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In Sacco and *in vitro* Evaluation of Heating and Formaldehyde Treated Protein Feed

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Abstract: Massive protein conversion into ammonia in the Rumen (RDP) should be avoided to prevent protein quality deterioration. Heating and formaldehyde treatments are commonly used to decrease Rumen-Degradable Protein (RDP), but it rarely used to protect local feed. We aimed to compare the effectiveness of heating and formaldehyde treatments in decreasing the RDP of local feed. The use of fistulated cattle, treatments, and procedures used in this study have been ethically cleared by the animal ethics committee, at IPB University. This study used a 5×3 factorial randomized block design with three replications with a sample size was 45. We used two factors; the 1st was high RDP feeds (TD = Tempe Dregs, BW = Brewery Waste, NSM = *Nigella sativa* Meal, SS = Soybean Seeds, CGF = Corn Gluten Feed) and the 2nd was treatment methods (unprotected, heating and formaldehyde). Ruminal degradation was determined using *in sacco* method. Three cannulated Frisian Holstein Bulls were fed twice daily with Elephant grass and concentrate at a ratio of 60:40 (DM basis). *In vitro* digestibility was evaluated using two two-stage Tilley and Terry *in vitro* methods. Parameters observed include nutrient content, *in sacco* degradation and ruminal degradation kinetics of dry matter, organic matter, crude protein (DM, OM, and CP), and *in vitro* digestibility. The results showed that protecting protein changed the nutrient content of feeds (1-6%). The DM and OM disappearance had the same pattern. The effective degradation of DM, OM, and CP significantly ($p<0.05$) decreased up to 17 and 27% in heating and formaldehyde treatments, respectively, compared to unprotected. Heating did not significantly affect the *in vitro* digestibility. Formaldehyde treatment had the lowest DM, OM, and CP *in vitro* digestibility (51.39, 74.25, and 45.90%). Heating treatment was effective in reducing the RDP of feed without interfering with feed digestibility.

Keywords: Formaldehyde, Heating, *in vitro*, Rumen Degradable Protein

Introduction

The protein requirement in dairy livestock considers the crude protein requirement and pays attention to Rumen-Degradable Protein (RDP) content. The RDP content in feed ingredients varied depending on the type of feed, lignin content, maturity level at harvest time, and feed processing (Elizalde *et al*., 1999; Rahmat *et al*., 2021). RDP consists of Non-Protein Nitrogen (NPN) and true protein. NPN consists of Deoxyribonucleic Acid (DNA), Ribonucleic Acid (RNA), Ammonia (NH_3), peptides, and Amino Acids (AA). True proteins are degraded by enzymes of microbial origin in the rumen into peptides and AA and then deaminated into NH_3 or incorporated into microbial proteins. The excess RDP will be degraded to NH_3, absorbed and metabolized into urea in the liver, and then reused (recycle urea) or excreted through urine (Kim and Lee, 2021). Urea in urine is quickly hydrolyzed to NH_3, producing greenhouse gas emissions that adversely affect the environment (Chadwick *et al*., 2018). The protein requirement in livestock is not only supplied by Microbial Protein Synthesis (MPS) but also by RUP. The optimal RDP to rumen undegradable protein (RUP) ratio in dairy cattle rations was 60:40 (NRC, 2001). The selection of feed ingredients in the formulation of dairy rations must be

precise with the recommended RDP: RUP ratio to optimize nitrogen efficiency.

A previous study showed that some protein feeds such as Soybean Seeds (SS), Corn Gluten Feed (CGF), Tempe Dregs (TD), Brewery Waste (BW) and *Nigella sativa* Meal (NSM) have a high protein degradation rate (>60%) (Rosmalia *et al.*, 2021; Belanche *et al.*, 2013). High RDP feeds need to be protected to be utilized optimally. However, excessive protection contributes to insufficient digestion and excretion through feces. Some standard protection methods include heating, chemical processes, and combinations. Heating changes the chemical composition, the ratio of α-helix protein structure to β-sheet and protein subfraction and decreases protein degradation within the rumen through protein denaturation, Maillard reactions, and cross-linking bonds (NRC, 2001; Doiron *et al*., 2009). The temperature and heating duration must be appropriate to protect proteins and there is no overheating that can cause an increase in the fraction of Acid-Detergent Crude Protein (ADICP) as an indication of protein damage (Vaga *et al*., 2017). Heating at 120°C for 40 and 60 min with an autoclave increased the ratio of α-helix protein structure to β-sheet (Doiron *et al*., 2009).

The method of protection using chemical agents is divided into three categories; chemicals by form cross-linking bonds (aldehydes); chemicals that change the structure of proteins by denaturation (acids, alkalis, and ethanol); and chemicals that bounds proteins without changing their structure (tannins) (Broderick *et al*., 1991). Formaldehyde binds to proteins and prevents proteolysis under the ruminal pH, but the bonds will be released when acidic conditions are in the abomasum (Kumar *et al*., 2015a). The use of formaldehyde at 1% could protect proteins from rumen degradation without affecting the microbial activity of the rumen (Suhartanto *et al*., 2014).

Considering the importance of protein and their RDP fraction in ruminant nutrition, therefore finding an effective method to adjust the RDP fraction through the protection of highly degraded feed protein is necessary. Most of study have been conducted using temperate feedstuff which has protein characteristics quite different from tropical feedstuff. Based on our current understanding, there exists a paucity of information about effective methods for protecting tropical protein feedstuff. Thus, we aimed to compare the effectiveness of heating and formaldehyde treatments in protecting proteins from rumen degradation of local feed using *in sacco* and *in vitro* studies.

Materials and Methods

Animal and Experimental Design

All animal procedures were performed according to the animal ethics committee (No. 047/KEH/SKE/XI/2021), IPB University. This study was conducted in the field laboratory of, the faculty of animal science, IPB University, Bogor, West Java, Indonesia. Three rumen-cannulated Frisian Holstein Bulls (368.5 kg) were used in this study. The bulls were housed in a stall barn with ad libitum water. The feed was given twice daily at 7:00 am and 2:00 pm. The ratio used in this study was a mix of elephant grass and concentrate with a ratio of 60:40 DM, similar to the feeding pattern described by Despal *et al*. (2022). The experimental design used a 3×5 factorial randomized block design and three replications. Factor 1 was the protection technique of the protein (unprotected, heating, and formaldehyde), while factor 2 was high RDP feeds (tempe dregs; TD, brewery waste; BW, *Nigella sativa* meal; NSM, soybean seeds; SS and corn gluten feed; CGF).

Samples, Heating, and Formaldehyde Treatment

The high RDP local feeds consisting of TD, BW, NSM, SS, and CGF (Rosmalia *et al.*, 2021; Belanche *et al.*, 2013) were carried out using the heating and formaldehyde method as described by Rosmalia *et al*. (2023). The heating process consisted of 30 min preconditioning autoclave (GEA LS-50 LJ, China) followed by heating at 120°C for 60 min. After heating, the samples were left at room temperature (25°) for 48 h. One liter of formaldehyde solution (1% v/v) was used to soak 1 kg of each feed for one hour, then sun-dried for two days. All feeds were ground to pass a 2 mm sieve for *in sacco* procedures and a 0.5 mm sieve for *in vitro* procedures.

In Sacco Degradability Procedures

The *in-sacco* degradability procedures were conducted to determine the degradability of dry matter, organic matter, and protein (DM, OM, and CP), following the method by Rosmalia *et al*. (2021). Before analysis, nylon bags (Ankom, 5×10 cm with 50 µ pore size) were labeled, oven-dried at 60°C for one hour, and then weighed. 5 g of each feed was inserted into nylon bags in triplicates and incubated in the rumen. Incubation was performed at 0, 3, 6, 9, 12, 24, and 48 h and followed the all-in method. The 0 h bags were not incubated in the rumen but only rinsed under tap water. The bags were washed with tap water, dried at 60°C for two days, and weighed. The residues were analyzed for DM, OM, and CP (AOAC, 2005).

In vitro Digestibility Procedures

In vitro, two stages by Tilley and Terry (1963) were performed to evaluate DM, OM, and CP digestibility. The rumen fluid of the three rumen-cannulated Frisian Holstein Bulls was taken and filtered through double layers of cheesecloth and put into a pre-conditioned heating thermos. The feed samples (0.5 g) were placed in a 100 mL fermentor tube. The McDougall buffer solution of 40 and 10 mL of rumen fluid was added into the fermenter tube. The Mcdougall buffer solution was made by mixing 9.8 g of $NaHCO_3$; 4.65 g of $Na_2HPO_4.2H_2O$; 0.57 g of KCl; 0.47 g

of NaCl; 0.12 g of $MgSO_4.7H_2O$; 0.04 g of $CaCl_2$ for each L of aquadest. The tube was bubbled with CO_2 and capped with a rubber stopper. Next, the tube was incubated for 48 h in a water-shaker bath (Memmert SV-1422, Germany) at 39°C. After 48 h incubation, microbial activity was halted by adding mercury chloride ($HgCl_2$). The samples were centrifuged at 3500 rpm/15 min. The residues were mixed with 50 mL of pepsin-HCl solution and incubated at 39°C/48 h. Then, the samples were filtered through a preweighed Whatman no.41 filter paper (diameter 90 mm, CAT No. 1441-090, Cytiva, China) linked to a vacuum pump. Collected residues were oven-dried at 105°C/48 h to determine DM, then incinerated at 550°C/4 h to determine OM. The CP of residue was analyzed using the Kjehdahl method (AOAC, 2005). The difference between the initial sample and the residue was calculated to get the DM, OM, and CP digestibility.

Calculation and Statistical Analysis

The kinetics degradation of DM, OM, and CP were estimated using a nonlinear equation proposed by Ørskov and McDonald (1979). Data obtained from *in sacco* and *in vitro* were analyzed using analysis of variance (ANOVA) by SPSS software (version 22; IBM SPSS Statistics Inc., USA). The difference between the treatments was considered significant when $p<0.05$ then further tested using Duncan's multiple range test.

Results and Discussion

Nutrient Contents

Nutrient content which includes DM, ash, CP, Ether Extract (EE), Crude Fiber (CF), Nitrogen Free-Extract (NFE), and Total Digestible Nutrient (TDN) in high RDP-protected feeds are presented in Table 1. High RDP-protected feeds include TD, BW, NSM, SS, and CGF. The DM content of feed TD and BW was low compared to the others. Both TD and BW are food processing by-products which had high water content. The high moisture content has an impact on the low DM content of the feed.

Table 1: Nutrient contents of high RDP feeds

		Treatments				
Parameters (%)	Feeds	Unprotected	Heating	Formaldehyde	Means	SEM
DM	TD	11.06	10.91	10.95	10.97	9.510
	BW	25.30	25.14	25.23	25.22	
	NSM	90.57	90.60	88.79	89.99	
	SS	89.21	90.99	90.47	90.22	
	CGF	92.80	88.27	89.38	90.15	
	Means	61.79	61.18	60.96		
Ash	TD	2.30	2.22	2.29	2.27	0.439
	BW	4.37	4.38	4.51	4.42	
	NSM	6.54	6.88	6.62	6.68	
	SS	6.50	6.65	6.34	6.50	
	CGF	6.06	5.86	5.46	5.79	
	Means	5.15	5.20	5.05		
CP	TD	12.35	12.81	11.39	12.18	2.832
	BW	23.88	24.67	24.76	24.44	
	NSM	38.95	39.45	40.33	39.58	
	SS	38.85	37.90	39.33	38.70	
	CGF	21.19	20.40	20.63	20.74	
	Means	27.05	27.05	27.29		
EE	TD	2.15	1.66	1.28	1.70	1.604
	BW	6.59	7.40	7.33	7.10	
	NSM	4.28	2.86	2.73	3.29	
	SS	18.91	16.64	18.11	17.89	
	CGF	2.51	2.64	2.65	2.60	
	Means	6.89	6.24	6.42		
CF	TD	32.68	33.96	33.87	33.50	2.662
	BW	20.18	18.67	18.19	19.01	
	NSM	4.39	4.45	4.57	4.47	
	SS	11.23	12.34	11.52	11.70	
	CGF	9.91	10.26	12.22	10.80	
	Means	15.68	15.94	16.07		
NFE	TD	50.52	49.36	51.17	50.35	3.045
	BW	44.98	44.89	45.21	45.03	
	NSM	45.83	46.36	45.76	45.98	
	SS	24.51	26.46	24.69	25.22	

Table 1: Continue

	CGF	60.32	60.83	59.04	60.06	
	Means	45.23	45.58	45.17		
TDN[1]	TD	55.74	54.27	53.76	54.59	3.206
	BW	73.07	75.28	75.53	74.63	
	NSM	85.00	83.20	82.94	83.71	
	SS	90.95	87.50	89.84	89.43	
	CGF	80.56	80.56	78.47	79.86	
	Means	77.06	76.16	76.11		

TDN[1] was calculated using the equation described by Indah *et al*. (2020); DM = Dry Matter; CP = Crude Protein; EE = Ether Extract; CF = Crude Fiber; NFE = Nitrogen Free-Extract; TDN = Total Digestible Nutrient; TD = Tempe Dregs; BW = Brewery Waste; NSM = *Nigella sativa* Meal; SS = Soybean Seeds; CGF = Corn Gluten Feed

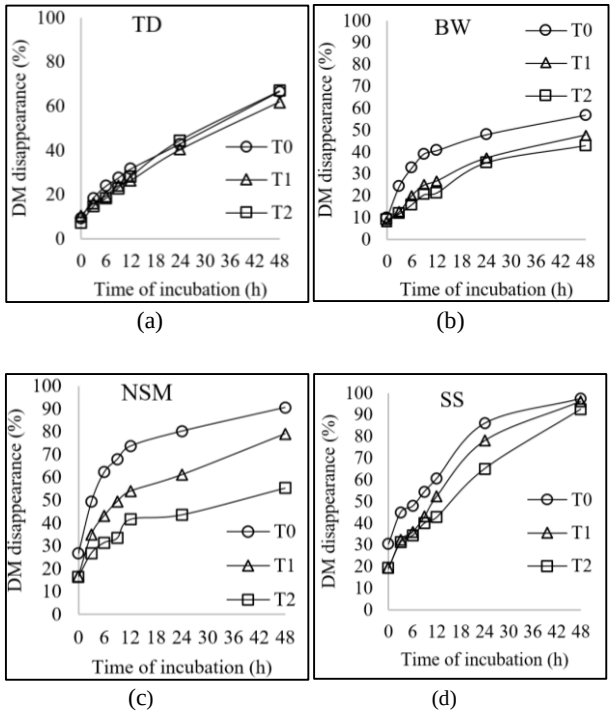
TD has lower CP but higher CF due to the predominance of the soybean epidermis component in the pulp of TD, which has a CF of 35.67% (Auza *et al*., 2017). The crude fat in SS was higher, while the NFE content was lower than in other feeds. SS generally have a moisture content of 9%, 30-40% protein, 30% carbohydrates, and 20% fat, whose value varies depending on location and climate (Cabanos *et al*., 2021). In general, there was a change in nutrient contents with the treatment of protein protection, both with heating and formaldehyde protection. However, changes in each nutrient component only range from 1-6%, except for the crude fat content for TD and NSM. There was a decrease in fat content (7-9%) due to protection treatment. The fat oxidation process caused a high decrease in fat content in TD and NSM due to the protein protection method (Prabakaran *et al*., 2018). Heating changes the structure of the cell wall and releases fats that are more sensitive to oxidation and volatile so fat content decreases (Troegeler-Meynadier *et al*., 2014).

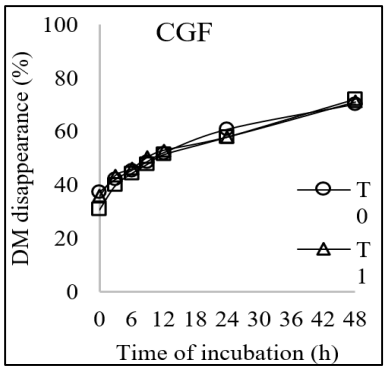
Ruminal in Sacco Disappearance and Kinetics Degradation

Figure 1 illustrates the percentage of DM loss for high RDP feeds at different incubation times. Each feed exhibits a distinct rate of DM degradation. TD and CGF demonstrated similar levels of DM degradation before and after treatments. There was a decrease in DM degradation observed for BW, NSM, and SS after treatments. The formaldehyde treatment resulted in a significant ($p<0.05$) reduction in DM degradation for NSM compared to the heat treatment.

Table 2 displays the kinetics of DM in high RDP-protected feeds. A significant ($p<0.05$) interaction was observed between the type of feed and the protection method regarding the *a*-value. TD and BW exhibited lower values for each protection method. The highest *a*-value was found in heating-treated CGF. The *a*-value significantly ($p<0.05$) differs for each feed, with CGF having the highest mean value among SS, NSM, TD, and BW. The formaldehyde treatment resulted in a significantly ($p<0.05$) decreased *a*-value, followed by the heat treatment and unprotected protein. Formaldehyde treatment has been shown to reduce the soluble fraction (*a*) and degradation rate (*c*) of DM in rice bran, according to Martín-Tereso *et al*. (2009).

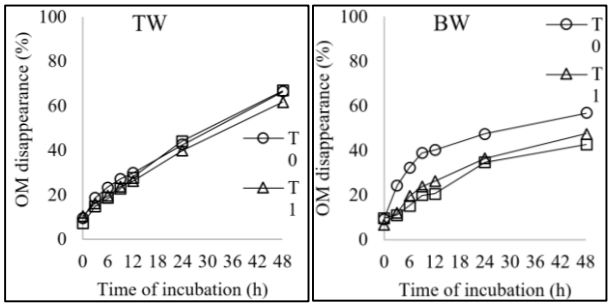
The type of feed and the protection method had a significant effect ($p<0.05$) on the *b*-value. The highest *b*-value was obtained for formaldehyde-treated TD. The average value of *b* significantly ($p<0.05$) differs for each type of feed, with TD and SS having the highest *b* values. The *b* value of BW in this study yielded similar results to Hatungimana and Erickson (2019). An interaction ($p<0.05$) was observed between the type of feed and protein protection methods regarding the $a + b$ values. Significant ($p<0.05$) differences in $a + b$ values were observed among each feed, with the presence of heat and formaldehyde treatment leading to a decrease in $a + b$ values. The degradation kinetics of *a* and *b* values were influenced by the interaction of protein and Neutral Detergent Fiber (NDF), nylon bag porosity and surface area, incubation time, and the ratio of sample to bag surface area (Liebe *et al*., 2018).





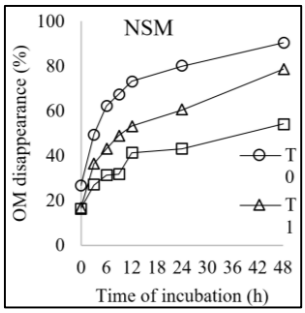
(e)

Fig. 1: Dry matter disappearance of; (a) TD; (b) BW; (c) NSM; (d) SS; (e) CGF

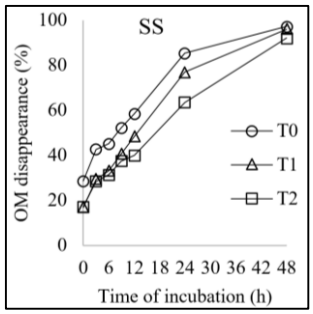


(a)

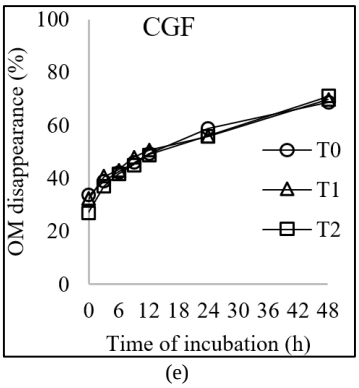
(b)



(c)



(d)



(e)

Fig. 2: Organic matter disappearance of; (a) TW; (b) BW; (c) NSM; (d) SS; (e) CGF

The results showed that EDDM was significantly ($p<0.05$) affected by feed type, protein protection method, and their interaction. Unprotected NSM and SS exhibited high EDDM values. The mean of DM on NSM, SS, and CGF showed a similar value but was higher than TD and BW. The protein protection treatment in each feed reduced the value of DM, with formaldehyde treatment resulting in a lower average DM compared to heating. Heating treatment using an autoclave at 120°C led to gelatinization which reduced the solubility of starch in the rumen and degradability (Srakaew *et al.*, 2021). The EDDM was determined by the distinctive characteristics of each feed, particularly the nutrient content in the cell content and cell walls (Wati *et al*., 2012).

The OM disappearance of the high RDP feed with treatments protected protein and unprotected is shown in Fig. 2. The pattern of OM degradation in high RDP feed closely resembles that of DM degradation. Since OM is a component of DM, they are interconnected. The rate of OM degradation in the rumen was known to be influenced by microbial protein synthesis and microbial population. Heat and formaldehyde treatments resulted in a reduction of OM degradation in BW, NSM, and SS at each incubation time. TD and CGF exhibited similar levels of OM degradation as the unprotected treatment.

Table 3 presents the kinetic degradation of OM degradation in high RDP-protected feeds. There was a significant ($p<0.05$) interaction between the type of feed and protein protection methods for *a* and EDOM values. The highest *a*-value was observed in unprotected CGF. CGF has the highest value, followed by NSM, SS, TD and BW. Protein protection affected the *a*-value in OM degradation's kinetics, resulting in a decrease compared to the unprotected feed (19-37%). The formaldehyde exhibited a lower value than the heat treatment. There was no interaction between the type of feed and protein protection methods for *b*, *a* + *b*, and *c* values. The degradation rate (*c*-value) of OM was not significantly different between feed types. The degradation rate in feed was affected by factors such as sample-to-bag area ratio and the interaction of NDF with pore size (Liebe *et al*., 2018).

The EDOM estimation was influenced by the feed type, protection protein treatment, and their interaction. The highest EDOM value was observed in unprotected NSM, while the lowest was found in formaldehyde-treated BW. The method of protein protection impacted the reduction of the EDOM value. In particular, the EDOM value was lower in formaldehyde than in heating treatment. Several factors, such as the nutrient content of the feed, rumen microbial activity and population, and rumen pH influence the extent of OM degradation (Bach *et al*., 2005).

Table 2: Kinetics degradation of dry matter in high RDP-protected feeds

		Treatments				
Parameters	Feeds	Unprotected	Heating	Formaldehyde	Means	SEM
a (%)	TD	11.29^{e}	10.98^{e}	7.74^{e}	9.73^{c}	2.824
	BW	11.36^{e}	8.03^{e}	7.97^{e}	9.12^{c}	
	NSM	28.51^{c}	20.36^{d}	18.10^{d}	22.32^{b}	
	SS	32.46^{bc}	18.25^{d}	19.91^{d}	22.43^{b}	
	CGF	37.37^{ab}	37.85^{a}	32.27^{c}	35.83^{a}	
	Means	23.61^{a}	20.34^{b}	17.20^{c}		
b (%)	TD	62.77^{ab}	46.99^{ab}	85.34^{a}	70.19^{a}	4.204
	BW	43.17^{ab}	43.66^{ab}	43.48^{ab}	43.44^{c}	
	NSM	58.97^{ab}	58.51^{ab}	38.14^{b}	51.87^{bc}	
	SS	83.29^{ab}	69.10^{ab}	40.51^{b}	61.92^{ab}	
	CGF	40.56^{b}	38.67^{b}	36.19^{b}	38.48^{c}	
	Means	55.93	52.06	48.73		
a + b (%)	TD	74.06^{ab}	57.97^{b}	93.08^{ab}	79.91^{a}	4.712
	BW	54.54^{b}	51.69^{b}	51.45^{b}	52.56^{b}	
	NSM	87.48^{ab}	78.86^{ab}	56.24^{b}	74.20^{a}	
	SS	115.75^{a}	87.34^{ab}	60.43^{a}	84.34^{a}	
	CGF	77.93^{ab}	76.52^{ab}	68.47^{ab}	74.31^{a}	
	Means	79.54^{a}	72.40^{ab}	65.93^{b}		
c (% h^{-1})	TD	0.04	0.03	0.02	0.03	0.011
	BW	0.11	0.05	0.04	0.06	
	NSM	0.13	0.08	0.07	0.09	
	SS	0.05	0.12	0.17	0.12	
	CGF	0.04	0.04	0.06	0.05	
	Means	0.07	0.07	0.07		
EDDM (%)	TD	33.05^{def}	27.10^{ef}	31.43^{def}	31.51^{b}	3.594
	BW	38.87^{cde}	27.56^{ef}	24.21^{f}	30.21^{b}	
	NSM	68.45^{a}	51.93^{b}	37.67^{de}	52.68^{a}	
	SS	66.61^{a}	51.11^{b}	41.68^{bcd}	51.45^{a}	
	CGF	52.60^{b}	52.85^{b}	50.44^{bc}	51.96^{a}	
	Means	50.87^{a}	44.42^{b}	37.08^{c}		

^{a,b}Significant different in the same row ($p<0.05$); *a* = rapidly soluble fraction (%); *b* = slowly degradable fraction and degraded at rate *c* (%); *c* = rate constant for degradation of b per h; EDDM = effective degradation of dry matter; TD = Tempe Dregs; BW = Brewery Waste; NSM = *Nigella sativa* meal; SS = Soybean Seeds; CGF = Corn Gluten Feed

Table 3: Kinetics degradation of organic matter in high RDP-protected feeds

		Treatments				
Parameters	Feeds	Unprotected	Heating	Formaldehyde	Means	SEM
a (%)	TD	11.23^{def}	11.09^{def}	7.67^{f}	9.86^{d}	2.545
	BW	10.61^{ef}	6.95^{f}	7.92^{f}	8.49^{d}	
	NSM	28.65	20.91^{c}	18.11^{c}	22.55^{b}	
	SS	30.42^{ab}	15.73^{cde}	16.80^{cd}	20.23^{c}	
	CGF	33.83^{a}	33.79^{a}	28.42^{b}	32.01^{a}	
	Means	22.41^{a}	18.16^{b}	15.71^{c}		
b (%)	TD	63.50	31.83	84.59	63.49^{ab}	4.679
	BW	43.83	44.79	46.05	44.89^{bc}	
	NSM	58.62	57.31	37.71	51.21^{bc}	
	SS	89.03	72.56	65.11	75.14^{a}	
	CGF	42.88	34.76	35.50	37.71^{c}	
	Means	57.47	49.42	52.99		
a + b (%)	TD	74.72	42.92	92.26	73.35^{b}	5.140
	BW	54.44	51.74	53.97	53.38^{c}	
	NSM	87.27	78.22	55.81	73.77^{b}	
	SS	119.45	88.28	81.91	95.36^{a}	
	CGF	76.71	68.55	63.92	69.72^{bc}	
	Means	79.88	67.59	68.69		
c (% h^{-1})	TD	0.04	0.07	0.02	0.04	0.009

Table 3: Continue

	BW	0.11	0.05	0.03	0.06	
	NSM	0.13	0.08	0.07	0.09	
	SS	0.04	0.12	0.12	0.09	
	CGF	0.04	0.06	0.08	0.06	
	Means	0.08	0.07	0.06		
EDOM (%)	TD	32.49^{fgh}	24.48^{gh}	31.11^{fgh}	29.96^{b}	3.541
	BW	38.57^{defg}	26.87^{gh}	23.79^{h}	29.74^{b}	
	NSM	68.26^{a}	51.61^{bc}	37.06^{efg}	52.31^{a}	
	SS	65.23^{ab}	48.69^{cde}	44.27^{cdef}	52.15^{a}	
	CGF	50.03^{cd}	49.73^{cd}	47.57^{cde}	49.11^{a}	
	Means	49.89^{a}	41.41^{b}	36.22^{c}		

^{a,b}significant different in the same row ($p<0.05$); *a* = rapidly soluble fraction (%); b = slowly degradable fraction and degraded at rate *c* (%); c = rate constant for degradation of b per h; EDOM = Effective Degradation of Organic Matter; TD = Tempe Dregs; BW = Brewery Waste; NSM = *Nigella sativa* Meal; SS = Soybean Seeds; CGF = Corn Gluten Feed

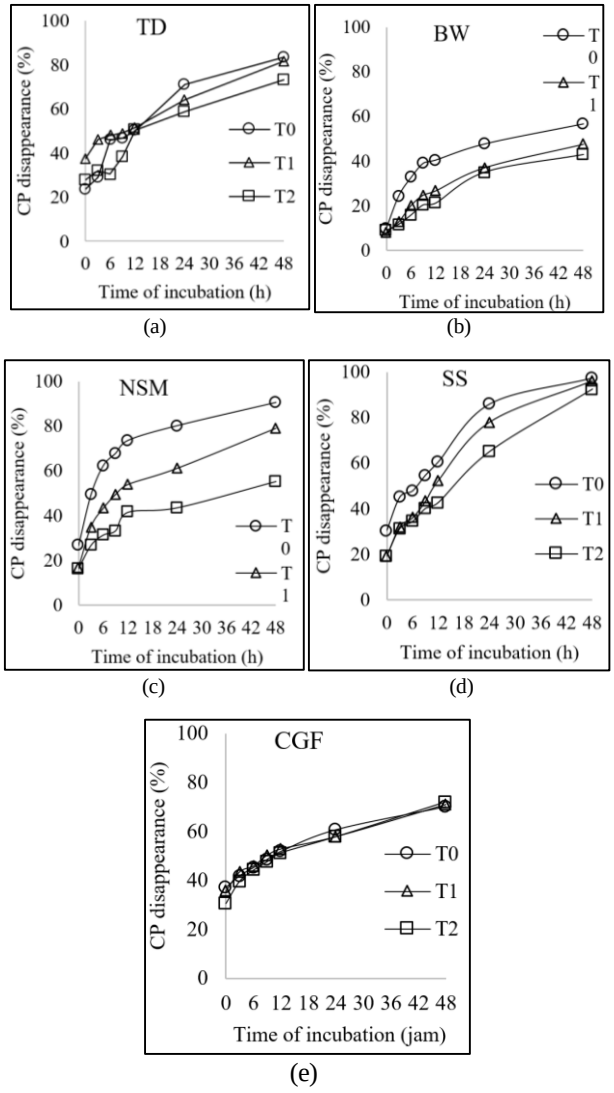


Fig. 3: Crude protein disappearance of; (a) TD; (b) BW; (c) NSM; (d) SS; (e) CGF

The CP disappearance on a high RDP feed is depicted in Fig. 3. CP degradation was higher at 0 h for BW, NSM, SS, and CGF in the treatment without protein protection. The 0 h degradation represented the amount of soluble protein without incubation in the rumen. The soluble protein decreased due to the protein protection method.

The kinetics degradation of CP in high RDP-protected feeds can be seen in Table 4. There was no interaction between the type of feed factor and the protection method on the CP kinetics values (*a*, *b*, *a* + *b*, and *c*). The feed type significantly ($p<0.05$) affected the value of *a*, *b*, and *a* + *b*, but did not affect the *c* value. The protein protection method did not affect the kinetic value of *a*, *b*, and *c*. CGF had the highest *a*-value, but the potentially degradable fraction of CP (*b* value) was the lowest. The formaldehyde treatment significantly ($p<0.05$) decreased the *a* + *b* value (20%). A previous study revealed that moist heating using an autoclave enhanced the nutritional value and protein utilization compared to dry heating. This improvement can be attributed to a decrease in the soluble protein component (*a*-value) and the *c*-value, resulting in an increased supply of RUP to the small intestine (Ahmad Khan *et al*., 2015). Molosse *et al.* (2022) also observed alterations in the DM and CP kinetics of cottonseed meal and peanut meal (autoclave heated at 127°C), increasing both the *b*-value and RUP value. Moderate heat treatment (125°C) altered the chemical profile of the protein, resulting in the formation of cross-linkages and an increase in the C fraction of protein in rapeseed meal (Haese *et al.*, 2022). Formaldehyde-treated SS reduced DM and CP's degradation rate (*c*) compared to untreated SS (Ehle *et al.*, 1982).

The EDCP was influenced by the type of feed, protein protection methods, and their interactions. The highest EDCP value was observed in unprotected CGF. Subsequently, the mean of EDCP values revealed that CGF consistently displayed elevated levels, followed by BW, SS, TD, and NSM in descending order. However, a previous study reported higher EDCP values for BW and NSM, with 76.62 and 96.70%, respectively (Rosmalia *et al*., 2021). The level of EDCP was affected by factors such as the structure and dimensions of the feed protein, the ratio of protein to NPN, the physicochemical properties of the protein, the presence of disulfide bonds, and cross-linking and anti-nutrient (Broderick *et al*., 1991).

Table 4: Kinetics degradation of crude protein in high RDP-protected feeds

		Treatments				
Parameters (%)	Feeds	Unprotected	Heating	Formaldehyde	Means	SEM
a (%)	TD	24.03	35.80	23.81	26.89^{bc}	4.166
	BW	35.22	42.62	35.05	37.63^{b}	
	NSM	31.59	28.47	14.73	24.93^{c}	
	SS	38.43	25.94	26.61	32.41^{bc}	
	CGF	71.35	62.94	61.08	65.12^{a}	
	Means	41.64	40.12	32.66		
b (%)	TD	67.90	67.49	47.57	60.18^{ab}	4.403
	BW	54.97	46.36	52.17	51.16^{ab}	
	NSM	60.74	39.04	28.85	42.88^{bc}	
	SS	66.87	81.83	55.24	65.49^{a}	
	CGF	25.65	23.81	39.38	29.61^{c}	
	Means	55.23	45.37	43.88		
a + b (%)	TD	91.94	103.29	71.38	87.07^{a}	4.314
	BW	90.19	88.98	87.21	88.79^{a}	
	NSM	92.33	67.51	43.58	67.81^{b}	
	SS	105.30	107.78	81.85	97.90^{a}	
	CGF	97.00	86.75	100.45	94.74^{a}	
	Means	95.35^{a}	87.01^{ab}	76.54^{b}		
c (% h^{-1})	TD	0.05	0.03	0.21	0.11	0.013
	BW	0.14	0.09	0.03	0.09	
	NSM	0.08	0.09	0.10	0.09	
	SS	0.08	0.05	0.08	0.07	
	CGF	0.05	0.14	0.07	0.09	
	Means	0.08	0.09	0.10		
EDCP (%)	TD	53.51^{cde}	56.85^{bcde}	46.40^{ef}	51.68^{c}	3.694
	BW	73.90^{abc}	65.96^{abcde}	52.03^{de}	63.96^{b}	
	NSM	65.65^{abcde}	51.10^{def}	30.20^{f}	48.98^{c}	
	SS	70.90^{abcd}	61.69^{abcde}	46.97^{ef}	61.39^{b}	
	CGF	81.41^{a}	78.36^{ab}	76.04^{ab}	78.60^{a}	
	Means	69.07^{a}	63.47^{b}	50.57^{c}		

^{a,b}significant different in the same row ($p<0.05$); a = rapidly soluble fraction (%); *b* = slowly degradable fraction and degraded at rate c (%); c = rate constant for degradation of b per h; EDCP = Effective Degradation of Crude Protein; TD = Tempe Dregs; BW = Brewery Waste; NSM = *Nigella sativa* Meal; SS = Soybean Seeds; CGF = Corn Gluten Feed

The protein protection method employed significantly ($p<0.05$) decreased the EDCP value, thereby highlighting the effectiveness of protein protection in curtailing protein degradation within the rumen. The extrusion process applied to legumes reduced EDCP and increased the proportion of protein digested in the small intestine (Solanas *et al*., 2005). Nasir *et al.* (2008) reported that heating SS at 140-145°C for 45 min reduced EDCP and total AA. The Maillard reaction, formed during the heating process, reduced the rate of degradation and EDCP in feed (Petit *et al*., 2002). Formaldehyde treatment resulted in lower EDCP values than the heating method, in which the extent of the reduction was 27 and 8%, respectively. The mechanism behind formaldehyde's action involves the formation of a methylol compound between Formaldehyde (HCHO) and the amino group of the protein, followed by a slow condensation reaction that generates cross-linking bonds between methylene and protein chains over time (Barry, 1976).

In vitro Digestibility

Table 5 presents DMD, OMD, and CPD for high RDP feeds. A significant ($p<0.05$) interaction was observed between the feed type and protein protection methods for DMD, OMD, and CPD. The DMD, OMD, and CPD differed among each feed type, with TD and CGF demonstrating higher DMD and OMD values. TD, SS, and CGF exhibited high CPD values. The CPD value obtained for SS using the heating method was lower than that reported by Castro *et al*. (2007), which was 79.40%. This discrepancy could be attributed to variations in the sources of soybean seeds used.

Table 5: *In vitro* digestibility of high RDP feeds

		Treatments				
Parameters	Feeds	Unprotected	Heating	Formaldehyde	Means	SEM
DMD (%)	TD	86.43^{a}	74.86^{abc}	63.72^{cd}	75.00^{a}	5.172
	BW	33.11^{gh}	31.08^{gh}	26.71^{h}	30.30^{d}	
	NSM	63.02^{cd}	58.91^{de}	42.48^{fg}	54.81^{c}	
	SS	81.27^{a}	67.67^{bcd}	48.67^{ef}	65.87^{b}	
	CGF	82.59^{a}	78.10^{ab}	75.34^{abc}	78.68^{a}	
	Means	69.28^{a}	62.12^{a}	51.39^{b}		
OMD (%)	TD	87.59^{a}	76.55^{abc}	65.77^{cde}	76.64^{a}	5.373
	BW	31.62^{hi}	29.70^{hi}	25.05^{i}	28.79^{d}	
	NSM	61.18^{de}	56.73^{ef}	39.47^{gh}	52.46^{c}	
	SS	80.37^{ab}	66.11^{bcde}	46.11^{fg}	64.19^{b}	
	CGF	81.85^{a}	77.03^{abc}	74.25^{abcd}	77.71^{a}	
	Means	68.52^{a}	61.22^{b}	50.13^{c}		
CPD (%)	TD	84.83^{ab}	76.58^{ab}	64.24^{b}	75.22^{a}	5.091
	BW	41.64^{c}	39.18^{c}	27.81^{c}	36.21^{c}	
	NSM	64.70^{b}	67.68^{ab}	30.84^{c}	54.41^{b}	
	SS	88.71^{a}	77.09^{ab}	41.38^{c}	69.06^{a}	
	CGF	73.17^{ab}	72.13^{ab}	65.21^{b}	70.17^{a}	
	Means	70.61^{a}	66.53^{a}	45.90^{b}		

^{a,b}significant different in the same row ($p<0.05$); DMD = Dry Matter Digestibility; OMD = Organic Matter Digestibility; CPD = Crude Protein Digestibility; TD = Tempe Dregs; BW = Brewery Waste; NSM = *Nigella sativa* Meal; SS = Soybean Seeds; CGF = Corn Gluten Feed

Protein protection significantly ($p<0.05$) impacted DMD, OMD, and CPD, reducing digestibility values. Formaldehyde treatment significantly ($p<0.05$) decreased DMD, OMD, and CPD values by 26, 27, and 36%, respectively, compared to unprotected treatment. The DMD and CPD values between unprotected and heating treatment tended to be similar. In contrast, their EDCP values differed, suggesting that the heating method reduces degradation in the rumen but did not affect post-rumen-digestion. Post-ruminant digestion of cottonseed meal was increased after heating treatment (Molosse *et al*., 2022). The heating process altered the chemical profile of the protein, protein subfractions, rumen degradation, post-rumen digestibility, and protein structure (such as changes in the ratio of α-helix and β-sheet) (Ahmad Khan *et al*., 2015). The effectiveness of the heating method was influenced by the temperature and duration of heating, with higher temperatures being more effective in reducing protein degradation than prolonged heating times (Tagari *et al*., 1986).

A previous study reported that formaldehyde treatment in corn reduced DMD by 30% in sheep (Oke *et al*., 1991). A previous study has found no significant reduction in nitrogen availability from formaldehyde-protected soybean meal (Subuh *et al*., 1994). Feeding formaldehyde-protected rape seed oil cake had been deemed safe for ruminants, as histopathological tests on goat tissues did not reveal any degenerative changes (Sahoo *et al*., 2006). The reduction of *in vitro* digestibility in formaldehyde treatment might be due to the high level of formaldehyde concentration used in this study. Hence, the EDDM, EDOM and EDCP values in formaldehyde treatment were lower than in heating treatment potentially inducing overprotection that rendered the protein indigestible, even in the post-ruminal. Kumar *et al.* (2015b) revealed that formaldehyde-protected mustard cake at a 2% level resulted in a reduction of DMD and OMD values up to 12%. Wulandari *et al.* (2022) suggested the use of 0.8% formaldehyde to protect proteins, such as soybean meal. Another study reported the bioavailability of protein in soybean meal was increased with 0.6% formaldehyde treatment (Yörük *et al*., 2006).

Conclusion

Heating and formaldehyde protein protection treatments exhibit the capacity to mitigate the degradability of dry matter, organic matter and protein in high RDP feeds through a reduction in the soluble fraction (a) and potential degradation ($a + b$). The formaldehyde method significantly decreases the digestibility of dry matter, organic matter, and protein (51.39, 50.13, and 45.90%). It is advisable to employ the heating method for protein protection in high RDP feeds, as it effectively reduces RDP without compromising feed digestibility. Importantly, this method is chemical-free and more practical for tropical smallholder farmers.

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Author's Contributions

Annisa Rosmalia: Conducted laboratory and data analysis, and wrote the manuscript.

Idat Galih Permana: Conceived the research idea, supervised and finalized the manuscript, and handled the correspondence.

Despal: Validated the results, supervised, and proofread the manuscript.

Toto Toharmat: Designed and supervised the experiment.

Ethics

The animal ethics committee, at IPB University reviewed and accepted the study's ethical standards (No. 047/KEH/SKE/XI/2021).

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