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Research Article
Effect of Non-Fiber Carbohydrate Sources with Formaldehyde Protected Soybean on *in vitro* Fermentability and Digestibility

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Abstract
Background and Objective: An optimal ratio of rumen degradable protein (RDP) and rumen undegradable protein (RUP), synchronized with energy availability and sulfur supplementation in a dairy ration, has been shown to enhance cow productivity. The research studied the influence of formaldehyde-protected soybean, corn (CO) and cassava meal (CM) non-fibre carbohydrate (NFC) and Na_2SO_4 supplementation on the fermentability and digestibility of dairy cattle rations. **Materials and Methods:** The experimental rations consisted of two types of NFC sources (CO and CM), each combined with soybean (SS), formaldehyde-protected soybean (PS) and formaldehyde-protected soybean with sulfur (PSS). Ration without soybean (WS) was used as a control. All types of rations have been tested for their fermentability, including pH, ammonia, total Volatile Fatty Acids (VFA), protozoa and rumen microbe analysis. Additionally, dry matter and organic matter digestibility (DMD and OMD) were assessed. The treatments were replicated four times. **Results:** The inclusion of NFC, protected soybean and sulfur supplementation in the ration did not have a significant effect ($p>0.05$) on pH, ammonia concentration, rumen bacteria, protozoa population and digestibility. However, the total VFA increased significantly in the CM rations combined with soybean compared to WS and CO rations. **Conclusion:** Adding cassava NFC, formaldehyde-protected soybean and Na_2SO_4 supplementation improved total VFA production while maintaining other fermentability and digestibility.

Key words: Cassava meal, corn, non-fiber carbohydrate, protected protein, sulfur

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Milk production is one of the factors affecting dairy cows' nutritional requirements. The preparation of dairy rations can be adjusted to milk production so the nutrients provided can be utilized efficiently[1]. The availability of feed nutrients and rumen microbial products has a significant impact on the productivity of ruminants. The optimum balance of rumen degradable protein (RDP) to rumen undegradable protein (RUP) ratio can enhance protein utilization, feed efficiency and reduce feed cost. The RDP:RUP balance recommended by the NRC dairy requirement is 60:40[2].

Soybean, a ruminant protein source, has high protein content but is highly degraded in the rumen. Its high crude protein content and easily degradable property in the rumen results in high ammonia concentration, leading to low protein efficiency. Thus, soybean protein needs protection to reduce degradability in the rumen but increase post-ruminant digestibility. One such method for protecting protein is formaldehyde treatment. Suhartanto *et al.*[3] stated that using formaldehyde at a 0.5-1% level could reduce dry and organic matter degradation.

Protected protein using formaldehyde treatment was cheaper and better than heat treatment. Formaldehyde did not induce a browning reaction, which can otherwise diminish the availability of essential amino acids such as lysine, histidine and methionine in the dietary amino acid intake. Additionally, heat treatment was observed to reduce the ruminal digestibility of arginine, cystine, lysine, phenylalanine and valine[4]. The principle of protein protection by formaldehyde is to form stable chemical bonds with proteins at ruminal pH levels (6-7) but unstable at low abomasum pH levels, thus breaking the formaldehyde bonds and allowing the protein to be digested in the small intestine[5]. The RUP supplementation increased the flow of non-ammonia and non-microbial nitrogen to the small intestine and, resulting in a 10% improvement in milk production[6].

In addition to ensuring protein adequacy, the RDP:RUP ratio and energy adequacy must also be considered to increase the efficiency of microbial protein synthesis[7]. One critical energy source for ruminants is carbohydrates, which comprise neutral detergent fiber (NDF) and non-fiber carbohydrates (NFC). Lu *et al.*[8] reported that feeds rich in NFC could enhance the utilization of ammonia nitrogen and microbial protein synthesis. However, the formulation of rations based on the RDP:RUP ratio and NFC requirements has not been implemented under smallholder tropical dairy farming. Rosmalia *et al.*[9] found that a 35% NFC level and RDP:RUP ratio in 60:40 resulted in high microbial protein synthesis.

Sulfur is an essential mineral for rumen microbes in synthesizing sulfur-containing amino acids, including cysteine, cystine, cystathionine, cysteic acid, homocysteine, taurine and methionine. The NRC[2] states that sulfur minerals require 0.14% to 0.26% of dry matter. According to Rosmalia *et al.*[10], RDP:RUP ratio of 60:40 with 0.2% sulfur supplementation was ideal for increasing ammonia concentration, total protozoa and bacteria population, as well as dry matter and organic matter digestibility. Maximizing nutrient efficiency through balancing RDP, NFC and sulfur supplementation has yet to be studied under smallholder tropical dairy farming. Therefore, this study aims to identify the optimum combination of soybean protein protection, NFC utilization and sulfur supplementation in dairy rations to improve ruminal fermentation and *in vitro* digestibility.

MATERIALS AND METHODS

Study area: This study was conducted at the Dairy Nutrition Laboratory, Department of Animal Nutrition and Feed Technology, Faculty of Animal Science, IPB University, Indonesia, from March to June, 2022.

Experimental ration: The experimental rations were composed of forage, concentrates and specific mineral supplements. The rations were formulated to maintain an RDP to RUP ratio of 60:40, following the recommendations provided by the National Research Council[2]. The primary sources of NFC in these rations were cassava meal and corn. Table 1 and 2 show the composition and nutrient content of these rations.

Soybean-protein protection: Soybean protein was protected using formaldehyde. The soybean was soaked in a 1% formaldehyde solution of 1:1 (w/v) for 1 hr. Then, the soybeans were dried under the sun for 48 hrs. The dried beans were ground using a laboratory grinder (Ossel E-250G-V2, China) and sieved with a 1 mm screen.

***In vitro* study:** Fermentability and digestibility were determined using two-stage Tilley and Terrys[11] methods. The experimental ration (0.5 g) was incubated with 10 mL rumen liquor and 40 mL McDougall's buffer solution to measure fermentability for 4 hrs. For digestibility measurement, the samples were incubated under ruminal for

Table 1: Feed ingredient and percentage composition of experimental ration

	CO				CM			
Feed	WS	SS	PS	PSS	WS	SS	PS	PSS
Elephant grass	50.00	50.00	50.00	50.00	50.00	50.00	50.00	50.00
Corn	7.50	7.50	7.50	7.50	0.00	0.00	0.00	0.00
Cassava meal	0.00	0.00	0.00	0.00	8.50	8.50	8.50	8.50
Rice bran	3.50	5.13	5.13	4.50	3.25	3.33	3.33	3.33
Wheat pollard	10.30	6.35	6.35	6.23	6.09	3.73	3.73	3.40
Molasses	8.65	7.50	7.50	7.50	4.00	4.00	4.00	4.00
Copra meal	4.00	7.74	7.74	7.50	9.15	9.15	9.15	9.15
Palm kernel meal	4.00	7.74	7.74	7.50	4.84	8.35	8.35	8.35
Soybean	0.00	5.00	0.00	0.00	0.00	5.00	0.00	0.00
Protected soybean	0.00	0.00	5.00	5.00	0.00	0.00	5.00	5.00
Corn gluten meal	2.48	1.75	1.75	1.75	2.36	2.23	2.23	2.20
Corn gluten feed	4.83	2.18	2.18	2.50	5.05	3.03	3.03	3.03
DDGS	3.65	1.27	1.27	1.50	5.58	1.60	1.60	1.53
DCP	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
$CaCO_3$	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Na_2SO_4	0.00	0.00	0.00	0.043	0.00	0.00	0.00	0.43

CO: NFC corn, CM: NFC cassava meal, WS: Without soybean, SS: Soybean, PS: Formaldehyde-protected soybean, PSS: Formaldehyde-protected soybean with sulfur, DDGS: Dry distilled grain with soluble and DCP: Dicalcium phosphate

Table 2: Nutrient content of the experimental ration (DM%)

	CO				CM			
Item	WS	SS	PS	PSS	WS	SS	PS	PSS
DM	91.57	91.04	91.17	92.06	91.57	90.05	90.28	90.43
Ash	11.79	11.60	12.03	11.91	11.68	11.68	11.61	11.92
Ether extract	3.35	3.42	3.93	3.41	3.50	3.61	4.20	4.04
Crude protein	13.38	12.62	13.45	15.73	12.82	13.22	12.63	13.29
RDP (DM%)	8.26	7.84	8.36	9.77	7.95	8.20	7.83	8.24
RUP (DM%)	5.12	4.78	5.09	5.96	6.16	6.35	6.06	6.38
Crude fiber	22.09	21.09	20.23	20.57	18.52	17.81	18.69	16.41
NFE	49.39	51.27	50.36	48.38	53.47	53.68	52.88	54.33
TDN	57.76	58.81	60.19	60.25	61.74	62.77	62.19	64.54

DM: Dry matter, RDP: Rumen degradable protein, RUP: Rumen undegradable protein, NFE: Nitrogen-free extract, TDN: Total digestible nutrient, CO: NFC corn, CM: NFC cassava meal, WS: Without soybean, SS: Soybean, PS: Formaldehyde-protected soybean and PSS: Formaldehyde-protected soybean with sulfur

48 hrs anaerobically and followed by 48 hrs of enzymatic incubation aerobically. After 4 hrs, the fermentability samples of incubation were terminated. The fermentation tube was centrifuged at 3500 rpm for 15 min. The supernatants were collected for the analysis of several parameters, including pH, ammonia concentration, total VFA, bacteria and protozoa population. The rumen pH was measured using a pocket pH Hanna HI198190 and the conway microdiffusion method was used to analyze the Ammonia (NH_3) concentration from the supernatant. Total Volatile Fatty Acids (VFA) were measured from the supernatant using a steam distillation method while calculating total bacteria and protozoa following Ogimoto and Imai's method[12]. In the first step in measuring digestibility, the experimental rations were anaerobically incubated with rumen fluid for 48 hrs to mimic the fermentation process. In the second step, centrifuged precipitates were added to the HCl-pepsin enzyme solution and incubated for 48 hrs to mimic the enzymatic digestion process. Then, the feed residues were collected using Whatman No. 41 filter paper and a vacuum pump. The *in vitro* digestibility can be calculated by subtracting the dry and organic matter (DMD and OMD) from the samples[13].

Statistical analysis: The data collected were analyzed using One-way Analysis of Variance (ANOVA). If there were significant differences in results ($p<0.05$), the analysis was continued with the Duncan's multi-range test (SPSS 25th version, IBM SPSS Statistics, USA).

RESULTS

The *in vitro* fermentation characteristics were shown in Table 3. The Table 3 shows that the addition of NFC (CO and CM), sulfur, protected and nonprotected soybean does not

Table 3: Effect of experimental ration on *in vitro* fermentation characteristics

	CO				CM					p-value		
Item	WS	SS	PS	PSS	WS	SS	PS	PSS	SEM	N	P	N×P
Rumen pH	6.59	6.60	6.68	6.59	6.54	6.54	6.57	6.60	0.029	0.016	0.194	0.329
NH_3 (mM)	4.25	5.45	4.23	4.28	4.52	5.28	5.01	5.07	0.141	0.101	0.052	0.461
Total VFA (mM)	108.70^{ab}	105.86^{ab}	96.19^{b}	99.49^{b}	108.91^{ab}	117.17^{a}	113.80^{a}	112.52^{a}	1.557	0.000	0.107	0.030
Protozoa (log cell mL^{-1})	6.38	6.44	6.50	6.53	6.36	6.56	6.40	6.28	0.036	0.369	0.559	0.301
Bacteria (log CFU mL^{-1})	11.36	11.41	11.19	11.19	11.38	11.80	11.41	10.93	0.077	0.576	0.147	0.520

^{abc}Means in the same row with different superscripts are significantly different ($p<0.05$), SEM: Standard error of mean, N: NFC sources, P: Unprotected and formaldehyde-protected soybean, N×P: Interaction between NFC and protein sources, CO: NFC corn, CM: NFC cassava meal, WS: Without soybean, SS: Soybean, PS: Formaldehyde-protected soybean and PSS: Formaldehyde-protected soybean with sulfur

Table 4: Effect of experimental ration on *in vitro* dry matter and organic matter digestibility

	CO				CM					p-value		
Item	WS	SS	PS	PSS	WS	SS	PS	PSS	SEM	N	P	N×P
DMD (%)	60.88	63.47	61.34	63.00	63.17	62.83	61.02	62.27	0.496	0.846	0.338	0.479
OMD (%)	64.01	66.20	64.27	65.76	65.93	65.38	63.87	64.76	0.509	0.919	0.427	0.484

SEM: Standard error of mean, N: NFC sources, P: Unprotected and formaldehyde-protected soybean, N×P: Interaction between NFC and protein sources, CO: NFC corn, CM: NFC cassava meal, WS: Without soybean, SS: Soybean, PS: Formaldehyde-protected soybean, PSS: Formaldehyde-protected soybean with sulfur, DMD: Dry matter digestibility and OMD: Organic matter digestibility

affect the pH value, Ammonia (NH_3) concentration, protozoa and bacteria population but affect the VFA concentration. The concentration of VFA produced in CM treatment is higher than the concentration of VFA produced in CO treatment. The results showed that the concentration of VFA is influenced by the interaction of NFC factors with both protected and nonprotected treatment groups. The effects of adding NFC, protected soybean and sulfur supplementation in rations on *in vitro* digestibility characteristics were shown in Table 4. The results showed that the treatments did not significantly affect the percentage of DMD and OMD.

DISCUSSION

The pH value plays an important role in microbial activity and rumen stability since it is one of the main factors in determining the appropriate environment for microbial growth and activity in the rumen[10]. The level of acidity (pH) of the rumen fluid describes the environmental conditions in the rumen and is a factor that determines the fermentation process. The pH value of rumen fluid is strongly influenced by the type, quantity and quality of feed consumed, the balance of macro and micronutrients, ecosystems and microbial populations of the rumen and the buffering capacity of livestock rumen[14]. The pH value in this study (6.54 to 6.68) shows that the level of acidity is within the normal range and is still under environmental conditions for microorganism growth and activity. Matthews *et al.*[15] also confirmed that the optimum rumen pH conditions range from 6-7. Appropriate pH conditions indicate that the rumen's growth process and microbial metabolism are not disturbed, so the digestive process is optimal[16].

The concentration of NH_3 in the rumen can be influenced by several factors, including the type of feed, protein solubility, protein degradation rate and protein levels in the ration[17]. The concentration of NH_3 in this study is not affected by NFC content, formaldehyde-protected soybean and sulfur supplementation[10,18]. While the previous study reported that protein protection in formaldehyde reduced protein degradation in the rumen thus reducing the ammonia value[19]. The concentration of NH_3 obtained in this study ranged from 4.23 to 5.45 mM, indicating that the concentration of NH_3 is still within the normal range, the protein can be degraded and ammonia is available for microbial growth. According to McDonald *et al.*[20], optimal NH_3 concentrations ranged from 4.9 to 17.6 mM. According to Davies *et al.*[18], the concentration of NH_3 in the rumen indicates the reshuffling of proteins entering the rumen and the process of protein synthesis by rumen microbes. Rumen microbes will reuse Ammonia (NH_3) for growth. Hence, the growth and increase of rumen microbes depend on the availability of NH_3 in the rumen, with a minimum ammonia concentration of 3.74 mM being required for microbial protein production.

Volatile Fatty Acids (VFA) result from the fermentation of organic matter in carbohydrates. The VFA is the primary energy source of ruminant livestock as well as the source of microbial protein formation carbon skeleton. Carbohydrates as an energy source in ruminants consist of fiber components, namely NDF (neutral detergent fiber) and NFC (non-fiber carbohydrate)[21]. The NFC consists of easily fermented carbohydrates, including starch and simple sugars. Kondo *et al.*[22] reported that NFC fraction is an important element in supporting the process of Adenosine Triphosphate (ATP) formation in the rumen for microbial protein formation. As the process progresses, ATP can be formed from structural carbohydrates or fiber fractions. However, it takes longer than NFC to degrade. The NFC can accelerate fermentation in the rumen and slow down the net degradation process in the rumen[23]. The concentration of VFA produced in NFC cassava treatment is higher than the concentration of VFA produced in NFC corn treatment. The NFC content in cassava is higher (84.62%) in comparison to corn (74.86%), resulting in higher concentrations of VFA[9]. The high NFC content indicates that cassava is easily degraded in the rumen. The concentration of VFA in this study ranged from 96.19 to 117.17 mM, indicating that the concentration of VFA is still within the normal range and NFC is easily fermented so that the energy produced is sufficient for microbial growth. Dieho *et al.*[24] reported that the concentration of total VFA with forage-based feed and concentrate ranged from 77 to 120 mM.

Rumen bacteria produce extracellular enzymes (amylase, cellulose, protease, lipase and phytase) that degrade the nutrients of feed in the rumen[25]. The mean value of total bacteria obtained in this study was relatively high, reaching 10.88-11.80 log CFU mL^{-1}. This value was still in normal conditions because the number of rumen bacteria could reach 10^{10}-10^{11} CFU mL^{-1} [26]. Each treatment contained relatively the same energy and protein. Thus, the total bacterial population in each ration treatment is not much different. Factors affecting rumen microbes' growth and activity are rumen temperature, rumen pH, availability of energy, protein and dry matter content[27]. While, Wu *et al.*[28] stated that high utilization of corn and Distillers Dried Grains with Solubles (DDGS) was associated with reducing ruminal bacteria.

Protozoa play a role in the rumen fermentation, particularly in the digestion of starch, which helps maintain a balanced rumen pH level. Besides, it can slow down the conversion of fermentable carbohydrates into lactic acid by rumen bacteria so that the pH can be controlled. Protozoa observations in this study ranged from 6.25 to 6.56 log CFU mL^{-1}, which indicates that the protozoa population was in normal condition[20]. In contrast, previous study reported that formaldehyde leads to cross-link formation with protein, making it difficult for protozoa to digest resulting in lower protozoa count[29].

Dry matter digestibility (DMD) is the ability of livestock to digest dry parts in the form of proteins, carbohydrates, fats and minerals in feed at a certain level of feed consumption. Organic matter digestibility (OMD) describes the ability of livestock to digest organic matter other than minerals in feed. Ruminants have microbes in the rumen with different levels of ability to degrade feed, resulting in differences in digestibility. The value of DMD produced in this study ranged from 58.50 to 63.16%, while the OMD ranged from 61.57 to 65.94%. This study's low crude protein content caused the value of DMD and OMD to be lower than reported by Lestari *et al.*[30], where DMD ranged from 66.46 to 70.71% and OMD ranged from 69.13 to 75.45%.

Sahroni *et al.*[12] reported that the RDP:RUP ratio with the same TDN level did not affect the DMD and OMD. Sulfur is essential for the synthesis of sulfur-containing amino acids (methionine, cystine and cysteine) for rumen microbial activity. Supapong *et al.*[31] reported that sulfur could be the primary limiting mineral for rumen fermentation efficiency and its main effect is to decrease the availability of microbial protein. However, this study is different from Rosmalia *et al.*[10], which reported that increasing the level of sulfur mineral supplementation leads to an increase in DMD and OMD values. This difference might be due to the high fiber content in the rations. Crude fiber negatively relates to digestibility value. Kumar *et al.*[32] also reported that supplementing crossbred cows using formaldehyde-treated mustard cake at level 1.2% did not affect the apparent digestibility (dry matter, organic matter, crude protein, ether extract, NDF, ADF and hemicellulose) but could improve milk production.

CONCLUSION

The cassava NFC, soybean, formaldehyde-protected soybean and Na_2SO_4 supplementation improve total VFA production while maintaining other fermentability and digestibility. For practicability, it was suggested to use cassava meal and soybean supplementation only for the best result obtained by all treatments.

SIGNIFICANCE STATEMENT

The novelty of this research is the use of formaldehyde protection method for soybeans as a protein source for ruminants, combined with cassava as a source of non-fiber carbohydrates (NFC) and the addition of sulfur to maintain fermentability and digestibility. The benefit of the research is to help farmers to find the best combination of energy and protein feed ingredients to increase dairy livestock productivity.

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