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PREFACE

In this edition, Volume 25 No 3, we proudly present articles from animal and veterinary sciences including genetics; reproduction, feed technology, and veterinary. The articles published in this edition are:

"Genetic Variants of Milk Protein Genes and Their Association with Milk Components in Holstein Friesian Cattle"; "Effect of Incubation Time During Sperm Sexing Process on Sperm Quality of Pasundan Bull"; "Use of Infrared Thermography for Identifying Physiological and Hematological Conditions of Young Sapera Dairy Goats"; "Effect of Combination of Follicle Size, FSH and Cysteamine on *In Vitro* Production of Sheep Embryos"; and "Synergistic Effect of Biofat and Biochar of Cashew Nutshell on Mitigate Methane in the Rumen".

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 2020

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Genetic Variants of Milk Protein Genes and Their Association with Milk Components in Holstein Friesian Cattle

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ABSTRAK

Asmarasari SA, Sumantri C, Gunawan A, Taufik E, Anggraeni A. 2020. Variasi genetik gen protein susu dan hubungannya dengan komponen susu sapi Friesian Holstein. JITV 25(3): 99-111. DOI: http://dx.doi.org/10.14334/jitv.v25i3.2502

Kandungan protein dalam susu sapi menjadi indikator kualitas susu sapi di masa yang akan datang. Atas dasar tersebut, perbaikan genetik untuk menghasilkan sapi perah Friesian Holstein (FH) menjadi penting dilakukan. Tujuan dari penelitian ini adalah untuk mengevaluasi varian genetik dari gen protein susu dan pengaruhnya terhadap sifat komponen susu sapi FH. Sapi yang diamati sebanyak 100 ekor. Sapi FH yang digunakan memiliki status fisiologis periode laktasi 1-3 dan bulan laktasi 1-12. Variasi genotipe gen protein susu diidentifikasi menggunakan metode RT-PCR *(Real Time-Polymerase Chain Reaction)*. Analisis komponen susu yang dilakukan meliputi kandungan protein, lemak, laktosa, dan *Solid Non Fat* (SNF) dengan menggunakan alat *Lactoscan*. Analisa genotyping dimulai dari ekstraksi DNA dan amplifikasi gen menggunakan metode RT-PCR. Hasil penelitian menunjukkan bahwa kadar protein susu dipengaruhi ($p<0.05$) oleh varian genetik gen CSN1S1-192 dan CSN2-67. Kadar lemak susu dipengaruhi ($p<0.05$) oleh varian genetik gen CSN1S1-192 dan CSN3. Sementara itu, kadar SNF susu dipengaruhi ($p <0.05$) oleh varian genetik gen CSN-BMC9215, CSN-BMC6334, CSN1S1-14618, CSN2_67, dan CSN3. Kadar laktosa susu dipengaruhi ($p<0.05$) oleh varian genetik gen CSN-BMC9215 dan CSN2-67. Disimpulkan bahwa varian genetik dari gen protein susu memiliki hubungan dengan komponen kimiawi susu sapi (protein, lemak, SNF, dan laktosa).

Kata Kunci: Varian Genetik, Gen Protein, Komponen Susu

ABSTRACT

Asmarasari SA, Sumantri C, Gunawan A, Taufik E, Anggraeni A. 2020. Genetic variants of milk protein genes and their association with milk components in Holstein Friesian cattle. JITV 25(3): 99-111. DOI: http://dx.doi.org/10.14334/jitv.v25i3.2502

Protein content in milk is an important indicator of milk. Accordingly, genetic improvement to produce Holstein Friesian (HF) dairy cattle is important. The objective of this study was to evaluate the genetic variant of milk protein genes and its effect on milk component traits of Holstein Friesian (HF). A total of 100 HF were used in this study. The HF cattle used have physiological status in the lactation period 1 up to 3 and lactation change of 1 up to 12 months. Genotype variants of milk protein genes were identified using Real Time-Polymerase Chain Reaction method. Analysis of milk component was carried out covering the component of protein, fat, lactose, and solid non-fat (SNF) by using a milk quality measuring device (Lactoscan). Genotyping of cattle blood samples consisted of DNA extraction, genes amplification using the RT-PCR method. The result showed that protein milk was significantly affected ($p<0.05$) by the genetic variants of CSN1S1-192 and CSN2-67 genes. Fat milk was significantly affected ($p<0.05$) by the genetic variants of CSN1S1-192 and CSN3 genes. Meanwhile, solid non-fat milk was significantly affected ($p<0.05$) by the genetic variants of CSN-BMC9215, CSN-BMC6334, CSN1S1-14618, CSN2_67, and CSN3 genes. Lactose milk was significantly affected ($p<0.05$) by the genetic variants of CSN-BMC9215 and CSN2-67 genes. It was concluded that genetic variants of the milk protein genes have an association with the component of cow's milk (protein, fat, solid non-fat, and lactose).

Key Words: Genetic Variant, Protein Genes, Milk Component

INTRODUCTION

Recently, genetic improvement in dairy cattle is not mainly to increase the amount of milk production but also to increase milk quality which is the high milk protein content. Milk has high-quality proteins, due to having sufficient amino acids to support the function of proteins in the human body (Davoodi et al. 2016). Franzoi et al. (2019) revealed that milk protein component is influenced by several factors, i.e., breed, lactation, season, and genetic polymorphism. Fresh milk with high protein is needed to meet the people nutrition requirement. The right component of milk protein is very useful to overcome health problems,

such as hypoallergenic. Besides, high protein milk is needed in the milk processing to produce high quality of dairy products. Therefore, genetic improvement is needed to produce Holstein Friesian cattle which have high milk protein carrier properties. One method for genetic improvement is a method of genotype selection. Genotype selection method in breeding programs is one of the newest methods to improve dairy cattle characters. Ozdemir et al. (2018) stated that the component of milk protein has a relationship with genetic polymorphisms. Genetic improvements to high protein milk will provide a fast and accurate selection response if the selection is applied at the DNA (nucleotide) level.

Ferretti et al. (1990) revealed that the protein component of cow's milk is controlled by two family genes of casein and whey. Casein, the largest component of milk protein (78-82%) is controlled by four casein genes, i.e., αs1-casein, β-casein, αs2-casein, and κ-casein. The location of these genes is very close to each other along 250 kilobases (kb) on chromosome 6 cattle (BTA 6q31). The close relationship, causing the casein gene inheritance often acts as a cluster. The protein component is predominantly produced by the αS1-casein (30.6%) and β-casein (28.0%) genes. Ferretti et al. (1990) stated that each gene was also known as CSN1S1, CSN2, CSN1S2, and CSN3 name genes. Farrell et al. (2004) explained that whey is a component of milk protein in a lower portion (12-18%). Laible et al. (2016) revealed that milk protein genes have the potential to be explored for genotypic selection to improve the cow's milk component. Bhat et al. (2019) added that the milk protein gene was reportedly relating to the nature of the cow's milk component. An association study between milk protein genes and milk components was carried out to see the impact of milk quality that occurs from the molecular breeding process in increasing protein component in milk based on milk protein genes. Holstein Friesian (HF) is a type of dairy cattle that is most widely imported by Indonesia. Many researchers stated that the milk component is an indicator of the quality of milk that determines the economic value of fresh milk (Davoodi et al. 2016; Thomas & Sasidharan 2015; Radika & Ajithkumar 2018; Ohlsson et al. 2017). Therefore, the aim of this study was to evaluate the genetic variants of milk protein genes and their effects on the milk component of HF cattle.

MATERIALS AND METHODS

Location and sample

This research was conducted at the dairy cattle experimental station in the Indonesian Research Institut for Animal Production which located at Ciawi-Bogor-West Java-Indonesia. A total number of 100 female HF cattle were used in this study with physiological status between 1-3 lactation periods and 1-12 months lactation. Milk samples were obtained after milking of individual cow, each sample was 500 ml. Analysis of milk component was carried out covering the component of protein, fat, lactose, and solid non-fat (SNF) by using a milk quality measuring device (Lactoscan). Milk sample collection for milk component traits analysis was done once a month, each sampling was done morning and evening. Sampling was carried out for 12 months of lactation with 3 periods of lactation. Blood samples were collected from lactating cows collected from the vena coxygea. Genotyping series consisted of DNA extraction from blood samples, genomic DNA binding, elution, primer design for amplification of milk protein genes of CSN1S1, CSN1S2, CSN2, and CSN3 was used Primer Express. Some factors that must be considered in the primer design, i.e., the primer temperature must be between 58-60^{o}C, choose a minimum of 3 best primer designs that at least likely to form a secondary structure (primer-dimers and harpin loops), check with BLAST (Basic Local Alignment Search Tool) to see the specificity of each set of target primers, the range of acceptable amplicons was 50 between 250 base pair (bp) but the most recommended was around 70-130 bp. The primer designs of the milk casein genes for the Real-Time PCR technique were shown in Table 1.

Real-Time Polymerase Chain Reaction (RT-PCR)

DNA extraction was carried out from fresh HF cattle blood samples using the illustra™ blood genomicPrep Mini Spin Kit. Detection of nucleotide diversity was carried out using RT-PCR with the composition of the reagents as in Table 2. The process of amplification and detection of diversity was carried out under pre-denaturation conditions at a temperature of 95 ºC for 20 seconds and 40 cycles consisting of denaturation of 95 ºC for 3 seconds and annealing at 60 ºC for 30 seconds.

Data analysis

Relationship of genetic variants of milk protein genes to the component traits of milk was carried out with a general linear model using SAS 9.1 software.

RESULTS AND DISCUSSION

Genetic variants of milk protein genes

Genetic variants of milk protein genes that were identified using the RT-PCR method were presented in Table 3. Based on the genotyping of the CSN1S1_192 locus of the αS1-casein gene, three genotypes have been

identified, i.e., AA, AG, and GG genotypes, therefore have two types of allele (A and G). Genotype identification showed that the highest frequency was AG genotype (0.67), followed by AA genotype (0.33) and GG genotype (0.00). Genotype identification for CSN2_9215 locus of the β-casein gene was identified and resulting in three genotypes, i.e., TT, TG, and GG genotypes, so that there were two types of alleles, i.e., T and G alleles. The study showed that the highest genotype was TG (0.40), followed by GG (0.25) and TT (0.25).

Meanwhile, for the CSN2_BMC6334 locus, three genotypes were identified, i.e., AA, AG and GG genotypes, resulting in two types of alleles, i.e., allele A and G. Observations on 100 HF cows showed that 25 heads of HF cows had AA genotypes, 44 heads had AG genotypes, and 31 heads had GG genotypes. Genotyping at the CSN2_67 locus of the β-casein gene resulted in three types of genotypes, i.e., AA, AC and CC genotypes; so that there were two types of alleles, i.e., alleles A and C. Genotype identification showed that the highest frequency was AC genotype (0.43), followed by CC genotype (0.35), and AA genotype (0.22).

Results of CSN1S2 locus genotyping, there were three types of genotypes, i.e., TT, TA, and AA genotypes, with two types of alleles, i.e. T and A. alleles. Genotype identification showed that the highest frequency was TT genotype (0.79), followed by TA genotype (0.16), and AA genotype (0.06). The CSN3 locus of the κ-casein gene produced 3 genotypes, i.e., GG (0.06), GT (0.36), and TT (0.58). For the CSN1S1_14168 locus, there were identified three types of genotypes, i.e., CC, CT, and TT, and two types of alleles were obtained, i.e. C and T alleles. Genotype identification showed that the frequency of CT genotypes was highest (0.47), followed by TT genotypes (0.30), while the lowest was CC genotype (0.23).

In this study, it was known that the A allele dominant at the milk protein genes. Volkandari et al. (2017) stated that polymorphic which AA genotypes and A allele at locus κ-casein were frequently commonly found in Holstein Friesian cattle. Zyiad & Fawzi (2014) reported that A and B genotypes were favorable alleles in Palestinian Holstein-Friesian cattle. Similarly, some researchers found that A allele in Holstein-Friesian was as dominant allele in milk protein genes (Volkandari et al. 2017; Barbosa et al. 2019; Huang et al. 2012).

Variant genetic of milk protein genes was influenced by cattle breed. Trakovickà et al. (2012) found that in the crossbred of Simmental and Holstein's cattle, A allele was frequently higher than the B allele. Meanwhile, Deb et al. (2014) reported that A allele more frequent than B allele in Frieswal cattle (HF x Sahiwal). Zepeda-Batista et al. (2015) added that B allele more frequent than A allele and E allele in Mexican Jersey cattle. Furthermore, Ren et al. (2013) revealed that the B allele was higher than the A allele. Many researchers from different countries reported that A allele was more dominant in milk protein genes at dairy cows than other allele (Djedovic et al. 2015; Brka et al. 2010).

Several researchers had proven that milk protein genes were highly polymorphic, containing very large amounts of SNP polymorphism (Schopen et al. 2011; Huang et al. 2012). Those studies informed that a direct relationship to protein from both single SNP and haplotypes in CSN1S1-CSN2-CSN1S2 with block haplotypes. In contrast, there was no significant relationship for a single SNP or haplotype in CSN3 blocks. This proves that CSN2 and CSN1S2 contain the highest locus in causing causative DNA variants (SNP). The most significant results were found for the CSN2_67 SNP C allele which was consistently related to protein superiority. SNP CSN2_67, as a substitution of C to A, on codon 67 in the ß-CN gene, caused histidine to replace proline in the amino acid sequence (Schopen et al. 2011; Huang et al. 2012; Cecchinato et al. 2018).

Association of genetic variants of milk protein genes on milk component traits

Examination of the effect of milk protein genes of CSN-BMC9215, CSN-BMC6334, CSN1S1-192, CSN1S1-14618, CSN1S2, CSN2-67, and CSN3 on milk protein levels are presented in Table 4. During 12 months lactation, it was known that genotypes of milk protein genes of CSN1S1-192 and CSN2-67 had significant effect ($p<0.05$) on protein levels of cow's milk. Milk protein levels from the AA genotype of CSN1S1-192 were higher (3.63%) than AG genotype (3.37%). Meanwhile, in the CSN2-67 gene, the highest levels of milk protein were obtained from the AA genotype (3.75%), then followed by the CC (3.73%) and AC (3.68%). Previously, Hamza et al. (2011) reported that CN genotypes had significant effect on milk protein component. Sigl et al. (2012) revealed that milk protein gene expression has close relationship to component of milk protein. Furthermore, Sigl et al. (2012) explained that the process of milk protein synthesis, including transcription, post-transcription, translation, and amino acid supply was controlled at various levels in mammary epithelial cells. The gene that codes for this protein is regulated by a complex interaction of peptides and steroids hormones, especially the lactogenic hormone prolactin, insulin, and hydrocortisone; and cell and cell-substratum

Table 1. Primer sequences of milk casein gene for the Real-Time Polymerase Chain Reactin (PCR) technique

Gene	Gene Bank	Position	Primary Sequence (5'->3')	Temperature (^{0}C)	Mutation
CSN1S1	X59856	26181	F: CCATCATTCTCTGACATCC	61.2	G>A
		Exon 17	R: AGGCAACAATATGCAGTC	61.6	
			VIC:CTCTGAGAACAGTGGAAAGACTACTAT GCC	74	
			FAM: CTCTGAGAACAGTGAAAAGACTACTAT GCC	70.7	
CSN1S2	M94327	13231	F: GCCGAATAAACATCCTGTCAACT	58	A>T
		Intron 13	R: CCCCTAAACAACCAGAGAGATTCA	59	
			VIC : CCTTCACCATAGTACT	67	
			FAM: TTCACCATAGTTCTAC	67	
CSN3	AY380228.1	13975	F: GAAGAGGTTAAACAGAAAGATCAATAAGATAG	58	G>T
		Intron 4	R: GACCAAAAATCATGTAGACAGTGTGA	58	
			VIC: AACATTTTGAGAGTCTAGGC	66	
			FAM: TTTGAGATTCTAGGCAAC	67	
CSN2	NW_001495211	9215	F:5'-CTTATGCACAATTATTTCACCACATG-3'	58	G>T
		Exon 1	R:5'-TCAGTATTTTTCCCTCATATGCTCAT-3'	58	
			VIC:5'- CTCATTTCACATCTTG-3'	67	
			FAM: 5'-TCACATCGTGTTTTTGA-3'	67	
CSN2	M55158.1	6334	F: 5'-CAGGATGATTGAGAGACATGTATGC -3'	59	A>G
		Intron 4	R: 5'-ACAGTCCATAGGGTCATACAGAGTTG-3'	59	
			VIC :5' -TGCAAAGTTGCTTCAG-3'	67	
			FAM :5'- CAAAGTTACTTCAGCCC-3' T	66	

Source: Huang et al. 2012

Table 2. Components and compositions reaction of the Real-Time Polymerase Chain Reactin (PCR) process

Compositions reaction	Compositions (µl)
DNA	5
Taqman GTXpress Master Mix (2x)	12.5
Custom Taqman SNP Genotyping Assays	1
PCR grade water	6.5
Total volume	20

Table 3. Genotype and allele frequencies of milk protein genes in Holstein Friesian cattle

Gene	N	Genotype Frequency			Allel Frequency	
CSN1S1_192	98	AA(0.33)	AG(0.67)	GG(0.00)	A(0.66)	G(0.34)
CSN-BMC9215	100	TT(0.25)	TG(0.40)	GG(0.35)	T(0.45)	G(0.55)
CSN-BMC6334	100	AA(0.25)	AG(0.44)	GG(0.31)	A(0.47)	G(0.53)
CSN2_67	98	AA(0.22)	AC(0.43)	CC(0.35)	A(0.44)	C(0.56)
CSN1S2	90	TT(0.79)	TA(0.16)	AA(0.06)	T(0.87)	A(0.13)
CSN3	90	GG(0.08)	GT(0.29)	TT(0.63)	G(0.22)	T(0.78)
CSN1S1_14168	100	CC(0.23)	CT(0.47)	TT(0.30)	C(0.47)	T(0.54)

interactions. Olenski et al. (2010) reported that there was a favorable genetic relationship of the A2 allele of the CSN2 gene with cow's milk protein. Ozdemir et al. (2018) reported that CSN1S1 gene affected milk protein component. Milk protein level was different between the CSN1S1 genotypes (Mangia et al. 2019). Zhou et al. (2019) stated that milk component traits were associated with the CN gene family, including CSN1S1 and CSN1S2. Bonfatti et al. (2010) explained that haplotypes that include CSN2 genes has been shown to influence milk protein component, suggesting that inheritance units can reach large genomic regions. Meanwhile, Huang et al. (2012) reported that the A and C alleles were associated with lower κ-CN concentrations. The other finding showed that there was relationship between the k-CN B allele and high protein component in Holstein breeds (Mohammadi et al. 2013). Furthermore, some researchers reported that B allele had a favorable and significant effect on milk protein components (Morkūnienė et al. 2016; Caroli et al. 2009). Relation of month lactation to protein component was reported by several researchers. In several previous studies, it was found that there was an inconsistency of the effect of lactation month on milk protein component. Some researchers (Jónás et al. 2016; Gurmessa & Melaku 2012) reported that milk protein component significantly influenced by lactation month. Meanwhile, Sudhakar et al. (2013) reported that the protein component in milk did not change in different lactation months. Çobanoglu et al. (2016) revealed that the highest protein component occurred in the first three months of lactation, where after that there was a decrease in protein component along with the increase in lactation month.

The effect of milk protein genes on milk fat levels are presented in Table 5. It was known that the genotype which affecting significantly ($p<0.05$) cow's milk fat levels were the genotype from CSN1S1-192 and CSN3 genes. The genotype of the CSN1S1-192 gene affected milk fat levels at four different lactation months, i.e., 2^{nd}, 7^{th}, 8^{th}, and 11^{th}. There was an inconsistency in the influence of the genotype of the CSN1S1-192 gene. At the lactation months 2^{nd} and 11^{th}, the AA genotype showed higher milk fat levels (4.17% and 4.16%) compared to AG genotype (3.89% and 4.08%). Conversely, at the lactation months 7^{th} and 8^{th}, the AA genotype (4.1% and 4.21%) of fat levels showed lower compared to AG (4.23% and 4.28%). Meanwhile, in the CSN3 gene, the highest levels of milk fat were obtained from the CG genotype (4.35%), and then followed by the TT (4.39%) and GT genotype (4.18%). Hamza et al. (2011) reported that CN genotypes had significant effect on milk fat component. Previously, Ardicli et al. (2018) reported that CSN1S1 genotypes were associated to milk fat. Besides, Dagnachew et al. (2011) stated that CSN1S1 had an association with milk fat. Le Parc et al. (2010) stated that CSN1S1 had the main function of the casein transportation efficiency from endoplasm to compartment Golgi. Bugeac et al. (2013) reported that

Tabel 4. The effect of milk protein genes (CSN-BMC9215, CSN-BMC6334, CSN1S1-192, CSN1S1-14618, CSN1S2, CSN2-67, and CSN3) on least squares means of milk protein component (%) in Holstein Friesian (HF) cattle for 12 months of lactation

Month of Lactation	Genotype																											
	CSN-BMC9215				CSN-BMC6334				CSN1S1-192			CSN1S1-14618				CSN1S2				CSN2-67				CSN3			
	CG	GT	TT	Sig	AA	GA	GG	Sig	AA	AG	Sig	CC	CT	TT	Sig	AA	TA	TT	Sig	AA	AC	CC	Sig	CG	GT	TT	Sig
1	3.58	3.62	3.66	NS	3.64	3.62	3.58	NS	3.59	3.57	NS	3.61	3.62	3.6	NS	.	3.62	3.62	NS	3.66	3.62	3.59	NS	3.65	3.47	3.66	NS
2	3.52	3.64	3.65	NS	3.54	3.54	3.37	NS	3.63	3.37	*	3.58	3.48	3.39	NS	3.89	3.62	3.47	NS	3.56	3.48	3.46	NS	3.71	3.46	3.67	NS
3	3.52	3.64	3.65	NS	3.54	3.54	3.37	NS	3.63	3.37	NS	3.58	3.48	3.39	NS	3.89	3.62	3.47	NS	3.56	3.48	3.46	NS	3.71	3.46	3.67	NS
4	3.53	3.55	3.59	NS	3.59	3.54	3.56	NS	3.61	3.53	NS	3.55	3.54	3.6	NS	.	3.52	3.55	NS	3.57	3.51	3.59	NS	3.57	3.47	3.45	NS
5	3.53	3.55	3.59	NS	3.52	3.55	3.58	NS	3.56	3.54	NS	3.5	3.56	3.62	NS	3.6	3.55	3.54	NS	3.49	3.55	3.58	NS	3.62	3.54	3.62	NS
6	3.55	3.57	3.56	NS	3.55	3.52	3.59	NS	3.6	3.55	NS	3.53	3.57	3.55	NS	.	3.49	3.56	NS	3.54	3.56	3.59	NS	3.54	3.63	3.62	NS
7	3.53	3.61	3.67	NS	3.67	3.55	3.56	NS	3.59	3.64	NS	3.62	3.57	3.55	NS	3.48	3.49	3.58	NS	3.66	3.57	3.55	NS	3.77	3.63	3.63	NS
8	3.76	3.79	3.83	NS	3.76	3.69	3.66	NS	3.67	3.71	NS	3.67	3.76	3.62	NS	3.61	3.7	3.71	NS	3.75	3.68	3.73	*	3.89	3.73	3.67	NS
9	3.82	3.88	3.89	NS	3.75	3.68	3.69	NS	3.68	3.7	NS	3.74	3.71	3.68	NS	3.55	3.7	3.71	NS	3.72	3.69	3.66	NS	3.56	3.84	3.86	NS
10	3.81	3.8	3.9	NS	3.87	3.82	3.8	NS	3.86	3.81	NS	3.86	3.8	3.71	NS	.	3.81	3.84	NS	3.87	3.81	3.82	NS	3.85	3.77	3.8	NS
11	3.58	3.62	3.66	NS	3.64	3.62	3.58	NS	3.6	3.56	NS	3.61	3.62	3.6	NS	.	3.61	3.62	NS	3.65	3.62	3.58	NS	3.7	3.51	3.73	NS
12	3.91	3.9	4.03	NS	4.03	3.87	3.93	NS	3.87	3.98	NS	3.93	3.96	3.83	NS	3.9	3.83	3.94	NS	4.08	3.99	3.93	NS	4.34	4	3.95	NS

Sig = Significance, NS = Non Significant difference (P>0.05), * = Significant difference (P<0.05)

Tabel 5. The effect of milk protein genes (CSN-BMC9215, CSN-BMC6334, CSN1S1-192, CSN1S1-14618, CSN1S2, CSN2-67, and CSN3) on least squares means of milk fat component (%) in Holstein Friesian (HF) cattle for 12 months of lactation

Month of Lactation	Genotype																									
	CSN-BMC9215				CSN-BMC6334				CSN1S1-192			CSN1S1-14618				CSN1S2				CSN2_67				CSN3			
	CG	GT	TT	Sig	AA	GA	GG	Sig	AA	AG	Sig	CC	CT	TT	Sig	AA	TA	TT	Sig	AA	AC	CC	Sig	CG	GT	TT	Sig
1	4.12	4.11	4.21	NS	4.17	4.11	4.14	NS	4.15	4.06	NS	4.08	4.14	4.2	NS	Nd	4.08	4.15	NS	4.18	4.15	4.1	NS	4.35	4.18	4.39	NS
2	4.04	4.14	4.17	NS	4.09	4.1	3.94	NS	4.17	3.89	**	4.05	4.06	3.97	NS	Nd	4.32	4.1	NS	4.06	4.07	3.95	NS	3.87	3.97	3.95	NS
3	4.04	4.14	4.17	NS	4.09	4.1	3.94	NS	4.17	3.89	NS	4.05	4.06	3.97	NS	4.32	4.1	4.01	NS	4.06	4.07	3.95	NS	3.87	3.97	3.95	NS
4	4.09	4.21	4.16	NS	4.12	4.19	4.11	NS	4.15	4.15	NS	4.17	4.12	4.15	NS	Nd	4.1	4.15	NS	4.1	4.19	4.12	NS	3.99	3.96	4.08	NS
5	4.17	4.22	4.09	NS	4.1	4.18	4.17	NS	4.14	4.13	NS	4.07	4.16	4.26	NS	4.1	4.09	4.13	NS	4.01	4.15	4.15	NS	4.16	4.07	4.28	NS
6	4.18	4.25	4.23	NS	4.22	4.17	4.24	NS	4.2	4.24	NS	4.23	4.2	4.2	NS	Nd	4.13	4.23	NS	4.21	4.25	4.19	NS	4.06	4.26	4.29	NS
7	4.18	4.18	4.3	NS	4.3	4.18	4.17	NS	4.1	4.23	*	4.26	4.2	4.14	NS	4.08	4.24	4.23	NS	4.31	4.18	4.19	NS	4.34	4.19	4.2	NS
8	4.2	4.27	4.38	NS	4.35	4.23	4.16	NS	4.21	4.28	*	4.25	4.29	4.15	NS	4.09	4.3	4.25	NS	4.37	4.23	4.22	NS	4.42	4.28	4.29	NS
9	4.35	4.36	4.28	NS	4.23	4.34	4.28	NS	4.29	4.35	NS	4.24	4.36	4.2	NS	3.55	3.7	3.68	NS	4.22	4.33	4.28	NS	4.14	4.3	4.38	NS
10	3.93	3.88	3.86	NS	3.84	3.9	3.95	NS	3.92	3.95	NS	3.87	3.96	3.82	NS	.	3.91	3.94	NS	3.82	3.88	3.95	NS	4.11	4.01	3.82	NS
11	4.12	4.11	4.21	NS	4.17	4.11	4.14	NS	4.16	4.08	*	4.08	4.14	4.2	NS	.	.	.		4.17	4.15	4.1	NS	4.18	4.09	4.3	*
12	4.46	4.63	4.6	NS	4.6	4.74	4.35	NS	4.56	4.39	NS	4.42	4.61	4.17	NS	4.12	4.86	4.54	NS	4.56	4.59	4.4	NS	4.85	4.72	4.26	NS

Sig = Significance, NS = Non Significant difference (P>0.05), * = Significant difference (P<0.05)

Tabel 6. The effect of milk protein genes (CSN-BMC9215, CSN-BMC6334, CSN1S1-192, CSN1S1-14618, CSN1S2, CSN2-67, and CSN3) on least squares means of milk solid non fat component (%) in Holstein Friesian (HF) cattle for 12 months of lactation

Month of Lactation	Genotype																										
	CSN-BMC9215				CSN-BMC6334				CSN1S1-192			CSN1S1-14618				CSN1S2				CSN2_67				CSN3			
	CG	GT	TT	Sig	AA	GA	GG	Sig	AA	AG	Sig	CC	CT	TT	Sig	AA	TA	TT	Sig	AA	AC	CC	Sig	CG	GT	TT	Sig
1	8.58	8.68	8.64	NS	8.63	8.61	8.65	NS	8.63	8.59	NS	8.6	8.58	8.72	NS	.	8.47	8.65	NS	8.63	8.7	8.54	NS	8.3	8.53	8.9	*
2	8.35	8.75	8.56	NS	8.62	8.59	8.35	NS	8.55	8.47	NS	8.59	8.47	8.43	NS	9.26	8.61	8.47	NS	8.05	8.52	8.33	NS	8.42	8.46	8.49	NS
3	8.39	8.23	8.46	NS	8.43	8.3	8.41	NS	8.43	8.36	NS	8.48	8.37	8.34	NS	8.53	8.5	8.36	NS	8.36	8.37	8.37	NS	7.88	8.4	8.32	NS
4	8.31	8.28	8.28	NS	8.3	8.25	8.3	NS	8.34	8.35	NS	8.27	8.24	8.38	NS	.	8.45	8.27	NS	8.32	8.22	8.49	NS	8.43	8.29	8.28	NS
5	8.53	8.41	8.43	NS	8.43	8.38	8.54	NS	8.38	8.45	NS	8.53	8.44	8.56	NS	8.57	8.7	8.43	NS	8.39	8.4	8.56	NS	8.59	8.48	8.48	NS
6	6.36	8.34	8.3	NS	8.29	8.3	8.4	NS	8.39	8.3	NS	8.37	8.31	8.36	NS	.	8.38	9.32	NS	8.3	8.34	8.37	NS	8.01	8.3	8.38	NS
7	8.33	8.44	8.61	**	8.61	8.43	8.31	**	8.4	8.47	NS	8.51	8.39	8.34	NS	8.22	8.46	8.41	NS	8.6	8.37	8.4	*	8.71	8.4	8.48	NS
8	8.47	8.36	8.66	*	8.64	8.44	8.42	NS	8.44	8.49	NS	8.48	8.58	8.3	*	8.19	8.47	8.43	NS	8.66	8.38	8.53	*	8.97	8.58	8.45	NS
9	8.28	8.33	8.41	NS	8.45	8.3	8.24	NS	8.31	8.33	NS	8.45	8.33	8.22	NS	7.92	8.42	8.29	NS	8.45	8.27	8.34	NS	8.4	8.47	8.15	NS
10	8.65	8.56	8.8	NS	8.75	8.65	8.62	NS	8.76	8.61	NS	8.72	8.61	8.48	NS	.nd	8.73	8.62	NS	8.75	8.6	8.67	NS	9.05	8.6	8.69	NS
11	8.56	8.68	8.67	NS	8.66	8.61	8.62	NS	8.63	8.57	NS	8.6	8.58	8.72	NS	.nd	8.48	8.65	NS	8.65	8.69	8.52	NS	8.69	8.53	8.91	*
12	8.79	8.84	8.78	NS	8.73	8.83	8.78	NS	8.55	8.8	NS	8.96	8.73	8.83	NS	9.07	8.75	8.95	NS	8.76	8.91	8.8	NS	7.87	8.67	8.81	NS

Sig = Significancy, NS = Non Significant difference (P>0.05), * = Significant difference (P<0.05), ** = Highly significant difference (P<0.01)

Tabel 7. The effect of milk protein genes (CSN-BMC9215, CSN-BMC6334, CSN1S1-192, CSN1S1-14618, CSN1S2, CSN2-67, and CSN3) on least squares means of milk lactosa component (%) in Holstein Friesian (HF) cattle for 12 months of lactation

Month Lactation	Genotype																									
	CSN-BMC9215				CSN-BMC6334				CSN1S1-192			CSN1S1-14618				CSN1S2				CSN2_67				CSN3			
	CG	GT	TT	Sig	AA	GA	GG	Sig	AA	AG	Sig	CC	CT	TT	Sig	AA	TA	TT	Sig	AA	AC	CC	Sig	CG	GT	TT	Sig
1	4.79	4.77	4.86	NS	4.87	4.77	4.79	NS	4.83	4.74	NS	4.78	4.81	4.82	NS	nd	4.81	4.8	NS	4.87	4.77	4.78	NS	4.79	4.7	4.92	NS
2	4.54	4.66	4.74	NS	4.78	4.6	4.56	NS	4.7	4.61	NS	4.81	4.58	4.61	NS	5.06	4.61	4.64	NS	4.76	4.58	4.57	NS	4.66	4.55	4.7	NS
3	4.73	4.55	4.69	*	4.69	4.58	4.73	NS	4.68	4.68	NS	4.74	4.66	4.67	NS	4.74	4.83	4.67	NS	4.66	4.64	4.76	NS	4.36	4.65	4.68	NS
4	4.63	4.62	4.65	NS	4.67	4.59	4.62	NS	4.66	4.67	NS	4.74	4.52	4.67	NS	.nd	4.66	4.64	NS	4.69	4.56	4.79	NS	4.72	4.63	4.66	NS
5	4.83	4.65	4.72	NS	4.75	4.62	4.83	NS	4.71	4.76	NS	4.82	4.71	4.9	NS	4.84	4.86	4.75	NS	4.73	4.66	4.87	NS	4.83	4.75	4.8	NS
6	4.77	4.73	4.75	NS	4.75	4.74	4.77	NS	4.79	4.73	NS	4.77	4.73	4.8	NS	.nd	4.78	4.75	NS	4.75	4.72	4.8	NS	4.58	4.72	4.77	NS
7	4.76	4.85	4.9	*	4.89	4.83	4.75	NS	4.82	4.85	NS	4.83	4.82	4.76	NS	4.68	4.82	4.81	NS	4.88	4.81	4.8	*	4.98	4.81	4.8	NS
8	4.89	4.86	5	NS	4.99	4.76	4.78	NS	4.91	4.91	NS	4.88	4.97	4.83	NS	4.73	4.9	4.87	NS	4.99	4.86	4.94	*	5.19	4.92	4.89	NS
9	4.68	4.74	4.77	NS	4.83	4.72	4.63	NS	4.72	4.69	NS	4.79	4.7	4.7	NS	4.5	4.81	4.7	NS	4.79	4.67	4.7	NS	4.81	4.72	4.62	NS
10	5.02	5.01	5.12	NS	5.09	5.04	5.02	NS	5.08	5.03	NS	5.08	5.01	4.92	NS	.	5.01	5.06	NS	5.08	5.03	5.03	NS	5.07	4.97	5.01	NS
11	4.78	4.77	4.86	NS	4.87	4.76	4.78	NS	4.82	4.73	NS	4.78	4.8	4.81	NS	.	4.81	4.8	NS	4.87	4.76	4.78	NS	4.91	4.76	4.92	NS
12	5.13	5.17	5.24	NS	5.23	5.08	5.15	NS	5.1	5.17	NS	5.09	5.19	5.04	NS	4.95	4.98	5.18	NS	5.28	5.23	5.15	NS	5.3	5.14	5.13	NS

Sig = Significancy, NS = Non Significant difference (P>0.05), * = Significant difference (P<0.05)

the genetic variants of CSN3 affected milk fat levels. Hristov et al. (2011) added that milk fat component in the Bulgarian black pied cattle was associated with the genotype from the CSN3 gene. Meanwhile, Komori et al. (2013) explained that CSN3 had a function in regulating the formation and stabilization of micelles. The structure and component of the morphometry of the milk fat globules (MFGs) were reported to be influenced by a genetic polymorphism in αs1-casein (CSN1S1) (Cebo et al. 2012). Fleming et al. (2017) reported that there was a positive correlation between the component of milk fat and the diameter of MFGs. The previous study showed that HF cow which had BB genotypes resulted in higher milk fat components than other genotypes (AA and AB) (Vidović et al. 2013). Relation of lactation stage to milk fat component reported by Salamonczyk (2013) that the highest of fat milk components was recorded in milk which was produced at the last lactation stage (>300 days). Meanwhile, Januś & Borkowska (2011) found that lower calorific value of milk due to lower fat component obtained in the first 100 days of lactation. Stoop et al. (2009) explained that stage lactation contributed to variation in milk fat component which caused by the different activity of fatty acid pathways.

The effect of the milk protein genes on solid non-fat milk levels are presented in Table 6. During 12 months lactation, it was known that genes genotype which had a significant effect ($p<0.05$) to the level of solid non-fat cow milk were genotypes for the CSN-BMC9215, CSN-BMC6334, CSN1S1-14618, CSN2_67, and CSN3 genes. The CSN-BMC9215 gene affected the levels of solid non-fat in the 7th and 8th lactation months. In the 7th lactation month, the highest solid non fat milk level was obtained from the TT genotype (8.61%), followed by the GT (8.44%) and CG genotypes (8.33%). In the 8th month, the highest level of solid non-fat milk was obtained from the TT genotype (8.66%), followed by the CG (8.47%) and GT genotypes (8.33%). Meanwhile, for the CSN-BMC6334 gene, the highest level of solid non-fat milk was obtained from the AA genotype (8.61%), followed by the GA (8.43%) and GG genotype (8.31%). In the CSN1S1-14618 gene, the highest level of solid non-fat milk was obtained from the CT genotype (8.58%), followed by the CC (8.48%) and TT genotype (8.3%). The CSN2-67 gene affected the levels of solid non-fat in the 7th and 8th lactation months. In those two months of lactation, it was found that the highest solid non-fat milk component was obtained from the AA genotype (8.6%), followed by the CC (8.4%) and AC genotypes (8.37%). The CSN3 gene influenced the level of solid non-fat milk at month lactation of 1st and 11th. At 1st lactation month, the TT genotype produced the highest levels of solid non-fat (8.9%), followed by the GT (8.53%) and CG genotypes (8.3%). Meanwhile, on the 11th lactation, the TT genotype produced the highest levels of solid non-fat (8.91%), followed by the CG (8.69%) and GT genotypes (8.53%).This finding differed with Hamza et al. (2011) who reported that CN genotypes had no significant effect on milk solid non fat (SNF) component. Previously, Anggraeni et al. (2017) reported that there was no significant effect of κ-casein genotypes on the component of milk solid non-fat.

The effect of variant genetic of milk protein genes on the milk SNF component was reported in the previous studies. Deb et al. (2014) in Frieswal cattle showed that AB genotype resulted in higher milk SNF component compared to AA genotypes. Furthermore, Gurses & Yuce (2012) added that AB genotype affected higher milk SNF component than AA genotype in East Anatolian Red cattle (Turkey cattle). Radhika & Ajithkumar (2018) revealed that the component of milk SNF decreased along the increase of the age of cow. The component of milk SNF was relatively high in the first month, then dropped to a low in the second month, then raised as lactation progresses.

The effect of milk protein genes on milk lactose levels are presented in Table 7. During 12 months of lactation observation, it was found that the genetic variants of milk protein genes which significantly affected ($p<0.05$) level of cow's milk lactose were the CSN-BMC9215 and CSN2-67 genes. The CSN-BMC9215 gene affected milk lactose levels in the 3rd and 7th lactation months. In the 3rd lactation month, the CG genotype produced the highest lactose levels (4.73%), followed by the TT (4.69%) and GT genotypes (4.55%). Meanwhile, in the 7th lactation month, the TT genotype produced the highest levels of lactose (4.9%), followed by the GT (4.85%) and CG genotypes (4.76%). The CSN2-67 gene influenced milk lactose levels in the 7th and 8th lactation months. In the 7th lactation month, the AA genotype produced the highest lactose levels (4.88%), followed by the AC (4.81%) and CC genotypes (4.8%). Meanwhile, at the 8th lactation month, the AA genotype produced the highest levels of lactose (4.99%), followed by the CC (4.94 %) and AC genotypes (4.86%). This finding was similar to Hamza et al. (2011) who reported that CN genotypes significantly affected milk lactose component. Relation of lactation stage to milk lactose component reported by Salamonczyk (2013), who reported that the component of milk lactose decreased along with lactation stage increasement. The first two lactation stages (1-100 and 101-200 days) resulted in highest milk lactose component. Sigl et al. (2012) reported that in observations of the first 20 weeks of lactation in HF cattle, it was found that the highest milk lactose component occurred at 7th week lactation. In general, genetic variants of milk protein genes was associated with the chemical component of milk, i.e., protein, fat, solid non-fat, and lactose. The results of this study open the opportunity of genetic improvement of HF cattle based on milk protein genes to improve milk components, not only milk protein components but also the other component of milk components (fat, SNF, and lactose).

CONCLUSION

Genetic variants of the milk protein genes have an association with the component of cow's milk (protein, fat, solid non-fat, and lactose). Protein milk was affected by the genetic variants of CSN1S1-192 and CSN2-67 genes. Fat milk was affected by CSN1S1-192 and CSN3 genes. Solid non-fat milk was affected by CSN-BMC9215, CSN-BMC6334, CSN1S1-14618, CSN2_67, and CSN3 genes. Lactose milk was affected by CSN-BMC9215 and CSN2-67 genes.

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Effect of Incubation Time During Sperm Sexing Process on Sperm Quality of Pasundan Bull

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ABSTRAK

Rasad SD, Solihati N, Winangun K, Yusrina A, Avicenna F. 2020. Pengaruh waktu inkubasi selama proses *sexing* sperma pada sperma kualitas Pasundan Bull. JITV 25(3): 112-119. DOI: http://dx.doi.org/10.14334/jitv/v25i3.2494

Tujuan dari penelitian adalah untuk menguji pengaruh waktu inkubasi terhadap kualitas semen *sexing* Sapi Pasundan. Separasi sperma menggunakan Bovine Serum Albumin (BSA) dengan konsentrasi 5% dan 10%, dengan perlakuan waktu inkubasi P1 = 45 menit, P2 = 60 menit dan P3. 75 menit. Penelitian ini menggunakan metoda Rancangan Acak Lengkap (RAL) dengan tiga perlakuan dan 6 ulangan. Data dianalisis dengan Duncan *test*. Parameter yang diukur adalah daya tahan hidup, membran plasma utuh, abnormalitas dan integritas DNA sperma *sexing*. Hasil penelitian menunjukkan waktu inkubasi berpenagruh (P<0,05) terhadap daya tahan hidup, tetapi tidak pada MPU, abnormalitas, dan integritas DNA. Waktu inkubasi 45 menit menghasilkan daya tahan hidup terbaik pada fraksi atas (4,33 hari) dan fraksi bawah (4,17 hari). Abnormalitas sperma pada fraksi atas antara 4,00%-4,50% dan fraksi bawah antara 4,10%-4,40%, sedang integritas DNA pada fraksi atas antara 98,16%-98,66% dan fraksi bawah antara 97,83-98,58%. Dari hasil penelitian dapat disimpulkan bahwa waktu inkubasi berpengaruh terhadap daya tahan hidup sperma, tetapi tidak terhadap MPU, abnormalitas dan integritas DNA sperma Sapi Pasundan hasil sexing

Kata Kunci : Waktu Inkubasi, Sapi Pasundan, *Sexing* Sperma

ABSTRACT

Rasad SD, Solihati N, Winangun K, Yusrina A, Avicenna F. 2020 Effect of incubation time during sperm sexing process on sperm quality of pasundan bull. JITV 25(3): 112-119. DOI: http://dx.doi.org/10.14334/jitv/v25i3.2494

The research was conducted to evaluate the effect of incubation time on viability, plasma membrane integrity, abnormality, and DNA integrity of sexed Pasundan's bulls sperm. The sperm sexing used 5% and 10% concentrations of Bovine Serum Albumin (BSA). A completely randomized design with three treatments and six replications was used in this study. The data were analyzed using variance analysis followed by Duncan's multiple distance test. Parameter evaluated were sperm longevity, plasma membrane integrity (PMI), abnormality, and DNA integrity of sexed Pasundan bulls sperm. Results showed that incubation time gave significant effect (P<0.05) on the longevity of sperm, but not on the PMI of Pasundan bulls sexed sperm. The incubation time of 45 minutes gave the highest value of longevity sperm on the upper layer (4.33 days) and the lower layer (4.17 days). Furthermore, the abnormality of sperm X in the upper layer was 4.00%-4.20% and the lower layer was 4.10%-4.40%. Meanwhile, the DNA integrity of an upper layer was 98.16%-98.66%, and the lower layer was 97.83%-98.58%. It is concluded that 45 minutes of incubation time significantly affected the longevity of sperm, but not plasma membrane integrity, abnormality, and DNA integrity of Pasundan bulls sexed sperm.

Key Words: Incubation Time, Pasundan Bull, Sexed Sperm

INTRODUCTION

Artificial Insemination technology could be increased in value using a sexed semen program that produces expected sex of calf, and this sex predetermination is commercially crucial in the farm (Kusumawati et al. 2017). Sperm sexing technology in livestock is an effort to increase the chances of certain birth of certain sex offspring according to the purpose of livestock raising. The application of sperm sexing technology could improve the efficiency of the Artificial Insemination (AI) program.

Sperm sexing is a method of separation between X chromosome bearing sperm (X-sperm) and Y chromosome bearing sperm (Y-sperm) to obtain a higher proportion of certain sperm to get a higher probability of birth of certain sex (Solihati et al. 2019). A direct method for sperm sexing in animals is based on the sorting of X and Y- bearing sperm before insemination. Sperm are separated into X and Y-

bearing populations based on the presence of X or Y chromosomes, respectively. The egg contains only X-chromosome then be fertilized by an X-sperm will produce female offspring, while if it is fertilized by a Y sperm, the offspring will be a male. Therefore, sexing spermatozoa could be used in conjunction with artificial insemination to produce expected offspring sex (Yadav et al. 2018) Even though sperm sexing contributes a great impact on breeding programs, it requires high cost and resulted in a low pregnancy rate especially when using the artificial insemination program (Carvalho et al. 2010). Separation of X and Y chromosomes could be done by the sedimentation method with Bovine Serum Albumin (BSA) solution. Carvalho et al. (2010) reported that the Y chromosome is smaller than the X chromosome. In previous studies, damage of spermatozoa membrane in the sexing process with albumin gradient could decrease the quality of spermatozoa, thus diluents for protecting the spermatozoa membrane to be in good quality are needed. The decrease in semen quality is due to physical and chemical effects on sperm cells during the separation process to obtain X or Y sperm.

The length of incubation is one factor influencing the sexed sperm quality. Incubation time is the time needed for the sperm to penetrate the BSA solution during the sexing process. Sperm containing an X chromosome is larger than those containing a Y chromosome. Different incubation times are estimated to affect the quality and longevity of X and Y sperm, but the information is still limited. Some researchers had performed sperm sexing with a 60-minute incubation period (Gunawan 2015; Hadi & Al-Tamimi 2013), and sorter incubation times for 20, 35 and 50 minutes (Sunarti, T Saili, et al. 2016), and 10, 20, 30 minutes (Situmorang et al. 2013), and 30, 60, 90, 120 minutes (Afriani et al. 2011).

One of the sperm quality results from the sexing process that needs to be considered is its viability, plasma membrane integrity, and abnormality because it is closely related to fertility. In addition to abnormalities, the DNA integrity of sperm also needs to be considered, because DNA is a genetic carrier for the offspring (Chowdhury et al. 2014). Based on the reasons above, this study was conducted to determine the effect of incubation time on sexed semen quality of Pasundan cattle.

MATERIALS AND METHODS

Experimental animals

This research was done in October - December 2018 at the Center of Beef Cattle Breeding Development and Artificial Insemination Institute, Cijeungjing, Ciamis. Samen was collected from seven five year Pasundan bulls, which was certificated from Indonesian Product Certification Institute. All bulls were maintained in semi-intensive cages, located in Cijeungjing, Ciamis, which was feed daily in form of forages and concentrates, as usual feeding in that center.

Semen collection and evaluation

BSA and BO solution was prepared one day before semen collection. Collecting semen was done twice a week and directly evaluated on macroscopic and microscopic quality.

Fresh semen was prepared and diluted using BO solution with a ratio of fresh semen and BO solution as 1:4. As much as 2 ml of 10%, and 5% BSA solutions respectively were slowly placed into tubes to make the BSA column. The diluted semen was put into the BSA column as much as 1 ml, so that the ratio of semen and BSA was 1: 4, respectively. The tubes were incubated into a water bath at 37ºC for 45 minutes, 60 minutes, and 75 minutes. During incubation, spermatozoa would penetrate the solution based on the speed of movement and form 3 layers: upper layer containing immotile sperm, the middle layer containing X sperm, and the lower layer containing Y sperm. One ml of the top layer was considered immotile and removed. Four milliliters of each BSA solution were separated and transferred into centrifugation tubes, then labeled as Y for the 10% BSA solution and X for the 5% BSA solution. Five ml BO solution was added to each centrifugation tube. They were centrifuged at 1800 rpm for 10 minutes. The centrifugation process formed 2 layers: the top layer was a supernatant that will be removed and the bottom layer was a pellet which was the result of sperm sexing. The tubes containing pellets were added by BO solution slowly through the tube wall. Semen was diluted with TRIS-egg yolk, then stored at 5º C as liquid semen.

The treatment of this research was incubation time and carried out after the sperm sexing process for 45 minutes incubation time (T_1), 60 minutes incubation time (T_2) and 75 minutes incubation time (T_3). There were 6 replications. The test is based on the number of bulls from which the sperm is taken. Dilution of semen used was TRIS-egg yolk.

The longevity of sperm observation (day)

The longevity of sperm was observed automatically using Computerized-Assisted Sperm Analysis (CASA) (AndroVision, MiniTube) connected to the microscope. The data was seen from the survival of sperm after treatment in units of days.

Plasma membrane integrity (PMI) (%)

Plasma membrane integrity was evaluated using a hypo-osmotic swelling test (HOS-Test) solution made

from 0.179 grams of 0.1 M NaCl dissolved into 100 ml of bidistilled water, semen samples were put into hypo-osmotic solution, then incubated for 30 minutes at 37ºC. After incubation, sperm were evaluated using an Olympus CX-21 binocular microscope with a 40x10 magnification. Sperm that have an intact plasma membrane is characterized by a swollen, circular tail, due to exposure to hypotonic solutions. Damaged sperm is characterized by a straight tail due to the absence of an osmotic reaction.

Abnormality of sperm (%)

Sperm abnormality is known through the differential coloring method using eosin dyes. The observed abnormalities are secondary abnormalities which include a severed tail, head without a tail, folded middle part. Observations were carried out under a microscope at 10x40 magnification. Abnormality of sperm was calculated from a minimum of 200 sperm.

DNA integrity (%)

This observation was seen by the Acridine Orange Test method and observed under a fluorescence microscope. Sperm with normal DNA shows greener fluorescence, while sperm with fragmented DNA will emit fluorescence that varies from yellow-green to orange. The percentage of sperm with whole chromatin was calculated by dividing the sperm count normal (green) with a total of 200 sperm counts observed.

Statistical analysis

The Completely Randomized Design (CRD) with three treatments and six replications was applied. Data were analyzed using variance analysis followed by Duncan's multiple distance test. The parameters of this study include viability, plasma membrane integrity, abnormality, and DNA integrity of the sexed sperm of Pasundan bull.

Table 1. The macroscopic and microscopic evaluation of fresh Pasundan bull semen

Parameter	Replication						Average
	1	2	3	4	5	6	
Macroscopic:							
Volume (ml)	8.00	5.50	5.00	6.00	5.00	5.50	5.831
Colour	Creamy white	Creamy white	Creamy white	Creamy white	Creamy white	Creamy white	-
Consistency	moderate	moderate	Aqueous	moderate	moderate	moderate	-
pH	6.40	6.40	6.40	6.40	6.40	6.40	6.40
Microscopic:							
Mass activity	++	++	++	+++	++	++	-
Total Sperm-Concentration per ejaculate (million/ml)	1,503.00	1,358.00	1,148.00	1,600.00	1,210.00	1,312.00	1,355.17±171.85
Sperm Motility (%)	70.00	70.00	70.00	70.00	70.00	75.00	70.83±2.04
Sperm Abnormalities (%)	3.00	3.00	3.00	2.50	2.00	2.50	2.70±0.40
DNA- Integrity (%)	98.00	99.00	98.00	98.00	99.00	98.00	98.33±0.47
Membrane plasma integrity (IPM)(%)	65.00	62.00	65.00	69.14	66.52	73.17	66.81±3.89

RESULTS AND DISCUSSION

Fresh semen evaluation

The evaluation of the fresh semen samples was done immediately after semen collection. The result of the evaluation is presented in Table 1. Table 1 shows the average semen volume was 5.83 ml. This result is in line with Feradis (2010); Kedia et al. 2014 and Anwar et al. (2015) which stated that the volume of bulls semen ranges from 5-8 ml /ejaculation. The color of semen in this study was creamy white, this is in line with Tan et al. (2014) and Solihati et al. (2018) which stated that the color of bull semen is milk-white and creamy.

Normal semen obtained had a distinctive odor, and thick consistency, this is in line with the result of (Kedia et al. 2014). The pH of the obtained semen was 6. The pH of fresh semen ranges from 6.4-7.8 (Ax et al. 2000). The mass activity of Pasundan bulls semen was ++ which was good because it looked like a big wave, many, dark, thick, and actively powered according to (Baharun et al. 2017). The sperm motility was 70.83% moved progressively, the motility of the individual was still in the normal range and in line with (Ax et al. 2000) who stated that the sperm motility of bull was 70%. Sperm concentration was 1.355 million/ml. This is in line with (Feradis 2010) who found the concentration of fresh semen of bulls was 1.000–2.000 million sperm cells per ml.

The percentage of sperm motility of Pasundan bulls was 70.83%. This result is lower than (Baharun et al. 2017) which was 82.41 ± 2.97%. The difference could be influenced by sperm maturity and the quality of plasma semen (Komariah et al. 2013) but these results were optimal according to Ax et al. (2000), the percentage of sperm motility must be more than 50%.

The percentage of spermatozoa abnormalities was 2.70%. This is smaller than the average abnormalities of Pasundan cattle of (Baharun et al. 2017) which was 11.13 ± 0.39%. According to Sujoko et al. (2009) abnormalities in ram sperm ranged from 5.00% to 35.00%, the abnormality of Pasundan bulls sperm in this study was very low. Abnormality is one indicator determining sperm quality. The abnormal morphology of sperm cells will affect the fertilization process, the high abnormality of spermatozoa will reduce fertility (Sujoko et al. 2009).

The IPM obtained in this study was 62.00% to 73.17% with an average of 66.81 ± 3.89%. This result was lower than that of Baharun et al. (2017), which was 84.89 ± 1.00%. The percentage of IPM that have optimum quality and requirements for further processing is ≥60.00% (Rizal et al. 2013; Shukla et al. 2013). The damage of plasma membranes cause decreased cell membrane integrity so that control of the transport system in sperm cells is disrupted which resulted in the decrease of metabolism, motility, and survival of spermatozoa (Purwoistri et al. 2013; Mishra et al. 2013).

Effect of incubation time on the longevity of sperm

The longevity of sperm was evaluated by looking at their motility daily until the motility reaches a minimum percentage of 40.00%. Table 2 shows the incubation time of 45, 60, and 75 minutes result in a significant effect ($P < 0.05$) on the longevity of sperm at the upper and lower layer. The 45 minutes of incubation time produced the longest longevity, while 75 minutes of incubation time produced the shortest longevity of sperm. This result was longer than that of Solihati et al. (2018) average longevity of sperm ranged between 13.875–19.625 hour. This result indicated that the longer the incubation time, the lower the longevity of sperm is.

The longevity of sperm is closely related to sperm motility because sperm longevity is obtained by observing sperm motility. The longevity of sperm at the upper layer was observed to a minimum of 40% motility. Decreasing the percentage of spermatozoa motility after sexing due to the long incubation time in the albumin layer, speed, and length of centrifugation which causes sperm losing a lot of energy thereby reducing the level of motility (Sudarma et al. 2014). The incubation process decreased the viability of the upper layer sperm from T1 to T3 because, during the incubation process, free radical formation occurs including hydrogen peroxide (H_2O_2) which is toxic and causes the damage of the plasma membrane, so that sperm motility decreases (Storey 2008). Hydrogen peroxide (H_2O_2) is needed for sperm motility and is useful in the capacitation process, so that sperm cells are produced naturally, but if the level is too high, it will cause lipid peroxidation. Hydrogen peroxide (H_2O_2) will react with polyunsaturated fatty acids that are in the sperm plasma membrane, causing lipid peroxidation. Damage of the plasma membrane due to lipid peroxidation directly decreases the motility of sperm. Decreased sperm survival is also due to the length of storage, because the longer storage time, lactic acid formed due to the process of sperm cell metabolism increases, and causes a high pH of the medium. Rizal & Riyadhi (2016) stated that sperm in anaerobic storage conditions (without oxygen) occurs in the process of cell metabolism which results, in the end, is lactic acid. The duration of storage causes a buildup of lactic acid in the media so that the pH of the media drops and results in the death of spermatozoa. Table 2 shows that Y sperm has a smaller and lighter head than X sperm, this is because Y sperm has a smaller amount of chromatin than X sperm, so it will have a direct impact

on sperm motility. Y sperm move faster than X sperm. Therefore Y sperm lose more energy quickly due to movement (Sunarti et al. 2016). The average motility of sperm in the lower layer is smaller than upper layer sperm, this is due to the lower layer sperm passing through two layers so that more energy is used, resulted in a decrease in motility, and viability. Sperm storage for a long time also caused motility to decrease, due to the presence of lactic acid resulting from cell metabolic processes, so that the pH of the medium is more acidic.

Effect of incubation time on plasma membrane integrity (PMI) of sperm after sexing process

The average PMI of sperm at the upper and lower layer after sexing using different times of incubation is presented in Table 3.

The sperm plasma membrane is the outer part that restricts between inside and external environments of sperm cells and rewarding in the process of metabolism of cells (Ode et al. 2010). Damage of the plasma membrane will affect the process of sperm metabolism so that it will affect the motility of sperm.

The results of the analysis of variance showed that the length of incubation did not significantly affect PMI in the upper layer.

Based on the observations, the percentage of plasma membrane integrity produced in this study after the sexing process did not differ from that of Sunarti et al. (2016). The average intact plasma membrane spermatozoa of Bali cattle after sexing using albumin of white egg and density gradients with each sexing time of 20 minutes, 35 minutes, and 50 minutes had no significant effect on PMI with an incubation time of 20 minutes, 35 minutes, and 50 minutes was 81.63± 28.93%, 81.14 ± 28.77%, and 80.57 ± 28.91%.

Sperm plasma membrane integrity is an important factor in sperm survival, because membrane damage will be followed by decreased of membrane integrity, motility, and spermatozoa fertility Sperm cell membrane phospholipids are known to contain polyunsaturated fatty acids that are so high that they react easily with free radicals. The function of phospholipids is to maintain membrane integrity and as a protection against environmental conditions (Diliyana et al. 2014). The long incubation time causes plasma membrane damage, this is because the longer the incubation time, the more polyunsaturated fatty acids present in the plasma membrane bind to free radicals, and lipid peroxidation occurred. So in this study, the incubation time treatment that produced the highest percentage of plasma membrane integrity was 45 minutes and the lowest was 75 minutes.

Effect of incubation time on the abnormality of sperm after sexing process

The effect of incubation time on the abnormality of sperm after sexing using BSA can be seen in Table 4. The result of the statistical test showed that incubation time was not significantly different on the abnormality of sperm after sexed in the upper and lower layer. This is may due to BSA content, diluents, incubation time and storage for 4 hours at 5^{o}C does not affect the morphological condition of Pasundan bull sperm.

The content of BSA can protect the sperm. In line with Uysal & Bucak (2007) which states that the composition of BSA is to protect the integrity of the spermatozoa membrane from environmental conditions such as heat or oxidative conditions. Bovine serum albumin (BSA) are extracellular cryoprotectants as a function of cell plasma membrane protectors. Bovine

Table 2. Effect of incubation time during sperm sexing process on the longevity of sperm

Replication	Treatment					
	Upper layer			Lower layer		
	T_1	T_2	T_3	T_1	T_2	T_3
	….……days ……			….……days ……		
1	4.00	4.00	3.00	5.00	3.00	3.00
2	4.00	3.00	3.00	4.00	4.00	3.00
3	4.00	4.00	3.00	4.00	4.00	3.00
4	4.00	4.00	3.00	4.00	4.00	3.00
5	4.00	4.00	4.00	4.00	3.00	4.00
6	5.00	4.00	4.00	4.00	3.00	4.00
Total	25.00	23.00	20.00	25.00	21.00	20.00
Average	4.17±0.41^{a}	3.83±0.41^{b}	3.33±0.52^{b}	4.17±0.41^{A}	3.50±0.55^{B}	3.33±052^{B}

P1: 45 minutes of incubation time, P2: 60 minutes of incubation times, P3: 70 minutes of incubation time
Different superscripts within row show significant differences. a,b upper layer; A,B lower layer

Table 3. Effect of incubation time during sperm sexing process on intact plasma membrane integrity of sperm

Replication	Treatment					
	Upper layer			Lower layer		
	T_1	T_2	T_3	T_1	T_2	T_3
	..……%.....			..……%.....		
1	55.00	54.42	53.64	5.00	3.00	3.00
2	50.75	49.41	41.96	4.00	4.00	3.00
3	53.00	47.39	46.40	4.00	4.00	3.00
4	50.00	46.00	43.50	4.00	4.00	3.00
5	57.50	55.50	53.50	4.00	3.00	4.00
6	63.21	53.75	52.70	4.00	3.00	4.00
Total	329,46	306.47	291.70	25.00	21.00	20.00
Average	54.91±4.91	51.08±4.00	48.62±5.31	4.17±0.41	3.50±0.55	3.33±0.52

Table 4. Effect of incubation time during sperm sexing process on the abnormality of sperm

Replication	Treatment					
	Upper layer			Lower layer		
	T_1	T_2	T_3	T_1	T_2	T_3
	...……..%.....			...……..%.....		
1	4.50	5.50	4.50	4.50	4.00	5.00
2	5.00	4.50	4.50	4.00	4.50	4.00
3	4.50	4.50	5.50	6.00	4.00	4.50
4	3.00	4.00	4.00	3.50	4.50	4.50
5	3.50	3.50	4.50	4.00	4.00	5.00
6	3.50	3.00	4.00	3.00	3.50	4.50
Total	24.00	25.00	27.00	25.00	24.50	27.50
Average	4.00 ± 0.70	4.20 ± 0.80	4.50 ± 0.50	4.20 ± 0.90	4.10 ± 0.30	4.60 ± 0.30

serum albumin is a granular protein (globular) with a molecular weight of 66 kDa, and has a composition of 20 amino acids, in terms of its amino acid content, BSA has a more complete content than plasma semen (Gadea 2003) and that BSA can maintain the condition of sperm of any environment, including during the incubation process. Li Y et al. (2008) stated that the composition and conformation of BSA consist of one BSA molecule in a single polypeptide chain with a molecular weight of about 66.000 g/mol2. Thus the complete content in the BSA can maintain the condition of spermatozoa in any environment, including during the incubation process, so that with any incubation length, BSA solution can minimize the risk of disability on the head or tail (Leach et al. 2011). This is related to the opinion of (Gosálvez et al. 2011), the condition of the imperfect sperm cell membrane will increase of the abnormality of sperm. The abnormality of sperm after incubation for 45, 60, and 75 minutes was still below 20% in all treatments. Generally, the abnormalities occur in the tail of sperm that inhibit movement and reduce sperm fertility, but as long as sperm abnormalities have not reached 20%, the semen can still be used for artificial insemination (Alawiyah & Hartono 2006; Gosálvez et al. 2011).

Effect of incubation time on DNA integrity of sperm after sexing

The average DNA integrity of sperm after separation using BSA is presented in Table 5. Results of the statistical test showed that the incubation time did not significantly different on the DNA integrity in the upper and lower layer. This is expected because the males are superior males that have been selected and maintained to a good standard so that the good quality semen is produced. Environment and feeding are very

influential during the process of spermatogenesis, especially in the phase of spermiogenesis. Besides, the DNA position is located inside the sperm.

Sperm themselves have a protective membrane, namely the plasma membrane, the outer membrane of the acrosome as well as the inner membrane of the acrosome. In this study, membrane damage due to incubation time tends to be less, because the sexing method used is BSA, where BSA itself can protect the integrity of the membrane from different environmental conditions. This is in line with Susilawati et al. (2014) which states that centrifugation can lead to damage cell membranes of sperms. Damage or a decrease in DNA integrity occurs when there is damage to the layers above, which will affect the integrity of DNA spermatozoa. This is thought to be a background factor in why the length of incubation does not affect the percentage of DNA spermatozoa integrity.

Table 5. Effect of incubation time during sperm sexing process on DNA integrity of sperm

Replication	Treatment					
	Upper layer			Lower layer		
	T_1	T_2	T_3	T_1	T_2	T_3
	...%.....			...%.....		
1	98.00	97.00	97.00	99.00	97.00	98.00
2	99.00	98.00	98.00	97.00	98.00	98.00
3	98.00	98.00	98.00	98.00	98.00	98.00
4	99.00	99.00	99.00	98.00	98.00	98.00
5	99.00	99.00	99.00	98.00	98.00	97.00
6	99.00	98.00	98.00	99.00	99.00	98.00
Total	592.00	589.00	589.00	589.00	588.00	587.00
Average	98.66 ± 0.47	98.16 ± 0.68	98.16 ± 0.68	98.16 ± 0.68	98.00 ± 0.57	9.83 ± 0.37

CONCLUSION

It is concluded that the incubation time significantly affected the longevity of the sexed sperm of Pasundan bull, but not on plasma membrane integrity, abnormality, and DNA integrity.

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Use of Infrared Thermography for Identifying Physiological and Hematological Conditions of Young Sapera Dairy Goats

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ABSTRAK

Pamungkas FA, Purwanto BP, Manalu W, Yani A, Sianturi RG. 2020. Aplikasi termografi inframerah sebagai indikator dalam penentu kondisi fisiologis dan hematologis kambing perah sapera dara. JITV 25(3): 120-130. DOI: http://dx.doi.org/10.14334/jitv.v25i3.2522

Termografi inframerah (IRT) merupakan salah satu solusi alternatif yang bisa digunakan untuk menggantikan metode invasif yang selama ini digunakan dalam memonitor parameter fisiologis dan hematologis kambing. Dalam konteks ini, penelitian ini bertujuan untuk mengevaluasi kondisi fisiologis dan hematologis kambing perah Sapera dara serta korelasinya dengan hasil penginderaan termografi inframerah. Empat ekor kambing perah Sapera dara dengan kisaran bobot badan 26-28 kg dipelihara pada kandang individu. Suhu permukaan kulit (Ts), suhu rektal (TR), suhu tubuh (Tb), denyut jantung (Hr), frekuensi respirasi (Rr), dan penginderaan termografi inframerah (area mata, mulut, hidung, kaki, tubuh bagian kanan, tubuh bagian kiri, vagina, dan vulva) diukur pada pukul 06.00-18.00 WIB dengan selang waktu pengukuran setiap 2 jam, sedangkan pengambilan sampel untuk pengukuran parameter hematologis dilakukan pada pukul 06.00 dan 18.00 WIB. Hasil penelitian menunjukkan adanya korelasi positif antara hasil penginderaan termografi IR pada berbagai bagian tubuh dengan parameter fisiologis (kecuali denyut jantung), sedangkan dengan parameter hematologis secara umum berkorelasi negatif. Korelasi tertinggi (r = +0.85) dimiliki oleh korelasi antara hasil termografi IR pada bagian kaki kiri belakang dengan suhu tubuh. Termografi inframerah secara umum dapat diterapkan untuk menilai kondisi fisiologis kambing terutama suhu tubuh.

Kata Kunci: Termografi, Inframerah, Parameter Fisiologis, Parameter Hematologis, Kambing Perah

ABSTRACT

Pamungkas FA, Purwanto BP, Manalu W, Yani A, Sianturi RG. 2020. Use of Infrared Thermography for Identifying Physiological and Hematological Conditions of Young Sapera Dairy Goats. JITV 25(3): 120-130. DOI: http://dx.doi.org/10.14334/jitv.v25i3.2522

Infrared thermography (IRT) is an alternative solution that can be applied to replace invasive methods currently used in the monitoring of goats' physiological and hematological parameters. This study was done to compare and correlate the physiological and hematological conditions of young Sapera dairy goats and their correlations with results obtained by IRT. Four young Sapera dairy goats (weight of 26-28 kg) were kept in the individual rearing cage. Skin surface temperature (TS), rectal temperature (TR), body temperature (TB), heartbeat (HR), respiration rate (RR), and IRT at eyes, mouth, nose, legs, left body, right body, vagina, and vulva were monitored from 6 a.m. to 6 p.m. in 2 h intervals. Blood samplings were done at the beginning and the end of the obsevation time. Results showed that IRTs at several body parts were positively correlated with physiological parameters, except for heartbeat. Negatively correlation was observed in hematological parameters. The highest correlation (r = +0.85) was observed in the correlation between the results of the left rear leg IRT on TB. It was concluded that IRT can be applied to examine goats' physiological conditions especially body temperature.

Key Words: Thermography, Infrared, Physiological Parameter, Hematological Parameter, Dairy Goat

INTRODUCTION

Body resistance to heat stress is an important factor for dairy goat to maintain optimal productivity according to genetic characteristics. Tyler & Ensminger (2006) report that heat-resistant animals can preserve their body temperatures in a normal range without changing their physiological and productivity status. Goat is a homeothermic animal which can control its body temperature constantly, even in an extreme environmental condition, and can adapt better to the hot environment than other animals (Kawabata et al. 2013; Naandam & Kojo 2014; Hasin et al. 2017; Ribeiro et al. 2018; Façanha et al. 2020).

However, dairy goats can also experience stresses such as physical, nutritional, psychological, and environmental stresses. The most frequently occurred environmental stress is heat stress that indicated by physiologically uncomfortable conditions for the animal (Gupta et al. 2013; Silanikove & Koluman 2015). Dairy goats experience heat stress when there is an unbalanced between heat production in the body or heat obtained from the environment and heat loss to the environment. Homeostatic responses that are generally occurred due to heat stress in goats include the increase in respiration rate, body temperature, and water consumption as well as the reduction in food intake (Gupta et al. 2013; Caulfield et al. 2014), immunity (Tao & Dahl 2013), and even animal death (Sarangi 2018).

On the other hand, several assessments on physiological and metabolic parameters in animal production commonly use invasive methods like the measurements of rectal temperature, respiration rate, and heartbeat as well as blood sample collection (Stewart et al. 2008; da Costa et al. 2015). These methods showed relatively inaccurate results due to an anxiogenic response from the procedure itself that makes difficulties in the interpretation of results (Soerensen & Pedersen 2015). Invasive methods are also subjective, as well as time, and require a lot of labor consumption in the identification of animal production parameters and have less consideration in animal welfare (Blokhuis et al. 2013). Therefore, the use of IRT becomes a good option in the monitoring of goats' physiological and hematological parameters.

IRT is a non-invasive method used in the measurements of heat transfer and blood flow patterns by detecting body temperature changes (Kammersgaard et al. 2013; Alsaaod et al. 2014; Nääs et al. 2014; Roberto et al. 2014; Tattersall 2016). The result obtained from IRT device allows direct monitoring in the temperature distribution at certain objects (Blanik et al. 2014) which helps in understanding the thermoregulation process (Ghahramani et al. 2016). Therefore, the present study aimed to compare and correlate the results obtained from the IRT method at several body objects with the other invasive methods for the assessments of young dairy goats' physiological and hematological conditions.

MATERIALS AND METHODS

This study was conducted at Goat Research Section, Indonesian Research Institute for Animal Production, Ciawi, Bogor, Indonesia situated at the elevation of 450-500 meters above sea level and rain intensity between 3500 and 4000 mm per year. Using animals in this study has been approved by the Experimental Animal Ethics Committee, Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture, the Republic of Indonesia with Registration No. Balitbangtan/Balitnak/Rm/04/2019.

Four young Sapera dairy goats with an average body weight of 26-28 kg were kept in the individual cages of 1.6 m x 1.0 m in size. The cage was located inside an asbestos-roofed building and each cage was made of metal with boarded floor and food container. All goats were fed by C-Prolac concentrate produced by PT. Citra Ina Feedmill Jakarta about 1600 grams per day and king grass silage at the same amount alternately. The feeding schedule was twice a day at 7 a.m. and 3 p.m. Drinking water was given to the animals two hours before feeding schedule using a water bucket provided in each cage.

Physiological responses

Physiological response parameters measured were skin surface temperature (TS), rectal temperature (TR), body temperature (Tb), heartbeat (Hr), and respiration rate (Rr). The physiological responses data were measured from 6 a.m. till 6 p.m. in 2 h intervals. TR was measured by inserting Omron rectal thermometer model MC-245 (Omron Healthcare Co. Ltd., Kyoto, Japan) to the rectum about 5 cm deep. TS were measured by Omron digital thermometer model MC-720 specified for measuring skin temperature (Omron Healthcare Co. Ltd., Kyoto, Japan) at four observation points, *i.e*. back (A), chest (B), upper leg (C), and lower leg (D). The average of TS mean was obtained using formula modified from McLean et al. (1983), while Tb was calculated according to McLean *et al*. (1983) as follows:

$$TS = 0.25 (A + B) + 0.32 C + 0.18 D$$

$$Tb = 0.86\ TR + 0.14\ TS$$

Hr is measured by placing the stethoscope near the left axillary bone, and then the heart rate calculation is performed for one minute. The Rr is measured by placing the stethoscope on the animal's chest, then the calculation of the amount of inspiration and expiration for one minute of respiration.

Hematological parameter profiles

Analysis in hematological parameters was carried out by collecting blood samples at the beginning and the end of heat measurement which was at 6 a.m. and 6 p.m. About 0.5 ml of blood samples were collected from a jugular vein after cleansing the area around the animal's neck with cotton dipped in 70% v/v alcohol so the veins can be easily identified and the blood collection area can be free from dung which may contaminate the samples. The target vein was gently

pressed using thumb until puffed. A 20G sterile syringe was employed and injected into the jugular vein at about 1-inch deep. The needle was connected with a vacutainer tube after the blood came out and the angle of the syringe was set to 45º. A little attempt may be required to find the correct needle position for discharging the blood. Each goat was returned to their crib after blood collection and no extra bleeding or inflammation should be confirmed.

Following blood collection, each tube was kept in the iced box and stored in the laboratory for further analysis. Vetscan® HM5 hematology analyzer (Abaxis, Inc. Union City, CA, United States of America) was employed to check all blood profiles including white blood cell (WBC), red blood cell (RBC), lymphocytes (LYM), monocytes (MON), neutrophils (NEU), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), and red blood cell distribution width (RDWC).

Infrared thermography

Infrared thermography (IRT) was performed using an IR camera FLIR A320 (FLIR Systems Co. Ltd., St. Leonards, NSW, Australia). IRT was employed for thermographic sensing purposes with an emissivity coefficient of 0.98 and carried out in 2 hours interval starting from 6 a.m. till 6 p.m. The IRT was taken at several observation points of each goat including its eyes, mouth, nose, legs, the left side of the body, the right side of body, vagina, and vulva at the distance of about 1 meter from the goats standing point (Fig. 1). All sensing data were saved in the memory card before transferred to a laptop for further analysis using ThermaCAM Researcher Profesional 2.10.

Data analysis

All collected data followed MIXED procedure from SAS (V. 9.1; SAS Institute Inc., Cary, NC, USA). A coefficient correlation between IR thermographic sensing and physiological responses as well as hematological parameters were calculated using the CORR procedure also taken from SAS.

RESULTS AND DISCUSSION

Body temperature, heartbeat, and respiration rate have been extensively examined in goats. Previous investigations using an invasive method in the physiological parameter assessment (Ogebe et al. 1996) showed a coherent association with results produce by a non-invasive method such as IRT (Roberto et al. 2014; Siva et al. 2014; Lecorps et al. 2016). Descriptive analyses of physiological parameters as well as IRT are described in Table 1. Skin temperatures measured by thermometer have values close to the same parameter measured by IRT, particularly at the front and rear legs. Meanwhile, the body temperatures measured physiologically showed almost similar results with body temperature measured by IRT at the vulva. It is important to note that rectal temperature (39.0°C) has a higher value compared to the IR measurement at the vulva (37.7°C). IRT showed high values at vagina and vulva, but low values at body and legs, whereas at eyes and nose it showed medium-ranged temperatures. However, IR measurements at the vagina and vulva showed a less temperature increment (2.1°C) at all observation times compared to the other body parts.

The results obtained from IRT at the leg, either front or rear, closed to the results obtained by invasive method i.e., about ±1°C higher than the skin temperature.

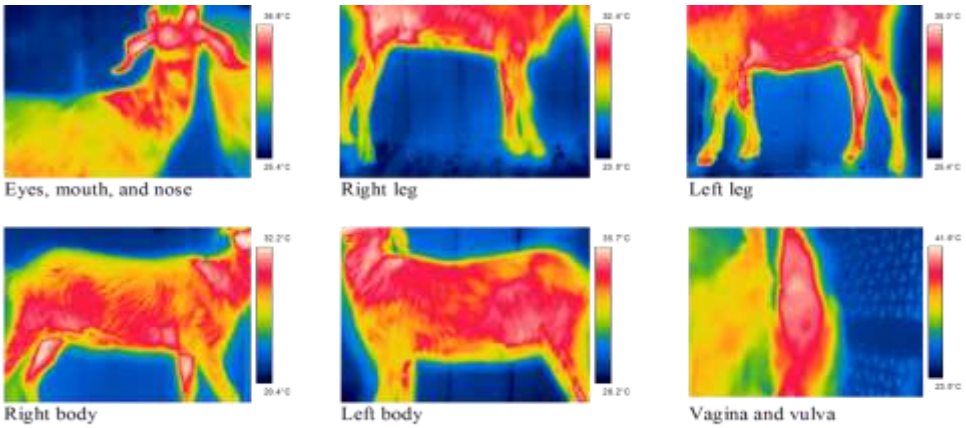

Figure 1. Image results from infrared thermography sensing at several body parts of young Sapera dairy goats

Table 1. Descriptive statistics of physiological condition and infrared thermography (IRT) sensing results of young Sapera dairy goats

Parameter	Mean ± SD	Minimum	Maximum
Physiological condition:			
TS (°C)	31.10 ± 1.84	27.90	33.50
TB (°C)	37.97 ± 0.42	37.10	38.30
TR (°C)	39.00 ± 0.29	38.60	39.30
HR (beat/minute)	95.50 ± 3.89	88.50	99.50
RR (times/minute)	52.64 ± 11.49	39.00	66.00
IRT (°C):			
Mouth and nose	35.90 ± 1.41	33.20	37.30
Right eye	35.57 ± 1.44	32.90	37.00
Left eye	35.73 ± 1.31	33.30	36.90
Right body	33.66 ± 2.26	29.80	36.40
Left body	33.67 ± 2.10	29.90	35.80
Right front leg	32.37 ± 2.27	28.50	34.90
Left front leg	32.43 ± 2.30	28.40	34.70
Right rear leg	32.60 ± 2.26	28.60	35.10
Left rear leg	32.63 ± 2.31	28.40	34.80
Vagina	36.70 ± 1.06	35.20	37.90
Vulva	37.67 ± 0.73	36.40	38.50

TS=Skin temperature; TB=Body temperature; TR= Rectal temperature; HR= Heartbeat; RR= Respiration rate

A similar finding was obtained by Paim et al. (2014) in the IRT at the neck of Santa Ines goat and its breed. In the other IRT studies, McManus et al. (2015) have identified results in a value of 31.29°C at the neck of crossbred goats of Dorset and Santa Ines as well as of 31.44°C at crossbred goats of Dorset and White Doper. The main reason for these similarities, as reported by Popoola et al. (2014), is that skin temperature is an adapted condition of the blood flow in the skin that ends up in the heat regulating process of the body and the skin, in which cattle usually release heat load from their bodies through skins at the neck, ears, and legs in a particular contribution to all body surface. Piccione et al. (2013) report that legs play an important role in the thermoregulation compared to the other body parts due to vasoconstriction in the legs can cause a better mechanism in the heat conversion.

The IRT result at the vulva (37.67°C) closed to the result obtained from body temperature measurement (37.97°C). Hoffmann et al. (2012) obtained a similar result in the IR thermographic sensing at cow's vulva (37.2°C). Different results have been reported by Stelletta et al. (2017) in which IRT at the vulva of Angora goats before lust synchronization is 36.78°C and decreases to 35.40°C at the end of the estrous period. Hoffmann et al. (2012) and Talukder et al. (2015) reported that vulva temperature was the indicator used to identify changes in body temperature as general features of animals' physiology and health. The difference in body temperature measured was due to the difference in the variety of goats used. Simoes et al. (2014) and Stelletta et al. (2017) reported an increase in vulva temperature during the estrous period until approaching ovulation, and then the vulva temperature decreased at the end of the estrous period. The absolute point of vulva temperature can be affected by an environmental condition such as relative humidity, wind speed, and solar radiation. According to Talukder et al. (2014), reduction in vulva temperature at the end of the estrous period is associated with a reduction in estrogen concentration.

The result found a higher rectal temperature compared to the IRT at the vulva. This difference can be acceptable due to rectal temperature measurement using a digital thermometer was considered as the best method to identify goats' condition. However, this method needs time and direct contact with the measured animals. Besides, the method of rectal temperature measurement using a digital thermometer depends on the depth of the penetration. Also, the type of the

thermometer as well as the stress level of the animals may affect the value of temperature obtained (Burfeind et al. 2010; Naylor et al. 2012; Hoffmann et al. 2012).

The IRT at vulva showed the highest temperature (37.67°C) but the lowest temperature increased (2.1°C) across the observation time compared to those measured at the other body parts. This finding agrees with the finding reported by Hooper et al. (2018) in which they found the highest value (38.29°C) at vulva using IRT compared to the other body parts and the lowest temperature increment (0.66°C) was found at observation time from 6 a.m. to 6 p.m. In this present study, the lower temperatures at body and legs than at the vagina and vulva were possibly affected by the high thermal isolation due to the difference in skin thickness and fur compactness (Arkin et al. 1991). Moreover, Bianchini et al. (2006) and Radon et al. (2014) suggested that skin and fur may influence heat transfer depending on their color, compactness, diameter, and depth, as well as on heat transmissivity and absorption.

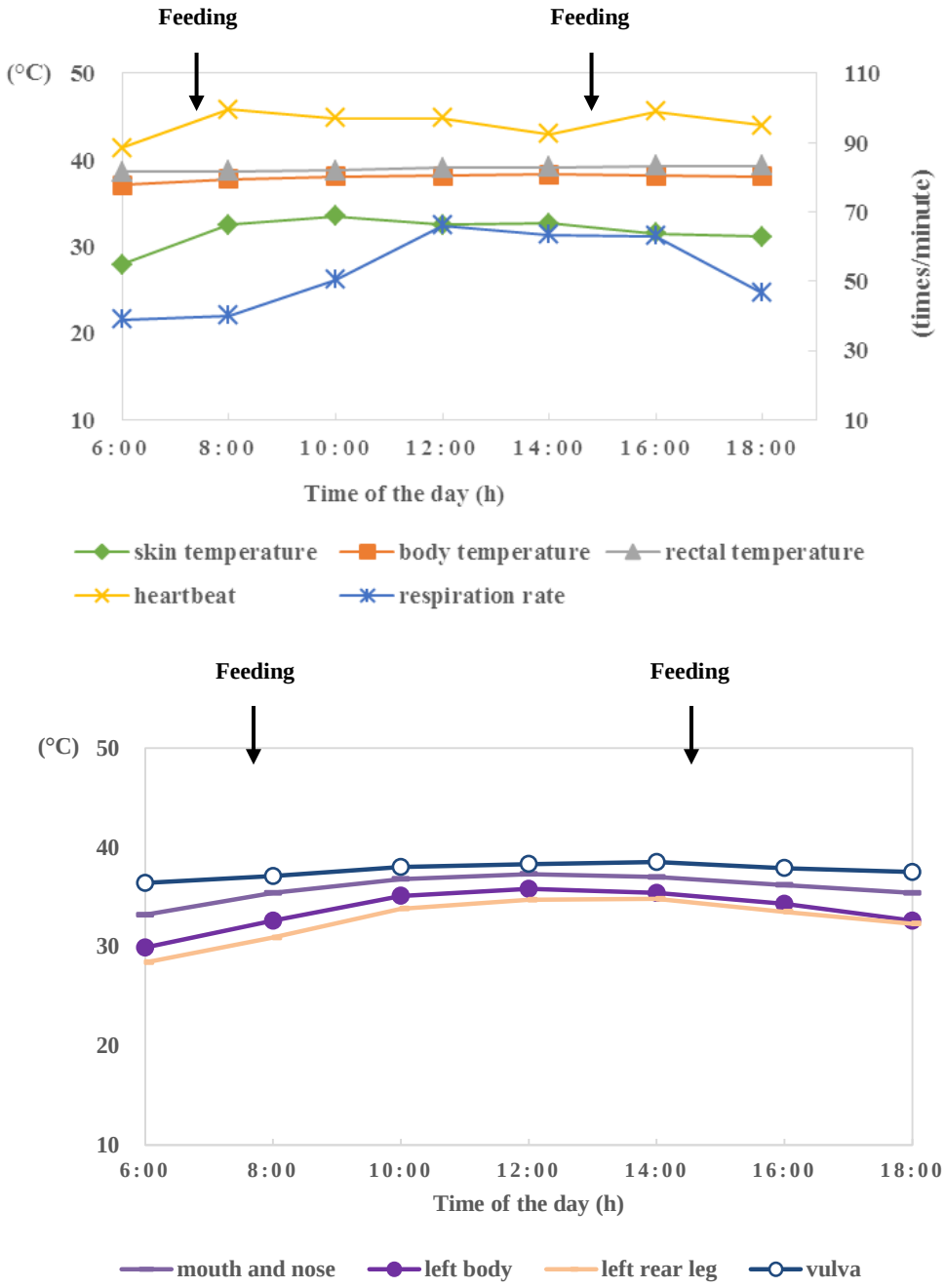

Figure 2. Changes in skin temperature, body temperature, rectal temperature, heartbeat, and respiration rate (A); IRT of mouth and nose, left body, left rear leg and vulva (B) of the goats during the observation

In general, results by IRT showed a similar curve pattern with the RR compared to the HR pattern along the observation time. The mean HR was 95 beats/minute and the RR was 52 times/minute. The IRT results at eyes, mouth, and nose, and the left/right body is in the range between the TS and TB (Fig. 2A). TR and TB have relatively stable changes and have an almost similar pattern with IR image results at the vulva. Similarly, the TS curve performs the same pattern with IRT at the left rear leg (Fig. 2B). All parameters showed significant increments in the feeding time at 7 a.m., but no increase in the feeding time at 3 p.m, except for HR which still showed a significant increase. HR showed two peaks during the whole day which occurred 1 hour after feeding time. A high increase was found in the respiration rate from 8 a.m. to 12 a.m., whereas a big decrease started from 4 p.m. to 6 p.m. (Fig. 2).

Two major peaks observed for the HR (Fig. 2) represents an increase in heat energy release due to feeding that usually occurs about 1 to 2 hours after feeding time. This condition is usually followed by an increase in the TS at several body parts (Montanholi et al. 2008) as is found also in this present study. The increase in heat energy release occurred for 3 to 4 hours after feeding (Puchala et al. 2007). This increase was associated with the protein mobilization process to trigger glucose synthesis (Lawler dan White, 2003). Puchala et al. (2007) suggested that the increase in an HR was caused by an intention to get food and hunger condition as a response of lateral *hypothalamus* and projected to the lateral *medulla* (as host for cells actuating autonomous system including parasympathetic vagal nucleus) and a group of sympathetic system cells in the bone marrow. Moreover, Olsson and Carlsson (1999) suggested that the provision of water and food at the same time may stimulate sensory input in the faring area caused by temporary activation of the sympathetic neural system which may increase HR.

In this study, the average HR about 95 (min 88.5 and max 99.5) beats/minute and RR of 52 (min 39 and max 66) times/minute were obtained. Afshar et al. (2005) reported an average RR and HR of Iranian female goats were as many as 20.2 and 114.2 times/minute, respectively. Hooper et al. (2018) obtained an average RR of Saanen goats about 49.5 times/minute, whereas de Lima et al. (2019) obtained the same from similar breed about 72.6 times/minute after giving birth. In another experiment, Phulia et al. (2010) found an increase in RR from 43.7 to 77.3 times/minute when Sirohi goats stood in a hot environment for six hours. HR and RR increased due to the effects of environmental conditions and body temperature. Increased respiratory action is an attempt to stabilize heat loss by evaporative cooling (Gupta et al. 2013), whereas increased HR is due to the increased muscle activity in combination with the increase in respiration speed and reduction in the peripheral resistance of blood vessel. The increased HR can cause increased blood flow to the body surface that could increase heat loss via conduction, convection, radiation, as well as diffusion from the skin (Marai et al. 2007; Spiers 2012; Okoruwa 2014). Al-Haidary et al. (2012) suggested that the RR could be used to estimate the negative effect of ambient temperature as an indicator of stress due to heat stress. Okoruwa et al. (2013) also suggested that the RR of more than 12-20 times/minute in goats and sheep could be used as an indicator of heat stress. Swollen breath is a mechanism of evaporation due to heat loss and respiration rate tends to follow heat loss because of that evaporation (Marai et al. 2007).

Evaluation in hematological parameters plays an important role to identify animals' prosperity because blood is an important indicator in response to stress due to heat (Okoruwa 2015). Hematological profiles of young Sapera dairy goats did not show significant changes across the observation time (from 6 a.m. to 6 p.m.). However, some changes were found in a few blood parameters, but the increments were very small (Table 2). The blood counts obtained in this present study were still in the reference hematological range for goats (Piccione et al. 2014; Arfuso et al. 2016) and higher values generally occurred in the morning. A study related to heat tolerance in goats has reported a decrease in few blood components in the evening such as HGB concentration (Correa et al. 2012).

Heat can reduce blood count due to hemodilution effect by which lots of water component will be transported by the blood circulation system to help evaporative cooling (Seixas et al. 2017). Almost similar HGB value was found in the experiment on West African Dwarf goats by Opara et al. (2010), whereas higher HGB, MCHC, and WBC values were found in the experiment on Etawah breed goats (Yupardhi et al. 2013) and on the crossbreeding of Saanen and Ettawah (Sarmin et al. 2019). Okonkwo et al. (2011) suggested that results on goat's hematology depended on the type and age of the goats. High HGB value tends to transport more oxygen to blood (Okonkwo et al. 2011). Similarly, higher erythrocyte (MCV, MCH, and MCHC) and WBC indices show a higher oxygen transport capacity on blood (Tsai et al. 2010) and a higher immune system to fight against infection agents (Piccione et al. 2014), especially on young goats than on does.

The associations between IRT results with physiological and hematological parameters are presented in Table 3 and Table 4. The results obtained from IRT sensing were positively correlated ($p < 0.0001$) with goats' physiological parameters (Table 3) such as TB ($r = 0.69$ to 0.85), TS ($r = 0.63$ to 0.76), and

Table 2. Hematological conditions of young Sapera dairy goats during study

Parameters	Observation time		Changes
	6 a.m.	6 p.m.	
RBC (10^6/µL)	15.58 ± 0.42	15.38 ± 0.79	-0.20
HGB (g/dL)	9.45 ± 0.35	9.35 ± 1.02	-0.10
HCT (%)	24.94 ± 0.87	24.63 ± 1.99	-0.31
MCV (fl)	16.25 ± 0.50	16.00 ± 0.82	-0.25
MCH (pg)	6.05 ± 0.17	6.08 ± 0.40	+0.03
MCHC (g/dL)	37.88 ± 1.02	37.93 ± 1.91	+0.05
RDWC (%)	35.30 ± 0.46	35.48 ± 1.40	+0.18
WBC (10^3/µL)	17.89 ± 2.15	17.02 ± 5.28	-0.87
LYM (10^3/µL)	8.39 ± 0.18	7.62 ± 2.17	-0.77
MON (10^3/µL)	0.15 ± 0.05	0.12 ± 0.03	-0.03
NEU (10^3/µL)	9.35 ± 2.03	9.28 ± 3.28	-0.07

RBC = red blood cell, HGB = hemoglobin, HCT = hematocrit, MCV = mean cell volume, MCH = mean cell hemoglobin, MCHC = mean cell hemoglobin concentration, RDWC = red blood cell distribution width, WBC = white blood cell, LYM = lymphocyte, MON = monocyte, NEU = neutrophil

TR (r = 0.48 to 0.67) as well as RR, except HR and hematology. A high positive correlation (r = 0.85) was found between IRT at the rear left leg with body temperature (Table 3). IRT was negatively correlated with hematological parameters (r = -0.01 to -0.71) as described in Table 4, except for neutrophil count (r = 0.32).

The data showed that IRT at different body parts have different correlations with skin temperature, rectal temperature, body temperature, heartbeat, and respiration rate as well (Fig. 2 and Table 3). The difference in the measured body parts is relevant to the heat storage and release (Kenny & Jay 2013). Fluctuations in rectal temperature and respiration rate across the observation time were also investigated by Hooper et al. (2018) with the lowest value occurred at 6 a.m. morning. Several studies (Paim et al. 2014; McManus et al. 2015; Seixas et al. 2017) reported that the lowest temperatures occurred in the morning and reached its peak at noon.

Temperature measurements at several body parts are important to identify the capacity of physiological adaptation of cattle to heat stress on the environment (Silanikove 2000). Experimental results showed a higher positive correlation between IRT testing with TB compared to that with TS or TR and no significant correlation was found between IRT with RR and HR. In this context, IRT is a possible non-invasive method to study temperature and metabolic response due to thermal stress on animals (Paim et al. 2013; Martello et al. 2016; Hooper et al. 2018). Evidence revealed that right and left legs, either front or back, showed a higher correlation with TB, TS, and TR. Piccione & Refinetti (2003) found a strong daily rhythm between TR and leg temperature due to the input rhythm from the suprachiasmatic nucleus at the *hypothalamus* that acts as a thermoregulating agent. D'Alterio et al. (2011) have described body extremity as the main factor in regulating heat storage or loss. Further, D'Alterio et al. (2011) report that goat's legs play important role in the mechanism related to heat loss by which these body parts have vein tissue rich in branches that make heat transfer be possibly mediated by the increase in blood flow.

A nonsignificant correlation between IRT result and HR was probably due to the absence of thermal pressure during the observation period. Another study suggests that heat stress in animals' cardiovascular systems may have an effect on the increase in heartbeat, and this effect is associated with the decrease in blood pressure that stimulates HR (Du Prezz 2000). In general, the results of IRT were negatively correlated with hematological parameters. Similarly, some studies found negative associations between ambient air temperature, as well as the temperature-humidity index (THI), and hematological parameters including RBC and HGB concentrations (Correa et al. 2012; Seixas et al. 2017). This finding was associated with hemodilution effect and low RBC count as well as HGB concentration in the animals' performing high adaptation capacity to environmental condition and showing a negative effect with heat released by their bodies (Seixas et al. 2017).

Table 3. Correlation data between physiological parameters of young Sapera dairy goats and infrared thermography (IRT) results

Physiological parameters	Nose	Right eye	Left eye	Right body	Left body	Right front leg	Left front leg	Right rear leg	Left rear leg	Vagina	Vulva
TS	0.74 ***	0.76 ***	0.67 ***	0.72 ***	0.74 ***	0.73 ***	0.74 ***	0.76 ***	0.75 ***	0.63 ***	0.63 ***
TR	0.51 ***	0.49 **	0.55 ***	0.48 **	0.51 ***	0.57 ***	0.60 ***	0.61 ***	0.67 ***	0.52 **	0.62 ***
TB	0.76 ***	0.76 ***	0.73 ***	0.73 ***	0.76 ***	0.79 ***	0.81 ***	0.83 ***	0.85 ***	0.69 ***	0.75 ***
HR	0.29 ns	0.30 ns	0.38 *	0.27 ns	0.26 ns	0.20 ns	0.19 ns	0.28 ns	0.24 ns	0.19 ns	0.20 ns
RR	0.43 *	0.46 *	0.53 **	0.44 *	0.43 *	0.41 *	0.37 ns	0.45 *	0.41 *	0.39 *	0.57 **

TS=Skin temperature; TR= Rectal temperature; TB=Body temperature; HR= Heartbeat; RR= Respiration rate

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = non signifiant

Table 4. Correlation data between hematological parameters of young Sapera dairy goats and infrared thermography (IRT) results

	Nose	Right eye	Left eye	Right body	Left body	Right front leg	Left front leg	Right rear leg	Left rear leg	Vagina	Vulva
RBC	-0.16 ns	-0.16 ns	-0.17 ns	-0.05 ns	-0.16 ns	0.15 ns	0.12 ns	0.01 ns	-0.01 ns	-0.03 ns	-0.02 ns
HGB	-0.21 ns	-0.19 ns	-0.18 ns	-0.08 ns	-0.19 ns	0.09 ns	0.09 ns	-0.04 ns	-0.04 ns	-0.14 ns	-0.17 ns
HCT	-0.32 ns	-0.26 ns	-0.31 ns	-0.18 ns	-0.25 ns	-0.04 ns	0.02 ns	-0.08 ns	-0.07 ns	-0.11 ns	-0.28 ns
MCV	-0.41 ns	-0.32 ns	-0.40 ns	-0.29 ns	-0.33 ns	-0.18 ns	-0.12 ns	-0.16 ns	-0.16 ns	-0.13 ns	-0.39 ns
MCH	-0.20 ns	-0.17 ns	-0.15 ns	-0.08 ns	-0.18 ns	0.03 ns	0.07 ns	-0.06 ns	-0.04 ns	-0.20 ns	-0.23 ns
MCHC	0.04 ns	-0.02 ns	0.06 ns	0.09 ns	-0.03 ns	0.22 ns	0.16 ns	0.03 ns	0.02 ns	-0.12 ns	0.08 ns
RDW	0.19 ns	0.18 ns	0.25 ns	0.17 ns	0.25 ns	0.02 ns	-0.03 ns	0.07 ns	0.07 ns	0.00 ns	0.09 ns
WBC	-0.14 ns	-0.13 ns	-0.16 ns	-0.05 ns	-0.18 ns	0.13 ns	0.10 ns	0.02 ns	-0.02 ns	-0.09 ns	-0.10 ns
LYM	-0.35 ns	-0.35 ns	-0.39 ns	-0.33 ns	-0.45 ns	-0.21 ns	-0.21 ns	-0.27 ns	-0.31 ns	-0.34 ns	-0.36 ns
MON	-0.69 ns	-0.64 ns	-0.59 ns	-0.49 ns	-0.52 ns	-0.44 ns	-0.40 ns	-0.48 ns	-0.45 ns	-0.60 ns	-0.71 ns
NEU	0.01 ns	0.03 ns	0.01 ns	0.14 ns	0.00 ns	0.32 ns	0.29 ns	0.19 ns	0.16 ns	0.08 ns	0.07 ns

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = non significant. RBC = red blood cell, HGB = hemoglobin, HCT = hematocrit, MCV = mean cell volume, MCH = mean cell hemoglobin, MCHC = mean cell hemoglobin concentration, RDW = red blood cell distribution width, WBC = white blood cell, LYM = lymphocyte, MON = monocyte, NEU = neutrophil

CONCLUSION

Infrared themography (IRT) shows a curve pattern that was almost the same as the physiological parameters in young Sapera dairy goats from the beginning until the end of the observation. Significant correlations were found between physiological parameters (except HR and hematological) of goats on thermography measurements results at several body parts. The highest positive correlation (r = 0.85) was found between TB and IRT of the left rear leg. Therefore, it was concluded that IRT can be applied to measure goats physiological conditions especially body temperature.

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Effect of Combination of Follicle Size, FSH and Cysteamine on *In Vitro* Production of Sheep Embryos

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ABSTRAK

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Hubungan partisipatif antara ukuran folikel, *follicle stimulating hormone* (FSH), dan sisteamin (agen antioksidan) berkontribusi dalam produksi embrio yang ditandai dengan jumlahnya yang melimpah dengan kualitas yang baik. Tujuan dari penelitian ini adalah untuk mengevaluasi efektifitas FSH, sisteamin dan ukuran folikel dalam produksi embrio *in vitro* oosit domba Awassi. Ukuran folikel dikategorikan menjadi: folikel kecil (1-2 mm) dan folikel besar (>2 mm). Oosit dimatangkan pada dua kadar FSH dan sisteamin yang ditingkatkan: A (40 ng/ml + 50 μM) dan B (60 ng/ml + 100 μM). Hasil interaksi bilateral menunjukkan perbedaan yang signifikan pada seluruh ukuran folikel (kelompok folikel besar) dan perlakuan pematangan (media B) pada tingkat fertilisasi (nilai tertinggi: 67,51%; p= 0,02), pembelahan (nilai tertinggi: 65,41%; p= 0,01), tahap sel 2-16 (nilai terendah: 2,29%; p= 0,0001), tahap blastokista (nilai tertinggi: 44,82%; p= 0,04), menuju tahap penangkapan morula (nilai terendah: 55,17%; p= 0,04) dan embrio Tipe I (nilai tertinggi: 52,87%; p= 0,03). Demikian juga, oosit matang dari kelompok folikel kecil (medium B) mencapai tingkat tertinggi pada tahap morula (56,60%; p= 0,03). Tidak terdapat perbedaan signifikan pada embrio Tipe II dan Tipe III. Untuk menghasilkan produksi yang tinggi dari embrio berkualitas tinggi, disarankan untuk menambahkan FSH dan sisteamin dengan kadar masing-masing 60 ng/ml dan 100 μM dalam media maturasi oosit *ovine* yang diperoleh dari folikel dengan diameter >2 mm.

Kata Kunci: Sisteamin, Ukuran Folikel, FSH, Produksi Embrio *In Vitro*, Domba

ABSTRACT

Mardenli O, Mohammad MS, Alolo AY. 2020. Effect of combination of follicle size, FSH and cysteamine on *in vitro* production sheep embryos. JITV 25(3): 131-138. DOI: http://dx.doi.org/10.14334/jitv.v25i3.2517

The participatory relationship among the follicle size, follicle stimulating hormone (FSH), and cysteamine (antioxidant agent) contribute to the production of embryos characterized by abundance and good quality. The aim of this study was to evaluate the efficacy of FSH, cysteamine and follicle size on *in vitro* embryo production of Awassi sheep oocytes. Follicles sizes were determined into two groups: small follicles (1-2 mm) and large follicles (> 2 mm). Oocytes were matured across two increasingly shared levels of FSH and cysteamine: A (40 ng/ml + 50 μM) and B (60 ng/ml + 100 μM). Results of the bilateral interaction showed significant differences across the follicle size (large follicles group) and the maturation treatment (B medium) in the rates of fertilization (highest value: 67.51%; p= 0.02), cleavage (highest value: 65.41%; p= 0.01), 2-16 cell stage (lowest value: 2.29%; p= 0.0001), blastocyst stage (highest value: 44.82%; p= 0.04), down to morula stage arrest (lowest value: 55.17%; p= 0.04) and Type I embryos (highest value: 52.87%; p= 0.03). Likewise, matured oocytes of small follicles group (B medium) attained the highest rate of morula stage (56.60%; p= 0.03). No significant differences were observed in Type II and Type III embryos. In order to obtain high yields of good quality embryos, it is advised to add FSH and cysteamine with levels of 60 ng/ml and 100 μM respectively to maturation medium of ovine oocytes obtained from follicles with a diameter > 2 mm.

Key Words: Cysteamine, Follicle Size, FSH, *In Vitro* Embryo Production, Sheep

INTRODUCTION

The deep understanding of the overall processes governing the development of ovarian follicles and oocytes inside the body (*in vivo*) has led to higher *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and cleavage rates in all animal species. Perhaps the emergence of the important trend in the *in vitro* embryo production (IVEP) technology which is the addition of hormones and anti-oxidant agents together in the maturation media was of great interest due to the positive participatory effect. The gonadotropic hormones (FSH, luteinizing hormone (LH) and chorionic gonadotropin (hCG)) are one of the most

important additives in IVM, as the use of these additives during maturation has led to a high rate in the embryos outcome (Kouamo & Kharche 2014; Kalita et al. 2019).

In the ovum pick-up (OPU) technique, FSH plays a key role in increasing the number of follicles and embryo outcome of cattle (Hasler 2014). Some studies indicated the possibility to increase the rate of twining birth rates in lactating cattle following FSH treatment at different stages of the estrus cycle (Situmorang et al. 2010; Situmorang et al. 2012). Beyond that, Aryogi et al. (2013) stated that with the increased levels of FSH, the Ongole cross breed cows with twin birth history could produce more than one of follicle de Graf in an estrus cycle.

Cysteamine is characterized by stability and antioxidativity. Due to the historical popularity and the primary role of cysteamine as an antioxidant agent, its usage has increased recently. However, this usage has also remained limited to specific concentrations, as are hormones. Within the close relationship that governs the follicle size and the size of the oocytes (diameter), it is noted that with the increased size of the follicle and the follicular fluid (FF) the diameters of the oocytes increase (Grabowska et al. 2016), and thus the oocytes acquire the developmental competence that makes them eligible to follow fertilization and subsequent cellular division of the embryos.

In Syria, Awassi sheep are considered a pure breed and of great economic importance because of the distinctive productive characteristics of meat and milk, and there was a need to establish a genetic bank to preserve the genetic resources of this breed. Hence, the aim of this study was to assess the combined effects of follicle size, FSH and cysteamine on the rates of fertilization and cleavage of Awassi sheep oocytes as well to studying the stage and embryo quality.

MATERIALS AND METHODS

Ovaries collection

Ovaries were collected from slaughterhouses located in the city of Aleppo during the reproductive season. Immediately after collection, the ovaries were kept in Dulbecco's Phosphate Buffer Saline (DPBS) and transported to the biotechnology laboratory at the Faculty of Agriculture within 20 minutes (Figure 1; A and B).

Follicle size determination

Follicles diameters were measured by a certain ruler and divided into two main classes: small follicles (SF): 1-2 mm and large follicles (LF): >2 mm.

Oocytes collection

Cumulus oophorus complexes (COCs) were collected in two consecutive ways (aspiration and slicing) as follows: for each group of follicles, the aspiration was done by aspirating the follicular fluid through a sterile 18-g needle attached to a 5 ml syringe containing a sterile saline solution. Next, the slicing method was applied to the same follicles from which the follicular fluid was aspirated by using a surgical blade. The dissected follicles were washed several times with Tissue Culture Medium-199 (TCM-199) supplemented with heparin. Obtained COCs in both ways were received in a Petri dish together (Figure 1; D).

FSH and cysteamine concentrations determination

The concentrations of both the FSH and cysteamine were determined according to two increasingly shared concentrations (Table 1).

Table 1. FSH and cysteamine levels determined in the study

Supplementation medium	FSH (ng/ml)	Cysteamine (μM)
A	40	50
B	60	100

Experimental design

The experiment was designed according to the two-factor design (2 follicle size classes × 2 maturation treatments) for several traits (IVF, cleavage, arrest, embryo stages and embryo quality). A focus was placed on showing the results of bilateral interactions among the factors involved.

IVM and IVF conditions

After adding FSH and cysteamine levels to TCM-199, IVM conditions were done as described elsewhere by de Oliveira Bezerra et al. (2019) with some modifications. In short, every 10-15 oocytes were cultured in TCM-199 supplemented with 10% fetal calf serum (FCS) and 50 μg/ml gentamycin sulfate. The selected oocytes were transferred to wells containing the maturation medium under mineral oil and then incubated for 27 hours at 38.5°C with 5% CO_2 and saturated humidity (Figure 1; C). Following incubation, the maturation stage was determined by the expansion of the cumulus cells and the detecting of the second polar body (Figure 1; E and F). IVF was done using

Tyrode's albumin lactate pyruvate (TALP) medium supplemented with 5 mg/ml bovine serum albumin (BSA), 0.2 mm sodium pyruvate, 25 mm sodium bicarbonate, 50 µl/ml penicillamine-hypotaurine-epinephrine (PHE) solution 10 mg/ml heparin and 13 mm of sodium lactate. Fresh semen was collected from proven rams (electrically) and centrifuged in a Percoll discontinuous gradient (1000×g for 10 minutes). The supernatant was discarded, and the pellet containing viable spermatozoa was re-suspended in 1 mm of TALP and centrifuged again at 300×g for 10 minutes. The spermatozoa were then diluted in TALP to achieve a final concentration of 1×10^6 sperm/ml. Every 10-15 oocytes were then transferred to a drop of fertilization medium and co-incubated with spermatozoa for 18–22 hours at 38.5°C, with 5% CO_2 and saturated humidity. The presence of zygote (pronucleus) was investigated under an inverted microscope (Figure 2; A).

In vitro culture (IVC)

Following IVF, the zygotes were washed twice in TALP and once in 50% TALP + 50% modified medium synthetic oviductal fluid (SOF), transferred to SOF supplemented with 50 mg/ml of amikacin, 5 mg/ml of BSA and 2.5% of fetal bovine serum (FBS) (de Oliveira Bezerra et al. 2019) and cultured at 38.5°C, 5% CO_2 and saturated humidity for 7 days. The presence of blastomeres (2-16 cell), morula and blastocyst stages were investigated under an inverted microscope (Figure 2; B, C and D).

Statistical analysis

After arranging the data (Microsoft Excel sheet) using a sophisticated computer, the data were analyzed by applying Pearson Chi-square of contingency tables in SAS Institute Inc. (2017) statistical package. The rates of different traits were presented as a percentage (%) and compared according to the Fisher exact test for each trait.

Embryo grading

Embryo quality was determined as previously described by Wintner et al. (2017) with some modification: Type I (excellent): the cells of the embryo are equal in size while fragmentation entirely is absent (Figure 3; A), Type II (good): the cells of the embryo are equal in size while minor fragmentation only could be seen (Figure 3; B), Type III (poor): the cells of the embryo are of equal or unequal size while fragmentation is moderate to heavy (Figure 3; C).

Reagents

The chemicals used were from Sigma Chemical Co (St. Louis, USA) unless mentioned otherwise.

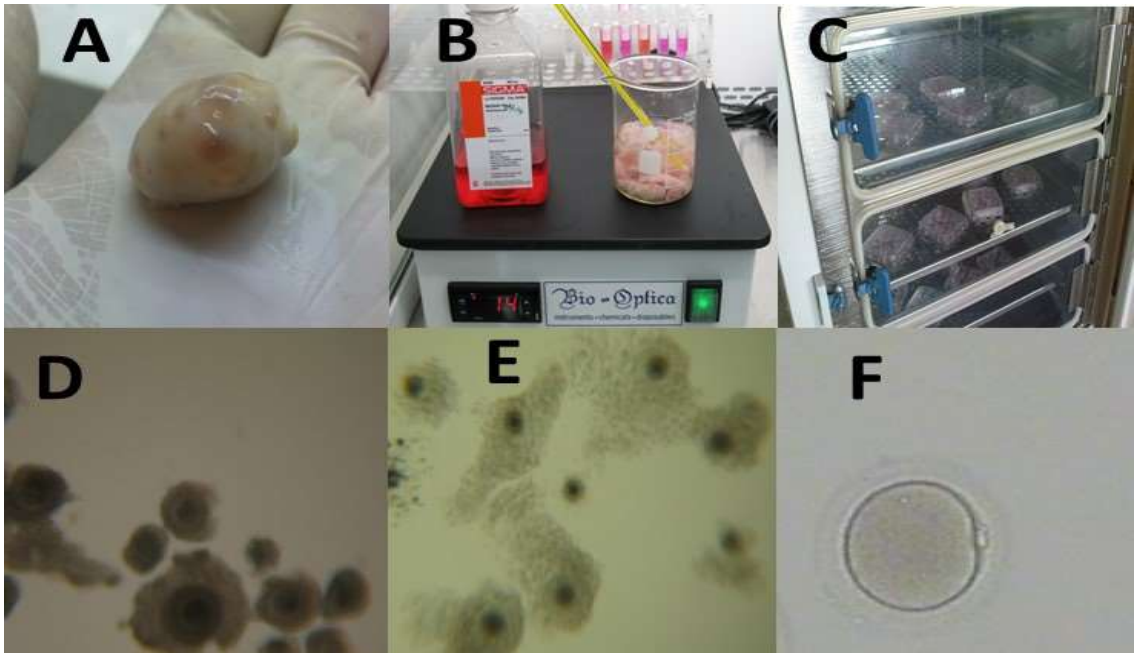

Figure 1. Ovaries collection, oocytes collection and IVM procedures. A: An Awassi sheep ovary. B: Preparing the ovaries for oocytes collection methods. C: Incubating the collected oocytes. D: Awassi sheep oocytes at GV stage. E: Matured oocytes with an expansion of cumulus cells. F: Matured Awassi sheep oocyte with a clear appearance of the second polar body

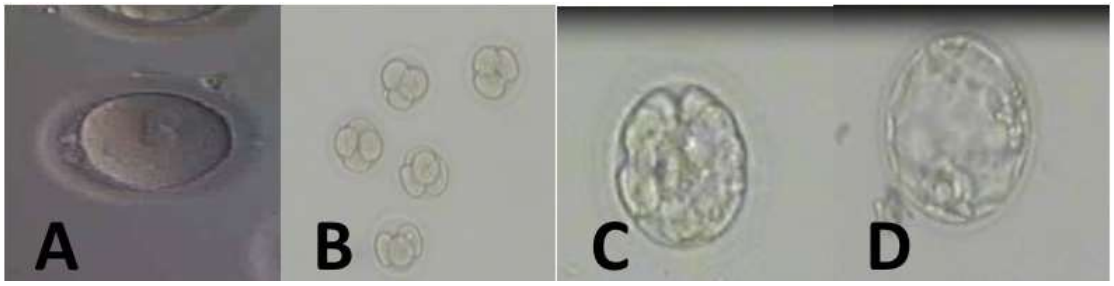

Figure 2. Embryo stages of *in vitro* Awassi sheep produced embryos. A: Zygote. B: Blastomeres (2-4 cell). C: Morula. D: Blastocyst.

Figure 3. Types of embryo quality of *in vitro* Awassi sheep produced embryos. A: Type I. B: Type II. C: Type III.

RESULTS AND DISCUSSION

Results

IVF and cleavage stages

Table 2 shows that matured oocytes in B medium achieved a high response in the IVF (p = 0.002; 67.51%; LF group) and cleavage stages (p = 0.01; 65.41%; LF group). In contrast, the lowest response was observed in oocytes that were matured in A medium (50.81%; SF group, 47.86%; LF group).

Embryo stage and arrest

As indicated in Table 3, the matured oocytes in B medium achieved the highest rates of morula (p = 0.03; 56.60%; SF) and blastocyst stages (p = 0.04; 44.82%; LF group). This was accompanied by achieving the lowest rates at the arrested 2-16 cell stage embryos (p= 0.0001; 2.29%; LF group) and the general rate of arrest (p = 0.04; 55.17%; LF group).

Embryo quality

The quality rates of the embryos ranged between 22.98% and 52.87% and only one significant difference was observed (Type 1), as the matured oocytes in B medium achieved the highest rates (p= 0.03; 52.87%; LF group), while the lowest rates were at embryos whose oocytes matured in A medium (32.14%; LF group) (Table 4).

Discussion

In the current study, the effectiveness of the combined effect of FSH, cysteamine, and follicle size in the studied traits was clearly featured (Tables 3, 4 and 5). The rise in IVF, cleavage and blastocyst rates can be attributed to the vital role resulting from the previous factors. In references, many studies examined the effect of FSH, cysteamine and follicle size separately. The vital roles of the previous factors can be summarized with some of the following points: in the ovary, with regard to FSH, the hormonal regulation plays the basic role in the occurrence of a number of changes that ultimately results in an increase in the size of the follicle, the follicular fluid and the diameter of the oocytes. The previous events would lead the oocytes to reach a stage that enables them to follow cellular divisions and pass the stage of fertilization and division in an optimal way. However, Gougeon (2010) indicated that the optimum size of the follicle in which the oocytes acquire the ideal developmental competence must be at least 2 mm.

The study of Lamb et al. (2011) proved that FSH plays an important role in improving oocyte developmental competence through the mutual relationship between FSH and hCG. In our study, one of the most important factor that plays the key role in determining the previous rates of maturation, fertilization and the ability to follow the cell division of early embryos is that the relationship that governs the follicle size, the diameter of the oocyte, the size of the follicular fluid and the dominant follicle (dynamic follicular wave), which ultimately leads to the retarding

Table 2. Rates of IVF and cleavage of Awassi sheep oocytes under the influence of both follicle size and maturation treatment factors

Source		Incubated oocytes	Fertilized oocytes		Cleaved oocytes	
Maturation treatment	Follicle size	No.	No.	%	No.	%
A	SF	185	94	50.81[a]	46	48.93[a]
A	LF	192	117	60.93[b]	56	47.86[a]
B	SF	201	105	52.23[a]	53	50.47[a]
B	LF	197	133	67.51[c]	87	65.41[b]
p			0.002		0.01	

Values in the same column with different superscript are significantly different at P< 0.05. A = FSH (40 ng/ml) + cysteamine (50 μM), B = FSH (60 ng/ml) + cysteamine (100 μM), SF = small follicles (1-2 mm), LF = large follicles (> 2 mm)

Table 3. Rates of embryonic stages and arrest of Awassi sheep oocytes under the influence of both follicle size and maturation treatment factors

Source		Embryo stage						Arrest (2-16 cell and morula stages)	
Maturation treatment	Follicle size	2-16 cell		Morula		Blastocyst			
		No.	%	No.	%	No.	%	No.	%
A	SF	16	34.78[b]	17	36.95[a]	13	28.26[a]	33	71.73[a]
A	LF	24	42.85[b]	19	33.92[b]	13	23.21[a]	43	76.78[a]
B	SF	6	11.32[c]	30	56.60[a]	17	32.07[a]	36	67.92[a]
B	LF	2	2.29[a]	46	52.87[a]	39	44.82[b]	48	55.17[b]
p		0.0001		0.03		0.04		0.04	

Values in the same column with different superscript are significantly different at P < 0.05. A = FSH (40 ng/ml) + cysteamine (50 μM), B = FSH (60 ng/ml) + cysteamine (100 μM), SF = small follicles (1-2 mm), LF = large follicles (> 2 mm)

Table 4. Rates of embryos types resulted from Awassi sheep oocytes under the influence of both follicle size and maturation treatment factors.

Source		Type I		Type II		Type III	
Maturation treatment	Follicle size	No.	%	No.	%	No.	%
A	SF	16	34.78[a]	15	32.60	15	32.60
A	LF	18	32.14[a]	21	37.50	17	30.35
B	SF	19	35.84[a]	16	30.18	18	33.96
B	LF	46	52.87[b]	21	24.13	20	22.98
p		0.03		NS		NS	

Values in the same column with different superscript are significantly different at P < 0.05. A = FSH (40 ng/ml) + cysteamine (50 μM), B = FSH (60 ng/ml) + cysteamine (100 μM), SF = small follicles (1-2 mm), LF = large follicles (> 2 mm), NS = not significant

inhibitory effect on the development of the follicles at the expense of the dominant follicle (Imron et al. 2016), and thus the negative effect on the developmental competence of oocytes in these non-developing follicles due to the presence of some growth inhibitors in the follicular fluid (FF).

During controlled ovarian stimulation, a close relationship was observed between weight-adjusted recombinant FSH (rFSH) dose and follicular growth, where a decrease in the variability of the follicular sizes and an increase in the rates of mature oocytes were observed (Abbara et al. 2019). On the other hand, the biochemical role of cysteamine can be summarized by neutralizing the harmful effects of reactive oxygen species (ROS) inside the cells, thus decreasing the damage caused by oxygen (O_2) in lipids, protein and nucleic acids during the early embryo development (Kitagawa et al. 2004). Besides, cysteamine stimulates glutathione synthesis (GSH), which in turn improves the developmental competence of oocytes (Gasparrini et al. 2006). In addition, recent studies highlight the important role of cysteamine in the positive effect on different stages of nuclear maturation of oocytes. In a study conducted by Mahmoud et al. (2016) the rates of metaphase II reached 76.2% (with 50% µm cysteamine) and 69.2% (without cysteamine) respectively. Not only that, cysteamine has a prominent role in up-regulating the expression of anti-apoptotic and down-regulating the expression of proapoptotic genes in early embryo stages (Elamaran et al. 2012). As a result, cysteamine has a long-term positive effect on oocyte performance in maturation, fertilization and subsequent development of early embryos (blastocyst stage). Perhaps these vital roles of cysteamine are the main reason for the low rates of arrest presented in our study (Table 3), specifically in embryos resulting from oocytes that have been matured in B medium (LF group). In general, the results of the current study related to the rates of cleavage and blastocyst stage can be compared with the results of some studies (Lojkić et al. 2016; Shabankareh et al. 2015; Muasa 2010; Lunardelli et al. 2016; Merton et al. 2013; Shabankareh & Zandi 2010; Ranjbar et al. 2018) summarized in Table 5 according to the studied factors.

The fertilization rates in the current study came low compared with the rates reached by Izumi et al. (2013) as those rates were 91.2, 90.9 and 85.3% at cysteamine levels of 100, 200 and 500 µM respectively (follicle size = 1 mm). On the other hand, the morula rates in our study were higher than those in the study of Shabankareh & Zandi (2010), as the rate was 50.2%. In the present study, the data of Table 4 indicated a significant difference in embryo quality (Type I; B medium; LF group). In fact, the quality index occupies a large area of importance in IVEP applications. Embryo quality is a basic and critical requirement in embryo transfer (ET) technology. In literature, many studies related to ET have emphasized that the

Table 5. Rates of cleavage and blastocyst stage resulted from maturation with different levels of FSH and cysteamine across different follicle sizes

Follicle size	FSH	Cysteamine	Cleavage	Blastocyst	Source
≤5 mm	75 IU	100 µM	39.5%	8.0%	Lojkić et al. (2016)
>5 mm			58.3%	24.7%	
3–6 mm	0.02 IU/m	-	79.1%	30.8%	Shabankareh et al. (2015)
6–9 mm		-	84.4%	33.6%	
10–20 mm		-	80.5%	38.7%	
1 -3 mm	1ul/ml	-	51.87%	12.07%	Muasa (2010)
> 3 -6 mm		-	55.88%	29.78%	
> 6 mm		-	84.87%	41.25%	
≤ 2 mm	0.5 µg	-	16.6%	40.2%	Lunardelli et al. (2016)
4-8 mm		-	19.0%	50.5%	
Different sizes	4 mg	0.1 mM	59.3%	16.6%	Merton et al. (2013)
2-8 mm	0.5 µg/ml	100 µM	84.5%	35%	Shabankareh & Zandi (2010)
2-8 mm	-	50 µM	48%	20%	Ranjbar et al. (2018)
	-	100 µM	48%	22%	

transferred embryos are of the first type (Type I) in the first importance level, and the Type II in the second importance level. However, the study of Wintner et al. (2017) reported that poor quality embryo does not negatively affect a good quality embryo when transferred together in a double ET. Nevertheless, studies related to embryo quality remained scarce. In contrast, some studies indicated some factors that play a fundamental role in determining the quality of embryos such as the reproductive status of females (Twigg-Flesner et al. 2014), nutritional status (Ashworth et al. 2009; Chundekkad et al. 2020), age (Hammami et al. 2013), reasons related to spermatozoa characteristics used in fertilization (Ervandi et al. 2013; Chapuis et al. 2017), semen quality (Kusumaningrum et al. 2015) and the type of protocol used for super ovulation (Sumantri et al. 2011).

CONCLUSION

Through the outputs of the current study, it was found that maturation of Awassi sheep oocytes originated from follicles with a diameter of more than 2 mm in TCM-199 supplemented with 60 ng/ml of FSH and 100 μM cysteamine resulted in a clear increase in embryos yields as well as in the Type I embryos.

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Synergistic Effect of Biofat and Biochar of Cashew Nutshell on Mitigate Methane in the Rumen

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ABSTRAK

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Salah satu cara untuk mengurangi emisi metana adalah dengan menggunakan aditif pakan yang berasal dari ekstrak tumbuhan yang mengandung senyawa metabolik sekunder. Penelitian ini bertujuan untuk mengevaluasi kombinasi biofat (BF) dengan biochar (BC) hasil pengolahan cangkang buah mete sebagai pakan aditif untuk menekan produksi CH_4 dan meningkatkan kinerja rumen secara *in vitro*. Penelitian ini menggunakan rancangan acak blok yang terdiri dari 6 perlakuan dan 4 ulangan. Perlakuan terdiri dari kombinasi biofat (BF) dengan biochar (BC) dalam rasio yang berbeda dan ditambahkan ke substrat sebagai berikut: Kontrol = substrat; BFBC1 = 0%BF: 100%BC; BFBC2 = 25%BF:75%BC; BFBC3 = 50%BF:50%BC; BFBC4 = 75%BF:25%BC; BFBC5 = 100%BF: 0%BC. Variabel yang diukur: produksi gas total dan CH_4, degradasi bahan kering (BK); bahan organic (BO) dan *neutral detergent fiber* (NDF), NH_3 dan konsentrasi *volatile fatty acid* (VFA) parsial. Hasil analisis menunjukkan penambahan kombinasi berbagai level BF dan BC menyebabkan penurunan secara sangat signifikan ($P<0,01$) terhadap produksi CH_4 di dalam rumen. Dibandingkan kontrol, produksi CH_4 turun pada BFB1 sebesar 11,50% BFBC2 36,85%, BFBC3 38,50% , BFBC4 41,84% dan BFBC5 26,07%. Kombinasi sampai pada level BFBC4 tidak meningkatkan kadar NH_3 secara nyata dibanding kontrol, tapi terjadi peningkatan produksi propionat dan total *volatile fatty acid* (VFA) secara signifikan di dalam rumen ($P<0.05$) pada penambahan kombinasi biofat dan biochar dibanding kontrol. Nilai degradasi BK dan BO sama dengan kontrol ($P>0,05$). Dapat disimpulkan bahwa efek sinergitas dari pakan aditif dalam menekan CH_4 dan meningkatkan produk fermentasi rumen adalah kombinasi BF dengan BC dengan rasio 75%BF:25%BC.

Kata Kunci: Cangkang Biji Mete, Biofat, Biochar, Kombinasi, *In Vitro*, Rumen

ABSTRACT

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One way to reduce methane emissions is by using feed additives derived from plant extracts containing secondary metabolic compounds. This study aimed to evaluate the effectiveness of combinations of biofat and biochar (bioindustrial products of cashew nut shells) as feed additive in reducing methane production and improving *in vitro* rumen fermentation. In this experiment, a randomized block design with 6 treatments and 4 replications was applied. The treatments were different combination of biofat (BF) and biochar (BC) as follows: Control= substrate only without addition of biofat or biochar; BFBC1 = 0%BF: 100%BC; BFBC2 = 25%BF:75%BC; BFBC3 = 50%BF:50%BC; BFBC4 = 75%BF:25%BC; BFBC5 = 100%BF: 0%BC. The measured variables were: total gas and CH_4 productions, dry matter (DM); organic matter (OM); and neutral detergent fiber (NDF) ruminal degradabilities, NH_3 and partial volatile fatty acid (VFA) concentrations. Result showed that the addition of combinations of biofat and biochar into the substrates resulted in significant decrease ($P<0.01$) of CH_4 production in the ruminal fluid. Compared to control, CH_4 production was lower by 11.50% (BFBC1), 36.85% (BFBC2), 38.50% (BFBC3), 41.84% (BFBC4) and 26.07% (BFBC5). All combinations except BFBC5 produced similar NH_3 concentration but significantly higher propionate and total VFA concentration in the *in vitro* rumen than control, dry matter degradability and organic matter degradability in the presence of combination of biofat and biochar at different ratios were similar to the control ($P>0.05$). In conclusion, the best combination in producing a synergistic effect as a feed additive to reduce methane, and enhance rumen fermentation products *in vitro* is BFBC4: biofat 75% and biochar 25%.

Key Words: Cashew Nut Shell, Biofat, Biochar, Combination, *In Vitro*, Rumen

INTRODUCTION

Methane is the second largest contributor after CO_2 to greenhouse gases in the atmosphere layer, and it has the capability of heat retention 23 times greater than CO_2. Livestock, especially ruminants, is one of the contributors to the accumulation of anthropogenic methane (about 28%). This is due to the process of methane formation or methanogenesis by archaea methanogen residing in the rumen through the reaction of CO_2 and H_2 to CH_4 (US EPA 2005; Cottle et al. 2011). Based on this, it is necessary to mitigate the emission of methane from ruminant livestock, which is not only related to the aspect of environmental conservation but also as an effort to optimize the productivity of ruminant livestock.

Nutrition strategies that have proven to be effective in methane emission mitigation are through direct inhibition of archea methanogen using ionophore compounds such as monensin (Gerber et al. 2013). However, the use of monensin or other types of antibiotics is constrained by the prohibition on the use of antibiotics as a feed additive in the diet. This led to the exploration of various natural compounds to reduce methane emissions (Jayanegara et al. 2013).

One way to reduce methane emissions is by using feed additives derived from plant extracts containing secondary metabolic compounds such as tannins (Bhatta et al. 2013), saponins (Wina 2012; Yuliana et al. 2014), essential oil (Patra & Yu 2012), and Cashew Nut Shell Liquid (CNSL) (Watanabe et al. 2010). Cashew plants (*Anacardium occidentale* Linn) are explored for their nuts, whereas the nut shells which are 45-50% of cashew fruits have not been utilized well. There are three bioindustrial products that have been developed from processing of cashew nut shell i.e CNSL or biofat, biochar and biosmoke. CNSL or biofat has been produced and used by industry **(**Rodrigues et al 2011), but biochar and biosmoke from cashew nut shell have not yet been reported. CNSL or biofat contain anacardic acid and its derivatives which are phenolic compounds to fatty acids (C_{15} = pentadecanoic acid) and exert antimicrobial activity **(**Gandhi et al. 2012). Biochar and biosmoke are products from pyrolysis process of the remaining shell after the biofat has been extracted.

In the previous experiments, these three products showed their potential activity to reduce methane production in the *in vitro* rumen fermentation (Saenab et al. 2018). However, the result showed that each product especially biofat and biosmoke reduced methane and followed by reduction of total gas and degradability of substrate in the rumen at addition of higher doses. Watanabe et al. (2010) concluded that CNSL was a methane reducing agent, but its effect on other parameters in the rumen has not shown significant results. Therefore, another substance may be used together with biofat to produce synergistic effects on rumen fermentation. Previous results (Saenab et al. 2018) concluded that addition of biofat (0.25 µl/ml), biochar (0.3 mg/ml) each showed reduced methane production without affecting feed degradability. The objective of the study was to evaluate the effect of different combinations of biofat with biochar as feed additive on reducing methane production and on improving rumen fermentation.

MATERIALS AND METHODS

Experimental procedures

The experiment was conducted from January to April 2016, at the Feed Laboratory of the Research Institute of Animal Production (IRIAP) in Bogor. The experiment has been approved by the Animal Welfare Commission of the Indonesian Agency for Agricultural Research and Development (Balitbangtan/Balitnak/Rm/05/2016). The cashew nut shells were obtained from farmers in Pati Regency, Central Java Province.

Bioindustrial products of cashew nut shell (biofat and biochar) were used as feed additive in this experiment. The method of preparing biofat (BF) and biochar (BC).

Cashew nut shells

Shells of cashew nut that have been separated from the nuts were dried under the sun. The dry shells were grinded into smaller particles with a blender and screened to obtain small sized particles (2 mm diameter). Then, the small sized cashew nut shell would be processed into bioindustrial products namely biofat and biochar.

Extraction to obtain biofat product

The prepared cashew nut shells were weighed (100 g) and then put into an erlenmeyer flask and added with 400 ml of hexane. Once submerged, the mixture (sample and solvent) were stirred well and then left on the table for 24 hours. The filtrate was then separated and 200 ml of new hexane was added to the residue. The filtrate was mixed together and was evaporated with a rotary evaporator at 40°C until the remained was dark brown thick oil and called Biofat.

Pyrolysis process to obtain biochar

The shell residue after biofat extraction was air-dried. It was then put into an activation tank (pyrolysis

tank) and tightly closed. Then, the pyrolysis furnace was started. The pyrolysis reaction took place at the pyrolysis reactor worked at 300^{o}C for 8 hours. Pyrolysis tank was connected with a long pipe. The furnace was turned off after 8 hours and left for cooling. Black residue inside the tank called charcoal or Biochar A complete feed for cattle consisted of Grass, *Gliricidia sepium* leaves, yellow corn, coconut cake, molasses, bran, urea, salt (NaCl), limestone ($CaCO_3$), and premix used as a substrate in the *in vitro* rumen fermentation. The CP and TDN of this complete feed was 15.63% and 69.7%, respectively (Saenab et al. 2018).

Buffer medium consisted of bicarbonate buffer solution, macro-mineral solution, micro-mineral solution, resazurin, distilled water, reducing solution and rumen fluid as described in Makkar (2003).

In vitro rumen fermentation

Treatments were different combinations of biofat (BF, 0.25 µL/mL) and biochar (BC, 0.3 mg/mL) as follows: a) Control (Substrate without any addition of Biofat or Biochar), b) Substrate + BFBC1= 0%BF:100%BC; c) Substrate + BFBC2= 25%BF:75%BC; d) Substrate + BFBC3= 50%BF:50%BC; e) Substrate + BFBC4= 75%BF:25%BC; f) Substrate + BFBC5= 100%BF: 0%BC.

Different combinations biofat and biochar were each added to the substrate. A total of 750 mg of substrate was weighed into the bottle. Rumen buffer solution (75 mL) was added and the rubber stopper was quickly applied on the bottle. The bottle was placed in the water bath and incubated at 39^{o}C for 48 hours. Rumen fluid was collected just before morning feeding from rumen of a fistulated Holstein Friesian cow fed with commercial concentrate and elephant grass. The total gas and methane production were recorded at 3, 6, 9, 12, 24, 30, 36, 48 hours of incubation. At the end of incubation, the supernatant was separated by filtration to obtain residue and supernatant. The residue was dried in the oven 105^{o}C for 24 hours and weighed. Ash content of feed and residue was determined according to method of AOAC (2005) and neutral detergent fiber (NDF) analysis was conducted following Van Soest et al. (1991) method without addition of amylase. The *in vitro* dry matter (DM) and organic matter (OM) of digested fractions were calculated from the dry matter and organic matter of initial sample minus those of residue. The DM or OM of digested fractions divided by the DM or OM of initial sample was calculated as *in vitro* dry matter or organic matter degradabilities. Other residue samples of *in vitro* incubation were digested using Neutral detergent solution to obtain residual NDF fraction. The NDF of digested fractions divided by the NDF of initial sample was calculated as *in vitro* NDF degradability. pH, NH_3 and VFA were measured after 4 hours of incubation. Ammonia content in the supernatant was determined using Conway microdiffusion technique. The supernatant for volatile fatty acid (VFA) analysis was kept in low pH by adding sulphuric acid. Volatile fatty acid products from fermentation was analysed by GC using gas chromatography *(Bruker Scion 436 GC*) with capillary column BR-Wax fame containing wall-coated open tubular (WCOT) used silica with the length of column 30 m x 0.32 mm imange diagnosis (ID). The carrier gas was Nitrogen 25 ml/min and the burning gas was hidrogen 30 ml/min. Injector temperature was 250ºC, while the column temperature gradient was 70 – 150^{o}C in 11 minutes. The detector used was Fingernail Imange Diagnosis (FID) with temperature of 275^{o}C.

Statistical Analysis

This study was done based on a randomized block design (RBD) with 6 treatments and 4 replications. The experimental data from different combinations of biofat and biochar and control (substrate without any addition of biofat or biochar) were analyzed separately by PROC GLM using SPSS Program Package 16. Further analysis using Duncan test was done for obtaining significant differences among treatments.

RESULTS AND DISCUSSION

Methane, total gas and ammonia productions, pH from *in vitro* rumen fermentation with the addition of biofat with biochar combination at different ratios are presented in Table 1.

Compared to control, the addition of biofat and biochar mixtures at different ratios caused a significant decrease ($P<0.01$) in the production of methane (Table 1). The production of methane was reduced by 11.50% (BFBC1), 36.85% (BFBC2), 38.50% (BFBC3), 41.84% (BFBC4) and 26.07% (BFBC5) compared to the control. The higher the level of biofat in the mixed of biofat and biochar, the less is methane produced, but when reaching 100% biofat (BFBC5), methane production significantly increased compared to BFBC 2,3,4. It seemed that there was a quadratic effect which indicates a synergistic effect of combination of biofat with biochar on methane reduction. The lowest methane production was observed at the biofat:biochar composition of 75:25%. Combination of two bioactive compounds to reduce methane production both *in vitro* or *in vivo* systems have been reported (El-Zaiat et al. 2014; Yogianto et al. 2014). El-Zaiat et al. (2014) reported that combination of Cashew Nut Shell Liquid (CNSL) and nitrate decreased methane production. It may be possible that CNSL and nitrate may have each different mechanism in reducing methane production so it synergistically depressed the process production of

Tabel 1. The effect addition of biofat:biochar combination at different ratios on methane (ml), total gas (ml), ammonia production (mm/g DM) and pH value in the *in vitro* rumen

Treatment	Level	Methane (ml)	Total Gas (ml)	NH_3 (mm/g DM)	pH
Control	0 %	45.15±3.42d	182.25± 13.52	7.6± 1.35b	6.70±0.00
1	BFBC1	39.25± 2.60c	144.17±20.01	8.1±0.68bc	6.68±0.01
2	BFBC2	28.47 ±5.94a	191.85±16.73	8.3±0.72bc	6.70±0.02
3	BFBC3	27.77 ±6.41a	194.87±7.09	8.1±0.87bc	6.74±0.01
4	BFBC4	26.25 ±3.27a	200.87±13.59	8.8±1.01bc	6.67±0.01
5	BFBC5	33.32 ±2.44b	190.87±16.97	5.9±0.52a	6.74±0.01

1: Level BFBC 1 (0%BF:100%BC), 2= BFBC2 (25%BF:75%BC), 3= BFBC3 (50%BF:50%BC), 4= BFBC4 (75%BF:25%BC), 5= BFBC5 (100%BF:0 %BC), DM= dry material
Different letters in the same column show significant (P<0.05) or very significant (P <0.01) difference. Statistical analysis of each product was tested separately against the control

Table 2. The effect of biofat/biochar and biofat/biosmoke combination at different ratios on molar proportion of acetate, propionate, butyrate, valerate, branched chain short chain fatty acids (BCVFA) and total volatile fatty acids (VFA) (mm) concentration, and acetate/propionate of feed incubated 48 hours in the *in vitro* rumen fermentation

Treatment	Level	Acetate	Propionate	Butyrate	Valerate	BCVFA	A/P	Total VFA (mm)
		.....mol/100mol.....						
Control	Control	62.06±2.40	20.14±2.20^{a}	10.33±1.75	2.18±0.76	5.27±0.24^{ab}	3.11±0.43^{c}	72.42±3.01^{a}
1	BFBC1	59.63±2.46	22.16±1.63^{ab}	11.73±1.77	1.93±0.41	4.88±1.39^{a}	2.71±0.29^{bc}	84.83±9.78^{ab}
2	BFBC2	55.59±4.33	22.87±2.16^{ab}	12.36±3.09	2.78±0.39	5.56±1.33^{ab}	2.45±0.34^{ab}	87.10±10.53^{b}
3	BFBC3	56.43±2.40	23.79±1.38^{bc}	10.31±1.34	2.89±0.80	6.58±0.69^{bc}	2.38±0.18^{ab}	84.47±5.53^{ab}
4	BFBC4	53.96±6.49	25.90±1.85^{c}	10.68±2.96	2.50±0.81	6.96±1.14^{c}	2.10±0.40^{a}	91.61±7.09^{b}
5	BFBC5	57.96±4.14	23.34±1.67^{bc}	11.86±1.69	1.80±0.35	5.03±0.86^{a}	2.50±0.34^{ab}	79.57±8.18^{ab}

BCVFA= branched chain short chain fatty acids, A/P= Acetate/Propionate ratio. 1: Level BFBC 1 (0%BF:100%BC), 2= BFBC2 (25%BF:75%BC), 3= BFBC3 (50%BF:50%BC), 4= BFBC4 (75%BF:25%BC), 5= BFBC5 (100%BF:0 %BC)
Different letters in the same column show significant (P<0.05) or very significant (P <0.01) difference. Statistical analysis of each product was tested separately against the control.

methane in the rumen. The synergistic effect of the combination of biofat and biochar on methane reduction may be explained as follows. Biofat reduced methane production through the major effect of anacardic acid as the main bioactive compounds in biofat. Anacardic acid is composed of phenolic and unsaturated fatty acids group **(**Lejonklev et al**.** 2013). Both groups were negatively affected rumen microbes as it was reported that phenolic group is able to suppress certain rumen microbes growth (Jayanegara et al. 2011; Hansen et al. 2012) and unsaturated fatty acids are toxic to ruminal microorganism (Maia et al. 2007). Biochar that added together with biofat may reduce methane production through its pores that absorbed gas from fermentation products including methane. Then, methane may be used by methane utilizing bacteria (methanotroph bacteria) which possibly live in the surrounding pores of biochar (Leng et al. 2012a; Leng et al. 2012b). Both substances, biofat and biochar with different mechanisms may work together and synergistically reducing methane in the rumen.

Table 1 shows the addition of different combination of biofat and biochar did not significantly increase (P>0.05) total gas production in the *in vitro* rumen compared to the control. Total gas was produced as the result of feed degradation by rumen microbes activity and it consisted of several gasses with the major CO_2 (about 65%), and methane (26%) followed by nitrogen (7%) and small amount of O_2, H_2 and H_2S (Yang 2017). The unaffected total gas produced in addition of biofat and biochar combination may be due to the absence of these additives affecting the activity and total population of rumen microbes that degrade feed. Even though this experiment did not measure gas composition and mcrA gene related to methanogens but the same material biofat/ CNSL was used by Mitsumori et al. (2014) showed that the addition of CNSL not only reduced methane, but also increased

hydrogen gas. Another experiment done by Shinkai et al. (2012) showed an inhibition activity toward methanogens by decreasing the copy and expression of mcrA in the rumen, hence, methane production was reduced. Therefore, in this experiment, total gas production was not changed but there may be a shift of gas composition due to combination of biofat and biochar.

Eventhough there was an increased on ammonia production at the BFBC1- BFBC4, but the increase was not significantly different from control (Table 1). At BFBC5 (100% of biofat and 0% biochar), however, a very significant decrease in ammonia production ($P<0.01$) occurred compared to other treatments and control. This explains that biofat at the level of 0.25 µL/mL (100% biofat) depressed feed protein degradation in the rumen. The level of ammonia increased at BFBC4 (75:25) was the highest in numeric compared to other treatments. It is interesting to note that 75% biofat in the combination with 25% biochar in BFBC4 did not negatively affect ammonia production, instead BFBC4 enhanced ammonia production. It shows that there is a synergistic effect on ammonia level caused by combination of biofat and biochar. Eventhough some phenolic was reported to decrease ammonia production in the rumen (Jayanegara et al. 2011; Kamra et al. 2012), biochar might be able to entrap ammonia in its pores so that it contributed higher effect on increasing ammonia level in the rumen.

Table 2 shows the combination of biofat and biochar caused a significant increase ($P<0.05$) on propionate and total VFA production in the rumen over the control. The increased propionate was consistent with the previous result (Saenab et al. 2018) when administration of biofat and biochar separately had caused a significant increase ($P<0.05$) on propionate production and total VFA in the rumen. The combination of biofat and biochar may cause a synergistic effect which resulted in a higher increase of propionate and total VFA. Higher increase of propionate was related to reduce methane production. There may be a competition in utilizing hydrogen by propionate producing bacteria and methanogens to form propionate and methane, respectively. A study by Watanabe et al. (2010) showed that population of several propionate producing rumen bacteria (*Selemonas ruminantium*, *Megasphaera elsdenii*,) increased in the presence of CNSL (biofat) while study of (Shinkai et al. 2012) showed that mcrA gene related to methanogens was depressed by the additions of CNSL (biofat). Eventhough the present experiment didnot measure the population of those bacteria and methanogens in the rumen, there may be possible that combination of biofat and biochar changed the composition of rumen bacteria toward higher propionate producing bacteria and lower methanogens resulted in higher propionate and lower methane produced in the rumen fermentation.

Meanwhile, the production of BCVFA (branched chain volatile fatty acid) showed a significant increase ($P<0.05$) only at the combination level of BFBC4 compared to control. BCVFA (isobutyrate and isovalerate) is a product from feed protein degradation in the rumen. This result indicates that addition of combination of biofat and biochar didnot negatively affect the process of protein degradation by rumen microbes. Protein as popypeptides in the feed will be degraded in the rumen by different microbes to become peptide, amino acids (normal chain amino acid and branched chain amino acids) and finally ammonia and branched chain fatty acids. These branched chain fatty acids (BCVFA) came from branched chain amino acids (valine, leucine and isoleucine) that were oxidatively deaminated, but its production depended on the type of protein source and level of inclusion in the diet (Apajalahti et al. 2019). Ammonia and branched chain fatty acids were the indicator for protein degradation, therefore ammonia and branched chain fatty acids were strongly correlated (Apajalahti et al. 2019). This experiment also showed that both ammonia and BCVFA were low concentration in the rumen with the addition of BFBC5 (100% biofat and 0% biochar). Addition of the lowest level biochar mixed with biofat seemed not only to reduce the negative effect of biofat but also give a beneficial effect on protein feed degradation, hence increase ammonia and BCVFA production.

The effect of ruminal dry material digestibility (DMD), organic material digestibility (OMD) and neutral detergent fibre digestibility (NDFD) from *in vitro* fermentation with the addition of biofat:biochar combination at different ratios are presented in Table 3.

Table 3 shows the result of DMD and OMD in the presence of combination of biofat and biochar at different ratios were the similar as the control ($P>0.05$) except at the BFBC4 (25:75%), in which DMD and OMD were slightly higher in numeric than those at other combinations. As it was shown in Table 1 and 2 that ammonia and BCVFA increased at BFBC4 addition, indicated that protein as part of organic matter could be degraded without any inhibition.

The NDF degradation decreased significantly at BFBC1, BFBC2 and BFBC5 compere to control. It seemed that fiber degrading bacteria in the rumen and protozoa were very sensitive to addition of biofat and biochar. It seemed that fiber degrading bacteria in the rumen and protozoa were very sensitive to addition of biofat and biochar. In the semi continous rumen fermentation done by Watanabe et al. (2010) and Oh et al. (2017) found that addition of CNSL/biofat

Tabel 3. The effect of dry material digestibility (ml/g sampel), organic material digestibility (ml/g sampel), and neutral detergent fibre digestibility (ml/g sampel), values of feed incubated 48 hours in the *in vitro* rumen fermentation with addition of biofat : biochar combination at different ratios

Treatment	Level	DMD (ml/g sampel)	OMD (ml/g sampel)	NDFD (ml/g sampel)
Control	0 %	72.15±1.69ab	75.21±0.81ab	54.41±2.58c
1	BFBC1	72.66±3.25ab	76.02±2.63b	41.57±2.33ab
2	BFBC2	73.79±1.36b	76.68±0.35b	42.97±1.57ab
3	BFBC3	74.57±3.39b	77.29±1.79b	46.20±2.98bc
4	BFBC4	75.91±3.13b	78.03±2.43b	46.58±1.53bc
5	BFBC5	69.25±4.44a	71.68±4.76a	38.44±5.85a

1: Level BFBC 1 (0%BF:100%BC), 2= BFBC2 (25%BF:75%BC), 3= BFBC3 (50%BF:50%BC), 4= BFBC4 (75%BF:25%BC), 5= BFBC5 (100%BF:0 %BC), DMD= dry material digestibility, OMD= organic material digestibility, NDFD= neutral detergent fibre digestibility
Different letters in the same column show significant (P<0.05) or very significant (P <0.01) difference. Statistical analysis of each product was tested separately against the control

reduced the population of *Ruminococcus flavefaciens*, *Ruminococcus albus* that represented fiber degrading rumen bacteria and also reduced the protozoa. Although in the present experiment did not observe or count the population of fiber degrading bacteria, it can be assumed that NDF degradation decreased may be due to the inhibition effect of biofat on the growth of those fibrolytic rumen microbes. But NDF degradation at BFBC3 and BFBC4 was not significantly different to control. Eventhough biofat and biochar negatively affected interestingly the combination biofat and biochar at level 50%:BF:50% BC and biofat and biochar at level 75%BF:25% BC could increase NDF degradation similar to that of control.

The phenolic compounds and unsaturated fatty acids in biofat were reported to negatively affect bacteria responsible for feed degradation in the rumen, even when biofat was combined with biochar, the negative effect of phenolic on feed degradation was reduced. This result was in agreement with Al Kindi (2015) who reported that the addition of biochar or activated charcoal together with tannin or phenolic containing leaves would eliminate the reducing effect of phenolic compounds on feed degradation especially fiber degradation.

The use of biofat from cashew nut shell as feed additive reduced methane and increased propionate but combination of biochar with biofat showed more beneficial effect on higher reduction of methane, higher propionate, BCVFA and ammonia production without disturbing feed degradation. It is suggested that the best combination of biofat and biochar of BFBC4 (75% BF:25%BC) obtained from *in vitro* trial should be evaluated as feed additives in the *in vivo* trial to observe the animal responses with different types of feed. Utilizing of biofat and biochar as feed additive, would give more value on cashew nut shell which previously considered as a waste with less value from cashew nut industry. It is also expected that combination of biofat and biochar as feed additive in livestock feeding would improve the environment to cleaner and greener one as it would reduce greenhouse gases.

CONCLUSION

It was concluded that the best combination of biofat and biochar that produced a synergistic effect on enhancing rumen fermentation products and reducing methane production was at the ratio of 75% Biofat:25% Biochar. It is suggested to conduct further studies on evaluating this combination of biofat and biochar as feed additive with different types of feed and study the animal responses (*in vivo* study).

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