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Fermentation Products, Microbial Protein Synthesis, and Hydrolytic Enzymes Activity of Rumen Fluids Local Beef Cattle Supplemented by Direct-Fed Microbials and Waru Leaf Flour

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ABSTRACT

The aim of this study was to evaluate the effect of Waru Leaf Flour (WLF) and Direct-Fed Microbials (DFM) supplementation on volatile fatty acid (VFA) and ammonia ($N\text{-}NH_3$) concentrations, total protozoa, microbial protein synthesis (MPS), and the specific activity of cellulase, amylase, protease in rumen fluids of local beef cattle. The study was conducted as an experimental investigation using a factorial Completely Randomized Design (FCRD). The first factor were three levels of DFM (P) mixed with rice straw (0%, 0.5%, and 1% dry matter of rice straw). The second factor consisted of three levels of WLF supplementation (D) in concentrates (0%, 0.24%, and 0.48% of dry matter concentrate). This resulted nine treatment groups, each it replicated three times.The diet composition consisted of rice straw and concentrate at 40:60 ratio (% dry matter). The interaction between DFM and WLF had a statistically significant impact ($p<0.01$) on the rumen fluid's total protozoa, N-NH3, and protein content. However, this interaction was not statistically significant ($P>0.05$) in relation to VFA, MPS, cellulase, amylase, and protease activity. While no significant interaction were observed for total VFA, cellulase, amylase, and protease, DFM supplementation had a statistically significant effect ($P<0.01$) on reducing enzymes activity, and total VFA ($P<0.05$). SDS-PAGE and zymography analyzes showed two protease molecules at all treatments with molecular weight of 144 kDa and 133 kDa, cellulase molecules at 62-67 kDa and 19-21 kDa respectively. The addition of DFM showed a new protease band, they had a measurement of 14 and 25 kDa.This study provides valuable insights into enzyme activities in rumen, particularly protease, cellulase, and amylase. The results suggest that the combination of 0.24% WLF and 0.5% DFM supplementation holds promise for optimizing rumen conditions and improving the performance of local beef cattle.

Keywords: DFM, Enzymes, Hibiscus, Metabolic-products, Rumen-fluids

Introduction

Indonesia continues to rely on the importation of cattle for fattening purposes in response to the ever-increasing demand for beef, which exhibits annual growth. According to Hanum and Setyari findings (2018), there is a persistent upward trend in the periodic importation of beef into Indonesia, indicating a significant dependence on these imports. The primary drivers behind this growing reliance on imports include the sluggish expansion of the domestic beef cattle population and limited local beef production capacity.

In the period spanning from 2020 to 2022, data illustrated that the growth rate of the local beef cattle population and the production of beef within Indonesia increased at 6.71% and 10.03% per year, respectively. Meanwhile, the national beef consumption demonstrated a substantially higher average growth rate reaching from 0.009 kg/capita/w in 2020 to 0.010 kg/capita/w in 2022 (BPS, 2023). The imbalance between beef production and consumption levels depicts Indonesia's inability to optimize the potential of beef cattle agribusiness in producing domestic beef. Therefore, efforts are necessary to reduce the import of young cattle through initiatives to enhance the productivity of local livestock breeds such as Bali cattle, Sumba Ongole cattle, and Madura cattle. The weight gain of local cattle breeds is relatively low (averaging 0.3 – 0.8 kg/day), based on data compiled from various sources on daily weight gain of local cattle breeds. For instance, Bali cattle exhibit a daily weight gain ranging from 0.320 to 0.810 kg/head/day (Wangi *et al*., 2017), while crossbred Ongole cattle show an average daily weight gain of 0.57 – 0.69 kg (Ngadiyono *et al*., 2019). Madura cattle, with an average weight gain

of 0.3 – 0.6 kg/day, possess an advantage in their adaptability to tropical environments and low-quality feed (Wiryawan *et al.*, 2017).

Increasing the productivity of local beef can be achieved through a feedlot management system. This method involves providing a high-concentrate diet to attain maximum productivity. The use of high-concentrate diets affects the rate of rumen microbial growth. One approach is through the application of direct-fed microbial (DFM) and saponins derived from Waru Leaf Flour (WLF). However, an increase in protozoa population due to DFM usage has a negative impact as it elevates methane production and reduces feed efficiency in the rumen. The use of synthetic anti-protozoa agents is no longer recommended due to the residues they leave in animal products. Hence, natural substances capable of addressing this issue are required. Saponins can lower protozoa populations and decrease methane production, resulting in safe and environmentally friendly end products (Ramdani *et al.*, 2017).

Waru leaves are among the plants containing anti-protozoa bioactive compounds such as quinoline, saponin, and fumaric acid. Bata *et al*. (2016) noted that Waru leaves contain 12.9 mg/g of saponin. Additionally, according to Bata *et al*. (2021) Waru leaves contain 3% saponin and 24.6% quinoline compounds with antiprotozoa properties. Waru leaves also contain sufficient bioactive fumaric acid and saponins, which act as protozoa defaunation agents. Widyawati *et al*. (2017) added that saponins as protozoa defaunation agents are inversely proportional to methane reduction as they inhibit the methanogenesis process.

The interaction between the active compounds in WLF and DFM is expected to maintain the stability of the rumen ecosystem. DFM supplementation is anticipated to stimulate Lactic Acid Utilizing bacteria (LUB), enhance nutrient absorption in the intestine, and support fermentative digestion processes such as total volatile fatty acid (VFA) and ammonia ($N\text{-}NH_3$) (ammonia), thereby increasing nitrogen utilization for microbial protein synthesis. This, in turn, has the potential to positively influence the performance of local beef cattle.

Materials and Methods

Rumen fluids collection. Rumen fluid was sourced from a minimum of three locally raised cattle at the Mersi Slaughterhouse promptly after the cattle's slaughter. The collection process comprised filling a thermos with water heated to 39°C, extracting the rumen fluids, and then transferring them into the thermos. This transfer was facilitated by employing a funnel covered with a 5-layered cheesecloth, following the method described by Afdal and Erwan (2013).

The mixing of DFM and WLF. The blending of Waru Leaf Flour (WLF) was carried out by mixing the leaf powder with concentrate until a homogenous mixture is achieved. The DFM mixing procedure involves adding the DFM to rice straw powder and then combining it with concentrate in a dry matter ratio of 40:60. The composition of the concentrate includes elements such as cassava waste (onggok), pollard, palm kernel meal, coconut meal, soybean meal, molasses, dolomite, salt, urea, and minerals.

The DFM contained *Azotobacter parpalii, Bacillus pumilus, B. stearothermophyllus, B. lentus, B. cereus, B. laterosporus, B. licheniformes, Corynebacterium pseudodiphtheriticum, Lactobacillus* sp., Micrococcus varians, *Pseudomonas fluorescens, Sarcina lutea, Staphylococcus epidermis, Streptococcus thermophyllus, Saccharomyces cereviseae, Phicia anomela* ($1{,}49 \times 10^7$ cfu/g bacterias, $4{,}50 \times 10^4$ cfu/g yeast). DFM produced by PT. Banyumas Raya Indonesia.

***In vitro* test.** The *in vitro* test is conducted to Tilley and Terry (1963) methods. After four hours incubation, 2 drops of $HgCl_2$ were added to stop the fermentation. The mixture then centrifuged at 5000 rpm for 20 minutes to separate the supernatant and residue. The supernatant were taken for analyzing VFA and $N\text{-}NH_3$ concentration, total protozoa, MPS, and enzymes activity.

Experimental design. The study utilizes an *in vitro* experimental approach. The design employed is a Completely Randomized Design (CRD) with a factorial pattern. The first factor comprises three DFM levels (0%, 0.5%, 1.0%), while the second factor encompasses three levels of Waru Leaf Flour (0%, 0.24%, 0.48%). Both DFM and WLF supplements were incorporated into the concentrate. In total, there are nine treatment combinations, each of which was replicated three times. The treatments were repeated three times with the result were 27 total treatment units. This research used three repetitions intending to increase accuracy and reduce the coefficient of variation.

The research parameters. Total VFA was measured by steam distillation method according to Kroman *et al.* (1967), $N\text{-}NH_3$ was analyzed using micro-diffusion Conway (Conway and O'Malley, 1942), total protozoa was quantified using the Sedgewick Rafter counting chamber (Ogimoto and Imai, 1981), microbial protein synthesis was determined according to Zinn and Owens (1995).

Rumen fluids protein concentration was measured by Bradford solution (Bollag and Edelstein, 1996), cellulase was assessed using Whatman No. 41 filter paper as substrate (Camassola and Dillon, 2012), protease was determined pursuant to Walter (1984), amylase was measured using the Bernfeld method (1955).

In-situ enzyme activity was assessed using zymography method (Bressollier *et al*., 1999), and protein molecular weight was determined through Native-PAGE with discontinous gel i.e. 4% stacking gel and 10% separating gel (Bollag and Edelstein, 1996).

Statistical analysis. The data was analyzed using analysis of variance (ANOVA), followed by a post hoc Honestly Significant

Difference (HSD) test in cases where interactions among treatments were observed. In instances where no interaction was detected, an orthogonal polynomial test was conducted, particularly when factor II exhibited a significant effect. If factor I demonstrated a significant effect, the analysis was extended with the application of the HSD test, following the guidelines by Steel *et al.* (1997).

Results and Discussion

Total VFA, N-NH_3, total protozoa and microbial protein synthesis (MPS)

The analysis of variance indicates that there is a significant interaction ($P < 0.01$) between P (DFM) and D (WLF) regarding total protozoa and N-NH_3. However, the interaction is not significant ($P > 0.05$) for VFA and MPS. Additionally, the increase in DFM level has a highly significant effect ($P < 0.01$), while it has a significant effect ($P < 0.05$) on VFA (Table 1).

The interaction of DFM and WLF showed varied responses, it increases the total protozoa and N-NH_3 production. Figure 1 illustrates that as DFM levels increase, the total protozoa in treatment D1 (without WLF) rose more quickly than in treatment D3 (0.48% WLF), which exhibits a slower increase. According to Philippeau *et al.* (2017), the use of DFM that was dominated by bacteria increases protozoa population. Bata and Rahayu (2017) noted that adding DFM would enhance total protozoa, but the addition of WLF might restrain protozoa growth due to the presence of antiprotozoal compounds like saponins and quinolines in Waru leaves, which were inhibit the growth of protozoa. Susanti and Marhaeniyanto (2014) mentioned that saponins can bind to sterols in protozoa cell membranes, causing them to lyseand reducing protozoa population. A decrease in protozoa population leads to reduced methane gas concentration (Rangubhet *et al*., 2017; Antonius *et al*., 2023), as around 26% of methanogenic bacteria are symbiotic with protozoa (Króliczewska *et al*., 2023).

Figure 2 show that at D2 (0.24% WLF), there's an initial decrease followed by an increase, but it still remains lower than D1 and higher than D3. This indicates that the addition of 1% DFM in 0.24% WLF supplementation does not significantly suppress total protozoa growth. Figure 2 illustrates that D3 is slower compared to D1, which is likely due to the inhibition of rumen bacteria caused by the addition of 0.48% TDW. These results corresponds to Patra and Saxena (2009), which stated that dosing is a factor affecting the effectiveness of Waru leaves, and excessive dosing can hinder bacterial growth. Additionally, Waru leaves contain antimicrobial compounds that hinder bacterial growth when doses are excessive. According to Bekuma (2019), the decrease in N-NH_3 concentration might be linked to reduced proteolytic bacteria and protein degradation due to the binding between protein and tannin compounds. Waru leaves contain tannins and antiprotozoa (saponins and quinolines). Bata and Rahayu (2017) reported that tannin content in Waru leaves was 8.93% respectively.

Treatment D2 shows a quadratic decrease with a maximum point at the addition of 0.68% DFM, followed by an increase of up to 1% DFM addition (Figure 2). This could be due to the increase bacterial activity resulting from the defaunation effect of saponins and quinolines from Waru leaves. Moreover, the addition of WLF (Waru Leaf Flavonoids) may contribute to maintaining

Tabel 1. Concentration of total volatile fatty acid and ammonia, total protozoa, and MPS on DFM and WLF supplementation

Variables	P1			P2			P3			Significance		
	D1	D2	D3	D1	D2	D3	D1	D2	D3	P	D	PxD
Total VFA (mM)	146,6	120,67	140	128,67	154	136	156	153,3	169,67	*	ns	ns
N-NH_3 (mM)	6,46	6,33	6,86	8,13	8,46	8,06	8,93	8,13	10,13	**	**	**
Total protozoa (10^{-3})	2,22	2,45	0,85	1,77	1,33	0,95	4,31	2,87	1,39	**	**	**
MPS (mg/100ml)	922[a]	942[a]	951[a]	921[a]	1036[a]	967[a]	954[a]	965[a]	954[a]	ns	ns	ns

DFM: direct fed microbials; WLF: waru leaf flour; MPS: microbial protein syntezis; P1: 0% DFM; P2: 0.5% DFM; P3: 1% DFM; D1: 0% WLF; D2: 0.24% WLF; D3: 0.48% WLF; P: Factor I (DFM); D: Factor II (WLF); PxD: Interaction of DFM and WLF; ns: non-significant.

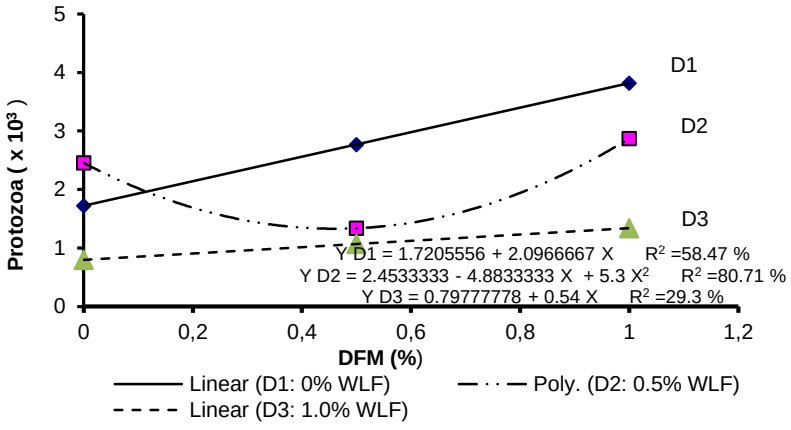

Figure 1. The Relationship between DFM addition and rumen total protozoa at various WLF levels.

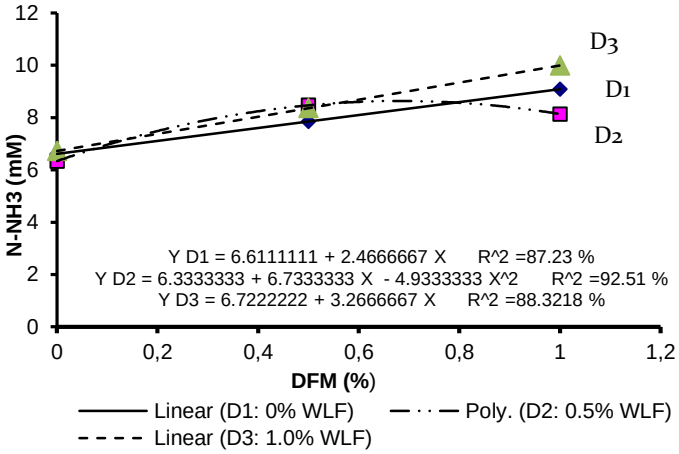

Figure 2. The relationship between DFM addition and N-NH_3 rumen at various WLF levels.

a consistent rumen pH. This is attributed to the presence of 7g/L of flavonoids in Waru leaves, as demonstrated by Bata *et al.* (2021). These flavonoids in the rumen serve as effective buffering agents. However, with the addition of 1% DFM, a decrease in N-NH_3 levels is observed. This might be due to the pH drop in the rumen, hindering N-NH_3 production as a result of increased lactat e-utilizing microbes present in DFM. This is not balanced by WLF addition, leading to a decrease in rumen pH that hampers fermentation processes and disrupts the rumen ecosystem.

WLF levels increased linearly along with the addition of DFM, this caused an increase in N-NH_3 production (Figure 2). DFM contains various types of lactic acid bacteria and bacillus sp. which contributes to increasing N-NH_3. Table 1 shows that the N-NH_3 concentration ranges from 6.4 to 10.13 mM. According to McMurphy *et al.* (2011), the optimal ammonia concentration for rumen microbial activity is greater than 4.86 mM. This indicates that the diets in this study was capable of serving N-NH_3 concentration for rumen microbial growth. Research by Suryani *et al*. (2015) reported that adding 1% DFM with Bacillus sp. Bacteria increased N-NH_3 levels up to 2 mM. The tendency for increased ammonia concentration might be due to the rise in protein content in the concentrate and the addition of DFM, along with the defaunation effect of antiprotozoal compounds in Waru leaves. In the rumen, proteins are hydrolyzed into peptides and amino acids by microbial enzymes. Subsequently, some amino acids undergo deamination to form ammonia, which is used by rumen microbes for microbial protein synthesis.

The analysis of variance indicates that the total VFA production was not significantly affected ($P > 0.05$) by TDW levels, but it was significantly affected ($P < 0.05$) by DFM levels (Table 1). The addition of DFM in P3 was higher than P1 and P2, but P1 and P2 do not differ significantly. This suggests that adding 1% DFM creates an optimal rumen condition for fermentation products compared to 0% and 0.5% DFM. This is likely due to the presence of *S. cerevisiae* and *A. oryzae* in the DFM, as reported by Suryani *et al.* (2015) that providing the probiotic *S. cerevisiae* in ruminant diet manipulate rumen microbes, enhancing fiber-degrading bacteria populations, digestion, and overall rumen fermentation performance.

Table 2. Total volatile fatty acid concentration on DFM and WLF supplementation

Treatments	D1 (mM)	D2 (mM)	D3 (mM)	Average
P1	146.67	120.67	140.00	135.8[b]
P2	128.67	154.00	136.00	139.6[b]
P3	156..00	153.33	169.67	159.7[a]
Average	14.80	142.70	148.50	

[a,b] Different superscripts at the same coloumn indicate significant differences ($P<0.05$) and highly significant ($P<0.01$).
DFM: direct fed microbials; WLF: waru leaf flour; P1: 0% DFM; P2: 0.5% DFM; P3: 1% DFM; D1: 0% WLF; D2: 0.24% WLF; D3: 0.48% WLF.

Probiotics, as dietary supplements in the form of live microorganisms administered directly to animals, can have a positive impact on the host by enhancing the balance of the rumen ecosystem. *S. cerevisiae* consumes oxygen within the rumen, thus promoting an optimal rumen environment, while *A. oryzae* produces enzymes such as α-amylase, α-galactosidase, proteinase, and β-glucosidase. These enzymes work to break down complex sugars into simpler molecules, thereby stimulating rumen microorganisms. McDonald *et al.* (2002) stated that the optimal range VFA required for optimal rumen microbial growth is 80-160 mM. Rumen microbes used VFA as the primary energy source for protein synthesis.

The addition of 0.5% DFM and 0.24% WLF effectively improved total VFA and N-NH_3 concentration and reduced total protozoa rumen.

Protein content and activities of protease, cellulase, and amylase

The analysis of variance indicates a significant interaction ($P < 0.01$) between P (DFM) and D (WLF) for protein content, but no significance ($P > 0.05$) for amylase, protease, and cellulase activities. The significant interaction ($P < 0.01$) indicates that the effect of DFM supplementation

Table 3. Protein concentration and activity of protease, cellulase and amylase at various DFM and WFL levels

Variable	P1			P2			P3			Significance		
	D1	D2	D3	D1	D2	D3	D1	D2	D3	P	D	PxD
Protein concentrtion (mg/ml)	0,0432	0,0442	0,046	0,0806	0,0816	0,1038	0,133	0,114	0,09	**	ns	**
Protease (U/mg)	21,05	21,11	19,61	11,31	11,38	8,69	6,71	8,01	10,22	**	ns	ns
Selulase (U/mg)	1,8303	1,7433	1,743	0,8773	1,0177	0,8163	0,577	0,685	0,898	**	ns	ns
Amilase (U/mg)	0,9873	1,0277	0,881	0,4797	0,5627	0,4443	0,358	0,394	0,567	**	ns	ns

[a,b] Different superscripts at the same coloumn indicate significant differences (P<0.05) and highly significant (P<0.01).
DFM: direct fed microbials; WLF: waru leaf flour; P1: 0% DFM; P2: 0.5% DFM; P3: 1% DFM; D1: 0% WLF; D2: 0.24% WLF; D3: 0.48% WLF; P: Factor I (DFM); D: Factor II (WLF); PxD: Interaction of DFM and WLF; ns: non-significant.

depends on the level of WLF, and vice versa; the effect of WLF levels on protein content depends on DFM supplementation. These two factors mutually influence and impact rumen fluid protein content.

The addition of DFM dosage has a highly significant impact ($P < 0.01$) on protein content, amylase, protease, and cellulase activity. However, increasing WLF levels do not have a significant effect ($P > 0.05$) on protein content and enzymes activity.

The interaction of DFM and WLF levels yields different responses in terms of rumen fluid protein content (Table 3). Treatment D3 (0.48% WLF) reaches an optimal point at a DFM level of 0.65% with a protein content of 0.10 mg/ml, followed by a decrease with increasing DFM levels. This protein content decrease is attributed not only to the presence of antimicrobial bioactive compounds in WLF but also to the fact that DFM contains bacteria with the ability to release antimicrobial compounds, such as *Sarcina lutea* (Dawes and Holms, 1957), Lactobacilli (Azzaz *et al*., 2016), and *Pseudomonas fluorescens* (Santoso *et al*., 2007). This suggests that the compounds might negatively impact bacteria, disrupting the microbial population balance in the rumen, and subsequently reducing the rumen fluids protein content.

The results of the protein content interaction in data P1, P2, and P3 (Table 3), with averages of 0.04 mg/ml, 0.08 mg/ml, and 0.112 mg/ml, are inversely related to the protease results for P1, P2, and P3 (Table 6), which are decreasing. This indicates an inverse relationship between protein content and protease activity. When protease levels increase, rumen protein content decreases. This is because proteins in the rumen are utilized by proteolytic bacteria, degraded into amino acids and ammonia-N, which are then used for microbial synthesis processes (Table 1). This condition also explains the reason why the addition of DFM and WLF did not affect MPS. Ramdani *et al*. (2017) informed that a decrease in protozoa population increases ammonia availability in the digestive tract. This suggests that a reduction in protozoa enhances the population of rumen bacteria, including proteolytic bacteria, and is supported by the addition of DFM dosage containing proteolytic bacteria. This is evident from the increasing NH_3 content with rising DFM levels (Table 1). However, due to the autolytic nature of protease, protease in rumen fluid undergoes degradation and loses its catalytic activity over time.

In treatment P1 (Table 4 and Table 5), the addition of DFM dosage has a highly significant impact ($P < 0.01$) compared to P2 and P3. This is likely because adding of DFM up to 1% can enhance the diversity and population of microbes in rumen fluid, which can utilize various nutrients present in the rumen, such as feed substrates, enzymes, and other dissolved nutrients. Rumen fluid serves as a source of protease and is presumed to lower enzyme activities like cellulase, amylase, and protease due to microbial proteases degrading the enzymes. As the density of rumen microbes increases, the protease content in the rumen also rises, potentially disrupting other enzymes (Malmuthuge and Guan, 2017).

Table 4. Activity of rumen fluids cellulase on various DFM and WLF levels supplementation

Treatments	D1 (U/mg)	D2 (U/mg)	D3 (U/mg)	Average
P1	1,83	1,74	1,743	1,771[a]
P2	0,877	1,017	0,816	0,903[b]
P3	0,577	0,685	0,898	0,72[b]
Average	1,094	1,147	1,152	

[a,b] Different superscripts at the same coloumn indicate significant differences (P<0.05) and highly significant (P<0.01).
DFM: direct fed microbials; WLF: waru leaf flour; P1: 0% DFM; P2: 0.5% DFM; P3: 1% DFM; D1: 0% WLF; D2: 0.24% WLF; D3: 0.48% WLF; P: Factor I (DFM); D: Factor II (WLF); PxD: Interaction of DFM and WLF; ns: non-significant.

Table 5. Activity of rumen fluids amylase on various DFM and WLF levels supplementation

Treatments	D1 (U/mg)	D2 (U/mg)	D3 (U/mg)	Average
P1	0,987	1,027	0,88	0,965[a]
P2	0,479	0,562	0,444	0,495[b]
P3	0,357	0,394	0,567	0,439[b]
Average	0,608	0,661	0,630	

[a,b]different superscripts at the same coloumn indicate significant differences (P<0.05) and highly significant (P<0.01).
DFM: direct fed microbials; WLF: waru leaf flour; P1: 0% DFM; P2: 0.5% DFM; P3: 1% DFM; D1: 0% WLF; D2: 0.24% WLF; D3: 0.48% WLF.

Table 6. Activity of rumen fluids protease on various DFM and WLF levels supplementation

Treatments	D1 (U/mg)	D2 (U/mg)	D3 (U/mg)	Average
P1	21,05	21,11	19,61	20,59[a]
P2	11,31	11,38	8,69	10,46[b]
P3	6,71	801	10,22	8,31[b]
Average	13,02	13,50	12,84	

[a,b] Different superscripts at the same coloumn indicate significant differences (P<0.05) and highly significant (P<0.01).
DFM: direct fed microbials; WLF: waru leaf flour; P1: 0% DFM; P2: 0.5% DFM; P3: 1% DFM; D1: 0% WLF; D2: 0.24% WLF; D3: 0.48% WLF.

Therefore, the degradation of feed by rumen microbes, particularly the attachment of cells to feed particles or substrates, is performed to enable enzymes to react directly with substrates without being damaged by proteases. This aligns with

McAllister *et al*. (2011) perspective that the rumen microbial process of substrate hydrolysis involves attaching to substrate particles. This implies that feed degradation occurs through cell membranes, with extracellular enzymes being secreted. Enzymes are proteins that are soluble in rumen fluids, which increases the potential for enzyme damage or hydrolysis. The phenomenon of decreasing enzyme activity in rumen fluids was found in the research of Budiansyah *et al*. (2022) who used enzymes in buffalo rumen fluids to improve the quality of palm kernel cake (PKC). There was no significantly effect on dry matter content (P>0.05) after PKC incubated by buffalo rumen fluid enzymes untill a level of 3.0% (v/w). It is thought that the enzymes in the rumen fluids have undergone hydrolysis or damage. The addition of 0.5% DFM increased the protein content of rumen fluids but the activity of protease, amylase and cellulase decreased by 50%.

Estimation molecular weight of hydrolytic enzymes

The determination of molecular weight of protease, amylase, and cellulase enzymes from the treatment WLF and DFM was carried out using the native page gel electrophoresis method, followed by confirming enzyme activities using zymography. This method is employed to separate and determine the quantity and size (molecular weight) of protein chains and protein subunit chains. The molecular weight is determined by calculating the Rf (ratio of protein molecule displacement to the total electrophoresis distance) and comparing it with the Rf value of the known molecular weight LMW (low molecular weight) marker. The distinction between native page gel and zymogram lies in the addition of substrates to the zymogram gel. Zymography is an electrophoresis technique designed to directly detect protease, cellulase, and amylase enzyme activities on the gel (in situ).

For this testing, four samples were used: P1D1 (control), P1D3, P2D3, and P3D3. These samples were selected based on the highest levels of total VFA, N-NH_3, and total protozoa variables. In the native page test of sample P1D1 (control), 7 bands were observed with molecular weights of 142, 123, 106, 60, 48, 29, and 19 kDa (Figure 3). Further confirmation is needed to determine the locations of protease, cellulase, and amylase activities. Therefore, zymography was utilized to confirm the active bands with enzyme activities. Based on the zymography results, protease molecules with a weight of 144 kDa and cellulase at 62 and 19 kDa were identified, but no amylase activity was found. This is consistent with the study by Gong *et al*. (2012), which indicates that cellulase with the addition of CMC substrate in rumen fluid has a molecular weight of around 63 kDa.

The bands forming clear zones in the zymogram gel represent areas where the casein substrate (protease) and CMC (cellulase) have been degraded by the enzymes. According to Mehrzad *et al*. (2005), Coomassie Brilliant Blue and Congo Red dyes bind to proteins containing aromatic side chain amino acid residues, forming blue and orange color complexes. Substrates on the gel that have been degraded by enzyme molecules leave clear zones as a mark of their degradation.

In the native page test of sample P1D3 (0% DFM, 0.48% WLF), seven bands were observed with molecular weights of 142, 114, 106, 86, 69, 34, and 20 kDa (Figure 4). Zymogram analysis was conducted to determine the molecular weight of protease, cellulase, and amylase. The protease molecule in P1D3 was found to have a molecular weight of 144 kDa, suggesting that the first band in the native page is the protease. Two bands with molecular weights of 67 kDa and 20 kDa were identified as cellulase molecules in the zymogram gel (Figure 4), corresponding to bands 5 and 7 in the native page. No amylase molecules were detected in the zymogram gel. When compared to the control P1D1, there is no difference in protease, while there is a difference in molecular weight for cellulase.

In the native page test of sample P2D3 (0.5% DFM, 0.48% WLF), eight bands were observed with molecular weights of 142, 132, 106, 86, 60, 34, 22, and 13 kDa (Figure 5). Based on zymogram analysis, protease molecules with

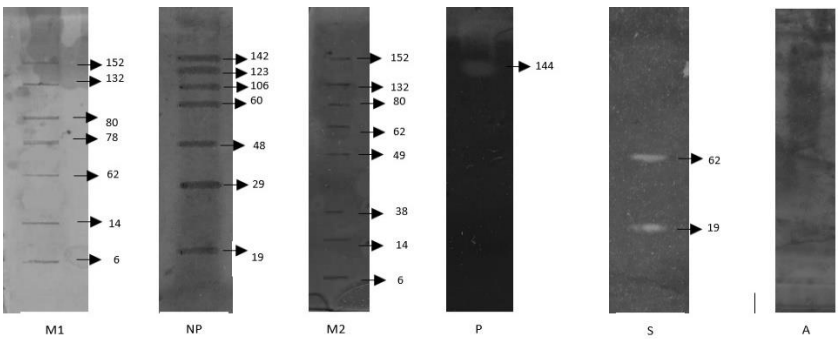

Figure 3. Native-PAGE and Zymography of P1D1 (0% DFM, 0% WLF). M1: Native PAGE Marker, M2: Zymogram Marker, NP: Native PAGE Sample, P: Protease, S: Cellulase, A: Amylase.

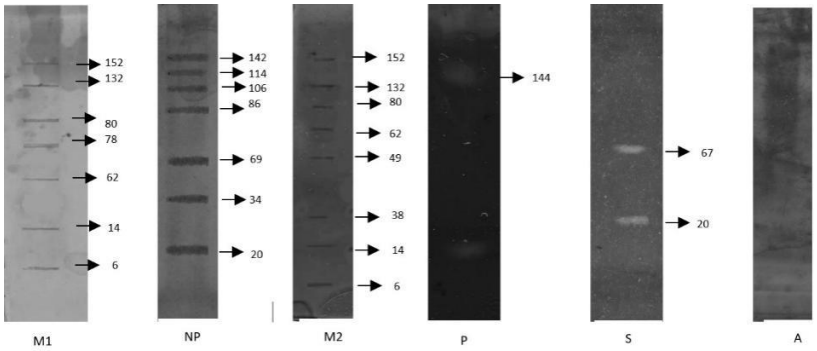

Figure 4. Native-PAGE and Zymography of P1D3 (0% DFM, 0.48% WLF). M1: Native PAGE Marker, M2: Zymogram Marker, NP: Native PAGE Sample, P: Protease, S: Cellulase, A: Amylase.

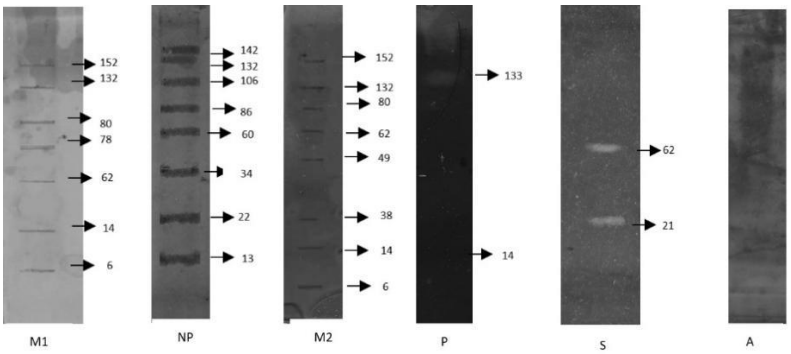

Figure 5. Native-PAGE and Zymography of P2D3 (0.5% DFM, 0.48% WLF). M1: Native PAGE Marker, M2: Zymogram Marker, NP: Native PAGE Sample, P: Protease, S: Cellulase, A: Amylase.

molecular weights of 133 and 14 kDa were identified. The protease molecules were found in the native page gel, likely corresponding to bands 2 and 7. Two bands of cellulase molecules with molecular weights of 62 kDa and 21 kDa were found in the zymogram gel (Figure 5). The cellulase molecules in the native page gel were bands 4 and 6. No amylase molecules were detected in the zymogram gel. When compared to the P1D1 (control) sample, there is a difference, particularly in the 14 kDa protease (Table 7). According to Kulkarni *et al*. (1999), protease enzymes with molecular weights of 14 to 35 kDa are classified as alkaline serine proteases.

In the native page analysis of the P3D3 sample (1.0% DFM, 0.48% WLF), eight bands were observed with molecular weights of 163, 142, 106, 80, 60, 36, 25, and 19 kDa (Figure 6). The zymogram analysis revealed two protease enzyme activity bands with molecular weights of 144 kDa and 25 kDa. These molecules correspond to bands 2 and 7 on the native page gel. Two cellulase enzyme activity bands were found with molecular weights of 62 kDa and 19 kDa, corresponding to bands 5 and 8 on the native page gel. However, amylase enzyme activity was not detected in the zymogram gel. When compared to the control (P1D1), differences were found, including a protease enzyme of 25 kDa in size (Table 7).

Supplementing DFM in concentrate (P treatment) is believed to enhance the diversity of protease molecules in the rumen fluid of cattle. Treatment P2 (0.5% DFM) and P3 (1.0% DFM) showed the presence of small-sized protease

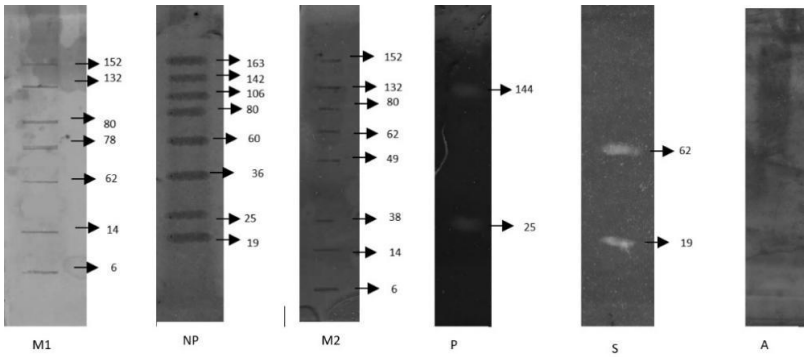

Figure 6. Native-PAGE and Zymography of P3D3 (1.0% DFM, 0.48% WLF). M1: Native-PAGE Marker, M2: Zymogram Marker, NP: Native PAGE Sample, P: Protease, S: Cellulase, A: Amylase.

Table 7. Estimation of protein molecular weight (kDa) in rumen fluids on DFM and WLF supplementation

Treatments	Native page (kDa)	Protease (kDa)	Selulase (kDa)	Amilase (kDa)
P_1D_1	142,123,106,60,48,29,19	144	62, 19	-
P_1D_3	142,114,106,86,69,34,20,12	144	67, 20	-
P_2D_3	142,132,106,60,48,22,13	133, 14	62, 21	-
P_3D_3	163,142,106,80,60,36,25,19	144, 25	62, 19	-

P_1D_1: 0% DFM, 0% WLF; P_1D_3: 0% DFM, 0.48% WLF; P_2D_3: 0.5% DFM, 0.48% WLF; P_3D_3: 1.0% DFM; 0.48% WLF.

molecules, namely 14 and 25 kDa (Table 7). This can be attributed to the presence of bacillus sp. bacteria in DFM. Studies by Lakshmi *et al.* (2018) confirmed that the protease activity of bacillus strain S8 resulted in small-sized molecules around 23 kDa. This is further supported by the research of Singh *et al*. (2012), where *Bacillus cereus* SIU1 exhibited a molecular weight of approximately 22 kDa. Junaidi *et al*. (2017) also reported that *Bacillus cereus* LS2B exhibited alkaline protease activity with molecular weights of 34 kDa, 17 kDa, and 13 kDa. Table 7 explains that in all four tested treatments, the zymogram did not display a clear zone of amylase activity. This might be due to the relatively short incubation time of the gel in the buffer after the native page. Arboleda and Fa (2007) explained that the minimum incubation time is 60 minutes. In this study, the incubation was carried out for only 30 minutes, which could potentially result in the absence of clear zones on the gel electrophoresis. Furthermore, Rajak (2005) highlighted the need for the addition of activators when analyzing amylase enzymes, such as metal ions (Ca, Mn, Mg, Fe) and non-metals like Na. The presence of activators can enhance the activity of amylase enzymes.

Conclusion

The study demonstrates that the supplementation of Direct-Fed Microbials (DFM) and Waru Leaf Flour (WLF) significantly impacts the rumen ecosystem of local beef cattle. The interaction between these supplements affects key parameters, including total protozoa, N-NH_3, total VFA production, and microbial protein synthesis. Additionally, the study provides valuable insights into enzyme activities, particularly protease, cellulase, and amylase. The results suggest that the combination of 0.24% WLF and 0.5% DFM supplementation holds promise for optimizing rumen conditions and improving the performance of local beef cattle, with potential implications for more efficient and sustainable beef production in Indonesia.

Conflict of interest

The authors declare that there are no conflicts of interest in the conduct of research until the writing of this manuscript. The authors have read and agreed to the contents of this manuscript.

Author's contribution

DN conducted to data collection and analysis, laboratory work, and manuscript writing (original). MB designed the experiments, validation and paper review process. SR provided laboratory support, contributed to paper review, validation, and manuscript development.

Ethics approval

There are no animal subjects in this article and informed consent is not applicable.

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