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RESEARCH ARTICLE

Preliminary Study on Rumen Degradation of Proteins Having Different Molecular Weights from Malunggay (*Moringa oleifera* Lam) leaves

Phoebe Lyndia T. Llantada[1]*, Renato SA Vega[2]

[1]*Philippine Carabao Center at Cagayan State University, Baung, Piat, Cagayan, 3527 Philippines.*
[2]*Institute of Animal Science, College of Agriculture and Food Science, University of the Philippines Los Baños, College, Laguna, 4031 Philippines.*

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Corresponding Author: Philippine Carabao Center at Cagayan State University, Baung, Piat, Cagayan, 3527 Philippines.

Abstract

This study aimed to assess the ruminal degradation of *Moringa oleifera* leaves using the in-sacco technique and SDS-PAGE analysis. Three drying methods (fresh, cool-dried, and oven-dried) were evaluated using two fistulated cows. Five-gram samples were incubated in the rumen for varying durations (6, 12, 24, 36, 48, and 72 hours), and SDS-PAGE was employed for protein analysis. The results revealed that most proteins in *M. oleifera* underwent degradation within the first 6 to 12 hours of incubation across all drying methods. However, cool-dried and oven-dried samples retained certain proteins even after 12 to 72 hours of incubation. Notably, proteins with molecular weights of approximately 120.0, 51.0, 37.6, and 26.583 kDa persisted in these samples, suggesting resistance to complete degradation in the rumen. These proteins may serve as a source of amino acids in the lower gastrointestinal tract, potentially influencing milk production in dairy animals. In conclusion, this study identifies specific *M. oleifera* proteins that resist ruminal degradation, highlighting their potential role in amino acid supply and their possible impact on milk production in dairy animals.

1. Introduction

In developing countries, ruminant production is a promising livestock sector that can significantly improve rural communities, as the feed for these animals does not necessarily compete with human consumption. Additionally, the continuous increase in population growth in these regions will drive higher demand for milk and meat. The relatively high price of beef and milk should encourage farmers to invest in raising ruminants.

In tropical regions like the Philippines, ruminant production remains a major challenge for both the government and the private sector. Identifying suitable feedstuffs that are rich in digestible protein, minerals, and vitamins continues to be a crucial issue in dairy and beef production.

In the country, *Moringa oleifera*, locally known as malunggay, is an abundantly available plant with potential as a feed source for both ruminants and monogastric livestock. It is commonly used as human food and is known for its high nutritional value. Additionally, due to its ability to enhance milk production in lactating mothers and its drought resistance, it serves as an alternative forage source for livestock, particularly during the summer when grass is scarce.

Moringa oleifera leaves contain 27.51% crude protein, 19.25% crude fiber, 2.23% crude fat, 7.13% ash content, 76.53% moisture content, 43.88% carbohydrate content, and 1296.00 kJ/g of energy (Ellis et al., 2008). Given its high protein content, *M. oleifera* leaves could serve as an affordable and effective alternative source of roughage and feed ingredients for livestock.

The use of alternative roughage sources like malunggay, which is high in protein, inexpensive,

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and locally available, would greatly benefit the cattle industry. Protein is the most expensive component of cattle feed and has the most complex structure among all nutrients. It plays a crucial role in maintenance, growth, reproduction, and lactation.

In ruminants, most dietary proteins are degraded by rumen microorganisms, producing ammonia and microbial protein. However, some proteins remain undigested by rumen microbes and are known as bypass proteins or rumen undegradable proteins (RUP). These proteins pass through the rumen and enter the abomasum and small intestine, where they are digested by proteolytic enzymes from the pancreas, ultimately releasing amino acids. According to Roxas (2006), incorporating high-quality protein in ruminant diets enhances bypass protein availability, ensuring that amino acids are utilized by the host animal rather than by rumen microorganisms.

Therefore, this study was conducted to determine whether malunggay protein can bypass ruminal degradation at 24, 36, 48, and 72 hours. The general objective of this study was to explore and identify the protein profile of malunggay leaves before and after ruminal degradation. Specifically, it aimed to (1) evaluate the molecular weight of proteins present in fresh, oven-dried, and cool-dried malunggay leaves using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), (2) compare the molecular weight of malunggay proteins exposed to the cattle rumen at 6, 12, 24, 36, 48, and 72 hours, and (3) determine if any rumen-indigestible peptides are present in malunggay samples after more than 12 hours of ruminal exposure using SDS-PAGE analysis.

2. Materials and Methods

2.1 Time and Place of Study

The research was carried out at the metabolism house of the Institute of Animal Science, College of Agriculture and Food Science, University of the Philippines Los Baños, College Laguna.

2.2 Plant Materials

The study focused on *Moringa oleifera* Lam, commonly known as the horseradish tree or malunggay in Filipino. Fresh malunggay leaves underwent cool drying and oven drying processes, resulting in green powder and greenish-brown powder, respectively. For each incubation period (6, 12, 24, 36, 48, 72 hours), and for each of the two animals (animal 1, animal 2), five grams of each sample (fresh, oven-dried, and cool-dried malunggay leaves) were enclosed in nylon bags (6.5 x 14 cm, mesh size 30-50µm), replicated three times.

Table 1. *Time and date of placing the samples inside the rumen.*

Incubation period (hrs)	Day	Time of placing nylon bags inside the rumen
72	1	8:00 am
48	2	8:00 am
36	2	8:00 pm
24	3	8:00 am
12	3	8:00 pm
6	4	2:00 am
Removal of bags	4	8:00 am

2.3 Ethical approval

All experimental procedures, including animal maintenance and sample collection, were conducted following the guidelines of the ethical committee of the University of the Philippines and as describe by the Animal Research: Reporting of In Vivo Experiments (ARRIVE) (Percie du Sert, et al., 2020).

2.4 Experimental animals and treatment

Two healthy fistulated cows with an average weight of 417.5 ± 12.5 kg were used in this study. The animals were fed with napier grass and concentrate twice daily, morning and afternoon. The rumens of both animals were used for the incubation of nylon bags containing various treatments (fresh, oven-dried, cool-dried) for each time duration (6, 12, 24, 36, 48, 72 hours). Nylon bags were sequentially placed in the rumen, anchored with a flexible nylon cord. The incubation schedule, as detailed in Table 1, ensured the systematic and timely retrieval of samples from the rumen.

2.5 Washing and Storing of Samples

Following incubation, nylon bags were immediately removed from the rumen, and washed under cold running water to halt fermentation and bacterial actions and eliminate adhering feed materials. After

thorough washing, the nylon bags were stored in the refrigerator to prevent protein denaturation.

2.6 Protein Suspension Preparation

The samples were thawed, weighed, and transferred to a mortar, where they were crushed with liquid nitrogen. They were then homogenized in a Tris-HCl solution and transferred to labeled microtubes, which were placed on ice and incubated overnight at 4°C.

Centrifugation was performed in three rounds (1,000g for 5 min, 4,500g for 10 min, and 14,000g for 25 min) at 4°C to separate the supernatant from the precipitate. Between rounds, samples were transferred to pentubes and kept on ice before another overnight incubation at 4°C.

The following day, sterilized tubes were prepared for denaturation. Each sample (80µl) was mixed with 20µl of buffer, vortexed, heated at 90°C for 5 minutes, and vortexed again before further analysis.

2.7 Protein Characterization

Denatured samples were transferred to fresh tubes and mixed with sample buffer. The mixture was vortexed and subjected to a water bath at 90°C for 5 minutes. Subsequently, protein samples were separated using SDS-PAGE under the following apparatus settings: a voltage of 200 volts, a run time of 50 minutes (MOPS buffer), and an initial current of 100–125 mA per gel, which decreased to 60–80 mA per gel by the end of the run. The Invitrogen Mini-Cell determined protein concentration, while Molecular Weight Standards from Invitrogen characterized the molecular weights of isolated proteins within the NuPAGE Gel.

2.8 Staining

After removing the cassette from the mini-cell, the gels were washed with deionized water. Each gel was carefully extracted and placed in a separate container with 100 mL of pure water, rinsed three times for 5 minutes each, with the rinse discarded after every step.

The gels were then soaked in ~20 mL of Simply Blue Safe Stain for 1 hour at room temperature with constant shaking. After staining, the stain was discarded, and the gels were washed with 100 mL of deionized water using a shaker for at least 1 hour. A second 1-hour wash was performed to enhance band visibility and reduce background noise. Finally, the bands were visualized by placing the gels on a whitesurface and were captured using a Canon® DSLR

2.9 Analysis of Band Intensities

Captured bands were uploaded, and protein band intensities at different molecular weights were measured using ImageJ® (NIH, 2009) software.

Table 2. *Means of the thawed weight of fresh, cool, and oven-dried samples of malunggay at varying incubation periods in cattle's rumen.*

Duration (hours)	Fresh	Cool Dried	Oven Dried	Mean
0	5.00	5.00	5.00	5.00
6	0.82	1.35	1.51	1.23
12	0.54	1.04	1.41	0.997
24	0.49	0.98	1.38	0.95
36	0.44	0.70	1.11	0.75
48	0.48	0.68	0.75	0.64
72	0.06	0.72	0.56	0.45

3. Results and Discussion

3.1 Protein Degradation Patterns in the Rumen

This study investigated the ruminal degradation of *Moringa oleifera* leaf proteins by comparing fresh, oven-dried, and cool-dried samples using electrophoresis rather than traditional proximate analysis. While proximate analysis provides a general assessment of feed composition, electrophoresis enables the identification of specific protein fractions and their degradation dynamics.

Thawed weight data (Table 2) revealed that fresh *M. oleifera* leaves exhibited the highest degradation rates, while oven-dried and cool-dried samples degraded more slowly. This suggests that moisture removal and increased dry matter content hinder microbial enzymatic activity in the rumen, leading to slower protein breakdown. These findings align with previous studies demonstrating that water activity influences microbial metabolism and proteolysis in ruminal environments (Henderson et al., 2015). Consequently, drying methods may modulate protein availability by altering microbial access to protein substrates.

3.2 Electrophoretic Profiling and Protein Identification

SDS-PAGE analysis (Table 3, Figures 2–5) identified seven distinct protein bands in *M. oleifera* leaves. Among these, three proteins were tentatively assigned based on molecular weight comparisons with standard markers: ATP Synthase/Ribulose biphosphate carboxylase (51 kDa), NADH dehydrogenase (37.6 kDa), Maturase K (26.583 kDa). The majority of proteins remain unidentified, highlighting the need for advanced proteomic characterization using mass spectrometry-based techniques such as LC-MS/MS to elucidate their biological functions and potential contributions to ruminant nutrition.

Table 3. *Putative Identification of Proteins at Various Molecular Weights (kDa) Incubated in the Rumen of Cattle, Based on UniProt Molecular Weight References and Protein Descriptions.*

Protein Number	Fresh Sample (kDa)	Cool Dry Sample (kDa)	Oven Dry Sample (kDa)	MW (kDa)	Putative Peptide from UniProt®
1	120	120	120	-	-
2	90	90	90	-	-
3	70	70	70	-	-
4	51	51	51	51	ATP synthase or Rubisco
5	37.6	37.6	37.6	37.6	NADH Dehydrogenase
6	26.583	26.583	26.583	26.583	Maturase K
7	17	17	17	-	-

A progressive reduction in band intensity with increasing rumen incubation time indicated extensive microbial degradation of *M. oleifera* proteins. This is consistent with proteolysis driven by ruminal microbial enzymes, leading to the hydrolysis of proteins into peptides and free amino acids for microbial assimilation (Van Amburgh et al., 2015).

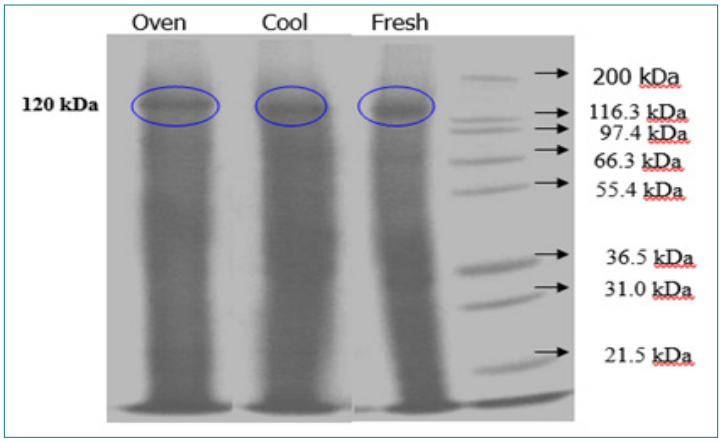

Figure 1. *Fresh, oven dry and cool dry Moringa oleifera leaves before rumen incubation with various molecular weights standard protein separated using 12% SDS-PAGE running gel.*

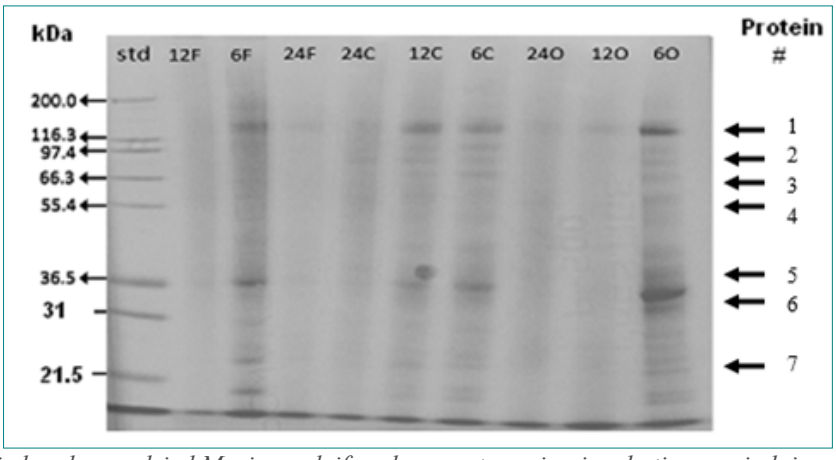

Figure 2. *Fresh, cool dried and oven dried Moringa oleifera leaves at varying incubation period, i. e. 6, 12 and 24 hours with molecular weight standard protein using 12 % SDS-PAGE running gel. (Animal 1)*

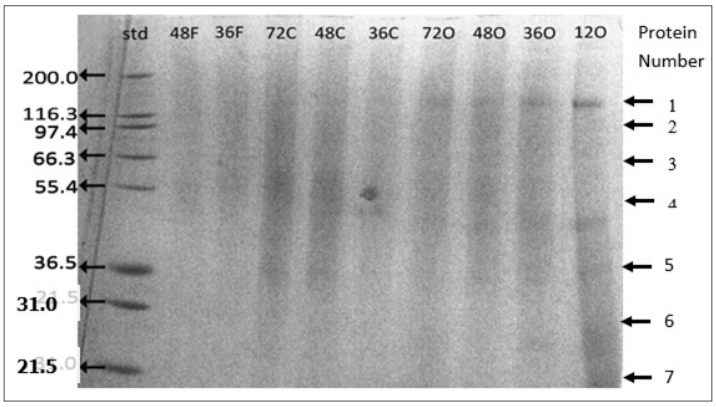

Figure 3. *Fresh, cool dried and oven dried Moringa oleifera leaves at varying incubation period, i. e. 36, 48 and 72 hours with molecular weight standard protein using 12 % SDS-PAGE running gel. (Animal 1)*

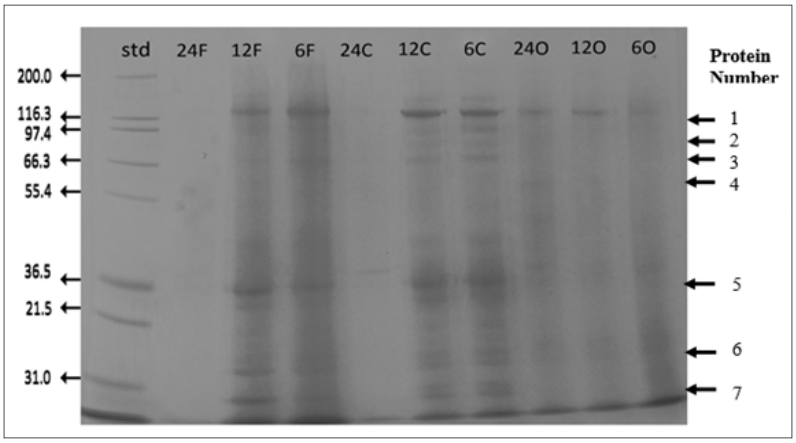

Figure 4. *Fresh, cool dried and oven dried Moringa oleifera leaves at varying incubation period, i. e. 6, 12 and 24 hours with molecular weight standard protein using 12 % SDS-PAGE running gel. (Animal 2)*

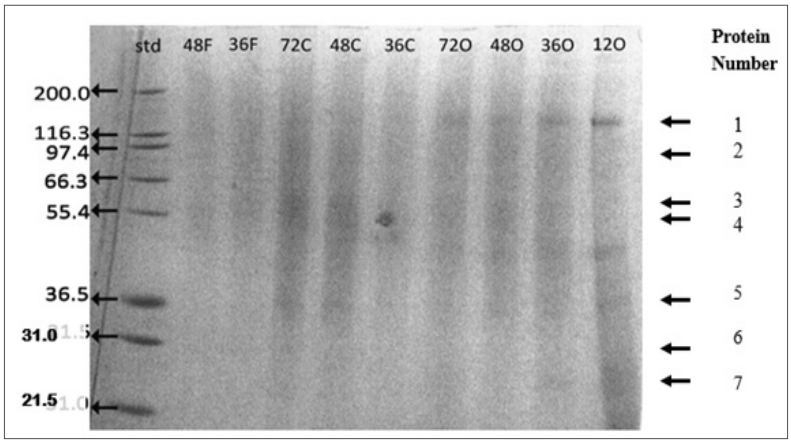

Figure 5. *Fresh, cool dried and oven dried Moringa oleifera leaves at varying incubation period, i. e. 36, 48 and 72 hours with molecular weight standard protein using 12 % SDS-PAGE running gel. (Animal 2)*

3.3 Differential Degradation Across Drying Methods

Protein degradation patterns varied depending on the drying method (Figures 6–12). The 120 kDa protein (Figure 6) degraded completely in fresh leaves after 12 hours, in cool-dried samples after 24 hours, and in oven-dried samples after 36 hours, suggesting a protective effect of drying on protein stability. Similarly, the 90 kDa (Figure 7), 70 kDa (Figure 8), and 17 kDa (Figure 12) proteins exhibited faster degradation in fresh and oven-dried samples compared to cool-dried samples. In contrast, the 37.6 kDa (Figure 10; NADH dehydrogenase) and 26.583 kDa (Figure 11; Maturase K) proteins were the most resistant to ruminal degradation, persisting up to 72 hours in dried samples. These findings suggest that certain proteins in *M. oleifera* may act as bypass proteins, escaping ruminal degradation and reaching the abomasum for post-ruminal digestion and absorption. The persistence of these proteins in dried samples indicates that drying may enhance protein

stability, potentially increasing rumen undegradable protein (RUP) fractions.

3.4 Potential Physiological Implications: The GH-IGF-1 Axis and Milk Production

Increased bypass protein availability in the abomasum could have significant physiological implications, particularly for growth hormone (GH) regulation via ghrelin secretion. Ghrelin, a GH secretagogue, is stimulated by amino acid availability and triggers GH release from the pituitary gland (Sugino et al., 2009). GH subsequently promotes insulin-like growth factor 1 (IGF-1) synthesis in the liver, which is critical for growth, lactation, and metabolic homeostasis (Bauman, 2014).

The potential role of *M. oleifera* in stimulating ghrelin-GH-IGF-1 signaling suggests that its protein composition may contribute to improved feed efficiency and milk production. While this hypothesis requires in vivo validation, it aligns with previous research showing that dietary protein quality directly affects GH and IGF-1 levels in ruminants (Elsabagh et al., 2020).

Although these findings highlight the potential of *Moringa oleifera* as a protein source in ruminant diets, further research is required to validate its effects on protein metabolism and production performance. Future studies should focus on proteomic characterization using LC-MS/MS to accurately identify the remaining *M. oleifera* proteins and their functional roles. Additionally, quantification of bypass protein fractions through high-performance liquid chromatography (HPLC) and protein sequencing is essential to determine their contribution to rumen undegradable protein (RUP). In vivo feeding trials should be conducted to evaluate the impact of *M. oleifera* protein fractions on ghrelin secretion, GH-IGF-1 axis activation, and milk yield. Moreover, optimizing drying methods to enhance RUP content while maintaining overall protein digestibility will be crucial for maximizing the nutritional benefits of *M. oleifera* in ruminant diets.

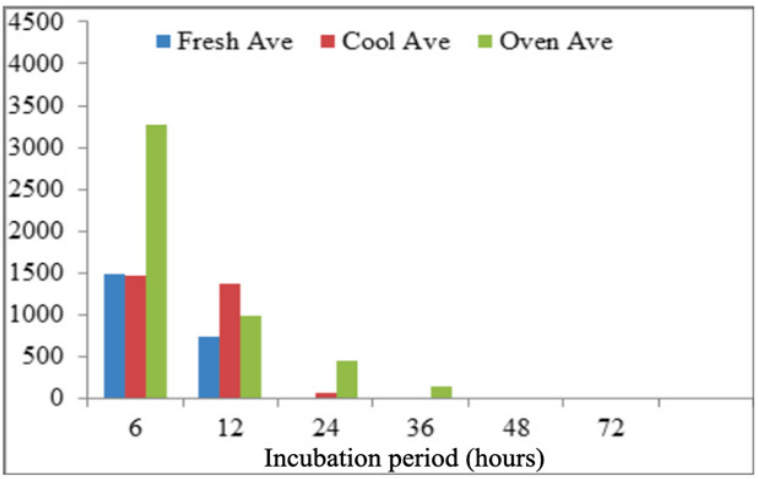

Figure 6. *Means of band intensity of 120 kDa protein in Moringa oleifera incubated at 6, 12, 24, 36, 48 and 72 hours in the rumen of cattle.*

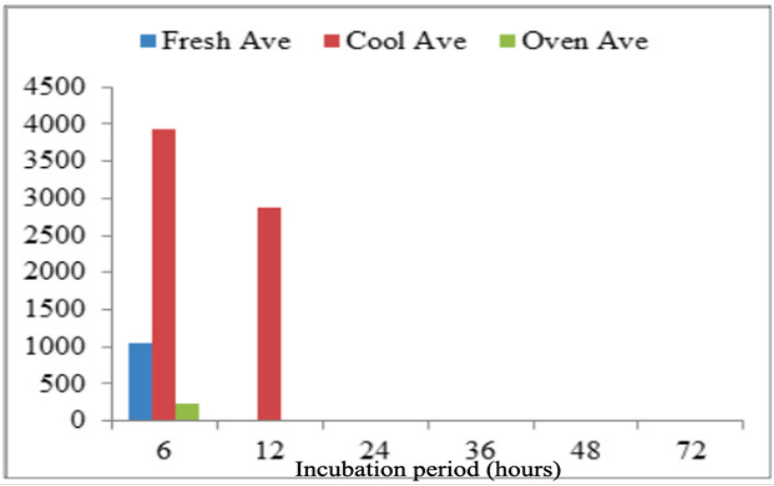

Figure 7. *Means of band intensity of 90 kDa protein in Moringa oleifera incubated at 6, 12, 24, 36, 48 and 72 hours in the rumen cattle.*

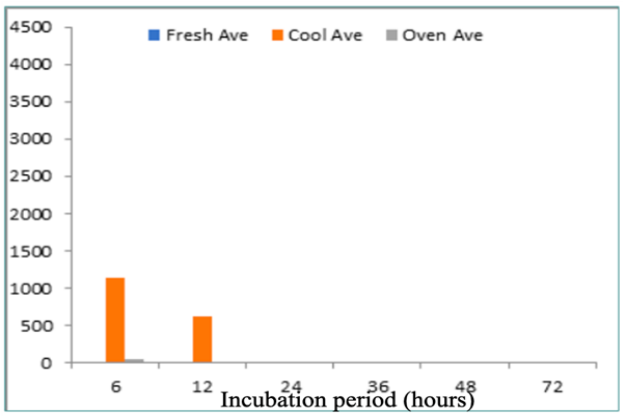

Figure 8. *Means of band intensity of 70 kDa protein in Moringa oleifera incubated at 6, 12, 24, 36, 48 and 72 hours in the rumen cattle*

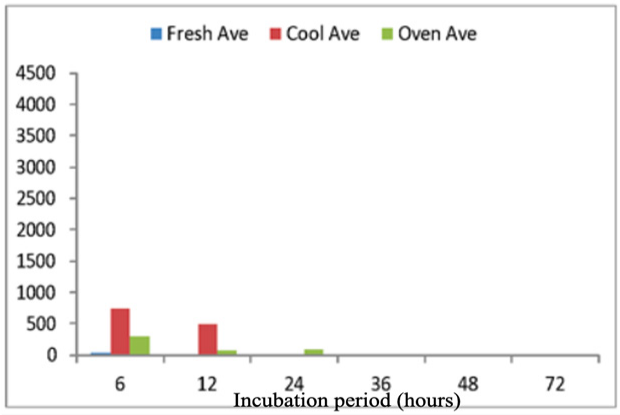

Figure 9. *Means of band intensity of 51 kDa protein in Moringa oleifera incubated at 6, 12, 24, 36, 48 and 72 hours in the rumen cattle.*

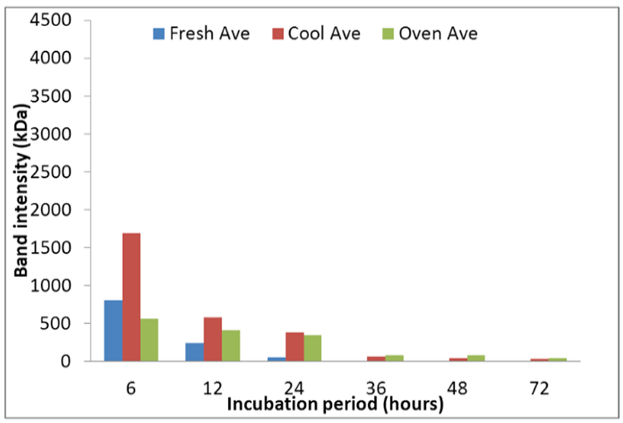

Figure 10. *Means of band intensity of 37.6 kDa protein in Moringa oleifera incubated at 6, 12, 24, 36, 48 and 72 hours in the rumen cattle*

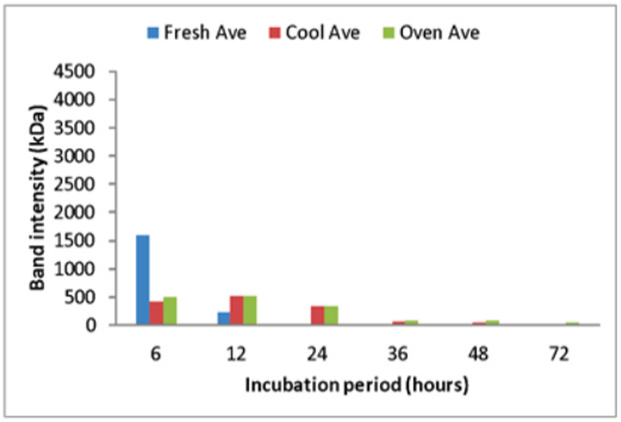

Figure 11. *Means of band intensity of 26. 283 kDa protein in Moringa oleifera incubated at 6, 12, 24, 36, 48 and 72 hours in the rumen cattle*

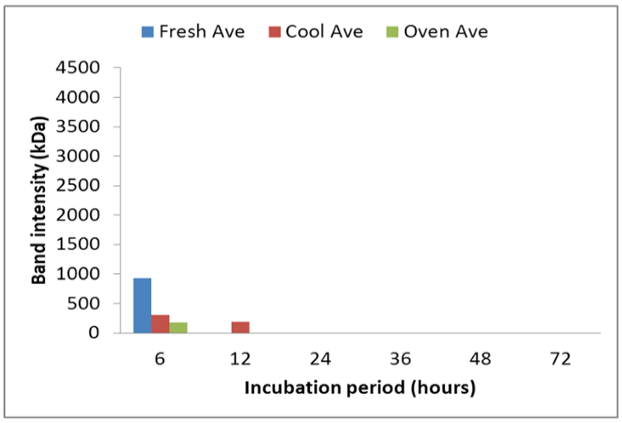

Figure 12. *Means of band intensity of 17 kDa protein in Moringa oleifera incubated at 6, 12, 24, 36, 48 and 72 hours in the rumen cattle.*

4. Summary and Conclusion

This study presents a novel approach to determining the molecular weights of *Moringa oleifera* (malunggay) proteins degraded in the rumen. Our findings indicate that *M. oleifera* proteins pass from the rumen to the lower gastrointestinal tract within 12 hours. Specifically, the 120, 51, 37.6, and 26.583 kDa proteins appear to escape microbial degradation. After 72 hours of rumen incubation, only two proteins, located at 37.6 and 26.583 kDa, remained undegraded. These putative proteins, identified as NADH dehydrogenase and Maturase K, may contribute to rumen-undegradable protein (RUP) fractions.

Furthermore, SDS-PAGE protein separation confirmed that while most proteins in *M. oleifera* leaves undergo degradation in the rumen within 72 hours, some remain intact for extended periods. The band intensity observed in the gels suggests that proteins with higher molecular weights degrade more slowly than those with lower molecular weights. These findings highlight the potential of *M. oleifera* as a source of bypass proteins, which may enhance post-ruminal protein availability in ruminants.

Recommendation

The potential role of *Moringa oleifera* (malunggay) in enhancing milk secretion should be further investigated through studies involving goats, sheep, cattle, or carabaos with cannulated abomasa. Future research should include measurements of blood ghrelin, growth hormone, the IGF complex, and milk production to determine the physiological effects of *M. oleifera* proteins. Furthermore, preliminary findings suggest that proteins with molecular weights of 120, 51, 37.6, and 26.583 kDa may escape rumen degradation, potentially serving as a source of amino acids that stimulate the secretion of endogenous growth hormone secretagogues. This mechanism could establish a link between the stomach and anterior pituitary GH secretion, ultimately influencing metabolic regulation and lactation performance. Therefore, further studies are essential to validate these findings and optimize the use of *M. oleifera* as a functional feed ingredient in dairy production.

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APPENDICES

Appendix 1. Protocol for SDS-PAGE:

1. Place the mini-gel buffer core into the lower buffer chamber.
2. Put the gel tension wedge into the lower buffer chamber.
3. Removed the gel cassette from the gel pouch.
4. Bathe with deionized water the gel cassette.
5. Peel the tape off the bottom of the cassette.
6. Pull the comb out of the cassette with care.
7. Place one or two gels in the Mini-Cell in such a way that the notched well side of the cassette faces the buffer core (if only one gel is being run, the molded buffer dam replaces the second gel cassette).
8. Clamp the gels in the lower buffer chamber by pulling the gel tension wedge forward.
9. Fill the upper buffer chamber with 1X running buffer (approximately 200ml), ensuring that all the wells are full of running buffers and that air bubbles are displaced from the wells.
10. Check for leakage of 1X running buffer from the upper to the lower chamber. In case of leakage, repeat steps 7 through 9.
11. Load (underlay) the samples into the wells with 20-µl pipette tips. (Note: use of special narrow pipette tips can maximize the volume that can be loaded by starting at the bottom of the well and slowly raising the tip during the loading process. Load unused wells with sample buffers used to prepare the samples. Be sure to load each lane with the same volume to prevent the appearance of uneven width of protein bands).
12. Fill the lower buffer chamber with 1X running buffer (approximately 600 ml).
13. With the power OFF, place the lid on the buffer core. The lid can be firmly placed only if the negative electrode is aligned correctly with the banana plug on the right.
14. Connect the electrode cables to the power supply.
15. Turn on the power supply and run gels, usually 130 volts for 90 min.
16. When the run is complete (dye front near bottom of gel), shut off the power supply, disconnect the electrodes, and remove the gels from the Mini-Cell.