

Lipid regulation and growth on native ram lambs in the south coast of West Java, Indonesia fed legume forages

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Abstract. Tanuwiria UH, Mushawwir A, Zain M, Despal D. 2023. Impact of protein source from legume forages on lipid regulation and growth on native ram lambs reared extensively in the south coast of West Java, Indonesia. *Biodiversitas* 24: 4183-4192. This investigation was carried out in three different locations to examine the impact of legume forage as a protein source on lipid regulation and growth. This study used 120 rams, consisting of 40 rams in each location (Garut, Cianjur, Sukabumi) spread along the South Coast of West Java. Physical environmental conditions were recorded during the study. Ram lambs at each location were divided into four groups; one group only received a basal diet (*Setaria/Setaria sphacelata*), and the other three groups were each given additional legumes (*Calliandra/Calliandra calothyrsus*, *lamtoro/Leucaena leucocephala*, and *moringa/Moringa oleifera*). Blood samples were collected every two weeks during the 16 weeks experiment. The blood sampling was handled based on standard procedures to prevent sample damage, and the body weight was measured every two weeks. Sample analysis related to lipid regulation and creatine kinase activity was measured using spectrophotometric techniques according to the instructions of the Kit Randox protocol and analysis of blood plasma fatty acids were analyzed by gas chromatography. The research results show that legumes as a protein source can regulate through molecular signaling to decrease lipid levels, saturated fatty acids, and lipid transport in the extracellular fluid, but increase unsaturated fatty acid and the growth rate of ram lambs. Based on the results of the current study, it can be a strong basis for the development of nutrigenomic aspects of protein for small ruminants, accompanied by studies of the ages of different ruminant samples.

Keywords: Lamb, legume forages, metabolism, protein

INTRODUCTION

Many factors play a role in determining the rate of growth in ruminants. Nutritional factors, both in quantity and quality, are the main factors (Speer et al. 2022), and environmental and genetic factors (Deng et al. 2022). The amount of protein digested in the rumen and which can pass through the rumen is one of the important factors determining growth. Previous studies showed that the source of protein in the ration affected its digestibility in the rumen (Coblentz and Akins 2021). Forage quality is also a major factor in providing protein for ruminants (Vahedi et al. 2021; Speer et al. 2022), although the use of forage from legumes has been popularly applied to sheep farmers (Kamil et al. 2020; Murshed et al. 2022) as well as other ruminants such as beef cattle and dairy cattle (Tanuwiria and Mushawwir 2020; Murshed et al. 2022), but variations in animal performance are very high. Many publications regarding protein from forage legumes that are degraded in the rumen or rumen degradable protein (RDP) have also been widely reported (Niyigena et al. 2021; Sejian et al. 2021). It was shown that RDP varies based on the type of legume consumed by ruminants, also showing a

different impact on post-absorption, rumen and nutrient metabolism and growth.

In a more interesting study, the type of forage protein source, such as legumes, and the amount of protein that can be absorbed on post-rumen or by intestinal villi absorptive cells is an important factor to consider. Protein digestibility using different legumes in ruminants has been reported by Jia et al. (2021), showing large variations. The results of this previous study confirmed that the chemical composition of legumes greatly determines their degradation in the rumen. The results of the same study were also shown by Hernawan et al. (2017) and Rahmania et al. (2022) that the chemical structure of legumes determines the proportion of protein that can be digested in the rumen to its absorption in the intestine. On the other hand, several types of forage legumes contain anti-nutritional compounds. The excessive consumption of anti-nutrients causes decreased nutrient absorption and is accompanied by decreased growth.

Previous studies have shown that legumes with high tannin content show low digestibility, as well as those with high fiber content (Tanuwiria et al. 2022), while protein digestibility is very influential on growth. In addition, several studies have also shown that an important factor that plays a role in determining the growing relationship

with legume protein is the composition of amino acids in the legume. A higher proportion of digestible amino acids (Vahedi et al. 2021; Deng et al. 2022) and essential amino acids (Hernawan et al. 2017; Rahmania et al. 2022; Tanuwiria et al. 2022) indicates a strong correlation to animal growth (Xu and Min 2022). In addition, the biomass storage potential of calliandra, lamtoro and moringa are 139.74 tons/ha, 127.73 tons/ha, and 142.76 tons/ha, respectively (Susilawati et al. 2019).

Although many reports have shown the impact of providing legumes as a protein source on growth, few studies show interactions between legume nutrients and growth. Growth is not determined by protein independently, and experiments on various types of experimental animals (Sejian et al. 2021) show that protein can regulate metabolism for other nutrients in body cells.

The results of a previous study reported by Zhao et al. (2022) showed a relationship between protein content in rations and fatty acid composition in muscle in pigs. Research on mice showed that there was an indication of a signaling relationship between feed proteins and fatty acid receptors (Park et al. 2021; Rahmania et al. 2022; Zhao et al. 2022). Selim et al. (2021) also reported fatty acid homeostasis due to giving high protein in experimental mice rations. Several previous researchers have also demonstrated the quantification of genes related to lipid homeostasis in pig cells by administering high protein in rations, among others (Adriani and Mushawwir 2020; Kharazi et al. 2022) that there was a decrease in the expression of the steroid receptor element binding protein-1 (SREBP-1) gene. The study by Lakhssassi et al. (2021) showed a decrease in the expression of proliferator peroxisome-activated receptor- γ (PPAR- γ) with higher protein consumption. The SREBP-1 and PPAR- γ decrease with low protein consumption, which decreases the rate of lipogenesis, leading to decreased organ growth.

The sheep population on the south coast of West Java reached 745,000 (Kharazi et al. 2022), although it showed a decline in reproductive performance. High temperatures, accompanied by high humidity, are reported along the south coast of West Java (Adriani and Mushawwir 2020). The failure of reproductive organ development can cause this decline. Tanuwiria et al. (2022a) reported a decrease in reproductive organ development due to protein deficiency and low lipid regulation (Park et al. 2021). High temperature is an important factor in reducing the growth rate of organs before sex maturity, and several researchers have shown decreased regulation with increasing temperature (Tanuwiria et al. 2022a; Mushawwir et al. 2020).

The results of studies using various legumes as protein sources for sheep have been widely reported by Lakhssassi et al. (2021). Still, no research shows the impact of feeding protein sources on sheep lipid regulation. This research is important due to the accuracy of the lipid metabolism rate affecting tissue growth, including muscle tissue, and the rate of steroidogenesis, and growth rate.

MATERIALS AND METHODS

Experiment animal and treatment

Young rams (ram lambs) were used in this experiment, aged 28-32 weeks, with an average body weight of 17.83 kg. The experimental animal of 120 ram lambs, scattered on the south coast of West Java Province, Indonesia, consisted of 40 ram lambs at each study site, namely Pameungpeuk (Garut District), Cidaun (Cianjur District) and Ujung Genteng (Sukabumi District). The average environmental temperature and humidity at the study site during the experiment were $31 \pm 2.1^\circ\text{C}$ and $84 \pm 3.5\%$. Temperature and humidity data were recorded continuously during the study using a "Digital Hygrometer Thermometer Data Logger USB Elitech RC-4HC Humidity".

The experimental ram lambs in each observation location were kept in a semi-intensive system. Experimental sheep were penned at night (05.30 pm-09.00 am) and given forage setaria (*Setaria sphacelata*) as a basal ration and experimental rations in the morning (06.00-09.00 am) before being pastured until the afternoon. Experimental sheep were grazed in the land around the location of the animal housing. Experimental sheep graze on land with dominant grass vegetation, freely choosing and with sufficient shade trees for shelter. While being pastured from 09.00 am-05.00 pm, the experimental sheep had access to sufficient drinking water to meet their drinking needs.

Forty ram lambs at each trial site were divided into 4 groups, each consisting of 10 experimental sheep. One group was only given a basal ration, namely setaria grass without legumes, and the other three groups were given a basal ration (BS) with the addition of 25% legumes each, namely calliandra (*Calliandra calothyrsus*), lamtoro (*Leucaena leucocephala*), and moringa (*Moringa oleifera*) for groups 2, 3, and 4 respectively.

All experimental ram lambs were given ear tag before the observation started; they could be separated based on the treatment group. Individual system cages are used while the animal samples are in the pen, equipped with a feed area at the front of the cage. This condition was possible to prevent ram lambs from feed fighting.

Examination of the health condition of the sheep selected as the trial sample, including physiological, hematologic conditions (erythrocyte, hemoglobin, hematocrit, and leukocyte levels) and worm infection, was carried out before the experiment started. Pre-trial health checks were implemented in this study to ensure animal samples were in good health and to minimize trial error rates.

Feeding, basal, and experimental diet

Basal and legume rations were fed every morning before grazing, and setaria was used as a basal ration in this experiment. The age of setaria cutting used ranges from 30-35 days. Setaria was obtained from each research location, which has been cultivated intensively. The nutrient composition of setaria from the three mixed study locations can be seen in Table 1.

Basal and trial rations were given every morning (06.00-08.30 am). A total of 2 kg of forage for each animal sample was given at the experiment start. And ad 100 g weekly until the end of the experiment for 16 weeks. The control group without legumes was given 100% Setaria grass, while the other group received legume forage treatment; each group received 75% setaria grass + 25% legumes of calliandra, lamtoro, and moringa. The composition of the nutrient legumes used in this study, based on the proximate and Van Soest analysis results, is shown in Table 2.

The withering of forage the day before being given to experimental animals has been carried out to prevent excess gas production in the rumen. Therefore, all forage ingredients (setaria grass and legumes) were collected the day before being given to the experimental animals. Calliandra, lamtoro, and moringa were given to all experimental sheep at three locations during the study, obtained from the same location.

Blood sampling and data collection

Blood samples from each sheep were collected at the start of the study and, after that, once every two weeks during the four months of the study. Furthermore, a 5 mL sterile tube containing EDTA and a 5 mL sterile syringe were used to collect blood samples from the jugular vein in the sheep's right neck. Before and after blood sampling, 95% alcohol review is used to prevent germ infection. Sterilization of alcohol paper that has been used by burning it is done to prevent unsterile waste. A special blood sample bag containing a cooler has been used to temporarily store the collected blood sample so the sample is not damaged.

A mobile centrifuge that had been prepared at the study site was used to separate blood plasma from each sheep sample that had been collected. Whole blood was separated from plasma using a rotational speed of 3,500 rpm for 5 minutes. The separated plasma was transferred into a 3 mL plasma cuvette coded using a 100-1000 µL micro fin. The plasma collected in a cuvette is stored in a sample box containing liquid nitrogen at -72°C before being brought to the laboratory for plasma chemical analysis.

Lipid composition and lipid transport analysis include cholesterol, LDL, apolipoprotein A, apolipoprotein B, apolipoprotein C, LDL, TAG (triglycerides), and NEFA (non-esterified fatty acids). Furthermore, a 5 µL of plasma sample was used to measure each parameter of lipid composition and lipid transport. The analysis was carried out according to the instructions and protocol in the Randox KIT (Randox UK); all reagents and solvents are available in the kit used. The reaction vessel between the sample, solvent, and reagent has used a 2 mL test tube. The solution that has formed a stable color is then pipetted into the spectrophotometer cuvette using a 100-1000 µL micro-pipe. Next, color absorption has been measured using a spectrophotometric technique. The spectrophotometer operation is based on the standard operational tools (Shimadzu UV Vis Spectrophotometer UV-1280).

The wavelength used was 550 λ, 550 λ, and 450 λ for cholesterol, triglyceride, and lipid transport respectively. The same method was also used to measure the activity of

the enzyme creatine kinase (CK) by spectrophotometer at a wavelength of 470 λ.

Determination of the level of fatty acid composition was carried out based on the total fatty acids in the blood plasma samples. Quantification of fatty acids was determined using gas chromatography (GC), and CP SIL 88 CB column (100 m60.25 mm, Chrompack-Varian, USA) was used as capillary GC. Setting the oven's temperature, installing hydrogen gas as a booster, and setting the injectors are based on the instructions and operational procedures (PerkinElmer Instruments, USA).

Body weight gain was measured every two weeks during the four months of the study. One by one, the sheep were weighed in the afternoon before being put into the pen. Digital scales type Sonic A12E, with a capacity of 500 kg with an accuracy of 0.01 kg have been used in this measurement. In overview, the steps of this research are shown in Figure 1.

Data analysis

Data tabulated with MS. Excel was analyzed using quantitative descriptive statistical analysis by Kruskal Wallis and Mann Whitney analyses to determine differences between groups. All data testing has been done with a 95% confidence level or a 5% degree of error (α 0.05). The statistical analysis application, SPSS IBM 2021 has been used to determine the effect of treatment on all parameters that have been measured, as well as to find out differences between treatment groups.

Table 1. Nutrient composition of setaria

Nutrient	(%)
Ash	21.64
Organic matter	78.36
Crude fat	4.51
Crude protein	21.01
Fiber	23.96
True Digestible Nutrient (TDN)	53.75

Table 2. Chemical composition of legumes used in this research

Composition (%)	Legume		
	Calliandra (<i>Calliandra calothyrsus</i>)	Lamtoro (<i>Leucaena leucocephala</i>)	Moringa (<i>Moringa oleifera</i>)
Water	9.70	15.09	4.56
Ash	7.34	7.00	9.94
Protein	27.84	27.91	24.50
Fiber	22.48	20.33	19.44
Fat	3.17	3.20	3.03
NFE	28.97	41.56	39.72
TDN	57.72	68.68	60.29
ADF	37.79	28.10	7.41
NDF	53.90	37.83	14.00
Lignin	6.64	2.17	1.32
Cellulose	31.15	25.93	6.59
Hemicellulose	16.11	9.73	6.59

DNFE: Nitrogen-Free Extracts; TDN: True Digestible Nutrient; ADF: Acid Detergent Fiber; NDF: Neutral Detergent Fiber

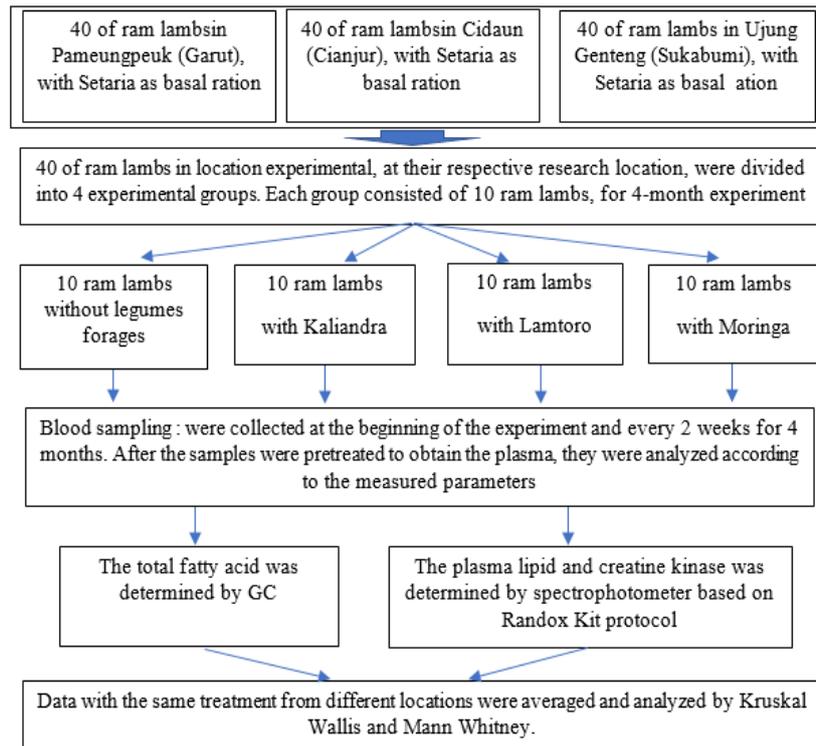


Figure 1. The overview of the experimental procedure

RESULTS AND DISCUSSION

The results of the current study indicate that feeding legumes as a source of protein can dictate lipid regulation and stimulate growth rate. It is believed that the proteins contained in legumes can interact with proteins that play a role in lipid regulation. In particular, this mechanism is presented through the results and discussion as follows:

Lipid regulation

The impact of legume administration on lipid composition and lipid transport activity in ram lamb blood plasma based on the results of this study is shown in Table 3. The current study showed that overall the administration of forage legumes as a protein source for ram lambs significantly ($P < 0.05$) suppressed lipid synthesis and its transport activity in the extracellular fluid. Lipid concentrations (CT, TAG, and NEFA) of the legume group were significantly lower ($P < 0.05$), compared to the legume group. This study reveals that legume protein can influence lipid metabolism in ram lambs cells. Previous researchers (Li et al. 2022; Zsolnai et al. 2022) in pigs have shown similar results to the current study; it has been reported that feeding high protein diets can reduce lipogenesis activity.

In Table 3, lipogenesis activity appeared to decrease with the provision of protein sources from legumes compared to the control group. In comparison, the CT

levels of the BS+K, BS+L, and BS+M groups were 156.63, 158.26, 172.47, respectively, significantly lower ($P < 0.05$) than the control (BS+0) of 204.34. Similarly, the other lipid levels (TAG and NEFA) and lipid transport (LDL, HDL, Apolp) also showed a decrease with the provision of protein sources.

A decrease in lipogenesis is also strongly associated with an increase in the expression of anabolic hormones, such as insulin, as a result of consuming a diet with higher protein. Studies reported by Adriani and Mushawwir (2020), Mushawwir et al. (2020), and Sun et al. (2022) show that increasing insulin will increase the absorption of glucose into the cytoplasm. An increase in glucose in the cytoplasm stimulates an increase in acetyl-CoA so that the activity of glucose-6-pyruvat dehydrogenase (G-6-PDH) decreases (Mushawwir et al. 2020; Li et al. 2022). Furthermore, a decrease in G-6-PDH causes a decrease in NADPH production (Sejian et al. 2021; Rahmania et al. 2022). Many previous researchers reported that a decrease in NADPH causes a decrease in the catalysis of malonyl-CoA into fatty acids and cholesterol (Zhu et al. 2022; Tanuwiria et al. 2022; Wang et al. 2022). Several previous researchers have demonstrated the role of PPARs in lipid regulation, including that lipid homeostasis is strongly maintained by PPARs. Higher protein consumption can decrease lipogenesis, but this gene regulates the rate (Kharazi et al. 2022; Toral et al. 2022).

Table 3. Composition of lipid and its transport in ram lambs without and with fed legume

Lipid (mg/dL)	Legume			
	BS+0	BS+K	BS+L	BS+M
CT	204.34±7.04 ^a	156.63±3.63 ^b	158.26±5.52 ^b	172.47±6.04 ^c
LDL	63.43±2.54 ^a	45.74±1.05 ^b	46.38±3.73 ^b	59.21±2.63 ^c
Apolp B	3.72±0.15 ^a	1.47±0.07 ^b	1.66±0.06 ^b	2.04±0.15 ^c
Apolp C	3.55±0.12 ^a	1.38±0.08 ^b	1.41±0.03 ^b	1.98±0.14 ^c
Apolp E	3.61±0.18 ^a	1.42±0.02 ^b	1.48±0.06 ^b	1.83±0.06 ^c
HDL	57.59±2.94 ^a	51.27±2.38 ^b	51.07±3.04 ^b	50.66±1.89 ^b
Apolp A-I	3.12±0.04 ^a	2.82±0.05 ^b	2.74±0.07 ^b	2.57±0.12 ^b
Apolp A-II	3.07±0.07 ^a	2.79±0.12 ^b	2.71±0.08 ^b	2.61±0.13 ^b
TAG	276.25±8.02 ^a	204.56±6.94 ^b	206.61±6.95 ^b	221.47±7.93 ^c
NEFA	84.36±4.52 ^a	69.43±4.03 ^b	71.57±4.08 ^b	72.31±4.06 ^b

Note: ^{a,b,c}average followed by different letter in a same row indicate significant differences ($P<0.05$); BS+0: Basal feed without legume; BS+K: Basal feed with 25% calliandra; BS+B: Basal feed with 25% lamtoro; BS+M: Basal feed with 25 moringa; CT: Cholesterol Total; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; Apolp: Apolipoprotein; TAG: Triglycerides; NEFA: Non-esterified Fatty Acid

In addition, the reduction in cholesterol is also triggered by a decrease in malic enzyme activity. Research by Speer et al. (2022) and Thomas et al. (2021) shows that high protein causes a decrease in malic enzyme activity, this decrease inhibits the formation of cholesterol from the precursor mevalonate acid. That is why there was a decrease in cholesterol levels in this study (204.34 without legume/control to 156.63, 158.26, and 172.47 with BS+K, BS+L, and BS+M, respectively). Several previous research results also showed that high protein rations also inhibited the expression of hydroxyl methyl glutaryl Co-A reductase (HMGCo-A reductase) (Mushawwir et al. 2020; Mushawwir et al. 2021). The HMGCo-A reductase enzyme plays a role in forming endogenous cholesterol (Zhao et al. 2022), research Jia et al. (2021) showed a decrease in HMGCo-A reductase activity and a decrease in cholesterol concentration in the circulating system and the extracellular plasma fluid.

As a result of the decrease in lipid metabolism in the current study, there is a decrease in lipoproteins which act as lipid transport in the extracellular fluid. A decrease in lipid transport from the tissues to the liver was indicated by a decrease in HDL and apolipoprotein A-I/II concentration in the ram lambs that received forage legumes (Table 3). The same indication also occurs for lipid transport from the liver to all tissues, namely by decreasing the concentration of LDL and apolipoproteins B, C, and E. Overall, this decrease in lipid transport can be an indication that the use of lipids in cells is more focused on cell growth and development of cells in each tissue, both in muscle and other tissues. Studies reported by Niyigena et al. (2021) and Adriani et al. (2021) show that providing higher protein in rations for growth phase animals causes the growth activity of cells to increase sharply, and the need for lipids is needed to support the biosynthesis of cells. The results of previous studies have also reported that lipid transport has decreased in animals with moderate muscle growth rates. This is characterized by decreased LDL and HDL activity and apolipoprotein (Toral et al. 2022).

This study also revealed that the anabolic pathway for TAG formation was decreased in the sheep group fed

forage legumes as a protein source. Table 3 shows that TAG level is 276.25 without legume (BS+0), significantly lower than group of BS+K, BS+L, and BS+M are 204.56, 206.61, and 221.47, respectively. A decrease in TAG indicates that the administration of this legume prevents premature growth of adipose tissue in sheep. One of the main factors causing the decrease in TAG is the decrease in the activity of enzymes important in the esterification and incorporation of fatty acid polymers.

Protein interaction with the activity of this enzyme has been reported (Mushawwir et al. 2020; Zsolnai et al. 2022) that providing high protein in the ration can reduce the activity of fatty acid synthase (FFA). Decreased FFA enzyme expression reduces the rate of fat and adipose tissue anabolism. The results of another study (Thomas et al. 2021; Zhu et al. 2022) reported that compensation with low-fat anabolism can increase the rate of muscle growth. This benefits ram lambs because tissue formation can be optimized before sexual maturity.

Several previous research reports have shown the molecular impact of protein administration on reducing fat synthesis. For example, research by Adriani et al. (2021) reported that high protein consumption led to decreased expression of genes related to lipid homeostasis in domestic fowl and ruminants. It was shown that the proliferator peroxisome-activated receptor- γ (PPAR- γ) gene dropped dramatically. The reduction of this gene causes an increase in the activity of the enzyme activated-fatty acid binding protein (A-FABP), thereby inhibiting the incorporation of fatty acids into TAGs.

Another pathway that causes a decrease in TAG is known that the PPAR- γ gene stimulates an increase in lipoprotein lipase (LPL) in domestic fowl (Kamil et al. 2020), dairy cattle (Tanuwiria and Mushawwir 2020; Li et al. 2022; Tanuwiria et al. 2022) small ruminants (Tanuwiria et al. 2022), broiler (Adriani et al. 2021). Therefore, high LPL activity causes catabolism or TAG degradation into free fatty acids. This inhibitory pathway can be the main pathway for reducing TAG synthesis in cells and is very effective in reducing blood TAG levels (Xu et al. 2022).

Although LPL causes an increase in the availability of free fatty acids (FFA) due to TAG catabolism, these fatty acids do not immediately circulate in the blood. Still, they are used by muscle cells and other tissues for tissue biosynthesis (Lakhssassi et al. 2021). Several research reports also show that this increase in free fatty acids simultaneously stimulates β -oxidation activity (Hernawan et al. 2017; Toral et al. 2022) and the formation of acetyl-CoA from the fatty acid pathway (Lakhssassi et al. 2021), as well as increases oxidation-reduction activity in mitochondria (Mushawwir et al. 2021a; Mushawwir et al. 2021b). This metabolic fact can also be why a decrease in TAG is accompanied by a decrease in NEFA based on the results of this study (84.36 without legume to 69.43, 71.57, and 72.31 with BS+K, BS+L, and BS+M, respectively). The Utilization of NEFA as an energy source also encourages efficiency in using volatile fatty acids to be converted into energy in the Krebs cycle.

Fatty acid profile

The impact of giving legumes on the concentration of fatty acids in ram lamb blood plasma based on the results of this study is presented in Table 4. The study results in Table 4 showed a decrease ($P < 0.05$) in C12:0 to C17:1 saturated fatty acid levels in the sheep group with forage legumes (except C14). On the other hand, levels of polyunsaturated long-chain fatty acids (PUFA) $> C18$ appeared to increase with the addition of forage legumes.

The results of this study confirmed and emphasized that protein consumption from legume forages can dictate the signaling of biosynthetic fatty acids (lipogenesis) in cells. This protein signal appears to stimulate the synthesis of long-chain unsaturated fatty acids and inhibit the synthesis of short-chain saturated fatty acids. The results of the reported study (Tanuwiria et al. 2011; Xu et al. 2022) suggested the ability of the protein to inhibit the expression of the sterol regulatory element binding protein-1 (SREBP-

1) gene. Low SREBP-1 levels lead to decreased activity of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) enzymes (Barbero et al. 2020; Toral et al. 2022).

These two enzymes (ACC and FAS) play a role in regulating the formation of fatty acids, especially short-chain fatty acids (SFA) (Sun et al. 2022). A Study by Barbero et al. (2020) showed that ACC functioned in catalyzing the malonyl-CoA formation from acetyl-CoA, thus, the concentration of malonyl-CoA decreased. Endogenously, in fatty acid biosynthesis, malonyl-CoA is the main precursor in the formation of fatty acids (Barbero et al. 2020; Lakhssassi et al. 2021; Sun et al. 2022).

In addition, the decrease in MUFA was also caused by the activity of the activated-fatty acid binding protein (A-FABP). An increase in A-FABP concentration is also related to signaling induction by protein (Tanuwiria et al. 2022). Giving higher protein in the ration also inhibits the expression of the PPAR- γ gene. Decreasing the gene's concentration triggers the enzyme A-FABP's activity (Tanuwiria et al. 2022a,b). The high activity of A-FABP causes the degradation of short-chain fatty acids to increase to acetyl-CoA through the β -oxidation pathway. That is why energy availability for muscle growth is not experiencing a shortage. The efficiency of using fatty acids as an energy source is very high because one fatty acid molecule can produce several moles of acetyl-CoA compounds. On the other hand, SFA breakdown can also be triggered by carnitine palmitoyltransferase 1 (CPT-1) activity. Previous studies reported that CPT-1 activity could increase with higher protein consumption for pigs (Sejian et al. 2021) and ruminants (Niyigena et al. 2021). CPT-1 increase can directly affect high protein in the ration, because protein stimulates ketogenic activity (Kharazi et al. 2022) and can also be caused by oxidation-reduction activity in mitochondria (Akakpo et al. 2022).

Table 4. Fatty acid (FA) composition of blood plasma in ram lambs without and with fed legum

Fatty acid (percentage of total FA)	Legume			
	BS+0	BS+K	BS+L	BS+M
C12:0	0.96±0.01 ^a	0.92±0.01 ^b	0.94±0.01 ^{ab}	0.95±0.01 ^a
C14:0	3.05±0.13 ^a	3.06±0.12 ^a	3.04±0.12 ^{ab}	3.04±0.09 ^a
C15:0	0.47±0.01 ^a	0.43±0.01 ^b	0.44±0.01 ^b	0.44±0.01 ^b
C16:0	21.94±1.06 ^a	20.53±2.64 ^b	20.57±2.04 ^b	20.62±2.04 ^b
C17:0	1.87±0.02 ^a	1.83±0.04 ^b	1.83±0.12 ^b	1.85±0.01 ^{ab}
C17:1	0.29±0.01 ^a	0.25±0.01 ^b	0.25±0.01 ^b	0.27±0.01 ^{ab}
C18:0	14.58±1.94 ^a	16.74±2.53 ^b	16.68±2.05 ^b	16.51±1.94 ^b
C18:1	29.48±3.05 ^a	33.63±2.79 ^b	33.27±2.94 ^b	32.71±2.96 ^b
C18:2	4.62±0.14 ^a	5.78±0.32 ^b	5.83±0.95 ^b	5.52±0.82 ^b
C18:3	1.79±0.02 ^a	2.83±0.01 ^b	2.89±0.11 ^b	2.74±0.13 ^b
C20:0	1.69±0.01 ^a	2.83±0.11 ^b	2.80±0.07 ^b	2.49±0.04 ^b
C20:4	4.03±0.14 ^a	5.17±0.38 ^b	5.09±0.73 ^b	4.63±0.07 ^b
C22:0	0.77±0.01 ^a	1.63±0.02 ^b	1.48±0.01 ^b	1.22±0.02 ^b

Note: ^{a,b}average followed different letter in a same row indicate significant differences ($P < 0.05$); BS+0: Basal feed without legume; BS+K: Basal feed with 25% calliandra; BS+B: Basal feed with 25% lamtoro; BS+M: Basal feed with 25% moringa

The result that contrasted with short-chain saturated fatty acids levels in this study was an increase in PUFA levels in the sheep group, which was given a protein source from forage legumes. Many previous studies have reported that protein administration is also associated with an increase in signaling potential through the enzymes desaturase (Tanuwiria et al. 2022) and elongase (Han et al. 2022), both of which are known to function in fatty acid chain elongation and double chain addition. Providing protein increases liver activity in forming monounsaturated fatty acids from saturated fatty acids (Han et al. 2022). This can be explained by the fact that the decrease in saturated fatty acids based on the results of this study (for example, the C12:0 level is 0.96 with BS+0 group to 0.92, 0.94, and 0.95 with BS+K, BS+L, and BS+M, respectively), can also be caused by an increase in the formation of PUFA from saturated fatty acids, by the activity of Δ^9 desaturase. Research Zhao et al. (2021) showed that Δ^9 desaturase activity in the liver increased to convert saturated fatty acids into unsaturated fatty acids due to high protein consumption.

It was also reported Trejo-Lopez et al. (2020) that Δ^9 desaturase in the endoplasmic reticulum catalyzes the conversion of palmitoyl-CoA or stearoyl-CoA to palmitoleoyl-CoA or oleoyl-CoA, respectively. Δ^9 desaturases and elongase activity, capable of inserting additional double bonds into monounsaturated fatty acids (Mavrommatis and Tsiplakou 2020; Trejo-Lopez et al. 2020; Mushawwir et al. 2021; Toral et al. 2022). This is why the concentration of polyunsaturated fatty acids is also increased by providing forage legumes as a source of protein.

Other research reported many scientific publications previously, stating that because animals have Δ^9 desaturase, an animal can regulate metabolism well for forming long-chain unsaturated fatty acids. This ability is shown, among other things, by its enzyme activity which can form Δ^9 (oleic acid) (Mushawwir et al. 2021) and other completely unsaturated fatty acids by combining elongation (Trejo-Lopez et al. 2020) and through chain desaturase (Xu et al. 2022). It is not a problem to supply long-chain fatty acids in rations. However, many studies have used double-chain fatty acids to stimulate other metabolic pathways, such as immunity and milk or egg production.

The impact of giving legume forage as a source of protein in increasing the concentration of long-chain unsaturated fatty acids is not only due to the activation of enzymes that are important in this metabolism. Another reason, based on previous research reports Zhu et al. (2022) state that despite a total decrease in fatty acids in the blood plasma, physiological mechanisms encourage the production of long-chain unsaturated fatty acids. It was stated that h acid is one of the micronutrients that play a role in maintaining the osmolarity of body fluids. That role is an important reason for the increase in the proportion of long-chain unsaturated fatty acids and the decrease in the proportion of saturated fatty acids.

Growth rate

The study revealed that the effect of giving legumes showed a difference ($P<0.05$) in the concentration of the enzyme creatine kinase every week of observation between groups of sheep without and with legumes (Table 5). An increase in CK concentration indicates an increase in its activity.

CK is an enzyme that catalyzes the conversion of creatine into creatinine. Each time this catalysis occurs, 1 mol of ATP is generated (Xu and Min 2022; Xu et al. 2022). This enzyme could be an indicator to measure the rate of muscle growth. Observations on the growth rate with increased CK levels have been reported by Tanuwiria et al. (2011), showing a strong correlation between the growth rate of muscle tissue and CK activity.

Muscle growth requires energy (ATP) to support the biochemical and physiological functions of the tissues during the growth process. The energy supply from the glycolysis process (Niyigena et al. 2019) is insufficient to meet the demand for ATP for the growth process (Han et al. 2022). One of the alternative energy solutions is the activating of the breakdown of creatine into creatinine (Kharazi et al. 2022; Adriani and Mushawwir 2020); the catalysis played by CK indicates the rate of this breakdown. The results of previous studies showed increased activation of CK (Mushawwir et al. 2021) to donate ATP (Hernawan et al. 2017; Jia et al. 2021) during the rate of muscle growth. If it is related to the CK concentration during the 16 weeks of observation, with the rate of body weight gain based on the study results (Figure 2), good linearity is seen. The body weight gain of the control group (BS+0) is 0.27 kg in week 2 to 5.03 in week 16. Body weight gain is higher in the BS+K, BS+L, and BS+M groups from 0.39 to 8.45, 0.32 to 7.85, and 0.32 to 6.84, respectively.

Table 5. Creatine Kinase (CK) concentration of blood plasma in ram lambs without and with fed legume

Week of observation	Creatine Kinase ($\mu\text{g/dL}$) - Legume			
	BS+0	BS+K	BS+L	BS+M
1	1.76 \pm 0.01 ^a	1.99 \pm 0.02 ^b	1.81 \pm 0.01 ^a	1.78 \pm 0.01 ^a
2	2.11 \pm 0.02 ^a	2.78 \pm 0.01 ^a	2.88 \pm 0.01 ^a	2.47 \pm 0.01 ^a
4	2.39 \pm 0.01 ^a	3.82 \pm 0.05 ^b	2.95 \pm 0.03 ^c	2.88 \pm 0.01 ^c
6	2.82 \pm 0.02 ^a	4.58 \pm 0.02 ^b	4.93 \pm 0.03 ^c	3.72 \pm 0.01 ^b
8	3.08 \pm 0.02 ^a	5.31 \pm 0.03 ^b	5.78 \pm 0.03 ^c	4.67 \pm 0.03 ^d
10	3.27 \pm 0.01 ^a	5.46 \pm 0.03 ^b	5.43 \pm 0.02 ^b	5.15 \pm 0.02 ^b
12	3.36 \pm 0.07 ^a	5.75 \pm 0.02 ^b	5.69 \pm 0.06 ^b	5.81 \pm 0.06 ^b
14	4.48 \pm 0.04 ^a	6.41 \pm 0.04 ^b	6.04 \pm 0.03 ^b	5.93 \pm 0.04 ^b
16	4.73 \pm 0.03 ^a	6.59 \pm 0.02 ^b	6.22 \pm 0.02 ^b	6.02 \pm 0.05 ^c

^{a,b,c}average followed different letter in the same row indicate significant differences ($P<0.05$); BS+0: Basal feed without legume; BS+K: Basal feed with 25% calliandra; BS+B: Basal feed with 25% lamtoro; BS+M: Basal feed with 25% moringa

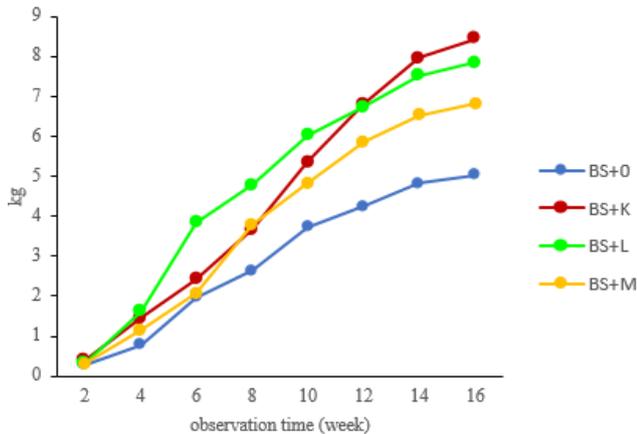


Figure 2. Average body weight gain on ram lambs reared semi-intensively during the observation, without and with the addition of legumes in the South Coast, West Java. BS+0 (basal feed without legume); BS+K (basal feed with 25% calliandra); BS+L (basal feed with 25% lamtoro); BS+M (basal feed with 25% moringa)

The results of this study showed that the highest growth rate was in the sheep group given calliandra. In contrast, the animal group without legume administration showed the lowest body weight gain rate. This growth rate was also followed by CK's activities in the supply of energy (ATP). Table 5 shows that the highest CK concentration at week 16 was in the sheep group given Kalianda, while the lowest CK level was in the group without legumes, namely 6.59 and 4.73 $\mu\text{g/dL}$ respectively. This difference in enzyme expression emphasizes that CK activity providing energy by catalyzing creatine is an alternative mechanism for supplying ATP for muscle tissue biosynthesis.

Overall the results of this study indicate that giving legumes can increase the proliferation rate of muscle cells, thereby increasing the volume and mass of the cells (Mushawwir et al. 2020; Mavrommatis and Tsiplakou 2020). Higher protein administration appears to prevent the earlier development of adipose tissue (Susilawati et al. 2019; Müller et al. 2022). Adipose development which is inhibited by protein signaling mechanisms (Susilawati et al. 2019; Selim et al. 2021; Vahedi et al. 2021; Murshed et al. 2022; Müller et al. 2022), can increase the diversion of nutrient utilization to become a precursor for the formation of muscle tissue. The study reported by Selim et al. (2021) showed that high protein intake maximizes the function of long-chain fatty acids in the growth of muscle cells. In addition, lipid anabolic activity appears to decrease with the signaling of proteins (Islam et al. 2021; Park et al. 2021; Akakpo et al. 2022). Mobilization of fatty acids for the growth of collagen tissue and muscle cells has also been reported by several researchers, Müller et al. (2022) in experimental rats, Jia et al. (2021); Xu et al. (2022); Han et al. (2022) in goats and sheep.

Based on this study and reports of previous studies, it can be explained that protein from legumes prevents fat deposits, so the manifestation of growth shown in this study is an increase in muscle mass, not an increase in

adipose tissue mass. Apart from protein being able to prevent fat deposits, protein is also able to prevent the expression of lipid-sensitive hormones (HSL) (Jia et al. 2021; Zhao et al. 2021; Zhao et al. 2022; Mushawwir et al. 2023). The decrease in HSL actually stimulates the mobilization of fatty acids towards muscle tissue and others. The availability of fatty acids in muscle is a good indication for supporting tissue biosynthesis. This mobilization is very appropriate for animals in the growth phase. Conversely, high-fat mobilization into muscle tissue for post-growth cattle stimulates lipogenic activity (Mushawwir et al. 2011; Vahedi et al. 2021).

In addition, the previous studies by Thomas et al. (2021), also showed that giving higher protein in the ration can increase insulin concentrations and insulin growth factor (IGF). Several reports have shown a close relationship between the hormones insulin (Murshed et al. 2022) and IGF (Jia et al. 2021) on growth rate. Furthermore, a publication by Purba et al. (2022) suggested that insulin and IGF are a group of anabolic hormones, capable of stimulating the growth of muscle tissue cells. Tissue protein synthesis in muscle appears to increase with increasing concentrations of the hormone insulin (Müller et al. 2022). Nonetheless, the balance of nutrients consumed that important is also revealed by (Coblentz and Akins 2021; Niyigena et al. 2021; Adriani et al. 2021; Zhao et al. 2022). In addition, insulin induced by high carbohydrate consumption appears to be less beneficial for muscle tissue growth (Mavrommatis and Tsiplakou 2020; Trejo-Lopez et al. 2020; Lakhssassi et al. 2021; Murshed et al. 2022) if it is not accompanied by high protein consumption (Zhao et al. 2022).

The different growth responses in ram lamb groups that received additional legumes could be due to the quality of the legumes. The quality of legumes is not only determined by their nutrient contents, but also by the anti-nutritional compounds. Anti-nutritional compounds contained in lamtoro have been reported by Vahedi et al. (2021) and Purba et al. (2022), it was stated that mimosine is an anti-nutritional compound found in lamtoro. In addition, the study (Niyigena et al. 2021) reported mimosin and excess blood urea nitrogen inhibition of animal growth.

Based on the results of this study, it can be concluded that the provision of forage legumes as a source of protein can regulate lipid regulation and affect the growth rate of ram lambs. This mechanism is taken as the ability of proteins in molecular signaling related to specific proteins that regulate lipid metabolism and growth. The administration of calliandra was the best legume compared to other legumes (lamtoro and moringa), indicated by the highest ram lamb growth rate compared to other groups of sheep.

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