Effect of Nutmeg Meal on Microbial Abundance of Biohydrogenation Process, and Fatty Acid Profile of Goat Rumen Fluid *In Vitro*

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Abstract. This study aimed to evaluate the effect of nutmeg meal as a source of phenol on the abundance of dominant microbes in the biohydrogenation process and the fatty acid profile of goat rumen fluid in vitro. This study used a randomized complete block design with four treatments and three replicates of in vitro batches, each replicate in duplicate. The dietary treatments consisted of elephant grass (40%), soybean meal (10%), with varying proportion of wheat pollard, canola oil and nutmeg meal hence, treatment P0 (40:10:45:5:0); P1 (40:10:40:5:5); P2 (40:10:35:5:15); P3 (40:10:30:5:10). Fermentation using the in vitro gas production method described by Menke and Steinggas with an incubation period of 48 hours. Parameters observed included microbial abundance and fatty acid profile of goat rumen fluid. The statistical analysis showed that adding nutmeg meal starting at the 5% level decreased the abundance of the main biohydrogenation process bacteria anaerovibrio, butyrivibrio fibrisolvens, and pseudobutyrivibrio. Total saturated fatty acid and stearate fatty acid concentrations decreased ($P<0.05$), while total unsaturated fatty acid, linoleic and oleic acid concentrations increased ($P<0.05$).

1 Introduction

The most popular source of animal protein in Indonesia is goat meat. Goat meat is an animal protein with lower fat and cholesterol than beef and lamb. [1]. Improving the quality of PUFA-rich goat meat can be done with feed manipulation by adding unsaturated fatty acid sources in the diets [2]. The source of unsaturated fatty acids derived from canola oil consists of 6%-14% α-linolenic acid, 50%-65% oleic acid, and <7% saturated fatty acids [3].

Feeding high unsaturated fatty acids in the rumen can undergo a biohydrogenation process. The bacteria butyrivibrio will hydrolyze the biohydrogenation process of linoleic unsaturated fatty acids in the rumen fibrisolvens into rumenic acid, and then rumenic acid will become vassenic acid [4]. Butyrivibrio proteoclasticus bacteria further hydrolyze vassenic acid into stearic acid, which is a saturated fatty acid [5]. The formation of saturated fatty acids in the rumen can be decreased by inhibiting fatty acid hydrolyzing bacteria [6].

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Utilization of plant secondary metabolite compounds is a natural method that can inhibit fatty acid hydrolyzing bacteria in the biohydrogenation process. [7]. Secondary metabolite compounds include flavonoids, tannins, and phenols [8]. Phenol compounds have antimicrobial abilities and form complex bonds with fats that can modify the rumen process of biohydrogenation and methanogenesis [9]. The mechanism of action of phenol compounds reduces the biohydrogenation process by binding to lipase enzymes and inhibiting lipolysis [10]. Phenol compounds in inhibiting the lipolysis process effectively reduce Anaerovibrio lipolytic bacteria and butyrivibrio fibrisolvens bacteria (gram-positive), which will hinder the saturation process to saturated fatty acids so that the opportunity is less for the concentration of conjugated linoleic and linolenic acid isomers to be hydrolyzed [11]

Plant secondary metabolite compounds can be found in nutmeg meal industrial waste, which is currently abundant and has not been utilized properly. Nutmeg meal is industrial waste from the essential oil distillation process, which contains 6.05% phenol secondary metabolite compounds, 0.035% flavonoids, and 0.6% tannin (pre-research). The addition of nutmeg meal as a phenol compound in animal feed has not been widely studied, and therefore further evaluation is needed to determine the effect of different levels of nutmeg cake in inhibiting fatty acid hydrolyzing bacteria and the rich acid profile produced in the rumen.

2 Materials and Methods

2.1 Research Design

The research used a randomized complete block design with a control feed and three treatments. In Vitro Fermentation was conducted three times, and each unit was replicated twice. The feed ingredients with high unsaturated fatty acid ration consisted of elephant grass, wheat pollard, soybean meal, canola oil, and nutmeg meal. The ratio composition was P0 (40:10:45:5:0); BP5 (40:10:40:5:5); BP10 (40:10:35:5:10); BP15 (40:10:30:5:15). The ratio of grass and concentrate was 40:60%. Nutmeg meal as a source of phenol was obtained from the distillation waste of the nutmeg essential oil factory in the Ungaran area. The total phenol content of nutmeg cake was 8.89 mg phenol/100mg per treatment BP5 0.44 mg phenol/100mg; BP10 0.88 mg phenol/100mg; and BP15 1.33 mg phenol/100mg. Rumen fluid was obtained from donor Kacang goats from Kentungan Slaughterhouse, Yogyakarta.

2.2 In Vitro Batch Fermentation

In vitro fermentation using the Menke and Steingass gas production method The collection of rumen fluid was carried out in the morning before the livestock is given feed, and the liquid is stored in a thermos at 39o C. 2 The rumen fluid was then filtered using four layers of gauze and mixed with buffer medium in a ratio of (1:2) in a 2000 ml Erlenmeyer flask with flushed with CO gas for 15 minutes. The mixed solution (buffer and rumen fluid) of 30 ml was transferred anaerobically into a syringe containing 300 mg of substrate (ration high in unsaturated fatty acids). Then the syringes were incubated in a water bath at 39o C for 48 hours. Observations of gas production fermentation were recorded at hours 0, 2, 4, 6, 8, 12, 24, 36 and 48. At 24 and 48, gas was taken using a syringe and put into a 10 ml vacutainer. At the end of incubation, the fermented liquid was poured into a 20 ml test tube and filtered with a glass wool crucible. Furthermore, the rumen fluid was centrifuged at 500g for 15 minutes to separate feed particles. The filtrate obtained was analyzed for pH, VFA content, microbial protein content, ammonia, and microbial abundance.

2.3 Rumen Fluid Fatty Acid Profile

Filtrate was derived from the 48 h incubation in vitro fermentation by the two-stage method [12] was taken and analyzed using gas chromatography.

2.4 DNA Isolation

15 ml of rumen fluid was centrifuged at 3000 rpm for 10 minutes; then the supernatant was taken and centrifuged again at 6000 rpm for 10 minutes. The precipitate was used as a sample to determine metagenomic diversity. DNA samples were extracted using the ZymoBIOMICS DNA mini kit protocol DNA mini kit catalog No. D4300.

2.5 Electrophoresis

The extracted DNA was confirmed by electrophoresis using 1% agarose medium dissolved in 30 ml of TBE 1x, then the solidified agarose was inserted into the tank, and 2 µL of blue juice mixed with six µL of isolated DNA was inserted into the agarose wells. Furthermore, the power supply is set at 100 volts. Running electrophoresis for 45 minutes, then the agarose gel is placed on a UV illuminator, and visualisation of DNA with UV light. Finally, if there is visible DNA, the DNA extraction sample is sent to Genetics Science.

2.6 Statistical Analysis

Fermentation parameters and fatty acid profile of rumen fluid were analyzed using analysis of variance with a complete randomized design in a unidirectional pattern; if there were significant differences, it was followed by Duncan's new multiple range test. Data analysis used IBM SPSS Statistics version 23. Microbial diversity was presented descriptively.

3 Results and discussion

The results showed that treatment BP5% had the lowest population of anaerovibrio bacteria, butyrivibrio fibrisolvens and pseudobutyrivibrio compared to treatment BP10% and BP15%. (Table 1). This indicates that phenolic compounds in BP5% were able to modulate anaerovibrio bacteria, butyrivibrio fibrisolvens and pseudobutyrivibrio which play a role in the process of lipolysis, biohydrogenation, causing bacterial abundance to decrease [5] the main bacterial species involved in the biohydrogenation process of the Butyrivibrio group belong to the genus butyrivibrio and pseudobutyrivibrio.

Table 1. Effect of different levels of nutmeg meal addition on microbial abundance (%) of goat rumen fluid in vitro.

Bacterial Abundance	Nutmeg Meal Level			
	P0	BP5%	BP10%	BP15%
Anaerovibrio	0,10	0,09	0,13	0,11
Butyrivibrio	0,34	0,19	0,32	0,32
Pseudobutyrivibrio	0,23	0,14	0,19	0,15

Adding polyphenols to the diet affects the abundance of rumen microbes related to the biohydrogenation process so that the rate of biohydrogenation in the rumen decreases [13]. The addition of different levels of phenolic sources of Paulownia leaf extract can inhibit the

biohydrogenation process by reducing the population of Ruminococcus albus and Butyrivibrio fibrisolvens bacteria to increase unsaturated fatty acids, including MUFA and PUFA [14]

The addition of nutmeg meal starting at the 5% level decreased (P<0.05) total saturated fatty acids and increased (P<0.05) total unsaturated fatty acids of rumen fluid in vitro. The addition of nutmeg meal at the 5% level was significantly different (P<0.05) from the 10% and 15% levels. Total saturated fatty acids decreased by 18.19%; 12.62%; 13.26%; total unsaturated fatty acids increased by 29.30%; 19.13%; 19.69% at 5%, 10%, and 15% BP levels shown in Table 2.

Tabel 2. Effect of adding different levels of nutmeg meal on the fatty acid profile of goat rumen fluid in vitro

Fatty Acid (% from fatty acid total)	Level addition nutmeg meal (%)			
	P0	BP5	BP10	BP15
Arkidat^{ns}	0,68 ± 0,10	0,53 ± 0,08	0,58 ± 0,10	0,60 ± 0,07
Heptadekanat^{ns}	1,30 ± 0,12	1,29 ± 0,07	1,32 ± 0,05	1,40 ± 0,24
Miristate*	3,01 ± 0,04^{a}	1,54 ± 0,34^{c}	1,49 ± 0,25^{c}	1,96 ± 0,07^{b}
Palmitate^{ns}	17,85 ± 0,59	16,71 ± 0,14	17,40 ± 0,98	17,45 ± 0,54
Pentadekanat^{ns}	0,82 ± 0,07	0,77 ± 0,06	0,78 ± 0,05	0,75 ± 0,02
Stearate	40,43 ± 1,24^{a}	32,99 ± 0,62^{c}	35,66 ± 0,75^{b}	34,87 ± 0,98^{b}
Cis-11-eicosenat^{ns}	0,68 ± 0,07	0,81 ± 0,03	0,82 ± 0,04	0,78 ± 0,13
Oleat*	13,22 ± 2,28^{b}	16,91 ± 0,32^{a}	16,10 ± 1,29^{a}	16,24 ± 0,98^{a}
Erucat^{ns}	6,37 ± 1,35	8,57 ± 0,57	7,28 ± 1,10	7,31 ± 1,52
Nervonat^{ns}	5,46 ± 0,30	5,99 ± 0,30	5,91 ± 0,56	5,58 ± 0,25
Palmitoleat*	0,57 ± 0,05^{b}	0,88 ± 0,25^{a}	0,82 ± 0,04^{ab}	0,83 ± 0,10^{ab}
Myristoleic acid methyl ester^{ns}	0,54 ± 0,29	0,80 ± 0,08	0,71 ± 0,12	0,73 ± 0,17
Cis-13,16-docosadienat^{ns}	3,23 ± 0,13	3,82 ± 0,33	3,68 ± 0,34	3,72 ± 0,43
Cis-5,8,11,14,17 eicosapentanat*	4,47 ± 0,21^{b}	5,49 ± 0,11^{a}	5,20 ± 0,47^{a}	5,41 ± 0,37^{a}
Linoleat*	1,36 ± 0,06^{c}	2,91 ± 0,06^{a}	2,25 ± 0,40^{b}	2,37 ± 0,20^{b}
SFA*	64,10 ± 0,85^{a}	53,83 ± 0,26^{c}	57,23 ± 0,74^{b}	57,03 ± 1,19^{b}
UFA*	35,90 ± 0,85^{c}	46,42 ± 0,69^{a}	42,77 ± 0,74^{b}	42,97 ± 1,19^{b}
MUFA*	26,84 ± 0,97^{c}	34,21 ± 0,90^{a}	31,63 ± 0,44^{b}	31,46 ± 0,75^{b}
PUFA*	9,06 ± 0,19^{c}	12,21 ± 0,25^{a}	11,14 ± 0,45^{b}	11,51 ± 0,48^{b}

Notes: *P<0.05; ns: non significance; P0: control without nutmeg meal; BP5: nutmeg meal 5%; BP10: nutmeg meal 10%; BP15: nutmeg meal 15%

Phenol compounds have antimicrobial properties capable of modulating rumen microbial activity and forming phenol complex bonds with lipids, thereby inhibiting the biohydrogenation process of saturated fatty acids in the rumen [9]. The mechanism of phenol compounds in reducing biohydrogenation by binding to lipase enzymes that will inhibit the process of lipolysis [10]. In line with research [15] the addition of 0-10% polyphenol source palm leaf extract can increase the concentration of C18:1 t-11 and C-9, t-11 CLA and reduce the concentration of C18:0 [16]. The addition of palm leaf phenolic sources in the ration can

reduce the rumen biohydrogenation process by increasing the concentration of unsaturated fatty acids, namely omega-3 fatty acids including C18: 3n-3 which results in healthier goat meat products.

Adding nutmeg meal at 5%, 10%, and 15% levels increased (P>0.05) oleic and linoleic acids compared to the control. The increase in oleic and linoleic acid concentrations of BP5% was higher than BP10% and BP15%. Phenol compounds can inhibit the isomerization and reduction phases of the biohydrogenation process, thereby increasing the composition of linolenic, linoleic, and oleic fatty acid bypasses [17]. Phenol compounds can also increase the synthesis of bioactive conjugated fatty acid isomers such as cis-9, trans-11 C18:2, and trans-11 C18:1 and reduce C18:0 [18].

Adding a nutmeg meal at 5%, 10%, and 15% levels decreased stearic acid by 19.94%, 13.21%, 15.46% compared to the control. The decrease in stearic acid concentration was highest in BP5% compared to BP10% and BP15%. The reduction of stearic acid is thought to be due to phenol compounds that can reduce the abundance of Butrurivibrio and Pseudobutyrivibrio bacteria, as shown in Table 2. [5] Butyrivibrio fibrisolvens and pseudobutyrivibrio bacteria play an essential role in biohydrogenation by hydrolyzing linoleate fatty acids into c9-t11 CLA (rumenic acid), which then becomes c18:1 t11 (vaccenic acid). Then there are butyrivibrio proteoclasticus bacteria hydrogenating vaccenic acid to 18:0 (stearic acid). A decrease in the abundance of Anaerovibrio bacteria can inhibit the lipolysis process so that lipolytic activity decreases, causing the free fatty acids produced for the biohydrogenation process to reduce. Adding Olea europaea L. fruit pomace kayak polyphenols increased the C18:2 cis-9 cis-12 and C18:3 cis-9 cis-12 cis-15 rumen fluid compared to the control [19].

4 Conclusion

The addition of nutmeg meal at a level of 5% inhibited bacteria that hydrolyze the biohydrogenation process with the abundance of anaerovibrio, butyrivibrio fibrisolvens and pseudobutyrivibrio at the lowest so that the concentration of saturated fatty acids stearate according to and unsaturated fatty acids oleic and linoleic increased.

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