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# Effect of Increasing Levels of *Gracilaria sp*. in Complete Feed on In Vitro Rumen pH, Total Volatile Fatty Acids, and Ammonia Concentrations

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**Abstract:** *This study evaluated the effects of increasing inclusion levels of Gracilaria sp. in complete feed on in vitro rumen fermentation characteristics, including pH, total volatile fatty acid (VFA), and ammonia ($NH_3$) concentrations. The experiment was conducted using a completely randomized design consisting of four dietary treatments and three replications. The treatments were increasing level of Gracilaria sp. in the diets, i.e. 0% (G0), 5% (G5), 10% (G10), and 15% (G15). Fermentation characteristics were evaluated using the first stage of the Tilley and Terry in vitro technique. Data were analyzed using one-way ANOVA followed by Duncan's Multiple Range Test. The inclusion of Gracilaria sp. significantly increased ($P<0.05$) total VFA and $NH_3$ concentrations but did not affect rumen pH. Total VFA concentration increased from 68.89 mM in G0 to 105.80 mM in G15, while $NH_3$ concentration increased from 7.82 mM to 10.75 mM. Rumen pH remained within the optimal range for microbial fermentation (6.86–7.00) across all treatments. It can be concluded that the inclusion of Gracilaria sp. up to 15% in complete feed improves rumen fermentation by increasing VFA and $NH_3$ production while maintaining a stable ruminal pH. The highest fermentation response was obtained at the 15% inclusion level.*

## I. INTRODUCTION

Feed is one of the most important factors determining livestock productivity because it directly influences animal growth, health, and production performance. High-quality diets must provide adequate amounts of essential nutrients, including protein, carbohydrates, lipids, fiber, minerals, vitamins, and water. To ensure balanced nutrient supply, complete feeds are increasingly used because they are formulated to meet the nutritional requirements of animals within a single ration. The use of locally available feed resources has attracted increasing attention as a strategy to improve feed quality while reducing production costs. Indonesia possesses extensive marine resources, with approximately 71% of its territory consisting of coastal and marine

ecosystems [1]. Among these resources, marine macroalgae have received considerable interest due to their diverse applications in food, pharmaceutical, cosmetic, and animal feed industries [2]. Recent studies have highlighted the potential of macroalgae as alternative feed ingredients because of their nutritional value and abundance in coastal regions [3].

One macroalgal species with promising potential as a feed ingredient is Gracilaria sp. This red seaweed contains high levels of carbohydrates (64–73%), moderate crude protein concentrations (9–17%), low lipid content (0.26–0.62%), and valuable minerals such as selenium and iodine [4]. Proximate analysis of *Gracilaria verrucosa* harvested from the waters of Takalar showed crude protein of approximately 8.27%, crude fiber of 5.40%, and substantial concentrations of macro-minerals, particularly potassium, suggesting its suitability as a nutritionally valuable feed resource[5]. Beyond its nutritional value, Gracilaria contains various bioactive compounds, including phenolics, flavonoids, alkaloids, and phlorotannins, which possess antioxidant properties and may influence rumen microbial activity [2,3].

In ruminants, ruminal fermentation by microorganisms produces volatile fatty acids (VFAs), which constitute the primary source of metabolizable energy for the host animal. Simultaneously, protein degradation generates ammonia ($NH_3$), which serves as a major nitrogen source for microbial protein synthesis. Therefore, dietary ingredients capable of stimulating microbial activity may enhance rumen fermentation efficiency [6]. Based on its nutritional composition and bioactive properties, increasing the inclusion level of *Gracilaria sp.* in complete feed may promote carbohydrate fermentation, increase VFA production, improve nitrogen availability through greater $NH_3$ production, and maintain ruminal pH within the optimal range for microbial growth.

Previous studies have demonstrated the potential of *Gracilaria species* to modify rumen fermentation. For example, Maia et al. [7] reported that *Gracilaria vermiculophylla* reduced methane production while maintaining normal VFA concentrations during in vitro fermentation. However, their study focused primarily on methane mitigation and utilized *G. vermiculophylla* as a fermentation substrate rather than as a component of complete feed. Information regarding the effects of incorporating *Gracilaria sp.* into complete diets at different inclusion levels on rumen fermentation characteristics remains limited, particularly for seaweed harvested from the coastal regions of East Nusa Tenggara, Indonesia.

Given the abundance of Gracilaria resources in this region and the lack of information regarding its utilization in ruminant feeding systems, further investigation is warranted. Therefore, this study was conducted to evaluate the effects of increasing levels of *Gracilaria sp.* in complete feed on in vitro rumen fermentation characteristics, specifically ruminal pH, total volatile fatty acid concentration, and ammonia concentration. The introduction of the paper should explain the nature of the problem, previous work, purpose, and the contribution of the paper. The contents of each section may be provided to understand easily about the paper.

## II. Materials and Method

### Study Site and Experimental Period

The study was conducted from September to October 2025. *Gracilaria sp*. samples were collected from the coastal area of Pasir Panjang Beach, Kupang, East Nusa Tenggara, Indonesia. Sample processing, including drying and feed preparation, was carried out in Binilaka, Oeltua Village, Taebenu District, Kupang Regency. Subsequently, in vitro analyses were performed at the Feed Chemistry Laboratory, Faculty of Animal Science, Marine and Fisheries, Universitas Nusa Cendana, Kupang, Indonesia.

### Materials and Experimental Diets

The *Gracilaria sp.* used in this study was harvested from the coastal waters of Kupang, East Nusa Tenggara. Complete feed diets were formulated using field grass and concentrate. The concentrate consisted of ground corn (60%), pollard (35%), and fish meal (5%). Proximate analyses were conducted to determine dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), crude fiber (CF), carbohydrate (CHO), nitrogen-free extract (NFE), and metabolizable energy (ME) content of each feed ingredient. The concentrate

component consisted of 60% ground corn, 35% pollard, and 5% fish meal. The chemical composition of feeds used in the present experiment is presented in Table 1.

Table 1. Chemical composition of feed ingredients

| Chemical composition | *Gracilaria* sp. | Field grass | Cencentrate | | |
|---|---|---|---|---|---|
| | | | Pollard | Cornmeal | Fishmeal |
| DM (%) | 96.597 | 94.579 | 92.337 | 92.716 | 91.325 |
| Ash (%) | 29.098 | 7.325 | 4.431 | 2.814 | 25.888 |
| OM (%) | 70.902 | 92.675 | 95.569 | 97.186 | 74.112 |
| CP (%) | 15.717 | 7.740 | 15.468 | 11.412 | 48.096 |
| EE (%BK) | 3.710 | 4.533 | 8.775 | 1.202 | 7.192 |
| CF(%BK) | 6.390 | 34.456 | 8.725 | 3.779 | 0.399 |
| CHO** (%) | 51.475 | 80.402 | 71.326 | 84.572 | 18.824 |
| NFE** (%) | 45.085 | 45.945 | 62.600 | 80.793 | 18.425 |
| GE MJ/ Kg DM | 13.980 | 17.332 | 19.082 | 17.807 | 17.367 |
| GE Kcal/ Kg DM | 3,328.63 | 4,126.69 | 4,543.34 | 4,239.76 | 4,135.06 |
| EM** Kkal/Kg BK | 2.565.96 | 2.379.53 | 3.642.31 | 3.526.29 | 3.042.57 |

Note : DM: dry matter; OM: organic matter; CP : crude protein; EE : cude fat; CF: crude fibre; CHO : carbohidrate; NFE: Nitrogen free extract; GE: gross energy, EM: metabolisable energy

**Experimental Design**

The experiment used a completely randomized design (CRD) with four dietary treatments and three replicates per treatment.

The treatments were:

- G0: 70% field grass + 30% concentrate
- G5: 5% Gracilaria sp. + 65% field grass + 30% concentrate
- G10: 10% Gracilaria sp. + 60% field grass + 30% concentrate
- G15: 15% Gracilaria sp. + 55% field grass + 30% concentrate

The inclusion levels of 5%. 10%. and 15% were selected to evaluate the progressive effects of Gracilaria supplementation without drastically altering the nutrient composition of the basal diet.

**Preparation of Gracilaria Samples**

Fresh *Gracilaria sp.* was collected manually from Pasir Panjang Beach, Kupang City, Indonesia. Only healthy and undamaged seaweed samples were selected. The collected seaweed was thoroughly washed with fresh water to remove sand. debris. and attached organisms. The samples were then air-dried until a stable moisture content was achieved. After drying. the seaweed was ground using a blender to obtain a uniform particle size suitable for incorporation into the experimental diets.

**Collection of Rumen Fluid**

Rumen fluid was obtained from healthy donor ruminants before the morning feeding. Fresh rumen fluid was collected using a stomach tube and immediately transferred into pre-warmed thermos flasks to maintain anaerobic conditions and temperature stability during transportation.

Upon arrival at the laboratory, the rumen fluid was filtered through four layers of cheesecloth to remove feed particles and obtain a homogeneous inoculum for *in vitro* fermentation.

**_In Vitro_ Fermentation Procedure**

*In vitro* fermentation was conducted following the first stage of the Tilley and Terry [8] technique. Approximately 0.5 g of each feed sample was weighed into fermentation tubes. Each tube received 40 mL of McDougall's buffer solution and 10 mL of freshly collected rumen fluid under continuous $CO_2$ flushing to maintain anaerobic conditions. The tubes were sealed and incubated in a shaking water bath at 39°C for 48 h. During incubation, tubes were gently agitated periodically to ensure adequate mixing between the substrate and fermentation medium.

At the end of the incubation period. fermentation was terminated by adding several drops of saturated $HgCl_2$ solution. Samples were then centrifuged at 2.500 rpm for 15 min. The resulting supernatant was collected for the determination of ruminal pH, Total volatile fatty acids (VFA) and ammonia ($NH_3$) concentrations.

**Parameters Measured**

Parameter measured included pH and the concentration total volatile fatty acids (VFA) and ammonia in the *in vitro* residues. The level of pH was measured immediately on the collected aliquot after incubation using a calibrated digital pH meter. Measurements were conducted promptly to avoid pH changes caused by exposure to atmospheric oxygen. Meanwhile. Total VFA concentration was determined using the steam distillation method according to standard rumen fermentation procedures. Ammonia concentration was determined using the Conway microdiffusion technique [9]. The $NH_3$ concentration was expressed in millimolar (mM) and calculated based on the volume and normality of sulfuric acid used during titration.

**Statistical Analysis**

Data were analyzed using one-way analysis of variance (ANOVA). When significant treatment effects were detected ($P < 0.05$), means were separated using Duncan's Multiple Range Test. Statistical analyses were performed using IBM SPSS Statistics version 25 (IBM Corp., Armonk, NY, USA).

## III. Results and Discussion

**Effects of *Gracilaria sp.* Inclusion on Total Volatile Fatty Acid Concentration**

The effects of increasing dietary inclusion levels of *Gracilaria sp.* on total volatile fatty acid (VFA) concentration are presented in Table 2. The inclusion of *Gracilaria sp.* significantly increased ($P<0.001$) total VFA concentration, with values rising progressively from 68.89 mM in G0 to 105.80 mM in G15. These results indicate that *Gracilaria sp.* enhanced ruminal fermentation activity and improved the availability of fermentable substrates for microbial metabolism.

Table 2. The effects of increasing dietary inclusion levels of *Gracilaria sp.* on pH, ammonia (NH3) and total volatile fatty acid (VFA) concentration

| Parameter | Treatment | | | | SEM | P-Value |
|---|---|---|---|---|---|---|
| | G0 | G5 | G10 | G15 | | |
| VFA | 68,891[a] | 80,572[b] | 95,600[c] | 105,803[d] | 4,089 | <0,001 |
| pH | 6,867[a] | 6,967[ab] | 7,000[b] | 6,867[a] | 0,078 | 0,093 |
| NH3 | 7,820[a] | 9,276[b] | 10,030[bc] | 10,755[c] | 0,488 | 0,002 |

Values bearing different superscript show significant differences (P<0.05) or a tendency to be significant (P= 0.05-0.1)

Volatile fatty acids are the primary end-products of carbohydrate fermentation in the rumen and constitute the major source of energy for ruminant animals [10]. Therefore. increased VFA production generally reflects enhanced microbial fermentation and greater utilization of dietary carbohydrates. The significant increase in VFA concentration observed in the present study suggests that the inclusion of *Gracilaria sp*. improved the fermentability of the complete feed.

This response is likely associated with the unique carbohydrate composition of *Gracilaria sp.* compared to field grass. Gracilaria contains lower crude fiber concentrations and a higher proportion of readily fermentable polysaccharides. including agar and galactan-based compounds [3]. These carbohydrates may be more accessible to rumen microorganisms than the structural carbohydrates present in mature tropical grasses. which are often rich in lignified cellulose and hemicellulose[11]. Consequently. the replacement of field grass with *Gracilaria sp.* may have increased the availability of fermentable carbohydrates and stimulated microbial fermentation.

The gradual increase in VFA concentration across treatments indicates that rumen microorganisms effectively utilized the additional substrates provided by *Gracilaria sp.* The highest VFA concentration observed in G15 suggests that the inclusion level of 15% had not yet exceeded the capacity of the rumen microbial ecosystem to utilize the available nutrients. Similar findings have been reported for other marine macroalgae. where dietary inclusion increased fermentative activity and improved nutrient degradation in vitro [11.12].

In addition to its nutritional value. Gracilaria contains various bioactive compounds. including polyphenols and antioxidant substances. which may influence rumen microbial populations and fermentation efficiency. Recent studies have suggested that certain seaweed-derived compounds can selectively modulate microbial communities. potentially improving fermentation efficiency without negatively affecting overall rumen function [3.4].

The VFA concentrations observed in this study (68.89–105.80 mM) were within the normal range reported for efficient ruminal fermentation. which generally ranges from 70 to 150 mM under in vitro conditions[13]. These findings indicate that the inclusion of *Gracilaria sp.* enhanced fermentation activity without disrupting normal rumen function.

The present results differ somewhat from those reported by [7], who found that supplementation with *Gracilaria vermiculophylla* had little effect on total VFA concentration. This discrepancy may be attributed to differences in seaweed species, inclusion level, substrate composition, and fermentation conditions. In the present study, *Gracilaria sp.* was incorporated directly into a complete feed formulation, thereby contributing substantially to the fermentable carbohydrate pool available to rumen microorganisms. The findings demonstrate that dietary inclusion of *Gracilaria sp.* stimulates ruminal fermentation and increases VFA production, suggesting improved utilization of dietary carbohydrates by rumen microbes.

**Effects of *Gracilaria sp.* Inclusion on Ammonia Concentration**

The concentration of ruminal ammonia ($NH_3$) increased significantly (P=0.002) with increasing inclusion levels of *Gracilaria sp.*, ranging from 7.82 mM in G0 to 10.75 mM in G15. This response indicates enhanced ruminal protein degradation and greater nitrogen availability for microbial growth.

Ammonia is a key intermediate in ruminal nitrogen metabolism. It is produced through microbial degradation of dietary protein and non-protein nitrogen sources and serves as the principal nitrogen source for microbial protein synthesis [14]. Consequently. ruminal $NH_3$ concentration is widely used as an indicator of nitrogen availability within the rumen ecosystem.

The increase in $NH_3$ concentration observed in this study is likely associated with the higher crude protein content of *Gracilaria sp.* compared with field grass. As the proportion of Gracilaria increased in the

diets. the supply of degradable protein available to rumen microorganisms also increased. resulting in greater ammonia production. This suggests that the protein contained in *Gracilaria sp.* was readily degraded under ruminal conditions.

The $NH_3$ concentrations recorded in this experiment remained within the optimal range reported for microbial growth and protein synthesis. It is suggested that ammonia concentrations above approximately 5 mM are generally sufficient to support maximal microbial activity[15]. Therefore. the values observed in the present study indicate adequate nitrogen availability for microbial fermentation.

The progressive increase in $NH_3$ concentration from G0 to G15 suggests a positive balance between protein degradation and microbial utilization of ammonia. Although $NH_3$ concentration increased significantly, no excessive accumulation was observed. This finding indicates that ammonia production remained synchronized with microbial nitrogen requirements. allowing efficient microbial utilization of degraded protein.

Furthermore, the increased $NH_3$ concentration may partially explain the enhanced VFA production observed in the present study. Adequate nitrogen availability is essential for microbial proliferation. particularly for cellulolytic and fibrolytic bacteria that contribute to carbohydrate degradation. Increased microbial growth resulting from improved nitrogen supply may therefore have contributed to the greater fermentation activity reflected by higher VFA concentrations.

The results are consistent with previous reports demonstrating that increased dietary protein availability enhances ruminal $NH_3$ concentration and microbial activity [14,16]. They also support the hypothesis that *Gracilaria sp.* can serve as a valuable nitrogen source in ruminant diets.

**Effects of *Gracilaria sp.* Inclusion on Ruminal pH**

The inclusion of *Gracilaria sp.* did not significantly affect ruminal pH ($P=0.093$), although a slight numerical increase was observed up to the 10% inclusion level. Ruminal pH values ranged from 6.86 to 7.00 across all treatments.

Maintaining an appropriate ruminal pH is essential for optimal microbial activity and fermentation efficiency. Most rumen microorganisms perform efficiently within a pH range of approximately 6.0–7.0, while cellulolytic bacteria generally require pH values above 6.2 to sustain fiber digestion [17]. Therefore, the pH values recorded in this study indicate favorable conditions for microbial fermentation.

The stability of ruminal pH despite increased VFA production suggests that buffering mechanisms remained effective throughout fermentation. Although VFA production increased substantially with higher Gracilaria inclusion levels. the concurrent increase in $NH_3$ concentration may have contributed to maintaining acid-base balance within the fermentation medium. Because ammonia is alkaline in nature, it can partially neutralize organic acids produced during fermentation. thereby preventing excessive declines in pH.

The slightly higher pH values observed in G5 and G10 may be associated with increased ammonia production resulting from enhanced protein degradation. However, at the highest inclusion level (G15). pH declined slightly despite elevated $NH_3$ concentrations. This reduction was likely due to the substantially greater production of VFAs, which increased acidity within the fermentation system. Nevertheless, pH remained well within the normal physiological range and therefore did not impair microbial activity.

These findings indicate that *Gracilaria sp.* can be included in complete feed formulations at levels up to 15% without negatively affecting ruminal pH. This is an important consideration because excessive acidification of the rumen can impair fiber digestion and reduce animal performance. The results suggest that Gracilaria supplementation enhances fermentation activity while maintaining a stable ruminal environment. The simultaneous increase in VFAs and $NH_3$ concentrations, together with the maintenance of normal ruminal pH. demonstrates that *Gracilaria sp.* improved ruminal fermentation efficiency without disrupting microbial homeostasis. This finding supports its potential use as an alternative feed ingredient for ruminant production systems, particularly in tropical coastal regions where seaweed resources are abundant.

## IV. Conclusion and Recommendation

The inclusion of *Gracilaria sp.* in complete feed increased total volatile fatty acids and ammonia concentrations while maintaining ruminal pH within the optimal range for microbial fermentation. The highest fermentation response was observed at the 15% inclusion level, indicating enhanced microbial activity and nutrient degradation. These findings suggest that *Gracilaria sp.* has considerable potential as an alternative feed ingredient for ruminants and may contribute to improving feed utilization in tropical livestock production systems. Further in vivo studies are recommended to evaluate its effects on feed intake, nutrient digestibility, animal performance, and economic feasibility under practical production conditions.

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