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PREFACE

In this edition, Volume 29 No 3, 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:
"Diversity of the Monoamine Oxidase A Gene in Beef Cattle"; "Improvement of Sexed Sperm Quality of Bali Bulls by Adding Palmyra (*Borassus flabellifer Linn.*) Fruit Water to Citrate-Egg Yolk Extender"; "Animal Welfare Study in Sheep Transported with Methionine Hydroxy Analog and Dextrose Supplementation"; "Biofilm Profile of Coagulase Negative *Staphylococci* Bacteria from Milk Isolate of Dairy Cows with Subclinical Mastitis"; and "Effect of extension programs on improving farmers' knowledge in the narrowing coastal area of Segara Anakan Lagoon, Indonesia".

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, September 2024

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LIST OF CONTENT

Diversity of the Monoamine Oxidase A Gene in Beef Cattle

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ABSTRAK

Siregar NA, Noor RR, Priyanto R, Ulum RF, Jakaria. 2024. Keragaman gen monoamine oxidase A pada sapi pedaging. JITV 29 (3):127-134. DOI:http://dx/doi.org/10.14334/jitv.v29i3.3414.

Monomamine Oxidase A (MAOA) merupakan gen yang turut mengontrol sifat-sifat agresif. Gen MAOA berperan dalam mengkodekan enzim monoamine oksidase A yang berperan dalam katabolisme neurotransmiter, termasuk dopamin, norepinefrin, dan serotonin. Penelitian ini bertujuan untuk mengidentifikasi keragaman gen SNP MAOA pada sapi potong dengan metode sekuensing. Penelitian ini menggunakan 127 sampel DNA sapi untuk identifikasi keragaman, antara lain sapi Bali, Limousin, Wagyu, PO, Madura, dan Wagyu-Bali (F1). Polimorfisme gen MAOA yang terletak pada promotor dan ekson 1 dianalisis menggunakan metode sekuensing. Frekuensi genotipe, frekuensi alel, nilai heterozigositas, dan keseimbangan Hardy-Weinberg dihitung menggunakan program PopGen32. Hasil penelitian menunjukkan bahwa gen MAOA yang terletak di daerah promotor memiliki enam SNP, salah satunya adalah SNP g.385G>A, sedangkan gen MAOA yang terletak di ekson 1 bersifat monomorfik. Metode PCR-RFLP digunakan untuk menyelidiki polimorfisme gen SNP g.385G>A MAOA menggunakan enzim restriksi RSaI. Gen MAOA terdeteksi pada 3 genotipe yaitu GG, GA, dan AA. SNP g.385G>A bersifat polimorfik pada sapi Bali, PO, Madura, dan silangan Wagyu-Bali (F1), sedangkan monomorfik pada sapi Limosin dan Wagyu. Penelitian lebih lanjut diperlukan untuk mengeksplorasi implikasi fungsional SNP g.385G>A dan hubungannya pada perilaku agresif sapi.

Kata Kunci: Sapi Pedaging, Keragaman Genetik, Gen MAOA, Single Nucleotide Polymorphism

ABSTRACT

Siregar NA, Noor RR, Priyanto R, Ulum RF, Jakaria. 2024. Diversity of the monoamine oxidase A gene in beef cattle. JITV 29 (3):127-134. DOI:http://dx/doi.org/10.14334/jitv.v29i3.3414.

Monoamine Oxidase A (MAOA) is a gene that controls aggressive traits. The MAOA gene plays a role in encoding the monoamine oxidase A enzyme, which plays a role in the catabolism of neurotransmitters, including dopamine, norepinephrine, and serotonin. This study aims to identify the diversity of the MAOA SNP gene in beef cattle using sequencing methods. This research used 127 cattle DNA samples to identify diversity, including Bali, Limousin, Wagyu, PO, Madura, and Wagyu-Bali (F1) cattle. MAOA gene polymorphisms in the promoter and exon 1 were analyzed using sequencing methods. Genotype frequencies, allele frequencies, heterozygosity values, and Hardy-Weinberg balance were calculated using the PopGen32 program. The results showed that the MAOA gene in the promoter region has six SNPs, one of which is SNP g.385G>A, while the MAOA gene in exon 1 is monomorphic. The PCR-RFLP method was used to investigate the SNP g.385G>A MAOA gene polymorphism using the RSaI restriction enzyme. The MAOA gene was detected in 3 genotypes: GG, GA, and AA. SNP g.385G>A is polymorphic in Bali, PO, Madura, and Wagyu-Bali cross (F1) cattle while monomorphic in Limousin and Wagyu cattle. Further studies are necessary to explore the functional implications of SNP g.385G>A and their relationship to aggressive behaviors in cattle.

Key Words: Beef Cattle, Genetic Diversity, MAOA Gene, Single Nucleotide Polymorphism

INTRODUCTION

Indonesia has diverse genetic resources for beef cattle, from local to introduced cattle. Bali cattle, one of the local cattle of Indonesia, are domesticated forms of wild Banteng (*Bos javanicus*), and they are noted for their adaptability to harsh environments (Anwar et al. 2017). However, they also exhibit behavioral traits such as defensive aggressiveness, especially when exposed to stressful conditions like transportation, unfamiliar environments, or improper handling. Bali cattle, in particular, exhibit a higher level of aggressiveness than introduced breeds, especially when restrained or threatened, making their management more challenging (Cooke 2014). This aggressiveness, combined with their hardiness, has driven interest in further research to balance productivity with behavior traits that impact cattle management practices.

The utilization of genetic resources in beef cattle aims to increase productivity, and proper management practices are essential, including effective livestock handling (Amiano et al. 2020). Cattle farming in

Indonesia often follows traditional methods, but modern practices emphasize improving productivity through better breeding, feeding, and handling techniques (Adinata et al., 2023). However, managing problematic livestock, especially those exhibiting aggressive behaviors, can significantly affect productivity. Aggressive behavior in cattle poses a risk to farmworkers and house workers, as it can lead to injury during routine activities like feeding, milking, or health checks (Lindahl et al. 2016). Many farms in Indonesia still rely on manual labor, and this risk has become more pronounced (Laya et al., 2024). Aggressiveness, such as headbutting or kicking, is especially dangerous when dealing with large animals like beef cattle (Titterington et al., 2022). This behavior disrupts management and reduces productivity by increasing stress levels in cattle and the handlers (Eusebi et al., 2018). In Indonesia, addressing these behavioral traits through selective breeding is a crucial strategy to reduce the risks associated with aggressive farming.

Several genes control aggressive traits in cattle, and the monoamine oxidase A (MAOA) gene is one of the key regulators, which are chemicals that transmit signals between nerve cells in the brain (Eusebi et al. 2021). Located on the X chromosome, the MAOA gene consists of 15 exons and 14 introns (Edgnülü et al. 2014). MAOA gene encodes the enzyme monoamine oxidase A, which is responsible for the catabolism of neurotransmitters like dopamine, epinephrine, norepinephrine, and serotonin (V and Husain RS 2017). These neurotransmitters regulate mood, emotions, stress response, and physical movements (Ferreira et al. 2015). After these chemicals send signals between neurons, the MAOA enzyme helps break them into inactive components. This process prevents an excessive buildup of neurotransmitters in the synapse, the gap between neurons. Some individuals carry a variant of the MAOA gene known as MAOA-L (low activity). This variant leads to a less effective enzyme, which slows the breakdown of neurotransmitters. As a result, neurotransmitters like serotonin and dopamine remain in higher concentrations in the brain, which can affect emotional regulation (Sacco et al. 2017). The MAOA enzyme is essential in brain areas such as the amygdala, which regulates emotions like fear and anger, and the prefrontal cortex**,** which is responsible for impulse control and decision-making. When MAOA activity is low, the brain's ability to regulate emotional responses is reduced, potentially leading to more aggressive or uncontrolled behavior (Yen et al., 2021). Mutations or disruptions in the MAOA gene can lead to imbalances in neurotransmitter regulation, which increases the likelihood of aggressive behavior in cattle (van Rhijn et al., 2022). This aggressiveness not only endangers farmworkers but also disrupts the overall management of the herd, causing delays in handling, reduced efficiency, and a higher risk of accidents (Peden et al. 2019).

Biomolecular technology can detect MAOA gene diversity more accurately. One biomolecular technique that can identify the diversity of the MAOA gene is Polymerase Chain Reaction - Restriction Fragment length polymorphism (PCR-RFLP) using restriction enzymes. RFLP can detect high levels of polymorphism and has been widely used to identify genes that produce essential traits (Khasanah et al. 2016). Exploration of MAOA gene diversity has been reported in several types of livestock, such as Lidia and Mexican Spanis cattle, which are known as aggressive breeds of cattle for agility and sport (Eusebi et al. 2020), local Indonesian sheep (Handiwirawan 2012), and Yorkshire (Chen et al. 2019), as well as in domesticated types of cattle such as Angus and Simmental cattle (Lühken et al. 2010). Based on the research results above, information on the MAOA gene has been carried out intensively in various livestock worldwide. However, studies of the MAOA gene in Indonesian beef cattle have never been conducted. Therefore, it is necessary to conduct research to identify and analyze the diversity of MAOA genes in beef cattle in Indonesia.

MATERIALS AND METHODS

Sample collection

The DNA samples used in this research were 127 cattle DNA samples from several representative beef cattle in Indonesia, that are 76 samples of native Indonesian cattle, namely Bali cattle, from BPTU-HPT Denpasar, Bali, and BPTP Kupang, NTT, and local cattle consisting of 15 PO cattle samples from UPTD Ciamis, West Java. The introduced cattle consisted of 6 samples of Wagyu cattle from BET Cipelang Bogor, West Java, and 9 Limousin cattle from BPTU-HPT Padang Mangatas, West Sumatra. Hybridized cattle consisted of 12 samples of Madura cattle from VBC Sapudi Island, Madura, East Java, and 8 Wagyu-Bali cross (F1) cattle from UPTD Kupang, West Nusa Tenggara. The DNA samples are from the collection of the Animal Molecular Genetics Laboratory, Faculty of Animal Husbandry, IPB.

DNA extraction and amplification

Extraction of cattle blood samples uses a DNA kit to obtain DNA samples and follows the procedure of the DNA kit used, namely Geneaid's Genomic DNA Mini Kit. The primers were designed from MAOA gene sequence data on the Ensembl web (www.ensembl.org) with accession number ENSBTAG00000016206. Primers were designed using Primer 3 (https://primer3.ut.ee/) and validated with Primer Stats (www.bioinformatics.org). The primer sequences that have been designed in the promoter fragment and exon 1

are the forward primers 5'-TAC ACA CCA CCT TGC ACT CA-3' and the reverse 5'-AGT GGA CTC TTG TGT GGA CA-3' with a length of 548 bp. The forward primer sequences 5'-TGT CCA CAC AAG AGT CCA CT-3' and reverse 5'-TCC ACA CTG ACC TGA GAT GC-3' were used to amplify a 341 bp long target sequence located at exon one position. 0.5-2 µL of DNA was put into the PCR tube, then 14 µL of premix solution was added, which consisted of 0.2 µL of forward primer and 0.2 µL of reverse primer, 6.1 µL of DW, and 7.5 µL of Red Master Mix. The mixture was incubated in a thermal cycler for the amplification process. The initial stage of the amplification process is the predenaturation stage at a temperature of 95ºC for 1 minute. The second stage consists of 35 cycles, each consisting of a denaturation process at 95ºC for 15 seconds, primary annealing at 57ºC for 15 seconds, and extension at 72ºC for 10 seconds. The final stage is primary elongation at 72ºC for 5 minutes. PCR product results were visualized using 1.5% agarose gel and 1% fluorosafe staining at 100 volts for 35 minutes.

Sequencing and PCR-RFLP

The PCR product results of the promoter fragment and exon 1 of the MAOA gene were sequenced using analysis services, namely the Macrogen Company, Seoul, South Korea. The PCR product of the promoter fragment and exon 1 of the MAOA gene was prepared in 20 µL per sample using forward primers for analysis. The genotype determination of the obtained single nucleotide polymorphisms (SNPs) was carried out using the PCR-RFLP technique using the RSaI restriction enzyme, which was incubated at 37 °C for 4 hours. The determination of cutting enzymes was carried out using the Nebcutter program (www.labtools.us/nebcutter-v2-0/). RFLP results were visualized using 2% agarose gel and 1.5% fluorosafe staining at 100 volts for 35 minutes. The marker used was 2 µL of 100 bp marker. The results of DNA electrophoresis in the form of bands are visible with the help of a UV transilluminator light.

Data analysis

The diversity of the MAOA gene in beef cattle samples was analyzed using the allele frequency, genotype frequency, heterozygosity value, and Hardy-Weinberg balance approach using the PopGen 32 application. Allele and genotype frequencies are calculated as follows:

$$X_i = \frac{\left(2n_{ii} + \sum_{j \neq 1} n_{ij}\right)}{(2N)}$$

$$X_{ii} = \frac{n_{ii}}{N}$$

Where X_i is the frequency of the allele, X_{ii} is the frequency of the genotype, n_{ii} is the number of individuals with genotype ii, n_{ij} is the number of individuals with genotype ij, and N is the total sample.

Estimating observed heterozygosity (Ho) and expected heterozygosity (He) is carried out to determine genetic diversity and estimate the balance of alleles in a population. The estimated heterozygosity value was calculated as follows:

$$H_o = \sum_{i \neq j}^{N} \frac{n_{ij}}{N}$$

$$H_e = 1 - \sum_{i=i}^{q} X_i^2$$

where Ho is the observed heterozygosity (population), He is the expected heterozygosity, n_{ij} is the number of heterozygous individuals, N is the number of individual observations, X_i is the allele frequency, and q is the number of alleles. The HWE was analyzed using the Chi-square test as follows:

$$X^2 = \sum \frac{(O - E)^2}{E}$$

X^2 is the Chi-square, O is the observed value, and E is the expected value.

RESULTS AND DISCUSSION

DNA amplification and SNP identification

The MAOA gene was successfully amplified in the promoter and exon 1 fragments with PCR product lengths of 548 bp and 341 bp, respectively. The results of MAOA gene amplification are presented in Figure 1.

The bands showing the appropriate size in gel electrophoresis after amplification using the PCR method indicate the success of the amplification reaction. Several factors can influence the success of amplification in the PCR method, namely the quantity and quality of the initial DNA used (Putra et al. 2021). The amount of DNA that is too low can produce less bright amplicon bands, while poor or degraded DNA quality can hinder the amplification process. PCR conditions such as annealing temperature, primer concentration, and number of amplification cycles also play an important role. Non-optimal settings of these parameters can result in low amplification efficiency (Hashim and Al-Shuhaib 2019).

Identification of the SNPs of the MAOA gene in the promoter resulted in the discovery of six new single nucleotide polymorphisms (SNPs) that differentiate Bali cattle from other cattle breeds, which can be used to characterize aggressive characteristics in Bali cattle. No SNP was found in the MAOA gene in the exon 1 fragment. The results of the six SNPs obtained are shown in Table 2.

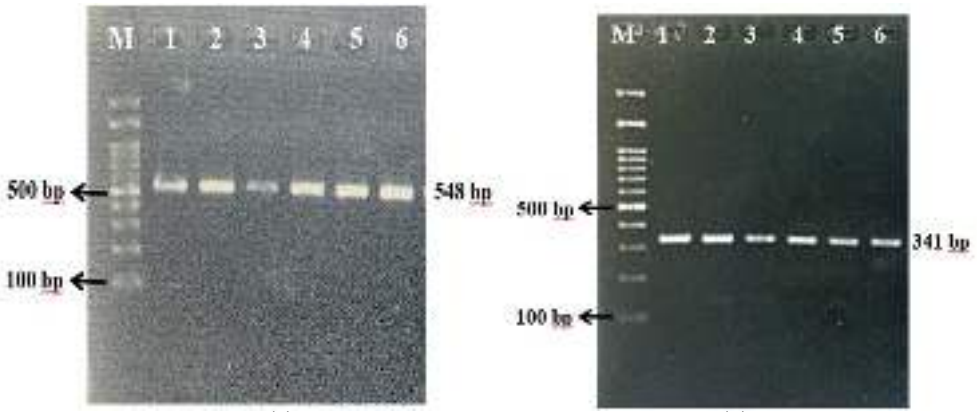

Figure 1. Electrophoresis visualization of PCR amplification results of the MAOA gene sequence promoter fragment (a) and exon 1 (b) on a 1.5% agarose gel; M: 100 bp marker; 1-3: Bali cattle samples, 4-6: Limousin cattle samples

Table 1. SNP identification on the MAOA gene promoter in Indonesian Bali cattle

SNP	Mutation	Restriction Enzymes
g.98A>C	Transversion	-
g.145G>T	Transversion	*Cac*8I
g.216A>C	Transversion	*Mse*I
g.340T>G	Transversion	-
g.349A>C	Transversion	-
g.385G>A	Transition	*Rsa*I

SNP= single nucleotide polymorphism

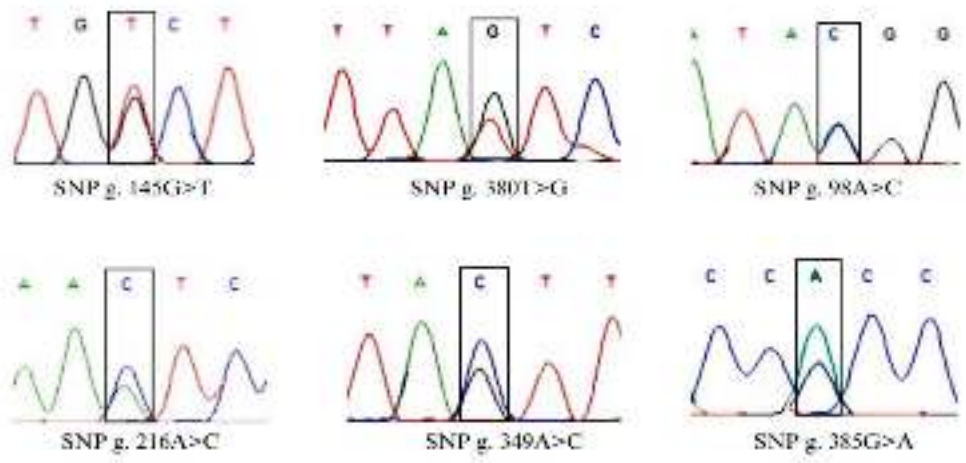

Figure 2. Partial sequencing maps and SNP identification promoter region MAOA gene in Bali cattle

SNP g.385G>A is a transition mutation, which is a replacement of a purine base with another purine base (G>A) or a replacement of a pyrimidine base with another pyrimidine base (C>T), while g.98A>C, g.145G> T, g.216A>C, g.340T>G, g.349A>C are transversion mutations, which is a replacement of a purine base with a pyrimidine base or vice versa (Setyani et al. 2021). Nucleotide changes in the promoter region do not change the amino acids.

Genetic diversity of the MAOA gene

RFLP was performed with a cutting enzyme after detecting an MAOA gene mutation in the promoter fragment. Based on the results of the six SNPs obtained, SNP g.385G>A was then analyzed using the PCR-RFLP technique, namely using the *RSa*I (GT|AC) enzyme (Figure 3). The PCR-RFLP results obtained three genotypes: GG, GA, and AA. The AA genotype has one

Figure 3. PCR-RFLP visualization of SNP g.385G>A MAOA gene promoter fragment on a 2.0% | *Rsa*I (5'-GT | AC-3') agarose gel. M: 100 bp marker; SNP g.385G>A: GG, AG, and AA

Table 2. Diversity of SNP g.385G>A MAOA gene in several breeds of beef cattle

Cattle	N	Genotype Frequency			Allele Frequency		Ho	He	χ^2 Chi-Square
		GG	GA	AA	G	A			
Bali	76	0.00	0.12	0.88	0.06	0.94	0.12	0.11	ns
Limosin	9	1.00	0.00	0.00	1.00	0.00	0.00	0.00	*
Wagyu	6	1.00	0.00	0.00	1.00	0.00	0.00	0.00	*
PO	15	0.40	0.60	0.00	0.70	0.30	0.60	0.44	ns
Madura	13	0.85	0.15	0.00	0.92	0.08	0.15	0.14	ns
WagyuxBali (F1)	8	0.00	0.75	0.25	0.38	0.63	0.75	0.50	ns

N= number of samples; Ho= observed heterozygosity; he= expected heterozygosity; χ^2 table (0,05:1) = 3,84; *= unexplained; ns = not siginificant (χ2 count < χ2 table)

band with a length of 548 bp, the GG genotype has two bands with a length of 358 bp and 190 bp, and the GA genotype has three bands with a length of 548 bp, 358 bp, and 190 bp. The diversity of SNP g.385G>A MAOA gene using the PCR-RFLP technique in several beef cattle breeds is presented in Table 2.

The findings from this research indicate that the single nucleotide polymorphism (SNP) g.385G>A in the MAOA gene, specifically in the promoter region, shows variation across different cattle breeds. The genotype associated with aggressive traits is often linked to polymorphic conditions in this region of the MAOA gene. In Bali cattle, the A allele frequency is significantly higher, with an actual frequency of 0.94, suggesting that this allele may contribute to the manifestation of aggressive behaviors. In contrast, the G allele is more common in other breeds, such as Limousin and Wagyu, where the MAOA gene is monomorphic, implying that these breeds may exhibit fewer aggressive tendencies due to the dominance of the G allele. The MAOA gene encodes the enzyme monoamine oxidase A, which plays a crucial role in neurotransmitter regulation, including serotonin, dopamine, and norepinephrine. These neurotransmitters affect mood regulation, emotional responses, and stress handling. Mutations or variations in this gene, particularly in the promoter region, can lead to abnormal levels of neurotransmitters, which may manifest in increased aggression (Ziegler and Domschke 2018). The higher frequency of the A allele in Bali cattle suggests that this variation in the MAOA gene could predispose these animals to more aggressive behaviors compared to monomorphic breeds for the G allele. Regarding breed-specific aggressive traits, Bali cattle, which exhibit a polymorphic condition for the SNP g.385G>A with a high A allele frequency, are likely more prone to aggression, which aligns with observations of Bali cattle's defensive aggressiveness in response tohandling stressful situations (Sari et al., 2021). In contrast, Limousin and Wagyu cattle, which are monomorphic for the G allele, are less likely to display such aggressive behaviors, likely due to selective breeding that has reduced genetic diversity in this region.

These findings highlight the importance of understanding genetic diversity when considering cattle management and breeding programs aimed at balancing productivity and behavior.

Heterozygosity values measure genetic diversity in populations, which can contribute to selection programs (Putri et al. 2021). The number of samples and alleles and their frequency influence the heterozygosity value. The heterozygosity value of SNP g.385G>A shows a Ho value that is higher than the He value in Bali, Wagyu, PO, Madura cattle, and Wagyu-Bali crosses (F1), which indicates high genetic diversity of the MAOA gene in Bali, Wagyu, PO cattle. , Madurese and Wagyu-Bali crosses (F1), while the Limousin and Wagyu cattle showed values that did not vary (0.00). The heterozygosity value ranges from 0 to 1. If the heterozygosity value is close to 0, then the heterozygosity value is low, whereas if it is close to 1, then the heterozygosity value is said to be high (Li et al., 2013). High observed heterozygosity indicates that the observed population has a high level of genetic diversity (Karimah et al., 2021). The research results showed that the Ho value of Bali cattle and Madura cattle tended to be in the low category, while the Ho value of PO cattle and Wagyu-Bali cross (F1) cattle tended to be in the high category. The adaptability obtained from natural selection in tropical areas causes limited genetic diversity in Bali and Madura cattle. In order to maintain good performance, Bali cattle need a suitable living environment that includes nutrition and climate. Significant differences between Ho and He values can indicate a genotypic imbalance in the analyzed population (Dairoh et al., 2022).

The balance of alleles in a population is seen based on the chi-square value calculated based on the difference in observed and expected genotype frequencies (Pratiwi et al. 2016). The population is in equilibrium if the calculated chi-square value is smaller than the table chi-square value (Ismail et al., 2020). The results of the analysis showed that Bali, PO, Madura, and Wagyu-Bali cross (F1) cattle were in Hardy-Weinberg equilibrium (equilibrium, P>0.05). The Hardy-Weinberg equilibrium value of Limousin and Wagyu cattle cannot be analyzed because they are monomorphic. In the equilibrium of a large population, allele frequencies will be stable from generation to generation, and there will be no influencing factors such as selection, migration, mutation, or genetic drift (Jakaria et al., 2023). A population is in genetic balance if the genotype frequencies (p2, 2pq, q2) and allele frequencies (p and q) are constant from generation to generation because the combination of gametes occurs randomly in a large population. Although polymorphisms in SNP g.385G>A have the potential to be candidate genetic markers, this study requires direct observation or measurement of aggression traits in the sampled cattle breeds. Therefore, further studies are needed to investigate the functional significance of SNP g.385G>A and their possible association with behavioral traits in cattle.

CONCLUSION

The results of this study found six specific SNPs, namely SNP g.98A>C, SNP g.145G>T, SNP g.216A>C, SNP g.340T>G, SNP g.349A>C, and SNP g.385G>A in the promoter region of the MAOA gene. In SNP g.385G>A, three genotypes were found using the PCR-RFLP method using the RSaI restriction enzyme, namely genotypes GG, GA, and AA. SNP g.385G>A is polymorphic in Bali, PO, Madura, and Wagyu-Bali cross (F1) cattle while monomorphic in Limousin and Wagyu cattle. Further studies are necessary to explore the functional implications of SNP G.385G>A and their relationship to aggressive behaviors in cattle.

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Improvement of Sexed Sperm Quality of Bali Bulls by Adding Palmyra (*Borassus flabellifer Linn.*) Fruit Water to Citrate-Egg Yolk Extender

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ABSTRAK

Hine TM, Nalley WM, Marawali A, Kihe JN, Kune P, Uly K. 2024. Peningkatan kualitas sperma sexing sapi bali melalui penambahan air buah lontar (*Borassus flabellifer* Linn.) dalam pengencer sitrat-kuning telur. JITV 29(3):135-142. DOI:http://dx.doi.org/10.14334/jitv.v28i3.3388.

Sexing sperma merupakan metode yang dikembangkan untuk memisahkan sperma berkromosom X dan Y yang selanjutnya digunakan untuk produksi anak sesuai dengan jenis kelamin yang dinginkan peternak. Namun demikian, proses sexing menyebabkan penurunan kualitas sperma yang disebabkan oleh terjadinya stres oksidatif akibat peningkatan radikal bebas yang berlebihan. Dengan demikian, ke dalam bahan pengencer perlu menambahkan antioksidan dalam upaya untuk menangkal pengaruh negatif dari radikal bebas terhadap kehidupan sperma. Penelitian ini dirancang dengan menambahkan air buah lontar (PFw), yang kaya akan antioksidan ke dalam pengencer sitrat-kuning telur (CEy). Tujuan penelitian ini adalah untuk mengevaluasi potensi PFw sebagai suplemen antioksidan alami dalam pengencer CEy dalam upaya meningkatkan kualitas sperma sexing sapi Bali. Sperma ditampung dengan menggunakan metode vagina buatan dari tiga ekor sapi bali jantan berumur 3-4 tahun. Sperma yang berkualitas baik (motilitas ≥70%, abnormalitas ≤20%) di-sexing dengan metode gradien albumin tiga lapis (5, 10, dan 15 persen) selama 20 menit, dan sperma yang berada pada lapisan albumin terbawah dipreservasi dalam pengencer: CEy, PFw, PFw-kuning telur (PFw-Ey), atau CEy-PFw. Hasil penelitian menunjukkan bahwa preservasi sperma dalam pengencer CEy-PFw menghasilkan kualitas sperma yang lebih tinggi (P<0,05) dibandingkan dengan ketiga pengencer lainnya, kecuali pada parameter abnormalitas sperma (P>0,05). Disimpulkan bahwa penambahan PFw ke dalam pengencer CEy berpotensi meningkatkan kualitas sperma sexing sapi bali.

Kata Kunci: Sapi Bali, Sitrat, Kuning Telur, Air Buah Lontar, Sperma Sexing

ABSTRACT

Hine TM, Nalley WM, Marawali A, Kihe JN, Kune P, Uly K. 2024. Improve the sexed sperm quality of Bali bulls by adding palmyra (*Borassus flabellifer* Linn.) fruit water to citrate-egg yolk extender. JITV 29(3):135-142. DOI:http://dx.doi.org/10.14334/jitv.v28i3.3388.

Sperm sexing is a technique designed to separate sperm carrying the X and Y chromosomes, which are then used for artificial insemination to generate offspring with the sex the breeder desires. However, the sexing process causes a decrease in sperm quality caused by oxidative stress due to an excessive increase in free radicals. Thus, to counteract the detrimental effects of free radicals on sperm life, antioxidants must be added to the diluent. This study was designed by adding palmyra fruit water (PFw) to citrate-egg yolk (CEy) diluent. This study aimed to evaluate the potency of PFw as a natural antioxidant supplement in CEy diluent to improve the quality of sexing sperm in Bali bulls. Sperm were collected using the artificial vagina method from three Bali bulls aged 3-4 years. Good quality sperm (motility ≥70%, abnormality ≤20%) were sexed with a three-layer albumin gradient method (5, 10, and 15 percent) for 20 minutes, and sperm that were on the bottom albumin layer were preserved in CEy, PFw, PFw-egg yolk (PFw-Ey), or CEy-PFw. The results showed that sperm preservation in the CEy-PFw diluent resulted in higher sperm quality (P<0.05) compared to the other three diluents, except for the sperm abnormality parameter (P>0.05). It was concluded that adding PFw into the CEy diluent could potentially improve the sexed sperm quality of Bali bulls.

Key Words: Bali Bulls, Citrate, Egg Yolk, Palmyra Fruit Water, Sperm Sexing

INTRODUCTION

Sperm sexing is a method designed to improve the proportion of desired sex calves in calf populations (Seidel 2014; Razmkabir 2018; Vishwanath & Moreno 2018). In animals, such as cattle, there are two kinds of sperm: sperm with X and Y chromosomes. When the X sperm fertilizes the egg during fertilization, a female calf is born, while the Y sperm fertilization results in a male offspring. Naturally, a cattle 's ejaculate contains an equal amount of each type of sperm, 50 percent of which have the X chromosome and 50 percent the Y chromosome (Rai 2018). When a farmer wishes to grow male calves, sexing technology can reduce the quantity

of X sperm and raise the fraction of Y sperm, and vice versa (Kumar et al. 2016; Rai 2018; Yadav et al. 2018; Rahman & Pang 2020).

Sexing treatment causes a decrease in sperm fertility. According to Purwoistri et al.'s study, sperm motility decreased from 70 percent in fresh semen to 53.5 - 63.0 percent after sexing, sperm viability decreased from 95.12 to 91.91-93.30 percent, and sperm abnormalities rose from 5.28 to 6.82-8.76 percent (Purwoistri et al. 2013). The quality of frozen sexed-sperm was also lower compared to non-sexed-sperm after thawing, with a motility of 31.4 vs. 36.0 percent and viability 75.89 vs. 81.70 percent, respectively (Mahfud et al. 2019); the pregnancy rate dropped from 59.09 in nonsexed-sperm to 41.17 percent in sexed-sperm (Bhat & Sharma 2020). One reason for the decline in sperm fertility is the presence of oxidative stress, which is brought on by an excessive rise in free radicals during the sexing process. Antioxidants must be added to sperm diluent to neutralize these free radicals (Rath et al. 2013; Spinaci et al. 2016).

Previous studies have employed palmyra fruit water (PFw) to preserve the semen of Bali and Sumba Ongole bulls (Hine et al. 2014; Kaka et al. 2024). On the fourth day of preservation, the progressive motility of Bali bull sperm in the PFw-egg yolk diluent reached 44 percent, a significant increase over citrate-egg yolk's (CEy) 30 percent (Hine et al. 2014). More investigation by Kaka et al. (2024) revealed that using a nanoparticle diluent made from a mixture of 75% PFw and 25% egg yolk on Sumba Ongole bulls sperm can maintain progressive motility until the seventh day of preservation, with a percentage of progressive sperm motility reaching 40.20 percent. The outcomes were similar to 40.50 percent for the Cauda Epididymal Plasma-3 diluent.

Palmyra fruit has a high antioxidant content and radical scavenging activity (57.32–83.25%) (Wijewardana et al. 2016; Kurian et al. 2017). The phytosterols, flavonoids, saponins, triterpenoids, phenols, alkaloids, and tannins (Renuka et al. 2018; Huynh Thi Le et al. 2020), vitamin E (Huynh Thi Le et al. 2020), and β-carotene (Wijewardana et al. 2016) are a few of the antioxidants found in palmyra fruit. The palmyra fruit extract contained a total of 104 g GAE/100 mg and 98.40 g QE/100 mg phenolics and flavonoids, respectively, vitamin E (52.15-55.12 mg/100 g) and β-carotene (617.55 -2647.19 µg/100 g) (Wijewardana et al. 2016; Huynh Thi Le et al. 2020). The range of 2.2-difenill-1-pikrilhidrazil (DPPH) radical inhibition was between 35 to 70 percent; the 3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) radicals' inhibition ranged from 40 to 75.5 percent. Palmyra fruit extract has a nitric oxide scavenging activity of 45 to 76 percent and a superoxide radical scavenging activity of 43 to 83 percent (Renuka et al., 2018). Additionally, the palmyra fruit has small amounts of fat (3.64–3.95%) and protein (3.50–4.09%) (Wijewardana et al. 2016), potassium (237.00-276.73 mg/100g), magnesium (211.61-293.62 mg/100g) and saponins (36.10–55.62 g/100g) (Abe-Inge et al. 2018).

This study aimed to evaluate the potency of PFw as a natural antioxidant supplement in CEy diluent to improve the quality of sexing sperm in Bali bulls.

MATERIALS AND METHODS

Animals

Three Bali bulls aged 3-4 years old kept in the Williams & Laura Foundation experimental pen, Kupang Tengah District, Kupang Regency, Indonesia, were used for semen collection. The bull received up to 10% of its weight in forage, 3 kg of concentrate daily, and unlimited water access.

Diluent preparation

There were four diluents prepared in this study, namely citrate-egg yolk (CEy), palmyra fruit water (PFw), palmyra fruit water-egg yolk (PFw-Ey), and citrate-egg yolk-palmyra fruit water (CEy-PFw) (Table 1). Citrate diluent was prepared as described by (Arifiantini et al. 2010), namely 2.9 g of sodium citrate dehydrate (Sigma Aldrich, W302600-SAMPLE-K) dissolved in 100 mL of aquabidest until homogeneous.

Table 1. Composition of diluent

Ingredient	Diluent			
	CEy	PFw	PFw-Ey	CEy-PFw
Sodium citrate dehydrate (g)	2.9	-	-	2.9
Aquades (mL)	100	-	-	50
Egg yolk (mL)	20	-	20	20
PFw	-	100	100	50
Penicillin G	1000	1000	1000	1000
Streptomycin sulfate (µg)	1000	1000	1000	1000

CEy= citrate-egg yolk, PFw= palmyra fruit water, PFw-Ey= palmyra fruit water-egg yolk, CEy-PFw= citrate-egg yolk-palmyra fruit water

Palmyra fruit water is taken from young palmyra fruits: the tip of a young palmyra fruit was cut with a sterile knife, and water was drawn into the Erlenmeyer tube using a 20 mL syringe (Hine et al. 2014). For each diluent, 20% egg yolk (v/v), 1000 IU penicillin G sodium salt (Sigma Aldrich, P3032-10MU), and 1000 µg Streptomycin Sulfate Salt (Sigma Aldrich, S1277-5G) per mL.

Semen collection and evaluation

Semen was collected utilizing the artificial vaginal technique (Sylla et al. 2015; Setiadi et al. 2022). Semen was assessed macroscopically for color, volume, consistency, and pH. Under a microscope (Zeiss) with a magnification range of 100 to 400x, sperm were evaluated microscopically for mass movement and percentage of progressive motility (0= non-motile, 100= 100% progressive motility); eosin-negrosin staining was used to determine the viability of the sperm (Haris et al. 2020), sperm concentration asses using a Neubauer hemocytometer counting chamber (Crespilho et al. 2017) and sperm morphology was measured by carbolfuchsin-eosin (Morrell et al. 2018).

Sexing and sperm preservation

An albumin gradient of 5%, 10%, and 15% is used in the sperm sexing method, as carried out by Ratnawati et al. (2020) with slight modifications. The four diluents (CEy, PFw, PFw-Ey, CEy-PFw) were utilized to prepare the three albumin gradients. Approximately 2 mL of a 5-10-15 percent albumin gradient should be placed in a test tube, and fill the test tube to just over the 5 percent albumin gradient with 2 mL of diluted semen (1:1), incubate for 20 minutes until the albumin gradient layer was formed. Put 2 mL of the albumin gradient at the bottom of the tube into a test tube with 3 mL of diluent (CEy, PFw, PFw-Ey, CEy-PFw), centrifuge for five minutes at 1500 rpm, remove 3 mL of the supernatant; after that, the final 2 mL of precipitate were diluted using one of the four diluents (1: 2) and kept at 3 to 8 ºC in the refrigerator.

Assessing the quality of sexed sperm

During the 96-hour storage period, the quality of the sexed sperm was assessed every 24 hours. Sperm motility was assessed by applying a 10 µL drop of diluted semen to a warm slide with a glass coverslip and monitored in five microscopic fields of view at 400X magnification. The motility score was computed from the average movement of the five visual fields. Sperm viability was assessed by eosin-nigrosin staining (Haris et al. 2020). 10 µL drop of diluted semen on a slide and 40 µl drop of nigrosin–eosin; it smears on a slide and dries quickly in the heating stage (80ºC). Observe 200 sperm cells in a total of 10 tiny fields of view. Sperm were classified as viable (unstained) or dead (stained). Sperm morphology was assessed using the same method as the viability evaluation procedure; however, the proportion of sperm with aberrant morphology was measured. The integrity of the acrosome was assessed by diluting 500 µl semen combined with 50 µl of 1% formaldehyde citrate in a test tube (Mughal et al. 2013). A phase-contrast microscope at 1000X examined the sperm (200 in total) for their usual apical ridge. Evaluating the integrity of the plasma membrane using the hypoosmotic swelling (HOS) test as described by (Ramu and Jeyendran 2013; Zubair et al. 2013). 200 sperm were counted for swelling/coiling of the tail under a 400X phase-contrast microscope observation.

Designing experiments and data analysis

This study, with four treatments and five replicates for 20 trial units, was built using a completely randomized design. Data were analyzed using ANOVA, followed by Duncan's test. Analysis was performed using SPSS 25 software.

RESULTS AND DISCUSSION

Motility, viability, and abnormalities of sexed sperm

After dilution, sperm motility and viability decreased slightly; however, sperm abnormalities increased in all treatments compared to fresh semen; this could be due to sperm stress during the sexing process, which lowers sperm quality. Nonetheless, there was no significant difference between the treatments ($P>0.05$) (Table 2).

Compared to PFw and PFw-Ey, CEy-PFw generated more viable and motile sperm from the first to the fourth preservation day ($P<0.05$). On the other hand, sperm motility and viability showed significant differences with CEy ($P<0.05$) only on the third and fourth days of preservation, respectively. On the fourth day of preservation, the sperm motility and viability in CEy-PFw diluent were noticeably higher than in CEy diluent (43.3 and 51.0 vs. 35.0 and 41.3%) (Table 2); this indicates that sperm motility and viability were improved by adding palmyra fruit water to the citrate-egg yolk diluent; this is possible due to the presence of several compounds in Palmyra fruit water that are highly advantageous for sperm life during preservation.

The reason for the low sperm motility and viability in PFw and PFw-Ey diluents is that they lack buffer elements, which help to keep the diluent's pH within the ideal range for sperm life, as well as protective elements

against cold shock in the form of lipoproteins and lecithin found in egg yolks. When measured on the fourth day of preservation, the pH of the PFw and PFw-Ey diluents dropped to 6.2, but the pH of the CEy and CEy-PFw diluents ranged from 6.4 to 6.7 (data not shown). Compared to the ideal sperm diluent pH of 6.8 to 7.2, the pH of PFw and PFw-Ey diluents is significantly lower (Liu et al. 2016). The proportion of abnormal sperm in fresh semen was 3.92%, which is still below the threshold of 20% for artificial insemination. Sperm abnormalities increased to 4.50–5.14% in all four diluents after being preserved for 4 days. From the post-dilution to the fourth day of storage, there was no discernible variation in the percentage of abnormal sperm across diluents ($P>0.05$); this implies that the variations in nutrient content among diluents have no effect on the percentage of abnormal sperm.

Sperm dilution is one method used to ensure sperm survival in an in-vitro environment. Dilution allows for maintaining the extender's pH and sperm metabolism; bacterial contamination is minimized, and cryogenic damage during preservation and cryopreservation can be suppressed (Malik et al. 2018; Raheja et al. 2018). A good sperm diluent should be able to keep the pH between 6.8 and 7.2 (Liu et al. 2016), provide energy (Mohamed et al. 2019), contain antioxidants to lessen stress brought on by free radicals (Mousavi et al. 2019), contain antibiotics to prevent contamination (Schulze et al. 2020), and act as an anti-cold shock (Amirat-Briand et al. 2010; Tariq et al. 2020). Diluents with these qualities can protect sperm life throughout storage and transportation, enabling their use in artificial insemination. Sperm extenders consist of two separate types: liquid and frozen. While frozen sperm can survive for years, liquid sperm can only last a few days (Johnston et al. 2012). Some scientists have created sperm diluents using a variety of components such as palmyra juice (Hine et al. 2014) and egg yolk (Filho et al. 2009), which are both sourced from plants and animals, which can sustain sperm quality, are readily available and inexpensive priced (Hine et al. 2014; Layek et al. 2016).

This study supports using PFw as a supplemental ingredient in the CEy extender. Adding PFw to the diluent will create a more favorable in vitro environment for sperm life; this is strongly tied to the existence of critical substances in PFw, including numerous antioxidants that help fight free radicals and carbohydrates that may serve as the sperm's potential energy source (Renuka et al. 2018; Behera & Nayak, 2022).

Palmyra fruit water has a high carbohydrate content of 10.96 percent (Haisya et al. 2011), which can be converted into glucose and fructose, two crucial energy sources for sperm movement and viability (Mukai & Okuno 2004). A study by Arifiantini and Purwantara (2010) demonstrated that sperm motility was improved when fructose was added to the citrate-yolk diluent. Since fructose and glucose are simple sugars with a low molecular weight, they may easily pass through cell membranes. Through the activity of an enzyme found within the cell, glucose and fructose may be metabolized into energy through the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (Ford 2006; Yi et al. 2008; du Plessis et al. 2015). The energy generated is subsequently used by sperm to sustain their viability and motility during preservation. ATP can be regenerated by adding a phosphoryl group from the extenders' carbohydrates or lipids. Thus, the quantity of energy produced for sperm life will depend on the amount of carbohydrates in the extender. According to Tourmente et al. (2015), the ATP compound in bovine sperm is closely related to sperm.

Palmyra fruit water also contains high levels of flavonoids and phenolics (Saranya and Vijayakumar 2016), which act as antioxidants to protect sperm against free radical attacks. Flavones and catechins are the most potent flavonoids that protect cells against reactive oxygen species (Panche et al. 2016) produced during metabolic processes. Reactive oxygen species cause damage to cell membranes, which is caused by lipid peroxidation. Damage to the cell membrane causes cell charge modification, changes in osmotic pressure, cell swelling, and death. Flavonoids can neutralize free radicals in various ways, such as by oxidizing them to create other harmless radicals, or they may scavenge superoxide and peroxynitrite directly, two radicals of highly reactive oxygen.

However, using Palmyra fruit water alone has not adequately preserved sperm survival during in vitro preservation, suggesting that sperm require other nutrients besides carbohydrates, which are available in Palmyra fruit water in sufficient concentrations. When combined with egg yolk in a citrate diluent, palmyra fruit water can enhance sperm quality more effectively than palmyra fruit water alone; i.e., motility, viability, plasma membrane integrity, and acrosomal integrity all rose by 38.33; 41.93; 42.32; 42.54 percent, respectively, which were observed on the fourth day of storage, suggesting that sperm requires the inclusion of egg yolk in the diluent.

Previous studies have listed several substances in egg yolks, including phospholipid, cholesterol, and low-density lipoprotein (LDL) (Layek et al. 2016; Anzar et al. 2019; Sun et al. 2020). Low-density lipoproteins work by stabilizing the sperm membrane and replacing phosphoproteins in the sperm membrane that have been disturbed during preservation, increasing cold tolerance to protect sperm from being damaged by cold shock, and increasing the cholesterol/phospholipid ratio to prevent the phospholipids in membranes from degrading (Lagace & Ridgway 2013; Stevenson et al. 2014). LDL-derived lipid-binding proteins in the yolk

Table 2. Viability, motility, and abnormality of sexed sperm of Bali bulls in four different extenders

Parameters	Treatments	Fresh semen	Post-dilution (Day 0)	Day 1	Day 2	Day 3	Day 4
Sperm motility(%)	CEy	79.5±4.32^{a}	77.5±2.74^{a}	67.5±2.74^{a}	57.5±2.74^{a}	45.0±3.16^{b}	35.0±4.47^{b}
	PFw	79.5±.4.32^{a}	77.5±2.74^{a}	28.3±4.08^{c}	7.5±2.74^{c}	5.0±0.00^{d}	5.00±0.00^{d}
	PFw-Ey	79.5±4.32^{a}	77.5±2.74^{a}	60.8±4.92^{b}	45.0±8.37^{b}	25.0±11.4^{c}	15.8±12.4^{c}
	CEy-PFw	79.5±4.32^{a}	77.5±2.74^{a}	69.5±3.94^{a}	60.7±3.56^{a}	53.3±2.58^{a}	43.3±2.58^{a}
	p-value	1.00	1.00	0.00	0.00	0.00	0.00
Sperm viability (%)	CEy	84.1±2.66^{a}	83.1±1.78^{a}	74.0±2.67^{a}	64.8±3.26^{a}	52.9±2.40^{a}	41.3±3.53^{b}
	PFw	84.1±2.66^{a}	82.2±2.06^{a}	35.1±5.31^{c}	15.8±1.93^{c}	11.9±1.82^{c}	9.09±0.56^{d}
	PFw-Ey	84.1±2.66^{a}	82.8±1.42^{a}	67.1±3.73^{b}	51.6±6.57^{b}	35.6±9.81^{b}	22.4±12.3^{c}
	CEy-PFw	84.1±2.66^{a}	83.4±1.58^{a}	77.0±3.32^{a}	67.6±4.12^{a}	57.8±5.42^{a}	51.0±2.12^{a}
	p-value	1.00	0.68	0.00	0.00	0.00	0.00
Sperm abnormalities (%)	CEy	3.92±0.35^{a}	4.06±0.78^{a}	3.94±1.01^{a}	4.17±0.61^{a}	4.46±0.69^{a}	4.50±0.51^{a}
	PFw	3.92±0.35^{a}	4.48±0.72^{a}	4.24±0.79^{a}	4.66±1.04^{a}	4.62±1.24^{a}	5.14±1.43^{a}
	PFw-Ey	3.92±0.35^{a}	4.16±0.43^{a}	4.13±0.61^{a}	4.31±0.99^{a}	4.61±1.00^{a}	4.67±0.61^{a}
	CEy-PFw	3.92±0.35^{a}	4.09±0.92^{a}	4.28±0.59^{a}	4.48±0.77^{a}	4.49±1.00^{a}	4.58±0.86^{a}
	p-value	1.00	0.75	0.87	0.79	0.99	0.63

CEy= citrate-egg yolk, PFw= palmyra fruit water, PFw-Ey= palmyra fruit water-egg yolk, CEy-PFw= citrate-egg yolk-palmyra fruit water. Different superscripts in the same column differ significantly (P<0.05)

Table 3. Plasma membrane and acrosome integrity of sexed sperm of Bali bulls in four different extenders

Parameters	Treatments	Fresh semen	Post-dilution (Day 0)	Day 1	Day 2	Day 3	Day 4
Plasma membrane integrity of sperm (%)	CEy	86.2±1.24^{a}	85.5±1.98^{a}	76.5±3.35^{a}	67.4±3.25^{a}	55.3±3.31^{a}	43.5±3.48^{b}
	PFw	86.2±1.24^{a}	84.3±0.20^{a}	39.4±5.83^{c}	20.2±6.51^{c}	14.9±2.19^{c}	11.2±0.92^{d}
	PFw-Ey	86.2±1.24^{a}	85.4±2.04^{a}	69.2±3.69^{b}	53.8±6.99^{b}	37.6±9.54^{b}	24.4±12.2^{c}
	CEy-PFw	86.2±1.24^{a}	85.7±1.95^{a}	79.3±3.32^{a}	70.2±4.92^{a}	60.1±6.22^{a}	53.5±3.01^{a}
	p-value	1.00	0.64	0.00	0.00	0.00	0.00
Acrosomal integrity of sperm (%)	CEy	87.4±0.66^{a}	86.2±1.72^{a}	76.9±3.28^{a}	67.7±3.87^{a}	55.9±3.31^{a}	44.4±3.62^{b}
	PFw	87.4±0.66^{a}	85.2±2.19^{a}	41.0±5.75^{c}	21.0±6.62^{c}	15.6±2.28^{c}	11.6±0.58^{d}
	PFw-Ey	87.4±0.66^{a}	85.6±2.34^{a}	70.1±3.91^{b}	54.6±6.65^{b}	38.5±9.58^{b}	25.1±12.1^{c}
	CEy-PFw	87.4±0.66^{a}	86.0±2.38^{a}	80.1±3.20^{a}	71.1±4.68^{a}	61.0±6.03^{a}	54.1±2.43^{a}
	p-value	1.00	0.87	0.00	0.00	0.00	0.00

CEy= citrate-egg yolk, PFw= palmyra fruit water, PFw-Ey= palmyra fruit water-egg yolk, CEy-PFw= citrate-egg yolk-palmyra fruit water. Different superscripts in the same column differ significantly (P<0.05)

protect sperm during freezing (Tarig et al. 2017; Raheja et al. 2018). The sperm protection mechanisms of LDL are as follows: 1). The plasma membrane of the sperm is attached to LDL phospholipids, which stabilize the membrane (Naz et al. 2018); 2) egg yolk phospholipids replace damaged plasma membrane phospholipids during sperm preservation and cryopreservation (Layek et al. 2016); and 3) seminal plasma protein interacts with LDL plasma protein (Manjunath 2018), which is responsible for the degradation of phospholipids and sperm cholesterol. Phospholipididylcholine (lecithin), the primary phospholipid in egg yolks, and lipoproteins help protect sperm against cold shock (Alvarez-Rodríguez et al. 2013). When sperm are cooled and frozen, the cholesterol-to-phospholipid ratio in the sperm membrane is disrupted mainly through the excretion of

cholesterol and the production of various reactive oxygen species (Raheja et al. 2018).

Plasma membrane and acrosome integrity of sexed sperm

Following dilution, the integrity of the plasma membrane and acrosome revealed no discernible variation (P>0.05) between the treatments; this is due to the sperm not having gone through the cooling process, which could have harmed the sperm membrane. Lipid peroxidation induces modifications in the membrane structure of sperm during the cooling process from room temperature to 5^0C. The reduction in sperm plasma membrane and acrosome integrity across all diluents indicates this. The integrity of the acrosome dropped from 87.4 to 11.6-54.1 percent on the fourth day of preservation, and the plasma membrane integrity dropped from 86.3 percent in fresh semen to 11.2-53.5 percent (Table 3).

CEy-PFw produced the highest levels of plasma membrane and acrosome integrity compared to the other three treatments, and from the first to the fourth day of preservation, it differed significantly from PFw and PFw-Ey (P<0.05). Only on the fourth day of preservation were discernible differences with CEy (P<0.05); this demonstrates that combining CEy with palmyra fruit water can result in a more optimal medium condition for preserving the integrity of the sperm's plasma membrane and acrosome during preservation.

Palmyra fruit water contains high levels of flavonoids and phenolics (Saranya and Vijayakumar 2016), which act as antioxidants to protect sperm membranes against free radical attacks. Flavones and catechins are the most potent flavonoids that protect cells against reactive oxygen species (Panche et al. 2016) produced during metabolic processes. Reactive oxygen species cause damage to cell membranes, which is caused by lipid peroxidation. Damage to the cell membrane causes cell charge modification, changes in osmotic pressure, cell swelling, and death. Flavonoids can neutralize free radicals in various ways, such as by oxidizing them to create other harmless radicals, or they may scavenge superoxide and peroxynitrite directly, two radicals of highly reactive oxygen.

This research confirms the effectiveness of palmyra fruit water as a component of citrate-egg yolk extender for sperm preservation in Bali bulls' sexed sperm. However, more research is needed on some sperm fertility issues when used for artificial insemination.

CONCLUSION

Palmyra fruit water has the potential to be a natural antioxidant supplement in citrate-egg yolk diluent to improve the sexed sperm quality of Bali bulls.

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Animal Welfare Study in Sheep Transported with Methionine Hydroxy Analog and Dextrose Supplementation

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ABSTRAK

Baihaqi M, Nuraini H, Jayanegara A, Manalu W. 2024. Studi kesejahteraan domba dalam kondisi pengangkutan yang disuplementasi dengan meionin hidroksi dan dekstrosa. JITV 29(3):143-150. DOI:http://dx.doi.org/10.14334/jitv.v29i3.3449.

Transportasi ternak dapat berdampak negatif pada kesejahteraan hewan dan performa setelah transportasi. Penelitian ini bertujuan mengevaluasi efek suplementasi metionin hydroxy analogue (MHA) dan dekstrosa sebelum transportasi pada status kesejahteraan hewan domba lokal. Penelitian menggunakan 27 domba jantan ekor tipis dengan bobot badan rata-rata 21,8±2,0 kg, berusia 10-12 bulan, yang dibagi secara acak dalam tiga perlakuan: domba tanpa transportasi dan pemberian suplemen (K: kontrol); domba yang diangkut dengan sistem transportasi tanpa pemberian suplemen (P1); domba yang diangkut dengan sistem transportasi dan diberi suplementasi aditif (P2: MHA 0,5 g/kg bobot badan dan dekstrosa 0,5 g/kg bobot badan). Domba P1 dan P2 diangkut selama 8 jam menggunakan kendaraan komersial yang biasa digunakan oleh peternak. Hasil penelitian menunjukkan bahwa domba di kelompok P2 secara signifikan memiliki penurunan bobot badan yang lebih rendah dibandingkan dengan P1 dan memiliki waktu pemulihan yang lebih cepat (P<0,05). Domba di kelompok P2 juga menunjukkan tingkah laku pascatransportasi yang lebih baik daripada P1, dan tidak berbeda nyata dibandingkan dengan kelompok kontrol. Kelompok P2 memiliki rasio N/L yang signifikan lebih rendah daripada P1 dan sebanding dengan kelompok kontrol (P<0,05). Domba pada kelompok P2 juga memiliki hormon kortisol dan glukosa darah yang signifikan lebih rendah daripada kelompok P1, yang menunjukkan peningkatan status kesejahteraan domba pada kelompok P2 dan Kontrol. Oleh karena itu, dapat disimpulkan bahwa suplementasi MHA dan dekstrosa sebelum transportasi meningkatkan status kesejahteraan domba selama transportasi.

Kata Kunci: Kesejahteraan Ternak, Dekstrosa, *Methionine Hidroxy Analogue*, Domba, Pengangkutan

ABSTRACT

Baihaqi M, Nuraini H, Jayanegara A, Manalu W. 2024. Animal welfare study in sheep transported with methionine hydroxy analog and dextrose supplementation. JITV 29(3):143-150. DOI:http://dx.doi.org/10.14334/jitv.v29i3.3449.

Livestock transportation adversely affects animal welfare and performance during subsequent production periods. This research aimed to evaluate the effects of methionine hydroxy analog (MHA) and dextrose supplementations before transportation on the animal welfare status of sheep. The study used 27 thin-tailed male sheep with an average body weight of 21,8±2,0 kg, 10-12 months of age, which were divided randomly into three treatments: sheep without transportation and additional supplement (K: Control); sheep transported without additional supplement (P1); sheep transported with additional supplement (P2: MHA 0.5 g/kg body weight and dextrose 0.5 g/kg body weight). Sheep in groups P1 and P2 were transported for 8 hours using commercial vehicles usually used by local farmers. The results showed that sheep in the P2 group had significantly lower body weight loss than those in the P1 group and faster recovery time (P<0.05). Sheep in the P2 group also demonstrated better post-transportation behavior than those in P1, with no significant difference from the control group. The experimental sheep in the P2 group had a significantly lower N/L ratio than those in P1 and was comparable to the control group (P<0.05). The P2 group also has significantly lower cortisol hormone and blood glucose concentrations than P1, which indicates improved sheep welfare status in P2 and Control groups. Therefore, it can be concluded that supplementation of MHA and dextrose before departure improves sheep's welfare status under transportation.

Key Words: Animal Welfare, Dextrose, Methionine Hydroxy Analogue, Sheep, Transportation

INTRODUCTION

The supply chain for small ruminants (sheep and goats) in Indonesia involves lengthy transportation times and multiple stakeholders. Additionally, facilities and infrastructure remain significant challenges, such as the condition of transportation during the shipment of livestock. In developed countries, the effects of transportation on livestock productivity and performance, welfare, carcass quality, and meat quality have been reviewed by several researchers (Miranda-de la Lama et al. 2014; Rey-Salgueiro et al. 2018). Animal welfare can be evaluated using output indicators expressed by livestock, such as physiological conditions,

production/body weight, behavior, and other factors (Broom 2019). However, research on sheep transportation is often conducted in temperate climates, which differ climatologically from tropical climates, using better transportation modes and road infrastructure, generally over shorter distances or travel times. Several researchers have also reported research on goat transportation in hot subtropical areas (Rey-Salgueiro et al. 2018; Biobaku et al. 2018). Other researchers have studied goat transportation in wet tropical areas in Malaysia (Raghazli et al. 2021), but using relatively short transportation times, namely two and six hours of travel. Preliminary studies in Indonesia conducted by Baihaqi et al. (2017) on sheep transportation concluded that the weight loss of livestock during 20 hours of transportation reached 11.59±2.75 percent of the initial weight. This weight loss is still higher than the transportation standards in developed countries such as Australia (5%) (Department of Agriculture and Water Resources 2023). A study by Lendrawati et al. (2020) stated that thin-tailed sheep transported for 12 hours could tolerate the adverse effects of transportation compared to the control group. Nevertheless, animal welfare during transportation in Indonesia has not been extensively studied. Some studies have been conducted to minimize the negative effects of livestock transportation, including providing supplements/additives to livestock before transportation, such as vitamin C (Ahmad Mir et al. 2019), electrolytes (Gupta et al. 2020), Selenium-Methionine and Cromium-Methionine (Mousaie et al. 2014), or molasses (Lendrawati 2020).

Methionine is an essential amino acid that contains a sulfur group and acts as a precursor for carnitine, creatine, cysteine, homocysteine, and succinyl-CoA. Additionally, methionine plays a role in lipid metabolism and activating enzymes related to antioxidants such as methionine sulfoxide reductase A (Blachier et al. 2020). Methionine is also involved in the biosynthesis of glutathione, a crucial molecule for combating oxidative stress (Martínez et al. 2017). Research on the use of methionine in the form of methionine hydroxy analog (MHA) has been applied to reduce heat stress in broiler chickens (Erfani et al. 2021) and dairy cows (Jacometo et al. 2016). Providing methionine as protected MHA is crucial to avoid rumen degradation in ruminant livestock. However, trials of MHA administration in transported sheep are still rare. They provided energy sources before transportation, which is expected to reduce transportation stress. Experiments with protected glucose administration in dairy cows showed improved insulin and plasma glucose concentrations (Sauls-Hiesterman et al. 2020). Based on this information, glucose supplementation in dextrose is expected to increase blood glucose reserves to minimize transportation stress and losses. Dextrose is another name for glucose, a monosaccharide with the chemical formula $C_6H_{12}O_6$ (Townshend et al. 2019). Dextrose is commonly marketed in powder form as a sweetener and nutritional supplement for food and feed. This simple sugar is thought to supply energy to livestock experiencing fatigue. Research conducted by Baihaqi et al. (2022) using methionine or dextrose separately at 0.24 g/body weight improved physiological responses, weight recovery time, and behavior in sheep. However, these studies have not significantly reduced body weight loss after 6 hours of transportation. We hypothesized that combining dextrose and MHA will more effectively reduce stress and body weight loss due to transportation. Based on this information, this research aimed to evaluate the effects of MHA and dextrose administration on the welfare status of local sheep transported under wet tropical climate conditions.

MATERIALS AND METHODS

Materials

The research used 27 male thin-tailed sheep with an average body weight of 21.8±2.0 kg and aged 10-12 months. The materials included protected methionine hydroxy analog (MHA), dextrose, grass feed, and commercial concentrate. The experimental sheep were housed in individual pens size 120x60 cm, and transportation was carried out using a pickup truck commonly used by local farms. Equipment used included sheep weighing scales, a stethoscope, a rectal thermometer, blood sample tubes, and CCTV to record the behaviors of the sheep in pens after transportation.

Research procedure

The experimental protocol of this study was approved by the Institutional Animal Ethics Committee of the School of Veterinary Medicine and Biomedical Sciences, IPB University, in 2022 (No 031/KEH/SKE/IX/2022). The study used three treatments: first, sheep that were not transported and without additional supplement (K: control); second, sheep transported without additional supplement (P1); and third, sheep transported with additional supplement (P2: MHA 0.5 g/kg body weight and dextrose 0.5 g/kg body weight). Each treatment had nine replicates, with one sheep per replicate. The sheep were adapted to individual pens for 2 weeks before the trial. The experimental sheep in the P2 group were given supplements for five days before transportation. MHA and dextrose were diluted in 100 ml of water and orally administered to the sheep every morning at 07:00 a.m. Sheep in the treatment P1 and P2 were unloaded randomly into the vehicle. The sheep were transported for approximately 8 hours with no rest stop from and to

the Small Ruminant Laboratory, Faculty of Animal Science, IPB University.

Body weight loss measurement

The sheep were weighed twice: first, before loading onto the transport vehicle to record the initial body weight, and second, after unloading, to record the final body weight after transportation. Weighing was performed using a hanging scale.

Measurement of physiological responses

The sheep's physiological responses, including heart rate, respiratory rate, and rectal temperature, were measured right after unloading it from the vehicle.

Measurement of blood profiles and metabolites

Blood samples were collected from sheep after unloading from the vehicle using a syringe from the jugular vein in the neck, with a volume of 5 ml. Three milliliters of blood were collected into tubes containing anticoagulants for blood profile analysis, while two milliliters of blood were collected into tubes without anticoagulants for metabolite analysis. The blood profiles included measurements of erythrocytes, hemoglobin, hematocrit, leukocytes, percentage of neutrophils and lymphocytes, as well as the neutrophil/lymphocyte ratio, using a hematology analyzer (MEK-6550J/K Celltax Nihon Kohden). Cortisol levels were measured using a competitive Enzyme-Linked Immunosorbent Assay (ELISA) method based on the procedures provided in the ELISA kit for cortisol hormone (Cat: EIA 1887 DRG, Instrument GmbH, Germany). Meanwhile, glucose levels (mg dL^{-1}) were analyzed using an enzymatic method with KIT number 11219.

Measurement of recovery time

Recovery time was measured by weighing the sheep daily at 06:00 a.m. before feeding. Recovery time is the number of days the sheep need to regain their pre-transportation body weight.

Behavior observation

Behavior was recorded using closed-circuit television (CCTV) recording equipment with two cameras installed on the ceiling of the pen to capture the sheep's activities. Observations were made for five hours after transportation based on Baihaqi et al. (2022). The parameters observed included eating, drinking, standing, walking, and lying/resting behaviors. The frequencies of these behaviors were calculated using CowLog 3.0.2 software (Pastell 2016).

Experimental design and data analysis

This study used a completely randomized design with three treatments (K/control: sheep without transportation and additives supplementation; P1: sheep transported without additives supplementation; and P2: sheep transported with the MHA and dextrose supplementations). Each treatment had nine replicates (9 sheep). The data were analyzed using analyses of variance.

RESULTS AND DISCUSSION

Body weight loss and recovery period of sheep

Body weight loss is an indicator of stress in sheep during transportation. The sheep treated with methionine and dextrose (P2) showed significantly different body weight losses and percentage of body weight losses compared to transported sheep without additives (P1) ($P<0.05$), but not significantly different from the control sheep ($P>0.05$). The data on body weight loss are presented in Table 1.

Table 1 shows that P2 sheep experienced improvements in mitigating body weight loss, which is confirmed by data showing no significant difference from control sheep ($P>0.05$) without transportation. The transportation process can cause stress in sheep, which in turn can lead to body weight loss. The level of body weight loss varies depending on the duration of transportation and other factors. Body weight loss can also be influenced by several factors, including body heat expenditure through sweating, respiration, urination, and defecation, which sheep perform to maintain homeostasis (Xu et al. 2023). The supplementation of MHA and dextrose improved the condition of transported sheep by reducing body weight loss due to transportation., and this demonstrates that the combined supplementation of MHA and dextrose has a better effect than separate supplementation, as observed in previous studies (Baihaqi et al. 2022). MHA can prevent oxidative stress and plays a role in lipid metabolism by activating enzymes related to antioxidants (Elango 2020). Furthermore, methionine mitigates the negative impacts of heat stress on protein degradation by upregulating genes associated with protein synthesis and downregulating those involved in protein breakdown, thereby enhancing protein retention (Erfani et al. 2021). Methionine is an essential amino acid crucial for protein synthesis and methylation processes and as a precursor for cysteine synthesis (Martínez et al. 2017). However, methionine is often degraded by rumen microorganisms,

reducing its availability for absorption in the small intestine. MHA, a more stable form of methionine, resists microbial breakdown in the rumen, ensuring a higher bioavailability of methionine for the animal (Solis-Cruz et al. 2022). This stability is particularly beneficial during transportation when sheep experience reduced feed intake and increased metabolic demands. Meanwhile, dextrose is a glucose monosaccharide that can be an energy source during transportation. Supplementing with dextrose ensures a readily available energy source, helping to maintain metabolic functions and prevent energy deficits that can lead to weight loss and weakened body condition. Therefore, combining MHA and dextrose can reduce stress and minimize weight loss during sheep transportation.

Transportation can cause body weight loss in livestock. The recovery period is the period needed for sheep to regain their initial body weight before transportation. Table 1 shows that sheep in the P2 treatment had a faster recovery time to their initial body weight than P1 sheep ($P<0.05$); this may be due to the higher daily weight gain in sheep in the P2 treatment compared to those in the P1 group. Methionine is an essential amino acid animals require for average growth and health. Animals must obtain methionine through their diet as they cannot synthesize this substance in their bodies. Methionine is one of the limiting amino acids for protein synthesis in growing ruminants (Wu et al. 2022). Protein synthesis in ruminant livestock produces peptides, amino acids, and ammonia (NH3). Protein is essential for animals, and a deficiency in amino acids can prevent protein synthesis, causing other amino acids to be converted into energy substrates or fats. The daily weight gain in sheep receiving MHA treatment is higher because damaged body tissues from transportation can be repaired more quickly. Therefore, the increased methionine amino acid in MHA sheep contributes to daily weight gain in sheep. Another factor is the administration of dextrose, a source of energy that livestock needs. Dextrose supplementation indicates that the energy stored by livestock is sufficient to support body weight gain. Energy sources such as rumen-protected sugar can alter the microbial diversity in the rumen, influencing both the fermentation pattern and microbial metabolism (Wang et al. 2021). The improvements observed with MHA and dextrose administration indicate that the welfare status of the sheep improves compared to those without additive administration.

Physiological responses of sheep

Physiological responses are indicators of stress and animal welfare in livestock. These can be measured through respiratory rate, heart rate, and rectal temperature. Physiological responses are reactions of livestock to various environmental changes. The physiological responses of the sheep in this study are shown in Table 2.

Table 2 shows that the P2 treatment resulted in significantly lower respiratory rates and heart rates compared to P1 ($P<0.05$) and was not significantly different from the control treatment ($P>0.05$). Transportation can induce stress in sheep, as evidenced by elevated heart and respiratory rates. The response is

Table 1. Body weight loss and recovery period of transported sheep among treatments

Variable	Control	P1	P2
Initial body weight (kg)	21.09±1.6^{a}	22.43±2.1^{a}	22.07±2.3^{a}
Final body weight (kg)	20.45±1.5^{a}	21.02±2.1^{a}	21.28±2.7^{a}
Weight loss (kg)	0.62±0.3^{b}	1.41±0.3^{a}	0.79±0.2^{b}
Weight loss (%)	3.04±1.3^{b}	6.33±1.5^{a}	3.62±0.9^{b}
Recovery time (days)	3.22±1.2^{b}	5.55±1.1^{a}	3.33±1.3^{b}

Values within the same row followed by different letters are significantly different ($P<0.05$). Control= sheep that were not transported and without additives; P1= sheep transported without additives; P2= sheep transported with supplementation of MHA at 0.5 g/kg body weight and dextrose at a dose of 0.5 g/kg body weight

Table 2. Physiological responses of transported sheep among treatments

Variable	Control	P1	P2
Respiration rate (minutes^{-1})	110.91±8.6^{b}	130.31±6.1^{a}	113.07±4.3^{b}
Heart Rate (minutes^{-1})	53.51±2.5 ^{b}	61.24±2.3^{a}	54.52±2.1^{b}
Rectal temperature (^{0}C)	39.40±0.10^{a}	39.51±3.34^{a}	39.70±2.25^{a}

Values within the same row followed by different letters are significantly different ($P<0.05$). Control= sheep that were not transported and without additives; P1= sheep transported without additives; P2= sheep transported with supplementation of MHA at a dose of 0.5 g/kg body weight and dextrose at a dose of 0.5 g/kg body weight

attributed to heat stress and the animals' attempts to dissipate body heat via respiration and blood circulation. Several studies have also documented increased heart and respiratory rates due to transportation (Navarro et al. 2020; Zhang et al. 2020). Supplementation of protected methionine in ruminants can reduce heat stress and improve the efficiency of thermoregulation (Davidson et al. 2024); this suggests that administering MHA and dextrose contributes to enhancing the welfare status of transported sheep.

Blood profile and metabolites of sheep

The hematological blood profile of the treated sheep can be seen in Table 3. Table 3 shows significant differences in neutrophil, lymphocyte profiles, and the neutrophil-to-lymphocyte (N/L) ratio ($P<0.05$). The N/L ratio is considered an indicator of stress levels in livestock (Carbillet et al. 2019). This study reveals significant differences between the control and treatment groups (P1 and P2). Transported sheep showed a significant increase in neutrophils and a decrease in lymphocytes, leading to a higher N/L ratio. These findings are consistent with the study by Marcato et al. (2021), which reported that transported calves experienced an increased proportion of neutrophils and a decreased proportion of lymphocytes in the blood.

The increase in neutrophils occurs because animals release cortisol in response to stress, triggering neutrophilia and increasing the number of neutrophils in the blood. The next step is lymphopenia, a decrease in blood lymphocytes, leading to an increased N/L ratio in animals experiencing transportation stress (Pascual-Alonso et al. 2017). In sheep receiving dextrose and methionine, an improvement in the N/L ratio was observed compared to the untreated group, indicating the positive impact of these treatments on transported sheep, with N/L values not significantly different from the control group (Table 3).

Blood metabolites of sheep

Blood metabolites, including glucose, creatinine, total protein, and cortisol hormone levels, are animal welfare indicators. This study's glucose and cortisol hormone profiles showed significant differences ($P<0.05$). The blood metabolite profiles are presented in Table 4. Table 4 shows that transported sheep receiving additives had significantly lower glucose and cortisol hormone profiles compared to sheep that did not receive additives ($P<0.05$). Sheep in P2 treatment also did not significantly differ from the control group's glucose or cortisol hormone levels ($P>0.05$), indicating improvement in sheep welfare status in P2. An increase in cortisol levels indicates stress in livestock. The increase in cortisol in P1 suggests that these sheep experienced more stress than the control and P2 groups, in line with the findings of Pascual-Alonso et al. (2017), which reported a doubling of blood cortisol levels in transported sheep. Stress in sheep leads to increased cortisol levels. Cortisol stimulates gluconeogenesis, the breakdown of glycogen in body tissues into glucose needed for energy, which can result in elevated blood glucose levels, as observed in this study. This observation is supported by Xiao et al. (2024), who also found that increased glucose levels were associated with elevated cortisol hormone levels.

Behavior of sheep

Behavior is a key indicator of animal welfare. Animals with good welfare status will express normal behavior. In this study, sheep behavior was observed for five hours after transportation. The behavioral data are presented in Table 5.

Table 5 indicates that sheep receiving the MHA and dextrose supplement exhibited a higher percentage of standing behavior compared to the control ($P<0.05$), which suggests that the MHA and dextrose supplementation

Table 3. Haematological blood profiles of transported sheep among treatments

Variables	Control	P1	P2
Haematocrit (%)	32.96±2.7^{a}	34.98±5.4^{a}	33.46±5.6^{a}
Erythrocytes (10^5/dL)	10.22±0.1^{a}	10.75±1.5^{a}	9.84±2.0^{a}
Haemoglobin (g/dL)	8.98±0.7^{a}	9.41±1.1^{a}	8.92±1.4^{a}
Leukocytes (10^3/dL)	21.02±6.5^{a}	29.01±12.1^{a}	26.17±6.4^{a}
Neutrophils (%)	63.66±8.7^{a}	77.55±9.4^{b}	72.11±8.2^{ab}
Lymphocytes (%)	33.72±4.1^{b}	22.00±3.2^{a}	27.44±7.5^{ab}
N/L Ratio	2.08±0.8^{a}	4.42±1.5^{b}	3.30±1.4^{ab}

Values within the same row followed by different letters are significantly different ($P<0.05$). Control= sheep that were not transported and without additives; P1= sheep transported without additives; P2= sheep transported and supplemented with MHA at 0.5 g/kg body weight and dextrose at a dose of 0.5 g/kg body weight dextrose

Table 4. Blood metabolites profiles of transported sheep among treatments

Variable	Control	P1	P2
Glucose (mg/dl)	85.93±17.02[b]	119.30±26.34[a]	87.21±8.27[b]
Creatinine (mg/dl)	2.43±0.91[a]	1.60±0.87[a]	1.68±0.05[a]
Total protein (g/dl)	6.80±0.72[a]	6.13±1.19[a]	6.23±1.04[a]
Cortisol Hormone (ng/mL)	5.27±3.70[b]	16.78±3.67[a]	7.13±2.19[b]

Values within the same row followed by different letters are significantly different (P<0.05). Control= sheep that were not transported without additives; P1= sheep transported without additives supplementation; P2= sheep transported supplemented with MHA at 0.5 g/kg body weight and dextrose at a dose of 0.5 g/kg body weight

Table 5. Percentage of sheep behaviour during five hours after transportation among treatments

Behavior	Control	P1	P2
Eating	22.21±2.4[a]	16.92±4.3[a]	24.52±1.7[a]
Drinking	3.41±3.1[a]	6.07±2.6[a]	5.27±1.3[a]
Standing	39.17±2.5[a]	29.4±3.1[b]	40.02±4.7[a]
Walking	13.04±2.2[a]	7.92±1.1[b]	9.17±2.1[ab]
Resting	22.16±4.6[b]	39.68±5.3[a]	21.02±2.3[b]

Values within the same row followed by different letters are significantly different (P<0.05). Control= sheep that were not transported and without additives; P1= sheep transported without additives; P2= sheep transported with supplementation of MHA at 0.5 g/kg body weight and dextrose at a dose of 0.5 g/kg body weight

supplementation positively affects the energy levels and physical condition of the sheep. Consequently, P2 sheep could stand for longer periods compared to P1 sheep. It is hypothesized that P1 sheep lost more energy and experienced greater stress during transportation, resulting in a shorter standing duration than the control and P2 groups. MHA may act as an antioxidant to prevent transportation stress (Del Vesco et al. 2015), while dextrose provides additional energy to minimize energy loss during transportation. The resting behavior of P1 sheep was significantly higher than the control and P2 groups (P<0.05), indicating that P1 sheep expended more energy during transportation and required more rest afterward. Transportation stress can induce stress in sheep, and stressed sheep require longer rest periods to recover their energy. Resting behavior serves to conserve energy used by the animal (Pascual-Alonso et al. 2017). Post-transportation rest effectively lowers cortisol levels and helps animals recover from travel stress (Pascual-Alonso et al. 2017).

CONCLUSION

The supplementation of MHA and dextrose to sheep transported for 8 hours effectively reduced body weight loss, shortened recovery time, decreased respiratory and heart rates, and lowered blood cortisol and glucose levels. Furthermore, the sheep with these supplements exhibited better behavior than those without additives. These findings indicate that sheep receiving MHA and dextrose supplementation before transportation have a better welfare status than those without additives.

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Biofilm Profile of Coagulase Negative *Staphylococci* Bacteria from Milk Isolate of Dairy Cows with Subclinical Mastitis

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ABSTRAK

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Staphylococcus sp. merupakan bakteri patogen penyebab mastitis subklinis. Bakteri ini terbagi menjadi kelompok bakteri *coagulase negative Staphylococci* (CoNS) dan kelompok bakteri *coagulase positive Staphylococci* (CoPS). Bakteri CoNS merupakan kelompok flora normal pada kulit manusia dan hewan, walaupun demikian beberapa penelitian telah membuktikan bahwa bakteri CoNS merupakan mikroorganisme yang paling banyak diisolasi dari susu sapi perah penderita mastitis subklinis. Kemampuan untuk membentuk biofilm merupakan faktor virulensi yang penting bagi bakteri CoNS. Deteksi pembentukan biofilm dilakukan pada 54 sampel bakteri CoNS berupa Bahan Biologi Tersimpan (BBT), yang diisolasi dari susu sapi perah penderita mastitis subklinis dengan hasil uji *California mastitis test* (CMT) positif 2 (++). Deteksi pembentukan biofilm dilakukan secara kualitatif dengan metode *Congo red agar* (CRA) dan *test tube* (TT). Hasil konfirmasi secara fenotipe, menunjukan bahwa 54 isolat (100%) merupakan bakteri CoNS. Hasil deteksi pembentukan biofilm menunjukkan hasil bahwa 51 dari 54 isolat (94,44%) positif membentuk biofilm. Sehingga, dapat disimpulkan bahwa bakteri CoNS memiliki kemampuan membentuk biofilm sebagai salah satu bentuk perlindungan diri dan faktor virulensi.

Kata Kunci: *Coagulase-negative Staphylococci*, Faktor virulensi, Mastisis Subklinis

ABSTRACT

Nadissa ARH, Sarasati W, Adi IC. 2024. Biofilm profile of coagulase-negative *Staphylococci* bacteria from milk isolates dairy cows with subclinical mastitis. JITV 29(3):151-160. DOI:htpps://dx.doi.org/10.14334/jitv.v29i3.3363.

Staphylococcus sp. is a pathogenic bacteria that causes subclinical mastitis. These bacteria are divided into coagulase-negative *Staphylococci* (CoNS) and coagulase-positive Staphylococci (CoPS). CoNS bacteria are a group of normal flora on human and animal skin. However, several studies have proven that CoNS bacteria are the most commonly isolated microorganisms from the milk of dairy cows with subclinical mastitis. The ability to form biofilms is an important virulence factor for CoNS bacteria. Detection of biofilm formation was carried out on 54 samples of CoNS bacteria in the form of Stored Biological Material (BBT), which were isolated from the milk of dairy cows with subclinical mastitis with positive California mastitis test (CMT) 2 (++). Detection of biofilm formation was performed qualitatively by Congo red agar (CRA) and test tube (TT) methods. Phenotypic confirmation results showed that 54 isolates (100%) were CoNS bacteria. Biofilm formation detection results showed that 51 out of 54 isolates (94.44%) were positive for biofilm formation. Thus, it can be concluded that CoNS bacteria have the ability to form biofilms as a form of self-protection and virulence factor.

Key Words: Biofilm, Coagulase-negative Staphylococci., Subclinical Mastitis, Virulence Factor

INTRODUCTION

Mastitis is one of the causes of decreased milk production in dairy cows (Panjuni et al. 2021). Clinical manifestations of mastitis are divided into clinical mastitis and subclinical mastitis (Suwito et al. 2021). Subclinical mastitis in Indonesia reaches 97-98%, while clinical mastitis only reaches 2-3% (Nisa et al. 2019). This physical clinical symptom does not appear, causing dairy cows with subclinical mastitis to become a reservoir that infects other dairy cows (Pribadi et al. 2020).

Staphylococcus sp. is the most common pathogenic bacteria found in cases of subclinical mastitis (Suwito et al. 2021). These bacteria are divided into two groups based on their ability to produce coagulase enzymes: coagulase-negative *staphylococci* (CoNS) and coagulase-positive *staphylococci* (CoPS). Identification of pathogenic microorganisms for the incidence of subclinical mastitis in dairy cattle is still concentrated in CoPS bacteria, such as Staphylococcus aureus, rather than CoNS bacteria, such as *Staphylococcus epidermidis* (Windria et al. 2016). CoNS bacteria are normal flora of the skin. This emerging bacterial pathogen causes

mastitis in dairy cows in countries such as Germany, Africa, Iran, and Egypt (Hosseinzadeh and Dastmalchi Saei 2014).

Mastitis infection caused by CoNS bacteria is subclinical but persistent (Cheng and Han 2020). CoNS bacteria can produce biofilms, an important factor in their pathogenicity (Goetz et al. 2017). The presence of biofilms causes bacteria to become more persistent on milking tools and hands, which are portals of entry for bacterial infections (El-Jakee et al. 2013). The formation of biofilms also causes an increase in the resistance and adaptation of CoNS bacteria to the environment in the animals' udders, so it becomes a problem in the treatment and prevention of subclinical mastitis in dairy cows (De Buck et al. 2021).

Biofilm is a collection of multicellular microorganisms in a matrix of extracellular polymeric substances. The existence of biofilms causes bacteria to be more able to attach to the surfaces of biotic and abiotic objects, as well as thickening the protective layer of bacteria (França et al. 2021). Bacteria in the biofilm can carry out synergism or positive interactions between bacteria, thus causing an increase in the pathogenicity of these bacteria (Espiritu and Villanueva 2022). This condition can lead to failure to treat subclinical mastitis in dairy cows, increasing antibiotic resistance and threatening animals and humans.

The ability to form bacterial biofilms can be detected quantitatively or qualitatively. The Congo red agar (CRA) method and the test tube method (TT) are qualitative test methods that can be used to detect the ability to form biofilms of CoNS bacteria. The CRA and TT methods have the advantage of fast processing time and easy result analysis because they are only based on visual changes in the media (Furtuna et al. 2018).

Information and research on CoNS bacteria related to the incidence of subclinical mastitis in dairy cattle in Indonesia is still very limited. CoNS bacteria have virulence factors that cause subclinical mastitis in dairy cows, namely their ability to form biofilms that need to be studied. This study was conducted as a test to detect the ability of biofilm formation as a virulence factor from CoNS bacteria from milk isolates of dairy cows with subclinical mastitis.

MATERIALS AND METHODS

Sample

The research sample used was stored biological material of 54 samples isolate bacteria, which had been isolated from milk samples of dairy cows with subclinical mastitis with positive California mastitis test (CMT) results of 2 (++). The bacterial isolates used as samples were stored in STGG media (skim milk, tryptone, glucose, glycerol) and 30% glycerol.

Method

Confirmation of Stored Isolates by Phenotype

A bacterial culture was carried out on blood agar plate (BAP) media to obtain CoNS bacterial colonies. This procedure was carried out by taking 1 dose of inoculum from the sample and then inoculating it on BAP media. The agar plate was then incubated at 37°C for 24 hours. Three types of hemolysis zones can form on BAP media, namely β-hemolysis (clear zone), which is formed due to complete lysis of erythrocytes, α-hemolysis (brownish green zone), which is formed due to bacteria reducing erythrocyte hemoglobin to methemoglobin, and γ-hemolysis. Or non-hemolytic (Almwafy et al. 2020).

Gram staining was performed to see the morphology of the bacteria. The smear preparation for Gram staining was made on an object glass by taking 1 dose of inoculum from BAP media and fixing it. The smear preparation was dripped with crystal violet for 2 minutes and then rinsed with running water. The smear preparation is then dripped with Lugol's solution, left for 30 seconds, and rinsed with running water. The next step is to fade the dye on the smear using 96% alcohol, then rinse it with running water. The smear preparation was stained again by dripping safranin dye, left for 2 minutes, rinsed with running water, and dried. Observations were made under a microscope using an objective lens magnification of 100x using immersion. CoNS bacteria are Gram-positive, non-motile, non-spore-forming cocci that form tetrads or in pairs but can also occur individually, in irregular groups (forming grapes), or in short chains composed of three or four cells (Becker et al. 2014).

Bacterial isolation was carried out on the differential media of mannitol salt agar (MSA). The procedure was carried out by taking 1 dose of inoculum from the results of bacterial culture on nutrient agar (NA) media, then inoculating it into MSA media and then incubating it for 24 hours at 37°C. CoNS bacteria cannot ferment mannitol, so the bacteria will grow without causing a color change (from red to yellow) in the media (Ryman et al. 2021).

The catalase test was performed to differentiate the genera *Staphylococcus* sp. and *Streptococcus* sp. The test will be carried out by dripping liquid hydrogen peroxide (H_2O_2) on an object glass, and mixed it with the bacteria from the BAP media. A positive result is indicated by the presence of gas bubbles (O_2) produced by *Staphylococcus* sp. because it breaks down hydrogen peroxide (Hayati et al. 2019).

A coagulase test was performed to detect the presence of coagulase enzymes. This test procedure was carried out by taking bacterial isolates with loops from BAP media, putting them into 1 ml of nutrient broth in a tube, then incubating them for 24 hours at 37°C. 1 ml of rabbit blood plasma is put into the media using a syringe and then incubated for 24 hours. The results of the CoNS bacterial test did not show plasma clots that congealed like a gel in the tube (Becker et al. 2014).

Biofilm detection with the Congo Red Agar (CRA) method

CRA media was prepared with 37 grams/liter of brain heart infusion broth (BHIB), 50 grams/liter of sucrose, 10 grams/liter of agar base, and 0.8 grams/liter of Congo red indicator. Congo red indicator was prepared as a concentrated solution and then autoclaved at 121°C for 15 minutes. Add Congo red indicator into brain heart infusion agar with sucrose stored in a petri dish and autoclaved at 55°C. CRA plate was inoculated with CoNS and then incubated at 37°C for 24 – 72 hours. A positive result in the CRA test method is indicated by the formation of black-pigmented colonies with a rough consistency and black zones around the colonies. In contrast, a negative result is indicated by the formation of reddish-pigmented colonies with a smooth consistency (Furtuna et al. 2018).

Biofilm detection with the Test Tube (TT) method

The bacteria were inoculated into a tube containing 10 ml of Tryptone Soya Broth and 1% glucose, then incubated for 24 – 30 hours at 37°C. The tube is washed with phosphate-buffered saline (pH 7.3) and dried. The dry tube was then fixed with Bunsen and stained with 0.1% crystal violet for 10 minutes. Wash the tube with distilled water, then dry it by storing the tube upside down at room temperature. Observations were made by observing whether or not a blue layer was formed on the bottom and wall of the tube. If a ring was found at the boundary of the liquid, it was ignored because it is not an indicator of biofilm formation (Benachinmardi et al. 2017). Interpretation of the results in this study was not carried out by scoring or using a nephelometer, only visual observations were made, and the results were divided into positive and negative. The formation of a blue coating on the walls and bottom of the tube indicates that the bacteria form biofilms.

Data analysis

The ability to form biofilms of CoNS bacteria was detected using qualitative methods, CRA and TT, after confirming stored isolates to determine the test sample. The data obtained is the change in color of the test medium (CRA or TSB) after inoculating the bacterial sample. The results of the study were analyzed descriptively, then presented in a tabular form containing the positive and negative results of the tests, conclusions, and the percentage of CoNS bacteria from milk isolates of dairy cows with subclinical mastitis which can form biofilms. CoNS bacteria are considered to have the ability to form biofilms if the results of one or both tests are positive. The data obtained was then calculated using Microsoft Excel and the Cohen's Kappa value was determined for its reliability value.

RESULTS AND DISCUSSION

Phenotypic test

In this study, the microscopic images obtained from isolates of CoNS bacteria were coccus-shaped bacterial colonies grouped irregularly, non-motile, and purple (Table 1). This result is similar to that of Ahmadunissah et al. (2021), which stated that *Staphylococcus* sp. belongs to the group of Gram-positive bacteria. The grouping of bacterial types is based on the outermost layer of the bacterial wall, namely lipopolysaccharide, which only Gram-negative bacteria have, as well as the thickness of the peptidoglycan layer (Panawala 2017). The cell wall of Gram-positive bacteria has a thicker peptidoglycan layer than Gram-negative bacteria. It contains teichoic acid (TA), an anionic polymer of polyol phosphate repeating units (Kho and Meredith 2018). Bacterial colonies turn purple because alcohol causes the cell walls to dehydrate and their pores to shrink, causing the crystal violet and lugol complexes to bind to the peptidoglycan layer on the bacterial cell wall (Thairu et al. 2014).

This study showed that samples of the CoNS bacterial isolates reacted positively to produce the catalase enzyme, which was indicated by the formation of bubbles (Table 1). These results are similar to research by Kartini (2020), which stated that Staphylococcus sp. can produce catalase enzymes so that it can break down hydrogen peroxide (H_2O_2) into water (H_2O) and gas (O_2). Catalase is an antioxidant enzyme that plays a role in the defense of bacteria against oxidative stress by catalyzing the decomposition of H_2O_2(Yuan et al. 2021). The catalase enzyme in milk can come from bacteria or mammary glands (Eslami et al., 2015). Increased activation of the catalase enzyme will go hand in hand with an increase in the number of somatic cells in milk, which indicates mastitis (Zeinhom et al. 2013).

The coagulase test results in this study showed that 54 isolates (100%) had a negative reaction (Table 1). The coagulase enzyme protects bacteria from host immune cells by binding and activating prothrombin, forming a pseudo capsule of fibrin as a protective barrier for

Table 1. Phenotypic test results of bacterial isolates from milk of dairy cattle suffering from subclinical mastitis

Test	Results	Sample (n)	Percentages (%)
	β	33	61.11
Hemolysis Type	α	0	0
	γ	21	38,89
Gram Staining	Coccus +	54	100
	Coccus -	0	0
Mannitol Salt Agar	+	46	85.19
	-	8	14.81
Catalase	+	54	100
	-	0	0
Coagulase	+	0	0
	-	54	100
Total		54	100

α= Alpha, β= Beta,γ= Gamma, Coccus += Coccus Gram Positive, Coccus -= Coccus Gram Negative, += Positive, - = Negative

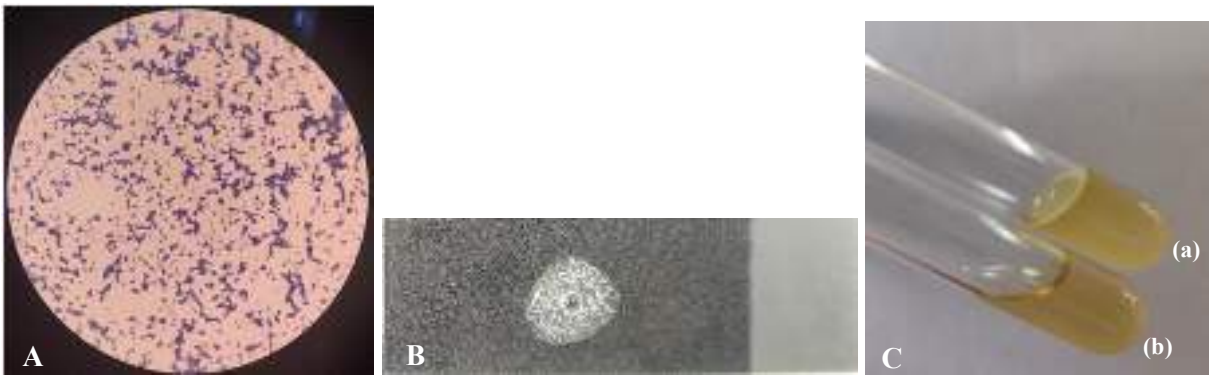

Figure 1. Phenotypic test results for coagulase negative Staphylococci (CoNS) bacteria **(A)** Gram Staining Results with 100x magnification, (B) Catalase Test Results, (C) Coagulase Test Results (a) Positive, (b) Negative (Personal Documentation)

bacteria (Tam & Torres 2019). CoNS bacteria do not produce this enzyme, so they are considered less pathogenic than CoPS bacteria (Roman et al. 2023). The results of this study showed that 21 of 54 samples (38.89%) were γ-hemolytic (Figure 2A), while 33 of 54 samples (61.11%) were β-hemolytic (figure 4b). 18 out of 21 isolates (85.71%) that were γ-hemolytic and 28 out of 33 isolates (84.85%) that were β-hemolytic could ferment mannitol (figure 5a). Three isolates that were γ-hemolytic (14.29%) and 5 isolates (15.15%) that were β-hemolytic could not ferment mannitol (Figure 3B).

This study's results differ from Kim et al. (2019) statement that CoNS bacteria are non-hemolytic, so they do not form a hemolysis zone on BAP. Research Organji et al. (2018) stated that the CoNS bacterial species could not ferment mannitol, contradicting this study's results. According to Vanderhaeghen et al. (2014) and Nocera et al. (2021), the ability to hemolyze and ferment mannitol possessed by CoNS bacteria will differ for each species.

Culture results on BAP and MSA media from 54 isolates showed results that led to several species of CoNS bacteria. 3 isolates (5.56%) were γ-hemolytic and could not ferment mannitol, leading to *Staphylococcus epidermidis*, while 18 isolates (33.33%) were γ-hemolytic and fermented mannitol, leading to *Staphylococcus sciuri, Staphylococcus equorum,* and *Staphylococcus hominis*. 5 isolates (9.26%) were β-hemolytic and could not ferment mannitol, leading to *Staphylococcus schleiferi*, while 28 isolates (51.85%) were β-hemolytic and fermented mannitol, leading to *Staphylococcus aureus, Staphylococcus haemolyticus, Staphylococcus saprophyticus* and *Staphylococcus xylosus* (Tabel 2).

The formation of a hemolysis zone by bacteria occurs because bacteria produce exoproteins, namely hemolysin, a toxin (Nasaj et al. 2020). Bacteria produce hemolysin as a form of self-defense from the host's immune system and release iron from red blood cells, which is needed as a source of energy for growth (Divyakolu et al. 2019). Research Heo et al. (2020), stated that the production of hemolysin in CoNS bacteria will differ in each strain of its species due to various

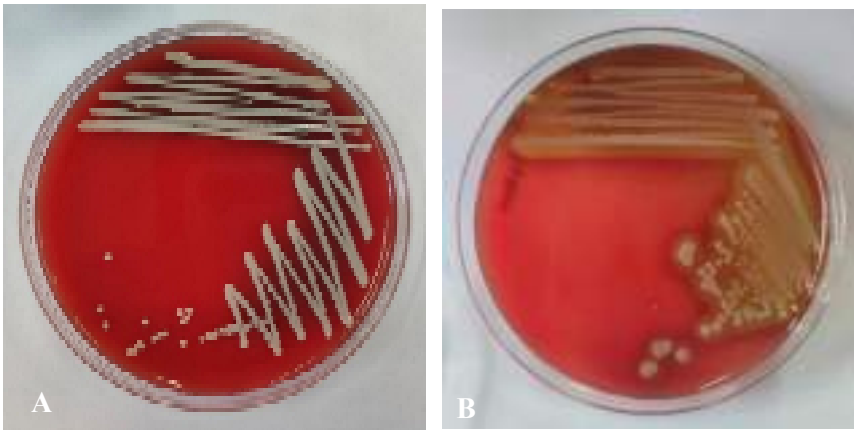

Figure 2. Coagulase-negative *Staphylococci* (CoNS) Colonies on Blood Agar Plate (BAP) Media. (A) γ-hemolysis colonies, (B) β-hemolysis colonies. (Personal Documentation).

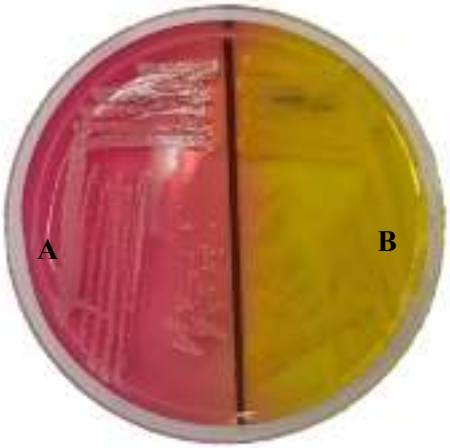

Figure 3. Coagulase-negative *Staphylococci* (CoNS) Colonies on Mannitol Salt Agar Plate (MSA) Media. (A) negative mannitol fermentation, (B) positive mannitol fermentation. (Personal Documentation)

Table 2. Species of coagulase-negative *Staphylococci* (CoNS) bacteria based on the results of hemolysis and mannitol fermentation zones from stored isolates from milk of dairy cattle suffering from subclinical mastitis

Results	Number	Suspected CoNS Bacteria
γ-hemolysis and MSA (-)	3	*Staphylococcus epidermidis* (Pinheiro et al. 2015)
γ-hemolysis and MSA (+)	18	*Staphylococcus sciuri* (Beims et al. 2016; Cirkovic et al. 2017) *Staphylococcus equorum* (Thakur et al. 2017) *Staphylococcus hominis* (Thakur et al. 2017)
β-hemolysis and MSA (-)	5	*Staphylococcus schleiferi* (Yarbrough et al., 2018)
β-hemolysis and MSA (+)	28	*Staphylococcus aureus* (Thakur et al. 2017) *Staphylococcus haemolyticus* (Pinheiro et al. 2015) *Staphylococcus saprophyticus* (Ayeni et al. 2017) *Staphylococcus xylosus* (Jeong et al. 2016)

β= Betta, γ= Gamma, MSA= Mannitol Salt Agar

hemolysin genes, namely hld, hlg, hlb, and hla. According to Azih & Enabulele (2013), the *Staphylococcus haemolyticus* bacterial strain is a CoNS bacterium that is predominant in producing hemolysin, while the CoNS bacterial strain that tends not to have the ability to hemolyze is *Staphylococcus epidermidis*.

The ability to produce hemolysin is one of the virulence factors of bacteria (Motamedi et al. 2018). Hemolysin plays an important role in infectious processes caused by bacteria because this toxin has a cytotoxic effect and can cause lysis in eukaryotic cells (Pakshir et al. 2017). Hemolysin can cause tissue damage and activation of the inflammatory response in the host (Zhao et al. 2020). Mannitol salt agar (MSA) is a differential media for bacteria (Boipai et al. 2020). This media contains about 7.5 – 10% salt (NaCl), so only bacteria with a high salt tolerance can grow, such as *Staphylococcus* sp. (Urip et al. 2022). Positive results on MSA media were obtained because bacteria fermented mannitol into organic acids, thus changing the pH indicator, namely phenol red in the media, to bright yellow (Rahmi et al. 2015). Bacteria's ability to ferment mannitol is regulated by the enzyme mannitol-1-phosphate dehydrogenase (M1PDH) (Virgianti et al. 2019).

CoNS bacteria are normal microflora of mammals' skin and mucous membranes (Thilakavathy et al. 2015). In dairy cows, this bacteria is commonly found at the tip of the teats, with the predominant species including *Staphylococcus haemolyticus* and *Staphylococcus equorum* (De Visscher et al. 2016). *Staphylococcus epidermidis* and *Staphylococcus aureus* are normal bacterial species on human skin, but in dairy cows, these bacteria are among the most common pathogenic bacteria isolated on the skin of the teat area (Chotigarpa et al. 2018; Brown and Horswill 2020). *Staphylococcus hominis* is another CoNS bacterium that belongs to the normal microflora of human skin (Azimi et al. 2020).

Infection by CoNS bacteria in cases of subclinical mastitis in dairy cows can occur during the milking process. Bacterial colonies on the skin can invade the mammary glands during this process (Chotigarpa et al. 2018). Bacteria can come from the milking hands or the equipment used because bacteria can form biofilms and tick and survive on the hands and equipment (El-Jakee et al. 2013). Decreased host resistance can also trigger an imbalance in the normal microflora in the body, which can lead to infection (Purwanti et al. 2018).

Detection of biofilm formation

Total of 49 out of 54 isolates (90.74%) showed positive results to form biofilms, while 3 isolates (5.56%) showed negative results when tested with the CRA and TT methods. 5 out of 54 isolates (9.26%) showed negative results when tested with the CRA method, but 49 isolates (90.74%) showed positive results with the same test method. More positive results were obtained with the TT test method, namely 51 isolates (94.44%), with fewer negative results, namely 3 isolates (5.56%) (Tabel 3). Reliability or The Cohen's Kappa coefficient (κ) value between the CRA and TT methods obtained in this study was 0.732, so it can be said that both methods are reliable.

The results in CRA test method in this study has almost the same results as the study of Gurler et al. (2022) and Abed et al. (2022). Differences in the percentage of positive results can be caused by differences in temperature or incubation time and the species of CoNS bacteria detected.Optimal positive results were obtained after incubation for 72 hours at 37 °C, according to the study of Cho et al. (2022). During incubation, the pH of the media decreased due to polysaccharides produced by bacterial metabolism and the degradation of sucrose. The polysaccharide then reacts with the Congo red indicator in the media, causing the pigmented bacterial colonies to turn black (Figure 4A) (HRV et al. 2016). The sucrose content in the media affects the production of EPS (extracellular polymeric substances) in bacteria. When the sucrose concentration is low, the diffusion of black pigment from the colonies will also be reduced (Normanita 2020).

The detection of biofilm formation using the TT method showed positive results in 51 of 54 samples (94.44%). This study has almost the same results as Raksha et al. (2019) and Schönborn et al. (2017). The difference in the percentage of positive results could be due to differences in temperature, incubation time, and the species of CoNS bacteria detected. Optimal positive results were obtained after incubation for 30 hours, according to research by Kusumaningrum et al. (2020). The biofilm formed by bacteria will adhere (adhesion) to

Table 3. Concordance between CRA and TT methods in detecting biofilm formation capability of coagulase negative *Staphylococci* (CoNS) isolate milk of dairy cattle suffering from subclinical mastitis

Method		*Congo Red Agar*		Total
		Positive	Negative	
Test Tube	Positive	49	2	51
	Negative	0	3	3
Total		49	5	54

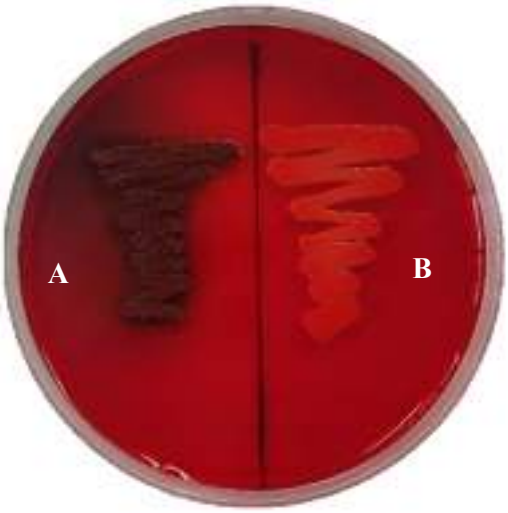

Figure 4. Coagulase-negative *Staphylococci* (CoNS) Colonies on Congo Red Agar (CRA). (A) positive biofilm, (B) negative biofilm. (Personal Documentation)

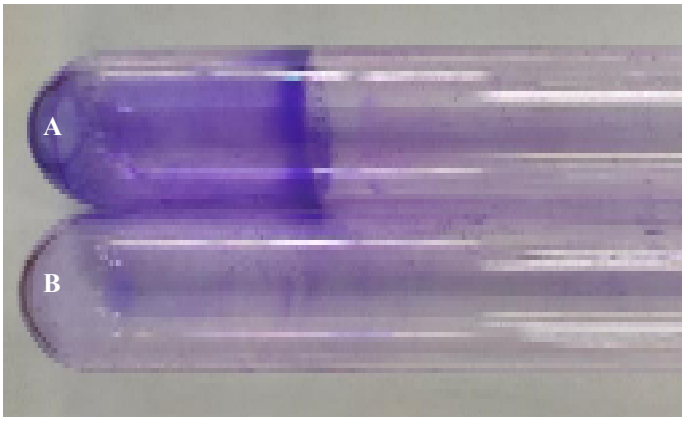

Figure 5. Biofilm Detection Test Results on Coagulase-negative *Staphylococci* (CoNS) Bacteria with the *Test Tube* (TT) Method. (A) positive biofilm, (B) negative biofilm. (Personal Documentation)

the tube wall so that when stained with crystal violet (Figure 5A), a ring will form at the bottom of the tube, and a bluish-purple inner layer of the tube (Tariq et al. 2021). This method uses Crystal violet as a dye because it can bind to negatively charged molecules to color the bacteria and the resulting matrix (Amador et al. 2021).

CoNS bacteria are commensal bacteria on human and animal skin. They are considered less pathogenic because they do not have virulence factors like CoPS bacteria and are rare in clinical pathology discussions (Argemi et al. 2019). The ability to form biofilms is an important virulence factor for CoNS bacteria, and this ability makes bacteria attach to abiotic and biotic layers and form defenses from host phagocytosis, chemotaxis, and antimicrobial agents (Shrestha et al. 2017; Manandhar et al. 2021). PIA regulates bacterial biofilm formation polysaccharide intercellular adhesin) encoded by the icaABCD (intercellular adhesion ABCD) gene (Kord et al. 2018). Biofilms that have formed are difficult to destroy, so treatment requires high doses of antibiotics or removing the infected part (Di Somma et al. 2020).

CONCLUSION

Detection of the profile of biofilm formation in CoNS bacteria derived from milk from subclinical mastitis dairy cows with a positive CMT value of 2 (++) using CRA, and TT on isolates showed that 51 out of 54 isolates (94.44%) were positive for forming biofilms.

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Effect of Extension Program on Improving Farmers' Knowledge in the Narrowing Coastal Area of Segara Anakan Lagoon, Indonesia

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ABSTRAK

Sulastri E, Triatmojo A, A'yun AQ, Tatipikalawan JM. 2024. Pengaruh program penyuluhan terhadap peningkatan pengetahuan peternak di wilayah pesisir penyempitan Laguna Segara Anakan, Indonesia. JITV 29(3):161-171. DOI:http://dx/doi .org/10.14334/jitv.v29.i3.3391.

Sedimentasi Laguna Segara Anakan menyebabkan banyak masyarakat di Kampung Laut meninggalkan profesi nelayan dan memulai profesi lain untuk menyambung hidup. Beternak domba menjadi alternatif profesi baru sebagai sumber pendapatan keluarga. Program penyuluhan merupakan salah satu upaya untuk membantu masyarakat beradaptasi dan meningkatkan pengetahuan mereka terhadap profesi baru tersebut. Penelitian ini bertujuan untuk menganalisis pengaruh penyuluhan terhadap peningkatan pengetahuan peternak tentang praktek pengawetan hijauan dan pencegahan penyakit scabies. Wawancara dilakukan pada 215 peternak untuk mengetahui peningkatan pengetahuan peternak yang dihitung menggunakan skor saat melakukan pretest sebelum dan post-test setelah program penyuluhan dilakukan. Uji T dilakukan untuk menganalisis perbedaan tingkat pengetahuan peternak sebelum dan sesudah kegiatan penyuluhan. Faktor yang mempengaruhi peningkatan pengatahuan diuji dengan analisis regresi, dimana variabel independent meliputi karakteristik peternak. Hasil penelitian menunjukkan bahwa sebelum dilakukan penyuluhan, peternak memiliki tingkat pengetahuan yang rendah terkait pengawetan hijauan dan pencegahan penyakit scabies, dan terdapat kecenderungan bahwa terjadi peningkatan jumlah peternak yang memiliki level pengetahuan tinggi. Peningkatan pengetahuan tentang pencegahan penyakit scabies lebih tinggi dibandingkan pengetahuan tentang pengawetan hijauan ($P\leq0,01$). Tingginya indeks pengetahuan peternak tentang pencegahan penyakit scabies disebabkan karena peternak tidak memiliki pengetahuan sebelumnya terkait penyakit scabies. Pendidikan peternak dan pengalaman beternak domba berpengaruh nyata terhadap pengetahuan peternak tentang pengawetan hijauan ($P\leq0,01$ dan $P\leq0,05$). Penelitian ini juga menemukan bahwa usia peternak berpengaruh terhadap pengetahuan peternak tentang pencegahan penyakit scabies ($P\leq0,10$). Dapat disimpulkan bahwa pengetahuan peternak tentang pengawetan hijauan dan pencegahan penyakit scabies menjadi lebih baik setelah mengikuti program penyuluhan.

Kata Kunci: Penyuluhan, Peternak, Pengawetan Hijauan, Pencegahan Penyakit Scabies

ABSTRACT

Sulastri E, Triatmojo A, A'yun AQ, Tatipikalawan JM. 2024. Effect of extension program on improving farmers' knowledge in the narrowing Segara Anakan Lagoon coastal area in Indonesia. JITV 29(3):161-171. DOI:http://dx/doi.org/10.14334/jitv.v29.i3.3391.

Segara Anakan Lagoon sedimentation has caused many people in Kampung Laut to change their profession from fishery and start other profession to survive. Sheep farming became an alternative to generate the family income. The extension program is one of the efforts to adapt and improve farmers' knowledge regarding sheep farming. This study aimed to analyze the effect of extension on improving the knowledge of farmers. The study instrument were used to measure the knowledge level of farmers was a questionnaire of pretest and post-test. Respondents were ramdomly sampled resulting in 215 farmers who participated in extension program. The differences of the farmers' knowledge level before and after extension program implemented were analized using t-test. Multiple regression analysis were carried out to determine the effect of characteristics on farmers' knowedge. Results showed that before the extension program was running no farmers had sufficient knowledge of forage preservation and scabies prevention. The trend indicated that the number of farmers with a high level of knowledge increased after the extension intervention. Improving knowledge on scabies prevention was higher than forage preservation ($P\leq0.01$). Farmers' education and experience in sheep farming significantly influenced their knowledge on forage preservation ($P\leq0.01$ and $P\leq0.05$). This study also found that farmers' age would affect their knowledge on scabies prevention ($P\leq0.10$). It can be concluded that farmers' knowledge on forage preservation and scabies prevention improved after participating in the extension program.

Key Words: Extension, Farmers, Forage Preservation, Scabies Prevention

INTRODUCTION

The Segara Anakan area is an estuarine lagoon composed of several ecosystems that are tightly interconnected and located in the Central Java Province, Indonesia. The Segara Anakan Lagoon plays a vital role in the productivity of Java south coastal waters. Biological resources inside the lagoon can provide a livelihood for the locals in the form of brackish fishery (Sanjatmiko 2021; Sulastri et al. 2020). Therefore, fishing is the main source of income for the locals who live near the coast. However, this ecosystem is undertreated by continuing sedimentations.

The white areas in Figure 1 indicate land or sedimentation that keeps expanding every year, whereas the grey area represents the water of Segara Anakan Lagoon that keeps decreasing. The reduction of the wet area caused by sedimentation in 38 years, from 1978 to 2016, is 2703.7 ha, and the rate of land formation is 71.15 ha per year (Rose et al. 2016). The sedimentation rate in Segara Anakan Lagoon during the dry season is 0.067 m/s, which increases to 1.61 m/s during the rainy season (Ihsan et al. 2018). The material flowing from the Citanduy river at a rate of 7.4 million tonnes per year allows for an expansion in the sedimentation area (Sari et al. 2016).

Nevertheless, given the evident continuous sedimentation in Segara Anakan Lagoon, many of these locals have been compelled to seek alternative employments outside fishing. Residents should consider additional employment because they can no longer rely on fishing for a living. Sheep farming is a new enterprise that has greatly aided the livelihood of locals (Hegde 2019).

Most of the sheep farming in the study region is conducted by smallholder farmers with limited access to information, experience and economic resources because livestock is a recent activity for them. Hence, farmers need assistance from institutions that are reliable and accessible (Gayatri et al. 2016). Extension could provide a strategy by transferring relevant knowledge and technology to farmers (Masephula & Olorunfemi 2023; Rokhani et al. 2021). It is also a process that provides lessons to farmers. In the context of the learning process, extension aims to change the knowledge, skill and attitude of farmers.

The extension programme should be customised to the problems and needs of the community. In this study, extension programme's issues are determined on the basis of field circumstances, in which sheep's average daily gain is low. A lack of knowledge in animal feed technologies, such as silage and hay production, is found based on observations from focused group discussions. Furthermore, scabies is frequently found in Segara Anakan Lagoon, which is often neglected by farmers because the disease does not cause harm to livestock.

Acceptance of cultural and technological changes on rural farms is essential for greater agricultural productivity. Thus, farmers have no alternative apart from learning and implementing scientific agricultural techniques to substitute their traditional methods (Umeh et al. 2018). Farmers' reluctance to respond favourably to new innovations or technologies might cause delayed developments in agriculture (Etwire et al. 2017).

In introducing innovations, farmers tend to seek the success of a programme rather than the process (Jost et al. 2016). The extension can be provided as a systematic process to help farmers analyse their present and expected future situations, become aware of their problems and increase their knowledge. For example, upon acquiring specific knowledge related to certain problems through extension, farmers can find solutions and consequences and act on possible alternatives (Skaalsveen et al. 2020).

The level of farmers' knowledge on potential hazards of the disease is important in preventing outbreaks (Hundal et al. 2016). The need for training interventions to increase awareness on disease and health risks (Qui et al. 2024). A serious education mobilisation should be initiated immediately in cooperation with various institutions to educate farmers and increase their awareness about the issue (Robertson 2020).

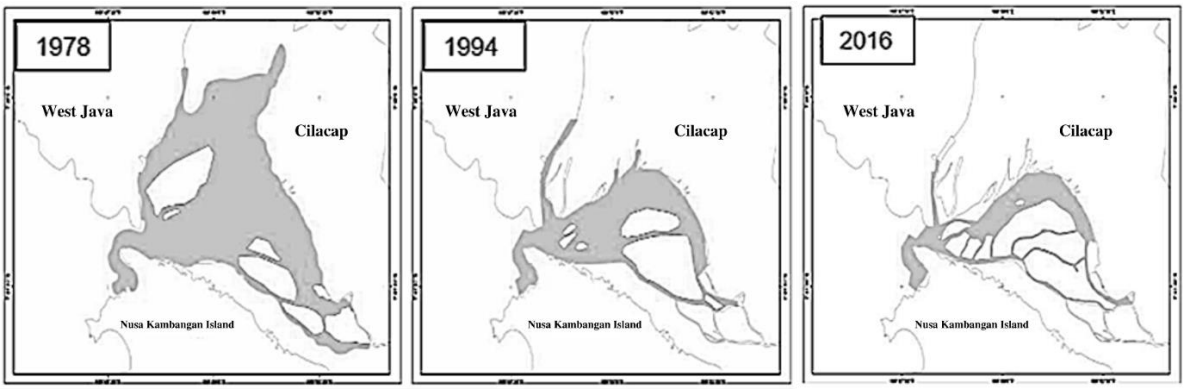

Figure 1. Changes in land profiles of Segara Anakan Lagoon (images processed from multi-temporal land satellite (Rose et al. 2016)

In addition, the level of farmers' knowledge is frequently distinctive, as each individual has distinct characteristics and expectations. The level of knowledge improvement is influenced by farmers' socioeconomic characteristics (Triveni et al. 2018; Xu & Zhang 2021). Hence, understanding how farmers' knowledge levels differ is critical, and the extension may achieve the best possible knowledge improvement by tailoring the content and delivery methods of information on good or modern agricultural practices in a specific area.

In assessing the performance of the extension programme, the improvement and factors that affect the knowledge level about sheep farming must be determined. Knowledge improvement can be determined by conducting pre-tests and post-tests on the extension process (Hosking et al. 2015). However, farmers' characteristics are considered as factors that affect the improvement of their level of knowledge. Hence, this paper discusses the effect of extension on improving the knowledge of farmers on forage preservation and scabies prevention.

MATERIALS AND METHODS

Research design

The study was carried out as descriptive methodology, to describe, explore, records and interprets conditions that exist. The data on farmers' knowledge were collected from before and after extension program conducted. The extension programme was a package of result demonstration and group discussions regarding sheep farming practices on forage preservation and scabies prevention (De Jesus & Buenas 2023).

Selection of sample

The study was conducted in 2023 at Kampung Laut Sub-district, Cilacap Regency, Central Java Province, Indonesia (Figure 2). A random sampling design was used to select a sample of 215 farmers who raise fewer than 20 heads of sheep. The sample size was determined on the basis of the participation of the farmers in the extension programme. A total of 215 farmers intensively participated in the extension programme on forage preservation and scabies prevention in sheep farming practices. These issues were determined on the basis of the analyses of needs at the stage of analysing learners through observations of sheep farming practices currently conducted by farmers.

Assessment of knowledge score

A lack of knowledge in animal feed technologies, such as silage and hay production, is found based on observations from focused group discussions. Furthermore, scabies is frequently found in Segara Anakan Lagoon, which is often neglected by farmers while this disease caused low of livestock productivity. The knowledge scores of farmers were measured on the basis of their pre-test and post-test results during the extension programme. Forage preservation was composed of seven question items (Table 1), and scabies prevention contained three question items (Table 2). The score was collected from the right answers. The maximal value for each question was one.

Farmers' interviews were carried out to collect data. Knowledge score was used to determine farmers' knowledge index regarding sheep farming practices, such as the type of forages, hay-making material, hay-making process, characteristics of good hay, silage-making materials, silage-making steps and characteristics of good silage, and scabies prevention, such as the causes of scabies, symptoms of scabies and medication of scabies. Knowledge level was measured by using a knowledge test. For quantitative measurements, the concept of scaling method was mostly used. The level of knowledge of the respondents was determined on the basis of whether they possessed low, medium or high knowledge (De la Fuente et al. 2017).

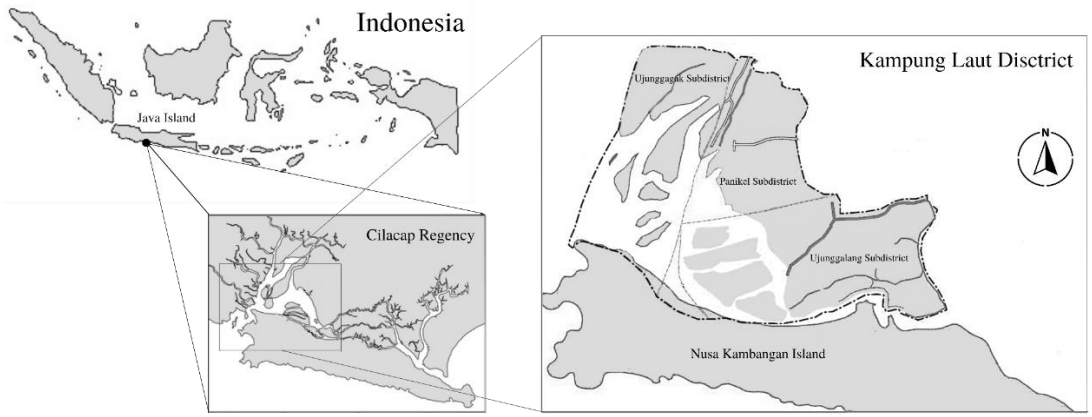

Figure 2. Location of Segara Anakan, Central Java, Indonesia

Table 1. Pre- and post-test questions on forage preservation

No	Subjects	Number of Correct Answers	Score for a Correct Answer	Maximum Score for A Question
1	Types of forage • Kangkung/*Ipomoea aquatica Forsk* • Kalanjana grass/*Pennisetum purpureum* • Cassava leaves/*Manihot esculenta* • Gamal/*Gliricidia sepium* • Calliandra/*Calliandra calothyrsus*	5	0.20	1
2	Hay-making materials • Grass • Legume	2	0.50	1
3	Hay-making process • Chopping • Drying • Storing	3	0.33	1
4	Characteristics of good hay • Retaining green colour with some yellowing • Having few damaged leaves • Preserving the whole and clear forms • Not being too dry to prevent breakage	4	0.25	1
5	Silage-making materials • Forage • Rice bran • Molasses • Broken rice grain • *Onggok** • Plastic bag	6	0.17	1
6	Silage-making steps • Chop forage into 5–10 cm pieces using a cutting tool • Mix the chopped forage with the remaining silage-making materials thoroughly • Place the mixture into the silo (plastic bag) and tightly pack it to avoid any air space • Fill up the silo all the way to the top • Cover the silo tightly with plastic, and place weight on top of the cover • The ensiling process takes place for 3 weeks. Silage can be immediately consumed by livestock. Ensiled feed can be stored for 1–2 years in airtight storage.	6	0.17	1
7	Characteristics of good silage • Giving out acidic taste and odour • Retaining green colouring • Retaining clear texture • No mould, mucous and congealment	4	0.25	1
Maximum Score				7

*a by-product of cassava processing to produce tapioca

Table 2. Pre- and post-test questions on scabies prevention

No	Subjects	Number of Correct Answers	Score for a Correct Answer	Maximum Score for Each Question
1	Causes of Scabies *Sarcoptes scabiei* infection Poor hygiene	2	0.50	1
2	Signs of Scabies Decreased appetite Increased scratching Scabs on skin Falling fur Skinny	5	0.20	1
3	Medication for Scabies Clean scabs Apply sulphur on scabs	2	0.50	1
Maximal Score				3

Data analysis

The data collected were tabulated and summarised by calculating means, percentages and standard deviations using Stata™ software, version 16. The level of knowledge of farmers on feed preservation was calculated by categorising their score on the knowledge test as low (0–2.5), medium (2.6–5.0) and high (5.1–7.0). Furthermore, the knowledge level of farmers on scabies prevention was categorised as low (0–1.0), medium (1.1–2.0) and high (2.1–3.0). Furthermore, the knowledge index was calculated by using the following formula:

$$\text{Knowledge index} = \frac{\text{Score of correct responses}}{Total\ score\ of\ knowledge\ item}\ x100$$

The results of improving knowledge on feed preservation and scabies prevention were compared by using statistical analysis methods, such as tabulation, mean, percentage, f test and t test. Multiple regression analysis was used to examine the influence of farmers' characteristics on improving knowledge using the following formula:

$$Y_1 = a + b_1X_1 + b_2X_2 + b_3X_3 + e$$

$$Y_2 = a + b_1X_1 + b_2X_2 + b_3X_3 + e$$

where Y_1 is knowledge improvement of farmers on forage preservation, Y_2 is knowledge improvement of farmers on scabies prevention, a is constant and $b_{1,2,3,4}$ is coefficient, X_1 is age (year), X_2 is level of education (year), X_3 is experience in sheep farming (year), X_4 is sheep holding (head), and e is error.

RESULTS AND DISCUSSION

Improving the knowledge of farmers on forage preservation and scabies prevention

Sufficient knowledge on feeding animals is an important requirement in sheep farming. Farmers should have sufficient knowledge regarding the type of forage, hay-making material, hay-making process, characteristics of good hay, silage-making material, silage-making step and characteristics of good silage. Table 3 shows that before the extension programme, the average score on forage preservation was 1.79. Their average score tripled to 5.16 after the programme. The average improvement score reached 3.36.

The highest improvement of knowledge was achieved by the question items on characteristics of good silage, hay-making process, silage-making materials and characteristics of good hay, in which the improvement score reached more than 0.50. Farmers initially had limited knowledge on the hay-making process, characteristics of good hay and characteristics of good silage, as shown by the low scores in the pre-test. However, after the extension programme, their scores reached 0.65 for the hay-making process, 0.56 for the characteristics of good hay and 0.67 for the characteristics of good silage. Four correct answers were recorded for the characteristics of good hay, namely, retaining a green colour with some yellowing, having a small number of damaged leaves, preserving the whole and clear forms and being not too dry to prevent breakage. The farmers were able to recognise one of the

four answers. The silage-making steps consisted of six correct answers, two of which were identified by the farmers after extension. The characteristics of good silage consisted of four correct answers, two of which were identified by the farmers.

The average pre-test score of knowledge on hay-making process was 0.06, but the score increased to 0.65 after the intervention. The farmers reported that the explanation on the hay-making process was easy to understand, and they wanted to practice it immediately because of the straightforward process. The farmers initially scored an average of 0.05 on the characteristics of good silage, but this score improved to an average of 0.67 after the extension programme (Table 3).

The farmers were not aware of *Gliricidia sepium* and *Calliandra calothyrsus* as the forage prior to the extension because these species were not available in the area. These farmers only knew of *Ipomoea aquatica* Forsk, *Manihot esculenta* and *Pennisetum purpureum*. The average score of the farmers on this information was 0.61, but this score improved to 0.86 on the post-tests. This result indicated that the farmers now knew that *Gliricidia sepium* and *Calliandra calothyrsus* could be used in forage for their sheep.

The sheep farmers experienced a significant change in knowledge as evidenced by the paired-sample f-test analyses on the differences between the scores of pre-tests and post-tests. Each of the seven question items resulted in significant differences at $P \leq 0.01$. These results demonstrated that the knowledge of the farmers on forage preservation significantly improved after they participated in the extension programme.

Based on the pre-test scores, hay and silage were feed technologies that were not recognised by the sheep farmers along the coastal area. Most of these farmers did not know the production process and characteristics of good silage. After the extension, the farmers received information on such subjects, and their knowledge was improved.

Scabies is a common disease of sheep in the study area. However, farmers lacked adequate knowledge on this disease. The result showed that the average score on the pre-test yielded 0.55, but it increased to 2.11 after the extension. The average improvement score after the extension reached 1.56 (Table 4). The knowledge of farmers was improved by the questions regarding the causes of scabies, symptoms of scabies and scabies medication. The mean scores were low in the beginning at 0.14, 0.26 and 0.15, but such scores improved to 0.67, 0.70 and 0.74, respectively, on the post-tests. Before the extension, the average score on scabies prevention was 0.55 and reached 2.11 after the extension (Table 4).

Table 3. Level of knowledge regarding forage preservation

	Maximal score	Pre-test	Post-test	t value	Improving score
Types of forages	1	0.61 (±0.14)	0.86 (±0.18)	24.96***	0.25
Hay-making materials	1	0.53(±0.28)	0.95 (±0.12)	24.10***	0.42
Silage-making materials	1	0.24 (±0.23)	0.81 (±0.29)	27.80***	0.57
Hay-making process	1	0.06 (±0.16)	0.65 (±0.21)	39.12***	0.59
Silage-making steps	1	0.25(±0.15)	0.67 (±0.19)	31.30***	0.42
Characteristics of good hay	1	0.05 (±0.13)	0.56 (±0.18)	42.79***	0.51
Characteristics of good silage	1	0.05 (±0.12)	0.67 (±0.21)	41.49***	0.62
Total	**7**	1.79 (±0.09)	5.17(±0.97)		3.38 (±0.88)
F-value					72.675***

*** Significant at 1% level

Table 4. Level of knowledge regarding scabies prevention

Parameter	Maximal score	Pre-test	Post-test	t value	Improving score
Causes of scabies	1	0.14 (±0.23)	0.67(±0.26)	25.76***	0.53
Symptoms of scabies	1	0.26 (±0.20)	0.70 (±0.15)	29.74***	0.44
Medication of scabies	1	0.15 (±0.24)	0.74 (±0.28)	25.73***	0.59
Total	3	0.55 (± 0.40)	2.11 (±0.41)		1.56 (±0.54)
F value					14.031***

***Significant at 1% level

The farmers were able to identify at least one of the two correct answers for the steps in scabies medication, namely, cleaning the infected parts and applying sulphur topically. The knowledge of the farmers on other subjects of scabies prevention was also significantly improved.

The farmers who previously worked in fisheries did not have basic experience and knowledge on sheep farming. Furthermore, they started with poor practices, such as minimal sanitation in the sheepfold, which made the sheep vulnerable to scabies. Fortunately, the extension programme helps the farmers improve their knowledge on scabies prevention. They were able to identify at least one of the causes of this disease, namely, *Sarcoptes scabiei* infection and poor sanitation of enclosures. They were also able to identify at least two of the symptoms of scabies, namely, low appetite, high scratching, many scabs, falling fur and emaciation.

Before intervention on forage preservation, most of the farmers pursued a low category of knowledge (91.70%), followed by medium level (8.30%). No farmers showed a high knowledge level. Similarly, related to the issue of scabies prevention, farmers mostly fell under the category of low level (83.40%) and medium level (16.60%). The number of farmers in the high knowledge level increased after scientific intervention (Tables 5 and Table 6). Young et al. (2015) and Danso-Abbeam et al. (2018) also reported that agricultural extension can be used to improve farmers' knowledge and practices.

Comparison of the improvement of knowledge on forage preservation and scabies prevention

T-test analyses indicated a significant difference in knowledge index on forage preservation and scabies prevention issues. Table 7 describes that extension could improve knowledge on both issues, but knowledge improvement on scabies prevention is higher than that on forage preservation. The high knowledge index of scabies prevention amongst the farmers was due to the fact that the farmers did not have any prior knowledge on scabies. Traditionally, farmers tended to ignore scabies because no death associated to scabies has been reported. Prior knowledge provides a boost to learning, and it is related to the subsequent learning process (Wade & Kidd 2019).

Many farmers in the study area only provide grass and legume to their livestock. However, the availability of grass and legume is limited during the dry season, resulting in poor nutrition of livestock (Sudarman et al. 2016). Hay and silage can solve forage scarcity during the dry season. Converting forage into hay and silage not only preserves forage but also increases the nutrient content. Hay is forage preservation in a dry form with a moisture content of 20% to 35%. Hay production aims to maintain the dry matter and nutrients during the storage of forage. Hay can be stored as feed for 1 year or longer. Silage is forage preservation in its fresh state under an anaerobic condition, which allows fermentation (Muck & Collins 2020; Rotz et al. 2020). Silage-making utilises

Table 5. Knowledge condition on sheep farming practices for forage preservation

Condition	Category of knowledge		
	Low (0-2.50)	Medium (2.60-5.00)	High (5.10 -7.00)
Pre-test	121 (91.70%)	20 (8.30%)	0
Post-test	11 (4.60%)	43 (17.80%)	187 (77.60%)

Table 6. Knowledge condition on sheep farming practices for scabies prevention

Condition	Category of knowledge		
	Low (0-1.00)	Medium (1.10-2.00)	High (2.10 -3.00)
Pre-test	201 (83.40%)	40 (16.60%)	0
Post-test	2 (0.80%)	85 (35.30%)	154 (63.90%)

Table 7. Difference in knowledge index between forage preservation and scabies prevention

Extension Issues	Knowledge Index
Forage Preservation	49.24
Scabies Prevention	53.26
t-statistic	3.02***

*** p-value with a significant level at 1%

additives, such as molasses, concentrate and minerals, to catalyse fermentation.

Figures 3 and 4 show the growth in knowledge of each extension issue in detail. The highest knowledge improvement in forage preservation was regarding the characteristics of good silage (0.62) and hay-making process (0.57). In scabies prevention, the highest knowledge improvement was regarding the medication for scabies (0.59) and symptoms of scabies (0.44, Figure 4).

A high knowledge index indicated that the extension material was well communicated, and farmers were able to understand the added information. The topics were selected after a discussion with the farmers prior to the extension, indicating that the farmers were aware of the problems they were facing and motivated to find solutions. Extension is a group study activity amongst farmers to increase their knowledge. An extension is effective when it can provide farmers with relevant recommendations that are easily and timely accessible to overcome their challenges. The recent technology must also be pertinent to the socioeconomic conditions of farmers (Ebenehi et al.2018; Mapiye et al. 2021).

The philosophy of extension '*helping farmers to help themselves'* (Ghimire & Suvedi 2017). is highly relevant to the results of this study. The extension aimed to help farmers improve their knowledge on forage preservation and scabies prevention. The resulting knowledge improvement can help farmers solve their problems on forage provision and scabies prevention of their livestock. The farmers are expected to apply the new-found knowledge to increase the productivity, profitability and sustainability of their livestock (Wheeler et al. 2017).

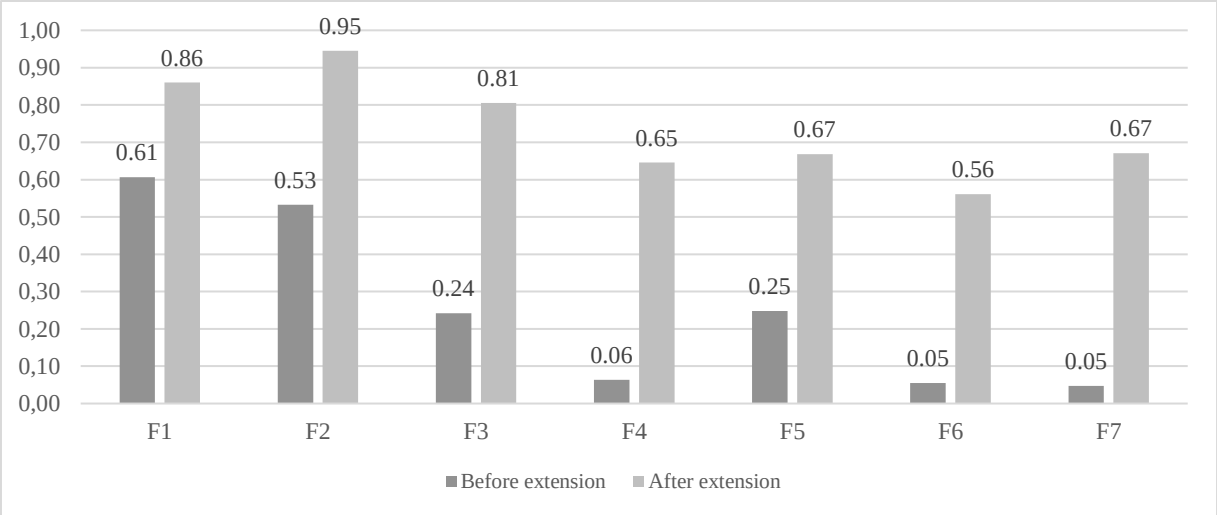

Figure 3. Level of knowledge on forage preservation before and after extension. F1= Types of forage; F2= Hay-making materials; F3= Hay-making process; F4= Characteristics of good hay; F5= Silage-making materials; F6= Silage-making steps; F7= Characteristics of good silage

Characteristic factor analysis on knowledge improvement of farmers

Farmers' education and experience in sheep farming significantly influences the knowledge improvement of farmers on forage preservation (P≤0.01 and P≤0.05). There was an increase in farmers' education and experience in sheep farming with an increase in knowledge improvement. This study found that farmers' age would influence the knowledge improvement of farmers on scabies prevention (P≤0.10). The result of characteristic factor regression analyses is described as follows:

1. $Y_1 = 12.96 + 3.04X_2 + 2.53X_3$
2. $Y_2 = 10.65 - 1.82X_1$

Productive farmers have some advantages of better working abilities, stronger stamina, and endurance, more open to new things, willing to try an innovation and share knowledge (Ibrahim et al. 2019). They also have a high probability of participation in agricultural extension activities (Suvedi et al. 2017). The result of this study showed that the knowledge improvement of the farmers on scabies prevention at a lower rate as they become older (Table 8).

Experience in sheep farming describes how long a farmer has been carrying out sheep farming in a matter of years. The knowledge improvement of farmers on forage preservation is higher with the longer of experience in sheep farming (P≤0.10). The longer the experience will enable the farmers to analyze innovations, which in turn increases the profits of their operations. Livestock experience plays a significant role in determining the success of farmers in improving the development of the farming business and the income of farmers. Previous studies also obtained results that farmers experience is found to be the potential factors of knowledge level (Duong et al. 2019; Šūmane et al. 2018).

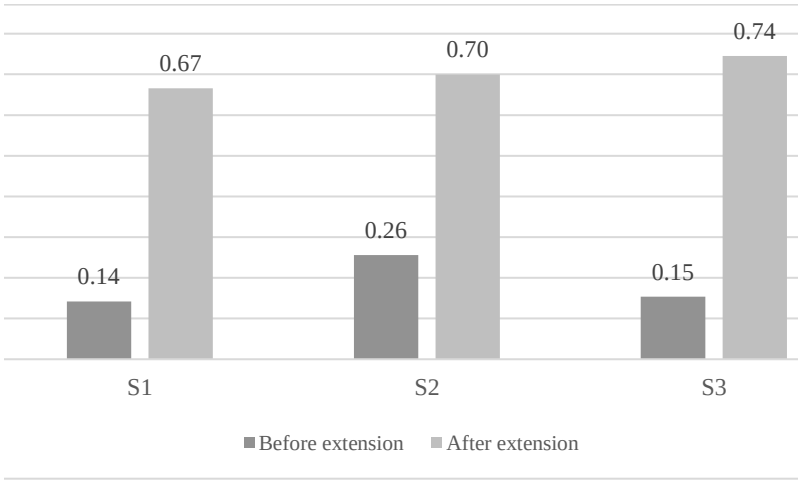

Figure 4. Level of knowledge on scabies prevention before and after extension**.** S1 = Causes of scabies; S2 = Signs of scabies; S3 = Medication of scabies

Table 8. Effects of characteristic factors on improving the knowledge of farmers

Variables	Forage Preservation		Scabies Prevention	
	Coefficients	p-value	Coefficients	p-value
Age	-0.62	0.53	-1.82	0.07*
Education	3.04	0.00***	1.16	0.24
Experience in sheep farming	2.53	0.01**	-0.53	0.59
Sheep holding	0.11	0.90	-1.07	0.28
	Constant = 12.96		Constant = 10.65	
	R Square = 0.06		R Square = 0.03	
	Std. Error = 0.69		Std. Error = 0.53	

*** *p*-value with a significant level at 1%; ** *p*-value with a significant level at 5%; **p*-value with a significant level at 10%

CONCLUSION

Farmers' knowledge on forage preservation and scabies prevention improved after participating in the extension program. The extension program proved to have a positive impact on the knowledge level of sheep farmers in Segara Anakan lagoon coastal area. The knowledge improvement were indicated through increasing the number of farmers with high level of knowledge after the extension intervention. Therefore, farmers should be provided many more opportunities to participate in the kinds of scientific intervention programs that can enhance their knowledge and skills.

Farmers' education and experience in sheep farming significantly influences the knowledge improvement of farmers on forage preservation. Farmers' age would influence the knowledge improvement of farmers on scabies prevention. It is critical to understand how farmers' knowledge levels differ, so that the extension may achieve the best possible knowledge improvement by tailoring the content and delivery methods of information on good or modern agricultural practices in a specific area.

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   The author's name is written entirely (without a degree) and typewritten by a CAPITAL letter. If the author is more than one person with a different address, Arabic numbers superscript should be given behind each name. The author's address should be written under the author's name, consisting of the institution's name and its complete address, made in line with the number of indexes on behalf of the author and typewritten by ITALIC.
3. **Abstract:**
   Abstracts are summaries of manuscripts, written in Indonesian or English, no more than 250 words, and stated in one paragraph. It includes background, purpose, material and methods, result, and conclusion. The author's name (in the CAPITAL form), publication year, manuscript title, and journal name are listed before abstract content with the layout as a reference. Keywords are listed under the abstract, a maximum of 5 words.
4. **Introduction:**
   Consists of research background, problems, efforts made, approaches taken to solve problems, and research objectives.
5. **Materials and Methods:**
   Elucidating clearly about materials used and method carried out. When animals are used as experimental materials, please indicate that it is performed according to animal ethics and welfare. See the ethical statement in the attachment.
6. **Results and Discussion:**
   It presents and discusses clearly and completely achieved research results based on the purpose. Development and discussion may be presented separately or united. The result description may be completed by concise tables and clear illustrations on a separate page. Tabel description (on top) and illustrations (on the bottom) should be clear and independent, so readers may easily understand the table without reading the text. The discussion consists of a description of the result, research mean, and benefit associated with the issue that will be solved. Measurement units, both in tables or illustrations, use the metric system.
7. **Conclusion:**
   It is the manuscript's final summary.
8. **Acknowledgment:**
   It can be written if needed.
9. **References:**
   The author is recommended to use the Mendeley Program (http://www.mendeley.com) and the citation style of Taylor & Francis - Council of Science Editors (author-da e). Mendeley's program aims to avoid mistakes in citations and reference writing. Cited references are preferably 80% of the primary article in the last ten years. They should not be from unpublished papers, except thesis and dissertation, such as practical guidance and research reports. The download is allowed if it is from an electronic magazine, genome database, or patent.

Citation in the references:
The literature in the reference is written alphabetically based on the author's name. The same author is written sequentially, starting from earlier order.

Example of reference writing

Primary paper:
Barbato O, De Felice E, Todini L, Menchetti L, Malfatti A, Scocco P. 2021. Effects of feed supplementation on nesfatin-1, insulin, glucagon, leptin, T3, cortisol, and BCS in milking ewes grazing on semi-natural pastures. Animals. 11:682. DOI:10.3390/ani11030682.

Book:
a. Alshelmani M, Abdalla E, Kaka U, Basit M. 2021. Advances in poultry nutrition research. In: Kumar Patra A, editor. Adv Poult Nutr Res. London (UK): IntechOpen; p. 19–32. DOI: 10.5772/intechopen.91547.
b. Reece W. 2015. Respiration in mammals. New Jersey (USA): Willey-Blackwell.
c. Van Soest P. 2018. Nutritional ecology of the ruminant. 2nd ed. New York (USA): Cornell University Press.

Proceeding:
Damayanti R, Wiyono A, Dharmayanti N. 2021. Pathogenicity study of ducks infected with a local isolate of highly pathogenic avian influenza-H5N1-clade 2.3. . In: Inounu I, Priyanti A, Burrow H, Morris S, Min R, Suhubdy, Sutaryono Y, editors. Proc 4th Int Semin Livest Prod Vet Technol. Bogor (Indones): Indonesian Center for Animal Research and Development; p. 277–288.

Thesis:
Mwasame DB. 2020. Analysis of the socio-

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

Electronic magazines:

Maranga B, Kagali R, Omolo K, Sagwe P. 2022. Effect of growth substrates on water quality, catfish (*Clarias gariepinus*) culture, and spinach (*Spinacia oleracea*) propagation under the aquaponic system. Livest Res Rural Dev.:82. http://www.lrrd.org/ lrrd34/9/3482mara.html.

Institution:

a. [PSA] Philippine Statistics Authority. 2016. Dairy Industry Performance Report, January – December 2015. Quezon City (Philiphine): Philippine Statistics Authority. P. 1-11
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 .....
d. The same author cited from 2 different papers: (Purwadaria et al. 2022a, 2022b).
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.

12. **Figure and graphic:**

a. The title uses Times New Roman with 1 space distance and 11 of the font size. It is a simple and clear sentence that is laid under the figure or graphic.
b. Line in the graphic should show clearly the difference between one and others if there is more than one curve.
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.

1. If the written manuscript is more than one, it needs approval from the other authors by enclosing the initial behind each name.
2. The complete manuscript is sent in three copies to the Editorial Board of IJAVS and its electronic file, or by online:

http://medpub.litbang.pertanian.go.id/ index.php/jitv.

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We hope this good collaboration would be continued in the future in improving IJAVS quality.

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