ISSN 0853-7380
E-ISSN 2252-696X

Accredited by the Ministry of Research and Technology /
National Agency for Research and Innovation
Decree Number: 85/M/KPT/2020

Jurnal Ilmu Ternak dan Veteriner

IJAVS *Indonesian Journal of Animal and Veterinary Sciences*

Volume 27
Number 3
September 2022

PUSAT PENELITIAN DAN PENGEMBANGAN PETERNAKAN
BADAN PENELITIAN DAN PENGEMBANGAN PERTANIAN
KEMENTERIAN PERTANIAN

JITV	Volume 27	Number 3	Page: 107-158	Bogor, September 2022	ISSN 0853-7380	E-ISSN 2252-6696X

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Indonesian Journal of Animal and Veterinary Sciences is published four times a year in March, June, September and December.

PREFACE

In this edition, Volume 27 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 SNP c.795A>G PLAG1 Gene and its Association with Birth Weight of Bali Cattle"; "Effectiveness of Various Glycerol Concentration as a Cryoprotectant in Frozen Semen of Pasundan Cattle"; "Assessment of Sperm Acrosome Status, Malondialdehyde and Aspartate Aminotransferase Enzyme Concentration of Frozen Semen from Limousin and Simmental Bulls in Different Commercial Diluents"; "Physiological Responses, Performance, Behaviour, and Welfare of Garut Sheep Reared in Semi-Intensive System in Indonesia"; "Mammalian Contribution to Transmission of *Schistosoma japonicum* Infection in West Lore, Poso, Central Sulawesi, Indonesia" and "Effect of *Lactobacillus casei* and Garlic Powder Administration on Broiler Performance, Immune Response and Blood Profile.

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 2022

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Jurnal Ilmu Ternak dan Veteriner

IJAVS *Indonesian Journal of Animal and Veterinary Sciences*

Volume 27, Number 3, September 2022 ISSN 0853-7380 E-ISSN 2252-696X

LIST OF CONTENT

Diversity of SNP c.795A>G PLAG1 Gene and its Association to Birth Weight of Bali Cattle

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(received 27-04-2022; revised 11-08-2022; accepted 11-08-2022)

ABSTRAK

Fahira A, Noor RR, Jakaria. 2022. Keragaman SNP c.795A>G gen PLAG1 dan asosiasnya terhadap bobot lahir pada sapi bali. JITV 27(3):107-113. DOI: http://dx.doi.org/10.14334/jitv.v27.i3.3045.

Gen PLAG1 merupakan salah satu gen yang berpengaruh terhadap pertumbuhan dan ukuran tubuh. Penelitian ini bertujuan menganalisis keragaman gen PLAG1 dan asosiasinya terhadap bobot lahir pada sapi bali. Total sampel yang digunakan sebanyak 104 sampel yang terdiri dari 66 sapi bali BPTU-HPT Denpasar dan 38 sapi bali BPT-HMT Serading yang masing-masing memiliki data bobot lahir. Analisis keragaman gen PLAG1 dianalisis menggunakan metode PCR-RFLP dengan enzim restriksi *Sac1*. Frekuensi genotipe dan alel, heterozigotas, dan keseimbangan *Hardy-Weinberg* dianalisis menggunakan program *Popgen32*. Asosiasi SNP c.795A>G gen PLAG1 dengan bobot lahir pada sapi bali dianalisis menggunakan *General Linear Model* (GLM). Amplifikasi gen PLAG1 menghasilkan 776 pb produk PCR dengan 2 alel. Genotipe PLAG1 gen terdiri dari AA (562 pb dan 182 pb), AG (562 pb, 182 pb, dan 104 pb), dan GG (562 pb dan 104 pb). Berdasarkan hasil penelitian, gen PLAG1 pada sapi bali bersifat polimorfik. Frekuensi alel pada sapi bali berada pada keseimbangan *Hardy-Weinberg*. Genotipe pada SNP c795A>G gen PLAG1 berasosiasi dengan bobot lahir pada sapi bali. Alel A memiliki pengaruh terhadap bobot lahir tinggi pada sapi bali dengan bobot lahir tertinggi terdapat pada genotipe AG.

Kata Kunci: Sapi Bali, PCR-RFLP, Gen PLAG1, SNP

ABSTRACT

Fahira A, Noor RR, Jakaria. 2022. Diversity of SNP c.795A>G PLAG1 gene and its association with birth weight of Bali cattle. JITV 27(3):107-113. DOI: http://dx.doi.org/10.14334/jitv.v27.i3.3045.

PLAG1 gene is one of those that regulate growth and body size. This study aimed to look at the PLAG1 gene polymorphism and its relationship to birth weight in Bali cattle using PCR-RFLP. The total sample used was 104 samples consisting of 66 Bali cattle from BPTU-HPT Denpasar and 38 Bali cattle from BPT-HMT Serading, each of which had birth weight data. PLAG1 gene polymorphism was analyzed using PCR-RFLP and the Sac1 restriction enzyme. The genotype and allele frequencies, heterozygosity, and Hardy-Weinberg equilibrium were all examined using Popgen32. General Linear Model was used to analyze the association of SNP 795A>G PLAG1 gene with birth weight in Bali cattle. Amplification of the PLAG1 gene resulted in 776 bp fragments and two alleles. The PLAG1 gene had three genotypes: AA (562 bp and 182 bp), AG (562 bp, 182 bp, and 104 bp), and GG (562 bp, 182 bp, and 104 bp). Based on the results, the PLAG1 gene in Bali cattle was polymorphic. The alleles frequency of Bali cattle was in Hardy-Weinberg equilibrium. The SNP c.795A>G PLAG1 gene genotype were associated with birth weight in Bali cattle. The A allele is a determinant of high birth weight in Bali cattle where the AG genotype has the highest birth weight.

Key Words: Bali Cattle, PCR-RFLP, PLAG1 Gene, SNP

INTRODUCTION

Indonesia has abandoned animal genetic resources (AnGR) that have been adapted to different environmental conditions. Beef cattle that have adapted to a specific environment resulted in genetic variation within and between groups of beef cattle. One of the beef cattle in Indonesia, namely Bali cattle, has a large population outside Java, especially in the eastern islands of Indonesia (Purwantara et al. 2012). Bali cattle are example of native Indonesian beef cattle with high diversity and are a genetic resource for native Indonesian cattle (Martojo 2012). Bali cattle are native Indonesian cattle resulting from the domestication of wild bull (Garick & Ruvinsky 2015). The advantages of Bali cattle are being able to adapt to an unfavorable environment (Astiti 2018), good reproductive ability (70–90% conception rate), and a high percentage of carcass (45-57%) (Purwantara et al. 2012; Ismail et al. 2014).

The genetic resources of beef cattle can be used to fulfill the needs of Indonesian meat consumption, but this potential has not been optimally increased due to slow growth rate (Sutarno & Setyawan 2015). However, this local beef cattle, that have adapted to Indonesia's tropical environment, have the potential to be developed and improved through a selection based on their breeding values of the same traits that have high economic values and by selecting the gene that is related to the growth traits such as Pleomorphic adenoma gene 1 (PLAG1) gene (Juma et al. 2016).

PLAG1 gene is involved in encoding a transcription factor that is extensively expressed during fetal development. PLAG1 gene works by influencing essential growth-related genes such as insulin-like growth factor 1 (IGF1), insulin-like growth factor 2 (IGF2), and growth hormone receptor (GHR) (Pereira et al. 2016). IGFI is a gene that regulates animal growth and reproduction (Fortes et al. 2013) and IGF2 is one of the genes that control cattle's growth and body size of cattle (Karim et al. 2011). Several studies have found that the PLAG1 gene is associated with the growth traits of cattle such as body size (Fortes et al. 2013) and carcass weight (Song et al. 2016). In addition, a study showed that the PLAG1 gene also affects birth weight in PO cattle (Hartati et al. 2015).

Several cattle breeds have been studied for the polymorphism of the PLAG1 gene such as Chinese cattle (Zhou et al. 2019), Holstein-Friesian (HF) × Jersey cattle (Karim et al. 2011), New Zealand Holstein Friesian cattle (Littlejohn et al. 2012), and Simmental (Song et al. 2016). Furthermore, characterization of the PLAG1 gene in Bali cattle has previously been carried out using the direct sequencing method and 7 SNPs, were found in exon 2, one of which was the c.795A>G SNP (Putra et al. 2021). However, polymorphism of the PLAG1 gene in beef cattle in Indonesia has infrequently been studied using the PCR-RFLP technique, so it is necessary to study the polymorphism of the PLAG1 gene, especially at SNP c.795A>G and its association with birth weight in Bali cattle using the PCR-RFLP technique.

MATERIALS AND METHODS

Samples

This research was carried out at IPB University's Faculty of Animal Science's laboratory of animal molecular genetics. In this study 104 DNA samples were used, 66 heads of Bali cattle at BPTU-HPT Denpasar, Bali Province and 38 heads of Bali cattle at BPT-HMT Serading, NTB, Indonesia. The 104 samples of Bali cattle used were cattle that had birth weight data. The birth weight data used was obtained from secondary data owned by BPTU-HPT Denpasar and BPT-HMT Serading. In addition, Bali cattle blood samples for DNA extraction were collected by authorized veterinarians.

DNA amplification

The National Center for Biotechnology Information (NCBI) provided the primer sequence used in this study under the accession number KP966078.1. Primer3 program was used to create the primer design. In addition, Primer Stat was used to evaluate the primer design with forward and reverse primers (5'-GTT AGG CTA GCA GCT TAG C-3' and 5'-CAG ATG ATC ACC ACC CTG-3') which will amplify exon 2 (region 1) of the PLAG1 gene in the c.795A>G SNP and produced a 776 bp PCR product.

PCR Thermal Cycler from Applied Biosystems was used to perform DNA amplification. The DNA amplification condition consisted of 3 stages, denaturation, annealing, and extension according to PCR conditions that matched the PLAG1 gene fragment. First, 1 µL of the extracted DNA sample was taken and then transferred to a 0.2 mL tube. Next, a DNA amplification reagent consisting of 6.1 µL Nuclease Free Water, 7.5 µL PROMEGA Green Master Mix, 0.2 µL forward primer, and 0.2 µL reverse primer was placed in a 1.5 µL tube then homogenized. Next, PCR reagents were distributed to the DNA samples, homogenized with a rotary mixer and placed into the PCR machine. DNA amplification was carried out under conditions of 95°C predenaturation for 1 minute, 95°C denaturations for 15 seconds, 60°C annealing for 15 seconds, and 72°C extensions for 10 seconds. DNA amplification process was performed up to 35 cycles. The PCR products were then electrophoresed using 1.5% agarose gel to verify the PCR results.

Genotyping using PCR-RFLP

PLAG1 gene was genotyped using selected single nucleotide polymorphisms (SNPs) and performed using the PCR-RFLP technique. 5 µl of PCR product were transferred to a 0.2 ml tube. First, the tube was inserted, then the mixing was made. The mixture consisted of 0.7 µl SacI enzyme buffer, 0.4 µl SaqI enzyme, and 0.9 µl DW. The mixture was incubated in an incubator for 4 hours at 65°C. In addition, 2% agarose gel was used to visualize 7 µl of incubated DNA.

Data analysis

Genotype and allele frequency was observed and expected heterozygosity, and Hardy-Weinberg equilibrium were calculated using the Popgene32 program (Yeh et al. 2000). The following formula was

used to calculate genotype and allele frequency (Nei & Kumar 2000).

$$X_{ii} = \frac{\sum_{i=1}^{n} ni}{N} \qquad X_i = \frac{(2nii + \sum_{j \neq 1} nij)}{(2N)}$$

where X_{ii} is genotype ii frequency, n_i is the number of individuals of genotype ii, X_i is allele i frequency, n_{ii} is the number of individuals of genotype ii, n_{ij} is number of individuals of genotype ij, and N is the total of sample. Observed and expected heterozygosity was determined using the following formula (Weir 1996):

$$Ho = \sum \frac{n_{ii}}{N} \qquad He = 1 - \sum_{i=1}^{n} p_{1i^2}$$

where Ho is observed heterozygosity, nii is the number of heterozygous individuals, N is the number of observed individuals, He is expected heterozygosity, p_{1i} is allele, i frequency in locus I, and n is the number of allele of locus 1.

Hardy-Weinberg equilibrium was calculated using the following formula (Hartl & Clark 1997):

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

where χ^2 is the *chi-square* value, O is thenumber of observations of genotype I, and E is the number of expected genotypes i.

Association between the genotype of the PLAG1 gene and birth weight was analyzed using the General Linear Model (GLM) and calculated using Minitab19 Program with the following mathematical model (Hou et al. 2019):

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where Y_{ij} is the observation value, μ is the general mean value, G_i is the genotype effect, and e_{ij} is the error effect.

RESULTS AND DISCUSSION

PLAG1 gene amplification

Amplification of the PLAG1 gene fragment was carried out using a PCR Thermal Cycler from Applied Biosystems and produced a PCR product length of 776 bp as shown in Figure 1. The temperature in the annealing process is the optimum temperature for the primer attachment process used by the DNA cutting point during the amplification process. Temperatures ranging from 55°C to 72°C are commonly used for optimal annealing (Innis et al. 2012). In producing optimal gene amplification, PCR optimization is required to use various PCR process conditions such as the type of DNA polymerase, concentration, temperature, and time (Langga & Kuswinanti 2012).

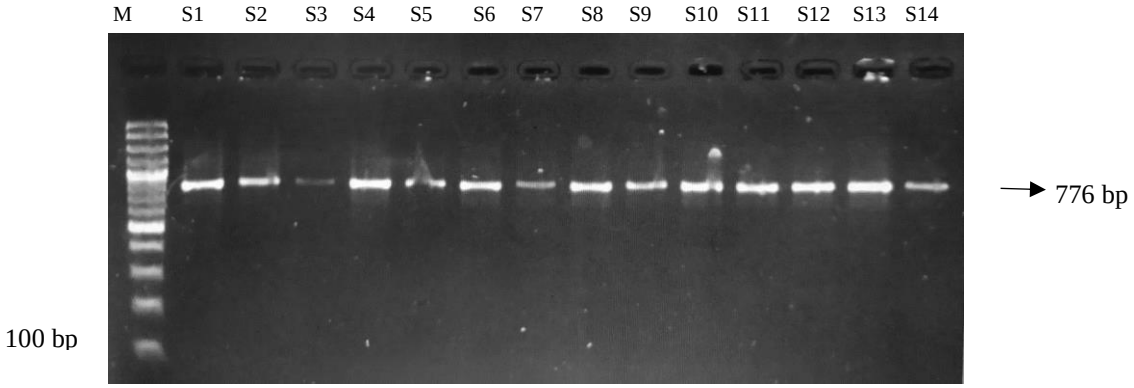

Figure 1. PCR product visualization (S1-S14: analyzed samples and M: marker 100 bp)

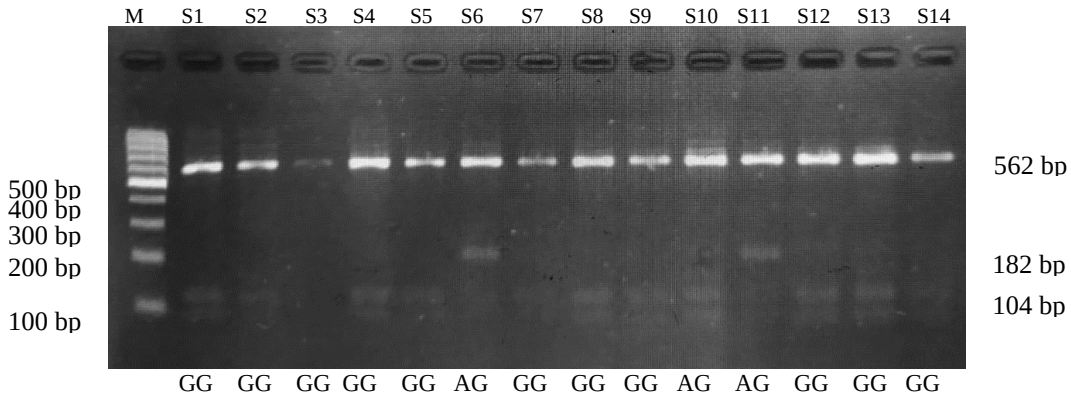

Figure 2. PLAG1 gene genotype visualization (S1-S14: analyzed samples and M: marker 100 bp)

Polymorphism of PLAG1 gene

Determination of genotype of the PLAG1 gene in Bali cattle was completed by the PCR-RFLP method using the Sac1 enzyme as a restriction enzyme. Restriction enzyme cut the DNA at certain specific sites. For example, the restriction enzyme Sac1 recognized the GAGCT|C cleavage site and cut it at 37°C. Three genotypes were produced by the results of cutting the PLAG1 gene fragment with the Sac1 enzyme. The AA genotype had two DNA fragments with product lengths of 562 bp and 182 bp. The AG genotype had three DNA fragments with product lengths of 562 bp, 182 bp, and 104 bp. Finally, the GG genotype had two DNA fragments with product lengths of 562 bp and 104 bp. DNA cleavage by restriction enzymes visualized with 2% agarose gel is presented in Figure 2.

Popgene32 program was used to calculate Bali cattle's analysis polymorphism PLAG1 gene. Table 1 shows the results of the analysis. Genotype frequency in Bali cattle at BPTU-HPT Denpasar were AA (0.03), AG (0.32), and GG (0.65), whereas at BPT-HMT Serading were AA (0.03), AG (0.16), and GG (0.82) with the highest genotype was GG in each location. In total, the genotype frequencies of AA, AG and GG in Bali cattle were 0.03, 0.26, and 0.70 with the highest genotype being GG. The G allele was the highest allele in BPTU-HPT Denpasar and BPT-HMT Serading. The highest allele frequency in Bali cattle in total was allele G (0.84). The allele of the PLAG1 gene in Bali cattle was polymorphic because its frequency was less than 0.99 (Volkandari et al. 2013). A population can be said to be polymorphic if it has more than 1 allele in 1 locus (Basyuni et al. 2012). Table 1 shows that Bali cattle observed and expected heterozygosity (Ho and He) values were less than 0.5 (50%).

Heterozygosity value that was less than 50% (0.5) indicates that a gene variation in the population is low (Dorji et al. 2012). Low gene variation in Bali cattle indicates an adverse selection or the influence of gene fixation caused by environmental factors (Rahmatullah et al. 2016). Estimating the heterozygosity value is used to calculate the genetic diversity level of a population that aims to assist the selection program. High heterozygosity indicates high genetic diversity in a population (Ulupi et al. 2014).

The Hardy-Weinberg equilibrium of the PLAG1 gene with Chi-Square (χ^2) in Table 1 shows that in Bali cattle, the PLAG1 gene frequency is in Hardy-Weinberg equilibrium because of χ^2 value < from χ^2 table (3.84). Therefore, the allele frequency in the population is relatively stable from generation to generation, the livestock population is large, and there is no mutation, selection, migration or genetic drift, gene flow, and meiotic drive, the population is in the Hardy-Weinberg equilibrium (Wang & Shete 2012). So, it is possible that the population in the two locations didn't experience migration, genetic drift, gene flow, or meiotic drive.

PLAG1 gene association with Bali cattle birth weight

Association of the PLAG1 gene with birth weight in Bali cattle was analyzed with General Linear Model (GLM) and calculated using the Minitab19 program. The results are presented in Table 2. The association analysis revealed that the SNP c.795A>G of PLAG1 gene had an association with birth weight in Bali cattle. The highest birth weight was shown in the heterozygous genotype (AG), and it had a significant effect (P<0.05) on birth weight compared to the AA and

Table 1. Genotype and allele frequencies, heterozygosity value, and χ^2 value of PLAG1 gene in Bali cattle breed

Location	N	Genotype frequency			Allele frequency		Ho	He	χ^2 value
		AA	AG	GG	A	G			
BPTU-HPT Denpasar	66	0.03	0.32	0.65	0.19	0.81	0.32	0.31	0.06^{ns}
BPT-HMT Serading	38	0.03	0.16	0.82	0.11	0.89	0.16	0.19	1.28^{ns}
Total	104	0.03	0.26	0.70	0.16	0.84	0.26	0.27	0.11^{ns}

N= Total samples, Ho= Observed heterozigosity, He= Expected heterozigosity, ^{ns}= not significant

Table 2. Association of the PLAG1 gene with birth weight in Bali cattle

Location	Genotypes			P value
	AA	AG	GG	
BPTU-HPT Denpasar	18.0 ± 1.4	20.0 ± 2.7	18.8 ± 1.8	0.08^{ns}
BPT-HMT Serading	17.0 ± 0.0^{ab}	18.2 ± 2.3^{a}	15.3 ± 1.9^{b}	0.01*

*= Significant at P<0.05, ^{ns}= Not significant. Different superscripts on the same row are different (P<0.05)

GG genotypes. The A allele is a determinant of high birth weight in Bali cattle. According to the location-based association analysis results, SNP c.795A>G of the PLAG1 gene was significantly associated with birth weight ($P<0.05$) in Bali cattle at BPT-HMT Serading. In contrast, BPTU-HPT Denpasar did not show a significant association. Another study showed that the SNP ss319607402A>G of the PLAG1 gene was associated with birth weight in New Zealand Holstein-Friesian cattle (Littlejohn et al. 2012). Furthermore, several studies stated that PLAG1 gene was associated with birth weight in PO cattle (Hartati et al. 2015) and birth weight in Brazilian Nellore cattle (Utsunomiya et al. 2013).

Birth weight is an economically essential characteristic in beef cattle, and it is usually the first trait measured in a calf (Utsunomiya et al. 2013). Genetics, feed, the cow's body weight, climate, the calf's sex, the environment experienced by the cow, and internal factors (conditions in the cow's uterus) are all factors that influence birth weight (Maylinda & Wahyuni 2020; Braz et al. 2021; Suwiti et al. 2022). The uterine environment may have an even more significant impact on fetal growth and birth weight than the parental genome (Sharma et al. 2009). However, several genes that are inherited and affect the embryo development are responses to environmental and genetic interactions experienced by the cows (Braz et al. 2021).

The difference in associations that occurred between the two locations indicated the influence of environmental conditions, namely the influence of temperature in the BPT-HMT Serading area, which was higher than the BPTU-HPT Denpasar, so that individuals with the heterozygous genotype (AG) could be better exposed and have a higher birth weight than homozygous genotypes (AA and GG). The result also indicates that the influence of genetic and environmental interactions resulted in a genotype being more adaptive in one environment than in another (Patriani et al. 2019). However, it can be seen that the birth weight at BPT-HMT Serading is smaller than at BPTU-HPT Denpasar. In order to survive the heat stress, cattle adapt to a smaller size (Syawal 2013).

PLAG1 gene is a zinc finger transcription factor of IGF2 that regulates growth and development and is found on chromosome 14 in cattle (Juma et al. 2016). PLAG1's effect on fetal growth and reproduction affecting birth weight is most likely due to the transacting regulation of the expression of insulin-like growth factors, specifically IGF2 (Voz et al. 2000). The insulin-like growth factor 2 (IGF2) gene encodes a placental and fetal growth factor that influences birth weight (St-Pierre et al. 2012).

Based on the results of this study of the PLAG1 gene's association with birth weight in Bali cattle, it is hoped that Bali cattle has the potential to be developed and increased its productivity through selection based on the breeding values of the growth traits that have high economic values, such as weaning and yearling weights. According to Boligon et al. (2009), birth weight positively correlates to growth characteristics such as weaning and yearling weights. Therefore, based on the results the AG genotype in the SNP c.795A>G of the PLAG1 gene can be used as a candidate marker assisted selection (MAS) for birth weight in Bali cattle in BPT-HMT Serading, NTB. However, further studies still need to be done to validate the results of the association to be used as a potential marker at BPT-HMT Serading with gene expression analysis.

CONCLUSION

SNP c.795A>G of the PLAG1 gene were polymorphic in Bali cattle. Furthermore, the Bali cattle's heterozygous genotype (AG) was significantly assocaited with birth weight at BPT-HMT Serading. Therefore, SNP c.795A>G of the PLAG1 gene has the potential to be used as a candidate for Marker Assisted Selection (MAS) in Bali cattle at BPT-HMT Serading.

ACKNOWLEDGEMENT

This study is supported by grants from the Ministry of Education and Culture's Master's Thesis Research Program.

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Effectiveness of Various Glycerol Concentrations as a Cryoprotectant in Frozen Semen of Pasundan Cattle

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(received 01-07-2022; revised 03-08-2022; accepted 03-08-2022)

ABSTRAK

Rasad SD, Solihati N, Winangun K. 2022. Pengaruh konsentrasi gliserol sebagai krioprotektan pada semen beku sapi Pasundan. JITV 27(3):114-121. DOI:http://dx.di.org/10.14334/jitv.v27i3.3066.

Penelitian dilakukan untuk mengetahui efektivitas gliserol sebagai bahan krioprotektan dengan berbagai konsentrasi terhadap kualitas semen Sapi Pasundan. Semen dikoleksi dari tujuh ekor pejantan Sapi Pasundan menggunakan vagina buatan setiap dua kali dalam satu minggu selama tiga bulan. Sampel semen selanjutnya ditambahkan bahan pengencer yakni TRIS-*Egg Yolk Extender* yang mengandung 20 % (v/v) kuning telur dan diberi perlakuan penambahan gliserol dengan lima konsentrasi berbeda (G5= 5%, G6= 6%, G7= 7%, G8= 8%, dan G9= 9%) kemudian dilakukan kriopreservasi. Rancangan Acak Lengkap (RAL) digunakan dalam penelitian ini untuk menguji pengaruh lima konsentrasi gliserol yang berbeda terhadap motilitas, Membran Plasma Utuh (MPU), Keutuhan Tudung akrosom (TAU), abnormalitas, dan laju pemulihan spermatozoa setelah proses kriopreservasi (post-thawing). Hasil evaluasi semen sapi Pasundan setelah proses pengenceran menunjukkan bahwa penambahan gliserol sebanyak 7% (G7) menghasilkan motilitas dan nilai TAU terbaik (83,68% dan 72,84%), penambahan gliserol sebanyak 7% dan 8% (G7 dan G8) menghasilkan nilai MPU terbaik (85,00% dan 84,50%), serta penambahan gliserol sebanyak 6%, 7%, 8%, dan 9% (G6, G7, G8, dan G9) menghasilkan nilai abnormalitas terendah (1%). Pada semen sapi Pasundan yang telah melalui kriopreservasi (*post-thawing*), penambahan gliserol sebanyak 7% (G7) menghasilkan nilai motilitas, TAU, MPU, dan laju pemulihan terbaik (54,49%, 38,57%, 54,29%, dan 72,28%). Sementara itu, penambahan berbagai konsentrasi gliserol tidak menunjukkan efek yang signifikan terhadap nilai abnormalitas spermatozoa setelah proses kriopreservasi. Secara umum, penambahan gliserol sebanyak 7% dalam pengencer semen menunjukkan hasil yang optimal sebagai bahan krioprotektan.

Kata Kunci: Krioprotektan, Gliserol, Sapi Pasundan, Kualitas Semen

ABSTRACT

Rasad SD, Solihati N, Winangun K. 2022 Effectiveness of various glycerol concentrations as a cryoprotectant in frozen semen of Pasundan cattle. JITV 27(3): 114-121. DOI:http://dx.di.org/10.14334/jitv.v27i3.3066.

This study was conducted to determine the effectiveness of Glycerol as a cryoprotectant with various concentrations on the quality of Pasundan cattle semen. Semen was collected from seven bulls of Pasundan Cow using an artificial vagina twice a week for three months. The semen sample was added with a TRIS-Egg Yolk Extender containing 20% (v/v) egg yolk and treated with the addition of Glycerol with five different concentrations (G5= 5%, G6= 6%, G7= 7%, G8= 8%, and G9= 9%) were then performed with cryopreservation. A Completely Randomized Design (CRD) was used to examine the effect of five different concentrations of Glycerol on motility, intact plasma membrane (IPM), the integrity of acrosome cap (IAC), abnormalities, and recovery rate (RR) of spermatozoa after cryopreservation (post-thawing). The results of diluted Pasundan cattle semen evaluation showed that the addition of 7% Glycerol (G7) resulted in the best motility and IAC values (83.68% and 72.84%), the addition of 7% and 8% Glycerol (G7 and G8) resulted in the best IPM values (85.00% and 84.50%). The addition of 6%, 7%, 8%, and 9% Glycerol (G6, G7, G8, and G9) resulted in the lowest abnormality values (1%). On the post-thawing Pasundan cattle semen evaluation, the addition of 7% Glycerol (G7) resulted in the best motility, IAC, IPM, and RR values (54.49%, 38.57%, 54.29%, and 72.28%). Meanwhile, adding various Glycerol concentrations did not significantly affect the abnormality value of post-thawing spermatozoa. Generally, the addition of 7% Glycerol in semen extenders shows optimal results as a cryoprotectant.

Key Words: Cryoprotectant, Glycerol, Pasundan Cattle, Semen Quality

INTRODUCTION

One of the local breeds of cattle in Indonesia is Pasundan cattle, which originated from the West Java region and spread in the buffer zone and the southern coast of the area (Aisah et al., 2017). As a local breed, Pasundan cattle have several superior traits, such as disease resistance, high feed quality, and climate change adaptability. Cattle is one of the livestock currently being developed to meet the national beef

needs (Arifin et al. 2014). Based on those characteristics, therefore, some efforts are required to increase the population of Pasundan cattle through artificial insemination (AI) programs.

Artificial insemination (AI) is one of the reproductive technologies and the most important technique that has been applied to improve the genetics of animals. The technology is widely used because several selected males could produce enough sperm to inseminate thousands of females recipient per year (Ax et al. 2000; Aurich et al. 2020). The success of an AI program depends on the quality of frozen semen. As we know, the semen cryopreservation process aims to maintain the quality of semen during the freezing and storage process. However, the freezing process of semen causes a decrease in the viability of spermatozoa cells due to cold shock and ice crystal formation. Undertaking a dilution process and adding the best cryoprotectant to the semen diluent can overcome the problems (Najafi et al. 2013; Mahendra et al. 2018). As it is required to optimize the semen quality of superior bulls, a dilution process is needed. The purpose of the semen dilution process is to increase the volume of semen and to maintain the survival of the sperm. One of the extenders commonly used for diluting cattle semen is the TRIS-Egg yolk. This choice is because the TRIS-Egg yolk has a good buffer capacity with low toxicity (Feradis 2010). Adding cryoprotectant substances to the TRIS-egg yolk extender can prevent a decrease in the quality of frozen semen (El-Sheshtawy & El-Nattat 2018; Hermansson et al. 2021).

Glycerol is the most common cryoprotectant agent used in semen cryopreservation. Moreover, applying Glycerol could prevent cell dehydration of spermatozoa cells and a buildup of H_2O molecules in the cells and minimize ice crystal formation during the freezing process (Gamal et al. 2016; Ma et al. 2022). Some research on using Glycerol as a cryoprotectant in cattle semen has been carried out (Gamal et al. 2016), and using 7% Glycerol was conducted to compare the viability and fertility of bovine semen diluted in Botu-Bov (BB) commercial extender with and without Glycerol as a cryoprotectant then cooled at 5^{o}C. Other research added 3, 5, and 11% Glycerol in buffalo semen (Fabbrocini et al. 2000), and their results showed that optimizing the timing of the Glycerol addition and the presence of energy source in the extender rendered a higher efficiency in the thawed spermatozoa of Mediterranean buffalo.

Other research indicated that by using different levels of Glycerol in sheep semen, as much as 5% can optimally maintain the semen quality (Rehman et al. 2013; Yánez-Ortiz et al. 2021), while Baharun et al. (2017); and Setiono et al. (2015) explained that the use of Glycerol in cow semen in a range of 5-7% could maintain the semen quality during the cryopreservation process. Based on the above description, this research was conducted to test the effectiveness of Glycerol as a cryoprotectant with various concentrations on the quality of Pasundan cattle semen.

MATERIALS AND METHODS

This study was carried out from October 2018 to September 2019 in the Artificial Insemination Center of Beef cattle, Cijeungjing-West Java, Indonesia. Fresh semen used in this research was collected from 7 Pasundan bulls (2-5 years old) that have characteristics of good semen quality. Parameters observed in this study were motility, intact plasma membrane (IPM), the integrity of acrosome cap (IAC), and abnormalities. These parameters were tested before and after cryopreservation. Recovery rate of spermatozoa was tested after cryopreservation (post-thawing).

Semen collection and initial evaluation

Semen was collected from each bull twice a week using an artificial vagina. Immediately after collection, the semen was evaluated using macroscopic and microscopic observations. Macroscopic evaluation is carried out by observing volume, color, smell, consistency, and pH. Microscopic qualities of fresh semen measured were sperm motility. Each ejaculate having less than 70% sperm motility was discarded.

Semen cryopreservation

Freshly collected semen was diluted in TRIS-Egg Yolk Extender. For every 100 mL, the extender contains 1.725 g Tris (hydroxymethyl) aminomethane crystal, 2.79 g Fructose crystal, 1.555 g Lactose, 0.95 g citrate monohydrate acid, 88 ml distilled water, 20% v/v egg yolk (Salamon & Maxwell 2017), and then treated with the addition of Glycerol with five different concentrations (G5= 5%, G6= 6%, G7= 7%, G8= 8%, and G9= 9%). Microscopic qualities of diluted semen measured were sperm motility, intact plasma membrane (IPM), intake of acrosome cap (IAC), and sperm abnormality. After dilution, the semen was packaged in a 0.25 ml straw with a motile sperm concentration of $25x10^6$, and the straw was equilibrated at 4^{o}C for 4 hours. After equilibration, straws were vaporized with liquid nitrogen at -80^{o}C for 9 minutes inside the Styrofoam box. Then, straws were plunged into a liquid nitrogen tank at -196^{o}C. Post-thawed semen evaluation is done by removing the straw from the liquid nitrogen tank and then stored at room temperature until the

semen melts completely. Then, the assessment is carried out by testing the same parameters as the diluted semen testing.

Evaluation of sperm motility

Sperm motility was calculated by calculating the total sperm concentration and the concentration of dead sperm. Semen concentration was measured by mixing 0.05 ml semen and 1 ml 3% Sodium Chloride and then calculated by using Hemocytometer (Neubauer chamber). Evaluation of semen concentration was done by counting the spermatozoa from five large squares. Semen concentration is the number of sperm cells that were calculated from five large squares cell $x10^7$ (Ax et al. 2000). Motile sperm are counted under a microscope with 400x magnification. The rate of motility was determined by percentage (Fabbrocini et al. 2000). The following formula calculated sperm motility:

$$Sperm\ Motility\ (Y) = \frac{\sum total\ sperm - \sum dead\ sperm}{Total\ Sperm} x\ 100$$

Evaluation of intact plasma membrane (IPM)

The intact plasma membrane (IPM) assessment was conducted using a Hypo Osmotic Swelling Test (HOST) solution prepared from 30 grams of NaCl dissolved in 100 ml of distilled water. Semen and HOS solution was homogenized with a ratio of 1:3 and then incubated at 37°C for 30 min. Furthermore, aliquots were smeared on the glass slide. IPM was evaluated using a 400x magnification microscope that observed at least 200 sperm cells. A circular-tail shape marked the intact membrane, while a straight-tail shape marked the damaged cell membrane. The calculation of the percentage of PMI used the following formula by Mitchell & Doak (2004):

$$\%IPM = \frac{\sum Intact\ plasma\ membrane\ of\ sperm}{200\ sperm\ cell} s\ x\ 100$$

Evaluation of intake of acrosome cap (IAC)

Evaluation of IAC sperm was conducted using 1% formalin fixation. The fresh or post-thawed semen was mixed with 1% formalin fixation with a ratio of 1:3. The observation was carried out on 200 sperm cells by using a microscope with 1000x magnification. The intake of the acrosome cap was characterized by a black line on the anterior portion of the head of sperm. The calculation of the percentage of IAC used the following formula (Mitchell & Doak 2004):

$$\%\ IAC = \frac{\Sigma Sperms\ with\ intact\ acrosome\ caps}{200\ sperm\ cells}\ x\ 100\%$$

Evaluation of sperm abnormalities

Spermatozoa abnormalities were evaluated using eosin-nigrosine dye. One drop of fresh semen was put on the end of the object glass using an ossicle, then one drop of 2% eosin-nigrosine solution was added near the fresh semen. Both were mixed and covered with an object glass. Fixation of the smear preparation used Bunsen. Observe using a microscope with a magnification of 400x (Arifiantini 2012). Spermatozoa that absorb color were declared dead. The number of sperm observed was at least 200 spermatozoa by the formula (Susilawati 2017):

$$Sperm\ Abnormalities = \frac{abnormal\ sperm\ count}{sperm\ count\ observed} x100\%$$

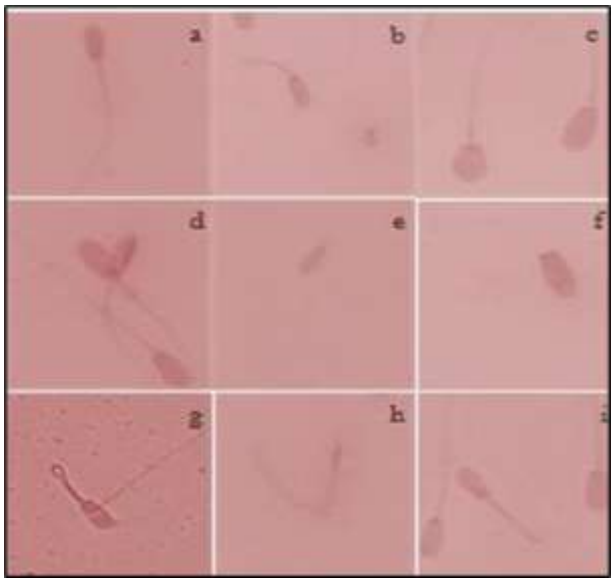

Figure 1. Normal and Abnormal Spermatozoa Morphology 1000x Magnification with Eosin-Nigrosine Solution. a) Normal Spermatozoa, b) Pear-shaped, c) Macrocephalus, d) Microcephalus, e) Detached Head, f) Head only, g) Circular Tail, h) Tail, and i) Stump Tail

Evaluation of recovery rate (RR)

Assessment of recovery rate was performed by comparing the data of post-thawing motility with fresh semen motility. The calculation of the percentage of recovery rate used the following formula (Arifiantini et al. 2005; Mahendra et al. 2018):

$$\%\ RR = \frac{\%post\ thawing\ motility}{\%fresh\ semen\ motility}\ x\ 100\%$$

Statistical analysis

This experimental study was analyzed using a completely randomized design (CRD). The study was conducted using five different Glycerol content

treatments (G5, G6, G7, G8, and G9) on extender cement and their effect on post-liquid semen quality. Each treatment is repeated six times. The data were analyzed using ANOVA followed by Duncan's multi-range test.

RESULTS AND DISCUSSION

Evaluation of semen before cryopreservation

Fresh semen was initially evaluated macroscopic and microscopically for its feasibility before treatment. The results of initial semen evaluations can be seen in Table 1. After the first assessment, semen that met the criteria was diluted with an extender and treated with various Glycerol levels. Based on table 1, the quality of fresh semen from Pasundan cattle macroscopically indicates normal conditions. The diluted semen was tested for microscopic quality. The results of diluted semen evaluations can be seen in Table 2.

Table 2 shows that the sperm motility of Pasundan cattle ranges between 83.68% for the highest value (G7) and 75.40% for the lowest value (G9). The results were higher than the other local cattle sperm motility. According to Aisah et al. (2017), Bali cattle semen had average motility of 64.65%. According to Romadhoni et al. (2014), Madura cattle had average motility of 70%. However, according to the results of Baharun et al. (2017), the average cow semen has a motility of 89.37%. Differences in the quality of semen can be caused by factors such as feed quality, weather conditions, livestock health, genetics, and livestock management programs (Ahirwar et al. 2018). However, based on the evaluation of the motility of semen of post-mortal cattle in this study, it was shown that fresh semen was feasible for the cryopreservation process.

Other results of the Fresh semen evaluation of this study showed that the fresh semen of Pasundan cattle had Intact Plasma Membrane (IPM) ranging between 85.00% and 84.50% for the highest value (G7 and G8) and 63.30% for the lowest value (G5). This result is not much different from other local species of cattle living in the tropics. Hapsari et al. (2018) stated that the fresh semen of Bali cattle at four years and seven years of age had an average percentage of IPM of 60.85% and 54.84%, respectively. The results of the other studies stated that the average IPM in the fresh semen of Madura cattle was around 78.83% (Romadhoni et al. 2014). Moreover, the results of this research showed that the Intake of Acrosome Cap (IAC) of fresh semen of Pasundan cattle ranges between 72.84% for the highest value (G7) and 68.29% for the lowest value (G5). The quality of IAC in this study is not much different from Bali cattle, which was 68.25% (Anwar et al. 2015). The quality of IAC in local cattle (*Bos sondaicus*) is still shallow compared to those in *Bos indicus* and *Bos taurus*. According to the results of Nofa et al. (2017), it was stated that fresh semen of Brahman and Limousine cattle had IAC of 90.85% and 90.40%, respectively.

Abnormality in this research's fresh semen of Pasundan cattle was very low, i.e., at 1-1.5% (Table 2). Research results were better than other local cattle sperm motility. Romadhoni et al. (2014) reported that fresh semen from Madura cattle had an abnormality of 4.5%. Research reported by Prastowo et al. (2018) showed a high percentage of abnormalities in the semen of Bali cattle that, reached 3.89%. Overall, the G7 treatment showed the best results on the motility and IAC of Pasundan cattle cement after dilution. The G7 and G8 indicate the best value in IPM while the abnormality value in each treatment was the same except for G5, which had a higher abnormality value.

Evaluation of Pasundan cattle post-thawing sperm motility

The quality of Pasundan Cattle post-thawing semen at a different Glycerol level could be seen in Table 3. Sperm motility of G7 had the highest sperm motility by 54.49% compared with other Glycerol levels.

Table 1. Initial Evaluation of Pasundan Cattle Fresh Semen

Bull	Volume (mL)	pH	Smell	Consistency	Color	Motility (%)
1	6.0	6.5	Specific	Viscous	White-Cream	84.34
2	5.5	6.5	Specific	Moderate	White-Cream	82.47
3	5.3	6.8	Specific	Viscous	White-Cream	81.78
4	4.5	6.5	Specific	Viscous	White-Cream	83.07
5	4.2	6.5	Specific	Viscous	White-Cream	81.25
6	4.5	6.5	Specific	Viscous	White-Cream	83.22
7	4.2	6.8	Specific	Moderate	White-Cream	81.69

Table 2. Microscopic quality of Pasundan cattle diluted semen

Level of Glycerol	Motility (%)	IPM (%)	IAC (%)	Abnormalities (%)
G5 (5%)	81.18[b]	63.30[a]	68.29[a]	1.5[b]
G6 (6%)	82.19[c]	70.00[b]	69.73[c]	1[a]
G7 (7%)	83.68[d]	85.00[d]	72.84[e]	1[a]
G8 (8%)	82.72[c]	84.50[d]	71.29[d]	1[a]
G9 (9%)	75.40[a]	72.00[c]	69.27[b]	1[a]

IPM= intact plasma membrane, IAC= intake of acrosome cap. G5= 5%, G6= 6%, G7= 7%, G8= 8%, G9= 9%

Table 3. Post-thawing Pasundan cattle microscopic semen quality

Level of Glycerol	Microscopic Quality			
	Motility (%)	IPM (%)	IAC (%)	Abnormality (%)
G5 (5%)	44.89[a]	36.86[ab]	46.86[a]	1.36[a]
G6 (6%)	53.41[d]	36.71[ab]	47.14[a]	1.14[a]
G7 (7%)	54.49[e]	38.57[b]	54.29[b]	1.07[a]
G8 (8%)	48.64[c]	35.86[ab]	51.86[ab]	1.64[a]
G9 (9%)	45.80[b]	33.29[a]	47.00[a]	1.07[a]

Differences in the superscription (a, b) in the same row show a significant difference (P<0.05). G5= 5%, G6= 6%, G7= 7%, G8= 8%, G9= 9%

According to Baharun et al. (2017), the frozen semen of Pasundan cattle added with 6% Glycerol produced lower post-thawed motility of 49.45%. Another study of Bali cattle frozen semen added with 8% Glycerol stimulated lower average post-thawed sperm motility of 51.88% (Nalley et al. 2016). Use of Glycerol more than 7% caused a decrease in post-thawed motility (Kulaksiz et al. 2013; Villaverde et al. 2013).

In each treatment, motility was lower than that of fresh semen (before treatment). A decrease in sperm motility occurs gradually, starting from dilution to thawing. Baharun et al. (2017) stated that a reduction in the quality of sperm of frozen semen could be caused by plasma membrane damage of sperm during freezing processes affecting sperm motility. This membrane is rolled to facilitate substances and ion exchanges needed for sperm metabolism to produce energy for sperm movement (Storey 2008).

Evaluation of integrity plasma membrane (IPM) of Pasundan cattle post-thawing sperm

The integrity of the plasma membrane is one of the semen quality determinants. Its fluid properties and flexibility are needed to help sperm flagella movement. Table 3 shows that the integrity plasma membrane (IPM) of Pasundan cattle decreased dramatically after freezing and thawing. The decrease in the plasma membrane and drastic temperature changes affect sperm membrane structures and characteristics (Rehman et al. 2013).

Table 3 shows that the differences in Glycerol levels in the dilution of semen of Pasundan cattle did not significantly affect the sperm IPM on G5, G6, and G8. However, G7 resulted in a higher percentage of IPM (38.57%); the lowest was G9 (33.29%). Hapsari et al. (2018) stated that the average IPM of post-thawed semen of Bali cattle (4 and 7 years old) was frozen with 7% Glycerol equal to 44.6% and 33.8%, respectively. The plasma membranes of sperm have unsaturated fatty acids, which are very susceptible to cryopreservation damage.

Research results showed a decrease in IPM of sperm of Pasundan cattle frozen semen between 36.86-42.14% after thawing. Villaverde et al. (2013) stated that 40 to 50% of fresh semen that has been frozen would be damaged in their plasma membrane. Using Glycerol in semen dilution could help to protect against plasma membrane damage due to osmotic pressure changes and mechanical damage because of ice crystals formation in the plasma membrane during freezing (Mahendra et al. 2018).

Evaluation of intake of acrosome cap (IAC) of Pasundan cattle post-thawing sperm

The acrosome of sperm is a part that plays a vital role in the fertilization process as a carrier of enzymes and genetic materials. This part of the acrosome equator is an important part of the spermatozoa, this is because the anterior part of this post acrosome initiates the merger with the oocyte membrane in the fertilization process (Susilawati 2017). The quality of IAC of Pasundan cattle sperm in this study decreased after the freezing and thawing. Table 3 shows that IAC dropped from the highest of 54.29% (G7) to the lowest of 47.00% (G9). Zekariya et al. (2011) stated that freezing and thawing processes negatively affected the integrity of sperm acrosomes because this process could change the structure of chromatin in the sperm DNA. The Duncan test showed that Pasundan cattle frozen semen with 7% Glycerol produced a higher percentage of IAC (54.29%) compared to other treatments (Table 3). Shah et al. (2016) stated that sperm acrosomes integrity in semen diluted with 7% Glycerol was higher than those diluted with DMSO without Glycerol.

Different results were shown by Villaverde et al. (2013), who indicated that the use of Glycerol could not improve the integrity of sperm acrosomes of cats, but

3% Glycerol gave a higher percentage of IAC compared with 5% and 7% Glycerol. Sperm acrosomes damage during the freezing process is due to changes in the acrosome membrane connected with the capacitation process of sperm, so the presence of Glycerol does not affect the integrity of sperm acrosomes.

Evaluation of Pasundan cattle post-thawing sperm abnormality

Evaluation of sperm abnormalities is classified into primary and secondary abnormalities. Primary abnormalities in sperm are more influenced by genetic factors, while secondary abnormalities are influenced by environmental factors (Toelihere 1985). Table 3 shows freezing and thawing did not increase abnormalities of Pasundan cattle sperms. The dilution process with various levels of Glycerol also did not affect the level of sperm abnormalities. The increase in sperm abnormality could be caused by its morphological development and handling processes. Observations showed that the average abnormalities of sperms of Pasundan cattle at each treatment were not significantly different (1.07%-1.64%). Post-thawed semen of Pasundan cattle is suitable for artificial insemination because the standard level of abnormality is below 20% (BSN 2008; Manjunath 2012).

Evaluation of recovery rate (RR) of Pasundan cattle post-thawing sperm

The recovery rate is one of the indicators of the successfulness of the freezing process of semen, which describes the rate of recovery of sperms after freezing (Pileckas et al. 2013; Bhat et al. 2020). The results showed that the addition of Glycerol at levels of 7% before the freezing process of Pasundan cattle semen could produce a high recovery rate of 72.28% (G7), respectively, compared to the other treatments. The result found in this research is higher than those reported by the other studies. Baharun et al. (2017) stated that frozen semen of Pasasundan cattle added with 6% Glycerol produced a recovery rate of 59.62%. Hapsari et al. (2018) report that the recovery rate in Bali cattle semen (4 and 7 years old) frizzed with 7% Glycerol showed the percentage of RR of 65% and 61.3%, respectively. Moreover, Aisah et al. (2017) and Yendraliza et al. (2019) stated that the average recovery rate of Bali cattle semen was 56% to 60%, while the remained 40% was thought to be damaged to cell death caused by temperature stress. Cell damage due to freezing could occur due to dehydration, increased electrolyte concentration, and the formation of intracellular ice crystals that can affect cell wall permeability. In the end, the spermatozoa lose their motility (Zelpina et al. 2012).

Table 4. Post-thawing Pasundan cattle sperm recovery rate

Level of Glycerol	Fresh Semen Motility (%)	Thawed Semen Motility (%)	Recovery Rate (%)
G_5 (5%)	81.18^b	44.89^a	55.30^a
G_6 (6%)	82.19^c	53.41^d	64.99^c
G_7 (7%)	83.68^d	54.49^e	72.28^d
G_8 (8%)	82.72^c	48.64^c	58.12^b
G_9 (9%)	75.40^a	45.80^b	55.36^a

G5= 5%, G6= 6%, G7= 7%, G8= 8%, G9= 9%

CONCLUSION

Diluted Pasundan cattle semen evaluation showed that adding 7% Glycerol (G7) resulted in the best motility. IAC values (83.68% and 72.84%), the addition of 7% and 8% Glycerol (G7 and G8) resulted in the best IPM values (85.00% and 84.50%), and the addition of 6%, 7%, 8%, and 9% Glycerol (G6, G7, G8, and G9) resulted in the lowest abnormality values (1%). On the post-thawing Pasundan cattle semen evaluation, the addition of 7% Glycerol (G7) resulted in the best motility, IAC, IPM, and RR values (54.49%, 38.57%, 54.29%, and 72.28%). Meanwhile, adding various Glycerol concentrations did not significantly affect the abnormality value of post-thawing spermatozoa. Generally, the addition of 7% Glycerol in semen extenders shows optimal results as a cryoprotectant that can protect and maintain the quality of spermatozoa during the cryopreservation process and ultimately was expected to increase the success of artificial insemination.

ACKNOWLEDGMENT

USAID supports this paper through Sustainable Higher Education Research Alliances (SHERA) Program - Center for Collaborative Research Animal Biotechnology and Coral Reef Fisheries (CCR ANIBIOCORE).

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Assessment of sperm acrosome status, malondialdehyde and aspartate aminotransferase enzyme concentration of frozen semen from Limousin and Simmental bulls in different commercial diluents

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(received 05-05-2022; revised 18-08-2022; accepted 18-08-2022)

ABSTRAK

Tahar MA, Komariah, Nuraini H, Maulana T, Gunawan M, Arifiantini RI. 2022. Pengujian status akrosom sperma, konsentrasi malondialdehid dan enzim aspartat aminotransferase dari semen beku sapi Limousin dan Simmental dalam berbagai pengencer komersial. JITV 27(3):123-130. DOI: http://dx.doi.org/10.14334/jitv.v27.i3.3049.

Kriopreservasi semen adalah proses pengawetan sel sperma pada suhu rendah, agar semen bekunya dapat digunakan di masa yang akan datang. Kualitas semen beku dipengaruhi oleh bahan pengencer. Tujuan dari penelitian ini adalah untuk membandingkan pengaruh pengencer komersial terhadap status akrosom, konsentrasi malondialdehid (MDA) dan enzim aspartat aminotransferase (AspAT) dari semen beku sapi Limousin dan Simmental. Semen yang digunakan dalam penelitian ini berasal dari sapi Limousin dan Simmental dengan 5 x penampungan (Faktor pertama). Prosedur satu langkah digunakan untuk metode pengenceran. Andromed®, Optixcell® dan Steridyl® digunakan sebagai pengencer (Faktor kedua). Data dianalisis dengan analisis varians (ANOVA) dilanjutkan dengan Tukey HSD dengan selang kepercayaan 5%. Hasil penelitian menunjukkan tidak terdapat interaksi (P>0,05) antara dua faktor terhadap status akrosom, kerusakan akrosom sperma Simmental dalam Steridyl® secara signifikan lebih rendah daripada yang lain (P<0,05), namun semua pengencer menunjukkan kerusakan akrosom sperma yang rendah. Penelitian juga menunjukkan tidak ada interaksi antara jenis pengencer dan rumpun pada konsentrasi MDA dan AspAT (P>0,05). Ketiga pengencer komersial yang digunakan memiliki kemampuan yang sama dalam melindungi status akrosom dan mempertahankan konsentrasi MDA dan AspAT pada pembekuan semen sapi Limousin dan Simmental. Ketiga pengencer komersial dalam penelitian ini dapat menjadi alternatif bahan pengencer semen beku sapi Limousin dan Simmental.

Kata Kunci: Status Akrosom, AspAT, MDA, Sapi Limousin dan Simmental

ABSTRACT

Tahar MA, Komariah, Nuraini H, Maulana T, Gunawan M, Arifiantini RI. 2022. Assesment of sperm acrosome status, malondialdehyde and aspartate aminotransferase enzym concentration of frozen semen from Limousin and Simmental bulls in different commercial diluents. JITV 27(3):123-130. DOI: http://dx.doi.org/10.14334/jitv.v27.i3.3049.

Sperm cryopreservation is the process of preserving sperm cells at low temperatures, so that its frozen semen can be used in the future. The quality of the frozen sperm is affected by the diluent. The objective of this study was to compare the effects of commercial diluents on acrosome status, malondialdehyde (MDA) and aspartate aminotransferase (AspAT) enzyme concentration of thawed Limousin and Simmental bull semen. Semen was collected twice weekly using an artificial vagina. The fresh semen processed into frozen semen had sperm motility of >70%. The one-step procedure was used for the dilution methods. Andromed®, Optixcell® and Steridyl® were used as diluents. Data were analyzed by analysis of variance (ANOVA) followed by Tukey HSD 5% confidence interval. The result showed no interaction (P>0.05) between two factors on acrosome status. The sperm acrosome damage of Simmental in Steridyl® was significantly lower than others (P<0.05), although all diluents showed low sperm acrosome damage. Also, no interaction between the type of diluent and breed on MDA and AspAT enzyme concentrations was detected (P>0.05). The results suggest that three commercial diluents have equal efficacy in protecting acrosome status and maintaining MDA and AspAT enzyme concentrations of frozen Limousin and Simmental bull semen. Therefore, all commercial diluents can be an alternative for Limousin and Simmental frozen semen.

Key Words: Acrosome Status, AspAT, MDA, Limousin and Simmental Bull

INTRODUCTION

Frozen semen of Limousin and Simmental is in great demand among breeders. Frozen semen of the two cattle in Indonesia is produced by the National Artificial Insemination Center (AIC) and the Regional AIC (RAIC). The quality of the frozen semen is influenced by the type of diluent (Zamuna et al. 2015). Homemade diluents are used in the AIC, while homemade and commercial diluents are used in the RAIC. Commercial diluents are more convenient to prepare, less time-consuming, and contain antibiotics according to international standards. Semen diluents for international trade must include a combination of the antibiotics gentamicin, tylosin, lincomycin, and spectinomycin (GTLS). These antibiotic combinations have been developed and are commonly used in the Americas and other European countries (Morrell & Wallgren 2014). The commercial diluent commonly used in RAIC is Andromed®, which contains soy lecithin. There are now several commercial diluents with different sources of lecithin; one of these is Steridyl®, has contains sterile egg yolk. The newest commercial diluent is Optixcell®, which contains liposomes.

Cryopreservation impairs lipid composition and sperm plasma membrane organisation, leading to the leakage of valuable intracellular enzymes, such as aspartate aminotransferase (AspAT) (Fraser et al. 2018), or energy substrates, such as adenosine triphosphate (ATP) (Fraser et al. 2007), ultimately resulting in cell death. Sperm undergo cold damage and cold shock at low temperatures above those that cause freezing, which reduces their viability (Gączarzewicz et al. 2010). In general, cold damage refers to damage caused in cells maintained at critical temperatures below those at which cells normally function. In contrast, cold shock refers to reduced viability caused by either a rapid drop in temperature or a sharp drop in temperature. There is some overlap between the effects of cold shock and cold damage on cell organelles, particularly cell membranes. After freezing and thawing the most commonly studied quality tests were sperm motility, viability, and plasma membrane integrity (Hidayati et al. 2018).

Assessment of acrosome status is still rarely performed, although it is important because it is related to sperm fertility. The sperm acrosome contains several enzymes that play an important role in penetrating the zona pellucida during fertilization (Prihantoko et al. 2020). Besides that Malondialdehyde (MDA) and AspAT concentrations of frozen sperm are important indicators of plasma membrane damage (Gączarzewicz et al. 2010). MDA concentration was caused by lipid peroxidation of sperm plasma membrane due to high reactive oxygen species (ROS) and decreased sperm quality (Subramanian et al. 2018). The objective of this study was to compare the effects of acrosome status, and concentration of MDA and AspAT enzymes in sperm from Limousin and Simmental bull semen, frozen in different commercial diluents. The finding of this study can be used as a consideration for the selection of alternative commercial diluents to improve the quality of frozen bull semen.

MATERIALS AND METHODS

This study was conducted between November 2021 and February 2022 in Ungaran AIC, Central Java. Laboratory of Biotechnology Research Center, National Agency for Research and Innovation, Cibinong. Reproductive Rehabilitation Laboratory, Department of Reproduction and Obstetrics, and Department of Internal Medicine and Clinical Pathology, Faculty of Veterinary Medicine, Bogor Agricultural University.

This study was performed according to the standard operating procedure (SOP) SNI ISO 9001:2015 No. 824 100 15084 at the Ungaran AI Center in Central Java. A veterinarian supervised all methods in this study. The ethics committee of Ungaran AI Center in Central Java provided ethical guidelines and approval for the reliable performance of bull semen collection.

Research design

This study was conducted in a factorial randomised block design with three types of commercial diluents (Andromed®, Optixcell®, and Steridyl®), two groups of cattle breeds (Limousin and Simmental), and five replicates. This study was an experimental laboratory. Samples in the form of fresh semen are processed into frozen semen. Limousin and Simmental use in this study was in the productive age (3-5 years old, weight around 800 kg), each consisting of three bulls. The bulls were kept intensively according to the RAIC SOPs. Semen collection was performed twice weekly using an artificial vaginal method by a bull master according to RAIC SOPs. The criteria for fresh semen used for this study is motility of 70%.

Preparation of the diluent

Each commercial diluent was mixed with aquabidest (according to the diluent brochure) in a ratio of 1:4 (Andromed®), 1:2 (Optixcell®) and 1:1.5 (Steridyl®). The diluent is then homogenised and placed in a water bath (35°C).

Macroscopic and microscopic evaluation of the fresh semen

The evaluation of the semen was performed according to Arifiantini (2012). Macroscopic evaluation

includes volume, the colour of semen, acidity (pH), and semen consistency. Microscopic evaluation is performed using a binocular microscope (Olympus CX23) and includes observation of sperm movement, sperm motility, sperm viability, sperm abnormalities, sperm concentration, and sperm plasma membrane integrity.

Sperm mass movement is observed by placing a drop of semen on a microscope slide and viewing it under a microscope at 100X magnification. Sperm motility was performed by dropping 10µl semen onto a microscope slide, diluting with saline in a 1:4 ratio, homogenising, and removing a drop, which was then covered with a coverslip. The sample was viewed under a microscope with 400x magnification. The assessment of progressive sperm motility is determined subjectively. The reported value ranges from 0-100% with a 5% scale (Susilawati 2013). Sperm viability and abnormalities were assessed using the eosin-nigrosin staining method. Furthermore, sperm plasma membrane integrity was assessed using a hypoosmotic swelling (HOS) solution. Finally, sperm concentration was measured using an SDM 6 photometer (Minitube, Germany).

Preparation of the semen cryopreservation

The dilution method used in this study was a one-step dilution, according to Arif et al. (2020), semen was diluted with the diluent at room temperature. Immediately after dilution, the semen was filled into a 0.25-mL mini-straw (Minitube, Germany) using an automated filling and sealing machine (automated Combisystem and Minijet printer; Minitube, Germany). After filling into the mini-straws, they were arranged on a freezing rack and equilibrated in a refrigerated chamber (4-5°C) for four hours. Freezing was performed in an automatic freezing machine (Minitube, Germany). The frozen semen is stored in a tank with liquid nitrogen for further storage time before evaluation.

Evaluation of sperm acrosome status in frozen-thawed semen

The acrosome status of sperm was assessed by the fluorescein isothiocyanate-peanut agglutinin (FITC-PNA) staining method according to Rajabi-Toustani et al. (2019) with slight modifications.

Evaluation of malondialdehyde and aspartate aminotransferase enzyme concentration in frozen-thawed semen

The evaluation of MDA concentration was performed using the thiobarbituric acid method. The MDA coefficient used was $1.56 \times 10^5\ M^{-1}\ cm^{-1}$, which is expressed in nmol MDA/ 10^8 of sperm (Sukmawati et al. 2015). AspAT enzyme concentration was evaluated using an automated chemical analyzer (VetScan®, Abaxis Inc, Union City) and a commercial kit (VetScan®, Abaxis Inc, Union City).

Data analysis

The data obtained were tabulated using the Microsoft Excel 2010 program and then processed using the Statistical Package for the Social Sciences (IBM SPSS® 25). The normality of the data of each assessment was tested using the Kolmogorov-Smirnov test method. The normally distributed data were then analysed using analysis of variance (ANOVA) with a significance level of 95%. If there was a significant difference, Tukey HSD was performed, and data were presented as means ± standard errors (SEM).

RESULTS AND DISCUSSION

The fresh semen from Limousin and Simmental cattle in this study had a volume of 7.83±0.63 mL and 7.49±0.37 mL, with a pH of 6.45±0.03 and 6.45± 0.04, respectively. The consistency of the semen was light to medium, and the colour was creamy to yellowish. Mass motility showed +++, and sperm motility was 80-90%. Sperm viability was 90.29±0.20% and 91.88±0.21% with membrane integrity of 89.40 ± 0.20% and 90.72 ± 0.21%, respectively. Sperm concentration was 1465.73±74.71 x 10^6/ml and 1775.67±83.68 x 10^6/ml, respectively. All bulls showed minor sperm abnormalities, 5.78±0.14% and 5.12±0.19%, respectively.

The quality of fresh semen from the two groups of bulls used in this study was very good and met the requirements for freezing fresh semen. Indonesian National Standard 4869-1:2017 states that frozen semen consists of fresh semen with minimum motility of 70% (BSN 2017). The semen quality of these bulls is traceable because the animals used are selected bulls kept in a bull rearing system according to the AIC SOP.

Assessment of sperm acrosome status of frozen limousin and simmental semen in different commercial diluents

Intact acrosome status is essential as it is closely associated with sperm fertility. The acrosome contains proteolytic enzymes that play a crucial role in the fertilization process but can be damaged during the freezing process (Lopes et al. 2021). The enzymes included acrosine, hyaluronidase, and corona-penetrating enzymes required for penetration into the cumulus oophorus and pellucid zone

Table 1. Acrosome status of Limousin and Simmental sperm are frozen in different commercial diluents.

Cattle breed	Diluents		
	Andromed®	Optixcell®	Steridyl®
Limousin (%)	93.84±0.60	95.65±0.40	97.22±0.52
Simmental (%)	95.88±0.31	96.40±0.46	97.63±0.36

Table 2.Acrosome status of sperm after frozen-thawed based on diluent or breed factor

Treatment		Acrosome status (%)
Diluent	Andromed®	94.86±0.45^{b}
	Optixcell®	96.03±0.31^{ab}
	Steridyl®	97.43±0.31^{a}
Cattle breed	Limousin	95.57±0.44^{b}
	Simmental	96.64±0.27^{a}

Different superscript letters following numbers in the same column indicate significant differences (P<0.05)

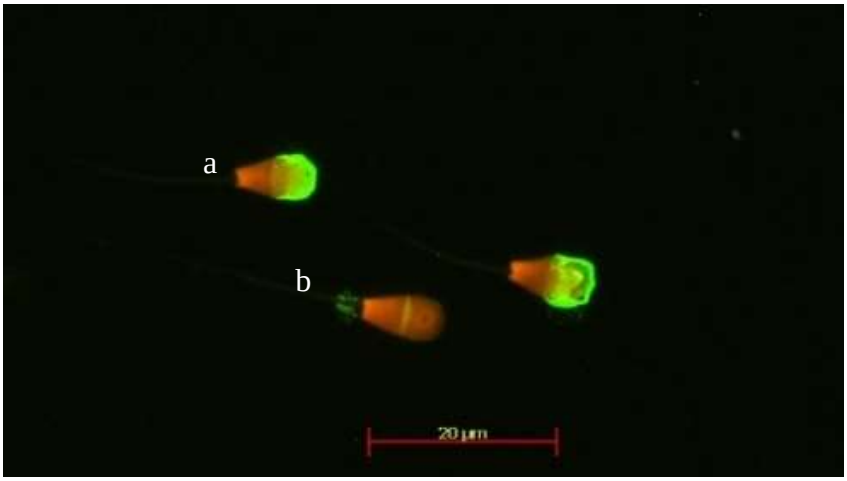

Figure 1. Sperm acrosome status using FITC-PNA staining. a. sperm with intact acrosome, b sperm with damaged acrosome

(Zaenuri et al. 2018) Detachment of the sperm acrosome marks the, acrosomal damage to the sperm head by the acrosomal reaction. A green glow on the sperm head indicated the intact acrosome status, while the incomplete acrosome status emitted a red colour (Figure 1). The results showed that there was no interaction (P>0.05) between the types of diluents and breed on the acrosome status of semen after thawing (Table 1).

The sperm acrosome status in this study was influenced by the type of diluent and the breed of cattle. Sperm frozen in Steridyl® diluent with lecithin from sterile egg yolk showed less acrosome damage than Andromed® (Table 2). This study showed that the degree of acrosome damage was influenced by the breed of cattle. Simmental had less sperm acrosome damage than Limousin (Table 2). In addition, the acrosome is located inside the spermatocyte and is protected by the plasma membrane. According to Sukmawati et al. (2015) and Indriastuti et al. (2020), breed and individual cattle have different sperm plasma membrane compositions. The cholesterol/phospholipid ratio and the degree of saturation of carbon chains differ among cattle breeds. Some of these differences cause cells to be sensitive to other cryopreservation methods, as evidenced by the decreased sperm quality after freezing, including damage to the acrosome. Furthermore, the freezing rate may also influence semen freezing success.

Previous studies have shown that sperm acrosome status after thawing in Limousin and Simmental cattle ranged from 88.40-97.09% to 86.60-97.91% but using different semen diluents (Nofa et al. 2018). The difference in semen acrosome status among cattle breeds in this study follows the statement of Üstüner et al. (2015).

Assessment of malondialdehyde concentration of frozen Limousin and Simmental semen in different commercial diluents

Evaluation of MDA concentration was essential to determine the level of lipid peroxidation in semen. Malondialdehyde is an aldehyde compound produced by the peroxidation of unsaturated fatty acids. MDA production results from increased reactive oxygen species (ROS) in cells (Tsikas 2017). The higher the MDA concentration, the greater the damage to sperm, resulting in low sperm quality (Sukmawati et al. 2015). The MDA concentrations of frozen-thawed Limousin and Simmental semen in different commercial diluents are shown in Table 3. The results showed no interaction (P>0.05) between the types of commercial diluents and cattle breeds in the concentration of MDA in frozen-thawed semen

The MDA concentration of thawed semen in the three commercial diluents was relatively low. The MDA concentrations of some crossbred cattle in Bioxcell® diluent, Tris egg yolk, and citrate egg yolk were 1.8±0.1 nmol/10^8, 2.5±0.1 nmol/10^8, and 2.6±0.0 nmol/10^8, respectively (Khumran et al. 2015) According to Dwinofanto et al. (2019), the MDA concentration of frozen-thawed Bali cattle semen in Tris egg yolk dilution was 6.8±0.3 nmol/10^8. MDA is the most commonly measured biomarker of oxidative stress, i.e. lipid peroxidation. The number of PUFAs that can contribute to MDA is much higher, and the amount of MDA in semen samples is an index of lipid peroxidation (Sharafi et al. 2015). In this study, the MDA concentration was very low in all cattle breeds and all diluents, ranging from 0.022 to 0.151 nmol/108; therefore, we can assume that the degree of lipid peroxidation in semen was also low. This result might be related to the number of PUPAs in the plasma membrane of both cattle breeds.

The assesment of aspat concentration of frozen Limousin and Simmental semen in different commercial diluents

The plasma membrane surrounds the entire cell and is directly affected by environmental changes. Another parameter related to the cell membrane, especially its integrity, was the activity of AspAT in seminal plasma. The concentration of enzyme activity in seminal plasma describes the status of sperm damage, especially of the midpiece (Tejaswi et al. 2016). The concentration of AspAT enzyme in frozen-thawed semen of Limousin and Simmental cattle in different commercial diluents are shown in Table 4. Analysis of variance showed that the type of commercial diluent and the breed of cattle and the interaction between the two factors has not affect the AspAT enzyme concentration in semen (P>0.05).

AspAT is an intracellular enzyme located mainly in the mid-piece of sperm tail (Soeparna & Arifiantini, 2013). Membrane damage in the tail, particularly in the midpiece, results in the release of the AspAT enzyme from the cell. The release of AspAT affects mitochondria; as a result, adenosine triphosphate (ATP) the production is halted, and spermatozoa stop moving (du Plessis et al. 2015). The release of AspAT from sperm into the seminal plasma is associated with increased sperm plasma membrane permeability. This condition leads to a decrease in the biological value of sperm (Frydrychová et al. 2010).

AspAT concentration in this study ranged from 75.00 to 90.33 U/L, and there was no interaction between cattle breed and diluents. The result of this study was almost the same as that reported by Hammad et al. (2019) from frozen Friesian-Holstein bull semen in Citrate-yolk diluents and was 75.20±1.11 U/L. In addition, Arif et al. (2020) reported that the AspAT concentration in frozen-thawed Limousin semen diluted in skim milk diluent was 8.33±1.14 U/L. The concentration of AspAT enzyme in fresh buffalo semen was also reported by El-Sharawy et al. (2017) and ranged from 57.7±0.82 U/L to 64.5±0.75 U/L. The three commercial diluents used in this study were able to maintain the normal configuration of the sperm plasma membrane; therefore, the release of the AspAT enzyme was still at a normal concentration.

Cryo-induced oxidative stress is associated with excess production of ROS resulting in biochemical and physical damage to the sperm membrane structures and subsequently leading to the reduced fertilising ability of sperm (Asadpour et al. 2021). All three commercial diluents contain antioxidants. The antioxidant content of the three diluents used in the study counteracted the effects of oxidative stress exceptionally well. This result is reflected in the low MDA and AspAT concentrations, so it was suspected that oxidative stress damage to the sperm plasma membrane was also low. This result follows the opinion of Hezavehei et al. (2018) that adding antioxidants to the diluent during the cryopreservation process can neutralise ROS and maintain the quality of thawed sperm. The function of antioxidants in the diluents was to add or remove an electron to neutralise ROS, stabilise free radicals, and inhibit oxidation (Ahmadi et al. 2016). Isnaini et al. (2019) confirmed that antioxidants must be used appropriately to maintain sperm quality during cryopreservation; high antioxidants may cause toxic effects in sperm cells.

A semen diluent contains an energy source and provides components to protect the sperm plasma membrane (Rizal & Riyadhi 2016). When sperm plasma membranes are not maximally protected, the

Table 3. Malondialdehyde concentration of frozen semen from Limousin and Simmental cattle frozen in different commercial diluents

Cattle breed	Diluents		
	Andromed®	Optixcell®	Steridyl®
Limousin (nmol/10^8)	0.040±0.010	0.039±0.011	0.025±0.007
Simmental (nmol/10^8)	0.151±0.115	0,043±0.013	0,022±0.005

Table 4. The concentration of AspAT enzyme in frozen-thawed semen of Limousin and Simmental cattle in different commercial diluents

Cattle breed	Diluents		
	Andromed®	Optixcell®	Steridyl®
Limousin (U/L)	86.67±8.25	90.33±7.54	75.00±20.31
Simmental (U/L)	80.33±4.33	90.33±5.84	76.67±2.91

absence of lecithin leads to sperm death to reduce cold shock (Nguyen et al. 2019). A drop in temperature from 37 ^oC to -60 ^oC can lead to cold shock, osmotic stress, and ice crystal formation. These factors can reduce the permeability of the sperm plasma membrane, potentially causing acrosomal damage (Ugur et al. 2019). The three commercial diluents in this study contain different sources of lecithin, and all have components sperm need to protect during freezing. Therefore, acrosome damage, MDA, and AspAT concentrations were low.

CONCLUSION

Commercial diluents with different lecithin-based ingredients showed equal efficacy in maintaining intact acrosome status and supported MDA and AspAT concentrations of thawed Limousin and Simmental semen. All commercially available diluents can be alternatives for Limousin and Simmental frozen semen.

ACKNOWLEDGMENTS

The authors express their gratitude and highest appreciation to the Ungaran Artificial Insemination Center for facilitating the research.

CONFLICT OF INTERESTS

The authors declare that they have no competing interests with respect to the material covered in the manuscript.

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Physiological Responses, Performance, Behaviour, and Welfare of Garut Sheep Raised in Semi-Intensive System in Indonesia

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(received 11-08-2022; revised 15-08-2022; accepted 19-08-2022)

ABSTRAK

Maulana YP, Ramdani D, Indrijani H, Yunasaf U, Mayasari N. 2022. Respon fisiologis, performa, tingkah laku dan kesejahteraan domba Garut yang dipelihara pada sistem semi-intensif di Indonesia. JITV 27(3):130-141. DOI:http://dx.doi.org/10.14334/jitv.v27i3.3068.

Peternakan domba di Indonesia terutama bergantung pada sistem intensif, yang sangat padat karya, dengan ruang gerak dan jumlah ternak yang terbatas. Negara-negara maju, di sisi lain telah mengembangkan peternakan sistem semi-intensif yang dapat meminimalkan jumlah pekerja dan dapat memberikan kesejahteraan hewan kepada dombanya. Penelitian ini bertujuan untuk membandingkan performans, respon fisiologis, perilaku, dan kesejahteraan domba Garut yang dipelihara menggunakan manajemen semi-intensif dengan penyediaan umbaran dan hanya kandang, rancangan percobaan uji-T menggunakan sepuluh ulangan (n= 10). Percobaan menggunakan domba betina sebanyak 20 ekor (umur satu tahun) dengan bobot rata-rata (± SD) (18,74±2,53 kg. Hasil penelitian menunjukkan bahwa tidak ada perbedaan (P>0,05) antara domba dengan akses ke umbaran dan tanpa akses terhadap konsumsi bahan kering (gram/ekor/hari) dan pertambahan bobot harian (gram/ekor/hari). Domba dengan akses ke umbaran memiliki status fisiologis yang lebih baik, terutama denyut jantung dan frekuensi pernapasan pada pagi dan sore hari (P<0,05) dibandingkan dengan domba dipelihara di kandang. Rasio N/L darah domba betina dengan pemberian umbaran menunjukkan hasil yang lebih baik (P<0,05), dan lingkungan pemeliharaan tidak menunjukkan perbedaan atau menimbulkan efek stres pada ternak dengan akses ke umbaran (P>0,05). Kesejahteraan domba lebih terpenuhi dengan menyediakan umbaran di peternakan domba, dan perilaku ternak juga lebih aktif ketika diberikan akses umbaran. Kesimpulannya bahwa sistem semi intensif dengan akses umbaran meningkatkan performa domba Garut sekaligus meningkatkan pemenuhan indeks kesejahteraan dan kesehatan fisiologisnya. Pendekatan ini harus dipromosikan ke seluruh Indonesia untuk pengelolaan peternakan yang lebih baik.

Kata Kunci: Sistem Peternakan, Domba Garut , Kesejahteraan Ternak, Kandang, Fisiologi

ABSTRACT

Maulana YP, Ramdani D, Indrijani H, Yunasaf U, Mayasari N. 2022. Physiological responses, performance, behaviour, and welfare of garut sheep raised in semi-intensive system in Indonesia. JITV 27(3):130-141. DOI:http://dx.doi.org/10.14334/jitv.v27i3.3068.

Sheep farming in Indonesia mainly relies on an intensive, labor-intensive system, with limited wiggle room and the number of livestock. On the other hand, developed countries have developed a semi-intensive system that can minimize the number of workers and provide animal welfare to their sheep. This study aimed to compare performance, physiological responses, behavior, and animal welfare of reared Garut sheep in semi-intensive management with an outdoor and full indoor pen, employing a T-test experimental design using ten replicates (n= 10). This experiment used 20 ewes sheep (one year old) with an average body weight of 18.74±2.53 kg. This experiment found no difference in dry matter intake (gram/head/day) or average daily gain (gram/head/day) between both treatments (P>0.05). Ewes with access to an outdoor pen had better physiological status, especially heart rate and respiratory rate, particularly in the morning and afternoon (P<0.05) compared to ewes with the full indoor pen. The ratio of blood N/L for ewes with an outdoor barn showed better results (P<0.05), and the rearing environment did not show any difference nor induce stress on livestock with access to an outdoor pen (P>0.05). Ewes' welfare increases, and they become more active after being provided access to an outdoor pen. A semi-intensive system with an outside enclosure enhances Garut ewes' performance, blood parameters, and welfare index.

Key Words: Farming System, Garut Sheep, Livestock Welfare, Pen, Physiology

INTRODUCTION

Previous studies showed housing system might affect sheep performance and welfare, especially in the tropical region (Sejian et al. 2021). Many farms, especially in less developed countries, continue to neglect animal welfare; this is related to poverty and social problems (Pinnillos et al. 2016) and other reasons

such as land limits, practical reasons, or to be safe from predator attacks. However, in terms of business benefits, scientific literature identifies employment benefits. It was discovered that improved animal welfare makes the animals safer and easier to handle, which results in a need for fewer workers, who are more satisfied, likely to have substantially less time off, and have fewer medical expenses (Sinclair et al. 2019). Farmers with low animal welfare awareness could affect their performance and decrease quality. Many livestock enterprises developed in Indonesia, such as sheep, mainly Garut sheep, have a significant potential to be developed, especially in West Java, Indonesia. In 2018, the largest sheep population was in West Java Province, with a total of 12.229.250 heads out of 17.833.732 sheep (Ditjen PKH 2021). Indonesia has a high opportunity for sheep husbandry development business to fulfill animal protein.

Sheep development in West Java and other provinces in Indonesia still depend on small-scale traditional farmers with a very labor-intensive environment. It limits animal freedom also the number of sheep population owned. Intensive livestock production is also one of the main reasons for biodiversity loss. Habitat change, such as natural to improved pastures and grassland to feed crops as one of the intensification consequences, could result in higher impacts. It also negatively affects water withdrawal, pesticides, or inorganic fertilizers. The farm that relies on grazing has the potential to defy this declining trend. Tälle et al. (2016) found that grazing generally had a more positive effect on the conservation value of semi-natural grasslands. Pasture-raised sheep products fill a premium niche. The production of pasture-raised sheep can fill a premium niche, have a minor negative environmental impact, and better animal welfare, including wildlife. The livestock rearing system significantly affects the welfare and comforting livestock during maintenance. Sheep farmers in Indonesia generally use an intensive rearing system with complete indoor maintenance. It could stress and emergence abnormal behavior affecting sheep production performance. Therefore some farmers practice a semi-intensive rearing system by providing access to an outdoor pen is a good choice for raising ewes. This system gives low stress and a better environment that increase productivity.

At this time, consumers also pay more attention to how the process of raising livestock they will consume. Many consumers, especially in developed countries, prefer to consume free-range raised livestock products. Consumer perceptions toward pasture-raised livestock are influenced mainly by their impact on the environment, and their health also depends substantially on the context of a purchase decision. Numerous consumer groups are prepared to pay more for pasture-raised attributes and the premium price of organic products (Stampa et al. 2020). Another reason is that consumers consider this rearing system better meet animal welfare. The provision of an outdoor pen in the rearing system has been regulated by institutions such as the USDA (United States Department of Agriculture) and Humane Farm Animal Care (HFAC). In certain countries such as America, The United Kingdom, and other European countries, where generally livestock products have their grades and with a certificate of recognition of free-range system by these institutions, livestock products could be sold at better prices without limiting livestock rearing welfare.

Access to open land (outdoor pen) in sheep farming increases lymphocyte proliferation in the body (Colditz & Hine 2016). The outdoor pen also supports the implementation and maintenance of livestock welfare during rearing (Colditz & Hine 2016). In addition to a semi-intensive rearing system of sheep while also paying attention to nutritional needs and health maintenance, a semi-intensive rearing system with access to an outdoor pen can provide good production results for livestock production (De Brito et al. 2017).

A semi-intensive rearing system providing access to an outdoor pen is suitable for sheep breeding. Open land increases interaction between rams and ewes, leading to a higher chance of conception. An open land provides a place for the animal to do more activities. It helps to manage ewes weight, which affects the giving birth process. A rearing system that provides access to an outdoor pen for sheep with some concentrate feed could increase the level of low nesfatin-1. It is good for reproductive and reproductive organ metabolism. Another result from this study was an increase in feed consumption in sheep with a good Body Condition Score (BCS) and an increase in milk production without affecting the ewe's weight (Barbato et al. 2021).

Use of free-range in sheep farming (Osoro et al. 2013; Gracindo et al. 2014) and welfare (Llonch et al. 2015; Grandin 2018; Munoz et al. 2019) have been studied a lot by other academics, but none of those previous studies have looked at the influence of provision of outdoor pen on sheep behavior affected by more fulfilled welfare, especially for Garut sheep. Thus, this study aims to evaluate and compare the physiological status, body weight, growth, conception rate, temperature humidity index, welfare index, and behavior of Garut sheep reared using a semi-intensive system where half subjects are provided with access to an outdoor pen, and other half was reared in indoor pens fulltime.

MATERIALS AND METHODS

Location and farming systems

This study was carried out at the Experimental Farm of Universitas Padjadjaran (UNPAD; 6.93^{o}S, 107.8^{o}E,

and 750 m above sea level), Sumedang Regency, West Java Province, Indonesia, between January 2021 to July 2021. The average temperature during the experimental period was 23.2^{o}C with a relative humidity of 82%. The outdoor pen used in this experiment was sized approximately 50 m^2 with a short (length 1-5 cm) field grass covering it and surrounded by an iron fence produced by NV Bekaert SA, Indonesia. Total 20 individual indoor pen-sized (1 m long × 0.7 m wide × 0.9 m high) parted by wood panels. The individual indoor pen is equipt with eyes and part-physical contacts.

Animals

The research ethics committee has approved animal care and experimental condition for sheep in this study of Universitas Padjadjaran (protocol No. 305/UN 6.KEP/EC/2021). Twenty ewes of 12±2 months old (Garut breed, Decree of Indonesian Agricultural Minister No. 2914/Kpts/OT.140/6/2011) were used in this experiment. Their initial average body weight was 19.5±2.5 kg (13.57% coefficient of variation, CV). The average body weight was 18.7±2.46 kg (12.82% CV) on day 10 of adaptation, and then the experiment was started here. Each ewe was randomly placed in an individual indoor pen with free access to feed and ad libitum clean water. Ten ewes had access to an external pen every day from 09.00 to 11.00 am (except during rain) (Outdoor Pen Treatment or OP). Another ten ewes were entirely confined in the indoor pen until day 70 of the experiment (Indoor Pen Treatment or IP). From day 71 to 138, each treatment modified the indoor pen into a communal pen-sized (2.5 m long × 4.2 m wide × 0.9 m high). Each indoor communal pen provided one ram for mating to measure ewes' conception rate. The exact time scheduled treatment was applied.

Animals feeding

All ewes were fed three times daily: 1 kg forage at 07:00 am, whole concentrate (500 grams) at 12:00 am, and 2 kg forage at 4:00 pm. The diet was mixed forage and designed concentrate. Concentrate consisted of 14.5 percent crude protein and 67 percent TDN (Total Digestible Nutrients). Feed ingredient composition and chemical content of concentrate can be seen in Table 1. Leftover forage and concentrate were collected and weighed before the next feeding time.

Data collection and measurements

Microclimate parameters

Characteristics studied were indoor pen temperature (Tip, ºC), outdoor pen temperature (Top, ºC), and relative humidity of both indoor and outdoor pen (RH, %) to compare microclimate in different farming methods. Tip, Top, and RH variables were measured and recorded manually every day at 07:00 am, 12:00 am, and 4:00 pm for 70 days using a digital room thermometer (HTC-2, Eagletech, China). The thermometer -50 to 70^{o}C (±1^{o}C accuracy) was used. RH measurement ranges between 20 and 99 % (±5 % accuracy). The thermometer was placed 1.5 m above the ground. It is not exposed to solar radiation and is shielded from the weather and radiation.

Physiology responses

Physiology responses affected by thermoregulatory variables such as respiratory rate (RR, breaths min^{-1}), heart rate (HR, beats min^{-1}), and rectal temperature (RT, ^{o}C) of each ewe were collected every Wednesday for 70 days of the experiment. Ewes were restrained in their indoor pen and controlled by holding the chin with both hands, then pressed by knees to prevent movement. The containment procedure took about 3 min per ewe. All ewes were adapted to the procedure and did not resist immobilization. RR was determined by monitoring flank movements and counting exhale of ewes for over 60 seconds with three repetitions assisted by a flexible stethoscope. HR was determined by positioning the same flexible stethoscope on the left thoracic region over the aortic arch and counting the heartbeat for 60 seconds with three repetitions. RT was measured by inserting a flexible tip digital clinical thermometer (MC-343F, Omron, China), with a measurement range of 32.0–42.0^{o}C, maximum indication error of ±0.1^{o}C, in the ewes rectum for 30 seconds. Thermoregulatory variables readings were carried out three times at 07:00 am, 12:00 am, and 4:00 pm. In addition, blood collection was also carried out on day 70 to test the neutrophil-lymphocyte ratio of blood as an additional stress test parameter. Additional parameters like red blood cells, white blood cells, and other hematological and blood chemical parameters were also done.

Animal performance

The performance of ewes was measured by collecting feed intake data daily. It counted the difference between offered and leftover in a gram of DMI (g/head/day). Bodyweight and growth of some specific body parts are being monitored and measured bi-weekly (Wednesday) until day 70.

Thermal comfort indices

Thermal comfort and environmental indices were calculated based on meteorological data recorded in an

Table 1. Ingredient composition and chemical content of the concentrate

Feed Ingredients	DM	CP	TDN	CF	Ca	P	Usage
	----------(%)----------						
Corn[1]	92.01	8.00	80.00	11.00	0.25	0.58	5
Dried cassava[1]	84.71	1.84	78.30	0	0.29	0.38	10
Chocolate skin[1]	80.00	14.50	47.00	33.00	-	-	15
Coconut Meal[1]	88.50	19.00	78.00	0	0.08	0.52	15
Palm Meal[1]	90.80	14.69	75.50	0	0.26	0.19	15
DDGS[2]	89.30	30.60	88.00	10.0	0.09	0.67	5
Wafer Crust[1]	90.00	13.60	70.00	5.00	0.50	0.30	15
Pollard[1]	89.57	16.41	74.83	5.86	0.13	1.29	19
Salt[1]	90.00	-	-	-	0.33	0.07	0.4
Lime[1]	100.00	-	-	-	39.00	-	0.4
Premix[1]	98.00	-	-	-	4.00	2.00	0.2

[1] Nurfitrini & Muhamad (2021); [2] USGC (2018)
DDGS= distillers dried grains with solubles, DM= Dry matter, CP= Crude protein, TDN= total digestable nutrient, CF= crude fiber, Ca= calcium, P= phosphorus

Table 2. Ethogram, description, and definition of observed behaviors

Behavior Explanation	Ethogram Classification		
	Behavior	Activity	Body Position
Animals are grazing with their heads down, and they can be stationary or moving	Grazing	Feeding / Grazing	Upright - Bow
Animals eat with their heads down on the available feed	Eat at the Feed Tray	Feeding / Grazing	Upright - Bow
Animals travel by taking progressive steps forward. The head position can be up or down.	Walk	Active	Upright
Animals are still and stand up straight. Behaviors are recorded only when the head is moving or chewing with the head held high.	Remastication - Standing	Active	Upright – Mouth movement
Animals lie down and are inactive with small head movements	Lying	Inactive	Kneel
Animals are still and stand up straight. Behaviors were recorded only when the head was held upright without movement.	Stand up	Inactive	Upright

Source Barwick et al. (2018)

Indoor and outdoor pen. The temperature and humidity index (THI) of the indoor and outdoor pens was determined, as described in the following equation by Thompson & Dahl (2012):

$$THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)]$$

where T is air temperature (^{o}C), RH is relative humidity (%), and THI is comfort index.

Behavior activities

Behavior activities were collected using a CCTV (closed-circuit television) placed in the outdoor and indoor pen. It was done to collect behavior data on day 70. Ewes were observed in four sessions from 09:30 to 09:45 am; then from 10:15 to 10:30 am; next from 2:30 to 2:45 pm; and last from 3:15 to 3:30 pm (±15 min each session). Data collected were the duration and frequency of behavior shown during each session. Details of behavior classification can be seen in Table 2.

Welfare index

Ewes' welfare index during rearing was assessed on day 70 of the study through a questionnaire based on the Welfare assessment from (EFSA 2014; Indonesia 2012). The answer was based on the farming system

and the condition of ewes from each treatment until the day of the assessment. A higher value denotes better satisfaction with livestock welfare. Six stable keepers completed the assessment, tallied, and compared the two treatments.

Conception rate

The conception rate of ewes was collected and measured using a conception test kit (Pregnadrop) by taking a sample of ewes urine on days 104 and 138. If the ewes' urine mixed and reacted with pregnadrop substance and became clear, that stated the ewes were pregnant; if not reacted, then ewes were not pregnant.

Statistical analysis

This study used a complete randomized design, with a t-test design for treatment structure. Three statistical measurements were used in this experiment for body weight, growth, dry matter consumption, conception rate; hematology parameters; and temperature humidity Index data were analyzed using an independent T-test. The variables for Independent T-test were computed as follows:

$$\boldsymbol{t} = \frac{\bar{x_1} - \bar{x_2}}{S_p\sqrt{\frac{1}{n_1}} + \sqrt{\frac{1}{n_2}}} \text{ with } \boldsymbol{S_p} = \sqrt{\frac{(n_1-1)s_1^2+(n_2-1)s_2^2}{n_1+n_2-2}}$$

where $\bar{x}1$ is the mean of the first sample; $\bar{x}2$ is the mean of the second sample; $n1$ is the sample size (i.e., number of observations) of the first sample; $n2$ is the sample size (i.e., number of observations) of the second sample; $s1$ is the standard deviation of the first sample; $s2$ is the standard deviation of the second sample; Sp is pooled standard deviation.

While welfare index data were analyzed using the Mann-Whitney test, the behavior parameter was analyzed using time management analysis. Data were analyzed in SPSS 26 statistical software. Statistical significance was assumed at P<0.05.

RESULTS AND DISCUSSION

Results

Microclimate parameters and thermal comfort index

This study did not find any interaction between indoor and outdoor pen farming systems for air temperature and relative humidity. However, the isolated effect of indoor pen farming systems was found. The air relative humidity was higher in IP than in OP (P= 0.001). It was also revealed that the RH value was higher than the typical reference for sheep due to the relatively humid area of the cage and the significant amount of rain that fell at the time of the study. A ventilator cage or an exhaust fan could be added to lower RH. A heating device could be added to lessen weather effects during heavy rainfall. As expected, IP showed 6% higher relative humidity compared with OP. Moreover, we did not find any differences between both systems regarding the air temperature (P= 0.630).

This study also did not find interaction in Temperature-Humidity Index (THI) between indoor and outdoor pen farming systems (P= 0.614). However, both treatments have a positive THI outcome, indicating that the environment in which the experiment was conducted is at a safe stage and suitable for use as a livestock development area, and can be used as a guide for farmers looking for a suitable spot to start a farm.

Thermoregulatory variables

Based on the interaction observed between the time of day and the farming system (P<0.05), the respiratory rate of ewes showed higher in IP compared to OP (Table 4). At noon, the difference between systems was not too high. It was only one breath per minute for every period of data collection. Increased respiratory rate was sufficient to keep ewes' rectal temperature in the physiological interval (Tables 4 and 5). For both IP and OP treatments, animals did not have any differences and were still normal (P>0.05). Heart rate for ewes in outdoor pen treatment (OP) was lower (Table 4) than for ewes in indoor pen treatment (IP) and showed a significant difference. These physiological parameters were associated with microclimate parameters (air temperature and relative humidity) (Table 3) that are much higher in indoor pen treatment (IP). It could cause disturbance and induce ewes to be more inclined to be stressed and increase heart rate and respiratory rate.

Hematology parameters

A significant difference (P<0.05) was found in Red Blood cells (RBC), Hemoglobin (Hb), hematocrit, Ratio of N/L (Neutrophil/Lymphocyte), and lymphocyte from both treatments. IP treatment has a higher RBC, Hb, and hematocrit, while OP treatment has a higher N/L ratio and lymphocyte. The lowest stress marker (e.g., ratio of N/L) indicated that an animal in a comfort zone or situation is 0.19 in a pregnant goat (Nareswari et al., 2021). This lower average N/L ratio compared to unpregnant ewes because progesterone hormone, active during pregnancy, stimulates the central nervous system to produce anti-stress. Logical markers-stress markers could be correlated with a physio, such as lower heart rate and respiratory rate of ewes with OP treatment. In this study, high THI in IP treatment has a higher

Table 3. Mean values (mean±SEM) of microclimate and thermal comfort index between treatments

Variables	Treatment		Normal reference range
	Indoor Pen	Outdoor Pen	
Air Temperature (°C)	23.27±0.16a	23.16±0.16a	20–30°C[1]
Air Relative Humidity (%)	85.19±0.79a	78.99±1.14b	≈60 %[1]
THI	65.22±0.13a	65.13±0.13a	Until 74[1]

[1] Santos et al. (2021)
Means with different letters (lowercase for rows) differ among themselves (T-test; P<0.05)

Table 4. Mean values (mean±SEM) of Respiratory rate (breaths min^{-1}) and heart rate between treatments throughout the day

Periods of the day	Respiratory rate (breaths min^{-1})		Heart rate (beats min^{-1})	
	Indoor Pen	Outdoor Pen	Indoor Pen	Outdoor Pen
07.00	24.40±0.31a	23.30±0.15b	101.30±2.49a	90.50±0.96b
12.00	25.40±0.31a	24.20±0.20b	115.10±2.05a	104.90±2.56b
16.00	25.20±0.29a	24.20±0.25b	111.30±1.87a	103.90±1.51b
Normal reference range	24 to 50[1]		84-135[2]	

[1] Singh et al. (2016) [2] Scott (2015)
Means with different letters (lowercase for rows) within farming systems differ among themselves (T-test; P<0.05).

Table 5. Mean values (mean±SEM) of rectal temperature (ºC), between treatments throughout the day

Periods of the day	Temperature (ºC)	
	Indoor Pen	Outdoor Pen
07.00	38.55±0.06a	38.54± 0.04a
12.00	38.78±0.05a	38.74±0.16a
16.00	38.86±0.06a	38.85±0.04a
Normal reference range	39.5-39.9ºC[1]; 38.3–39.9ºC[2]	

[1] Reece (2015), [2] Wojtas et al. (2014)
Means with different letters (lowercase for rows), within farming systems differ among themselves (T-test; P < 0.05).

risk of inducing stress. It showed that ewes in IP treatment have higher RBC, HB, and hematocrit, can cope with high oxygen needs and are more susceptible to pathogen microorganisms in the body. The immune system will be more responsive to guard against pathogen microorganisms in the body. Higher lymphocyte values in ewes after OP treatment demonstrated that they could be more disease resistant than the one with lower lymphocyte values. Increasing stress levels impact higher catecholamine secretion in ewe's body, and Catecholamines cause the hypothalamus to produce Corticotropin-Releasing Hormone (CRH), which causes the anterior pituitary to produce adrenocorticotropin (ACTH). Cortisol production is stimulated by adrenocorticotropic hormone leading to lymphopenia (low lymphocytes) and neutrophilia (high neutrophilia) in ewes (Satyaningtijas et al. 2014). This result also proves that ewes with access to outdoor pen (OP) treatment had a lower stress level than those from IP treatment.

Performance variable

From all performance parameters collected, only chest size from OP treatment shows a higher and more significant result (P<0.05). This study revealed that sheep receiving OP treatment had other behaviors that did not reduce their ability to grow; otherwise, they were nearly identical to ewes with IP treatment or had a greater ADG (Average Daily Gain) than ewes that confined fully in an indoor pen (IP). This big difference in the ewes can be seen in chest size, which was attributable to the dynamic behavior of ewes in the outdoor pen, which allowed more muscle to accumulate in the chest, resulting in a larger chest circumference.

Table 6. Mean values (mean±SEM) of various hematology parameters of ewes between treatment

Parameter	Treatment		Normal reference range
	Indoor pen	Outdoor pen	
RBC (10^{12}/l)	15.04±0.17a	14.02±0.37b	9-15 10^{12}/l[1]
Hb (g/dL)	13.96±0.26a	12.16±0.32b	9-13 g/dL[2]
Hematocrit (%)	38.87±0.81a	34.66±0.82b	27-30%[3]
MCV (fL)	25.40±0.62a	24.80±0.59a	-
MCH (pg)	8.34±0.94a	7.88±0.89a	-
MCHC (g/dL)	32.25±3.59a	35.38±0.41a	-
WBC (10^9/l)	15.51±1.21a	18.31±1.33a	-
N/L Ratio	0.77±0.05a	0.56±0.06b	≤1.5[4]
Neutrophil (10^9/l)	6.70±0.70a	6.71±0.79a	-
Lymphocyte (10^9/l)	8.73±0.61a	11.51±0.99b	-

[1]Scott (2015), [2]Gogaev et al. (2020), [3]Wojtas et al. (2014), [4]Oramari et al. (2014)
Means with different letters lowercase for rows), within farming systems differ among themselves (T-test; P<0.05). RBC= red blood cell, Hb= hemoglobin, MCV= mean corpuscular volume, MCH= Mean corpuscular hemoglobin, MCHC= mean corpuscular hemoglobin concentration, WBC= white blood cell, N/L Ratio= neutrophil/lymphocyte ratio

Table 7. Mean values (mean±SEM) of various performance parameters of ewes between treatment

Parameter	Treatment		P-value
	Indoor pen	Outdoor pen	
BW Day 0 (Kg)	18.04±0.73a	19.94±0.57a	0.057
BW Day 70 (Kg)	23.66±0.94a	25.69±0.83a	0.120
ADG (gram/day)	80.24±7.93a	82.28±8.31a	0.861
DMI (gram/head/day)	602.59±15.19a	617.25± 8.23a	0.407
FCR	8.49±1.19a	8.18±0.81a	0.827
Chest size Day 70 (cm)	67.03±1.16a	70.11±0.73b	0.037
Height Day 70 (cm)	62.20±1.89a	62.92±2.69a	0.498
Hip Width Day 70 (cm)	17.50±0.24a	17.59±0.31a	0.819

Means with different letters (lowercase for rows) within farming systems differ among themselves (T-test; P< 0.05). BW= body weight, ADG = average daily gain, DMI= dry matter intake, FCR= feed conversion ratio

Table 8. Mean rank (mean±SOR) of welfare index observation of ewes between treatment

	Treatment		P-value
	Indoor pen	Outdoor pen	
Welfare Index	83.36±7585.50a	99.64±9067.50b	0.026

Means with different letters (lowercase for rows), within farming systems differ among themselves (Mann-Whitney test; P<0.05)

Table 9. Mean values (mean±SEM) of conception rate of ewes between treatment

Day	Treatment		P-value
	Indoor pen	Outdoor pen	
104 (%)	70±0.15a	60±0.16a	0.660
138 (%)	80±0.13a	90±0.09a	0.556

Means with different letters (lowercase for rows) within farming systems differ among themselves (T-test; P<0.05)

Table 10. Observation result of ewes behaviors

Behavior	Treatment															
	Indoor pen								Outdoor pen							
	Duration (s)				Frequency				Duration (s)				Frequency			
Session	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Active	23.8	1.8	42.4	208	1.2	0	1.4	3.2	315.6	454.4	168.6	455.8	10	6.8	1.4	5.4
Inactive	869	898.2	822.6	629	2.6	0.2	2.2	2.4	187.6	143.4	717.6	325.6	7	4.4	1.2	2.6
Feeding	7.2	0	35	63	0.8	1.2	0.8	1.8	396.8	302.2	13.8	118.6	6	4.4	1	3

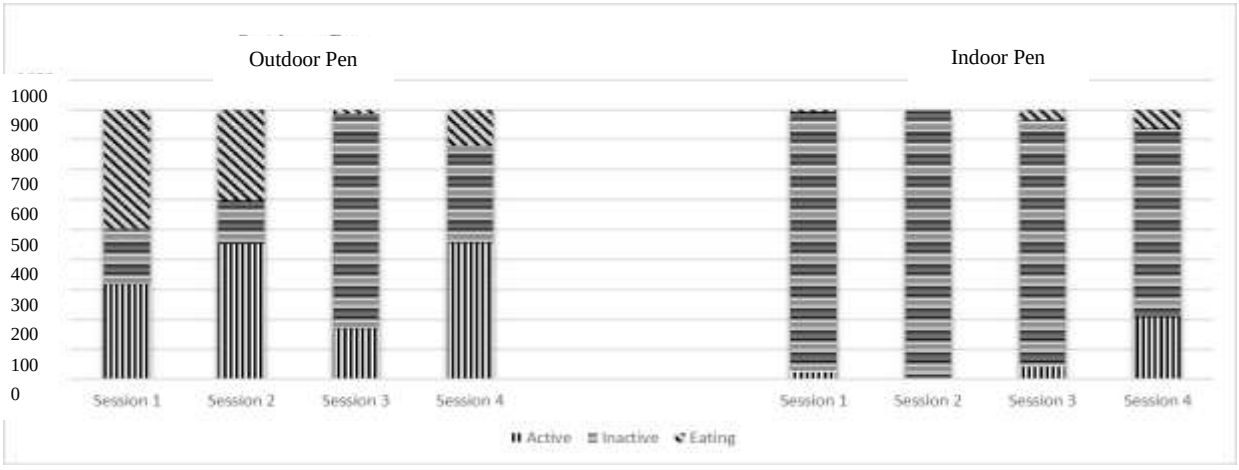

Figure 1. The behavior of ewes by duration

Welfare index observation

OP treatment showed higher results (P<0.05) in animal welfare. A higher index score of ewes in OP treatment demonstrates that providing an outdoor pen could better meet the needs and well-being of ewes without compromising livestock growth.

Pregnancy rate

There is no significant difference shown in the treatment for pregnancy rate (P>0.05) between indoor (IP) and outdoor pen (OP) farming systems. However, if we look at the progression from one measurement to the next, this study could be observed that IP treatment had 70% of the pregnant population in the first measurement, while OP therapy had 60%. Even so, in the second measurement, ewes with OP treatment had a 30% greater pregnancy rate than those with IP treatment, resulting in an overall pregnancy rate of 80% in IP treatment and 90% in OP treatment.

Behavior of ewes

There was an increase in inactive behavior and eating behavior of ewes in outdoor pen treatment. This phenomenon shows that indoor pens did not fulfill ewes need and welfare and have higher stress risk.

In this experiment, ewes behavior was observed in four distinct sessions to acquire a complete picture of the influence of being in an indoor and outdoor pen on ewes behavior. The first session was compelled from 09:30 until 09:45 am; then from 10:15 until 10:30 am; next from 2:30 until 2:45 pm; and last from 3:15 until 3:30 pm (±15 minutes each session). The observation of ewes behavior can be seen more clearly in Diagram 1. Indoor pen treatment was dominated by passive behavior, and the outdoor treatment had a higher result in active and feeding behavior. This observation demonstrates that providing an outdoor pen for ewes can increase their activity level, which increases as the area expands, allowing ewes to roam freely and partake in behavioral patterns as their instincts urge.

Discussion

Table 3 shows the value of indoor and outdoor pens in the Temperature Humidity Index (THI). THI was not significantly different (P>0.05), with an average value of 65.22 and 65.12 for indoor pens, respectively. Rana et al. (2014) calculated the THI value from temperature and relative humidity to determine the heat stress. THI table presents five animal comfort zones: 72< no stress,

72–78 mild stress, 78–89 severe stress, 89–98 very severe stress, and 98> dead animals. It could be concluded that the outdoor and indoor pen environment will not cause stress for ewes. THI results also support the ewes' body temperature, which is not significant despite the expenditure on the outdoor pen because the THI value is not much different and does not cause heat stress in ewes.

Table 4 shows data from observations of ewes' physiological status. It could be seen that ewes given access to an outdoor pen had lower respiratory rate per minute and heart rate or pulse rate per minute. It was also significantly different ($P<0.05$) in the morning, noon, and evening examinations (Table 4). This observation indicates that ewes tend to be more comfortable during rearing. Singh et al. (2016) stated that tropical sheep have a respiratory rate frequency ranging from 24 to 50 breaths per minute, varying from many breeds, and a heart rate ranging from 84 to 135 per minute (Scott 2015). Complete rearing treatment in an indoor pen showed that ewes were more stressed than in outdoor pens. This increase in respiration rate and heart/pulse rate is caused by an increase in oxygen demand by the body, so the supply of blood needs to be increased; this increase directly affects heart rate and respiration rate and is also due to the effect of decreasing blood pressure from peripheral vasodilation (Pachen et al. 2021).

Meanwhile, the rectal temperature of the two treatments in Table 5 shows that values ($P>0.05$) were not much different but still within the average level. Wojtas et al. (2014) said that ordinary sheep's body temperature under thermoneutral conditions ranges from 38.3–39.9^{o}C. This body temperature results from heat balance received and released by ewe's body.

Table 6 also shows significant differences in parameters ($P<0.05$). The number of red blood cells (RBC), amount of hemoglobin (Hb), and the number of ewes blood hematocrit. This difference itself is still within the average level, where the standard red blood cell value for sheep ranges from 9-15 10^{12}/l (Scott 2015; Gogaev et al. 2020), for blood hemoglobin values ($P<0.05$) range from 9-13 g/dL (Gogaev et al. 2020), and a hematocrit of 27-30% (Wojtas et al. 2014). The ewes' situation in an indoor pen, which is usually static, and lack of air movement may indeed cause stress in ewes, resulting in increased cell metabolism throughout the body and an increase in energy demand causing oxygen demand and accelerated erythropoiesis on the bone marrow. There was also an increase in the red blood cell. Hematocrit is the proportion of blood cells compared to plasma, so the increase in hematocrit is in line with the increase in erythrocytes. When the erythrocytes increase, hematocrit value will also increase, and vice versa. High hematocrit in the ewes' body is caused by erythropoiesis, indicating an increase in energy requirements in the body. The environment could affect stress, in this case, the restricted movement of Ewes. Providing access to an outdoor pen is one way to solve this issue. Another step that could be taken is to lessen the impact of other stress factors, such as inadequate feed, poorly ventilated cages, and many other factors.

According to Mills et al. (2012), when animals are frightened or stressed, spleen constriction is increased by epinephrine so that the movement of red blood cells in the blood becomes very vigorous, finally increasing the hematocrit value. This study showed that indoor pens had higher hematocrit values than outdoor pens. It was estimated due to heat stress, lack of freedom of movement, and not being accustomed to contact with humans. Pinto-Santini & Ungerfeld (2019) stated that the potential effect of a ewe's in-estrous phase could also increase the hormone cortisol, which raises stress in animals, and the effect of stress during blood collection can increase since ewes are frightened during the injection. These findings provide another explanation for this abnormal hematocrit value for both treatments. The number of blood erythrocytes causes the difference in hemoglobin in ewes' blood. This increase is in line with an increase in energy requirements resulting in amino acids forming Hb prioritized for energy synthesis so that Hb decreases. Breeding ewes are most likely to be exposed to heat stress. Narayan & Parisella (2017) stated that stress caused an increase in glucocorticoid production, primarily cortisol, which stimulates gluconeogenesis. Increased gluconeogenesis rate for energy fulfillment resulting Hb-forming amino acids (especially glycine and methionine) preferred to enter the Krebs cycle pathway for energy synthesis, which causes the rate of Hb formation to decrease (Duehlmeier et al. 2013).

Table 6 shows physiology from the blood test physiology results of ewes. N/L ratio showed significant differences ($P<0.05$). Maheshwari et al. (2013) said that changes in the ratio of neutrophils/lymphocytes (N/L) are indicators of assessing individual responses to environmental changes. In addition, Gjerstad et al. (2018) stated that the hypothalamic–pituitary–adrenal (HPA) axis regulates circulating levels of glucocorticoid hormones. When stress engages the HPA axis, a spike in glucocorticoid hormones helps the body get ready to deal with the stressor and recover from it. Metabolic processes modulation, immune system, reproduction, behavior, and cognitive functioning are a few of glucocorticoids' many consequences. One of the body's responses to stressors is known to increase glucocorticoid hormones for gluconeogenesis.

As a result, variations in the number of neutrophils and lymphocytes in the blood are one of the physiological measures used to assess livestock stress

levels, including sheep. Oramari et al. (2014) stated that the normal value of the N/L ratio in sheep is ≤1.5. The results of both treatments showed that the ewes were not classified as stressed. However, the value of the N/L ratio of ewes given access to an outdoor pen had a lower ratio value and showed a higher lymphocyte value. The lower value of lymphocytes in ewes' blood in the full indoor pen was due to environmental stress. It caused body ewes to activate the hypothalamic-pituitary-adrenal cortical system causing the hypothalamus to produce corticotrophin-releasing factor (CRF). CRF stimulates the pituitary to release the hormone ACTH (adrenocorticotropin hormone) then adrenal cortex tissue produces corticosteroid hormones. Corticosteroid hormones cause a decrease in lymphocyte levels. This decrease in lymphocytes causes a decrease in the immune system. Pascual-Alonso et al. (2017) stated that stress could cause the body's N/L ratio, cause an increase in hormones secreted by adrenal glands and increase the N/L ratio.

Table 7 shows ewes' growth and weight gain from the two treatments. There was no significant difference in parameters (P>0.05) except the chest circumference of ewes reared in an outdoor pen which showed a higher value between indoor and outdoor pens. This big difference in the ewes' chest circumference was attributable to active activity in an outdoor pen allowing more chest muscle accumulation. It resulted in a larger chest circumference. This claim is supported by research from Zhang et al. (2022), who found that exercising sheep could increase muscle metabolism. These findings are consistent with the findings of this study, which found that exercise in an outdoor pen led to more significant muscle development than indoor treatment. An increase in chest circumference indicated it. Both body weight, ADG (Daily Weight Gain), and other body measurements did not show significant differences.

Table 7 also shows data on livestock dry matter consumption and feed conversion ratio where there is no significant difference in parameters (P>0.05). These results indicate that the availability of outdoor pens for ewes does not affect dry matter consumption. This study also showed that sheep placement in outdoor pens does not affect livestock production but still pays attention to livestock welfare during rearing. Galindo et al. (2016) stated that until now, farming systems have focused on animal nutrition, basically comparing monoculture systems with systems combining grasses and legumes without regard for livestock welfare.

Table 10 shows the results of observing the behavior of ewes due to the provision of access to the outdoor pen. The results show that ewes with access to outdoor pens have higher active feeding behavior than ewes entirely reared in indoor pens. This higher dynamic behavior proves that ewes are more prosperous because they can behave according to their instincts, channel their desire to move and move, and interact without a hitch. In Figure 1, it can be seen that ewes that are entirely reared in indoor pens without having access to their outdoor environment behavior are dominated by inactive behavior wherein a total of four observations, the value is consistently above 70%; this passive behavior also proves that ewes tend to be more stressed due to not being able to move. The result is confirmed by observations of ewes physiological status, where the value is greater than the treatment in outdoor treatment.

Regarding self-breeding in an open environment, it has been shown that no relationship exists between dominance and mating behavior (Ungerfeld et al., 2019). Although it rarely happens, in the mating process with only one ram, there is a possibility that there is a dominating ewe, especially those with a larger and more aggressive body, which causes rivalry among ewes for a ram and can result in a lower pregnancy rate (González-Tavizón et al. 2022). Nevertheless, with the high active behavior of ewes in outdoor pen treatment and the continuous presence of a ram, there is a significant level of social interaction between ewes and rams. It is recommended that further investigations be conducted into the quality of newborn lambs, the miscarriage rate of ewes, and also the death rate of newborn lambs reared using this outdoor pen system, including the study of the ideal outdoor pen based on the area of observation or farm that can more boost livestock growth without compromising livestock welfare.

CONCLUSION

In a tropical country like Indonesia, providing access to an outdoor pen better influences handling stress in livestock. Also, it benefits their welfare, particularly in terms of freedom of mobility, interaction with one another, and an increase in dynamic behavior and feeding. Providing access to the outdoor pen has a positive effect on blood parameters.

ACKNOWLEDGEMENT

The authors would like to express gratitude to the Faculty of Animal Husbandry at Universitas Padjadjaran for allowing us to conduct this research, along with NV Bekaert SA, Indonesia, for providing research funds and equipment for this research.

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Mammalian Contribution to Transmission of *Schistosoma japonicum* Infection in West Lore, Poso, Central Sulawesi, Indonesia

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(received 04-04-2022; revised 18-08-2022; accepted 18-08-2022)

ABSTRAK

Budiono NG, Satrija F, Ridwan Y, Handharyani E, Murtini S, Mananta O. 2022. Kontribusi mamalia terhadap transmisi infeksi *Schistosoma japonicum* di Lore Barat, Poso, Sulawesi Tengah, Indonesia. JITV 27(3):142-151. DOI:http//dx.doi.org/10.14334/jitv.v27i3.3026.

Kajian tentang peran hewan domestik dalam penularan schistosomiasis japonica di Kecamatan Lore Barat Kabupaten Poso masih terbatas meskipun penyakit ini bersifat zoonosis. Penelitian ini menggunakan desain potong lintang untuk mengetahui prevalensi schistosomiasis pada mamalia domestik dan untuk mengidentifikasi kontribusi relatif penularan schistosomiasis dari masing-masing spesies mamalia di Kecamatan Lore Barat. Sampel feses diambil dari 209 ekor hewan (44 ekor sapi, tujuh ekor kerbau, dua ekor kuda, 86 ekor babi, dan 70 ekor anjing) dan telur *S. japonicum* diidentifikasi serta diukur intensitas infeksinya menggunakan metode Danish Bilharziasis Laboratory. Pemeriksaan 1852 sampel feses manusia dengan metode Kato-Katz dilakukan oleh Laboratorium Schistosomiasis Lengkeka. Pencemaran lingkungan dengan telur *S. japonicum* dan kontribusi relatif masing-masing spesies diukur. Prevalensi tertinggi infeksi *S. japonicum* pada hewan adalah pada kuda (100%; 2/2), sapi (54,55%; 24/44), dan babi (51,16%; 44/86). Prevalensi pada kerbau dan anjing masing-masing adalah 28,57% (2/7) dan 32,86% (23/70). Penyumbang utama pencemaran telur *S. japonicum* ke lingkungan adalah sapi (69,74%), diikuti babi (21,95%), dan kerbau (4,71%). Penelitian ini melaporkan tingginya prevalensi infeksi *S. japonicum* pada hewan (45,46%), sedangkan prevalensi schistosomiasis pada manusia rendah (0,59%).

Kata Kunci: Hewan, Koprologi, Manusia, *Schistosoma japonicum*, Zoonosis

ABSTRACT

Budiono NG, Satrija F, Ridwan Y, Handharyani E, Murtini S, Mananta O. 2022. Mammalian contribution to transmission of *Schistosoma japonicum* infection in West Lore, Poso, Central Sulawesi, Indonesia. JITV 27(3):142-151. DOI:http//dx.doi.org/10.14334/jitv.v27i3.3026.

Studies on the role of domestic animals in the transmission of schistosomiasis japonica in the West Lore Sub-district, Poso District, are still limited despite its importance as zoonosis. This study used a cross-sectional design to determine schistosomiasis prevalence in domestic mammals and identify the relative contribution of each mammalian species' schistosomiasis transmission in the West Lore Sub-District. Fecal samples were obtained from 209 animals (seven buffaloes, 70 dogs, 44 cattle, 86 pigs, and two horses). The Danish Bilharziasis Laboratory technique was used to detect the occurrence of *S. japonicum* egg in feces and the intensity of schistosomiasis infection. The examination of 1852 human fecal samples using the Kato-Katz method was carried out by the Laboratory of Schistosomiasis Lengkeka. The measurement of environmental pollution with *S. japonicum* eggs and the relative contribution of each species in the transmission was performed. The highest prevalence of *S. japonicum* infection in animals was in horses (100%; 2/2), cattle (54.55%; 24/44), and pigs (51.16%; 44/86). The prevalence in buffaloes and dogs was 28.57% (2/7) and 32.86% (23/70). Cattle (69.74%) were the main contributors to *S. japonicum* eggs contamination in the environment, followed by pigs (21.95%) and buffaloes (4.71%). This study reported a high prevalence of schistosomiasis in animals (45.46%) while low human schistosomiasis prevalence (0.59%).

Key Words: Animals, Coprology, Humans, *Schistosoma japonicum*, zoonosis

INTRODUCTION

Schistosomiasis is known as a neglected disease due to schistosome infections. The species infect humans are *Schistosoma japonicum*, *S. mansoni*, *S. haematobium*, *S. mekongi*, and *S. intercalatum* (Inobaya et al. 2014). The disease spreads in 78 tropical and subtropical countries worldwide, and the etiologic agents in East and Southeast Asia are *S. japonicum* and *S. mekongi*. Species of animals that can serve as reservoir hosts for *S. japonicum* are cattle, cats, dogs, horses, pigs, and rodents (Saelens & Gabriël 2020).

The disease is zoonotic as the etiological worm species infect humans and more than 40 species of mammals (Li et al. 2014). The cycle of schistosomiasis transmission involves humans (and other mammals) through contact with *S. japonicum* cercariae-infested area where the parasite and intermediate snail host inhabit (McManus et al. 2020). Infected non-human mammals can be the source of *S. japonicum* egg contamination into the environment during animal defecation (Lin 2019). Animals, including non-human mammals, are expelling their feces through open defecation around residential areas (Mohd Zain et al. 2015). Non-human mammals infected with *S. japonicum* contaminate the environment with *S. japonicum* eggs while defecating, and they become schistosomiasis spreaders to the intermediate host snails (Satrija et al. 2015). Lastly, due to this reason, the disease's life cycle is sustainable and can threaten public health as human disease and/or re-infection with *S. japonicum* can occur. Animals infected with *S. japonicum* may show decreased appetite, diarrhea, and weight loss. Animal deaths can occur in severe cases (Lin 2019). Information on the role of mammalian species, especially domestic animals that live close to humans in endemic areas of Indonesia, is still lacking.

Domestic animals are China's primary players in transmitting schistosomiasis (Li et al. 2014). Buffaloes have a role in transmitting the disease for more than 80% in an area of the country (Gordon et al. 2019). A previous study in China reported that the displacement of buffaloes from endemic regions could reduce parasite reproduction rates to below one (R_0<1). That study successfully inhibited schistosomiasis transmission from humans and bovines to the intermediate hosts (*Oncomelania hupensis* snails) (Borlase et al. 2021). Although 46 mammalian species are susceptible to *S. japonicum*, 13 of which live in Indonesia, only a few are important in transmission to humans (Satrija et al. 2015).

Schistosomiasis in Indonesia spreads over twenty-eight villages in two districts of Central Sulawesi Province. Six of these villages are in the Sub-district of West Lore, located in the Bada Valley. The sub-district was firstly reported as an endemic area of schistosomiasis in 2008 (Gunawan et al. 2012). Non-human mammal cases of schistosomiasis in West Lore have never been reported (Gunawan et al. 2012), except for a point survey in 2015 (Nurwidayati et al. 2015) that found three mice infected with *S. japonicum*. Therefore, non-human mammal surveillance and measuring each species' relative involvement in the environmental contamination with *S. japonicum* eggs are needed. This study is designed to determine both human and domestic mammal schistosomiasis prevalence, measure the index of *S. japonicum* contamination by mammals, and calculate the relative contribution of mammals in the transmission of schistosomiasis japonica. These efforts aim to prevent *S. japonicum* eggs from infecting definitive hosts in the environment. Endemic residents at high risk of *S. japonicum* infection must be well-versed in the dangers of the disease.

MATERIALS AND METHODS

Study design and location

A cross-sectional study was performed in the West Lore Sub-district, Poso District, Central Sulawesi Province, in the second semester of 2018. The sub-district is situated 750 m above sea level. The total population of the West Lore community in 2017 was 3300 people (BPS 2018). Most of the residents of West Lore are farmers. Six villages of the West Lore Sub-district are Tomehipi, Tuare, Kageroa, Lengkeka, Lelio, and Kolori. All villages were chosen to be the location of this study. The sampling coordinates were taken using a Global Positioning System (GPS) Garmin to map the spread of schistosomiasis in animals. This study also used secondary data of focal point *O. h. lindoensis* snail reported by Vector-Borne Diseases Research Unit of Donggala, National Institute of Health Research and Development. This study uses secondary data from 1852 samples of the West Lore Sub-district residents obtained from the Lengkeka Schistosomiasis Laboratory to determine human schistosomiasis prevalence. This study also made a distribution map of schistosomiasis using the QGIS application.

Ethical clearance

The study obtained ethical approval from the Institute for Research and Community Service of IPB University with no. 67/2017. Animal owners permitted the researchers to collect fecal samples from their animals with informed consent.

Fecal collection

Stool samples were taken from 209 animals (70 dogs, 86 pigs, two horses, seven buffaloes, and 44 cattle). With the help of local leaders, the researchers informed animal owners the day before sampling. The researchers asked animal owners not to release their animals for morning fecal collection by rectal palpation, the minimum weight of fecal samples from individual studied animals was 20 g. Fresh stool samples that fall around the animal or in the cage were collected for animals that cannot be palpated rectally. Interviews were conducted to obtain supporting data in the form of characteristics of the animal owner, data on the sex of

the animal, the age of the animal, the purpose of rearing the animal (for consumption animals, for sale, or working animals) collected from the animal owner, along with the time of taking stool samples. The feces were stored in plastic clips in an icebox at 2-8°C. The samples were held in the refrigerator until further examination in the laboratory. A positive diagnosis of infection by *S. japonicum* was carried out with the Danish Bilharziasis Laboratory procedure (Carabin et al. 2015; Budiono et al. 2019).

Danish Bilharziasis Laboratory (DBL) technique

The DBL technique is a combination technique that involves filtration and sedimentation. Briefly, 5 g of feces were weighed, dissolved in 50 mL of 0.9% NaCl, agitated, and sieved using graded mesh sieves (filter mesh sizes were 400 µm, 100 µm, and 40 µm). The mixture of samples retained in a 40 µm sieve was transferred into a Baermann glass that already contained 0.9% NaCl. The mixture was then kept for 10 minutes in the darkroom. The sedimented materials in Baermann glass were transferred to a test tube for centrifugation and made into a 2.25 mL mixture by transferred sedimented materials mixed with 0.9% NaCl. For the calculation of *S. japonicum* eggs, the mixture of 150 µL (of the 2.25 mL previous mixture) was transferred into the counting chamber and was added with 850 µL of 0.9% NaCl (to make 1 mL of total volume). The researchers counted *S. japonicum* eggs in three counting chambers to determine the number of eggs per gram of feces. The criteria for *S. japonicum* egg are based on morphological observation of eggs in fecal samples. The morphology of *S. japonicum* eggs is round, with a length and width of 70-100 µm x 50-70 µm. The eggs of *S. japonicum* are non-operculate, have a transparent shell, and have a minute- or rudimentary-lateral spine or knob that may be unnoticeable and difficult to see. If the number of counted eggs differed by >10% with the triple-slide calculation, the egg counting was reexamined (Carabin et al. 2015; Budiono et al. 2019).

Kato-Katz method

The laboratory officers examined human *S. japonicum* infection with the Kato-Katz technique, the technique recommended by the World Health Organization. Briefly, a nylon filter and standard volume plastic mold representing ±41.7 mg of feces were used (He et al. 2018). The nylon filter was used to sieve the feces. The sieved feces were placed in the plastic mold to get the feces weight of ±41.7 mg. Three slides were prepared from each regimented fecal sample, and each slide was examined under the microscope by a trained examiner. A total of 1852 fecal samples from the West Lore Sub-district residents were collected. The prevalence of human schistosomiasis was calculated by dividing the number of infected individuals by the number of examined individuals. In addition, the intensity of infection of each infected individual was calculated.

Total daily egg output

Total daily egg output (TDEE) for each animal species was measured with the following formula (Cao et al. 2016).

$$\text{TDEE} = \left(\frac{\text{total positive}}{\text{examined}}\right) \text{ x TA x P x E x F(g)}$$

where TA is total animals, P is prevalence, E is egg per gram of infected animal feces, and F is fecal weight in gram (g).

The total daily egg output from a host a day (EPD), which represents the potential for contamination by each species, with a slightly different formula as follows (Cao et al. 2016):

$$\text{EPD} = \text{N x P x EPG of infected animals x } \left(\frac{\text{F (g) per day}}{100}\right)$$

where N is the number of animals of a particular species, P is prevalence, and F is the fecal weight per day in gram (g).

Contamination index

The contamination index (CI) for each species was calculated using previously published formulas (Gordon et al. 2012; Gordon et al. 2015). The data needed are the arithmetic mean of eggs per gram of feces (EPG) from infected animals, number of infected animals, and weight of feces (g) with the following formula:

$$CI = A\ x \sum \text{infected animals x F (g)}$$

where A is the arithmetic means of EPG of infected animals and F is fecal weight in gram (g).

The daily weight of feces excreted by each cattle and buffalo is 25 000 g (Gordon et al. 2015), horse 15 000 g (Lawrence et al. 2003), dog 150 g (Brambillasca et al. 2010), pig 500 g (Huaynate et al. 2006), and human 250 g (Rose et al. 2015).

Relative transmission index

The relative transmission index (RTI) was calculated using data on the total number of individuals of each species, the prevalence of each species, the number of feces released by individuals of each species, in addition to the number of eggs per gram of feces of each species (EPG) are required (Cao et al. 2016). The formula of RTI is as follows:

$$\text{RTI} = \frac{\text{N x P x FE x EPG}}{\sum(\text{N x P x FE x EPG})}$$

where N is the number of individuals of particular species, P is prevalence, FE is the number of feces excreted per day (g), and EPG is the total egg per gram of feces.

Data analysis

An infected animal is an animal wherein the researchers found at least one of *S. japonicum* eggs on observation by the Danish Bilharziasis Laboratory method. The total number of eggs was counted using this method. Total egg count in the form of the arithmetic mean of eggs per gram of feces (AMEPG) was changed to the geometric mean of eggs per gram of feces (GMEPG) to indicate the intensity of infection (van Dorssen et al. 2017).

RESULTS AND DISCUSSION

The population of domestic animals and humans in the West Lore Sub-district was 1 623 and 2 520, respectively. The number of samples taken from animals was 209, and humans were 1852, while the overall prevalence of schistosomiasis in animals was 45.46% (95/209). The highest prevalence of *S. japonicum* infection in animals was in horses (100%; 2/2), cattle (54.55%; 24/44), and pigs (51.16%; 44/86). The prevalence in buffaloes and dogs was 28.57% (2/7) and 32.86% (23/70), respectively. Pigs had the highest arithmetic mean of eggs per gram of animal feces (17.02), and buffaloes had the lowest (1.0) (Table 1).

The prevalence of schistosomiasis in humans in West Lore is 0.59%. Schistosomiasis prevalence in humans in each village was 3.11% (6/193) in Tomehipi Village, 1.56% (4/276) in Kageroa Village, and 0.29% (1/347) in Tuare Village. No *S. japonicum* infection was found in the examined persons in the other three villages (Lengkeka, Lelio, and Kolori) (Table 1). The highest degree of *S. japonicum* infection in humans occurred in Kageroa Village (146 eggs per gram of feces or EPG), followed by Tuare Village (96 EPG) and Tomehipi Village (42.67 EPG). The cattle sampled came from all villages except Lengkeka Village. Sampled buffaloes came from Kageroa Village and Kolori Villages. There was one buffalo infected with *S. japonicum* in the two villages. The sampled horses came from Tuare Village, and all are positive for schistosomiasis. The samples of humans, pigs, and dogs were from all the study villages. The study found *S. japonicum*-infected animals from all villages surveyed, while human infection by *S. japonicum* from three villages, namely, Tomehipi, Tuare, and Kageroa (Table 2).

Based on the relative transmission index, this study estimated that the total number of *S. japonicum* eggs released from infected individuals (animals and humans) was 19 580 703 eggs. Cattle are the primary contributor species of schistosomiasis transmission in the West Lore Sub-district (69.74 %). Other species, pigs and buffaloes, contributed 21.95 % and 4.71 % of *S. japonicum* egg contamination. The contribution of the horse, dog, and human species is less than 2% (Table 1). The calculation of the contamination index showed that overall, infected cattle contributed the most (3 576 000) in contaminating *S. japonicum* eggs into the environment every day. The cow contamination index at the individual level was also the highest, with 149 000 *S. japonicum* eggs per day (Table 3).

Figure 1 shows the location of animals infected by *S. japonicum* and the intermediate host snail foci. Schistosomiasis remains a public health concern in Indonesia. Several decades of disease control programs have not succeeded in achieving disease eradication. The World Health Organization sets the elimination of schistosomiasis by 2025 (Deol et al. 2019). Continuous surveillance of schistosomiasis in humans, animals, and snails is the effort to achieve this target. A region targeting the elimination must ensure no new cases of schistosomiasis in humans, animals, and snails for five consecutive years (Fornillos et al. 2019).

Table 1 Relative Transmission Index of *S. japonicum* infection in humans and animals in West Lore Sub-district

Species	Cattle	Buffaloes	Horses	Pigs	Dogs	Humans
Total of the population at risk	168	129	4	987	335	2520
Total of examined individuals	44	7	2	86	70	1852
Total of positive individuals	24	2	2	44	23	11
Prevalence	54.55%	28.57%	100.00%	51.16%	32.86%	0.59%
The arithmetic means of eggs per gram of feces	5.96	1.0	5.5	17.02	3.69	85.1
Fecal weight (g)	25000	25000	15000	500	150	250
Total daily egg excretion	13654956	921382.5	330000	4297117.7	60929.8	316316.7
Relative transmission index	69.74%	4.71%	1.69%	21.95%	0.31%	1.62%

Table 2. Distribution of *S. japonicum*-infected hosts by village using the Danish Bilharziasis Laboratory (for animals) and Kato-Katz (for humans) techniques

Species	Cattle		Buffaloes		Horses		Pigs		Animals		Humans	
Village	+/N	%	+/N	%	+/N	%	+/N	%	+/N	%	+/N	%
Tomehipi	0/1	0	Na	Na	Na	Na	5/16	31.25	5/28	17.86	6/193	3.11
Tuare	13/22	59.09	Na	Na	2/2	100	12/19	63.16	7/16	43.75	1/347	0.29
Kageroa	9/17	52.94	1/3	33.33	Na	Na	8/10	80.00	8/13	61.54	4/256	1.56
Lengkeka	Na	Na	Na	Na	Na	Na	1/5	20.00	3/7	42.86	0/380	0
Kolori	0/2	0	¼	25	Na	Na	12/22	54.55	0/2	0	0/385	0
Lelio	2/2	100	Na	Na	Na	Na	6/14	42.86	0/4	0	0/291	0
Total	24/44	54.54	2/7	28.57	2/2	100	44/86	51.16	23/70	32.86	11/1852	0.59

+/N = Numbers of positive individuals/numbers of examined individuals

Table 3. Prevalence, the intensity of infection (arithmetic and geometric mean of eggs per gram of feces), and contamination index of *S. japonicum* in animals (DBL) and humans (Kato-Katz) in West Lore Sub-district

Species	Positive number	The arithmetic means of eggs per gram of feces	The geometric means of eggs per gram of feces	Overall contamination index	Individual contamination index
Cattle	24	5.96	5.11	3 576 000	149 000
Buffaloes	2	1.0	1.0	50 000	25 000
Horses	2	5.5	3.74	165 000	82 500
Pigs	44	17.02	8.11	374 440	8 510
Dogs	23	3.69	3.23	12 730.5	553.5
Humans	11	85.1	67.5	234 025	21 275

The peculiarity of *S. japonicum* infection from other *Schistosoma* infections is the wide range of definitive hosts, namely humans and other mammals. Domestic animals and wildlife can act as zoonotic reservoirs (Cao et al. 2016). Species' variety of final hosts makes disease control more difficult. Understanding the transmission dynamics of schistosomiasis in each species is critical for developing disease control programs (Satrija et al. 2015). Domestic animals infected with schistosomiasis, particularly cattle, horses, and buffaloes, can be the primary transmission source in another Indonesian endemic sub-district (Lindu) (Budiono et al. 2019). This study firstly reports that domestic mammals contribute to schistosomiasis transmission in the West Lore Sub-district.

Based on the roadmap for eradicating schistosomiasis and the Indonesian government's coordination with other stakeholders, control efforts are carried out to achieve the cessation of local disease transmission by 2020 with an effective surveillance system. The discontinuation of local schistosomiasis transmission means no *S. japonicum* infection in humans, animals, and snails for five consecutive years (2021-2025) (Kemenkes & Bappenas 2018).

This study reported the overall prevalence of schistosomiasis in animals using the Danish Bilharziasis Laboratory technique of 45.46% of the 209 animals examined. The animals examined include horses, cattle, pigs, buffaloes, and dogs. Animal schistosomiasis prevalence varies between 28.57%-100%. The results of the current study support previous studies that *S. japonicum* is zoonotic and can infect mammals other than humans (Li et al. 2019). In addition, the high infection rate in animals in this study (2018) can be the basis for the Indonesian government to develop a strategy for controlling animal schistosomiasis. A previous study (Zhang et al. 2019) highlighted that schistosomiasis control that does not involve animal control is not efficient. This study found animals infected by *S. japonicum* in all study villages: cattle, buffaloes, horses, dogs, and pigs. These *S. japonicum*-infected animals may be the source of desease

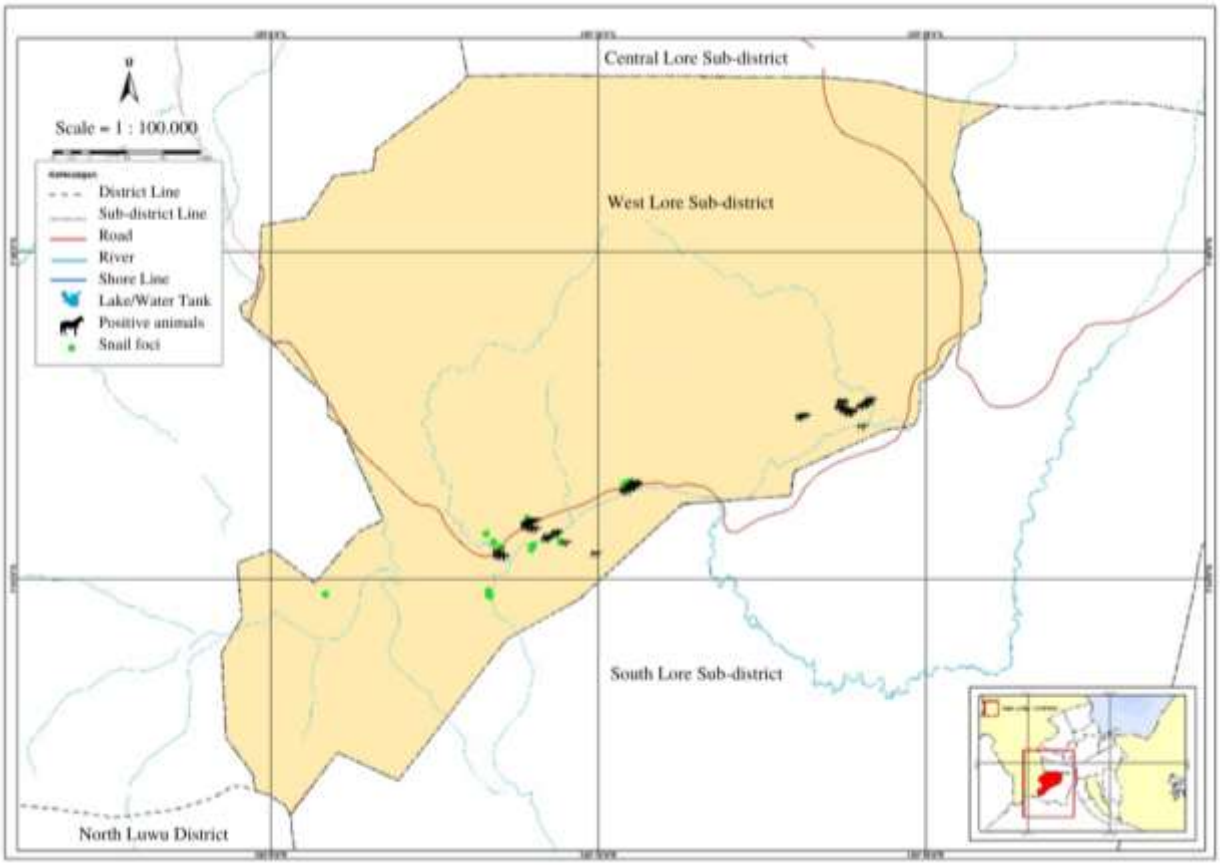

Figure 1. Distribution map of schistosomiasis in animals and intermediate host snails in West Lore Sub-district.

transmission in the West Lore Sub-district. This outcome is dissimilar from a previous study (2010) which failed to find *S. japonicum* infection in 90 animals examined (19 dogs, 55 pigs, one horse, three buffaloes, and 12 cattle) (Rosmini et al. 2014). Another survey did not find *S. japonicum* infection in animals in Lengkeka, Kageroa, Tuare, and Tomehipi villages, with samples of 17, 14, 30, and 31, respectively (Anastasia et al. 2019). The result variations may be due to the different number of samples in collection time and examination methods. Rosmini et al. (2014) found no *S. japonicum* worms in 14 successfully trapped and necropsied rats. No previous studies have demonstrated the presence of *S. japonicum* infection in non-human mammals in West Lore, except for a point survey by Nurwidayati et al. (2015) that reported *S. japonicum* infection in 3 mice. Such studies are still limited to rodents. In addition, the number of animals examined in the study is very limited (Nurwidayati et al. 2015). Previous studies by Rosmini *et al.* (2014) and Anastasia et al. (2019) used the formalin-ether centrifugation method to examine animal feces, while this study used the Danish Bilharziasis Laboratory technique. Detecting *S. japonicum* eggs in feces is still the gold standard in diagnosing schistosomiasis in human and animal species. Due to limited resources, this study used the DBL method, a coprology diagnostic test, to detect *S. japonicum* eggs in animal feces. Even though it has limitations, such as less than 80% sensitivity and 92% specificity for a single-day sample collection (Carabin et al. 2005), the technique has several advantages. The DBL technique's benefits are that it is (1) easy to perform; (2) a quantitative examination tool; and (3) not toxic; (4) it can differentiate between viable and unviable *S. japonicum* eggs; (5) be reexamined for quality control; and (6) be applied to diagnose other trematode infections (Carabin et al. 2005; Anh et al. 2008; Budiono et al. 2018; Lumain & Balala 2018).

Many animals live close to human settlements in West Lore, such as cattle, buffaloes, horses, dogs, and pigs. Most of these animals were raised by releasing them into the wild. Several possible explanations for the difference in results from previous studies are that the study used a different examination technique than the previous one, and the animal species examined differed between this study and prior studies. Theoretically, cross-infection between definitive host species can occur. Cross-infection frequency depends on the access of each animal species to *O. h. lindoensis* snail foci as the transmission site of the final host infection. Schistosomiasis transmission in an area is dynamic and complex due to each location's cultural and biological factors. Variation in yield differences occurs because of animal species' sensitivity to

infection. It can also cause infection variations in different areas (Cao et al. 2016). Previous studies found cattle and goats were more susceptible to *S. japonicum* infection than buffaloes (Liu et al. 2012; Cao et al. 2016).

Human schistosomiasis prevalence in 2018 was 0.59%. The prevalence decreased by 90% compared to the 2010 survey (5.9%; 88/1484) (Rosmini & Risti 2015) and when it was first discovered (2008) (0.8 %; 9/1067) (Satrija et al. 2015). There were variations of prevalence in different study villages in 2018. This study reports a variation in prevalence by village, from 0% in 3 villages (Lengkeka, Kolori, and Lelio) to 3.11% in Tomehipi. The coverage of the schistosomiasis survey in humans in 2018 was 73.49% (1852/2520), higher than the survey coverage in 2008 (38.2%; 1067/2793) and 2010 (61.1%; 1484/2427) (Satrija et al. 2015; Rosmini & Risti 2015). Reports of schistosomiasis cases in the West Lore Sub-district are continuing because of the continuous transmission in the area around the location of the water bodies. Sugiarto et al. (2011) found a significant relationship between human activities in endemic sites in water contaminated with *S. japonicum* cercariae. Most West Lore inhabitants carry out activities such as farming, allowing *S. japonicum* to infect them. Also, *S. japonicum* can infect various definitive host species, including humans, but its transmission is highly dependent on the availability and abundance of sensitive definitive hosts (Webster et al. 2016). In addition, there is a relation between re-infection with the presence of intermediate hosts (snails), which can be a source of transmission to humans (Mujiyanto & Jastal 2014; Pawakkangi et al. 2018).

This study firstly reports the contribution of domestic mammals to the transmission of schistosomiasis in West Lore. In total, infected animals and humans lay as many as 19 580 703 *S. japonicum* eggs per day based on the calculation of the relative transmission index. Infected cattle were the main contributor to the contamination of *S. japonicum* eggs to the environment (69.74%), while infected pigs and buffaloes contributed 21.95% and 4.71% of *S. japonicum* egg contamination. The succinct contribution of horses, dogs, and humans in disease transmission (<2%) was reported. This finding differs from the previous one in those buffaloes was the main contributor (62.3%) to schistosomiasis transmission in the Lindu Sub-district. Cattle and horses in the Lindu Sub-district contributed 24.03% and 11.18% to schistosomiasis transmission, respectively (Budiono et al. 2019). Differences in species contributing to disease at the two study sites may be due to location differences and variations in host availability in endemic locations.

In addition, the difference in disease transmission is closely related to the distribution of intermediate hosts (*O. hupensis* snails) (Xu et al. 2020). The different roles of definitive host species as reservoirs and their contribution to disease transmission can be the basis for disease control at the local level, which can vary from one area to another. Buffaloes and cattle are the main reservoir animals for *S. japonicum* infection in swamp and lake areas in China (Shao et al. 2013; Sun et al. 2017) and the Philippines (Gordon et al. 2015; Tenorio & Molina 2020). This study evaluates disease in domestic animals living in schistosomiasis endemic areas to determine their role in transmitting *S. japonicum* infection to humans. The overall prevalence of *S. japonicum* infection in animals in this study (42.11%) was lower than reported in Coronado (58.2%) (Tenorio & Molina 2020) and North Samar (62.1%) (Gordon et al. 2015) of the Philippines. Differences in results may occur due to sampling locations and examination techniques. The study in the Philippines used the formalin–ethyl acetate sedimentation-digestion (FEA-SD) method, which removed 70% of debris from cattle or buffalo fecal samples (Xu et al. 2012).

The contamination index measures the total *S. japonicum* eggs discarded by infected individuals in the study. The results showed that all infected cattle, pigs, horses, buffaloes, dogs, and humans emitted 3 505 500, 374 440, 165 000, 50 000, 12 730.5, and 234 025 *S. japonicum* eggs/day to the environment, respectively. Each cow lays 149 000 eggs per day, while each buffalo, horse, pig, and dog lays 25 000, 82 500, 8 510, and 553.5 *S. japonicum* eggs per day. Each infected human lays 21 275 *S. japonicum* eggs per day.

The presence of snails as *S. japonicum* intermediate hosts plays a role in infection sustainability and disease control complexity (Satrija et al. 2015). This study attempts to map the distribution of animals infected by *S. japonicum* (Figure 1). It also measures the proximity of infected animals to the location of the *O. h. lindoensis* snails as the study included location data of snail foci sourced from previous research publications (Mujiyanto & Jastal 2014; Pawakkangi et al. 2018). Based on observations during sample collection, the *O. h. lindoensis* snail foci adjacent to the site of the positively infected animals. The location of the snail focus is the home range of animals raised in a wild-free system. The map depiction supports that the infection of animals by *S. japonicum* occurs during activities (such as grazing, wallowing, drinking, and excretion) around the location of the intermediate hosts, *O. h. lindoensis* snails. The snail foci in West Lore Sub-district are close to residential areas and animal husbandry sites. The settlers living in West Lore mostly work as farmers, which allow them to contact the source of schistosomiasis transmission. This condition could be the reason for human infection with *S. japonicum* in endemic villages in West Lore, even though the government has implemented a mass (praziquantel) drug administration program.

The challenge of controlling schistosomiasis in the West Lore Sub-district is community behavior, and people in the area keep domestic animals traditionally. West Lore inhabitants raise animals with four different approaches. First, the animals are fully released. An example of this animal raising is horse and dog species. Second, animals are kept in a cage at night and released during the day. An example of this animal management is cattle and buffaloes. Third, the animals are in pens. Some pig owners are adopting this kind of animal raising. Last, the animals are tied, and the owners move them once or twice a day into (a) different place(s) to let them graze or find food. Some cattle, pig, and buffalo owners raise their animals with this approach.

These animal raising techniques allow domestic mammals to be infected with *S. japonicum* while the animals are doing activities around the water bodies that are also intermediate-host snail foci. Animals' continuous contact with *S. japonicum* cercariae in the snail foci during their activities, such as free grazing/eating, drinking, wallowing in water, defecating, and peeing, can be the possible cause of animal infection by the zoonotic worms. In addition, if the animals have already been infected, they can be the disease spreader. Infected animals defecating around the transmission sites, where the infected snail intermediate hosts (*O. h. lindoensis*) reside, can be the source of other animal and human infections. In addition, these animal rearing methods provide an opportunity for animal infection by *S. japonicum* and persistent disease infection sources. Some sub-district areas consist of land with swamps and puddles of water. The people of West Lore have free access to meadows with running water and grasslands that hold water. Also, domestic mammals in the sub-district have access to water bodies, and some species, such as cattle, buffaloes, and horses, graze in the pasture area. Dogs can roam in such areas as these animals are free-living and have no cages. Residents, free-roaming domestic animals, and rodents doing activities in schistosomiasis active transmission sites (*O. h. lindoensis* snail foci) are receptive to *S. japonicum* infection. Hence, the involvement of rodents and other wild animals in schistosomiasis transmission is still questionable. Further research opportunities are testing praziquantel efficacy in bovines to reduce animal schistosomiasis prevalence in the country.

CONCLUSION

The main contributors to the contamination of *S. japonicum* eggs to the environment were cattle (69.74%), followed by pigs (21.95%), and buffaloes (4.71%). The presence of *S. japonicum* infection in animals using the Danish Bilharziasis Laboratory technique in West Lore is still high, with the highest prevalence in animals (45.46%). On the contrary, the infected humans in the same area are scarce (0.59%). Mass chemotherapy of schistosomiasis in infected humans and animals still needs to be carried out.

ACKNOWLEDGEMENT

This study was funded by the Ministry of Education, Culture, Research, and Technology, the Republic of Indonesia, through the Master Leading to Doctorate for Outstanding Undergraduate (PMDSU) scheme. The authors also thanked the Health Office of the Poso District for permitting this study.

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Effect of *Lactobacillus casei* and Garlic Powder Administration on Broiler Performance, Immune Response and Blood Profile

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(received 08-12-2021; revised 19-08-2022; accepted 19-08-2022)

ABSTRAK

Mangisah I, Yunianto VD, Sumarsih S, Sugiharto S. 2022. Pengaruh *Lactobacillus casei* dan tepung bawang putih terhadap performa, respon imun dan profil darah ayam broiler. JITV 27(3):152-158. DOI: http://dx.doi.org/10/14334/jitv.v27i3.2961.

Penelitian bertujuan untuk mengkaji pengaruh pemberian campuran *Lactobacillus casei* dan tepung bawang putih (LGP) terhadap performa, respon imun dan profile darah ayam broiler. Ayam broiler umur sehari sebanyak 140 ekor dengan BB rata-rata 43.70±0.88 g ditempatkan secara acak dalam 20 unit percobaan. Penelitian didesain dengan rancangan acak lengkap dengan 4 perlakuan dan 5 ulangan. Perlakuan diberikan pada saat ayam umur 22-35 hari (fase finisher). Perlakuan yang diberikan adalah campuran *Lactobacillus casei* dan tepung bawang putih (LGP) dengan konsentrasi 0, 1, 2 dan 3%. Parameter yang diukur adalah profil darah dan bobot organ limfoid (bursa fabrisus, limpa, dan thymus) serta produksi karkas. Data yang terkumpul dianalisis ragam dan jika terdapat pengaruh nyata maka dilanjutkan dengan uji wilayah ganda Duncan pada taraf 5%. Hasil penelitian menunjukkan bahwa pemberian LGP memperbaiki respon imun, bobot karkas dan meningkatkan bobot badan akhir ayam broiler. Pemberian LGP 3% adalah konsentrasi terbaik dalam memperbaiki respon imun dan performa ayam broiler.

Kata Kunci: Darah, Broiler, Bawang Putih, Imun, *Lactobacillus*

ABSTRACT

Mangisah I, Yunianto VD, Sumarsih S, Sugiharto S. 2022. Effect of *Lactobacillus casei* and garlic powder on broiler performance, immune response and blood profile. JITV 27(3):152-158. DOI: http://dx.doi.org/10/14334/jitv.v27i3.2961.

This study aimed to examine effect of giving a mixture of *Lactobacillus casei* and garlic powder (LGP) on broiler performance, immune response and blood profile. One hundred and forty-day old broilers with an average body weight of 43.70±0.88 g were placed randomly in 20 experimental units. Completely randomized design was used in this study, with 4 treatments and 5 replications. The treatment was conducted when the chickens were 22-35 days old (finisher phase). The chickens were given a mixture of *Lactobacillus casei* and garlic powder (LGP) at concentration levels of: 0, 1, 2, and 3%. Parameters measured were blood profile, weight of lymphoid organs (bursa of Fabricius, spleen, and thymus), and carcass production. Data were analyzed for variance and if there was a significant effect, then Duncan's multiple range test was followed at the 5% level. Results showed that the administration of LGP improved the immune response, carcass weight and final body weight of broiler chickens. The administration of 3% LGP improved immune response and achieved the best broiler performance.

Key Words: Blood, Broilers, Garlic, Immune, *Lactobacillus*

INTRODUCTION

In Indonesia, most of broiler chickens are kept in open cages. Indeed, chickens in open cages are very susceptible to stress due to changes in environmental temperature and humidity. The short harvest age of broilers with fast growth makes the chickens susceptible to stress and disease infections. Stress can lower the body's immunity so that it is susceptible to several diseases and reduce growth, and ultimately affect the profits of farmers.

There are stressors that come from outside the chicken body, including environmental temperature, humidity, flock density, and transportation (Tamzil 2014). Causes of stress that come from within the body include infectious diseases and malnutrition (Das et al. 2012). An alternative to minimize the negative effect of stress and disease cases in broiler chickens is the administration of an antibiotic growth promoter. In many countries, the use of growth promoters in the form of antibiotics has been prohibited, because it makes microorganisms resistant and drug residues are found in livestock products.

Eliminating antibiotics in poultry feed leads to decreased growth performance and increased disease prevalence (Bilal et al. 2021). To overcome this

problem, many natural substances have been developed to maintain health and improve physiological functions, modulate the immune system, improve bacterial balance, modulate intestinal morphology, and increase growth rate (Sugiharto 2016). Among the alternative to antibiotic growth promoters are probiotics and prebiotics or a mixture of both (synbiotics). In this study, use of synbiotics was studied in broilers through a mixture of *Lactobacillus casei* (as probiotic) and garlic powder (as prebiotic).

Recent study showed that synbiotics can improve intestinal microflora and stimulate the growth of beneficial bacteria and improve broiler performance (Alagawany et al. 2021). The use of synbiotics has a better effect than the separate supplementation of probiotics and prebiotics on broiler (Sugiharto 2016). Several studies have shown the benefits of synbiotics in the improvement of the intestinal microbial ecosystem (Shanmugasundaram et al. 2019).

Use of synbiotics (*Lactobacillus spp.*, *Saccharomyces cerevisiae* and inulin) for broilers increased lactic acid production, short chain fatty acids, and commensal bacterial population, while it decreased branched chain fatty acids and pathogenic population in the intestine (Markowiak-Kopeć & Śliżewska 2020). The following increased feed conversion ratio warranted the use of synbiotics as feed additives in broiler chicken production (Shanmugasundaram et al. 2019). Other studies have shown that synbiotic supplementation in feed improve performance of broiler chickens under stress (Sohail et al. 2010; Cengiz et al. 2015; Kridtayopas et al. 2019).

The often-used probiotic in synbiotics is *Lactobacillus casei*, which has potential as a probiotic. These commensal bacteria are capable of producing lactic acid and hydrogen peroxide which can suppress the growth of intestinal pathogenic bacteria, provide favorable conditions for improving digestion and utilization of nutrients, and increasing feed efficiency. Study also revealed that the use of *Lactobacillus casei* may compensate for the deficiency of the endogenous antioxidant enzyme superoxide dismutase (SOD) when chickens are under stress (Hill et al. 2018). *Lactobacillus casei* like other lactic acid bacteria (LAB) requires a "food" substrate to support its growth, which is called a prebiotic. Among the ingredients that are classified as prebiotics are fructooligosaccharides and inulin.

Garlic *(Allium sativum*) contains high levels of fructooligosaccharides (FOS) and inulin, and has been shown to be useful as a prebiotic (Sunu et al. 2019; Mudannayake et al. 2015). FOS compounds in garlic bulbs are 1-2% (Dixit et al. 2018), while according to Sunu et al. (2019), FOS content in garlic bulbs is 3.34%. According to (Mudannayake et al. 2015), content of fructan type inulin based on fresh weight in garlic is 18.62%. Garlic, in addition to containing FOS and inulin as prebiotics, also contains various bioactive components, including phenolic compounds, organosulfur and saponins, which improve digestive tract health (Kothari et al. 2019). Garlic's organosulfur and phenolic compounds play a very important role as antioxidants to prevent cell and organ damage from the oxidation process (Kothari et al., 2019; Sunu et al. 2021).

Purpose of this study was to examine effect of synbiotics supplementation (*Lactobacillus casei* and garlic powder) on immune response, blood profile, and carcass production of broiler chickens during finisher period. This study is useful to provide information on synergistic work of *Lactobacillus casei* and garlic powder in improving health and productivity of broiler chickens in terms of immune response, blood profile, and carcass production.

MATERIALS AND METHODS

Production of a mixture of *Lactobcillus casei* and garlic powder (LGP)

The manufacture of synbiotic *Lactobacillus casei* and garlic powder begins with the process of making garlic powder. Garlic was peeled, washed, thinly sliced, and dried under the sun, then finely ground into garlic powder. Pure isolates of *Lactobacillus casei* were obtained from the Microbiology Laboratory of Gadjah Mada University. The isolates were rejuvenated using agar medium (MRSA) at the Animal Physiology Laboratory of the Faculty of Animal Husbandry and Agriculture. A loopful of bacteria was scratched into the medium so that it was slanted and then incubated for 48 hours at 38ºC. *Lactobacillus casei* bacteria with a concentration of 10^9 cfu/ml were then inoculated into 100 ml of 10% sterile skim milk solution and incubated for 24 hours at 37ºC. The incubation results were mixed with 400 ml of sterile 10% skim milk solution and incubated again for 24 hours at 37ºC. The next step, the bacteria were mixed with 1.5% garlic powder and then incubated again at the same temperature for 48 hours. Then, lactic acid bacteria concentration was calculated from the result using total plate count method. Furthermore, the mixture was called a mixture of *Lactobacillus casei* and garlic powder (LGP). LGP was then stored in refrigerator and ready to be applied as a treatment in this study.

Animal and feed experimental

All procedures used in this experiment were approved by the Animal Research Ethics Committee, Faculty of Animal Science, Faculty of Animal and

Table 1. Relative weight of lymphoid organs of broiler chicks

Items (% live body weight; unless otherwise noted)	Dietary treatments			
	Control	LGP1	LGP2	LGP3
Bursa of Fabricius	0.06± 0. 03^{c}	0.08±0.03^{b}	0.10±0.05^{ab}	0.13±0.02^{a}
Spleen	0.10±0.04	0.11±0.05	0.10±0.03	0.08±0.01
Thymus	0.29±0.07^{b}	0.40±0.07^{a}	0.34±0.10^{a}	0.40±0.04^{a}

^{a,b}Values with different superscript within the same row were significantly different. Control= broilers receiving ration without LGF; LGF1= broilers receiving 1% LGF; LGF2= broilers receiving 2% LGF; LGF3= broilers receiving 3% LGF

Table 2. Blood profile and performance of broiler chicks

Items	Dietary treatments			
	Control	LGP1	LGP2	LGP3
Erythrocytes (10^{6}/µL)	2.46±0.17^{b}	2.44±0.75^{b}	2.57±0.81^{ab}	2.79±0.64^{a}
Hemoglobin (g/dL)	7.62±0.16^{b}	8.34±0.75^{a}	8.42±0.81^{a}	8.70±0.64^{a}
Total Plasma Protein (g/dL)	2.16±0.09^{b}	2.52±0.27^{ab}	2.48±0.26^{ab}	2.72±0.44^{a}
Pack cell volume (%)	24.00±2.44^{b}	24.80±3.03^{b}	25.20±1.64^{b}	29.60±2.51^{a}
Leukocytes (10^{3}/ µL)	8.62±2.12^{b}	9.54±1.52^{ab}	10.87±1.25^{a}	11.12±1.80^{a}
Heterophils (10^{3}/ µL)	34.60±5.50^{a}	26.60±2.51^{b}	20.40±3.21^{c}	19.60±0.55^{c}
Lymphocytes (10^{3}/ µL)	60.60±3.51	65.60±12.50	68.20±8.29	68.40±2.61
H/L	0.57±0.13^{a}	0.39±0.04^{b}	0.31±0.05^{b}	0.30±0.0.7^{b}
Weight of carcass (g)	1251.20±24.49^{b}	1381.40±72.54^{a}	1292.60±35.14^{ab}	1304.80±27.81^{ab}
Final body weight (g)	1846.32±58.23^{b}	1947.88±112.91^{bc}	1954.80±149.54^{bc}	2024.54±85.57^{a}

^{a,b}Values with different superscript within the same row were significantly different. Control= broilers receiving ration without LGF; LGF1= broilers receiving 1% LGF; LGF2 =broilers receiving 2% LGF; LGF3= broilers receiving 3% LGF

Agricultural Sciences, Diponegoro University, No. 57-01/A-4/KEP-FPP. One hundred and forty-day old broilers with average body weight of 43.70±0.88 g were used in this study. The chickens were obtained from commercial hatcheries in Salatiga, Indonesia, were randomly assigned to 20 experimental plots. The chickens were reared for 35 days in open cages with litter floors, and were given commercial feed and drinking water on an ad libitum basis. Chickens were given ND B1 vaccine on day-4 and Gumboro on day-14 through eye drops. Lasota ND vaccine was given on day- 21 through drinking water. On days 22 to 35, the chickens were divided into 4 groups (treatments), each consisting of 7 birds. Each treatment had 5 replications. A completely randomized design was used in this experiment. Broilers chicken were receiving treatment feed containing the synbiotic of LGP (0, 1, 2, and 3%), namely: (1) Control= broilers receiving ration without LGP, (2) LGP1= broilers receiving 1% LGP in the ration, (3) LGP2= broilers receiving 2% LGP in the ration, and (4) LGP3= broilers receiving 3% LGP in the ration. Synbiotics were added to 50% of the ration for that day. The next 50% of the ration was given when the feed containing LGP has finished.

Parameter measurement

Parameters measured included performance, blood profile, immune response and carcass weight of broilers. A sample of 20 individuals was taken from the experimental unit. Blood was taken on day 35 from the wing vein with a 3 ml syringe in the morning, using tube containing an anticoagulant (EDTA). The blood samples were then analyzed for the number of red blood cells (RBC), white blood cells, hemoglobin (Hb), heterophils, and lymphocytes using a hematology analyzer referring to the instructions from PT. Prima Alkesindo Nusantara, Indonesia.

After the blood was drawn, the chicken was slaughtered. The abdomen was opened, and the physical condition of the digestive organs was observed. The intestines were pink, there were no fluid and lesions, and blood spots were not found. The liver was not fragile and the liver was red. The digestive organs were then removed and the lymphoid organs were weighed. Carcass weight was measured and recorded. The collected data was tested for variance and if there was a significant effect, then Duncan's multiple region test followed.

RESULTS AND DISCUSSION

Immune response

The mixture of LGP in the ration significantly affected the relative weight of bursa of Fabricius and thymus of broiler chickens (Table 1).

The weight of the lymphoid organs (bursa of Fabricius, thymus, and spleen) is an indicator of immune which is closely related to productivity. Result showed that the weight of bursa of Fabricius and thymus was significantly ($P<0.05$) affected by the administration of the synbiotics. Bursa of Fabricius weight in this study was in the normal range. According to Sugiharto et al. (2018), the range of thymus relative weights was due to the administration of multi-strain probiotics plus vitamins and minerals, which is 0.13–0.20% of body weight, while the thymus is 0.44-0.54%. Increase in weight of bursa of Fabricius and thymus reflects an increase in body's resistance. Size of thymus varies greatly, in which the relative size is greatest in newborn animals, while the absolute size is greatest at puberty. After maturity, thymus undergoes atrophy of the parenchyma and the cortex is replaced by fatty tissue. Thymus that undergoes rapid atrophy is due to a reaction to stress (Cowan et al. 2020; Sunu et al. 2021). Thymus is a T cell regulator that acts on primitive cells originating from the bone marrow and makes them immunologically capable of acting as an antibody-forming body (Cowan et al. 2020).

Increasing of the immune response is caused by an increase in lactic acid bacteria in digestive tract, which affects balance of the microbiota population in the digestive tract. Changes in the microbiota affect the morphology of intestinal wall and cause immune reactions (Rehman et al. 2020), which in turn affect development of immune organs. Commensal microbiota has been recognized as an important inductor for maturation and development of innate defense mechanisms and adaptive immune response of chickens (Brisbin et al. 2008). Immune response is also influenced by T and B lymphocytes, which may be associated with stimulating lymphatic tissue. Thymic atrophy can be caused by acute stress, proinflammatory cytokines, steroid and hormone disorders (Cowan et al. 2020). Recent research shows that the synbiotic (combination of *Enterococcus faecium*, *Pediococcus acidilactici*, *Bifidobacterium animalis*, *Lactobacillus reuteri*, and fructooligosaccharides) can be used as a growth promoter to reduce fear response and stress state of heat-stressed on broilers chicken (Mohammed et al. 2021).

Inclusion of LGP synbiotic leads to changes in intestinal microbial function and this is thought to affect synthesis of B vitamins. Immune regulation is influenced by specific B vitamins (Yoshii et al. 2019). Deficiency of this vitamin can cause hyper homocysteinemia which suppress immune system (Ahmad et al. 2019).

Blood profile

Blood profile of broiler chickens due to the administration of the mixture (LGP) in the finisher phase is presented in Table 2. The addition of 3% LGP gave the best effect on hemoglobin, erythrocyte, TPP, PCV, and leukocyte levels in finisher phase broilers reared in open cages. This result was in line with the findings of Beski and Al-Sardary (2015) that the administration of probiotics and synbiotics increased Hb, erythrocytes, and PCV, and decreased H/L in broiler blood. According to Sunu et al. (2021), hemoglobin value in broiler chickens given synbiotic *Lactobacillus acidophilus* and garlic extract resulted in an increase in Hb, by 7.91 -9.45 g/dL. Sugiharto et al. (2018), erythrocytes in broiler given probiotic was 1.94-2.07 (10^6/µL). Garlic powder contained in synbiotics is known to contain fructooligosaccharides (FOS). This can be useful as prebiotics to stimulate the growth of *Lacobacillus casei* probiotic bacteria in the digestive tract (Kothari et al. 2019), resulting in better digestive tract conditions. This is supported by the population of lactic acid bacteria (LAB) in the cecum of broiler chickens in the LGP treatment particularly at 3%, which was 6.4×10^{11} CFU/g, and has the highest number compared to other treatments. An increase in LAB caused an increase in of lactic acid pruction, which altered pH of digestive tract more acidic and suppressed growth of pathogenic bacteria in the digestive tract. According to Shanmugasundaram et al. (2019), synbiotic supplementation consisting of *Lactobacillus reuteri, Bifidobacterium animalis, Enterococcus faecium, Pediococcus acidilactici* and *fructooligosaccharide* reduced *Salmonella* colonization in the intestines in poultry. Recently, the use of synbiotics (*Lactobacillus spp., Saccharomyces cerevisiae* and inulin) in broiler chickens, increased lactic acid, short chain fatty acid, and beneficial bacteria populations, while decreasing branched chain fatty acids and gut pathogen populations (Śliżewska et al. 2020).The increase in LAB due to LGP supplementation was also caused by LAB being able to stimulate development of intestinal epithelial cells, so that it is more optimal in process of absorption of nutrients in feed including protein and Fe or iron, which are raw materials for hemoglobin and erythrocyte synthesis. Beski & Al-Sardary (2015) stated that giving synbiotics increases blood hemoglobin levels, because production of lactic acid from synbiotics makes pH condition of the small intestine more acidic so that absorption of Fe as a raw material for hemoglobin increases. Increased hemoglobin levels can also be

caused by increased production of vitamin B complex by bacteria in the small intestine (Yoshii et al. 2019). Vitamin B12 deficiency in livestock can cause weakness and anemia because vitamin B12 is involved in the formation of hemoglobin (Ahmad et al. 2019).

Total plasma protein increased with the increasing levels of LGP. This means that LGP synbiotics not only improve the condition of the digestive tract, but also increase the absorption of nutrients, especially protein as a raw material for blood synthesis. With the increase in total plasma protein due to administration of 1-3% LGP, the synthesis of immune organs and production of antibodies also increases. Blood plasma proteins play a role in maintaining colloid osmotic pressure, as a very fast substitute for amino acids, play a role in gluconeogenesis, transport minerals and hormones, and play a role in the formation of enzymes and the immune system in the body (Filipović et al. 2007).

The hematocrit value in the 3% LGP treatment was significantly higher than the other treatments. Packed cell volume (PCV) are very sensitive to the level of protein intake and protein digestibility by poultry (Saki et al. 2018). Administration of 1-3% LGP improves gut microbiota, lowers pH, and increases protein digestibility so that the availability of protein for red blood synthesis increases, which is indicated by an increase in PCV. Giving a combination of probiotics and prebiotics in feed significantly increased the PCV, RBC, and Hb of guinea fowl (Habibu et al. 2016). It was reported that hematocrit and red blood cell volume have a very large correlation with each other. If the average hematocrit decreases, the hemoglobin level becomes lower (Ahmad et al. 2019, Habibu et al. 2016).

Leukocytes in this study showed a significant increase ($P<0.05$). This means that giving LGP is able to modulate the number of white blood cells, which means that the production of antibodies increases to fight foreign objects in the body. The administration of LGP can increase the digestibility of nutrients, especially protein which is the material for the synthesis of blood components, including leukocytes. The increase in the number of leukocytes is also related to the immune system in reducing pathogen attacks. If the attack of pathogens increases, it will further increase the production of leukocytes in the blood (Sugiharto et al. 2018; Rehman et al. 2020). The response of broilers to stressors depends on the type of stress experienced. The increase in the number of leukocytes illustrates the presence of a humoral and cellular resistance response to pathogenic agents that cause disease. An increase in the number of leukocytes indicates an improvement in the body's defenses/ immune system. (Saki et al. 2018). Efficient activation of host defense and timely restoration of immune homeostasis are closely related to success against bacterial pathogens (Bayona et al. 2017). This research results indicate that the range of broiler chicken leukocytes was normal. According to Sunu et al. (2021), the range of leukocytes in broiler chickens treated with synbiotics (garlic extract and *L acidhophilus*) was $7.43x10^3$/ ml– $11.60x10^3$/ml.

The administration of LGP in finisher phase broilers significantly reduced blood heterophil levels. Heterophils are a type of granulocyte from leukocytes that act as the first leukocyte defense that appears during acute inflammation and infection. Lymphocytes which are agranulocytes of leukocytes have a role as advanced defense cells that play a role in lysing toxic cells (T lymphocytes) and forming immunity (B lymphocytes). The heterophil-lymphocyte ratio (H/L ratio) can be referred to as an indicator of stress in chickens (Saki et al. 2018). In this study, the number of lymphocytes did not decrease. This means that the administration of LGP is able to capture free radicals and suppress the hormone corticosterone so that the production of lymphocytes remains normal and does not decrease (Kridtayopas et al. 2019). The decrease in the H/L ratio in the group of chickens treated with LGP synbiotic may be influenced by the increase in the number of beneficial microbes in the digestive tract, which stimulates afferent neurons through a cytokine neurohumoral route and lead to reduced levels of corticosterone in the bloodstream (Sohail et al. 2010).

The H/L ratio in the 1-3% synbiotic treatment decreased significantly, a lower H/L ratio indicated an increase in body resistance. The number of lymphocytes is most commonly found in white blood cells and is considered an indicator of the health level of livestock because lymphocytes are the body's most important defense cells (Saki et al. 2018). Some researchers report that the H/L ratio is more acceptable as an indicator of mild or severe stress when compared to measuring corticosterone hormone levels (Sunu et al. 2019; Saki et al. 2018). The decrease in the H/L ratio in this study may be due to the effect of synbiotic LGP in inhibiting stress caused by nutrition and the environment. (Kleniewska & Pawliczak 2017) stated that synbiotics containing *Lactobacillus casei* with inulin are effective compounds to protect the body from oxidative stress damage. Synbiotics may have a positive effect on the blood plasma antioxidant capacity and the activity of selected antioxidant enzymes. Stress can stimulates the adrenal glands to secrete hormones such as direct effect estrone for has a direct effect to analyze a lymphatic cell which causes an increase in H/L ratio (Beski et al. 2015).

Administration of LGP synbiotic significantly ($P<0.05$) increased carcass production and final body weight. Giving 1-3% LGP resulted in the same carcass weight. Giving 3% LGP resulted higest final body weight. This was due to the role of *Lactobacillus casei* and garlic in the digestive tract, which causes an

increase in lactic acid bacteria, and improves microbial balance and better gut health. The gut microbiota plays a decisive role in the manipulation of intestinal epithelial proliferation, vitamin synthesis, and host energy metabolism. Changes in the microbiota can affect the morphology of the intestinal wall and cause immune reactions which in turn can affect the energy expenditure and development of chickens (Rehman et al. 2020). Administration of *Lactobacillus casei* DBN023 to chicks significantly increased their jejunum villi height, villi-to-crypt depth (V/C) ratio, and muscle thickness, enhanced gut mucosal immunity, regulates cytokine balance, and effectively reduces gut inflammation (Wang et al. 2019). The increase in gut villi height causes an increase in nutrient absorption for the synthesis of body tissue, and ultimately increases carcass weight and final body weight.

CONCLUSION

The administration of 3% LGP improved the weight of bursa fabricius, thymus, H/L ratio and yielded the best broiler performace.

ACKNOWLEDGEMENT

The Authors extend their gratitude to Universitas Diponegoro, for the valuable funding of "Program Riset Pengembangan dan Penerapan (RPP)", contract number: 233-93/UN7.6.1/PP/2021.

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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:

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