, quality, and in vitro maturation all improved with the wintertime, medium-sized follicles, and without CL (Elbaz et al. 2019). These various oocyte collection techniques did not impact the pace of oocyte nuclear maturation (Nikiforov et al. 2020). According to Mohammed et al. (2019), follicle size, nutrition level, and animal species all affect the composition of the follicular fluid, which can either encourage or impede oocyte maturation and continued embryonic development.

According to this study, there is no discernible difference in local buffalo's maturation percentage and rate depending on the method used to harvest the oocytes. According to this finding, which was corroborated by (Mehmood et al. 2011), buffalo oocytes retrieved from resected ovaries using the suction approach had a higher IVM rate than those obtained using the slicing procedures (Chaudhari et al. 2014). Buffalo oocyte maturation is influenced by a wide range of variables, including culture medium, recovery technique, oocyte quality, season of collection, and ovarian condition. It was possible to extract oocytes of every grade using the aspiration approach from samples taken from slaughterhouses, according to Hammood and Aliawy (2017), and it was also possible to mature oocytes in a lab. In contrast to a previous study by Saleh (2017), the suction approach produced fewer oocytes in this study, but they were more mature than those from other procedures. According to (Mahesh et al. 2014), slicing and slicing after aspiration were the earlier methods that produced more oocytes count with good quality that reached maturity condition in a well-considered degree when comparing various methods for oocytes collection in regards to the oocytes number, quality, and degree of maturation. As an alternative to aspiration using a syringe or vacuum pump, Ondho et al. (2024) assert that methods of slicing and puncturing the ovaries can be used. According to follicle size, nutrition level, and animal species, there are differences in follicular fluid compositions that either promote or impede oocyte maturation and further embryonic development (Mohammed et al. 2019). Leptin promotes the developmental competence of bubaline oocytes by modulating cumulus enabling factors and genes regulating pluries potency in the blastocyst (Gilchrist et al. 2016). Cumulus cells also play a crucial role in the substrate supply, transport, and production of chemical components for oocytes, such as microRNAs during oocyte maturation (Panda et al. 2017). Therefore, it may be concluded that the slicing method will yield more good quality cultivable buffalo oocytes than the aspiration method (Arul 2017).

The findings indicated that the buffalo oocyte collection method substantially impacted the number of oocytes produced. The aspiration plus slicing method was recommended for oocyte collection and showed larger COCs and a faster maturation rate.

CONCLUSION

The number, quality, and in vitro maturation of local buffalo were best suited by using oocytes that had been stored for 6 hours and cut into pieces and aspirated. Oocyte quantity was not significantly impacted by the ovary storage period, but quality and maturation rate were. Oocyte collection methods considerably impacted

oocyte number but had no discernible impact on oocyte quality or maturation rate.

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Effects of Different Levels of Fish Meal in the Diet on Carcass Traits and Meat Quality of Bali Cattle

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ABSTRAK

Tahuk PK, Nahak OR, Bira GF. 2024. Pengaruh level tepung ikan yang berbeda terhadap sifat karkas dan kualitas daging Sapi Bali jantan. JITV 29(2):79-90. DOI: http://dx/doi.org/10.14334/jitv.v29i2.3431.

Penelitian ini bertujuan untuk mengetahui pengaruh penggunaan tepung ikan sebagai sumber protein dalam pakan komplit terhadap sifat-sifat karkas dan kualitas daging sapi bali jantan yang digemukkan. Ternak yang digunakan adalah 15 ekor sapi Bali jantan berumur 2-2,5 tahun dengan kisaran berat badan awal 180-200 kg. Ternak dibagi menjadi tiga kelompok dengan ulangan setiap perlakuan 5 ekor ternak. Ketiga perlakuan tersebut masing masing adalah T1 ternak mendapat pakan komplit dengan level tepung ikan 4%; T2 ternak mendapat pakan komplit dengan level tepung ikan 8%; dan T3 ternak mendapat pakan komplit dengan level tepung ikan 12%. Data dianalisis sesuai prosedur ANOVA. Hasil penelitian menunjukkan bahwa bobot potong, bobot karkas, dan bobot non karkas perlakuan T3 lebih tinggi (P<0,05) dibandingkan dengan T2, sedangkan T1 relatif sama dengan T2 dan T3. Persentase karkas dan non karkas, bobot dan persentase daging, serta kadar air dan protein daging relative sama diantara ketiga perlakuan. Kandungan lemak daging perlakuan T2 lebih tinggi dibandingkan dengan perlakuan T1, sedangkan kandungan lemak daging perlakuan T2 relatif sama dengan perlakuan T1 dan T3. Kandungan kolagen daging T1 lebih tinggi (P<0,05) dari T3, sebaliknya perlakuan T2 relatif sama dengan perlakuan T1 dan T3. Kolesterol daging perlakuan T1 dan T2 relatif sama dan lebih rendah (P<0,05) dari perlakuan T3; sedangkan nilai pH, susut masak, daya ikat air, dan keempukan daging relatif sama di antara perlakuan. Disimpulkan bahwa peningkatan level tepung ikan hingga 12% dalam pakan komplit memberikan kontribusi positif terhadap performans ternak yang ditunjukkan dengan tingginya bobot karkas yang dihasilkan dengan kualitas fisik dan kimiawi daging yang optimal.

Kata Kunci: Sapi Bali, Karkas, Pakan, Tepung Ikan, Kualitas Daging

ABSTRACT

Tahuk PK, Nahak OR, Bira GF. 2024. Effects of different levels of fish meal in the diet on carcass traits and meat quality of Bali cattle. JITV 29(2):79-90. DOI: http://dx/doi.org/10.14334/jitv.v29i2.3431.

This study aims to ascertain how fattened Bali bulls' carcass characteristics and meat quality are affected by using fishmeal as a protein source in full feed. Fifteen male Bali cattle, ages two to three and with beginning body weights ranging from 180 to 200 kg, were the animals employed. Livestock were divided into three groups, with 5 animals replicated in each treatment. The cattle were split into three groups: T1 cattle were fed a complete diet containing 4% fishmeal; T2 cattle were fed a complete diet containing 8% fishmeal; and T3 cattle were fed a complete diet containing 12% fishmeal. The ANOVA method was used to analyze the data. The findings demonstrated that compared to T2, the T3 treatment's slaughter weight, carcass weight, and non-carcass weight were higher (P<0.05), although T1 was essentially equal to T2 and T3. Across the three treatments, the percentages of meat and non-meat, weight and meat percentage, moisture content, and meat protein were all comparatively close. While the beef fat content of the T2 treatment was comparatively equivalent to that of the T1 and T3 treatments, it was higher than that of the T1 treatment. T1 meat had a higher (P<0.05) collagen concentration than T3. However, the T2 treatment was identical to the T1 and T3 treatments. While the pH, cooking shrinkage, water binding capacity, and meat softness were all somewhat consistent across treatments, the amount of cholesterol in the meat of treatments T1 and T2 was significantly lower (P<0.05) than that of treatment T3. Based on the high carcass weight generated with the best possible physical and chemical quality of meat, it can be determined that adding 12% more fishmeal to the entire feed improves animal performance.

Key Words: Bali Cattle, Carcass, Feed, Fish Meal, Meat Quality

INTRODUCTION

The demand for protein by the Indonesian people continues to increase every year, demanding the availability of adequate meat. Nationally, the domestic beef supply needs to be increased, as indicated by the high number of beef imports. According to (Agus & Widi 2018), the increasing demand for meat has not been

matched by domestic beef production, the supply of which is less than 60% of the national demand for beef. The imbalance between supply and demand for beef has increased imports of live cattle and frozen meat to fulfill national demand in the short and medium term.

This condition illustrates that more than the domestic beef supply is needed to fulfill the community's needs. Bali cattle are superior local beef cattle that can provide meat to meet the demand for meat and other animal protein sources from poultry and other ruminants. Improving fattening management is one of the alternatives farmers can pursue to produce the maximum amount and quality of meat.

Cattle raising in East Nusa Tenggara play important role in the daily life of local people including to fulfill nutritional needs, to generate cash income, to develop social relationships, and to maintain religious activities (Firman & Nono 2021). Especially on Timor Island, farmers have been fattening beef cattle (Balinese cattle) for generations. The farmers depend on raising cattle, in addition to farming, to fulfil their family needs, so raising Balinese cattle has become an integral part of the local community's life (Saili 2020).

Despite being an integral part of the local community's life, cattle fattening by farmers is still faced with the problem of a lack of feed availability, both in quality and quantity, due to seasonal influences (Lamidi & Ologbose 2014). In the rainy season, livestock growth is positive, indicated by higher body weight gain due to sufficient feed obtained by livestock. Conversely, livestock growth is negative in the dry season, often resulting in death.

According to Ntakyo et al. (2020), prolonged drought in the dry season has adverse effects on cattle productivity, such as reduced growth and milk production (Lamidi and Ologbose 2014). Meanwhile, (Lamidi and Ologbose 2014) stated that a feed shortage in the dry season reduces livestock growth, lacks workforce livestock, and does not maximize livestock production and reproduction. As reported by (Rauf et al. 2015), grazed Balinese cattle produced a daily weight gain of 0.148 ± 0.069 g/head/day; while Balinese cattle that received rice bran and cocoa husk as supplementary feed produced a daily weight gain of 0.207±0.149, and 0.138±0.101 kg/head/day, respectively.

Feed availability that is not continuous throughout the year not only has a direct impact on feed availability and livestock productivity but also hurts livestock rearing activities carried out by farmers, where fattening activities are not carried out throughout the year due to limited feed given to livestock. Therefore, the problem of feed availability that is not continuous throughout the year needs to be solved so that the productivity of Bali cattle fattened by farmers can be increased, both in the rainy and dry seasons (Martojo 2012). In addition, farmers can carry out fattening activities throughout the year without worrying about a decline in animal performance. One of the solutions offered to overcome the problem of feed shortage is the production of complete feed by utilizing forage that is quite abundant in the rainy season. If complete feed production can be developed properly, there is a sufficient stock of feed ingredients available with adequate nutrition to meet the needs of livestock during feed shortages. According to (Beigh et al. 2017), the full feed consists of a quantitative mixture of all dietary constituents that have been thoroughly blended to prevent separation and selection. It is fed as the only source of nutrients, with the exception of water, and is designed in a specified proportion to meet the needs for each nutrient. In addition, the use of complete feed is advantageous because forage and concentrates are presented together to livestock to increase feed palatability and minimize the nature of feed selection by livestock (Tahuk et al. 2020). To ensure animal performance, the balance of nutrients, such as protein and energy, in complete feeds is important.

Protein is an essential nutrient because its sufficiency can ensure maximum muscle tissue synthesis. Fishmeal is a potential protein source for feed to fulfill livestock needs. It is a naturally balanced feed ingredient high in protein, energy, and minerals. In addition, fishmeal is a natural source of vitamins such as choline and biotin and vitamins B12, A, D, and E and includes trace elements such as selenium and iodine.

The quality of fishmeal depends on the raw materials used and the processing method. Fishmeal is a source of easily digestible protein, omega-3 long-chain fatty acids (EPA and DHA), and other essential vitamins and minerals. The quality of the nutrient composition found in fishmeal sets it apart from other dietary supplements, particularly the content of essential amino acids (Ween et al. 2017), long-chain polyunsaturated omega-3 fatty acids (Shahidi and Ambigaipalan, 2015), and mineral (calcium, phosphorus) (Canti et al., 2023).

Applying fishmeal in rations can improve the performance of buffalo and cattle (Kumar et al., 2018) because fishmeal is of better quality than other protein feed sources, such as soybean meal or a combination of soybean meal and urea. Applying fishmeal in the ration can also increase consumption and nutrient digestibility in late-gestational-phase Bali cows (Hartati et al., 2015). In fattened Bali bulls, the use of fishmeal and its impact on productivity has yet to be well informed. Therefore, this research is essential to obtain that information.

MATERIALS AND METHODS

Location, livestock, and feed

The research was conducted at the University of Timor Campus. In contrast, the research results were applied

at the Nekmese Farmers Group, Usapinonot Village, West Insana District, North Central Timor, East Nusa Tenggara. Analysis of the physical and chemical quality of meat was conducted at the Meat Laboratory, Faculty of Animal Husbandry, Gadjah Mada University, Yogyakarta, while analysis of meat cholesterol content was conducted at the Feed Chemistry and Biochemistry Laboratory, Faculty of Animal Husbandry, Gadjah Mada University, Yogyakarta. The study used 15 Bali cattle aged 2–2.5 years. The initial body weight ranged from 180–200 kg, with an expected weight gain of 0.75 kg/head per day. Feed offered field grass, ground corn, bran pollard, rice bran, and fish meal, arranged as a complete ration. Fishmeal was purchased from a local feed shop.

The study used 15 Bali bulls in a complete randomized design (CRD) unidirectional pattern, divided into three treatment groups with five bulls each. The groups were: T1, receiving a ration with 11% CP, 72% TDN, and 4% fishmeal; T2, with 13% CP, 72% TDN, and 8% fishmeal; and T3, with 15% CP, 72% TDN, and 12% fishmeal. A mineral premix was added to meet the livestock's needs. The study had two stages: adaptation and data collection. The feed ingredients and nutrient content are detailed in Tables 1 and 2. The adaptation stage lasted 14 days (2 weeks), and the data collection stage lasted 12 weeks. Rations were given twice daily: in the morning at 08.00 WITA (local time) and in the afternoon at 16.00 WITA (local time). Drinking water was provided *ad libitum*.

Parameters and their measurements

Variables and measurements included slaughter and empty body weights, carcass weights and percentages, and the amount of meat produced. Physical composition measured included meat pH, cooking loss, water holding capacity/water binding capacity (%), and meat tenderness. The chemical composition of meat measured

Table 1. Feed Ingredients (%) and Nutrient Composition in Complete Feed

Feed Ingredients	Proportion (%)	Nutrient content (%)	
		CP	TDN
T1			
Native grass	30.00	2.10	17.40
Milled corn	89.00	4.60	37.80
Rice bran	13.00	0.90	6.60
Pollard bran	11.00	1.80	8.10
Fish meal	4.00	2.10	2.00
Total	**100.00**	**11.50**	**72.00**
T2			
Native grass	30.00	2.10	17.40
Milled corn	42.00	4.60	37.80
Rice bran	9.00	0.60	4.60
Pollard bran	11.0	1.80	8.10
Fish meal	8.00	4.20	4.10
Total	**100.00**	**13.30**	**72.00**
T3			
Native grass	30.00	2.10	17.40
Milled corn	42.00	4.60	37.80
Rice bran	5.00	0.30	2.50
Pollard bran	11.00	1.80	8.10
Fish meal	12.00	6.30	6.10
Total	**100.00**	**15.10**	**72.00**

T1= complete diet containing 4% fishmeal, T2= full diet containing 8% fishmeal, T3= complete diet containing 12% fishmeal CP= crude protein, TDN= total digestible energy

Table 2. Nutrient contents of feed ingredients and complete ration for Bali cattle fattening

Feed Ingredients	Nutrient Content										
	DM	OM	CP	EE	CF	CHO	NFE	TDN (%)	GE		ME
	(%)	(% DM)							MJ/kg.DM	Kcal/kg.DM	Kcal/kg.DM
Native grass	90.67	82.318.39	2.77	1.39	35.66	78.16	42.50	51.09*	14.67	3492.05	2053.42
Fish meal	91.03	70.15	55.67	8.92	4.89	5.55	0.66	64.66*	17.50	4166.17	2958.92
Milled corn	88.19	86.91	9.32	4.89	1.71	72.70	70.98	94.99*	16.53	3929.38	3792.41
Pollard bran	87.62	82.97	18.50	5.47	6.73	59.00	52.73	83.34*	16.56	3942.08	3408.55
Rice bran	90.05	76.32	8.60	9.68	18.29	58.04	39.75	91.18*	15.43	3673.09	2868.76
Ration											
T1	88.05	80.47	14.87	5.54	14.07	60.06	45.99	78.93**	15.88	3781.88	3014.04
T2	87.36	79.63	15.59	5.06	14.17	58.99	44.82	77.84**	15.71	3740.13	2951.32
T3	87.46	70.58	16.67	3.40	14.86	59.51	44.65	76.75**	15.50	3689.39	2843.36

DM=dry mater; OM=organic matter; CP=crude protein; EE=extract enter; CF=crude fiber; CHO=carbohydrates; NFE=nitrogen free extract, calculated by the equation: NFE=[100 – (ash content + CF content + EE content + CP content)] %; TDN= Total digestible nutrients; GE = gross energy; ME= energy metabolism; T1= Complete ration with CP content of 11%, TDN of 72%; T2= Complete ration with CP content of 13% and TDN of 72%; T3= Complete ration with CP content of 15% and TDN of 72%. *= Total digestible nutrients, calculated by the equation of (Hartadi et al. 1980)**= Total digestible nutrients, calculated by the equation of (Wardeh 1981)

included the percentage of protein, water, fat, collagen, and meat cholesterol (mg/100g).

The slaughter weight was determined by weighing the cattle just before slaughtering and feeding for ± 24 hours. Drinking water was provided ad libitum during the fasting period. Carcass weight (hot carcass) was determined (Tahuk et al. 2020; Tahuk et al. 2018) from the weighing of carcasses after slaughter, obtained from the difference between slaughter weight and non-carcass weight. The hot carcass percentage is calculated from the ratio of hot carcass weight to slaughter weight multiplied by 100 percent. The equation is carcass percentage (%) = hot carcass weight (kg)/cut weight (kg) x 100%. Meat weight (kg) is obtained after the decomposition of the carcass. The percentage of meat is obtained from the ratio of the weight of the meat to the weight of the hot carcass. The equation is meat percentage (%) = meat weight (kg)/carcass weight x 100% (Tahuk et al. 2020; Tahuk et al. 2018).

The physical composition of meat measured includes pH, water holding capacity, cooking loss, and tenderness. Determination of pH and water holding capacity and cooking shrinkage use according to the procedure of Povše et al. (2015). The equation for calculating cooking shrinkage (%) is A - B/A x 100%, where A = weight before heating (grams) and B = weight after heating (grams). The procedures Warner-Bratzler breaking power test, according to Povše et al. (2015), was used to determine meat tenderness.

The chemical composition of meat was measured, including moisture, fat, protein, and collagen content. The test method used was near-infrared spectroscopy according to Osborne's instructions (Prieto et al. 2017) by utilizing the Meat Analyzer Food Scan device, which reads at a wavelength of 800–1400 nm. The measurement of meat cholesterol used the Liebermann-Burchard method according to the instructions of (Keklik et al. 2018), where the readings used a spectrophotometer at a length of 680 nm.

Data analysis

Data were analyzed using the variance analysis procedure of a completely randomized design (CRD) (Drebee 2018). SPSS 26 software was used to simplify and accelerate the analysis.

RESULTS AND DISCUSSION

Slaughter weight and empty body weight

A high slaughter weight of livestock indicates effective management during fattening, but it does not guarantee the quality of carcasses. According to the study results (Table 3), animals in the T3 treatment had higher slaughter and empty body weights compared to the T2 treatment. In contrast, the T1 treatment was similar to the T3 treatment. Specifically, T3 treatment cattle had a 24.311% higher slaughter weight than T1 and 36.759% higher than T2 treatment cattle. Additionally, the empty body weight of T3-treated animals was 16.015% higher than T1-treated animals.

The results of this study illustrate that the use of fishmeal as a protein source up to 12% in complete feed still shows positive performance in livestock. There is a tendency to decrease the slaughter weight in T2 cattle due to variations in individual responses that still need to be optimal for the complete feed. However, increasing the level of fishmeal in the ration can increase the slaughter weight of Bali cattle. The slaughter weight of T3-treated animals was higher due to a significant increase in body tissue synthesis compared to the T1 and T2 (Table 3). The increase in slaughter weight is because livestock's protein and energy needs have been met, which contributes to the synthesis of body tissues, resulting in a high slaughter weight. The slaughter weight of the T1 and T2 was lower than the report of (Tahuk et al. 2020), who obtained the slaughter weight of Bali bulls fattened with complete feed containing *Gliricidia sepium* leaves ranging from 219,250±14,245 to 239,000±21,280 kg.

In contrast, T3 had a higher slaughter weight than the report of Tahuk et al. (2020). Similarly, the slaughter weight of T1 and T2 was lower than the report of (Tahuk et al. 2018b), who obtained a slaughter weight of 228.60-251.20 kg in Bali bulls received different CP levels. However, the cattle in T3 showed a higher slaughter weight than the report of Tahuk et al. (2018b). The initial live weight, time of rearing, the ADG, and feed quality influenced the difference in slaughter weight in this study.

Carcass yields

The carcass weight (Table 3) of animals in T3 was higher than those on T2 treatment ($P<0.05$) but relatively the same as T1. Similarly, the T1 showed results that were not much different from the T2 treatment. On the other hand, the carcass percentages of the three treatment groups showed relatively similar results. The non-carcass weight of T3 animals was higher ($P<0.05$) than that of T1 and T2; conversely, T1 and T2 produced relatively the same carcass weight. The increase in carcass and non-cass weight of T3 was 24.031% and 26.552% higher than that of T1 and 45.236% and 26.552% higher than that of T2. Although the three treatment groups were statistically similar, there was a tendency for the percentage of carcasses and non-carcasses of T3 to be 0.365% higher and 0.495% lower than T1, 5.977% higher, and 7.171% lower than T2.

Both slaughter weight and carcass weight influence the value of dressing percentage. A higher proportion of

carcass weight to slaughter weight increases the dressing percentage. This study found that using fishmeal at levels of 4%, 8%, and 12% in complete feed had a similar effect on dressing percentage. The dressing percentages observed in this study were higher than those reported by Tahuk et al. (2020), who found dressing percentages ranging from 50.61±1.595% to 51.140±0.512% in young Bali cattle fed complete feed with *Gliricidia sepium* leaves as a protein source. Similarly, Tahuk et al. (2018b) reported dressing percentages ranging from 54.07±2.39% to 55.61±0.93% in Bali cattle reared on smallholder farms. These results indicate that high-quality feed can maximize carcass production in Bali bulls during the accelerated growth phase.

This study's non-carcass weight of T3 (Table 3) was relatively high due to the higher slaughter weight. An increase in slaughter weight not only contributes positively to the carcass produced but also impacts the development of the non-carcass component of the livestock. This study's weight and percentage of non-carcass produced were still relatively high. The livestock grazing for approximately 12 hours before slaughter has not significantly reduced animal tract contents. In addition, this condition is related to the development of non-carcass components during the rearing phase. Non-carcass weight is closely related to the feed ingredients obtained and their digestibility. When the digestibility of feed is low, it contributes to an increase in the contents of the digestive tract, resulting in higher non-carcass components. This study observed that the fiber fraction of the ration and its constituents was high, resulting in increased fiber fraction consumption (Table 1 and 2). As a result, the ability of rumen microbes to degrade was not maximized.

Meat weight and percentage

The results showed that the weight and meat percentage of the three treatment groups were relatively similar (P>0.05) among treatments. However, T3 had a higher carcass weight than the other two treatments. Each animal in the T3 treatment had a weight and percentage of meat that were 16.67% higher and 14.83% lower than those in T1, respectively. In contrast, T3 produced 17.67% more meat than T2, but its meat percentage was 17.87% lower.

The meat weight in this study was not influenced by fishmeal (P>0.05). The weight and percentage of meat produced in this study were lower than the report of Tahuk et al. (2020), who obtained the weight and percentage of meat ranging from 75.39±4.86 kg to 86.00±8.76 kg (68.62±0.62 to 71.72±1.47%), respectively, who obtained using complete feed containing *Gliricidia sepium* leaves as a protein source. The meat weight of this research report was also lower than that of Tahuk et al. (2018), who obtained a meat weight ranging from 84.98 to 93.16 kg and a meat percentage of slaughter weight ranging from 35.67 to 37.35% from Bali bulls fattened on smallholder farms.

Meat quality

Protein content

The results showed that the meat protein content (%) of Bali cattle given fishmeal as a protein source was relatively similar (P>0.05) (Table 4). The treatment cattle showed the highest meat protein, ranging from 22.71±0.23 to 22.97±0.73% (Table 4). The meat protein content of the three treatment groups was not significantly different. This condition illustrates that feed protein at the level of 11–15% of the ratio used is optimal to fulfill basic life so that it can be utilized for body tissue synthesis when the quality of nutrients obtained by livestock increases, the increase in nutrient quality has a positive impact on increasing energy utilization for fat and protein deposition (Park et al. 2018).

The meat protein content of Bali cattle in this study was within the normal range. Thus, the use of fishmeal as a source of protein in rations with 11–15 percent CP has a similar and insignificant effect on meat protein deposition. This condition illustrates that the animal feed protein obtained in the three treatments is optimal for maximum meat protein synthesis. In addition, the relatively same age impacts the rate of protein synthesis, which is not much different either. According to Bulkaini et al. (2020), the rate of protein synthesis in Bali cattle decreases with age. In growing cattle, the protein synthesis and degradation rate increases, and the protein synthesis rate often exceeds protein degradation. The rate of synthesis and degradation in animals diminishes as they get closer to maturity, eventually settling at a low and balanced rate Kutay et al. (2024).

The meat protein of these Bali bulls was lower than the report of Tahuk et al. (2020), who obtained meat protein ranging from 22.89±0.44 to 23.58±0.26% in Bali bulls that received *Gliricidia sepium* leaves as a protein source. Differences influence the content of meat protein levels in several research reports on animal genetics and differences in feed used. The results of this study indicate that fattening Bali bulls using fishmeal as a protein source produces relatively normal meat protein. Forage feed is generally high in fiber and low in energy, resulting in low carcass fat content but increased protein and water content in meat (Baik et al. 2023).

Moisture content

Moisture content determines the quality of beef, whereas fresh beef with a moisture content of 65–80% is

highly perishable (Li et al. 2018). The meat moisture content (%) of male Bali cattle fed complete feed with fishmeal as a protein source was about the same across treatments, ranging from 72.51±0.54 to 72.79±0.56% (Table 4). The relatively low-fat meat content in T1, T2, and T3 closely correlates with the moisture content of meat in the three treatment groups. Despite the T3 having a higher fat content than the T1, the resulting fat content value did not significantly differ, impacting the water content of the produced meat and remaining relatively the same (P>0.05) among treatments.

The relatively similar moisture content of the meat in this study indicates that the quality of Bali beef produced was average. Generally, moisture content in meat has a negative relationship with fat content; as fat content increases, moisture content decreases. According to Geletu et al. (2021), moisture content in meat is an essential component because it determines the quality of the meat. Water makes up approximately 75% of the weight of beef, making it the component with the most significant quantitative impact on surface appearance, color, and texture. In cattle, meat moisture content typically ranges from 70.54 to 77.64, averaging 74.09% (Abdelwhab and Mohammed, 2019), and 72.76±0.47% to 73.32±0.64% in Bali cattle (Tahuk et al., 2020). The difference in moisture content from several

Table 3. Carcass characteristics of Bali cattle fattened with complete feed containing fish meal as a protein source[1]

Carcass characteristics	T1[2]	T2[2]	T3[2]	SEM	P Value
Initial body weight (kg)	189,00±24,64	158,33±31,56	195,33±22,19	9.53	0.263
Slaughter weight (kg)	208.75±41.40^{ab}	189.75±18.46^{a}	259.50±39.20^{b}	12.67	0.048
Empty body weight (kg)	187.26±39.18^{ab}	163.61±14.06^{a}	217.25±33.98^{b}	10.46	0.100
Carcass weight (kg)	121.30±31.39^{ab}	103.59±11.49^{a}	150.45±26.19^{b}	8.66	0.066
Non-carcass weight (kg)	87.45±10.09^{a}	86.16±7.26^{a}	109.05±13.59^{b}	4.21	0.024
Dressing percentage	57.59±3.60	54.54±1.04	57.80±2.05	0.78	0.167
Percentage of non-carcasses	42.41±3.60	45.46±1.04	42.20±2.05	0.78	0.167
Meat weight (kg)ns	57.00±13.98	56.500±10.85	66.50±7.724	3.22	0.398
Meat percentage (%)ns	47.14±3.32	54.62±9.19	44.86±6.01	2.14	0.148

[1]Data are presented as mean±SD; [2]T1= Complete ration with 11% crude protein, 72% total digestible nutrient; T2= complete ration with 13% crude protein and 72% total digestible nutrient; T3= complete ration with 15% CP and 72% TDN. Different superscripts on the same line indicate differences (P<0.05); ^{ns} = nonsignificant

Table 4. Chemical and physical composition of meat of male Bali cattle fed with complete diets containing fishmeal as a protein source[1]

Meat Chemical Composition	T1[2]	T2[2]	T3[2]	SEM	P Value
Protein (%)ns	22.97±0.73	22.77±0.23	22.71±0.23	0.13	0.72
Water (%)ns	72.50±0.54	72.79±0.56	72.78±1.16	0.21	0.85
Intramuscular fat (%)	3.42±0.53b	3.98±0.41ab	4.24±0.35a	0.15	0.07
Collagen (%)	1.91±0.06b	1.59±0.06ab	1.64±0.27a	0.14	0.05
Cholesterol (mg/100g)	52.34±4.36a	54.33±6.22a	62.74±2.71b	0.82	0.03
Physical Composition of Meat					
pH of means	5.55±0.13	5.45±0.13	5.57±0.07	0.03	0.17
Cooking loss (%)ns	27.39±1.24	26.49±0.45	26.92±1,67	0.34	0.61
Water holding capacity (WHC,%)ns	34.42±6.29	38.75±3.89	37.50±2.24	1.29	0.41
Breakability (tenderness)ns	8.60±0.81	8.26±0.61	8.13±0.49	0.18	0.59

[1]Data are presented as mean±SD; [2]T1= Complete ration with 11% crude protein, 72% total digestible nutrient; T2= complete ration with 13% crude protein and 72% total digestible nutrient; T3= complete ration with 15% CP and 72% TDN. Different superscripts on the same line indicate differences (P<0.05); ^{ns} = nonsignificant

research reports is due to differences in animal genetics and feed used and variations in fat deposits from each animal. Differences in moisture content across studies are due to variations in animal genetics, feed, and fat deposits.

Intramuscular fat content

The deposition of meat fat demonstrates that the body uses the excess nutrients to meet basic life needs, particularly energy. Meat fat content (%) in Bali cattle fed complete feed containing fish meal as a protein source showed that T3 was higher than T1 ($P<0.05$) but relatively the same as T2 ($P>0.05$). The intramuscular fat content of T3 was 24.05% higher than T1 and 6.57% higher than T2 (Table 4). Intramuscular fat content (Table 4) illustrates that using fish meals in complete rations positively affects fat tissue synthesis due to the sufficient energy obtained by livestock. The complete ration formulation in this study contains enough protein and energy to fulfill basic living and production needs. Once basic living needs have been met, the excess energy is utilized to increase production, including muscle and fat tissue synthesis.

Fat deposits in cattle are determined by whether or not livestock obtains enough feed. Feeding with high energy content can increase fat deposits. Livestock with limited feed impacts the low-fat deposits produced (Schumacher et al. 2022). Using high-concentrate feed in livestock in the finishing phase can shorten the production cycle and increase fat deposition (Patino et al. 2015). Dietary energy levels increase intermuscular fat, reducing the amount of heat-resistant connective tissue. In addition, increased intramuscular fat results in increased juiciness, tenderness, and aroma characteristics of meat (Kutay et al. 2024).

Carcass fat content in this study was lower than the report of (Tahuk et al. 2020), who obtained carcass fat levels ranging from 4.77±0.65 to 5.61±0.47% in Bali bulls fed complete feed containing *Gliricidia sepium* leaves as a protein source. Fat deposition in livestock is influenced by various factors, including the amount and type of feed given to livestock, genetics, gender, and environment (Schumacher et al. 2022). In addition, Piao and Baik (2015) reported that the season significantly affects fat deposition in cattle. Heat stress (HS) or cold stress can affect food intake, heat production, and nutrient partitioning priorities, reducing animal performance. According to (Patino et al. 2015), the intramuscular fat content of bulls reared on pasture with energy supplementation is lower when compared to cattle reared in confinement with forage and concentrate feeding ratio of 50:50. The intermuscular fat content of meat is influenced by nutritional factors including metabolism, digestibility and absorption of fat, and availability of glucose or soluble sugars (Park et al. 2018). It was also explained that triglyceride synthesis is a critical factor for IMF deposition, while triglyceride hydrolysis decreases IMF deposition; in addition, manipulation of digestion and absorption of dietary fat in the small intestine can increase IMF deposition; similarly, maximum starch utilization is essential for IMF deposition and can be achieved by optimal starch fermentation in the rumen and maximum starch digestion and absorption in the small intestine.

Collagen

The collagen levels can affect the value of meat by limiting its tenderness and cooking convenience (Bruce and Roy 2019). The collagen content affects meat's tenderness and cooking convenience. The collagen levels in the study were 1.91±0.06% for T1, 1.58±0.06% for T2, and 1.64±0.29% for T3. T3's meat collagen was 14.271% lower than T1's but 4.127% higher than T2's. Statistically, T1 had more collagen than T2 ($P<0.05$), but T2 was not significantly different from T1 or T3 ($P>0.05$). Variations in collagen distribution in Bali beef were observed, with T1 having higher collagen due to lower fat content.

Collagen content varies by sex, age, and meat type within the same carcass and is influenced by fat content. The collagen levels were lower than Tahuk et al. (2020) reported, who found 2.40±0.21% to 2.53±0.44% in Bali bulls fed with *Gliricidia sepium* leaves. Differences in intramuscular fat are influenced by cattle age and feed quality. According to Bruce and Roy (2019), factors such as the age at slaughter, use of growth steroids, and cattle breed affect collagen quality. Collagen plays a crucial role in cooked beef; when heated, it shrinks and causes fluid loss, making it less tender (Wiśniewski et al., 2021).

Cholesterol

The impact of cholesterol content on consumer health is a severe issue in beef production. Therefore, beef's high-fat content has become a discussion topic for beef consumers due to its associated health effects, such as cardiovascular disease (Troy et al. 2016). According to Bronzato and Durante (2017), red meat consumption is considered a dietary risk factor for cardiovascular diseases (CVD). Most of the risk of red meat intake has been related to saturated fat and cholesterol content. As shown in Table 4, adding 12% more fishmeal raised the cholesterol level in beef ($P<0.05$) to 62.74±2.71 mg/100g, compared to 4% and 8% fishmeal levels, which raised the cholesterol levels to 52.34±4.36 mg/100g and 54.33±6.22 mg/100g, respectively. The increase in cholesterol level of T3 was 19.89% compared to T1 and 15.48% compared to T2. This condition illustrates that increasing the level of fishmeal not only

increases muscle tissue synthesis, spurring livestock growth but also contributes to an increase in the proportion of fat, including meat cholesterol.

The cholesterol content of meat produced in this study is higher than the cholesterol content of Bali beef reported by (Suryanto et al. 2014), ranging from 38.75 ± 4.27 to 38.75±2.63 to 42.00±4.97 mg/100g, and the report of (Tahuk et al. 2020), who obtained Bali beef cholesterol, ranging from 28.79±4.42 to 29.77±3.16 to 33.69±1.21 mg/100g. However, the cholesterol content of the results of this study is lower than that of (Abdelwhab & Mohammed 2019), who obtained a cholesterol content in beef of 74.50±6.73 mg/100g. The livestock's type of feed, age, and genetics influence the difference in meat cholesterol content.

Physical quality of meat

Cooking loss

Cooking loss is one of the parameters that determines the meat quality, which is related to consumer acceptance. Cooking loss combines liquid and soluble matter lost from the meat during cooking. Besides, cooking loss is a critical factor in the meat industry as it determines the technological yield of the cooking process. From a nutritional perspective, cooking loss brings about the loss of soluble proteins, vitamins, and different supplements (Pathare and Roskilly 2016).

The findings showed that the cooking loss (%) of Bali cattle that were fed a complete diet with fishmeal as a protein source was low and pretty much the same across treatments (P>0.05) (Table 4). The low cooking loss illustrates that the nutrients in the beef still survive (and are protected) well during the cooking process. The WHC, which remained relatively high in this study, and the meat's ultimate pH content, which remained within the normal range, also contributed to the low cooking loss of the meat. The cooking loss value of T3 animals was lower than that of T1 by 1.71% and T2 by 1.61%, respectively.

The use of 4, 8, and 12% fishmeal levels in complete rations with CP levels of 11, 13, and 15% and TDN of 72% positively impacted the cooking loss of Bali cattle. The high energy content of feed in this study increased the synthesis of intramuscular fat (marbling) in meat, which impacted the protection of fluids during the cooking process. Increased intermuscular fat content in meat will reduce the amount of heat-resistant connective tissue, improving the juiciness, tenderness, and aroma characteristics of meat (Kutay et al. 2024). In addition, temperature and cooking time determine the cooking shrinkage value of meat (Ježek et al. 2019). The low cooking loss value is closely related to the type of feed consumed by the animals. Feeding with a high energy content can incrae intramuscular fat synthesis (marbling), which protects fluids during cooking (Tahuk et al. 2018).

The amount of cellular membrane damage influences the amount of cooking loss in meat, the amount of water that escapes from the meat, the shelf life of the meat, protein degradation, and the ability of the meat to bind water, as well as genetic factors and feed given to cattle (Strydom et al. 2016). The cooking shrinkage produced in this study was lower (better) than the report of Ninu (2017), who obtained cooking losses of 33.88 and 28.49% in the meat of 2- and 3-year-old male and female Bali cattle, respectively. Nevertheless, it is better than Tahuk et al.'s (2018b) findings, which found that cooking losses in Bali cattle raised on smallholder farms with varying feed protein levels ranged from 37.60±0.88 to 40.50±1.11%. Thus, it can be said that using fishmeal as a protein source in fattening Bali cattle can improve the quality of the meat produced.

Water holding capacity (WHC)

Water holding capacity is one of the indicators of meat quality, as seen from the ability of meat to bind water. Water holding capacity is the amount of water that meat can hold during cutting, heating, grinding, and pressing, as well as during transport, storage, and cooking. Therefore, water-holding capacity is an essential criterion in quality assessment in meat processing (Warner 2014).

The results showed that the water-holding capacity (%) of Bali cattle meat fed with complete feed containing fishmeal as a protein source was relatively similar among treatments (Table 4); this illustrates that the use of fishmeal at levels of 4, 8, and 12 percent in complete feeds containing CP 11, 13, and 15% with TDN 72% in fattened Bali Bulls did not hurt the water-holding capacity of meat. The rations used in T1, T2, or T3 were optimized for meat protein deposits, thus contributing positively to the WHC of the meat. The nutritional effect of feed significantly influences the value of meat's water-binding capability (Watanabe et al., 2018). Furthermore, there is a stronger correlation between the pH level of meat and water binding ability than between intramuscular fat content. Therefore, the normal WHC in this study is thought to be influenced by the pH of the meat, which is still within the normal range. Jankowiak et al. (2021) reported that the pH value strongly correlated with water-holding capacity, water loss, meat tenderness, and water and protein content(Jankowiak et al. 2021). The value of water binding capacity obtained in this study is higher than the report of (Tahuk et al. 2020) who obtained water binding capacity ranging from 29.54±5.69% to 32.34±26.26% in Bali bulls fattened

with complete feed containing *Gliricidia sepium* leaves as a protein source; as well as the report of (Tahuk et al. 2018b) who obtained WHC ranging from 14.93±1.60 to 15.79±2.48% in Bali cattle fattened in smallholder farms.

Tenderness

Meat tenderness value can describe the quality of meat, which can be known from the Warner Blatzler (WB) breaking power value. The study showed the tenderness (kg/cm2) of T1 at 8.60±0.81, treatment T2 at 8.26±0.61, and T3 at 8.13±0.49. We did some statistical testing and found that when different amounts of fishmeal were added to the complete diet, the average meat tenderness from Bali bulls was about the same across treatments (Table 4). The relatively similar meat tenderness values of the three groups of treated animals are related to influencing factors such as higher water binding capacity and lower cooking shrinkage and are not much different. Connective tissue content, sarcomere length, and myofibrillar damage are the primary sources of variation in meat tenderness (Strydom et al. 2016). The significant determinants of meat tenderness are connective tissue and cross-links, myofibrillar integrity, sarcomere length, protein denaturation, and intramuscular fat (Warner et al. 2022).

Meat tenderness was not affected by bulls' growth on pasture with energy supplementation or in pens with a 50:50 forage concentrate ratio. However, the amount of collagen in meat is closely correlated with high and low meat tenderness scores. When the collagen level is high, the meat will be less tender (Patino et al. 2015). In this study, the variation in collagen content was not much different, which resulted in relatively the same meat tenderness value.

Thus, the difference in the tenderness value of the fattened Bali bulls in this study is also expected to be caused by the difference in the presence of collagen cross-linking in the meat, which is not much different. Collagen solubility negatively correlates with meat tenderness, especially in aged cattle. An increase in collagen with low solubility can reduce meat tenderness (Li et al. 2018). It is also reported that the effects of species, age, and muscle type contribute to variations in meat tenderness due to the influence of collagen. Tenderness in meat is one of the variables whose value is largely determined by cooking. In addition, the tenderness of the meat is largely determined by the anatomical-histological structure of the muscle, especially the connective tissue sheath in the muscle, the activity of meat tenderization, as well as meat preparation, temperature, and cooking time (Ježek et al. 2019).

The meat tenderness of the three groups of cattle in this study was lower than the report of Ninu (2017), who obtained meat breakage values of 4.10 kg/cm2 and 4.09 kg/cm2 (more tender), respectively, for male and female Bali cattle aged 2–3 years. Similarly, this tenderness value is lower and almost the same as the report of Tahuk et al. (2018b), who obtained the tenderness of Bali beef in smallholder farms ranging from 5.58±0.79 to 8.80±0.86 kg/cm2 and lower than the report of Tahuk et al. (2020), who obtained tenderness of 4.42±0.82 to 6.32±1.42 kg/cm2 in Bali bulls fattened with complete feed containing *Gliricidia sepium* leaves as a protein source. The difference in tenderness value is caused by differences in feed quality, cattle growth pattern, muscle type, pH, intramuscular fat (IMF), and total and soluble collagen content (León-Ecay et al. 2022).

CONCLUSION

Using fishmeal as a protein source in complete feed generally positively affected fattened Bali bulls. The body weight gain was exceptionally high, especially when the cattle received a proportion of fishmeal at a ratio of 12%. The improved growth performance led to an increase in carcass and meat production. Therefore, it is recommended that farmers and animal husbandry practitioners consider using fishmeal as a potential protein source to enhance the performance of fattened Bali bulls.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this published article, neither the funding nor the content.

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Utilization of Sago Waste Fermented by *Neurospora* sp. as Alternative Corn Substitution on Laying Duck Production

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ABSTRAK

Matitaputty PR, Nurfaizin, Ralahalu TN, da Costa MA. 2024. Penggunaan limbah sagu yang difermentasi dengan *Neurospora* sp. sebagai alternatif pengganti jagung terhadap produksi itik petelur. JITV 29(2):91-96. DOI:http://dx.doi.org/10.14334/jitv.v29i2.3216.

Persediaan jagung sebagai sumber energi dalam ransum ternak di wilayah Maluku masih memiliki kendala sehinga perlu dicari penggantinya. Salah satu pakan alternatif yaitu adalah penggunaan ampas sagu yang difermentasi dengan *Neurospora* sp. Tujuan pengkajian ini mengetahui pengaruh penggunaan ampas sagu yang difermentasi *Neurospora* sp. sebagai pakan alternatif terhadap produktivitas itik petelur. Materi yang digunakan adalah itik lokal betina Maluku sejumlah 120 ekor umur umur 22 minggu yang dibagi menjadi 4 perlakuan. Masing-masing perlakuan mendapat 5 ulangan yang terdiri dari 6 ekor pada setiap unit perlakuan. Empat perlakuan yang diberikan yaitu T0 (penggunaan fermentasi ampas sagu 0% dalam ransum), T1 (10 % dalam ransum), T2 (20 % dalam ransum), T3 (30 % dalam ransum), T4 (40 % dalam ransum) dilakukan selama 14 minggu. Berdasarkan penelitian dihasilkan penggunaan pakan ampas sagu terfermentasi *Neurospora* sp. terdapat beda (P<0,05) pada konsumsi, produksi telur dan konversi ransum pada perlakuan T0, T1, T2 dan T3 terhadap T4., Sedangkan pada kualitas telur yang meliputi berat telur, kandungan karoten kuning telur; dan bau tidak berbeda nyata. Kesimpulan dari pengkajian ini adalah penggunaan Ampas sagu yang difermentasi dengan *Neurospora* sp. hinga level maksimal 30 % mampu mensubstitusi penggunaan jagung dalam ransum tanpa mengurangi produktivitas itik.

Kata Kunci: Itik Petelur, *Neurospora* sp., Ampas Sagu

ABSTRACT

Matitaputty PR, Nurfaizin, Ralahalu TN, da Costa MA. 2024. Utilization of sago waste fermented by *Neurospora* sp. as alternative corn substitution on laying duck production. JITV 29(2):91-96. DOI:http://dx.doi.org/10.14334/jitv.v29i2.3216.

The corn stock as a feed energy metabolism source in the Moluccas region is still an obstacle, so there is a need to find a substitution. One of the alternative feeds used is sago waste fermented by *Neurospora* sp. The study aimed to determine the effect of sago waste fermented by *Neurospora* sp. as alternative feed productive for laying ducks. The material used was 120 local Moluccas laying ducks aged 22 weeks divided into 5 treatments. Each treatment consisted of 5 replications of 6 laying ducks in each treatment unit. There were five dietary treatments with the inclusion of fermented sago weste: T0 (0%), T1 (10%), T2 (20%), T3 (30%), and T4 (40%). A feeding trial was carried out for 16 weeks. Results showed that the treatments using fermented sago waste by *Neurospora* sp. significantly affected (P<0.05) consumption, production, and feed conversion efficiency. At the same time, egg quality regarding egg weight, yolk carotene content, and odor were not significantly influenced. In conclusion, the maximum level of the use of *Neurospora* sp. fermented sago was 30% in feed without reducing the general performance of the ducks.

Key Words: Laying Duck, *Neurospora* sp., Sago Waste

INTRODUCTION

Duck is one of the poultry productions that has been reared traditionally. Products like eggs benefit food and income (Daud et al. 2020). Optimal egg production can be achieved by laying ducks under sufficient nutritious feed. There needs to be more than corn as raw material stocks in the Moluccas region to maintain duck farming. It is imported, making the prices relatively expensive. Furthermore, most of the duck business problems in Moluccas are due to low productivity, as farmers need to feed ducks properly according to optimal nutrient requirements.

However, corn used as a duck's energy source can be substituted with another local feed ingredient. A considerable stock of cheaper sago waste is available

close to the farms. However, there have been problems with high levels of crude fiber of 9.22-10.50% and low crude protein of 0.92-1.01% (Uhi 2018). Fermentation technology using *Neurospora* sp. is one solution to reduce high crude fiber feed (Adli et al. 2021). *Neurospora* sp. produces cellulase enzymes that degrades crude fiber (Kanti dan Sudiana. 2016). *Neurospora* sp. is usually used to make fermented sago waste called "oncom," one of the traditional foods in Indonesia, which is also found in Waimital Village, Seram Western District, Moluccas Province. Orange conidia is a unique characteristic of *Neurospora* sp. That can be seen in the surface of "oncom" (Kenyamu et al. 2014; Nurfaizin and Matitaputty 2016; Munasik et al 2023) The substrate fermented by *Neurospora* sp. can increase crude protein and carotene content. It was reported that feed containing fermented sago waste could effectively maintain the body weight of growing ducks (Uhi 2018).

The fermented sago waste contained crude protein 4.56-4.58%, crude fiber 5.49-6.25%, extract ether 0.71-0.73%, and metabolizable energy 3,508-3,860 kcal/kg (Uhi 2018). Carotenoids are effective as antioxidants, preventing free radical oxidation and increasing the carotene in the yolk (Bovšková, et al 2014; Kenyamu, et al. 2014)

Based on the information above, it is hypothesized that sago waste fermented by *Neurospora* sp. can be compatible with duck feed. The study aimed to determine the effect of sago waste fermented by *Neurospora* sp. on the productivity of local laying ducks.

MATERIALS AND METHODS

Material

This experiment used 120 local laying ducks, aged 22 weeks and with an average body weight of 1296,50 g/head. The ducks were divided into 4 dietary treatments, with 5 replicates per treatment. Each replicate consisted of 6 birds kept intensively in a 1 m x 1 m cage for 14 weeks. Diets (Table 1) and drinking water were offered *ad libitum*.

Neurospora sp. fermentation of sago waste

Two kilograms of sago waste were mixed with 600 ml of tofu waste and water until the percentage of water content was 60% and stirred until smoothly homogenous. Then, the mixed material was steamed at 100ºC for about 45 minutes and cooled at room temperature. Nine percent of the inoculum of *Neurospora* sp. was added and stirred smoothly and homogeneously. The material was then incubated for 10 days. The fermentation product was harvested, dried in the sun and grind, and stored for further utilization.

Experimental diets

The experimental diets were analyzed proximately for dry matter, crude protein, crude fiber, crude fat, non-nitrogen-free extract, ash, Ca, and P at the Indonesian Research Institute for Animal Production Laboratory at Bogor Regency. For total carotene content, the diet samples were analyzed at the Indonesian Agricultural Postharvest Research and Development Laboratory in Bogor City, according to (AOAC 2009).

Experimental design

The experiment was conducted using a completely randomized design (CRD) with five treatments and four replicates consisting of six birds each. The treatments are T0=Control (farmer diet), T1=10 % Sago Waste fermented by *Neurospora* sp. used in the diet, T2=20 % Sago Waste fermented by *Neurospora* sp. used in the diet, T3=30 % Sago Waste fermented by *Neurospora* sp. used in the diet, and T4=40 % Sago Waste fermented by *Neurospora* sp. used in the diet.

Measurements of variables

Duck day production, feed consumption, and feed conversion were measured during the experimental treatment observation period of 14 weeks. Duck day production was calculated by the number of eggs produced divided by the number of ducks on the same day. Feed given and left was recorded to calculate daily feed intake. The feed conversion ratio was calculated by dividing the total feed weight consumed by the total egg weight on the same day. The egg yolk color index was observed by comparing egg yolk color using the Roche Egg Yolk Color Fan (Bovšková et al. 2014; Pane et al. 2023). Spectrophotometric methods with isooctane solvent and observed absorbance of light with a wavelength of 450 nm were used to determine the total carotene content of yolk (AOAC 1990). An organoleptic test on egg odor compared to the palatability of fishy odors was conducted involving adult untrained panelists of 30 people consisting of 10 men and 20 women. Panelists rated egg odor by scaling from 1 to 5. Scale 1 was very fishy, 2 for fishy, 3 for rather fishy, 4 for less fishy, and 5 for not fishy.

Data analysis

The nutrient content of sago waste fermented by *Neurospora* sp. was analyzed descriptively. Egg production and qualitative data were analyzed using ANOVA, and any mean differences were further tested using the Duncan Test (Mattjik and Sumertajaya 2013). The values were expressed as means, and statistical significance was judged at the probability of $P<0.05$. Overall, data analysis was performed using SPSS 18 software.

Table 1. Ingredient and nutrient content of experimental diet

Ingredients	Treatment				
	T0[1]	**T1**	**T2**	**T3**	**T4**
	----------%----------				
Corn	40.00	30.00	20.00	10.00	0.00
Sago waste fermented by *Neurospora* sp, (%)	00.00	10.00	20.00	30.00	40.00
Ricebran (%)	30.00	26.00	26.00	25.00	25.00
Fish meal (%)	10.00	11.00	11.00	12.00	12.00
Coconut meal (%)	17.00	18.00	18.00	18.00	18.00
$CaCO_3$(%)	00.00	4.00	4.00	4.00	4.00
Premix (%)	3.00	1.00	1.00	1.00	1.00
Total (%)	100.00	100.00	100.00	100.00	100.00
Moisture content (%)	11.95	12.62	12.07	11.98	12.69
Crude protein (%)	19.27	19.08	19.08	19.51	19.31
Crude fiber (%)	4.61	4.33	3.42	3.93	6.89
Crude fat (%)	3.13	2.96	1.72	1.63	2.78
Gross energy (kal/Kg)	3778	3567	3625	3518	3430
Ca (%)	1.74	3.36	2.87	2.83	4.01
P (%)	0.42	0.57	0.56	0.48	0.72
Total Carotene, (%)	0.24	0.20	0.22	0.39	0.37

T0= Basal diet without fermented sago waste; T1= Diet contained 10% fermented sago waste, T2= Diet contained 20% fermented sago waste, T3= Diet contained 30% fermented sago waste, T4= Diet contained 40% fermented sago waste

RESULTS AND DISCUSSION

Nutrient content of *Neurospora* sp.-fermented sago waste

The proximate and carotene analyses are presented in Table 2. Based on the analysis, it is known that Neurospora sp. ferments sago waste products changed nutrition content, especially crude protein, crude fiber, and total carotene of feed.

Crude protein content in sago waste increased during *the Neurospora* sp. fermentation process (from 1.4% to 3.28%). After fermentation by Neurospora sp., the crude fiber content of sago waste decreased from 11.56% to 7.02%. *Neurospora* sp. improved sago waste nutrition content through fermentation, which produced protease and cellulose enzymes to improve sago waste's nutrition content. Cellulolytic enzymes break cellulose bonds of the substrate, which causes the content of crude fibers to decrease. (Li et al. 2014) reported that *Neurorospora* crassa showed protease and cellulase enzyme activity. Based on (Kanti da&n Sudiana. 2016), *Neurospora* crassa can also synthesize and secrete enzymes involved in cellulose degradation and increased protein content of material during the *Neurospora* fermentation process. *Neurospora*-fermented stuff was utilized better in animal feeds (Kanti & Sudiana 2016).

The total carotene content increased due to fermentation (from 0.033% to 0.742%). The carotene was produced during *the Neurospora* fermentation process. The color of the carotene was orange. Increased carotene caused by fermentation with *Neurospores* could synthesize geranyl diphosphate (GGDP) until it eventually became β-carotene (Gedela et al. 2015; Liu et al. 2016). *Neurospora* produces intracellular carotenoid pigments stored in conidia, which causes an orange color (Kenyamu & Mappiratu 2014). *Neurospora* is potentially used in the feed industry as it produces yellow-to-orange carotenoids (Gmoser et al. 2018).

Ducks productivity

There was no significant ($P>0.05$) effect of T0, T1, T2, and T3 dietary treatments on feed consumption, egg production, and feed conversion ratio. Treatment T4, containing 40% fermented sago waste, significantly ($P<0.5$) reduced food consumption, reduced egg production, and increased feed conversion ratio compared to ducks on T0, T1, T2, and T3.

Table 2. Nutrient Content of *Neurospora* sp. fermented sago waste

	Sago waste	Sago waste fermented by *Neurospora* sp.
Moisture Content (%)	12.30	10.34
Crude protein (%)	1.40	3.28
Crude fiber (%)	11.56	7.02
Crude fat (%)	0.25	1.19
Ash (%)	11.76	14.32
Ca (%)	0.32	0.37
P (%)	0.02	0.07
Total Carotene (%)	0.033	0.742

Table 3. The productivity of laying ducks under experimental diets

	T0[1)]	T1	T2	T3	T4
Feed Consumtion (g/day)	115.04±6.47 [b2)]	114.19±6.01 [b]	114.80±6.28 [b]	114.49±5.49 [b]	111.86±8.92 [a]
Duckday (%)	53.68±14.19[b]	54.38±14.04[b]	54.05±15.26[b]	56.55±17.61[b]	50.15±12.62[a]
Feed conversion ratio	3.65±1.05 [b]	3.61±1.14 [b]	3.66±1.26 [b]	3.53±1.41 [b]	3.95±1.09 [a]

T0= Basal diet without fermented sago waste; T1= Diet contained 10% fermented sago waste, T2= Diet contained 20% fermented sago waste; T3= Diet contained 30% fermented sago waste, T4= Diet contained 40% fermented sago waste Values on the row with difference superscripts are significantly different (P<0.05)

Feed consumption of all treatments of ducks under T0, T1, T2, and T3 were significantly different (P<0.05) from feed consumption of ducks on T4. Using 40% level sago waste fermented by *Neurospora* sp. affected decreasing duck consumption, caused by crude fiber in the T4 treatment, which is relatively high at around 6% compared to the other treatments. According to (Novieta, et al. 2023) bulky crude fiber content made the digestive tract feel full and more quickly stopped eating, so the consumption of diet decreased; according to (Zulkarnain, et al. 2017) research explained that sago waste fermented sago waste was used as an energy source in broiler chicken diet. Therefore, feedstuff, considered an energy source for poultry, must contain highly digestible carbohydrates (Sugiyono, et al. 2015).

Feeding Neurospora sp. fermented sago waste seemed to cause a slight increase in egg production but was insignificant (P>0.05). However, T3 caused the duck to produce more eggs than the duck in T4, which showed that using *Neurospora* sp. fermented sago waste at 30 % of the diet will produce good egg production. All nutrient content in the T0, T1, T2, and T3 except T4 have been capable of fulfilling nutrient requirements to produce maximal local duck egg production. According to (Otay, et al. 2014), high egg production can be obtained by feeding based on nutritional ducks' requirements. Compared to treatment with standard diets, using sago waste fermented by Neurospora sp. as an alternative feed in Maluku province did not affect laying duck production except on T4. Gunawan et al. (2015) reported a level of 5% fermentation of sago waste on a diet substituted with a commercial quail diet without decreasing egg production.

Feeding Neurospora sp. fermented sago waste at 30% (T3) provided the lowest feed conversion ratio to laying ducks. A high feed conversion ratio means that feed consumed by ducks could have been utilized more optimally for egg production. According to (Fanani, et al. 2017), The higher the feed conversion the less economical the use of the feed otherwise, the lower the conversion rate the smaller it is, the more economical it is.

Egg quality

Egg quality data as affected by feeding *Neurospora* sp. sago waste by *Neurospora* sp. Are presented in Table 4. Feeding *Neurospora* sp. fermented sago waste was not significantly affected (P>0.05). The egg weight of 54.79-56.38 g/egg was the average duck egg weight at 22-30 weeks old. Therefore, the use of *Neurospora* sp. Fermented sago waste in local duck feed was obviously no harm to egg weight. The duck egg weight was affected by many factors, such as genetics, age, and nutrition (Widiyaningrum, et al. 2016). Based on (Lupita, et al. 2019), the average local Magelang duck eggs weigh about 53,66-57.84 g/egg at the age of 20 weeks old.

Table 4. The quality of eggs as affected by experimental diets

	T0[1)]	T1	T2	T3	T4
Egg weight (g/egg)	56.38±3.43	56.26±3.44	56.27±2.82	56.17±3.18	54.79±4.22
Total carotene of yolk (ppm)	105.65±19.88	118.12±12.27	146.51±45.15	178.07±63.59	144.09±70.79
Egg yolk color index	8.38±1.11	8.31±1.48	8.25±2.14	8.31±1.10	8.27±0.49
Odor scale	3.54±0.98	3.89±0.89	3.64±1.21	3.79±1.11	3.32±0.76

T0= Basal diet without fermented sago waste; T1= Diet contained 10% fermented sago waste, T2= Diet contained 20% fermented sago waste; T3= Diet contained 30% fermented sago waste, T4= Diet contained 40% fermented sago waste

Feeding local Maluku ducks with 15% sago waste resulted in a minimum egg weight of 40g (Matitaputty & Bansi 2018).

Dietary treatment did not significantly affect the total egg yolk carotene (P>0.005). It is assumed that experimental diets contained sufficient carotene to maintain the quality of duck yolk egg. According to Mulyadi,, et al. (2017), factors that affect egg yolk color are carotenoids and other pigment substances in the diet. Pigments forming egg yolk color come from yellow feed colors like corn. Carotenoids consumed from feed and other biofortification were saved in peripheral tissues to change the orange pigment and the egg yolk. However, the quantity and quality of feed consumed would affect egg production and physical-chemical quality (Moreno, et al. 2016).

Utilization *of Neurospora* sp-fermented sago waste resulted in a good index production of egg yolk color. The egg yolk color index of duck feed dietary treatments (T0, T1, T2, T3, and T4) was not significantly affected (P>0.05). Increasing the yolk color score of the treatment will cause a preferable product and affect the chemical composition of the egg yolk. The yolk color is one of the attractions for duck egg consumers. According to Pane et al. (2023), a high egg yolk color score describes a high carotene content of the egg yolk.

The odor of duck eggs was not significantly different (P>0.05) due to all treatments. The duck eggs' odor was rather fishy (scale 3.32-3.89)—the odor was identified with the sense of smell (nose). The odor test is essential in providing an assessment of the acceptability and quality of a product. In general, the quality of duck eggs is in the range of fishy to rather fishy (Gunawan 2019). Strong fishy products would make consumers not interested in consuming the product (Qomaruddin & Afandi, 2017). The fishy odor of eggs is related to duck feed consumption (Sundari et al. 2020).

CONCLUSIONS

The *Neurospora* sp-fermented sago waste in diet effectively increased egg productivity. To some extent, the 30% *Neurospora* sp-fermented sago waste was compatible with substituting corn in feed without reducing ducks' productivity.

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Gastroprotective Effects of *Curcuma zedoaria* in Ethanol-Induced Gastric Ulcers of Experimental Rats

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ABSTRAK

Sutardi LN, Mustika AA, Andiyanto, Handharyani E, Winarto A, Rahma A, Nabilah AS, Rahman FA. 2024. Efek gastroprotektif dari *Curcuma zedoria* terhadap kejadiaan gastritis ulcerative pada tikus yang diinduksi dengan etanol. JITV 29(2):97-102. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3183.

Gastritis merupakan salah satu masalah kesehatan global. Kejadian ulkusa dapat dikarakterisasi dengan melihat adanya inflamasi pada lapisan epitel mukosa lambung. Kerusakan di lambung dapat terjadi karena kelebihan sekresi asam lambung, pepsin, dan kehadiran *Helicobacter pylori*, stress, kebiasaan merokok, meminum alcohol, pola makan yang tidak teratur. Infeksi. Dan akibat penggunaan *non-steroidal anti-inflammatory drugs*. Penelitian ini bertujuan untuk mengetahui efek gastroprotektif infusa *Curcuma zedoaria* pada tikus yang mengalami kerusakan mukosa lambung akibat diinduksi HCl/ethanol. Sebanyak 16 tikus Sprague Dawley dibagi menjadi 4 kelompok secara acak: kelompok kontrol negatif (tanpa pemberian terapi), kelompok kontrol positif (terapi dengan omeprazol), P1 (infusa temu putih 30%), dan P2 (infusa temu putih 60%). Parameter penelitian yang dievaluasi adala lesio mukosa lambung, degenerasi sel, kerusakan intraseluler, dan nilai pH. Skor ulkus dan kerusakan mukosa lambung dievaluasi melalui inflamasi mukosa lambung dan pemeriksaan histopatologi. Pemberian infusa temu putih dapat menurunkan indeks ulkus secara signifikan ($P<0.05$) melalui pencegahan terjadinya lesion mukosa lambung, erosi, degenerasi seluler, dan nilai pH juga naik secara signifikan. Hasil penelitian ini menunjukkan bahwa infusa temu putih memiliki efek gastroprotektif yang dapat digunakan untuk mendukung penggunaan obat tradisional.

Kata Kunci: *Curcuma zedoria*, Gastritis, Temu Putih

ABSTRACT

Sutardi LN, Mustika AA, Andiyanto, Handharyani E, Winarto A, Rahma A, Nabilah AS, Rahman FA. 2024. Gastroprotective effects of *Curcuma zedoaria* in ethanol-induced gastritis ulcers of experimental rats. JITV 29(2):97-1021. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3183.

Gastritis is one of the most common health problems of humans in the world. Gastric ulcer is mostly characterized by inflammation of the epithelial cells lining the gastric mucosa. Stomach injury may occur due to excessive secretion of stomach acid, pepsin, *Helicobacter pylori*, stress, smoking habit, alcohol consumption, irregular eating pattern, infection, and the use of non-steroidal anti-inflammatory drugs. This study aimed to explore the gastroprotective effects of *Curcuma zedoaria* infusion (CZI) on HCl/ethanol-induced gastric mucosal damage in rats. A total of 16 Sprague Dawley rats were randomly divided into 4 groups: negative control (without treatment), positive control (treated with omeprazole), P1 (CZI 30%), and P2 (CZI 60%). Several endpoints were evaluated, including gastric mucosal lesions, cellular degeneration, intracellular damage, and pH value. The gastric mucosal injury and ulcer score were determined by evaluating the inflamed gastric mucosa and by histopathological examination. After the treatment animal with CZI significantly ($P<0.05$) decreased the ulcer index by preventing gastric mucosal lesions, erosion, and cellular degeneration, and the value of pH value was increased ($P<0.05$). These results demonstrate that CZI has significant gastroprotective properties which support its use in traditional medicine.

Key Words: *Curcuma zedoaria*, Gastritis, White Turmeric

INTRODUCTION

Gastritis is one of the most common health problems in the human population. Gastritis is an inflammatory disorder that may be caused by various factors. Necrosis and desquamation of epithelial cells of the stomach may appear and may further lead to stomach ulcers ((Beiranvand & Bahramikia 2020)). Stomach constructive factors are the secretion of mucin, peptide, prostaglandin, and vascularization. Stomach destructive factors are the secretion of stomach acid and peptides, *Helicobacter pylori*, stress, smoking habit, alcohol,

irregular eating pattern, infection, and non-steroidal anti-inflammatory drugs (Chiou et al. 2010); Beiranvand & Bahramikia 2020)).

According to the World Health Organization (WHO), the prevalences of gastritis incidence are as follow: Canada (35.0%), China (31.0%), France (29.55%), England (22.0%), and Japan (14.5%). Gastritis in Indonesia is on the sixth place with the number of cases being 33,580 (60.86%) for inpatients, and on the seventh place with the number of cases 201,083 for outpatients. The gastritis case prevalence in Indonesia for several cities are: Medan reaches 91.6%, Jakarta 50%, Denpasar 46%, Palembang 35.5%, Bandung 32%, Aceh 31.7%, Surabaya 31.2%, and Pontianak 31.2% (Mustakim et al. 2022). The disease may affect family economy value due to relatively high expenses for gastritis treatment (Joish et al. 2005).

Many studies have been conducted to develop a suitable drug to control gastritis problems. Herbal medicines are known as an alternative approach to treat gastric ulcer. Several herbal preparates have been investigated recently, such as *Origanum syriacum L.*, *Gardnia jasminoides Ellis,* Korean red ginseng, *Kalopanax pictus*, *Curcuma zedoria* and other such plants (Lee et al. 2009; Oyagi et al. 2010; Afify et al. 2012; Sohn et al. 2015; Gozali et al. 2022). *Curcuma zedoaria*, also known as white turmeric, is a traditional herb in Indonesia (Gozali et al. 2022). The plant is known to be effective to treat gastrointestinal disorders, such as nausea, gastroparesis, and gastric atony. It also prevents gastric ulceration caused by stress and HCl (Kimura et al. 2013).

The purpose of this study was to evaluate the gastroprotective effects of white turmeric infusion for acute gastritis through the assessment of inhibitory effects on stomach mucosal lining cell damage, irritated by HCl/ethanol as well as the anti-inflammatory activity in stomach tissue. This research used acute gastritis animal models in vivo. Finally, it is expected to provide scientific information on the efficacy of white turmeric infusion as a gastroprotective agent for gastritis alternative treatment. This information could also be used as the basis of standardized herbal drug development from white turmeric infusion in controlling gastritis.

MATERIALS AND METHODS

Curcuma zedoaria rhizomes were used in this research as an infusion and were obtained from Tropical Biopharmaca Research Center, IPB University, Bogor. Chemicals and reagents included ethanol 60%, HCl 0.3 M, omeprazole, male *Sprague Dawley* (DW) rats (Laboratory Animal Management Unit, School of Veterinary Medicine and Biomedical Sciences, IPB University), ketamine (Ket A 100®, Peru), xylazine *(Dormi-Xyl*®2, Peru), pH universal strop test*,* graduated alcohol (PT. Brataco, Indonesia), xylol (Merck, German*)*, buffered neutral formalin (BNF) 10% (Delta Lab, Indonesia), Hematoxylin-Eosin (HE) (Merck, Germany), Phosphate Buffer Saline (PBS) (Sigma-Aldrich, American), distilled water (PT. Brataco, Indonesia), and gum. Laboratory equipment was rodent cages (UPHL FKH IPB, Indonesia), scale (Camry, Indonesia), automatic tissue processor *(Sakura*®, USA), rotary microtome, and light microscope (Olympus®, Japan).

Preparation of white turmeric infusion

The infusion was produced by washing and slicing fresh white turmeric rhizomes, followed by boiling in an infusion pan with the ratio between white turmeric and distilled water being 1:10. Boiling was performed at 90°C for 15 minutes until a final concentration of an infusion at 10% was obtained. The white turmeric rhizomes infusion was then diluted to 30% and 60% concentrations.

Gastritis induction in rats

The animals used in this experiment were male Sprague Dawley rats weighing 180-200 grams. The animal feed followed a standard nutrition content according to the Indonesian Food and Drug Authority (Badan Pengawas Obat dan Makanan Republik Indonesia-BPOM RI). Drinking water was provided *ad libitum*. Rats were kept in a normal living environment at 25-28°C and 35-60% humidity. The rats were acclimatized for 14 days.

Sixteen rats were randomly assigned to four groups, 4 rats in each, and fasted for 18h prior to oral dosing with the normal saline solution (negative group/KN), omeprazole (a standard treatment control at a dose of 20 mg/kg bw; positive group/KP), and CZI at concentration 30% (P1) and 60% (P2). Ninety minutes later, all the rats were orally administered 5 ml/kg bw of a mixture of ethanol 60% and 0.15 M HCl solution. All rats were then anesthetized with ketamine and xylazine to reduce pain. Rats were killed by cervical dislocation 90 minutes after the administration of HCl/ethanol solution. The stomach was quickly removed and fixed in 10% neutral formalin solution for 1h, opened by an incision along the greater curvature (Al-Quraishy et al. 2017; Nam & Choo 2021).

Macroscopic lesions of the stomach mucosal lining cells

All rats were necropsied for pathological examination and tissue collection of the stomach. The

stomachs were released from the abdomen and incised to measure the acidity of stomach fluid content using a pH universal strip test (merck universal pH indicator). The stomachs were then washed with physiological NaCl to observe the stomach mucosal lining cells. The stomachs were then fixed in buffer-neutral formalin 10% for histopathological examination. The macroscopic evaluation score was based on gastric mucosa hemorrhage, hyperemic, and loss of rugae (score 0-3; 0 no lesions, 1 mild, 2 moderates, 3 severe).

Microscopic examination

The fixed tissues of the stomach were trimmed at the corpus (glandular) region and set into a tissue cassette. The samples were dehydrated and cleared in an automatic tissue processor with graduated alcohol and xylol. The tissues were put in a paraffin block, cut by rotary microtome, and attached to the object glass. Rehydration was performed followed by staining with hematoxylin and eosin. The slides were examined under the microscope and assessed for histopathological changes such as congestion, mucosal erosion, and inflammation (score 0-3; 0 no lesions, 1 mild, 2 moderate, 3 severe).

Data analysis

The data analysis process of this study included two stages. The first stage included a descriptive analysis to describe the distribution of the data. The second stage included hypothesis testing with ANOVA.

Animal ethics approval

Ethical approval for this study was obtained from The Animal Ethics Committee, School of Veterinary Medicine and Biomedical Sciences, IPB University under certificate Number: 020/KEH/SKE/VII/2022.

RESULTS AND DISCUSSION

The stomach is characterized by folded villi mucosae known as the rugae. The mucosae of the stomach are formed by 3 layers including the epithelial, propria, and muscular layers. The administration of ethanol to rats may cause mucosal damage to the stomach. The injury of stomach mucosae is commonly followed by lowering the pH level of the stomach content, where the normal pH value of the stomach is around 6.5 (Beiranvand & Bahramikia 2020). The present study shows that the negative control was the most acidic stomach content among all treatment groups. The pH value of stomach content (Table 1) are as follows: the negative control (KF) is 1.5, the positive control dosed with omeprazole (KP) is 5.25, the white turmeric 30% infusion (P1 30%) is 3, and the white turmeric 60% infusion (P2 60%) is 3.75. Omeprazole is a drug often used to increase the pH value of the stomach and is regarded as an effective drug to treat stomach acid (Mahdayana et al. 2020; Lazebnik et al. 2021). Omeprazole works by inhibiting the acid-activated proton pump so that it then forms covalent bonds with H+, K+, and ATPase via disulfide bonds which in turn inhibits acid pump enzymes (Shin & Kim 2013). The same mechanism is thought to occur in the use of white turmeric which is known to contain curcumin. Because curcumin can act as a secretion of gastric acid through inhibition of H+/K+ATPase in gastric parietal cells, resulting in a decrease in gastric pH in rats (Kim et al. 2005; Mei et al. 2009; He et al. 2015). Table 1 shows that the white turmeric infusion was effective to control stomach acid problems.

Macroscopically shows that stomach ulcer, congestion, and hemorrhage were not found in the positive control and omeprazole group (Figure 1). The negative control showed congestion, hemorrhage, and ulcer formation on the surface of the stomach mucosa (Figure 1). The stomach of the P1 group was suffering from general hyperemia without hemorrhage, and only a few hyperemias were seen in the P2 group. The rugae of the KN group showed fewer rugae and flattened stomach mucosal. On the other hand, many stomach rugae were noted in the omeprazole group (KP group) and P2 group (*Curcuma zedoria* infusion in 60%), although the P2 group did not have as much a positive control. Identical damage was observed macroscopically in the stomach that did not receive treatment (KN) (Fig. 1a). The stomach of KN rats had the lowest pH value and the highest macroscopic damage of 5.50±1.00 (Table 1). The data supported microscopically (Figure 2) shows that hyperemia and massive infiltration of inflammatory cells in the mucosal region followed by desquamation of mucosae epithelial cells were found in the negative control group with the score 8.67±0.58. Based on Table 1, it is known that macroscopically through assessing the severity of the incidence of hemorrhage, hyperemia, and loss of mucosal folds, it is known that giving CZI 30% compared to giving CZI 60% or treatment using omeprazole, not significantly different ($P<0.05$). Further microscopic observations revealed that giving a CZI of 60% had a better effect compared to rats given a CZI of 30%. It seems that the treatment of stomach ulcers depends on the concentration of white turmeric infusion where a higher concentration of white turmeric infusion was dosed will lead to a better condition of stomach recovery.

The local defense of stomach mucosae is an important factor that protects the mucosa cells from irritants. Several components of mucosal defense are the mucosal blood flow, mucus and bicarbonate secretion,

Table 1. Results of gastric pH, macroscopic and microscopic observations

Kelompok	pH	Skor Makro	Skor Mikro
KN	1.50±0.58^{c}	5.50±1.00^{a}	8.67±0.58^{a}
KP	5.25±0.50^{a}	4.25±1.89^{ab}	4.33±0.58^{bc}
P1	3.00±0.00^{b}	2.50±1.29^{b}	5.00±1.00^{b}
P2	3.75±0.96^{b}	3.00±1.16^{ab}	3.00±0.00^{c}

Gastric macroscopic is assessed by parameters of hemorrhage, hyperemia, loss of mucosal folds (score 0-3; 0 no lesio, 1 mild, 2 moderate, 3 severe). Gastric microscopy was assessed by parameters of congestion, erosion, and inflammation (score 1-3; 1 mild, 2 moderates, 3 severe). Different superscript letters show significant differences (P<0.05)

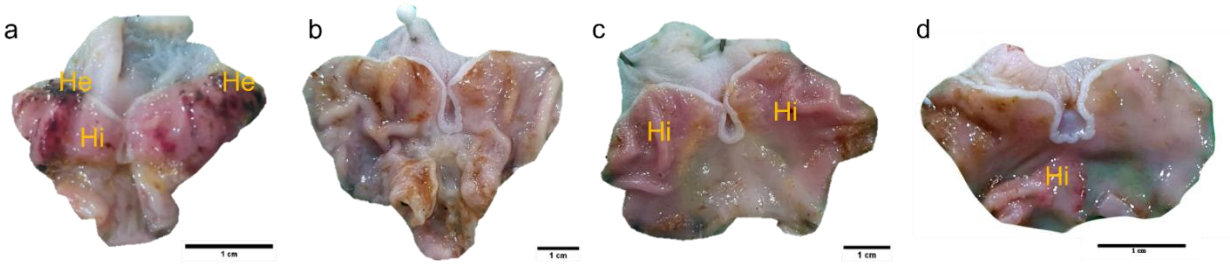

Figure 1. Gross pathology. The effect of white turmeric (*Curcuma zedoaria*) infusion on *Sprague Dawley* (SD) rat stomach suffering from gastritis on treatment groups: a). negative control b). positive control (omeprazole), c). infusion 30%, d). infusion 60%. He: hemorrhage, Hi: hyperemia

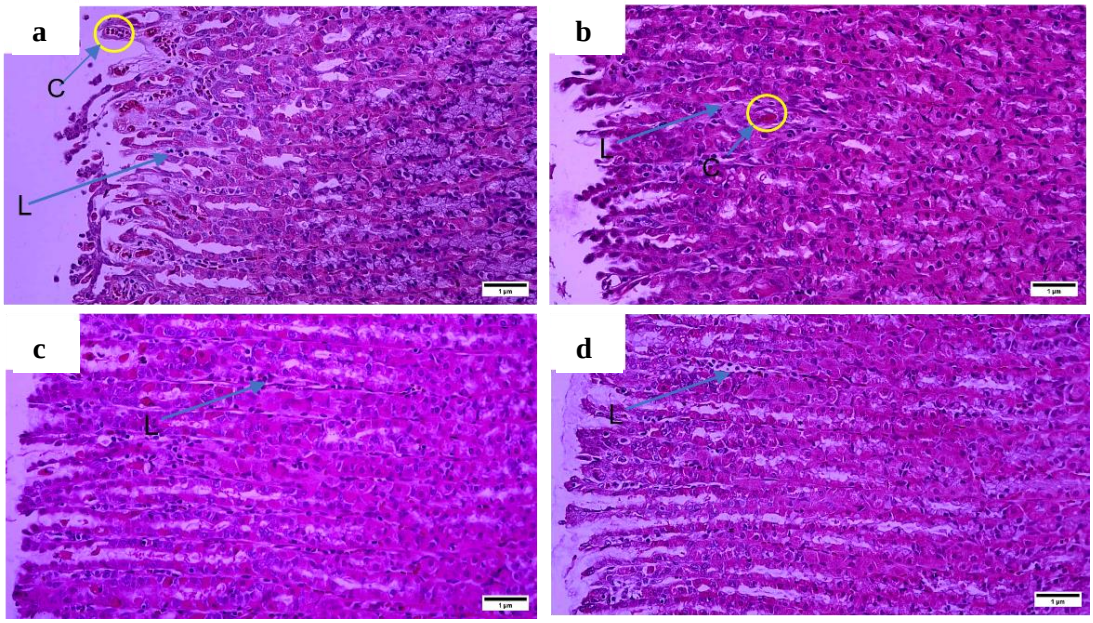

Figure 2. Histopathology of white turmeric (*Curcuma zedoaria*) infusion on the stomach of Sprague Dawley (SD) rats suffering from gastritis, in treatment groups: a). negative control, b). positive control (omeprazole), c). infusion 30%, d). infusion 60%. (staining: HE, magnification: objektive 20x). C: congestion, L: lymphocyte

prostaglandin, and the proliferation of mucosal cells (Laine et al. 2008). If this mucosal defense mechanism is affected followed by the presence of an irritant agent such as ethanol, stomach mucosal damage may occur.

Ethanol is known to cause severe effects in the stomach, such as acute mucosal inflammation, mucosal hyperemia, edema, bleeding, erosion, and stomach mucosa ulceration. Alcohol dehydrogenase found in the stomach can catalyze ethanol into acetyl aldehyde, and oxidation xanthine which would catalyze ethanol into free radicals. The free radicals will then increase the production of reactive oxygen species (ROS) that triggers endothelial cell damage (Chen et al. 2019). The combination of HCl and ethanol may stimulate stomach acid secretion, necrosis, and apoptosis of stomach mucosa through the destruction of the local mucosa

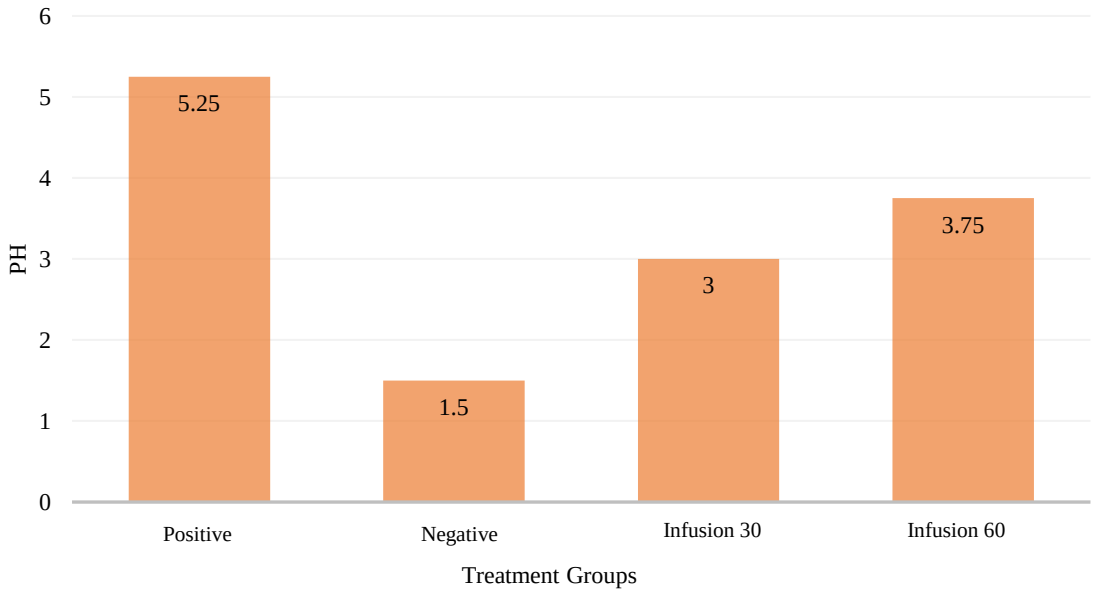

Figure 3. Rat Stomach pH Value

defense system (Bujanda 2000; Sohn et al. 2015). The high number of lymphocyte cells microscopically indicates the inflammation of the stomach. The results of the microscopic assessment carried out sequentially for negative, and positive controls, CZI 30% and CZI 60% were 8.67±0.58, 4.33±0.58, 5.00±1.00, and 3.00±0.00.

Curcuma zedoaria is reported to contain several bioactive including tannins, flavonoids, saponins, alkaloids, terpenoids, carbohydrates, and steroids (Azam et al. 2017). The flavonoid compound in white turmeric acts as an antioxidant containing hydroxyl radicals and superoxide which protect the lipid membrane and prevents cell damage (Czekaj et al. 2018; Gozali et al. 2022). The flavonoid compound, quercetin, contained in white turmeric could also possibly have a protective effect on the mucosa by lowering oxidative stress and increasing the activity of the antioxidant enzyme (Coşkun et al. 2004). The curcumin content in *Curcuma zedoaria* also has anti-inflammatory activity by inhibiting the production of NO and expression of iNOS induced by LPS, as well as the expression and activation of cyclooxygenase-2 (COX-2) (Pan et al. 2000; Hong et al. 2004). These could be the reasons that few inflammations were noted in rats given white turmeric infusion prior to gastritis induction.

CONCLUSION

Curcuma zedoaria can inhibit the stomach-damaging effect of ethanol and HCl administration. Based on the macroscopic and histopathological features of rat stomachs given white turmeric infusion, it was found that stomach injuries decreased along with the increase of white turmeric concentration that was given.

ACKNOWLEDGEMENT

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Improving Storage Stability and Kinetics of Pasundan Bull Sperm Encapsulation Using Alginate Solid Beads

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ABSTRAK

Manan MA, Khan A, Samsudewa D, Pamungkas FA, Abbas F, Khan BN, Haidari K, Zulfiqar H, Darussalam I, Widaningsih W. 2024. Peningkatan stabilitas penyimpanan dan kinematik enkapsulasi *solid bead* alginat pada sperma sapi Pasundan. JITV 29(2):103-113. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3427.

Penelitian ini bertujuan untuk mengembangkan proses enkapsulasi sperma sapi pasundan dan menyelidiki apakah enkapsulasi *solid bead* alginat dapat meningkatkan kemampuan bertahan hidup sperma dalam penyimpanan suhu dingin. Proses enkapsulasi dilakukan dengan meneteskan sperma-alginat 0,5% dan 0,25% kedalam larutan yang mengandung 1,5% kalsium klorida ($CaCl_2$), lalu dibiarkan mengendap selama satu menit sehingga sperma berasa dalam enkapsulasi *solid bead* alginat. Enkapsulasi *solid bead* alginat dan yang tidak dienkapsulasi dalam pengencer kuning telur tris dengan alginat 0,25% dan 0,5% diamati 0, 24, 48, 72, dan 96 jam penyimpanan pada 5°C. Parameter yang diamati selain viabilitas dan integritas membran spermatozoa, juga mencakup parameter motilitas total, motilitas progresif, dan kinematika spermatozoa yang diukur menggunakan sistem *computer-assisted sperm analysis* (CASA). Data yang diperoleh dianalisis secara statistik menggunakan *analysis of variance* dan *Duncan Multi Range Test*. Hasil penelitian menunjukkan bahwa meskipun proses awal enkapsulasi menghasilkan penurunan motilitas total, motilitas progresif, dan nilai kinematika, enkapsulasi *solid bead* alginat lebih stabil dibandingkan sperma yang tidak dienkapsulasi hingga 4 hari penyimpanan pada suhu 5 °C. Pergerakan spermatozoa pada enkapsulasi *solid bead* alginat dibatasi karena viskositas media alginat tanpa mengganggu viabilitas dan integritas membran spermatozoa. Dapat disimpulkan bahwa enkapsulasi *solid bead* alginat dalam semen sapi pasundan dapat meningkatkan stabilisasi spermatozoa selama proses penyimpanan.

Kata Kunci: Alginat, Kinematika, Sperma Sapi Pasundan, Preservasin, Alginate Solid Bead Enkapsulasi

ABSTRACT

Manan MA, Khan A, Samsudewa D, Pamungkas FA, Abbas F, Khan BN, Haidari K, Zulfiqar H, Darussalam I, Widaningsih W. 2024. Improving storage stability and kinetics of Pasundan bull sperm encapsulation using alginate solid beads. JITV 29(2):103-113. DOI: http://dx.doi.org/10.14334/jitv.v29i2.3427.

The study aims to develop a pasundan bull sperm encapsulation process and investigate whether alginate solid bead encapsulation improves sperm lifetime and survivability in cold storage. In order to make sperm encapsulation, 0.5% and 0.25% sperm-alginate droplets were added to a solution containing 1.5% dissolved calcium chloride (CaCl2) in physiological saline, and droplets were allowed to settle for one minute, resulting in sperm embedded in solid beads of alginate matrix. Solid beads and unencapsulated sperms in a diluent of tris egg yolk with 0.25% and 0.5% alginate were assessed following 0 h, 24 h, 48 h, 72 h, and 96 h of refrigerated storage at 5°C. The observed parameters, in addition to sperm viability and membrane integrity, also include total motility, progressive motility, and sperm kinematics, which were measured using computer-assisted sperm analysis (CASA) systems. To determine if differences amongst data were statistically significant, analysis of variance was used, and the Duncan Multi Range Test was performed. The result showed that although the initial process of encapsulation resulted in a decrease in total motility, progressive motility, and kinematics value, alginate solid bead encapsulation was found to be more stable than unencapsulated sperm during storage at 5 °C for up to 4 days. The movement of spermatozoa is restricted to the viscosity of the alginate medium without disturbing the membrane's viability and integrity. It can be concluded that alginate solid bead encapsulation in pasundan bull semen can improve sperm stabilization during refrigerator storage.

Key Words: Alginate, Kinematics, Pasundan Bull Sperm, Preservation, Solid Bead Encapsulation

INTRODUCTION

The Pasundan cattle, endemic to Indonesia, primarily occupy the Southern coast of West Java and the boundary along the Northern Priangan region, retaining its importance as an emblematic breed among the Sundanese people (Arifin et al. 2019). According to 2015 research by the West Java Central Statistics Agency, there was a significant reduction in the Pasundan cattle population, dropping from 50,000 to 40,000 heads in 2013 (Dwitresnadi et al. 2015). Improvements in the breeding process and the genetic quality of livestock are necessary to maintain the population. As part of these efforts, Artificial Insemination (AI) employing frozen semen has been identified as a vital technology to raise the population and genetic traits of Pasundan cattle (Widyastuti et al. 2022). Agriculture Regulation No. 10/Permentan/PK210/2016 mandates that 60% of AI procedures must employ indigenous breeds (Baharun et al. 2023).

Artificial Insemination uses treated semen, both liquid and frozen. This procedure involves the dilution of semen using centrifugation in an appropriate extender, followed by further preservation and freezing for cryogenic storage (Galarza et al., 2023). However, during these storage processes, sperm incur issues arising from temperature-induced biomolecular changes, physiological imbalances, and oxidative damage. These factors may reduce sperm viability, motility, and functional fertilization capability (Sharafi et al., 2022).

Improving sperm resistance against stress and enhancing sperm lifetime during storage would have tremendous practical advantages for the breeding business. In contrast, it improves the period available for transit and usage for artificial insemination. One feasible method would be to preserve sperm cells inside microcapsules to shield them from environmental stress (Pruß et al., 2022). Within the discipline of zootechnics, the first concept for encapsulating male gametes is dedicated to facilitating a regulated discharge of spermatozoa. Over the past 30 years, researchers have actively investigated the encapsulation process and increased its application to several animal species (Perteghella et al. 2015a). Increased fertility and prolificacy can be ensured by sperm encapsulation, which can keep a high concentration of sperm in the uterus during estrus (Gordon 2017).

Alginate encapsulation of sperm is vital in reproductive technology and animal reproduction. The gelation process frequently uses calcium chloride (Feyzmanesh et al., 2022). A significant advantage of alginate encapsulation is its efficacy in enhancing sperm viability during preservation or cryopreservation. Sperm encapsulation has been a focal point in bovine and pig breeding efforts, mainly aimed at enabling more prolonged sperm release within the female reproductive system after a single insemination (Roca et al. 2016; Alm-Kristiansen et al. 2018). Solid bead encapsulation, a novel technique for sperm encapsulation, was introduced to create a regulated semen delivery system, mainly for stallion semen (Pruß et al., 2022). This experiment involved dropping semen that contained calcium chloride into an alginate solution. A semi-permeable calcium alginate gel membrane is formed when calcium ions diffuse out of the droplets and react with the alginate. This one-step method yields capsules containing spermatozoa that have stability and vitality preserved (Pruß et al. 2022).

On the other hand, most research has evaluated semen properties using traditional techniques, which provide little and frequently inconsistent information regarding sperm motility across laboratories (Alipour et al. 2017). Objective computer-assisted sperm analysis (CASA) systems are often used to analyze sperm motility, including sperm kinematics. These methods enable the precise and repeatable evaluation of sperm motility characteristics (Knox 2015). Using a CASA system, sperm kinematics are assessed by taking pictures of individual sperm cells. After that, the system tracks the cells' trajectories using algorithms to describe their movement (Van de Hoek et al., 2022).

This work aimed to design a procedure for alginate solid bead encapsulation optimized for pasundan bull sperm. Additionally, the research intended to determine whether encapsulation leads to enhanced stability during cold storage and maximizes sperm survival in contrast to unencapsulated by assessing sperm motility and kinematics using CASA.

MATERIALS AND METHODS

Animals

The study was conducted at the Center for Artificial Insemination Breeding and Development of Beef Cattle in Ciamis, West Java, Indonesia, and authorized by Ethical Clearance and Foreign Research Permit, Directorate of Management for Research and Innovation Permit and Scientific Authorities, BRIN No. 011/KE.02/SK/01/2023. The research involved three Pasundan bulls aged 3-6 years, with body weights ranging from 380 to 430 kg. The bulls were fed a diet of Pennisetum purpureum grass up to 10% of body weight and commercial concentrate up to 1% of body weight, retaining 16% crude protein, fed twice a day, and water provided ad libitum.

Semen collection and evaluation

The semen was collected using an artificial vagina once a week from each pasundan bull, from 07.30 AM to 10.00 AM, following the Operational Standard

Procedure of the Center for Artificial Insemination Breeding and Development of Beef Cattle. After collection, the semen samples were immediately subjected to microscopic and macroscopic evaluation.

Unencapsulated sperm

Semen was collected using an artificial vagina, and ejaculated semen was immediately evaluated following collection and diluted with Tris Egg Yolk (TEY) extender in alginate with concentrations of 0.25% and 0.5% (Kumar et al. 2019). The diluted semen was packaged in a corning tube (15 ml). The samples were then placed in a beaker glass with a water jacket cooled from 37 °C to 5 °C and stored at 5 °C. Microscopic evaluation of unencapsulated sperm was determined after diluting with sodium alginate and Tris Egg Yolk (TEY) extender and after storage at 5 °C for four days.

Sperm alginate encapsulation in solid bead structures

Microencapsulation procedures developed for canine and stallion sperm (Shah et al. 2011; Pruß et al. 2022) were used with minor modifications. Briefly, the sperm diluted with the TEY diluent was supplemented with 0.25% and 0.5% alginate. The sperm was forced through a 21-gauge needle attached to a 5 mL syringe into a beaker glass containing the 1.5% calcium chloride ($CaCl_2$) dissolved in physiologic saline. The distance between the tip of the needle and the surface of the calcium chloride solution was maintained at 8.5 cm to ensure the shape of the microcapsule. The sperm suspension immediately upon contact with the calcium chloride solution solidified the entire droplet to form a high-viscosity microgel. The microgels were swayed gently and allowed to react for 30 sec. A schematic presentation of the procedures resulting in a calcium-alginate solid bead is presented in Figure 1.

The microgels were then collected and rinsed two times with physiologic saline. After that, sperm/alginate solid bead capsules were transferred to TEY diluent and packaged in a Corning tube (15 ml). The samples were then placed in a beaker glass with a water jacket cooled from 37°C to 5°C and stored at 5°C. Microscopic evaluation of sperm was determined after encapsulation and storage at 5°C for four days.

To assess sperm evaluation, shell structures/spheres were pierced, using a 100 µL plastic micropipette tip to aspirate sperm residing in the inner core. Sperm inside alginate solid bead capsules were recovered by repeated gentle pipetting of the concentrated bead-containing solution, disintegrating the beads. Recovered sperm were diluted in TEY diluent, and the sperm was ready for evaluation.

Microscopic evaluation of sperm

Sperm concentration was evaluated using a photometer (SDM 6, Minitub, Germany). Semen with ≥70% progressive motility of sperm, a concentration of sperm $\geq 500 \times 10^6$ ml^{-1}, and morphologically abnormal sperm ≤20% were used for the experiment. Computer-Assisted Sperm Analysis (CASA) using the Hamilton Thorne IVOS II ver. 12.1 was utilized to analyze sperm motility. An 8 µl semen sample was diluted with 4 ml of 0.9% NaCl. The combination was mounted on a slide, covered with a coverslip, and viewed using a microscope with a 10x10 objective magnification linked to a computer containing CASA. Five fields of view were tested, and the motility was evaluated on a scale from 0%

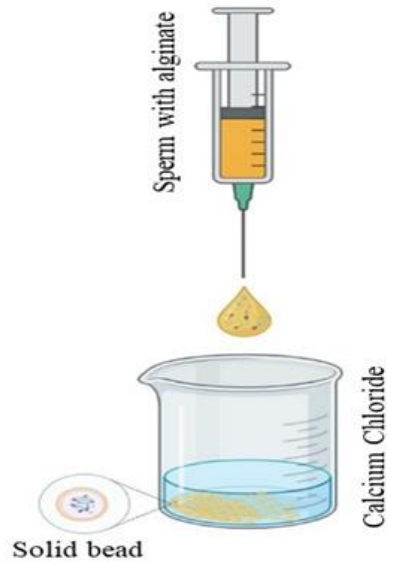

Figure 1. Formation of alginate solid bead encapsulation

Table 1. Sperm kinematic descriptors and their corresponding definition (Barbas et al. 2018)

Kinematic Descriptor	Measurement Unit	Descriptor Definition
Curvilinear velocity (VCL)	(μm/s)	The average path velocity of the sperm head along its true
Straight-line velocity (VSL)	(μm/s)	The average path velocity of the sperm head along its true trajectory per unit time
Average path velocity (VAP)	(μm/s)	The average velocity of the sperm head along its average trajectory per unit time
Linearity index (LIN)	(%)	The ratio between VSL and VCL (x 100)
Straightness index (STR)	(%)	The ratio between VSL and VAP (x 100)
Wobble coefficient index (WOB)	(%)	The ratio between VAP and VCL (x 100)
Amplitude of lateral head displacement (ALH)	(μm)	The average value of the extreme side-to-side movement of the sperm head in each beat cycle
Beat cross-frequency (BCF)	(Hz)	The frequency with which the actual sperm trajectory crosses the average path trajectory

to 100%. The CASA system measured different motility characteristics, including total motility (TM), progressive motility (PM), fast motility (FM), slow motility (SM), local motility (LM), and immotile (IM). The kinematic variables assessed were the curvilinear velocity (VCL), straight-line velocity (VSL), average pathway velocity (VAP), linearity (LIN), straightness (STR), wobbles (WOB), the amplitude of lateral displacement (ALH). They beat cross frequency (BCF), shown in Table 1**.**

Sperm viability was assessed using eosin-nigrosin staining, a technique adapted from Barth and Oko (Chenoweth, 2022) with modification. The integrity of the plasma membrane was asses using a hypoosmotic swelling solution (HOS test) consisting of 0.735 g Na citrate, 1.351 g fructose, and 100 ml distilled water. (Baldaniya et al. 2021). The solution was incubated at 37°C for 30 minutes, and then 10 μL of the mixture was placed on a microscope slide and examined at 400× magnification.

Statistical analysis

Data were analyzed using Statistical Analysis Software (SAS Institute, Cary, NC, USA). ANOVA (analysis of variance) was used to determine if there were differences among statistically significant data, and the Duncan Multi-Range Test was performed. Data are presented as mean values ± standard deviations. Differences are considered statistically significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

The total motility and kinematic values of sperm are rapid, slow, circle, local, and immotile between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points. These are presented in Figure 2, Tables 2 and 3. The results showed that the total motility value of sperm, both encapsulated and unencapsulated, decreased during the storage process for up to 96 hours. The encapsulation process showed lower total motility values than the unencapsulated sperm, especially for up to 48 hours refrigerated at 5°C ($P<0.05$). However, encapsulated sperm did not experience a significant decrease in total motility values compared to unencapsulated during refrigerator storage; this can be seen from the total motility values found to be numerically superior in encapsulation 0.25% alginate groups after 96 hours refrigerated at 5°C without statistical differences.

The slow motility value of encapsulated sperm at 0 hours at room temperature is lower than that of unencapsulated sperm. However, after 96 hours of refrigerated storage, the decrease in the slow motility value of sperm is less than that of the unencapsulated sperm. The immotile value influences the total motility value. The immotile values after 96 hours at refrigerated storage were found to be numerically superior in the encapsulated 0.25% alginate group, followed by unencapsulated 0.25% alginate, encapsulated 0.5% alginate, and unencapsulated 0.5% alginate groups without statistical difference, respectively.

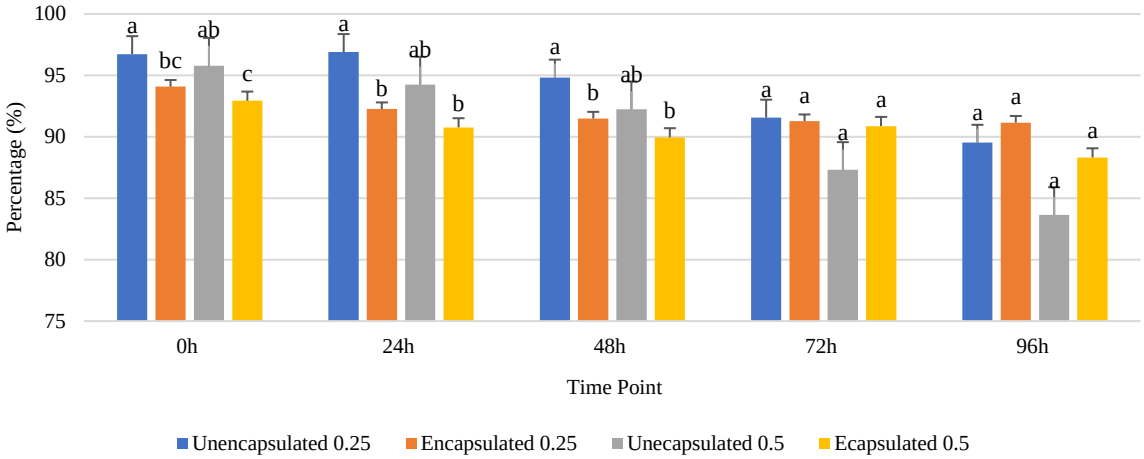

Figure 2. Total motility of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points of 0h at room temperature and 24h, 48h, 72h, 96h refrigerated at 5 °C. Statistical significance difference (P≤ 0.05) was shown with the different superscripts

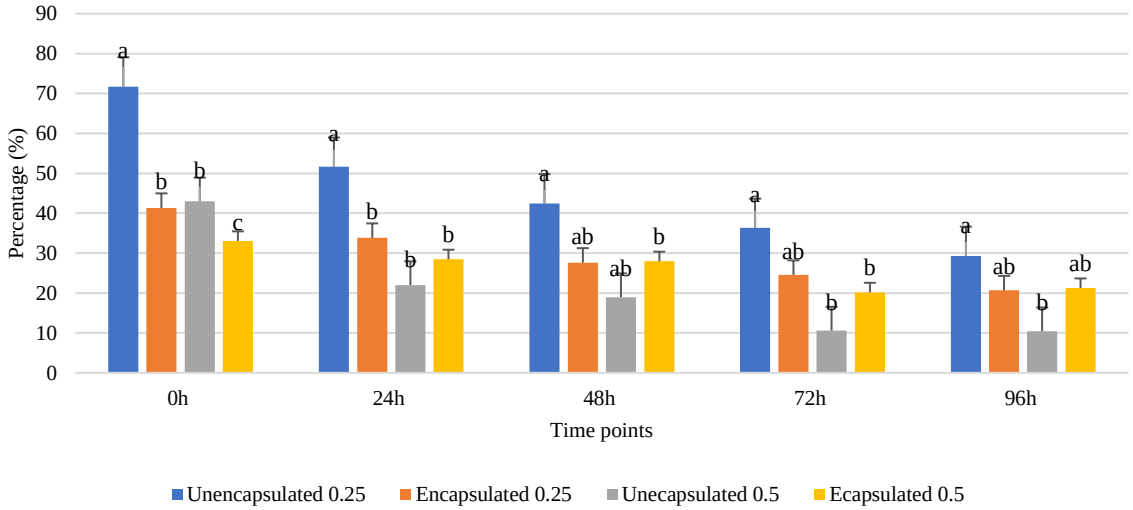

Figure 3. Progressive motility of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points of 0h at room temperature and 24h, 48h, 72h, 96h refrigerated at 5 °C. Statistical significance difference (P≤0.05) showed with the different superscripts

The results of the analysis of the kinematic value of sperm at the time of 0 h storage showed that the values of VCL, VSL, VAP, and ALH in the unencapsulated 0.25 group were higher, followed by the encapsulation 0.25, unencapsulated 0.50. They encapsulated 0.50 alginate groups with a statistical difference, respectively. However, after 96 h of refrigerated storage, VCL, VSL, VAP, and ALH values showed no significant difference and found that the unencapsulated group 0.5 had the lowest value. I f we look deeper, it turns out thatduring the storage process, the unencapsulated group experienced a more drastic decrease in VCL, VSL, VAP, and ALH values than the encapsulated group.

LIN, WOB, and BCF values at 0 h storage indicate that the encapsulated group is higher than the unencapsulated group. However, after 96 h of refrigerated storage, the values of LIN, WOB, and BCF showed no significant difference. Similarly, the STR values from 0h to 96h storage showed no difference statistically.

Alginate solid bead encapsulation is a viable strategy to reduce harmful effects following sperm preservation. During the preservation process, many spermatozoa die or are seriously damaged. Refrigerated semen storage causes structural and functional changes in sperm as indicative of a natural aging process influenced by storage temperature, time of day, and species (Wiebke et al., 2022). When freshly ejaculated sperm is rapidly cooled from body temperature to temperatures below 5°C, a cold shock occurs, resulting in a loss of sperm viability. Cold shock during the period of adaptation and storage of spermatozoa at low temperatures leads to changes in the composition of the spermatozoa membrane (Wiebke et al. 2022); this

Table 2. Evaluation of sperm motility between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at the different time points of 0h at room temperature and four days refrigerated at 5°C

Parameters	Groups	Time Points				
		0h	24h	48h	72h	96h
Fast Motility	Unencapsulated 0.25	2.64±0.38^{a}	1.03±0.30^{a}	1.02±0.36^{a}	0.71±0.26^{a}	0.57±0.21^{a}
	Encapsulated 0.25	1.62±0.32^{a}	0.90±0.26^{a}	0.55±0.21^{b}	0.30±0.09^{b}	0.08±0.04^{b}
	Unencapsulated 0.5	0.20±0.04^{b}	0.14±0.01^{a}	0.24±0.12^{b}	0.15±0.03^{b}	0.12±0.06^{b}
	Encapsulated 0.5	0.45±0.12^{ab}	0.70±0.10^{a}	0.54±0.28^{b}	0.17±0.00^{b}	0.16±0.05^{b}
Slow motility	Unencapsulated 0.25	69.10±0.95^{a}	50.60±3.79^{a}	41.42±6.33^{a}	35.59±6.99^{a}	28.74±6.68^{a}
	Encapsulated 0.25	37.31±1.65^{bc}	32.58±4.21^{b}	26.68±5.88^{ab}	24.07±3.75^{ab}	20.52±3.84^{ab}
	Unencapsulated 0.5	42.76±1.47^{b}	21.88±2.89^{b}	18.72±4.76^{b}	10.46±2.00^{b}	10.32±3.88^{b}
	Encapsulated 0.5	32.49±3.12^{c}	27.53±4.48^{b}	27.08±6.98^{ab}	19.82±3.36^{b}	20.96±5.79^{ab}
Circular Motility	Unencapsulated 0.25	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}
	Encapsulated 0.25	2.40±0.65^{a}	0.38±0.14^{a}	0.34±0.14^{a}	0.18±0.09^{a}	0.05±0.02^{a}
	Unencapsulated 0.5	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}
	Encapsulated 0.5	0.12±0.08^{b}	0.33±0.21^{a}	0.23±0.09^{ab}	0.17±0.06^{a}	0.14±0.08^{a}
Local Motility	Unencapsulated 0.25	25.00±0.66^{b}	45.28±4.28^{c}	52.38±6.47^{b}	55.26±5.79^{b}	60.22±5.15^{a}
	Encapsulated 0.25	52.75±1.11^{c}	56.45±2.32^{b}	60.88±4.67^{ab}	66.73±3.52^{a}	70.50±4.28^{a}
	Unencapsulated 0.5	52.82±1.82^{b}	72.23±3.20^{a}	73.27±4.52^{a}	76.7±1.45^{a}	73.20±2.35^{a}
	Encapsulated 0.5	59.88±2.37^{a}	62.31±2.99^{b}	61.99±5.81^{ab}	70.72±2.44^{a}	67.06±5.48^{a}
Immotile	Unencapsulated 0.25	3.27±0.47^{c}	3.10±0.32^{b}	5.18±0.99^{b}	8.44±2.07^{a}	10.48±2.38^{a}
	Encapsulated 0.25	5.92±0.41^{ab}	9.75±2.27^{a}	11.51±2.01^{a}	8.72±0.86^{a}	8.85±1.05^{a}
	Unencapsulated 0.5	4.22±0.51^{bc}	5.75±0.37^{ab}	7.76±1.21^{ab}	12.70±2.32^{a}	16.36±4.20^{a}
	Encapsulated 0.5	7.06±1.12^{a}	9.24±1.91^{a}	10.05±1.80^{a}	9.13±1.08^{a}	11.69±0.90^{a}

Statistical significance difference ($P \leq 0.05$) is shown with the different superscripts along the column.

Table 3. Kinematic study of unencapsulated and encapsulated sperms of pasundan bulls

Time	Groups	Kinematic parameters							
		VCL (µm/s)	VSL% (µm/s)	VAP (µm/s)	STR (%)	LIN (%)	WOB (%)	ALH (µm)	BCF (Hz)
0h	Unencapsulated 0.25	54.67±1.20^{a}	31.25±0.65^{a}	29.44±2.78^{a}	0.95±0.00^{a}	0.57±0.01^{c}	0.61±0.01^{c}	1.45±0.03^{a}	0.61±0.01^{c}
	Encapsulated 0.25	38.05±1.21^{b}	26.91±2.11^{a}	28.01±2.10^{a}	0.96±0.00^{a}	0.71±0.03^{a}	0.74±0.03^{a}	0.98±0.02^{b}	0.74±0.04^{a}
	Unencapsulated 0.50	32.78±0.93^{b}	20.63±0.80^{b}	17.99±2.89^{b}	0.95±0.00^{a}	0.63±0.01^{bc}	0.67±0.01^{bc}	0.98±0.03^{b}	0.67±0.01^{bc}
	Encapsulated 0.50	25.04±4.46^{c}	16.92±5.45^{b}	17.81±2.83^{b}	0.95±0.00^{a}	0.68±0.01^{ab}	0.72±0.01^{ab}	0.75±0.09^{c}	0.72±0.01^{ab}
24 h	Unencapsulated 0.25	42.56±3.17^{a}	22.69±1.98^{a}	20.03±4.68^{a}	0.93±0.00^{b}	0.53±0.01^{b}	0.57±0.00^{b}	1.24±0.04^{a}	0.57±0.01^{b}
	Encapsulated 0.25	28.96±6.04^{b}	17.61±3.69^{ab}	18.61±3.86^{a}	0.95±0.00^{a}	0.61±0.02^{a}	0.65±0.02^{a}	0.90±0.15^{b}	0.65±0.04^{a}
	Unencapsulated 0.50	23.57±1.58^{b}	13.67±1.16^{a}	15.45±2.23^{a}	0.93±0.00^{b}	0.58±0.01^{a}	0.63±0.01^{a}	0.81±0.03^{b}	0.63±0.02^{a}
	Encapsulated 0.50	26.13±4.81^{b}	16.14±2.84^{ab}	17.05±2.99^{a}	0.95±0.00^{a}	0.62±0.01^{a}	0.66±0.01^{a}	0.79±0.12^{b}	0.66±0.03^{a}
48 h	Unencapsulated 0.25	39.02±4.83^{a}	20.33±2.72^{a}	16.47±4.40^{a}	0.93±0.01^{a}	0.52±0.01^{b}	0.56±0.01^{b}	1.15±0.10^{a}	0.56±0.01^{b}
	Encapsulated 0.25	24.93±6.06^{a}	16.92±4.57^{a}	16.63±4.47^{a}	0.94±0.01^{a}	0.61±0.04^{a}	0.66±0.04^{a}	0.75±0.15^{a}	0.66±0.04^{a}
	Unencapsulated 0.50	20.59±3.64^{a}	12.03±2.05^{a}	10.98±2.94^{a}	0.92±0.01^{a}	0.59±0.00^{a}	0.64±0.01^{a}	0.71±0.08^{a}	0.64±0.01^{a}
	Encapsulated 0.50	22.05±7.16^{a}	13.75±4.53^{a}	14.47±4.69^{a}	0.95±0.01^{a}	0.62±0.01^{a}	0.66±0.01^{a}	0.71±0.20^{a}	0.66±0.02^{a}
72 h	Unencapsulated 0.25	33.81±5.75^{a}	17.70±3.05^{a}	16.29±3.50^{a}	0.92±0.01^{a}	0.53±0.01^{b}	0.57±0.01^{c}	1.06±0.14^{a}	0.57±0.01^{b}
	Encapsulated 0.25	24.35±3.55^{ab}	14.73±2.62^{a}	15.67±2.69^{a}	0.94±0.01^{a}	0.60±0.04^{ab}	0.64±0.04^{ab}	0.74±0.08^{b}	0.64±0.07^{ab}
	Unencapsulated 0.50	17.41±2.41^{b}	9.95±1.21^{a}	10.63±1.21^{a}	0.92±0.00^{a}	0.58±0.02^{ab}	0.63±0.01^{ab}	0.62±0.07^{b}	0.63±0.02^{ab}
	Encapsulated 0.50	20.21±2.98^{b}	12.54±2.33^{a}	13.32±2.31^{a}	0.94±0.00^{a}	0.61±0.02^{a}	0.66±0.02^{a}	0.65±0.07^{b}	0.66±0.04^{a}
96 h	Unencapsulated 0.25	29.07±5.79^{a}	15.30±6.67^{a}	13.24±3.61^{a}	0.91±0.01^{a}	0.52±0.01^{a}	0.57±0.01^{a}	0.91±0.13^{a}	0.62±0.01^{a}
	Encapsulated 0.25	20.02±2.42^{ab}	10.33±6.67^{a}	12.59±2.25^{a}	0.93±0.01^{a}	0.58±0.04^{a}	0.62±0.03^{a}	0.74±0.08^{ab}	0.62±0.06^{a}
	Unencapsulated 0.50	14.46±4.19^{b}	9.91±3.74^{a}	11.69±2.44^{a}	0.90±0.01^{a}	0.58±0.02^{a}	0.64±0.02^{a}	0.54±0.12^{b}	0.64±0.04^{a}
	Encapsulated 0.50	21.54±3.90^{ab}	15.85±6.68^{a}	14.04±2.98^{a}	0.94±0.02^{a}	0.60±0.04^{a}	0.64±0.04^{a}	0.71±0.07^{ab}	0.64±0.07^{a}

VCL= curvilinear velocity; VSL= straight-line velocity; VAP= average pathway velocity; STR= straightness; LIN= linearity; WOB= wobbles; ALH= amplitude of the lateral head displacement; BCF= beat cross frequency. The data is shown in the form of Mean±S.E at the ($P<0.05$)

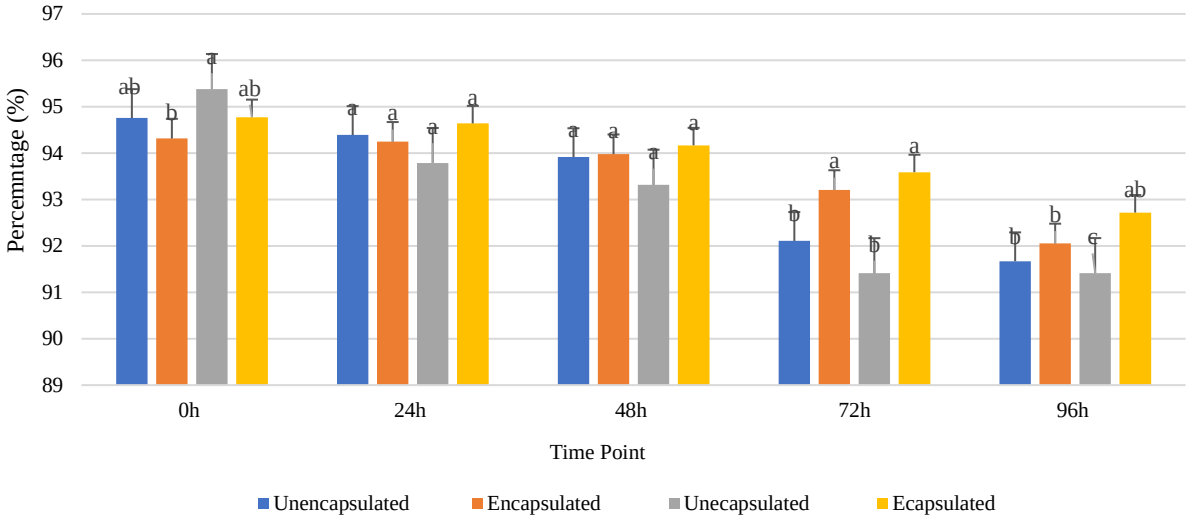

Figure 4. Plasma membrane integrity of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points of 0h at room temperature and 24h, 48h, 72h, and 96h refrigerated at 5 °C. Statistical significance difference (P≤0.05) showed with different superscripts.

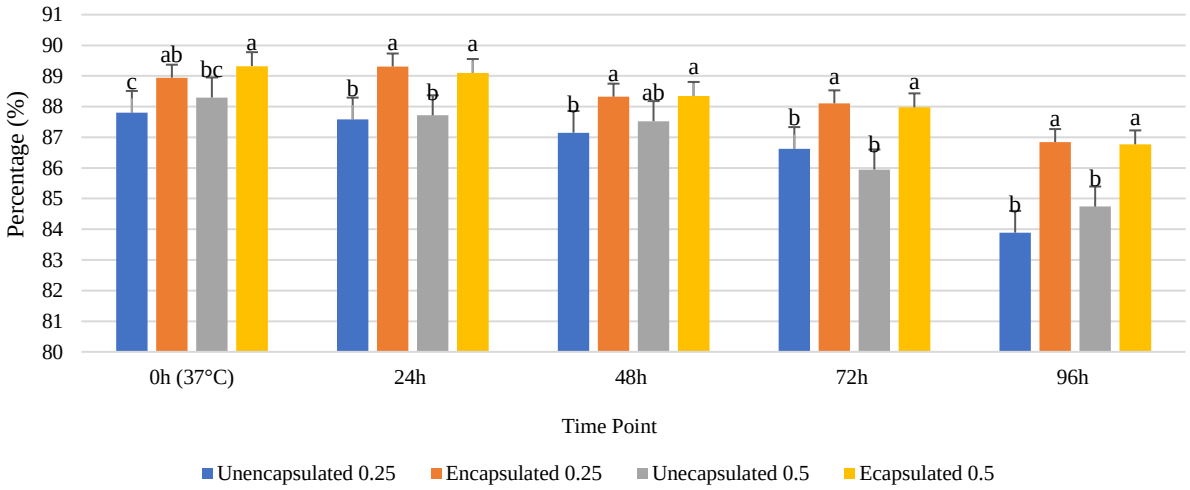

Figure 5. Sperm viability between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points of 0h at room temperature and 24h, 48h, 72h, and 96h refrigerated at 5°C. Statistical significance difference (P≤0.05) showed with the different superscripts

indicates that cold shock changes the composition of the membrane lipid bilayer and the fluidity of the plasma membrane (Gunde-Cimerman et al. 2014).

The cold sensitivity of spermatozoa changes with time and temperature (Paschoal et al., 2020). Other essential factors include seminal plasma (Höfner et al. 2020), aging during incubation and diluents used (Rahman et al. 2023), and the cholesterol content (Batissaco et al. 2020). The inner sperm membrane is more sensitive to damage from cold shock. The consequence of cold-induced membrane, dilution, and cooling during sperm preservation increases the permeability of the plasma membrane, resulting in a change in phase transition and the entry of free calcium ions from the external environment into the cell, inducing capacitation processes and making cell membranes more fusogenic and unstable (Bernabò et al. 2018).

Although the initial encapsulation process decreased total motility, progressive motility, and kinematics value, alginate solid bead encapsulation was found to be more stable than unencapsulated sperm during storage at 5 °C for up to 4 days. The primary purpose of sperm encapsulation is to increase the stability of sperm during cold storage (Gosálvez et al. 2021). In addition, encapsulation has explicitly been tried to facilitate prolonged sperm release in the female reproductive tract after insemination (Perteghella et al. 2015b; Perteghella et al. 2017; Pruß et al. 2022). A decrease in total motility, progressive motility, and kinematics value in the initial

process of encapsulated sperm may be caused by alginate increasing the medium viscosity; this was evident from the decrease in sperm velocity upon incubation in sperm alginate solid bead encapsulation.

In the results, it was observed that sperm motility and kinematics value decreased in alginate solid bead encapsulated sperm as compared to unencapsulated sperm, which was in line with the study by Pruß et al. (2022). One of the factors that seems to be causing sperm motility and kinematics to decline is the presence of alginate particles on the surface of the sperm. Alginate is widely used as a gelling agent, and it was anticipated that it would increase the viscosity of extenders, leading to lesser values for sperm velocities (Kumar et al. 2019). Sperm motility is actively regulated by the fibrous sheaths that make up the cytoskeletal structure of sperm flagella (Lehti & Sironen 2017). Retaining the alginate residue on the sperm may hinder its ability to move its tail actively and decrease its motility (Pruß et al. 2022). Sperm motility was found to be reduced by Torre et al. (2000) when encapsulated in porcine semen with 0.5% alginate; this was attributed to the presence of residual alginate particles in the sperm. Furthermore, Ebel et al. (2023) report that the concentration of crosslinking agents, type of alginate, total amount of alginate, method used to encapsulate cells, and procedure moderating hydrogel disintegration can affect how successfully alginate hydrogel is formed and can independently affect sperm motility.

Figure 3 shows the progressive motility values of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points. The results showed that the progressive motility value of sperm, both encapsulated and unencapsulated, decreased during the storage process for up to 96 hours. The encapsulation process showed lower progressive motility values than the unencapsulated sperm at 0 hours at room temperature. However, after storage in a refrigerator at 5°C for up to 96 hours, the progressive motility was highest in the unencapsulated 0.25% alginate group was followed by the encapsulated 0.25% alginate, encapsulated 0.5% alginate, and unencapsulated 0.5% alginate groups, respectively.

The plasma membrane integrity value of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points is presented in Figure 4. Similar to the total and progressive motility values, the plasma membrane integrity value, encapsulated and unencapsulated, decreased during the storage process for up to 96 hours. While the plasma membrane integrity value was highest in the unencapsulated 0.5% alginate group, a statistical difference was found between the unencapsulated 0.5% alginate and the encapsulated 0.25 and 0.5% alginate groups ($P<0.05$). No significant difference was found between the groups at 24 and 48 hours. However, at 72 and 96 hours, the encapsulated groups had significantly more intact sperm than the unencapsulated groups; this can be seen from the plasma membrane integrity values that were numerically superior in the encapsulation 0.5% alginate group than the unencapsulated groups.

Figure 5 presents the viability value of sperm between the unencapsulated and encapsulated groups (0.25% and 0.5% alginate) at different time points. As with the previous parameter, the viability value, both encapsulated and unencapsulated, decreased for up to 96 hours during storage. The viability values were numerically superior in the encapsulated groups than the unencapsulated groups at all point times.

Although sperm motility is hampered by the viscosity of the alginate in the encapsulation process, the results of the present study indicate that alginate encapsulation maximized the viability and membrane damage during the storage process for up to 96 hours. Sperm viability was observed to be more significant in the alginate-encapsulated groups than in the unencapsulated groups based on the results of the present experiment. Alginate is known to be a biodegradable polymer with high biocompatibility. It also has a remarkable ability to form a three-dimensional matrix around cells, like the extracellular matrix. It is possible to maintain the appropriate cell viability in various in vitro and in vivo settings using this porous matrix (Pahlevanzadeh et al., 2020). Alginate has a unique structure that facilitates the transport of nutrients and signaling molecules. It was established that poly (propylene fumarate)-co-alginate blocks the entrance of ROS (Tram et al. 2020).

In comparison to previous studies on human and canine sperm encased in solid beads, the present study shows that using alginate encapsulation improves sperm vitality during long-term preservation (Gosálvez et al. 2021). Additionally, Kumar et al. (2019) used alginate to enhance the buffalo semen extender and then used a programmed biological freezer to cryopreserve the samples. Their results showed that supplementing with alginate increased sperm vitality while maintaining membrane integrity.

CONCLUSION

Overall, this study demonstrates the feasibility of pasundan bull sperm in alginate solid bead encapsulation structures to improve sperm stabilization. The sperm integrity and viability remain unaffected until 4 days of storage at 5°C. Although the initial encapsulation process decreased total motility, progressive motility, and kinematics value, alginate solid bead encapsulation was more stable than unencapsulated sperm during storage at 5 °C for up to 4 days. The movement of spermatozoa is restricted to the viscosity of the alginate medium without disturbing the membrane's viability and integrity.

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Estimating the Genetic Situation of Native Upper Egypt Subpopulations of Rabbits Using Microsatellite Markers

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ABSTRAK

Emam AM, Makhlouf MM, Faid-Allah E. 2024. Estimasi situasi genetik subpopulasi kelinci lokal Mesir Hulu menggunakan penanda mikrosatelit. JITV 29(2):114-124. DOI:http://dx.doi.org/10.14334/jitv.v29.i2.3428.

Penelitian ini bertujuan untuk mengeksplorasi keragaman genetik pada empat subpopulasi kelinci asli Mesir Hulu menggunakan penanda mikrosatelit. Sebanyak 247 sampel biologis dikumpulkan dari individu subpopulasi kelinci asli Mesir Hulu (NUER) yang tidak berkerabat di 77 pedesaan dan dilakukan pencirian genotipe melalui 31 lokus mikrosatelit. Empat ratus sembilan puluh enam alel tercatat di antara 4 subpopulasi NUER dengan sekitar 43% tercatat sebagai alel privat. Subpopulasi Luxor menunjukkan nilai rata-rata jumlah alel terbesar adalah 19,012, kekayaan alel adalah 8,009, dan alel privat adalah 133. Nilai negatif dari koefisien *inbreeding* tercatat di Qena dan Luxor (masing-masing -0,084 dan -0,134) Tentang 45% lokus memberikan kandungan informasi polimorfik tinggi (PIC) dan 58% tidak signifikan dalam keseimbangan Hardy –Weinberg (HWE). Tumpang tindih antara Asyut dan Sohag tampak pada analisis diskriminan komponen utama (DAPC). Secara umum, kami menyimpulkan bahwa klasifikasi ditemukan menurut arah geografis pada subpopulasi selatan (Qena dan Luxor) dan utara (Asyut dan Sohag). Kecuali itu, subpopulasi selatan (Qena dan Luxor) menunjukkan variasi genetik yang tinggi. Penelitian ini dapat digunakan sebagai dokumen pendukung bagi para peneliti di bidang peternakan kelinci dan pertanian kelinci di tingkat nasional dan daerah.

Kata Kunci: Keragaman Genetik, Kelinci Lokal, Mesir Hulu, Mikrosatelit

ABSTRACT

Emam AM, Makhlouf M, Faid-Allah E. 2024. Estimating the genetic situation of native Upper Egypt subpopulations of rabbits using microsatellite markers. JITV 29(2):114-124. DOI:http://dx.doi.org/10.14334/jitv.v29.i2.3428.

This study aimed to explore genetic diversity in four native upper Egypt subpopulations of rabbits using microsatellite markers. A total of 247 biological samples were collected from unrelated individuals of native Upper Egypt rabbit (NUER) subpopulations across 77 rural villages and were genotyped via 31 microsatellite loci. Four hundred ninety-six alleles were recorded among the 4 NUER subpopulations, with about 43% being private. Luxor's subpopulation exhibited the most significant values of the mean number of alleles, which was 19.012, allelic richness was 8.009, and private alleles were 133. The negative values of the inbreeding coefficient were recorded in Qena and Luxor ($^{-}$0.084 and $^{-}$0.134, respectively). About 45% of loci gave highly polymorphic information content (PIC), and 58% were insignificant in Hardy –Weinberg equilibrium (HWE). The overlapping between Asyut and Sohag has appeared in the discriminant analysis of principal components (DAPC). Generally, we concluded that the classification is based on geographical directions to southern subpopulations (Qena and Luxor) and northern (Asyut and Sohag). Except that, the southern subpopulations (Qena and Luxor) showed high genetic variation. This study could be used as supporting documents for researchers in rabbit breeding and agriculture at national and regional levels.

Key Words: Genetic Diversity, Native Rabbits, Upper Egypt, Microsatellite

INTRODUCTION

Local farm animal breeds play an essential role in sustainable agriculture for rural and fragile societies in developing countries (Datta et al. 2024). They have adapted throughout generations to abiotic stresses such as endemic illnesses and parasites, the capacity to survive extended periods of feed and water scarcity, and tolerance to heat stress (Mapiye et al. 2019). Egyptian native rabbits are one of the essential local farm animal breeds widely distributed in rural areas for self-sufficiency under the backyard familial system (Mostafa et al. 2020; Youssef et al. 2021). Genetic maintenance generating and amelioration strategies for Egyptian rabbit genetic resources may benefit from this data in the future (Allam et al. 2024). Limited studies were conducted to investigate genetic variability deeply for farm animals in the Upper Egypt strip, characterized by a hot and dry climate (Galal 2021).

Genetic markers are considered the best methods for genetic evaluation and structure differentiation in codominant and highly polymorphic livestock

(Loukovitis et al. 2023). Microsatellites, as genetic markers, are considered simple sequence repeats, short tandem repeats, and simple sequence length polymorphisms (Yadav et al. 2024). They are widespread and have become a requirement for farm animals' genetic evaluation, which could contribute to achieving food security and protein basket variability (Kasarda et al. 2020). Several factors are affected by genetic variation, like genetic drift, migration, mutation, and selection (Kardos et al. 2021).

Several genetic markers were used in genetic studies of rabbits, such as Sequence-related amplified polymorphism (SRAP) (Mohamed & Abdelfattah 2018), mitochondrial DNA (mtDNA) (Emam et al. 2020); simple sequence repeat (Adeolu et al. 2021) and single-nucleotide polymorphisms (Ballan et al. 2022). Microsatellites have been efficiently used in the evaluation of the genetic status of commercial lines rabbits (Jochová et al. 2017; Omotoso et al. 2019; Adeolu et al. 2021), and native rabbit populations in North African countries (Ben Larbi et al. 2014; s

MATERIALS AND METHODS

Ethics approval

This study followed the guidelines set via The Institutional Animal Care & Use Committee, IACUC; Menoufia University (IACUC reference № is MUFAG/F/AP/04/23).

Samples collection

Seventy-seven rural villages belonging to Upper Egypt governorates (Asyut, Sohag, Qena, and Luxor) were surveyed to collect 247 biological samples (hair bulbs and tissues) of native rabbits (Figure 1; Table 1). Samples were collected from January 2022 to March 2023. Every governorate was regarded as a subpopulation. The rabbits in the research were unrelated; if they were, they came from just one parent (buck or dosage), and the offspring were only evaluated as one individual. Rabbit hair samples were plucked with a bulb and maintained in tiny plastic bags, while tissue samples were kept in Eppendorf tubes containing 95% ethanol.

Laboratory procedures

The DNA extraction was conducted using an alkaline lysis protocol for the hair bulb and tissue samples (Cinelli et al. 2007). Initially, the quality of DNA was assessed by 0.8% agarose gel. After passing the quality control step, 31 microsatellite loci (Invitrogen, France) for rabbit biodiversity were studied on the purified DNA. The PCR reaction was performed on 5 multiplexes according to the PanelPlex Software (Ann Arbor, MI, USA). The condition of PCR for each multiplex is shown in Table 2. The quality of PCR products was then checked using agarose gel (2%). The sizes of the fragments were determined using the genetic analyzer (ABI PRISM 3730 XL; Applied Biosystems, Foster City, CA, USA). Genotyping was read by GeneMapper® Software 5 (Applied Biosystems, Foster City, CA, USA).

Data analysis

The GENAIEX 6.4.1 program (Peakall & Smouse 2006) calculated the following: analysis for each subpopulation and locus; analysis for each subpopulation and locus; mean number of observed alleles (MNa) for each subpopulation; the number of alleles for each locus (Na); the number of private alleles (Pa) for each locus; and observed and expected heterozygosity (H_o and H_e) for each subpopulation and locus. In addition, the percentage of molecular variance was estimated by the previous program. The values of allelic richness (Ar), inbreeding coefficient among populations and loci (F_{IS}), pairwise genetic differentiation among populations (F_{ST}), and reduction in heterozygosity due to inbreeding for each locus (F_{IT}) were calculated using the FSTAT 2.9.3.2 program (Goudet 2002). The program of Cervus 3.0.6 was used to calculate the polymorphic information content (*PIC*) and Hardy-Weinberg equilibrium (HWE) for each locus (Kalinowski et al. 2007). The ape (version 3.5) was used to achieve the neighbor-joining (NJ) tree, and the Adegenet Package (version 1.3-5) was used to achieve the discriminant analysis of principal components (DAPC) via the R program (R Core Development Team 2008). The STRUCTURE program 2.3.4 (Pritchard et al. 2000) was used to estimate the population structure by using an analysis of Bayesian clustering. The estimation was based on independent runs with 100000 Markov Chain Monte Carlo (MCMC) iterations and a burn-in of 50000 steps, adhering to the rule of $1 \leq K \leq 8$ (K= number of assumed clusters). The ΔK statisticas was calculated by Evanno et al. (2005) method.

RESULTS AND DISCUSSION

Genetic variability among 4 NUER subpopulations

The genetic variability among the 4 NUER subpopulations (Asyut, Sohag, Qena, and Luxor) is presented in Table 3. The results were characterized by a superior value of MNa (15.999). This value was higher than the MNa of the European domestic rabbits (MNa=3.136) (Alves et al. 2015) and some commercial rabbit lines in Egypt (MNa= 4.980) (El-Aksher et al. 2017). Moreover, more MNa was found in Luxor and Qena than in others. It is an essential indicator of genetic

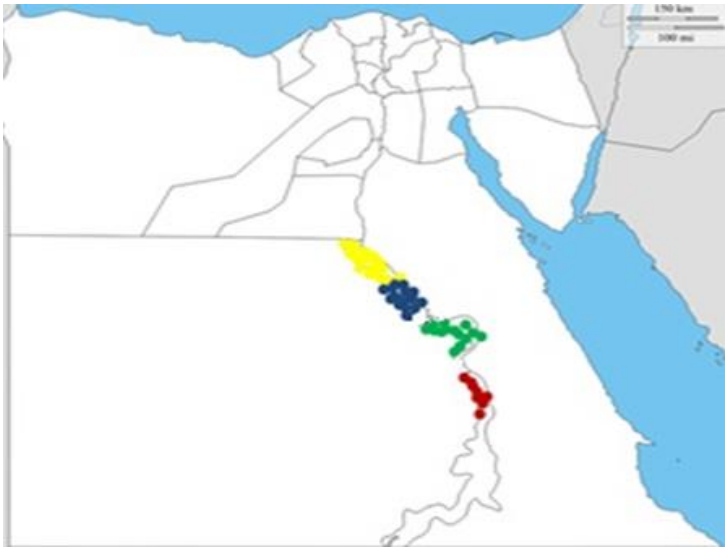

Figure 1. Locations of samples for the four subpopulations are Asyut (*yellow*), Sohag (*blue*), Qena (*green*), and Luxor (*red*)

Table 1. Biological samples geographical location

Geographical coordinates	Village	Center	Governorate
Asyut	Dairut	El Matawaa	27°34'56"N 30°50'49"E
		El sharaqwaa Bahary	27°34'54"N 30°45'46"E
		Dashloot	27°33'51"N 30°42'18"E
	El Ghanayem	El Amry	26°56'66"N 31°47'21"E
		Al Azayzaa	26°54'51"N 31°32'20"E
		Deer El Janadlaa	26°56'57"N 31°35'17"E
	El Qusiaa	Tanaghaa	27°12'27"N 30°30'31"E
		Aramya El Khudary	27°30'45"N 30°31'27"E
		Deer El Qaseer	27°30'55"N 30°31'42"E
		El Saraqnaa	27°24'21"N 30°46'49"E
		Elshiekh Dawood	27°26'24"N 30°49'37"E
	Abnun	Al Sawalem	27°14'41"N 31°09'32"E
		Nazlet Qadaieh	27°20'19"N 31°09'36"E
		Alma'abda	27°19'48"N 31°02'24"E
		Bani Mahmediat	27°19'09"N 31°03'36"E
		Deer sho	27°17'18"N 31°08'49"E
	Manfalout	El Hawatka	27°15'31"N 31°06'11"E
		Hamma	27°17'38"N 30°56'09"E
		Bani Shaquier	27°20'51"N 30°44'11"E
		Bani Magraa	27°16'45"N 30°56'14"E
		Arab El Amaiem	27°17'28"N 30°53'35"E
	Asyut	Awlad Ebrahim	27°09'08"N 31°13'16"E
		El Hasanni	27°15'08"N 31°03'25"E
		Alnamayssah	27°09'02"N 31°14'56"E

Geographical coordinates	Village	Center	Governorate
		Manqabaad	27°12'09"N 30°06'40"E
		Alwaan	27°12'44"N 30°02'32"E
	El Fatteh	El Kalabaat	27°11'42"N 31°18'35"E
		Deer Basra	27°09'34"N 31°16'59"E
		Jazereet Alakraad	27°12'04"N 31°08'21"E
		Taal Awlaad Serraj	27°10'56"N 31°13'47"E
	Abu Tij	Abu Kherss	27°00'09"N 31°14'32"E
		Baquraa	27°06'02"N 31°17'30"E
		Dakraan	26°57'52"N 31°17'31"E
	Sahel Saleem	El Afardaa	27°02'39"N 31°22'39"E
		Deer Tassa	27°02'02"N 31°24'16"E
		El Zarabbii	26°58'49"N 31°16'02"E
	Badary	Hamameyaa	26°55'20"N 31°28'47"E
		Temmaa	26°56'34"N 31°26'08"E
	Sidfa	Koom Abu Hajer	26°55'01"N 31°31'21"E
		Magries	26°58'46"N 31°22'16"E
		Bani Feez	26°57'04"N 31°27'23"E
Sohag	Tima	El Aghanaa	26°51'02"N 31°20'31"E
		El Hadiqaa	26°51'41"N 31°23'45"E
		Mashtaa	26°51'57"N 31°28'39"E
	Tahta	Banjaa	26°48'22"N 31°28'51"E
		Nazlet Alqadii	26°45'10"N 31°24'52"E
	Juhaynah	El Tolayhat	26°44'16"N 31°27'43"E
		Nazzah	26°42'19"N 31°28'55"E
		El Harafshaa	26°43'57"N 31°28'41"E
		Nazlet Ali	26°43'17"N 31°25'05"E
	Saqultah	El Galawyah	26°45'34"N 31°27'23"E
		El Faraseyah	26°42'44"N 31°37'21"E
		Bani Wasel	26°39'16"N 31°41'56"E
		Na'ge Hammed	26°57'29"N 31°41'32"E
	Al Maraghah	Eqsas	26°40'52"N 31°37'04"E
		Al Hariedyah	26°42'21"N 31°34'27"E
		Shandawell	26°39'19"N 31°36'10"E
	Akhmim	Abar Malek	26°36'25"N 31°43'45"E
		Al Salamouney	26°37'31"N 31°45'36"E
		Pardiess	26°17'09"N 31°57'46"E
		Halafy	26°19'54"N 31°58'34"E
Qena	Abu Tesht	El Alimat	26°14'31"N 31°59'56"E
		El Qaleia	26°05'00"N 32°10'06"E

Geographical coordinates	Village	Center	Governorate
		El Maharza	26°10'43"N 32°07'19"E
	Farshut	El Dahsaa	26°03'28"N 31°08'51"E
		Koom Al Bagaa	26°05'35"N 32°10'27"E
	Nagaa Hammadi	Salamiah	26°04'15"N 32°16'04"E
		Al Sha'aina	26°05'48"N 32°19'46"E
		Bahjourah	26°02'35"N 32°10'27"E
	Qus	El Harajiaa	25°65'47"N 32°46'32"E
Luxor	Al Zayteyah	El Madawed	25°44'25"N 32°42'37"E
	Luxor	El Qarnaa	25°43'05"N 32°38'37"E
		El Daeyah	25°39'10"N 32°35'36"E
	Armant	El Domeqrat	25°44'25"N 32°42'37"E
		El Rayanah	25°44'25"N 32°42'37"E
	Esna	El Adaymah	25°14'14"N 32°35'22"E
		El Dayabyah	25°38'01"N 32°50'16"E

Table 2. Multiplexes contain and PCR reaction conditions

Multiplex	Locus	Accession Number	Repeat Pattern	Temperature	Time	Cycles
1	INRACCDDV0101	AJ874443	$(TG)_{12}$	95	15 m	
	INRACCDDV0106	AJ874448	$(CA)_{14}$	95	30 s	8X
	INRACCDDV0108	AJ874450	$(CA)_{13}$	63 - 56 (↓10)	1 min	
	INRACCDDV0139	AJ874479	(TG)16	72	45 s	
	INRACCDDV0016	AJ874380	(CA)13	95	30 s	35X
	INRACCDDV0172	AJ874510	(AC)14	55	30 s	
	INRACCDDV0176	AJ874514	$(TC)_{11}(TG)_{13}AG(TG)_3$	72	45 s	
	INRACCDDV0203	AJ874540	$(GT)_{16}$	60	20 m	
	INRACCDDV0119	AJ874461	$(GT)_{16}$	95	15 m	
2	INRACCDDV0140	AJ874480	$(TG)_{14}$	95	30 s	7X
	INRACCDDV0157	AJ874497	$(GT)_{12}$	61 - 55 (↓10)	45 s	
	INRACCDDV0201	AJ874538	$(TG)_{14}(AG)_{10}$	72	45 s	
	INRACCDDV0087	AJ874430	$(TG)_{14}$	95	30 s	37X
	INRACCDDV0089	AJ874432	$(CA)_{14}$	55	45 s	
				72	1 min	
				60	20 m	
				10	∞	
3	SAT03	J03744	$(TC)_{22}$	95	15 min	
	SAT04	M33582	$(TC)_{13}(N)_2(TC)_2TG(TC)_7$	95	30 sec	32x
	SAT05	X99887	$(TC)_{23}TTT(CT)_5$	60	30 sec	
	SAT07	X99888	$(TG)_{14}$	72	45 sec	8x
	SAT08	X99889	$(CT)_{14(}GT)_8TT(GT)_5$	95	30 sec	

Multiplex	Locus	Accession Number	Repeat Pattern	Temperature	Time	Cycles
				53	30 sec	
				72	45 sec	
				60	20 min	
				12	∞	
4	INRACCDDV0102	AJ874444	$(AC)_{18}$	95	15 m	
	INRACCDDV0104	AJ874446	$(GT)_{14}$	95	30 s	9x
	INRACCDDV0169	AJ874508	$(CA)_{17}$	59 - 54 (↓10)	30 s	
	INRACCDDV0192	AJ874530	$(TG)_{11}$	72	30 s	
	INRACCDDV0205	AJ874542	$(TG)_{17}$	95	30 s	31x
	INRACCDDV0228	AJ874561	$(TG)_{12}$	54	45 s	
	SAT13	X99892	$(GT)_{13}$	72	45 s	
				95	30 s	9x
				53	30 s	
				72	45 s	
				60	20 m	
				10	∞	
5	INRACCDDV0182	AJ874520	$(TG)_{20}$	95	15 m	
	INRACCDDV0185	AJ874523	$(AC)_{13}$	95	30 s	8X
	INRACCDDV0259	AJ874589	$(GT)_{14}(GA)_{9}$	63 - 56 (↓10)	45 s	
	INRACCDDV0313	AJ874634	$(TG)_{7}(AC)_{10}GC(AC)_{6}GC(AC)_{8}$	72	45 s	
	INRACCDDV0040	AJ874400	$(TG)_{16}$	95	30 s	39X
				56	30 s	
				72	45 s	
				60	20 m	
				10	∞	

variability (Vajed Ebrahimi et al. 2017; Hoban et al. 2022). Likewise, the mean value of Ar (7.036) was higher than the Algerian native rabbits (2.349) (Bouhali et al. 2023). The elevated levels of Ar were found in the southern subpopulations (7.501 in Qena and 8.009 in Luxor), which is a reliable marker of a population's capacity for evolution and conservation (Bora et al. 2023). In addition, The positive relationship between warm conditions and genetic variety contributes to the archetype of mutations, which are the inherent source of increasing genetic variety (Teixeira and Huber 2021; Bora et al. 2023). On the other hand, our results indicated that in the southern subpopulations (Qena and Luxor), the H_o was higher than H_e, with negative FIS values. In contrast, the northern subpopulations (Asyut and Sohag) have H_o smaller than H_e, while they have positive F_{IS} values. Consequently, current results agree with some findings in rabbit (Abdel-Kafy et al. 2018) and pig (Zorc et al. 2022) populations. It could be due to the prosperity of genetic variables in isolated breaking effects (Ismail et al. 2018).

Polymorphism of loci

Genetic variability for each locus in all subpopulations is presented in Table 4. A total of 496 alleles were recorded in this study. Previous studies of local rabbits found 119 alleles in Tunisia (Ben Larbi et al. 2014) and 120 alleles in Egypt (Emam et al. 2017). The INRA192 locus exhibited the most significant alleles (30), while INRA172 was the lowest (5). Our results on NUER subpopulations showed that 45 % of loci were highly formative of *PIC* (>0.5). Also, several studies reported a high percentage of formative *PIC* (El-Aksher et al. 2016; Emam et al. 2017; Lai et al. 2018). Moreover, 58% of the loci were not significant in terms

Table3. Parameters of genetic variation calculated for 4 NUER subpopulations

Subpopulation	N	MNa±SD	Ar±SD	H_o±SD	H_e±SD	F_{IS}±SD
Asyut	75	12.130±0.398	5.901±0.333	0.928±0.046	0.681±0.036	0.214[a]±0.027
Sohag	64	15.200±0.387	6.734±0.368	0.933±0.048	0.767±0.046	0.096[b]±0.019
Qena	59	17.633±0.371	7.501±0.356	0.992±0.028	0.770±0.039	-0.084[c]±0.020
Luxor	49	19.012±0.407	8.009±0.352	0.999±0.011	0.777±0.010	-0.134[c]±0.012
Mean values		15.995±0293	7.036±0.352	0.963±0.033	0.749±0.014	0.023±0.011

Number of samples (N). Mean number of observed alleles (MNa), Stander deviation (SD), number of private alleles (Pa), mean observed and expected heterozygosity (Ho and He), allelic richness (Ar), inbreeding coefficient (F_{IS}). Value followed by different superscripts (a, b, and c) within the last column are significantly different (P≤0.05)

Table 4. Parameters of genetic variation for each locus among 4 NUER subpopulations

Multiplex	Marker	Locus	Na	H_o ±SD	H_e±SD	F_{IS}	F_{IT}	F_{ST}	*PIC*	HWE
Multiplex-1		INRA101	7	0.931 ±0.016	0.816 ±0.015	0.796801	0.275193	0.087062	0.811	*
		INRA106	6	0.972 ±0.014	0.758±0.026	0.319937	0.231384	0.067089	0.655	**
		INRA108	14	0.981 ±0.023	0.954±0.020	0.032704	0.224597	0.077195	0.501	***
		INRA139	16	0.761 ±0.139	0.918±0.024	-0.195980	-0.481000	0.564966	0.400	NS
		INRA016	8	0.901 ±0.013	0.789±0.023	0.494116	0.471729	0.59179	0.727	*
		INRA172	5	0.750 ±0.116	0.922±0.031	0.528080	-0.381610	0.032544	0.666	**
		INRA176	18	0.688 ±0.123	0.862±0.033	-0.415720	-0.340160	0.053375	0.311	NS
		INRA203	18	0.975 ±0.020	0.769±0.035	-0.299580	-0.184580	0.088492	0.202	NS
Multiplex-2		INRA119	24	0.924 ±0.015	0.795±0.038	-0.095980	-0.153590	0.082622	0.050	NS
		INRA140	7	0.753±0.097	0.977±0.043	0.427974	-0.189930	0.103951	0.5675	**
		INRA157	23	0.927 ±0.037	0.867±0.025	0.022136	0.100973	0.09857	0.501	***
		INRA201	23	0.967 ±0.024	0.888±0.041	-0.26914	-0.133650	0.106755	0.112	NS
		INRA087	23	0.992 ±0.002	0.830±0.034	-0.20547	-0.131800	0.061119	0.109	NS

Multiplex	Marker	Locus	Na	H_o ±SD	H_e±SD	F_{IS}	F_{IT}	F_{ST}	*PIC*	HWE
Multiplex-3		INRA089	7	0.898 ±0.031	0.745±0.041	0.694213	0.19397400	0.110386	0.719	*
		Sat3	17	0.969 ±0.001	0.824±0.027	-0.21352	-0.071910	0.116693	0.185	NS
		Sat4	17	0.94 ±0.035	0.825±0.037	-0.21087	-0.080910	0.107322	0.182	NS
		Sat5	15	0.988 ±0.038	0.759±0.025	-0.21087	0.119284	0.1508	0.502	NS
		Sat7	15	0.961 ±0.040	0.842±0.030	-0.18753	-0.070230	0.098775	0.145	NS
		Sat8	8	0.948 ±0.011	0.896±0.017	0.399282	0.115500	0.112063	0.521	***
Multiplex-4		INRA102	13	0.942 ±0.035	0.812±0.029	0.133165	0.148562	0.067462	0.502	***
		INRA104	10	0.973 ±0.025	0.794±0.028	0.208797	0.263542	0.034577	0.508	***
		INRA169	25	0.954 ±0.043	0.798±0.041	-0.253130	-0.184170	0.055028	0.109	NS
		INRA192	30	0.992 ±0.021	0.797±0.022	-0.255490	-0.115080	0.111834	0.104	NS
		INRA205	26	0.682±0.154	0.945±0.041	-0.384830	-0.209360	0.126708	0.090	NS
		INRA228	8	0.747±0.169	0.983±0.035	0.446278	0.132374	0.142971	0.703	*
		Sat13	19	0.978±0.014	0.767±0.051	-0.210870	0.190921	0.133732	0.107	NS
Multiplex-5		INRA182	8	0.978±0.021	0.837 ±0.054	0.489048	0.185160	0.080593	0.711	*
		INRA185	19	0.948±0.017	0.903±0.0547	-0.309260	-0.165210	0.110023	0.111	NS
		INRA259	18	0.952±0.014	0.842±0.026	-0.186870	-0.136010	0.042851	0.103	NS
		INRA313	26	0.827±0.111	0.941±0.033	-0.25421	-0.149480	0.083503	0.126	NS
		INRA040	23	0.777±0.101	0.969±0.040	-0.11421	-0.169480	0.083503	0.100	NS
Mean Values			16	0.902±0.049	0.852±0.033	0.023194	-0.02242	0.122076	0.359	

Na= number of observed alleles; H_o and H_e= mean observed and expected heterozygosity standard deviation (SD); PIC= polymorphic information content per locus; HWE= Hardy-Weinberg Equilibrium. Differentiation among populations (F_{ST}), reduction in heterozygosity due to inbreeding for each locus (F_{IT}), reduction in heterozygosity within each breed due to inbreeding . (F_{IS});*P≤0.05; **P≤0.01;*** P≤0.001; NS= Non-Significant

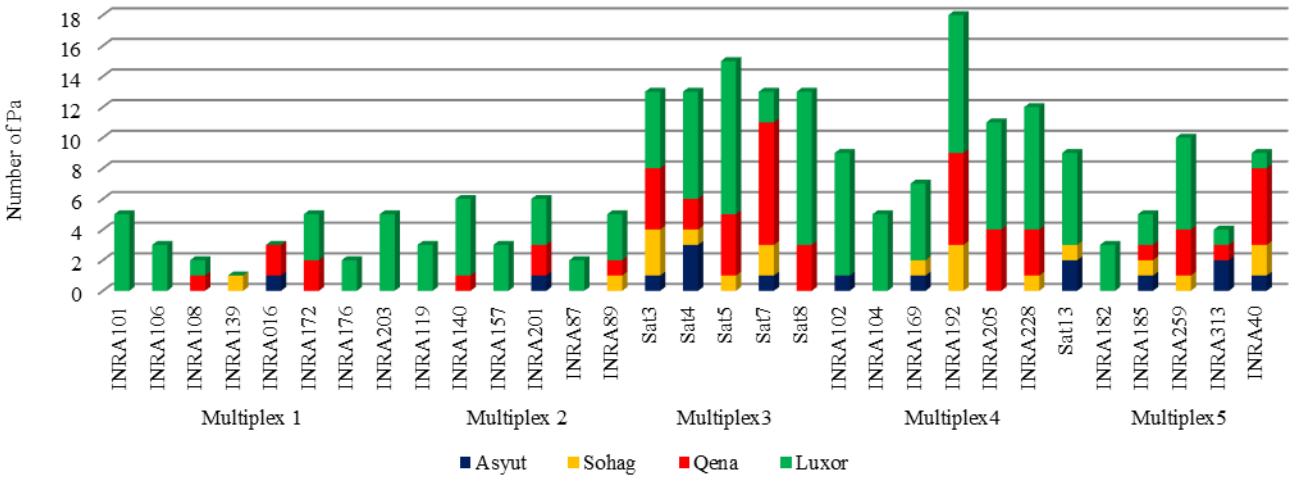

Figure 2. Distribution of private alleles (Pa) among subpopulations and microsatellite markers

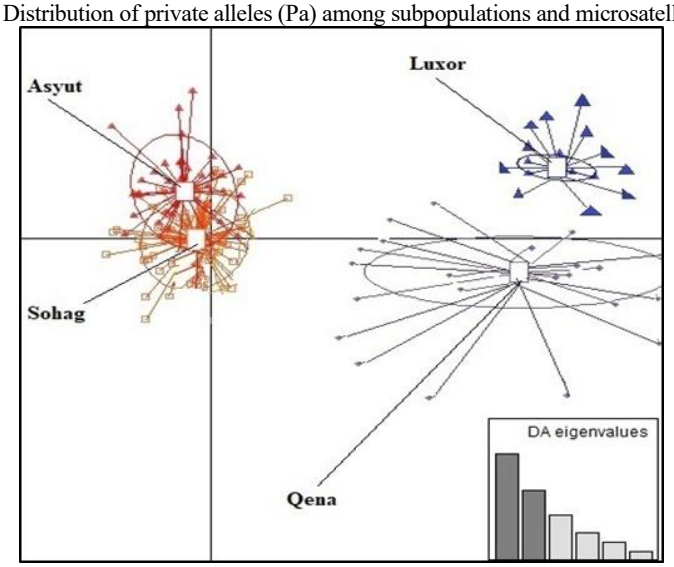

Figure 3. Analysis of discriminant analysis of principal components (DAPC) for NUER Subpopulations

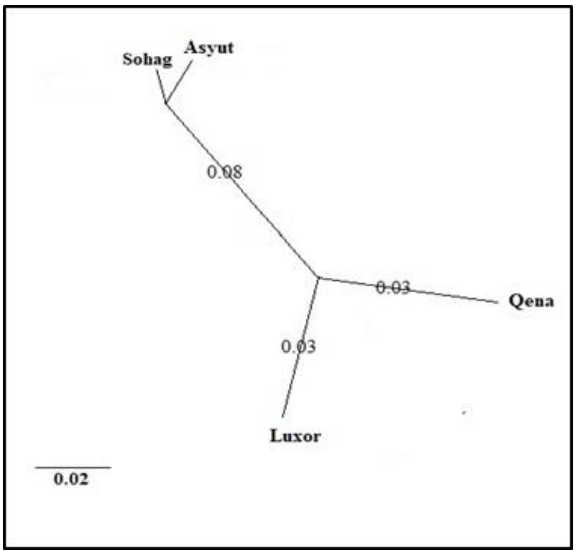

Figure 4. Neighbor-joining tree (NJ) for NUER Subpopulations

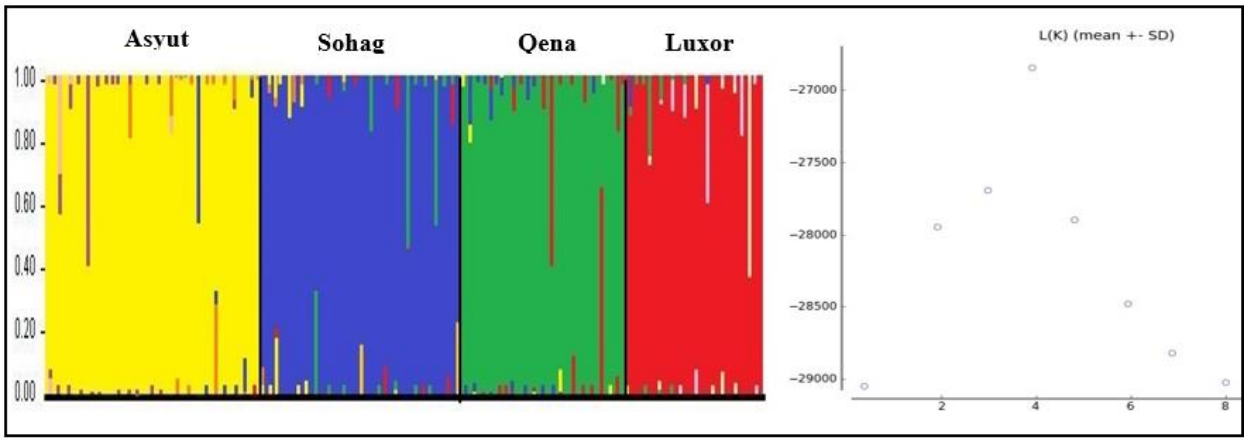

Figure 5. Estimated native Upper Egypt rabbits (NUER) subpopulation structure. In each K, the colors represent the percentage of each cluster in each rabbit population. ΔK calculated from K=1 to K=8

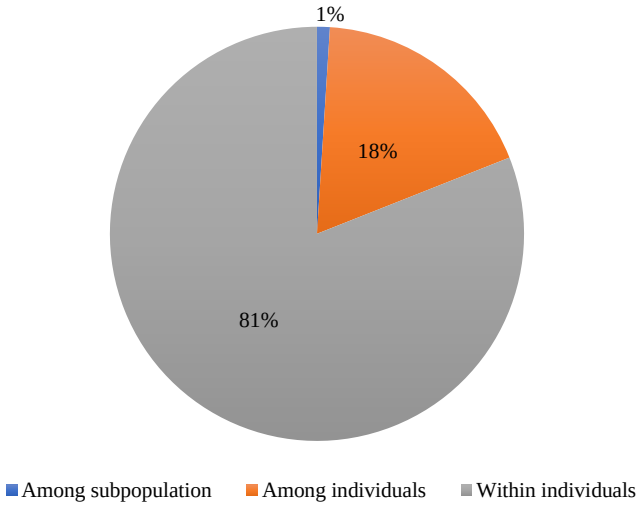

Figure 6. Percentage of molecular variations for NUER subpopulations

of HWE, which is characteristic of the absence of inbreeding situations in the majority of subpopulations, according to (Roden et al. 2023) and natural genetic selection (Demiray et al. 2024).

This study recorded about 43% of alleles (220) as Pa (Figure 4). The highest Pa is recorded in Luxor (133), while the lowest is in Asyut (15). The affluence of Pa was characterized in the NUER subpopulations. The highest values of private alleles were recorded by the subpopulation of Luxor (133) and SAT5 locus (15). The variety of private alleles strongly indicates the absence of a genetic bottleneck position (Holmes et al. 2023). studies reported a high percentage of formative PIC (El-Aksher et al. 2016; Emam et al. 2017; Lai et al. 2018). Moreover, 58% of the loci were not significant in terms of HWE, which is characteristic of the absence of inbreeding situations in the majority of subpopulations, according to (Roden et al. 2023) and natural genetic selection (Demiray et al. 2024).

This study recorded about 43% of alleles (220) as Pa (Figure 2). The highest Pa is recorded in Luxor (133), while the lowest is in Asyut (15). The affluence of Pa was characterized in the NUER subpopulations. The highest values of private alleles were recorded by the subpopulation of Luxor (133) and SAT5 locus (15). The variety of private alleles strongly indicates the absence of a genetic bottleneck position (Holmes et al. 2023).

Genetic differentiation and structure of NUER subpopulations

In Figure 3 and Figure 4, the NUER subpopulation was classified into two main groups: north (Asyut and Sohag) and south (Qena and Luxor). Genetic overlapping was observed in the northern subpopulation due to geographical proximity between the last and first points (less than 9 km). In contrast, the southern subpopulations expressed far greater separation for separation between the last point in the north of Qena and the first point of Luxor in the south (about 50 km). The categorization of rabbits according to geographical direction was reported in several studies on rabbits (Alda

& Doadrio 2014; Ben Larbi et al. 2014; Emam et al. 2017; Jochová et al. 2017; Iannella et al. 2019; Cheptanui 2022). The highest K values and ΔK for different clustering when K=4. Likewise, the approved value of ΔK was equal to the population number for previous studies (Emam et al. 2016; Dudu et al. 2020).

NUER subpopulation's molecular variance percentage

The estimation of molecular variance percentage for the NUER subpopulations is shown in Figure 6. The result revealed that the percentage of variance among subpopulations was only 1%, whereas 18% and 81% among and within individuals, respectively. The low variation among subpopulations (1%) is convincing evidence for random mating among populations and the limitation of inbreeding (Adeolu et al. 2021). It was a good sign for allowing a population to adapt and survive in shifting environmental conditions (Pavlova et al. 2017; Ma et al. 2020). Previous results agree with El-Aksher et al. (2016) and Adeolu et al. (2021) but do not match with Bouhali et al. (2023).

CONCLUSION

This study demonstrated the current genetic situation of the NUER population through a deep investigation of four subpopulations for the first time. The subpopulations of NUER shed light on the rich genetic variability with the absence of inbreeding and bottleneck positions. Towards the south in Qena and Luxor, genetic variability parameters increased more than in the north (Asyut and Sohag). The overlapping was observed in the northern subpopulations. The current study could be used to document rabbit genetic resources in the Upper Egypt strip in national and international reports. It could reflect the state's interest in local farm animal interest according to the climate change plan. Moreover, it opens the field of interest in local farm animals in rural and fragile areas as efforts to guarantee food security and improve livelihoods there. Additionally, similar studies should be replicated on distinct species in this vital area from Egypt's hot-dry regions.

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Proceeding:
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Thesis:
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economic contribution of donkey ownership and use to houshold livelihoods in Kiambu country, Kenya (Thesis). Nairobi (KE). University of Nairobi

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Institution:

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b. [FAO] Food and Agriculture Organization. 2021. Gateway to dairy production and products. Food Agric Organ United Nations. [accessed August 10, 2021]. https://www.fao.org/dairy-production-products/production/feed-resources/en/.

Patent:

Raab RM, Lazar G, Shen B. 2022. AGRIVIDA Inc, assignee. Engineered phytases in animal feed. 2022 Feb 8.

10. **Citation in text:**

The citation consists of the author's last name and publication year.

Example:

a. One author: ..... ranging from 84 to 135 per minute (Scott 2015). Scott 2015 stated.....
b. Two authors: ..... in glucocorticoid production, primarily cortisol (Narayan & Parisella 2017). Narayan & Parisella (2017) stated that stress caused an increase in ……
c. Three authors or more: ..... in milk production without affecting the ewe's weight (Barbato et al. 2021). Barbato et al (2012) reported .....
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e. The author with the same family name is written consecutive: (Dawson J 1986; Dawson M 1986).
f. Several different authors are written consecutively: (Damayanti et al. 2021; Leonard et al. 2022; Motlagh RK 2022).
g. Institution: FAO (2021).....

11. **Table:**

a. The standard word used is Times New Roman with 1 space distance and 11 of the font size.
b. The title is a simple, clear, and understandable sentence without reading the manuscript.
c. Each column from the table should have a heading. Its unit is separated from the title by a comma, in parentheses, or at its bottom.
d. The table description is written under the table with 1 space distance and 11 of the font size. The data source is written under the table or in the table in its header.
e. The dividing line is made in the form of horizontal.

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c. Clear contrast figure with proportionate size and high resolution to present the best performance.
d. Write a figure or graphic source under the title.

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