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Research Article

Increasing the *In Vitro* Enzymatic Activity of Cellulase and Amylase from Beef Cattle Rumen Fluid Supplemented with *Moringa oleifera* Leaves and Sulfur

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Abstract

Background and Objective: A combination of feed with *Moringa oleifera* can be used for the optional development of rumen microbes which will provide th protein to host animal. An *in vitro* study was conducted to assess the activity of cellulases, protease and amylase enzymes from beef cattle rumen fluid supplemented with *Moringa oleifera* leaves and sulfur. **Methodology:** The experiment used a Completely Randomized Design (CRD) of 3×2 variables including three concentrations of *Moringa oleifera* leaves (0, 10 and 20%) and two levels of sulfur supplementation (0 and 0.4%), producing six treatments. Each treatment was replicated four times for a total of 24 experimental conditions involving 4 h of *in vitro* incubation each. Rumen fluid derived from bulls was sourced immediately after slaughter. *Moringa oleifera* leaves were obtained from Kutasari village. The activities of cellulases, protease and amylase enzymes were measured. **Results:** The results demonstrated that the interaction between *Moringa oleifera* leaves and supplemental sulfur significantly increased ($p<0.05$) cellulases activity and highly significantly increased ($p<0.01$) amylase activity but had no significant effect ($p>0.05$) on protease activity. Increased supplementation with *Moringa oleifera* leaves decreased protease activity. **Conclusion:** The supplementation of bovine rumen fluid with 20% *Moringa oleifera* leaves and 0.4% sulfur resulted in the highest measured activities of cellulases and amylase.

Key words: *Moringa oleifera*, sulfur, enzymatic activities of protease, cellulases and amylase

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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

Livestock productivity is largely determined by the quality and quantity of feed consumed. Feed quality includes the content of nutrients, such as energy, protein, minerals and vitamins and the content of anti-nutritive substances such as tannins, lignins and other secondary compounds[1]. Ruminants obtain their required amino acids from three sources: The proteins in feed that escape fermentation in the rumen, endogenous proteins derived from the peeling walls of the gastrointestinal tract and microbial proteins that are synthesized in the rumen[2].

Given the important role of rumen microbial protein as a supplement for the host animal, efforts to maximize the production of microbes in the rumen by optimizing conducive environmental conditions are necessary[3]. However, farmers in Indonesia, especially in rural areas, use agricultural waste as forage for their livestock, which is less than optimal support for the development of rumen microbes that provide protein for the host animal. Cattle reared on traditional farms generally experience feed shortages, as the amount of feed provided is usually less than the amount needed, low quality and rarely supplemented with additional food or concentrates[4]. This shortage can be ameliorated through the supplementation of feed with protein sources such as *Moringa oleifera* leaves that contain high levels of protein (25.1-25.3%) and all essential amino acids, vitamins and minerals in high concentrations[5].

To improve the performance of rumen microbes, sulfur, which functions as a cofactor in the synthesis of rumen microbial protein, must be added[3]. Sulfur is a mineral that plays an important role in increasing microbial activity and affects fermentation processes in the rumen. Supplementation with sulfur is indispensable for the synthesis of amino acids that contain sulfur, i.e., methionine, cystine and cysteine[6]. *In vitro* studies have shown that the supplementation of beef cattle feed with up to 15% *Moringa oleifera* leaves can improve the rumen ecosystem[7]. Therefore, supplementation with both *Moringa oleifera* leaves and sulfur is expected to improve rumen microbial activity, which in turn increases enzymatic activity.

Rumen fermentation processes will not occur in the absence of microorganisms that produce enzymes to digest food products. Therefore, protein synthesis by rumen microbes is an indicator of the kinematics of rumen fermentation[8]. *Moringa oleifera* leaves are one source of nitrogen for the synthesis of proteins by rumen microorganisms. This synthesis is more effective with the presence of sulfur as a cofactor because sulfur is a component of the amino acids cysteine and methionine[9-11] and sulfur is an important component of rumen bacteria[12]. Information on the combined use of *Moringa oleifera* leaves and sulfur to increase the activity of cellulases and amylase in bovine rumen fluid has not been reported. It is therefore necessary to conduct a study of the proper combination of *Moringa oleifera* leaves and sulfur to increase the activity of cellulases and amylase in bovine rumen fluid.

MATERIALS AND METHODS

The research material used in this study is the fresh rumen fluid of beef cattle taken from a slaughterhouse immediately after slaughter. Feed consisting of grass and concentrate in proportions of 40:60, respectively, was provided to the animals. The concentrate consists of rice bran, cassava waste, coconut cake, corn flour and mixed minerals. Feed with a proportion of grass to concentrate of 40:60 contains 92.38% dry matter, 12.55% crude protein, 16.52% crude fiber, 10.33% ash and 58.88% Nitrogen Free Extract (NFE). *Moringa oleifera* leaves were dried using an oven at 60°C for 2×24 h and then mashed. The nutrient contents of concentrate, elephant grass and *Moringa oleifera* leaves are listed in Table 2

This study used *in vitro* experiments[13] and a Completely Randomized Design (CRD) of 3x2 variables. The first variable was the level of *Moringa oleifera* leaves (0, 10 and 20%, based on the concentration of dry matter) and the second variable was the level of sulfur supplementation (0 and 0.4%, based on the concentration of dry matter). Thus, six treatments were evaluated and each treatment was replicated four times for 24 sets of experimental conditions. *In vitro* incubation was performed for 4 h. The six treatments were P1 = feed

Table 1: Nutritional content of elephant grass and powdered *Moringa oleifera* leaves

	Feed stuff		
Nutrients	Concentrate	Elephant grass	*Moringa oleifera*
Dry matter (%DM)	89.69	96.41	93.41
Protein (%DM)	13.25	11.50	20.36
Fat (%DM)	8.55	4.59	9.37
Crude Fiber (%DM)	11.32	24.31	4.14
Ash (%DM)	7.04	15.27	9.62
Nitrogen free extract (%DM)	60.25	44.33	56.50

Analyses were conducted in the Laboratory of Animal Nutrition and Feed, Department of Animal Science, Jenderal Soedirman University (UNSOED), April, 2015

consisting of 40% grass, 60% concentrate (without *Moringa oleifera* leaves or sulfur) (basic feed); P2 = P1+0% *Moringa oleifera* leaves+0.4% sulfur; P3 = P1+10% *Moringa oleifera* leaves+0.0% sulfur; P4 = P1+10% *Moringa oleifera* leaves+0.4% sulfur; P5 = P1+20% *Moringa oleifera* leaves+0.0% sulfur; P6 = P1+20% *Moringa oleifera* leaves+0.4% sulfur.

Time and location: The study was conducted from May, 2016 until June, 2016 in the Laboratory of Animal Nutrition and Food Sciences (INMT Lab), Faculty of Animal Science, Jenderal Soedirman University (UNSOED) Purwokerto, Central Java, Indonesia.

Collection of *Moringa oleifera* leaves: *Moringa oleifera* leaves collected from Kutasari village, Baturaden Regency, Purwokerto were dried at room temperature (25±4°C) for 7 days. The dried leaves were then milled using a blender[13].

Rumen fluid collection: Rumen fluid was obtained from male beef cattle slaughtered in Bantarwuni Slaughterhouse in Purwokerto. Rumen was taken from animals immediately after slaughter and squeezed and filtered using calico cloth. The resulting rumen fluid was placed in a warm thermos and immediately transported to the Laboratory of Nutrition and Feed (INMT Lab) Faculty of Animal Science, Jenderal Soedirman University (UNSOED), where CO_2 was added.

***In vitro* experiments:** *In vitro* digestion was performed using a 250 mL Erlenmeyer flask filled with 2 g of sample. The sample was then added to 16 mL of rumen fluid and 24 mL of McDougalls solution, with a pH 6.5-6.9. The mixture was shaken and CO_2 was passed through the mixture for 30 sec. Before being placed into a water shaker bath, the flask was covered with ventilated rubber. The flask was then incubated in the water shaker bath at a temperature of 39°C for 4 h. After 4 h, three drops of HgCl were added to the fermentation flask and the flask was centrifuged in a Qynamica Type Velocity 14® centrifuge at 12,000 rpm. The supernatant was stored in a freezer and enzymatic activities were assessed; cellulases activity was analyzed using a method of Camassola and Dillon[14], protease activity was analyzed using a method described by Walter[15] and amylase activity was analyzed using a method described by Bernfeld[16]. The absorbance of cellulases enzymes was determined using a UV spectrophotometer (HITACHI U-3900®) at a wavelength of 550 nm. The absorbances of protease and amylase were read at wavelengths of 578 and 540 nm, respectively.

Variables measured: The variables measured were the enzymatic activities of (1) Cellulase[14], (2) Protease[15] and (3) Amylase[16].

Statistical analysis: Data were analyzed using one way analysis of variance followed by a polynomial orthogonal test[17].

RESULTS AND DISCUSSION

The average cellulases, protease and amylase activities measured are listed in Table 2. This study demonstrates that the interaction between *Moringa oleifera* leaves and supplemental sulfur significantly increased ($p<0.05$) cellulases activity and highly significantly increased ($p<0.01$) amylase activity but had no significant effect ($p>0.05$) on protease activity. However, increasing levels of *Moringa oleifera* supplementation decreased the activity of protease ($p<0.01$) in accordance with the equation Y = 17.362-0.3018X with a determination coefficient (r^2) of 0.8.

Cellulases activity following supplementation with *Moringa oleifera* leaves without the addition of sulfur (0%) was linear, following the equation Y = 8.3208+0.3233X with a coefficient of determination (r^2) of 0.95, while the addition of 0.4% sulfur resulted in activity following the equation Y = 9.9904+0.2996X with a coefficient of determination (r^2) of 0.95 (Fig. 1). The activity of cellulases in rumen fluid supplemented with *Moringa oleifera* leaves and 0.4% sulfur was higher than that of fluid with *Moringa oleifera* leaves but without sulfur supplementation.

Table 2: Mean enzymatic activities of cellulases, protease and amylase and protein levels in rumen fluid

Treatments	Cellulases enzymatic activity (U mg^{-1} protein)	Protease enzymatic activities (U mg^{-1} protein)	Amylase enzymatic activity (U mg^{-1} protein)
P1	8.1181±0.50	18.2473±1.11	0.3643±0.0106
P2	12.2780±0.18	16.1729±1.91	0.1842±0.0169
P3	11.9543±0.63	14.4065±0.31	0.9917±0.0591
P4	12.4124±0.26	14.8906±0.90	0.9067±0.0871
P5	14.5845±0.62	12.0365±0.67	0.7953±0.0829
P6	16.2653±0.62	10.3103±0.85	1.1548±0.0571

P1: Basal feed (40% elephant grass, 60% concentrate) without *Moringa oleifera* leaves or sulfur, P2 = P1+0% *Moringa oleifera* leaves+0.4% sulfur, P3 = P1+10% *Moringa oleifera* leaves+0.0% sulfur, P4 = P1+10% *Moringa oleifera* leaves+0.4% sulfur, P5 = P1+20% *Moringa oleifera* leaves 0.0% sulfur, P6 = P1+20% *Moringa oleifera* leaves+0.4% sulfur

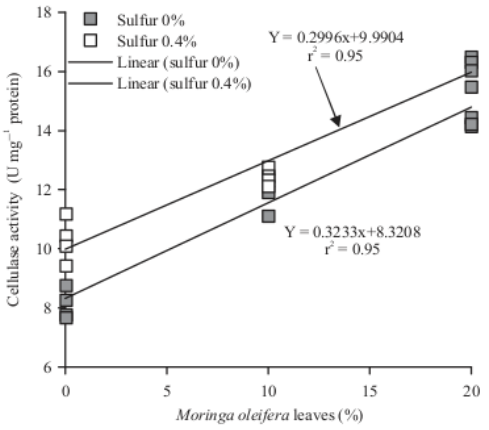

Fig. 1: Effect of the interaction between *Moringa oleifera* leaves and sulfur on cellulases activity in rumen fluid

Supplementation with 0.4% sulfur, when combined with up to 20% *Moringa oleifera* leaves, increases the activity of cellulolytic enzymes. This occurs because this combination provides sufficient cofactor availability for microbial protein synthesis and provides optimal environmental conditions in rumen fluid for cellulolytic microorganisms. The ability of sulfur to increase the activity of cellulases is supported by the work of Bal and Ozturk[18], which hypothesizes that sulfur also supports crude fiber degradation in the rumen and stimulates the growth of cellulolytic bacteria.

Higher levels of *Moringa oleifera* leaf supplementation in feed led to increased cellulases activity. This is likely due to the availability of ammonia, fiber and cofactors for microbial protein synthesis, which is also higher with greater supplementation. The protein content of the *Moringa oleifera* leaves used in the study is quite high (20.36%). The high protein content of *Moringa oleifera* leaves is in accordance with the study of Bey[19], who found that *Moringa oleifera* leaves have a high protein content (27.1%) and are a plausible source for the high availability of ammonia in the rumen fluid, which results in increased microorganism activity, including cellulolytic microorganisms. This conclusion is supported by a study conducted by Suhartati *et al.*[7] showing that feed supplementation with *Moringa oleifera* leaves up to 15% increases the concentration of ammonia and the bacterial count in the rumen fluid[7]. The availability of ammonia also affects the pH stability under neutral conditions. RAGFAR[20] states that cellulolytic microorganisms work optimally at a neutral pH between 6.3-6.8. Supplementation with *Moringa oleifera* leaves also increases crude fiber levels. According to Budiansyah[21], increasing the concentration of dietary fiber increases cellulolytic activity. Therefore, the use of higher concentrations of *Moringa oleifera* leaves increases cellulolytic activity.

The effect of supplementation with *Moringa oleifera* leaves on protease activity is linear (Fig. 2), following the equation Y = 17.362-0.3018X with a determination coefficient (r^2) of 0.80. The equation indicates that higher concentrations of *Moringa oleifera* leaves decrease protease activity. These results are in agreement with the study of Bijina *et al.*[22], who found that *Moringa oleifera* leaves contain high levels of protease inhibitory activity. Among the different parts of *M. oleifera* leaves tested, crude extract isolated from mature leaves and seeds showed the highest level of trypsin inhibition. Among the various extraction media evaluated, crude extract prepared in phosphate buffer resulted in maximum recovery of protease inhibition, with high inhibitory activity toward serine proteases such as thrombin, elastase, chymotrypsin and cysteine. Bijina *et al.*[8] reported that glycine, glutamic acid, alanine, proline and aspartic acid are the major amino acids constituting the inhibitor protein. Maximal activity was observed at pH 7 and at 40°C. Because an *in vitro* study was conducted using a temperature of 40°C and a pH between 6.8 and 7, the conditions required for inhibitor activity were fulfilled. Hence, increasing concentrations of *Moringa oleifera* leaves were accompanied by decreasing protease activity.

The effect of supplementation with *Moringa oleifera* leaves without the addition of sulfur on amylase activity is quadratic, following the equation Y = 0.3643+0.1039X-0.0041X2, with a coefficient of determination (R^2) of 0.96 and a peak of P (12.67, 1.024). The effect of supplementation with *Moringa oleifera* leaves and 0.4% sulfur was linear, following the equation Y = 0.2633+0.0485X with a coefficient of determination (r^2) of 0.91 (Fig. 3). Feed supplemented with *Moringa oleifera* leaves without sulfur (0%) led to increased amylase activity at supplementation levels up to 12.67% and amylase activity declined at higher levels. The 12.67% supplementation level provides growth factors such as minerals, vitamins and amino acids for amylolytic microorganisms that contribute to carbon and ammonia skeletons. These growth factors lead to an increased mass of amylolytic microorganisms along with increased amylase activity. Different results are found at higher levels of *Moringa oleifera* supplementation; amylase activity decreases. Adisakwattana and Chanathong[23] found that phenolic compounds, flavonoids and tannins in *Moringa oleifera* leaves have the ability to inhibit the activity of some amylolytic enzymes such as α-glucosidase and

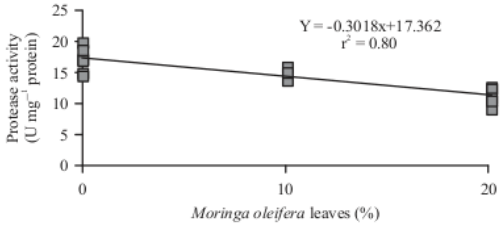

Fig. 2: Effect of *Moringa oleifera* leaves on protease activity in rumen fluid

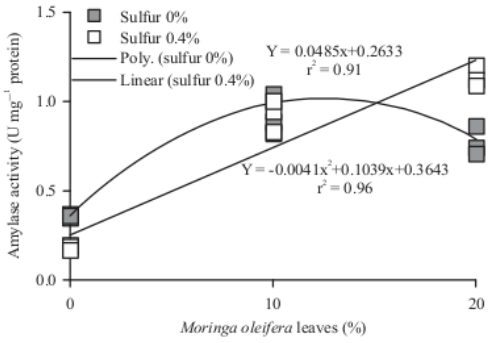

Fig. 3: Effects of the interaction between *Moringa oleifera* leaves and sulfur on amylase activity

α-amylase. Thus, supplementation with high levels of *Moringa oleifera* leaves decreases amylase activity.

In contrast, the combination of *Moringa oleifera* leaves and 0.4% sulfur continues to increase amylase activity with increasing levels of *Moringa oleifera* leaves. This is because the supplementation of 0.4% sulfur increases the synthetic activity of microorganisms including amylolytic microorganisms. Komizarczuk and Durand[24] found that sulfur is an important growth factor for rumen microorganisms. Sulfur is required by rumen microbes for the synthesis of sulfur-containing amino acids. Sulfur content in the microbial biomass can reach approximately 8 g kg^{-1} of dry matter and is primarily contained in proteins[25]. Therefore, the combination of *Moringa oleifera* leaves and 0.4% sulfur provides an excellent growth factor for microorganisms and increases amylase activity.

The increased activity of cellulases and amylase following supplementation with *Moringa oleifera* leaves and sulfur is promising for beef cattle farming, because it can increase livestock productivity. Increased livestock productivity leads to higher incomes and better farmer welfare. The results of this study can be applied directly to the farming of beef cattle and recommend the addition of 20% *Moringa oleifera* leaves and 0.4% sulfur (based on the concentration of dry matter) into the feed.

CONCLUSION

The results of this study conclude that supplementation of cattle feed with 20% *Moringa oleifera* leaves and 0.4% sulfur (based on the concentration of dry matter) resulted in the highest measured activities of cellulases and amylase.

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