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Reproductive Performance of Garut Ewes Fed Sorghum-Indigofera after Stimulation with Pregnant Mare Serum Gonadotropin

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ABSTRACT

This study aimed to evaluate the effect of mixed feed sorghum-Indigofera on Garut ewes reproductive performance stimulated with Pregnant Mare Serum Gonadotropin (PMSG) hormone. Twenty-eight multiparous Garut ewes were grouped based on feed type (local forage (LF) vs. mixed feed sorghum-Indigofera (SI)), for a 30-day acclimatization period with a completely randomized design. Following acclimatization, all animals were synchronized with PGF2α (i.m.) twice, 11 days apart. Half the population of each group was stimulated with 500 IU PMSG after the second injection. Estrous characteristics were observed for seven days, followed by natural mating. Pregnancy status and the number of fetuses were detected 30 days after mating using ultrasound (USG), and reproductive efficiency was evaluated after parturition. Data during acclimatization were analyzed using a two-sample t-test, while the reproductive performances used a General Linear Model (GLM). Estrous characteristics showed no interaction ($p>0.05$) between feed types and PMSG stimulation. The onset of estrous in the SI treatment occurred 9.33 hours faster ($p<0.05$) than in the LF treatment. Meanwhile, the duration of estrous in the group stimulated with PMSG was 18.67 hours longer ($p<0.05$) than without PMSG. The vulva temperature at standing heat was 38.16±0.69 °C ($p>0.05$), with the pregnancy rate ranging 42.85%-100%. Interestingly, the SI group treated with or without PMSG had a larger litter size (1.43-4.50) compared to the LF group (1.25–1.33). It is concluded that feeding with SI had improved reproductive success, with the highest number of ewes giving birth. Additionally, the ewes fed SI with PMSG had the largest litter size.

Keywords: Garut ewes; indigofera; PMSG; reproduction; sorghum

INTRODUCTION

The litter size of Garut ewes within the traditional husbandry system remains low (±120%), with a relatively high rate of embryonal loss (30%) (Khotijah *et al.*, 2015). Dixon *et al.* (2007) stated that fetal mortality in the prenatal phase could reach 19.9%. This condition can be attributed to both genetic and environmental factors. Fertility and fecundity rates have often been considered as characteristics carried from ewes (Murphy *et al.*, 2020). However, male genetic and non-genetic factors can also influence the litter size and reproductive performance (Holler *et al.*, 2014). Furthermore, the libido and sperm quality conditions of bucks can affect the lambing rate of ewes (David *et al.*, 2015).

Low feed quality in small-scale farming in Indonesia is one of the environmental factors leading to high reproductive failure. Addah *et al.* (2012) reported that the high rate of fetal losses and lamb mortality, as well as the low birth weight and growth of pre-weaned lambs in the tropics, could be due to the quality of the natural forage, which varies considerably with the season. In addition, the intrauterine growth trajectory is largely influenced by nutrition rather than genetics during pregnancy.

Feed improvement can be undertaken to increase the reproductive performance of ewes (Hayati *et al.*, 2021). Moreover, improved litter sizes can be achieved through hormonal stimulation (Mohazer *et al.*, 2012). However, the sheep are genetically prolific and are controlled by the single gene of *FecJ* (Davis, 2005). Basically, it is possible to increase the litter size by controlling the content of follicle-stimulating hormone (FSH) in the blood circulation (Sirjani *et al.*, 2012). An exogenous gonadotropin hormone application such as Pregnant Mare Serum Gonadotropin (PMSG), which has similar characteristics and function as FSH and luteinizing hormone (LH) (Tirpan *et al.*, 2019), will stimulate the follicle growth and ovulation that can, in turn, improve the estrous quality and litter size (Türk *et al.,* 2008).

Forage types and feed processing techniques are the environmental factors that can affect animal reproductive performance. Sorghum and Indigofera forages are feed ingredients with high nutrient content and palatability (Abdullah, 2010). Moreover, sorghum and Indigofera forage contain antioxidants such as flavonoids (Jevcsák & Sipos, 2016) and carotenoids (Hutapea *et al.,* 2018) that can support the development of oocyte quality and litter size. The antioxidants in the feed may improve the antioxidant status in the reproductive tract, which will improve immunity status during the gestation period to support the development of the fetus until the end of the gestation period and result in heavier birth weight (Fassah *et al.,* 2015).

To our knowledge, forage sorghum and Indigofera have great potential to improve the reproductive performance of ruminants. However, there is relatively little data on whether a mix of the forage with the PMSG hormone can increase the litter size and reproductive performance of Garut ewes. Further research on the application of mixed feed and reproductive technology is required to increase the Garut sheep population in Indonesia. Mixed feed based on sorghum-Indigofera (SI) forage with high nutrient and antioxidant levels is expected to improve follicle development, ovulation rate, estrous characteristics, and pregnancy rate. Moreover, it is also expected to support the more optimum performance of the PMSG hormone. Therefore, this study aimed to evaluate the effect of mixed feed based on sorghum and Indigofera forage on the reproductive performance of Garut ewes after stimulation with the PMSG hormone.

MATERIALS AND METHODS

Research Location, Animals Model, and Experimental Design

This study was conducted in a community farm center involving Garut sheep farmers in Majalengka District, West Java, Indonesia (6°48’50.0”S 108°13’35.8”E; 141 m ASL) from March to September 2021. Ethical permission for this study was obtained from the Animal Commission on Ethics, Faculty of Veterinary Medicines, IPB University (approval number: 011/KEH/SKE/V/2021). Twenty-eight multiparous Garut ewes were used for the experiment with the following criteria: normal and healthy body condition, 2–3 years old, body weight ±30 kg, and not pregnant. The experiment was carried out in two phases (acclimatization and a reproductive observation period). A completely randomized design was employed for both experiment periods. During the acclimatization period, the ewes were divided into two treatment groups with 14 replications. In the reproductive observation period, the ewes were divided into four treatment combinations with a 2 x 2 factorial arrangement, with seven ewes in each experimental unit.

Acclimatization Period and Performance of Pre-mating Ewes

The acclimatization period lasted for 30 days, during which all animals were confirmed as not being pregnant via an ultrasound scan. The animals were randomly assigned to individual cages and divided into two feed treatment groups: 1) 100% local forage (LF) (n=14); and 2) 50% forage sorghum silage + 40% Indigofera forage hay + 10% rice bran (SI) (n=14). Samples of the experimental feed were analyzed for dry matter (DM), ash, crude protein (CP), ether extract (EE), crude fiber (CF), and minerals according to AOAC (2005), and gross energy (GE) was determined by bomb calorimetry (Lima *et al.,* 2011). The DM content was determined using AOAC method 973.18, while the organic matter and ash were determined according to AOAC method 942.05. Samples for the DM analysis were dried in a conventional oven at 105 ^{o}C, while the organic matter and ash samples were measured by ashing in a furnace at 550 ^{o}C for 16 h. The CP content was measured using the 984.13 methods (Kjeldahl) to determine the quantity of the total N. The CP content was calculated by multiplying the total N by 6.25. Furthermore, ether extract was measured according to EE method 920.39 dissolved by petroleum ether as the organic solvent. Meanwhile, CF was determined according to AOAC method 978.10 by boiling the samples in 1.25% H_2SO_4 and 1.25% NaOH. Finally, the mineral content was determined with method 965.09A using the atomic absorption spectrophotometer. The nutrient content of the feed was obtained based on the proximate analysis conducted at the Integrated Laboratory of the Faculty of Animal Science, IPB University, as presented in Table 1.

The field grasses used in this study comprised the local forages found around the farmers’ fields, including vine legumes that the village farmers mostly use to feed their animals. The predominant species of local forages and legumes include *Cynodon dactylon* (Kawatan Grass), *Cynodon Plectostachyus* (African Grass), *Brachiaria decumbens* (BD Grass), *Brachiaria ruziziensis* (Ruji Grass), *Panicum repens* (Lampuyang Grass), *Digitaria decumbens* (Pangola Grass), *Paicum maximum* (Benggala Grass), *Leersia hexandra* SW. (Swamp Rice Grass), *Centrosema* (Centro), and *Calopogonium mucunoides* (Asu Beans).

On the other side, the sorghum crop used in this experiment was sweet sorghum variety Samurai 1 strain Zh-30, which was irradiated by gamma rays at a wave of 30 Gy and produced by the National Nuclear Energy Agency of Indonesia (BATAN). Meanwhile, the Indigofera plant used in this experiment was the *Indigofera zollingeriana* species, a leguminous type (Suharlina *et al.,* 2016a). The sorghum was harvested 70 days after planting (dap) and Indigofera was harvested at the end of each 50-day period. LF and SI were served in proportions equivalent to 4% and 3% of body weight in DM per day, respectively. LF was fed in a proportion greater than 3% due to the relatively high variation in its nutrient content. The exaggerated feed amount was hoped to meet the minimum requirement of 3% of body weight. Feed was served twice daily, in the morning and evening, while drinking water was given *ad libitum*. The variables observed in this phase were nutrient intake and blood cholesterol content. Dry matter intake (DMI) was measured by subtracting the remaining or uneaten amount of dry matter feed from the total amount of dry matter feed served to the experimental sheep, according to Moate *et al*. (2021). Meanwhile, CP and total digestible nutrients (TDN) intake were calculated using the CP and TDN content measured by proximate analysis and multiplying with DMI. The DMI, CP, and TDN intakes were measured daily for 30 days. Blood was taken from the jugular vein using a venoject during the final week of the acclimatization period to determine blood cholesterol levels. The serum was then measured spectrophotometrically using an Olympus AU 2700 autoanalyzer (Tokyo, Japan) in combination with commercially available specific kits (Budak *et al.*, 2011).

Hormonal Treatment

Hormone application was undertaken on the 31st day after the acclimatization period. All groups of ewes were synchronized using $PGF_{2\alpha}$ (Lutalyse®, Zoetis, USA). $PGF_{2\alpha}$ was injected twice, with an interval of 11 days (Bukar *et al.*, 2012), and contained 12.5 mg dinoprost tromethamine (Mekuriaw *et al.*, 2016). Furthermore, 500 IU PMSG (Folligon®, Intervet International B,V., Boxmeer, The Netherlands) was administered intramuscularly (i.m.) to half of the ewe population in each feed treatment group (n=7) at the time of the second $PGF_{2\alpha}$ injection (Hussein *et al.*, 2021). The treatment group was managed as follows: 1) LF without PMSG stimulation (control); 2) LF + PMSG stimulation; 3) SI without PMSG stimulation; and 4) SI + PMSG stimulation (Figure 1).

Estrous Detection and Ewe Mating

Signs of estrous were observed and recorded after estrous synchronization twice a day, i.e., in the morning and afternoon, for seven days. Changes in vulvar temperature were measured following the estrous observation schedule using an infrared thermometer. The onset of estrous was recorded as the ewes displayed standing heat when the bucks attempted to mount. Meanwhile, mated ewes were marked with paint to prevent mating repetition. The mating process was performed naturally with selected bucks, which were chosen based on several criteria: healthy, 2–3 years old, minimum weight of 60 kg, high libido, and proven through resulting offspring. A total of three bucks were chosen to mate 28 ewes. The estrous duration was calculated when the ewes showed standing heat for the first time until they rejected a teaser buck's attempt to mount.

Pregnancy Diagnosis and Reproductive Efficiency

A pregnancy diagnosis for a ewe was made using transrectal ultrasonography (USG) with a 7.5 MHz linear probe (SSD500 model, ALOKA Co. Ltd., Japan) on the 30th day after the mating. The parameters

Table 1. Proximate analysis nutrient content of local forage and mixed feed sorghum-Indigofera

Feed types	Feed nutrient								
	DM (%)	Ash (%)	CP (%)	EE (%)	CF (%)	Ca (%)	P (%)	TDN (%)	GE (Cal/g)
LF (acclimatization period)	11.33	12.22	15.32	2.25	23.64	1.81	0.45	53.60	3630
LF (pregnancy period)	13.73	17.11	15.33	4.59	25.32	2.11	0.47	54.12	3831
LF (lactation period)	30.88	11.89	12.60	2.18	22.90	0.83	0.45	51.00	3396
SI	26.45	10.34	19.82	1.91	30.57	1.48	0.56	59.35	4423

Note: LF= Local forage; SI= Sorghum-Indigofera; DM= dry matter; CP= Crude protein; EE= ether extract; CF= Crudefiber; Ca= Calcium; P= Phosphorus; GE= Gross energy.

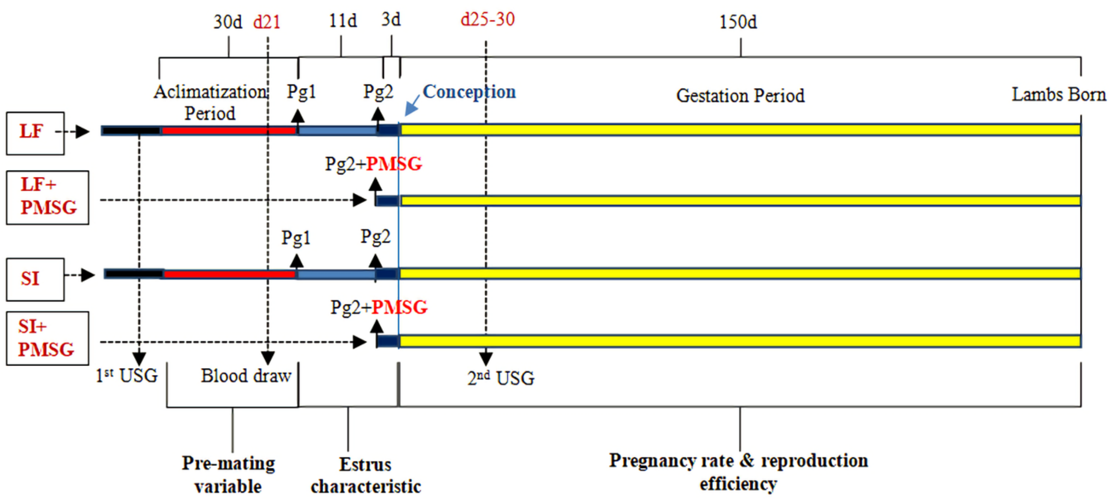

Figure 1. The scheme of experimental flow treatment of mixed feeding sorghum-Indigofera with or without PMSG in Garut ewes. LF= local forage; SI= Sorghum-Indigofera; Pg1&2= PGF2α administration; PMSG= Pregnant mare serum gonadotrophin administration; USG= Ultrasonography.

observed in this research were pregnancy rate, which was confirmed by finding an embryonic sac, the average number of embryos, the number of lambs born, fetus loss and abortus number, average number of living fetuses per pregnant ewe, and litter size. Several parameters were calculated using the following formulas:

Pregnancy rate= [(Total USG-detected pregnant ewes)/ Total mated ewes] x 100%

Embryo loss= (Total USG-detected number of embryos) – (number of lambs born + abortus)

Total number of embryos=numbers of lambs born + embryo loss

USG accuracy= [(Number of USG-detected embryos) / Total number of embryos] x 100%

The average number of embryos=
Total number of embryos / Total pregnant ewes

The average number of live fetuses per pregnant ewe=
Number of lambs born / Total pregnant ewes

Litter size= Number of lambs born / Number of parturition ewes

Statistical Analysis

The DMI and blood cholesterol level data were analyzed using a two-sample t-test. Meanwhile, the estrous characteristics and reproductive efficiency were analyzed using a General Linear Model (GLM) at the 95% confidence level. The data were presented in mean ± standard error (SEM) value. The data were processed using SPSS 25.0 version (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Feed Nutrient, Nutrients Intake, and Blood Cholesterol Level

The LF nutrient content was observed three times during the experiment, namely during the acclimatization, pregnancy, and lactation periods. The energy and protein content of LF decreased during the dry season, which coincided with the lactation period of the ewes. However, the nutrient content of the SI feed remained constant during the study as the feed ingredients used and their compositions were similar. The CP, CF, TDN content and the GE of the SI feed were higher than the LF (Table 1). During the acclimatization period, the DM and TDN intakes of SI feed were 0.51 and 0.23 kg lower ($p<0.05$) than those of LF, respectively, while the CP intake from both feeds showed no differences ($p>0.05$). However, this difference in DM and TDN intake had no significant effect ($p>0.05$) on the blood cholesterol content of the Garut ewes, despite the slight difference in numbers of 3.12 mg/dl (Table 2).

Estrous Characteristics

Only one ewe did not respond after being treated with prostaglandin. The onset of estrous in the ewes in the SI treatment group was 9.33 hours earlier ($p<0.05$) than in the LF treatment group. Meanwhile, the duration of estrous in the ewes in the PMSG stimulation group was 18.67 hours longer ($p<0.05$) than in the treatment group without PMSG stimulation (Table 3). This finding shows no interaction ($p>0.05$) between the type of feed and PMSG administration between treatments. The average temperature of the vulva at the heat peak showed no significant difference ($p>0.05$), which was in the range of 38.16±0.69 °C.

Pregnancy Rate and Reproductive Efficiency

The detection of pregnancy using ultrasound in the Garut ewes on the 30th day after mating had low accuracy for determining the number of fetuses in cases with more than three fetuses. This situation was confirmed at parturition, wherein more fetuses were born compared to the ultrasound result. The highest pregnancy rate occurred in the group of animals fed with SI without PMSG stimulation (100%). Meanwhile, the lowest pregnancy rate was found in the SI group treated with PMSG (42.85%); however, this group also had the highest litter size. Both ewe groups fed with LF, i.e., those with and without PMSG treatments, obtained lower than average pregnancy rates compared to the

Table 2. Nutrients intake, blood cholesterol, and body weight of Garut ewes during the acclimatization period

Variables	Treatments		p value
	LF	SI	
Number of ewes (N)	14	14	-
Number of ewes analyzed (n)	6	6	-
Dry matter intake (kg/head/day)	1.30±0.10[a]	0.79±0.05[b]	0.001
Crude protein intake (kg/head/day)	0.20±0.02	0.16±0.01	0.059
Total digestible nutrient intake (kg/head/day)	0.70±0.05[a]	0.47±0.03[b]	0.005
Blood cholesterol (mg/dl)	66.13±5.56	69.25±3.95	0.099
Initial body weight (kg/head)	32.93±2.74	32.67±1.55	0.936
Final body weight (kg/head)	35,75±2,52	35,33±1.97	0.899
Average daily gain (kg/head)	0.09±0.04	0.09±0.02	0.936

Note: LF= local forage; SI= Sorghum-Indigofera; Mean in the same row with different superscripts differ significantly ($p<0.05$).

groups fed with SI without PMSG treatment (57.14 and 71.42% vs. 100%).

The group fed on SI without PMSG treatment showed the highest pregnancy rate and the total number of fetuses. Meanwhile, the ewes stimulated with PMSG recorded an average of two fetuses. The highest fetus loss was obtained from the LF+PMSG treatment (63.64%), followed by the SI, SI+PMSG, and LF treatments, with total fetus losses of 30.77%, 25%, and 16.67%, respectively. The SI treatment was more effective in maintaining a higher average of living fetuses (76%) than the LF treatment (52.94%).

The average number of fetuses was significantly affected ($p<0.05$) by either feed or PMSG treatment, with statistically no interaction between both treatments ($p=0.067$) (Table 4). The average numbers of fetuses in the LF and SI treatment groups were 1.80±0.60 vs. 2.50±1.35. This indicated that the SI treatment was more effective than LF. On the other side, the average numbers of fetuses in the non-stimulated and stimulated PMSG treatment groups were 1.73±0.47 vs. 2.88±1.36, respectively. This result revealed that the PMSG was better than the non-PMSG treatment in terms of increasing the number of fetuses.

The number of live fetuses at parturition statistically showed no significant differences and no interaction between the feed and PMSG treatment ($p>0.05$). Interestingly, however, the result of this experiment revealed an interaction between the SI and PMSG treatment ($p=0.011$). The SI group treated with PMSG obtained an average litter size of 4.5, which was higher than the other treatment groups (<1.43) (Table 4).

DISCUSSION

Nutrient intake is related to how efficiently feed is used to support optimum body metabolism. The results of the present study revealed that the DMI and TDN of

Table 3. Estrous characteristics of Garut ewes fed Sorghum-Indigofera after PMSG stimulation

Variables	LF		SI		Significance level (p-value)		
	Without PMSG	PMSG	Without PMSG	PMSG	Feed	PMSG	Interaction
Number of ewes (head)	7	7	7	7	-	-	-
Estrous response (%)	86 (6/7)	100 (7/7)	100 (7/7)	100 (7/7)	-	-	-
Estrous onset (hour)	44.00±9.80 (n=6)	40.00±8.76 (n=6)	31.20±9.86 (n=5)	34.50±9.00 (n=4)	0.042	0.934	0.391
Estrous duration (hour)	56.00±16.00 (n=4)	85.50±12.37 (n=4)	63.60±14.45 (n=5)	73.60±3.58 (n=5)	0.718	0.004	0.117
Vulva temperature at heat (^oC)	38.09±0.72	38.53±0.56	37.76±0.63	38.26±0.73	0.243	0.072	0.910

Note: LF= local forage; SI= sorghum-Indigofera; n= population; PMSG= Pregnant Mare Serum Gonadotropin.

Table 4. Pregnancy rate and reproductive efficiency of Garut ewes fed Sorghum-Indigofera after PMSG stimulation

Variables	LF		SI		Significance level (p-value)		
	without PMSG	PMSG	without PMSG	PMSG	Feed	PMSG	Interaction
Number of ewes (head)	7	7	7	7	-	-	-
Number of pregnant ewes (%)	57.14 (4/7)	71.42 (5/7)	100 (7/7)	42.85 (3/7)	-	-	-
Number of USG-detected embryonic on 30 days after mating (head)	6	11	13	7	-	-	-
Number of lambs born (head):							
Single	3	2	5	0	-	-	-
Twins	2	2	2	0	-	-	-
Triplets	0	0	3	6	-	-	-
Quintuplets	0	0	0	6	-	-	-
Total lambs	5	4	10	12	-	-	-
Embryo loss	1	7	3	0	-	-	-
Abortus	0	0	0	3	-	-	-
Total number of fetuses (head)	6	11	13	12	-	-	-
USG accuracy level (%)	100 (6/6)	100 (11/11)	100 (13/13)	58.33 (7/12)	-	-	-
Average number of embryos	1.50±0.29 (6/4)	2.20±0.20 (11/5)	1.86±0.14 (13/7)	4.00±1.00 (12/3)	0.01	0.001	0.067
Average number of living fetuses per pregnant ewe	1.25±0.25 (5/4)	0.80±0.37 (4/5)	1.43±0.30 (10/7)	3.00±1.73 (9/3)	0.076	0.383	0.126
Litter size	1.25±0.25^a (5/4)	1.33±0.33^a (4/3)	1.43±0.30^a (10/7)	4.50±1.50^b (9/2)	0.006	0.008	0.011

Note: LF= local forage; SI= Sorghum-Indigofera; PMSG= Pregnant Mare Serum Gonadotropin. Mean in the same row with different superscripts letters show interaction ($p<0.05$).

SI feed were lower than for LF (Table 2). However, SI feed did not significantly affect average body weight gain compared to LF. This indicated that the digestion efficiency of the SI feed was better than LF, which may be associated with the high energy content of the SI feed (Table 1). These findings are supported by Sileshi *et al*. (2021), who reported that the DM consumption of sheep was affected by the feed energy content. Furthermore, it was stated that the feed consumption decreased along with the increasing dietary energy levels; in other words, the animal will stop eating when its energy needs are met.

Nutrients are required not only to increase body weight but also for the body's metabolism and reproductive processes. Protein and energy factors are among the factors that affect the body's condition and readiness for the reproductive process. Ebrahimi *et al*. (2007) stated that the ideal composition of protein and energy content in feed would help maintain the rumen environment and microbial growth in optimum condition, affecting sheep productivity.

Meanwhile, protein is required in the process of cell regeneration and is usually obtained from feed intake. Protein comprises various amino acids that have specific roles in growth and reproduction. The proximate analysis of the feed used in this research showed that the SI feed had a higher protein content than LF (Table 1). The high protein content of SI feed is thought to derive from the Indigofera forages. Suharlina *et al*. (2016b) reported that Indigofera could produce high CP biomass and have a better digestibility rate than other legumes. Abdullah (2010) further reported that Indigofera had a protein content of approximately 29.76%–29.83% and a low level of tannins (0.09%–0.65%). In addition, Indigofera contains the carotenoids antioxidant (Hutapea *et al.,* 2018) alongside complete amino acids (Palupi *et al.,* 2014). Indigofera has an amino acid score close to soybean meal, meaning it has potential as a protein source in the feed (Palupi *et al.,* 2014). These factors presumably play an important role in supporting the better quality of reproductive performance in the SI-fed group.

It is generally reported that energy is required to maintain optimum metabolic processes in the body, including the absorption level of other nutrients and the reproductive process. Interestingly, the energy content of the SI feed in this result was higher than LF. The high energy content of SI feed is predicted to be generated from sorghum forage silage (4.37 Mcal/kg DM) (Colombini *et al.,* 2015) and rice bran and to account for as much as 18.70–19.17 Mcal/kg DM (Shi *et al.,* 2015). It is strongly suspected that the animals treated with SI feed had more energy for the reproductive process. The result of this experiment is supported by Kusina *et al.* (2001), who reported that feeding with moderate energy levels (0.53 MJ ME kg^{-1} $W^{0.75}$) had a better influence on the estrous characteristics and reproductive performance of goats than feeds with low energy levels.

The parameters of reproductive quality in pubertal animals are generally measured by steroid hormone concentrations, which are synthesized from cholesterol. Cholesterol is a lipid that can be obtained from feed. It is present in the bloodstream and is considered vital to living organisms (Puvača *et al.,* 2016). Cholesterol serves to build cell membranes and produce various hormones essential for proper body function, such as estrogen, progesterone, and aldosterone (Ahmad *et al.,* 2004). While the cholesterol content in SI tends to be higher than LF (Table 2), the cholesterol content in the LF feed in the present study was still higher than in sheep fed with tropical browse plants (±60.68 mg/dl), as reported by Astuti *et al.* (2011). This discrepancy is probably due to the composition and nutritional content of the forage used.

Although cholesterol plays an important role as a steroid hormone precursor that affects the estrous quality, our findings showed no significant differences in cholesterol levels among the treatment groups. As such, estrous characteristics are not predominantly affected by cholesterol but may be influenced by the energy content of the feed, especially SI feed (Table 1). A previous study showed that using a flushing technique in the short-term with high energy (4–10 days) before the estrous synchronization could improve the glucose, insulin, and IGF-1 production in the ovary (Habibizad *et al.,* 2015). These important components will induce the FSH-receptor synthesis, leading to an increase in follicle development and ovulation rate (Guo *et al.,* 2019).

Protein concentration is a further factor affecting reproductive function (Webb *et al.,* 2004). Indigofera is a legume with a proven high protein content of around 1.67% arginine amino acid (Palupi *et al.,* 2014). Arginine is nitric oxide (NO) precursor that affects the ovary function through the NO system. The NO system is involved in the steroidogenesis process, hypothalamic-hypophysis-gonad action, oocyte production, ovulation, and luteolysis (Dixit & Parvizi, 2001). These compounds are also thought to be why the ewes in the SI treatment had a faster onset of estrous.

In this study, the group stimulated with PMSG had a longer estrous duration. This was thought to be associated with the half-life of PMSG in the body, which is approximately 40 to 125 hours (Tirpan *et al.,* 2019). Exogenous FSH from PMSG caused high levels of estrogen in the bloodstream (Dogan & Nur, 2006) and continued to stimulate ovarian activity, thereby causing a negative feedback effect on the pituitary that suppresses LH secretion (McCartney *et al.,* 2002). Continuous follicle stimulation without LH secretion will result in persistent follicles and thus may result in longer estrous duration.

This study proved that SI feed increased the fertility of ewes. The highest pregnancy rate (100%) in the SI feed treatment group indicated that this feed type has a good quality to support the reproductive process. Dupont *et al.* (2014) stated that feed consumption and body conditioning score (BCS) strongly influenced the endocrine system and fertility level. The high energy and protein levels in SI feed treatment were associated with an increased pregnancy rate and the ability to maintain twin fetuses after PMSG stimulation up to birth. Muñoz *et al*. (2009) reported that increasing feed nutrients during the gestation period can increase the live weight of the ewes, BCS, maintenance of pregnancy up to birth, fetal size, and reduce the risk of death of the fetus and lamb after birth.

Furthermore, several studies have reported that forage sorghum and Indigofera contain antioxidants (Hutapea *et al*., 2018) along with amino acids such as arginine (Palupi *et al.,* 2014), which may contribute to the reproductive efficiency of ewes. Sejian *et al.* (2014) reported that the antioxidants in feed could help reduce stress in ewes while also increasing fertility and the prolific level. In addition, arginine could improve the reproductive performance and uterine environment for pregnancy maintenance in sheep (Lassala *et al.,* 2010; Wu *et al.,* 2013). Arginine supplementation during pregnancy could also increase the nutrients in the fetus (Thureen *et al.,* 2002) and prevent fetal growth retardation in malnourished ewes (Lassala *et al.,* 2010).

The high fetal loss in LF with PMSG treatment (63.64%) can be influenced by either genetic or environmental factors. The incidence of fetal loss in this study was higher than the 19.9% reported by Dixon *et al*. (2007), and various sheep were reported to have multiple embryonal and fetal losses. Overall, the incidence of fetal loss was 3%–4%, with occurrences every 20-day interval after 25 days of the gestation period (Dixon *et al.,* 2007). Nutrient and hormone manipulation factors can also influence reproductive function. The application of low feeding to meet the animal nutrient requirement can decrease insulin and serum leptin levels (Caldeira *et al.,* 2007; Tsiplakou *et al.,* 2012), in addition to P4 concentration, which may influence the fetal survival rate (O'Callaghan *et al.,* 2000).

Moreover, using PMSG treatment to increase the litter size was ineffective when applied to the animals fed with fluctuating nutrient content (LF). This was proved by the fact that the litter size in the LF group was smaller than the SI group with PMSG treatment (1.33±0.33 vs. 4.50±1.50). Grazul-Bilska *et al*. (2012) reported that inadequate feeding of ewes had a negative effect on follicle quality and resulted in lower follicle ovulation, thus resulting in less morula and blastocyst formation. The high energy and protein content in SI feed are likely synergistically related to the high litter size after PMSG treatment. It has been revealed that nutrition has a stimulatory effect on follicle numbers, and the high energy and/or protein in diets may increase the ovulation rate in ewes (Ocak *et al.,* 2006; Koyuncu & Canbolat, 2009).

Interestingly, the results of this study show that pregnancy failure only occurred in PMSG-stimulated ewes. The incidence rate was higher in the LF treatment (40%) compared to the SI (33%) treatment (Table 4). The possibility of pregnancy failure may be related to high estrogen concentration through continuous stimulation due to the longer half-life of PMSG. High estrogen concentration will counteract the effect of progesterone; thus, the pregnancy will be terminated. Khafaji (2018) reported a loss of embryonic incidence after synchronized progestagen-PMSG due to insufficient P4 production to maintain pregnancy. Furthermore, Gordon (1997) reported that more than 63% of pregnancy failures occurred after the progestagen-PMSG synchronization protocol. However, the result of this study indicated that the SI feed combined with PMSG stimulation resulted in better maintenance of twins than the LF feed ($p<0.05$) and showed a strong interaction between SI feed and PMSG stimulation ($p=0.011$). To our knowledge, this is the first study to prove that mixed feed SI in combination with PMSG stimulation results in improved reproductive performance with the largest litter size in Garut ewes.

CONCLUSION

Garut ewes fed with SI showed increased reproductive success, with the highest number of ewes giving birth until parturition. In addition, the ewes fed on SI with PMSG stimulation had the largest litter size. To further improve the pregnancy rate in PMSG treatment, it is suggested that progesterone is given at the beginning of the pregnancy.

CONFLICT OF INTEREST

Luki Abdullah serves as an editor of the Tropical Animal Science Journal, but has no role in the decision to publish this article. The authors also declare that there are no financial, personal, or other relationships with any other person or organizations related to the material covered in the manuscript.

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