



Comparative Physiological Assessment of Three Natural Feed Additives on *In-vitro* Rumen Fermentation Efficiency

Ahmed A. Ismail^{1*}, Marwa A. Ibrahim², Mahmoud A. Abdl-Rahman¹, Francois A. Sawiress¹

1. Department of Physiology, Faculty of Veterinary Medicine, Cairo University, Egypt
2. Department of Biochemistry, Faculty of Veterinary Medicine, Cairo University, Egypt

* Corresponding author: Ahmed A. Ismail, e-mail address: ahmedthebestnasser@gmail.com

1. Abstract

This study investigated the impact of three natural feed additives: Sodium bentonite, *Moringa oleifera*, and red seaweed (*Jania Granifera*) on *in vitro* rumen fermentation characteristics, methane mitigation, enzyme activity, and microbial population dynamics. Rumen fluid was collected from Baladi sheep and incubated in gas-tight syringes with feed supplemented with the respective additives. After 24 hours of incubation, Key fermentation parameters were evaluated, including total gas and methane production, pH, ammonia concentration, total and individual volatile fatty acids (VFAs), extracellular cellulase activity, microbial biomass, and microbial efficiency. Quantitative PCR was employed to quantify the relative abundance of total bacteria, cellulolytic bacteria, and methanogenic archaea.

The results demonstrated that red seaweed supplementation significantly reduced methane production (~34%) and the abundance of methanogenic archaea (to 0.38-fold compared to the control). However, it lowered total VFAs and cellulase activity. *Moringa* supplementation enhanced gas production and microbial yield, and maintained high cellulase activity and acetate production, suggesting improved microbial growth. Sodium bentonite enhanced fermentation efficiency and propionate production, exerting moderate methane suppression. Although all additives increased the relative abundance of cellulolytic bacteria, discrepancies were noted between bacterial counts and enzyme activities, suggesting potential metabolic inhibition by bioactive compounds in the additives.

In conclusion, the findings support the targeted use of these natural additives to improve ruminal fermentation efficiency, modulate the ruminal microbial ecosystem, and reduce methane emissions to minimize the effects of methane on global warming.

Key words: Baladi sheep, *Jania Granifera*, Methane, *Moringa oleifera*, Sodium bentonite.

2. Introduction

Methane (CH₄) is a potent greenhouse gas and ranks as the second most significant contributor to climate warming, following carbon dioxide [1]. In terms of heat-trapping capability, a single methane molecule has a greater impact than a CO₂

molecule; however, methane has a relatively short lifespan in the atmosphere, ranging from 7 to 12 years, whereas CO₂ can persist for several centuries or longer [2].

Both natural sources and human activities contribute to atmospheric





methane. Approximately 60% of current methane emissions are attributed to human activities [3]. The primary anthropogenic sources include agriculture (especially livestock), fossil fuel extraction and use, and the decomposition of organic waste in landfills [4]. Natural processes account for the remaining 40% of methane emissions, primarily from wetlands, wildfires, and termites [5].

Among agricultural sources, ruminant livestock are a major contributor to methane emissions due to enteric fermentation [6]. In the rumen, methane is produced by methanogenic archaea that scavenge hydrogen (H_2) and carbon dioxide (CO_2) produced by other ruminal microorganisms, resulting in methane (CH_4) production [7].

Additionally, compounds such as formic acid and methylamines produced by other rumen microbes serve as alternative substrates for methanogenesis [8].

The interaction between methanogens and other ruminal microbes, including protozoa, bacteria, and fungi, is crucial for maintaining fermentation stability, primarily through interspecies hydrogen transfer [9]. This symbiotic interaction prevents hydrogen accumulation, which would otherwise inhibit the fermentation of carbohydrates [10].

The modulation of ruminal fermentation using natural (e.g., plant extracts, seaweed) and inorganic additives (e.g., clays like bentonite) has gained attention as a strategy to enhance nutrient utilization efficiency and simultaneously reduce greenhouse gas emissions, particularly methane [11], [12].

This study evaluated the effects of bentonite, moringa (*Moringa oleifera*), and seaweed (*Jania Granifera*) supplementation compared to a control group on various fermentation parameters, gas and methane production, pH, ammonia concentration,

total volatile fatty acids (TVFA), individual VFAs, enzyme activity, and microbial efficiency.

3. Materials and Methods

3.1. Sample Collection

The ruminal contents of 5 slaughtered sheep (Baladi breed), fed ration consisting of Tipn and wheat grains in a ratio of (50:50) as shown in table (1), were collected to prepare the treatment systems, which were then transferred into a thermos flask that had been prewarmed to the lab [13].

Then, Ruminal fluids were passed through four cheesecloth layers (straining) into a separate flask, pre-gassed with CO_2 that is oxygen-free. The particle-free fluid is then mixed with McDougall's buffer in the proportion 1: 2 (v/v) solution [14].

Incubation was carried out in a CO_2 incubator for 24 hours, previously set at a temperature of $39^\circ C$ at 5% CO_2 level [15].

3.2. Preparation of Treatment Systems and In-Vitro Fermentation

Two hundred milligrams of the feed sample were weighed into 60 mL calibrated plastic syringes with pistons lubricated with Vaseline. Thirty mL of buffered rumen fluid was put into the syringes, and three treatment systems were prepared for each sample in a quintuplicate syringe per treatment: sodium bentonite (4% of dry matter intake DMI) [16], *Moringa oleifera* (7% of DMI) [17], *Jania Granifera* (3% of DMI) [18] and negative control (no additives). Moringa leaves were purchased from the local market (Haraz, Cairo, Egypt), while Sodium bentonite was purchased from El Aksa company. Seaweed was collected from Alexandria shores. Both seaweed and Moringa were identified by Prof. Abo Shanab Sanaa in the Botany Department, Faculty of Science, Cairo University





3.3. pH determination

Following the end of the incubation period (24 hours), the fluid samples were taken out and dispensed into plastic bottles, and the pH was measured right away using a pH meter (JENWAY 3510).

3.4. Determination of the Individual VFA Proportions and Total VFA Concentrations

To ascertain the overall VFA concentrations and individual VFA proportions, 5 mL of fermentation fluids were mixed with 1 mL of 25% metaphosphoric acid, centrifuged at 7,000 x g for 10 minutes, and Supernatants were kept in a deep freezer (-20°C) until analysis was done.

Steam distillation is used to measure the total concentrations of VFAs [19].

Molar proportions of VFAs were determined using High Performance Liquid Chromatography (YL 9100) [20].

3.5. Measurement of Ammonia Nitrogen Concentration

Two milliliters of 0.2 N HCl were added to 5ml of fermented fluid sample, and then the samples were centrifugated for 20 minutes at 5000×g, the supernatant was then frozen at (-20°C) till examined spectrophotometrically [21].

3.6. Determination of Methane in Total Gas

Methane is measured after immediately being collected from each syringe using portable gas analyzer (testo 317-2) to detect its concentration in fermentation gas by micromole using a

catalytic bead sensor, where methane is oxidized on a heated catalytic surface (bead), releasing heat which causes a change in electrical resistance proportional to gas concentration [22].

3.7. Determination of Extracellular Cellulase Activity

The fermentation fluid samples were first centrifuged at 3,000x g for 20 minutes to separate the supernatant. Then, 0.5 mL of the supernatant (crude enzyme) was mixed with 0.5 mL of a 1% carboxymethyl cellulose (CMC) solution in 0.05 M sodium citrate buffer. This mixture was incubated at 55°C for one hour without shaking. After that, the reaction was stopped by boiling for five minutes. The boiled samples were then centrifuged at 7,000 × g for five minutes. The amount of reducing sugars in the supernatant was measured using a colorimetric method. One unit of enzyme activity was defined as the amount of enzyme that produces 1 micromole of glucose equivalent per minute [23].

3.8. Determination of In-Vitro True Substrate Degradability (TSD)

True degradability in vitro was ascertained after emptying the remaining contents of each individual syringe into a beaker; the syringes were carefully washed with neutral detergent solution (NDS). The content was dissolved for one hour using NDS to solubilize microorganisms and only gain undigested feed. The remaining content was subsequently filtered, dried for two hours at 130° C, and weighed. The weight of the substrate was then used to calculate the degradability [24].

TSD = weight of feed before incubation - feed residues after NDS incubation

3.9. Stoichiometric Estimations





1) Acetate / propionate (A / P) ratio calculation from measured acetate and propionate values.

2) Fermentation efficiency (FE) =

$$\frac{0.622 A + 1.092 P + 1.56 B}{A + P + 2B} \times 100$$

Where A, P, and B are the moles of acetic, propionic, and butyric. The result is expressed in percentage [24].

3) Microbial biomass production (MBP), Microbial yield (Y_{ATP}), and efficiency of microbial protein production (EMPP)

4) Microbial biomass production was then estimated [25]:

$$MBP = TSD - (\text{gas volume} \times SF)$$

Where TSD is true substrate degradability, and SF represents a stoichiometric factor 2.2

Once the MBP figure is known, the efficiency of microbial protein synthesis (EMP) can be calculated according to the equation [26]:

$$EMP = \frac{TSD - (\text{gas vol} \times SF)}{TSD}$$

Microbial yield (Y_{ATP}) was calculated as the mass of microbial cells produced (in milligrams) per millimole of ATP generated during the fermentation of carbohydrates to VFAs. Moles of ATP generated per mole of short-chain VFAs and methane, which are 2 for acetate, 3 for Propionate, 3 for butyrate, and 1 for methane [27].

$$Y_{ATP} = \frac{\text{microbial biomass production (mg)}}{\text{Number of ATP generated (mmol)}}$$

3. 10. Quantitative Analysis of Bacterial Populations by Real-time PCR (qPCR)

Total DNA from rumen fluid was extracted through the QIAamp UCP Pathogen Mini Kit (Cat. No. / ID: 50214). The purity and concentration of DNA in the extracted sample were determined with a nano spectrophotometer at A260/A280 [28].

The qPCR was performed to measure the population of total bacteria [29], cellulolytic bacteria [30], and methanogens [31]. Microbial DNA was amplified from total DNA with specific primers as described in table (2).

Bacterial populations were determined in the samples by SYBR green quantitative PCR assay. Real-time qPCR (Applied Biosystems StepOnePlus Real-Time PCR System Thermo Fisher Scientific, Foster City, CA, USA) was performed using Bio-Rad iCycler iQ Multicolor real-time PCR detection system (Bio-Rad Laboratories, Inc.) with fluorescence detection of SYBR Green dye. The amplification condition was as follows: one cycle at 95°C for 3 min for initial denaturation and then 40 cycles of 95°C for 30 s followed by annealing and extension at 60°C for 1 min. Detection of the fluorescent product was set at the last step of each cycle. Relative quantification was expressed as proportions of total rumen bacteria [32].

Total bacteria at different time points are shown for reference. The numbers of bacteria were expressed as the relative number of bacteria at 0 h, which was taken as 1.00 (100%).

3. 11. Statistical Analysis

The Statistical analysis of the data was performed using a one-way analysis of variance (ANOVA) test. To compare the means of different treatments, the least significant difference (LSD) test was applied at a significance level of 5% [33].





4. Results

4.1. Gas and Methane

It was observed among all treatments that the moringa-supplemented group exhibited the highest gas output (39.1 mL), surpassing even the control (36.2 mL). On the other hand, bentonite resulted in the lowest gas production (33 mL), Seaweed supplementation showed an intermediate gas value (34.5 mL), similar to the control. Methane production was drastically reduced by red seaweed. The methane level in the seaweed group (152.6 μ mol) was 34% lower than the control (230.9 μ mol). Bentonite (205.8 μ mol) and moringa (214.3 μ mol) also contributed to methane reduction, but to a lesser extent, as shown in table (3).

4.2. Measurement of pH, TVFA, and Ammonia Nitrogen

All treatments kept pH within the ideal physiological range for rumen microbes (6.88–7.12). Moringa caused a slight alkalization (pH 7.12), while seaweed slightly lowered the pH (6.88). Both control and bentonite maintained near-neutral values (6.97 and 7.05, respectively). The control (1063.3 μ mol) and moringa (1040.5 μ mol) groups recorded the highest TVFA values. In contrast, bentonite (1033.5 μ mol) caused a modest reduction, and seaweed showed a significant decrease (918.9 μ mol).

Regarding ammonia concentrations, bentonite demonstrated the most potent ammonia-reducing effect (12.6 mg/dL), followed by moringa (14.11 mg/dL) and seaweed (15.16 mg/dL), compared to the control (16.74 mg/dL), as summarized in table (4).

4.3. Fermentation Efficiency, A/P Ratio, and Molar Proportions of IVFAs

It was estimated that Bentonite achieved the highest efficiency (79.60%), followed closely by seaweed (78.94%),

both exceeding control (77.63%) and moringa (77.34%). Regarding the A/P ratio, Bentonite (1.63) and seaweed (1.75) significantly reduced the A/P ratio compared to the control (2.04), while Moringa increased the A/P ratio to (2.14).

Acetate levels were highest in moringa (57.6%) and the control (54.3%). Propionate was most abundant in bentonite (30.3%) and seaweed (28.9%), validating the observed A/P ratio trends. Butyrate, which is important for epithelial health, was elevated in seaweed (14.3%) and moringa (13.4%), conversely, bentonite reduced butyrate (10.4%), as illustrated in table (5).

4.4. Cellulolytic Activity and True Substrate Degradability (TSD)

Concerning cellulolytic activity, the control group recorded the highest value (8.68 mmol/min), closely followed by moringa (8.29 mmol/min), Bentonite showed a moderate reduction (7.60 mmol/min), In contrast, seaweed exhibited markedly suppressed cellulase activity (5.7 mmol/min), as shown in table (6). The highest TSD was seen in control (97.64 mg) and moringa (96.52 mg), while bentonite (91.9 mg) showed a mild reduction. Seaweed significantly lowered TDS (80.3 mg).

4.5. MBP, EMP, and Y_{ATP}

It was recorded that Bentonite was the highest MBP (19.3 mg) and EMP (0.211), outperforming even the control (MBP = 18 mg, EMP = 0.184). Moringa, despite its favorable effects on TVFA and cellulolytic activity, showed lower MBP (10.5 mg) and EMP (0.108). Seaweed exhibited the poorest performance in both MBP (4.48 mg) and EMP (0.037).

Interestingly, bentonite produced the highest microbial yield per mmol ATP generated Y_{ATP} (7.23 mg/mmol ATP), while seaweed showed the lowest Y_{ATP} (1.82 mg/mmol ATP) as seen in table (7).





4.6. Microbial Populations

The quantification of specific microbial groups sheds light on how treatments influence the microbial ecology of the rumen. Total bacterial abundance showed slight increases across all additives, with red seaweed reaching the highest relative level (1.27).

Bentonite (1.22) and moringa (1.17) also supported a significant bacterial increase. Cellulolytic bacteria were significantly enriched in all treatments compared to the control (1.00), with seaweed showing the most pronounced increase (2.90), Moringa (1.73), and bentonite (1.64). Methanogenic archaea, key players in enteric methane emissions, were significantly suppressed by all treatments. The most notable reduction was seen in seaweed (0.38), followed by moringa (0.73) and bentonite (0.79), compared to the control (1.00), as summarized in table (8)

5. Discussion

Rumen fermentation pattern is the key to animal metabolism, production, and reproduction. In the present study, the aim was to improve the fermentation pattern with three natural feed additives. It was estimated that moringa supplementation resulted in the highest gas production, followed by the control, then seaweed, and the least amount of gas volume was in the bentonite group. The high fiber and protein contents of moringa may have stimulated microbial metabolic activity, leading to increased gas production [34]. Seaweed's relatively lower gas production might be attributed to the presence of secondary metabolites like phlorotannins, which can inhibit microbial fermentation [35]. The lowest gas production was in the bentonite treatment due to its adsorbing ability [36].

As expected, the red Seaweed supplementation significantly reduced methane production (nearly 34% reduction) compared to control, bentonite, and

moringa. This supports earlier reports that certain seaweed, notably species like *Asparagopsis taxiformis*, contain bromoform and other halogenated compounds that inhibit methanogenesis by Inhibition of methyl-coenzyme M reductase (MCR) [37], [38]. Bentonite's slight reduction in methane might be due to its cation exchange capacity and adsorbing effect, which influences microbial communities [36], [39]. Moringa's moderate methane reduction aligns with its tannin and saponin content, known as methane suppressants [40].

The optimum pH is very detrimental in the rumen microbial ecosystem; hence, the pH values (6.8–7.2) remained within the optimal range for ruminal microbial activity, especially bentonite treatment [10]. Moringa treatment showed the highest pH, possibly reflecting a buffering effect due to its mineral composition [41], [42]. Seaweed slightly reduced pH, which could be due to altered fermentation pathways [43].

Ammonia nitrogen is the main indicator of proteolysis and protein turnover, so lower ammonia levels suggest reduced proteolysis or enhanced microbial protein assimilation and vice versa. Ammonia levels were lower in the bentonite and seaweed groups compared to the control. Bentonite likely adsorbs free ammonia ions, decreasing the concentration [44]. Moringa also reduced ammonia moderately, consistent with its high condensed tannin content that binds proteins and reduces deamination [45]. The moderate reduction by seaweed may have resulted from bioactive compounds [46].

TVFA concentrations reflect the end products of carbohydrate fermentation and are indicative of energy availability to the host animal. TVFA levels were highest in the groups of control and moringa. Seaweed supplementation significantly reduced TVFA production, which agrees with





previous studies [12]. Reduced TVFA could be attributed to a methanogenesis inhibition mechanism causing altered fermentation pathways [7]. The bentonite group moderately reduced TVFA due to its ability to adsorb volatile fatty acids or inhibit fermentation by binding microbial enzymes or cofactors [36].

The acetate-to-propionate (A/P) ratio is a key indicator of rumen fermentation pattern. Lower ratios favor gluconeogenic propionate production, while higher ratios indicate more fibrous, acetate-dominant fermentation. Acetate production was highest in the moringa group, while Propionate was highest in the bentonite and seaweed groups. However, Butyrate was highest in the seaweed group. An increased (A/P) ratio was observed in moringa groups as Moringa leaves are rich in fiber that selectively favors the growth of acetogenic and fibrolytic bacteria [47]. On the other hand, increased propionate production and decreased acetate: propionate (A/P) ratio (observed in bentonite and seaweed groups) are indicative of a shift toward more glucogenic VFA profiles, beneficial for animal productivity as was previously mentioned [8], [11], [48].

Cellulase activity directly reflects the capacity of the microbial community to degrade structural polysaccharides. Cellulase activity was highest in the control and moringa groups [49]. Seaweed supplementation markedly reduced cellulase activity, possibly due to inhibitory compounds like phlorotannins [46], [50].

True substrate degradability (TSD) reflects the fermentation end products and gives an indirect measure of fermentation completeness. TSD followed a similar pathway to fermentation parameters. The seaweed group showed lower TSD, which correlates with lower microbial fermentation intensity [51]. Fermentation efficiency represents the proportion of substrate fermented and fermented

products. Fermentation efficiency was highest with bentonite and seaweed, suggesting that methane mitigation was achieved without greatly compromising fermentation [52], [53]. Although the fermentation efficiency value in seaweed was high, while cellulolytic activity, TSD, MBP, and microbial yield were low and this may be due to high propionate and low methane as Fermentation efficiency formula gives higher weight to propionate and butyrate, so increased propionate can mathematically increase Fermentation efficiency even if total VFAs value is low [24].

Microbial biomass production (MBP) and efficiency of microbial protein synthesis (EMP) are critical indicators of how well the rumen microbiome converts dietary energy into microbial mass. Seaweed reduced microbial biomass and efficiency, consistent with reduced fermentation intensity and possible microbial inhibition [54], [55]. Moringa showed moderate MBP and EMP values, reflecting improved nitrogen utilization without drastic changes in fermentation [56]. Only bentonite increased MBP and EMP, as Bentonite can adsorb ammonia, confirmed by ammonia nitrogen value, as it provided the lowest ammonia levels, which may indicate less nitrogen wastage and more nitrogen incorporation into microbial biomass [44]. Bentonite supplementation led to the highest microbial yield, confirming the enhanced MBP [39]. Moringa showed a moderate reduction in microbial yield as the MBP value was low, in addition to its higher values of acetate and methane compared to the bentonite group, which means an increased number of ATP generated, and microbial yield is inversely proportional to the number of ATP generated [57]. On the other hand, seaweed treatment showed the least microbial yield, as it increased propionate production (3 ATP) at the expense of acetate (2 ATP), which increased the





number of ATP generated even more than the moringa group [58]. Seaweed also inhibits microbial enzymes and suppresses cellulolytic and fermentative metabolism [46]. The observed increase in cellulolytic bacterial abundance in all experimental incubation systems, in contradiction with the cellulolytic activity, suggests microbial inhibition despite high count, which may be attributed to bioactive compounds such as halogenated compounds (e.g., bromoform) in red seaweed [59] or tannins and saponins in moringa [47]. These bioactive compounds can inhibit (MCR) enzyme activity, suppress fermentation, or redirect hydrogen away from acetate/TVFA production.

In the case of bentonite, it's characterized by adsorbing toxins and buffering pH, yet it can bind nutrients or enzymes, reducing fermentation efficiency [39].

Overall, the increase in cellulolytic bacteria doesn't necessarily represent functionally dominant species (like *Fibrobacter succinogenes* or *Ruminococcus albus*). Instead, less efficient or inactive strains may inflate population count without an increase in fiber degradation or VFA output [10].

Most notably, the marked reduction in methanogenic archaea aligns with previous findings on the antimethanogenic effects of certain feed additives, particularly seaweed like *Asparagopsis taxiformis* due to the bromoform content, while clay minerals of bentonite demonstrated similar reductions in methane production due to alteration of the ruminal redox environment, suppressing archaea [44], [60]. These results support the hypothesis that integrating such additives can shift the rumen microbial ecosystem in favor of reduced methane emissions without negatively impacting total microbial activity. The selective inhibition of archaea could lead to improved hydrogen utilization by alternative microbial pathways, such as

propionate production or biohydrogenation [8].

The total bacterial population showed an increase in all treatment groups, indicating that while specific microbial groups like methanogens were greatly reduced, the overall bacterial density increased thanks to the abundance of cellulolytic bacteria that were positively affected, which reflects a balanced microbial ecosystem, which is crucial for maintaining efficient fermentation and nutrient utilization [10].

6. Conclusions

The comparative evaluation of sodium bentonite, *Moringa oleifera*, and red seaweed revealed distinct effects on rumen fermentation parameters, methane mitigation, and microbial dynamics under in vitro conditions. Among the tested additives, *Moringa oleifera* supplementation resulted in the highest total gas production and supported enhanced cellulolytic activity, volatile fatty acid production, and microbial yield, reflecting efficient microbial growth. However, its effect on methane reduction was modest. Sodium bentonite significantly improved fermentation efficiency, microbial biomass production (MBP), and propionate levels, contributing to a lower acetate-to-propionate (A/P) ratio, a favorable shift for energy metabolism. It also demonstrated the strongest ammonia-reducing capacity, supporting improved nitrogen utilization.

Red seaweed was the most effective in suppressing methane production (~34% reduction), primarily through marked inhibition of methanogenic archaea and a notable increase in cellulolytic bacterial populations. However, this was accompanied by reductions in cellulase activity, total VFAs, and substrate degradability, suggesting a trade-off





between methane suppression and fermentation intensity.

Overall, each additive displayed unique advantages: Moringa for promoting microbial growth and fiber degradation, bentonite for improving fermentation efficiency and nitrogen metabolism, and red seaweed for effective methane mitigation. These findings support the selective application of these natural feed additives to optimize rumen function while contributing to environmentally sustainable ruminant production systems. It's clear that the in vivo impact of these additives varies from the in vitro effect, but they also have a significant economic impact regarding feed conversion rate and growth rate, especially as they are commercially available. Further studies utilizing a mix of these additives may offer better potential in methane mitigation without altering rumen fermentation patterns.

Conflict of interest: Nothing to declare

7. References

1. IPCC. Climate Change 2014: Synthesis Report. Geneva: IPCC; 2014.
2. Ciais P, Sabine C, Bala G, Bopp L, Brovkin V, Canadell J, et al. Carbon and Other Biogeochemical Cycles. In: Climate Change 2013: The Physical Science Basis. Cambridge: Cambridge University Press; 2013.
3. Saunio M, Stavert AR, Poulter B, Bousquet P, Canadell JG, Jackson RB, et al. The Global Methane Budget 2000–2017. *Earth Syst Sci Data*. 2020;12(3):1561–1623.
4. EPA. Global Anthropogenic Non-CO₂ Greenhouse Gas Emissions: 1990–2040. EPA Report 430-R-21-003; 2021.
5. Conrad R. The global methane cycle: recent advances in understanding the microbial processes involved. *Environ Microbiol Rep*. 2009;1(5):285–92.
6. Beauchemin KA, Kreuzer M, O'Mara F, McAllister TA. Nutritional management for enteric methane abatement: a review. *Aust J Exp Agric*. 2008;48(2):21–27.
7. Morgavi DP, Forano E, Martin C, Newbold CJ. Microbial ecosystem and methanogenesis in ruminants. *Animal*. 2010;4(7):1024–36.
8. Ungerfeld EM. Metabolic hydrogen flows in rumen fermentation: principles and possibilities of interventions. *Front Microbiol*. 2020;11:589.
9. Janssen PH. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim Feed Sci Technol*. 2010;160(1–2):1–22.
10. Russell JB, Rychlik JL. Factors that alter rumen microbial ecology. *Science*. 2001;292(5519):1119–22.
11. Beauchemin KA, McGinn SM. Methane emissions from cattle fed corn- or barley-based diets. *J Anim Sci*. 2006;84(3):653–60.
12. Cieslak A, Szumacher-Strabel M, Stochmal A, Oleszek W. Plant components with specific activities against rumen methanogens. *Animal*. 2013;7(S2):253–65.
13. Callaway TR, Martin SA. Effects of a *Saccharomyces cerevisiae* culture on ruminal bacteria that utilize lactate and digest cellulose. *J Dairy Sci*. 1997;80(9):2035–44.
14. McDougall EI. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochem J*. 1948;43(1):99–109.
15. Abdel-Rahman MA, El-Sayed AE, Khat tab HM. Effects of some medicinal plant extracts on in vitro gas production, rumen fermentation and methane production. *Egypt J Nutr Feeds*. 2009;12(2 Suppl):303–16.
16. Khalifeh R, Rouzbehan Y, Rezaei J. The effect of sodium bentonite and monensin on





- rumen fermentation parameters and nutrient digestibility in sheep. *J Anim Physiol Anim Nutr.* 2012;96(6):915–21.
17. Yang C, Ruan Z, Yu K, Wang M, Feng Y. Effect of *Moringa oleifera* leaves supplementation on growth performance, rumen fermentation, and microbial diversity in goats. *Anim Sci J.* 2019;90(4):512–21.
 18. Youyoung L, Kim JY, Park J, Kim KH, Lee HG. Effect of red seaweed (*Jania rubens*) supplementation on rumen fermentation and methane emission in vitro. *Animals.* 2022;12(2):234.
 19. Eadie JM, Hobson PN, Mann SO. A note on some comparisons between the rumen content of barley-fed steers and that of young calves also fed on high concentrations. *J Anim Prod.* 1967;9:247.
 20. Mathew S, Sagathevan S, Thomas J, Mathen G. An HPLC method for estimation of volatile fatty acids of ruminal fluid. *Indian J Anim Sci.* 1997;67(9):805–7.
 21. Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. *Clin Chem.* 1962;8(2):130–2.
 22. Rieger P. Non-dispersive Infrared (NDIR) gas sensors: principles, design, applications, and advantages. Winsen Sensor Co.; 2019. p. 2–5.
 23. Miller GL, Blum R, Glennon WE, Burton AL. Measurement of carboxymethylcellulase activity. *Anal Biochem.* 1960;1(2):127–32.
 24. Blümmel M, Steingass H, Becker K. The relationship between in vitro gas production, in vitro microbial biomass yield and 15N incorporation and its implications for the prediction of voluntary feed intake of roughages. *Br J Nutr.* 1997;77(6):911–21.
 25. Grings EE, Blümmel M, Sudekum KH. Forage quality and strategic supplementation for ruminants. *J Anim Feed Sci.* 2005;14(Suppl.1):191–208.
 26. Johnston JD, Tricarico JM. Manipulation of the rumen microbial ecosystem to improve livestock productivity and reduce methane emissions. In: *Proc 22nd Ann Symp Biotechnol Feed Ind.* Lexington, USA: Alltech Inc.; 2006. p. 83–91.
 27. Isaacson HR, Hinds FC, Bryant MP, Owens FN. Efficiency of energy utilization by mixed rumen bacteria in continuous culture. *J Dairy Sci.* 1975;58(11):1645–59.
 28. Singh KM, Pandya PR, Tripathi AK, Patel GR, Parnerkar S, Kothari RK, et al. Study of rumen metagenome community using qPCR under different diets. *Meta Gene.* 2014;2:191–7.
 29. Denman SE, McSweeney CS. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol Ecol.* 2006;58(3):572–82.
 30. Koike S, Kobayashi Y. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol Lett.* 2001;204(2):361–6.
 31. Denman SE, Tomkins NW, McSweeney CS. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol Ecol.* 2007;62(3):313–22.
 32. Yeoman CJ, Fields CJ, Lepercq P, Ruiz P, Forano E, White BA, et al. In Vivo Competitions between *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus* in a Gnotobiotic Sheep Model Revealed by Multi-Omic Analyses. *mBio.* 2021;12(2):e03533-20.
 33. Snedecor GW, Cochran WG. *Statistical Methods.* 8th ed. Ames, IA: Iowa State University Press; 1989.





34. Albores-Moreno S, Guevara-Fefer P, Robledo-Padilla FJ, Soto-Navarro SA, Calderón-Leyva G. Nutritional evaluation of *Moringa oleifera* leaves as an alternative protein source for ruminants. *Trop Anim Health Prod.* 2019;51(5):979–87.
35. Kinley RD, de Nys R, Vucko MJ, Machado L, Tomkins NW. The red macroalga *Asparagopsis taxiformis* is a potent natural antimethanogenic that reduces methane production during in vitro fermentation with rumen fluid. *Anim Prod Sci.* 2020;60(3):282–89.
36. Mallo JJ, Rioperez J, Honrubia MP. The addition of natural bentonite to diet improves nutrient digestibility and growth performance in pigs. *Anim Res.* 2002;51(6):517–26.
37. Li X, Norman HC, Kinley RD, Laurence M, Wilmot M, Bender H, et al. *Asparagopsis taxiformis* decreases enteric methane production from sheep. *Anim Prod Sci.* 2018;58(4):681–88.
38. Roque BM, Brooke CG, Ladau J, Polley T, Marsh LJ, Najafi N, et al. Effect of the macroalgae *Asparagopsis taxiformis* on methane production and the rumen microbiome assemblage. *Anim Microbiome.* 2019;1(1):3.
39. Iqbal MF, Yang Y, Ahmed S, Younas U, Qiu D. Use of bentonite to improve performance of livestock. *Animals.* 2020;10(11):2152.
40. Jayanegara A, Goel G, Makkar HPS, Becker K. Reduction in methane emissions from ruminants by plant secondary metabolites: a meta-analysis. *Livest Sci.* 2015;162:117–28.
41. Makkar HPS, Francis G, Becker K. Bioactivity of phytochemicals in some lesser-known plants and their effects and potential applications in livestock and aquaculture production systems. *Animal.* 2007;1(9):1371–91.
42. Gunun P, Wanapat M, Anantasook N, Kang S. Changes of microbial population in the rumen of swamp buffaloes as influenced by coconut oil and mangosteen peel powder supplementation. *Livest Sci.* 2016;185:88–93.
43. Newbold CJ, Lopez S, Nelson N, Ouda JO, Wallace RJ, Moss AR. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation in vitro. *Br J Nutr.* 2006;96(3):417–30.
44. Khatera SA, Hussein AM, Khafaga AF, Basiouni S, El-Naby ASA. Effect of bentonite and probiotics on growth performance and biochemical blood parameters in ruminants. *J Anim Feed Sci.* 2018;27(4):358–63.
45. Ahnert S, Dickhoefer U, Schulz F, Susenbeth A. Influence of ruminal nitrogen balance on microbial protein synthesis and nutrient digestion in goats. *Livest Sci.* 2015;177:48–54.
46. Chagas JC, Silva JP, Pereira DH, Oliveira AS, de Jesus RS, da Silva RR, et al. Influence of phlorotannins on ruminal fermentation. *Animals.* 2019;9(11):957.
47. Makkar HPS, Becker K. Nutritional value and anti-nutritional components of whole and ethanol extracted *Moringa oleifera* leaves. *Anim Feed Sci Technol.* 1996;63(1–4):211–28.
48. Dijkstra J, Oenema O, Bannink A. Dietary strategies to reducing methane emissions from ruminants. *Animal.* 2011;5(2):176–83.
49. Pellikaan WF, Stringano E, Leifeld J, Kemp B, Harriman M, Merry RJ, et al. In vitro assessment of the effects of condensed tannins on rumen fermentation. *Anim Feed Sci Technol.* 2011;165(1–2):30–41.
50. Wang Y, McAllister TA, Acharya S, Wittenberg KM, Krause DO. Impact of phlorotannins from brown algae on rumen





- microbial fermentation. *J Appl Phycol.* 2021;33(1):385–96.
51. Pilajun R, Wanapat M, Wachirapakorn C. Effects of coconut oil and mangosteen peel supplementation on rumen fermentation. *Livest Sci.* 2010;127(2–3):187–93.
52. Li Z, Wright ADG, Liu H, Wang Y. Reducing methane production in the rumen using feed additives. *Anim Feed Sci Technol.* 2017;234:1–9.
53. Belanche A, Ramos-Morales E, Newbold CJ. Contribution of rumen protozoa and bacteria to fermentation. *Front Microbiol.* 2020;11:1813.
54. McAllister TA, Newbold CJ. Redirecting rumen fermentation to reduce methanogenesis. *Aust J Exp Agric.* 2008;48(2):7–13.
55. Tomkins NW, Colegate SM, Hunter RA. A bromochloromethane formulation reduces methane production from cattle fed grain or forage diets. *Anim Prod Sci.* 2009;49(8):701–8.
56. Shingfield KJ, Bonnet M, Scollan ND. Recent developments in altering the fatty acid composition of ruminant-derived foods. *Animal.* 2013;7(S1):132–62.
57. Patra AK. Effects of essential oils on rumen fermentation, microbial ecology and ruminant production. *Asian J Anim Vet Adv.* 2011;6(9):873–88.
58. Baldwin RL. *Modeling Ruminant Digestion and Metabolism.* London: Chapman & Hall; 1995.
59. Machado L, Magnusson M, Paul NA, Kinley RD, de Nys R, Tomkins NW. Identification of bioactives from the red seaweed *Asparagopsis taxiformis* that promote antimethanogenic activity in ruminants. *Anim Prod Sci.* 2014;54(10):1272–84.
60. Asmare B, Demeke S, Tolemariam T, Tegegne F, Hailemariam D, Blümmel M. Effects of clay minerals on rumen fermentation and methane production in vitro. *Trop Anim Health Prod.* 2016;48(6):1093–8.





Table (1): Chemical analysis of the fed ration on a dry matter basis

Chemical analysis	% of dry matter
Crude fiber	29.5
Crude proteins	11.8
Ether extract	3.4
Nitrogen-free extract	36.2
Total ash	12.2

Table 2: primers used in qPCR

Bacteria species	Sense primer	Antisense primer
Total bacteria	CGGCAACGAGCGCAACCC	CCATTGTAGCACGTGTGTAGCC
Cellulolytic bacteria	CCCTAAAAGCAGTCTTAGTTCG	CCTCCTTGCGGTTAGAACA
methanogens	GAGGAAGGAGTGGACGACGGTA	ACGGGCGGTGTGTGCAAG

Table 3: Effect of three natural feed additives on Total gas production (ml) and Methane production (μmol)

Treatment	Gas (mL)	Methane (μmol)
Control	36.2 ^(b) ± 0.58	230.9 ^(a) ± 0.769
Bentonite	33 ^(c) ± 0.71	205.8 ^(c) ± 0.377
Moringa	39.1 ^(a) ± 0.40	214.3 ^(b) ± 0.508
Seaweeds	34.5 ^(b) ± 0.50	152.6 ^(d) ± 0.469

The previous Data represent the mean ± S.E., n = 5, p < 0.05.
Means with different superscripts are significantly different.





Table 4: Effect of three natural feed additives on pH, TVFA (μmol) and Ammonia nitrogen (mg/dL)

	pH	TVFA (μmol)	Ammonia (mg/dL)
Control	6.97 ^(b) ± 0.030	1063.3 ^(a) ± 0.689	16.74 ^(a) ± 0.150
Bentonite	7.06 ^(a) ± 0.033	1033.5 ^(c) ± 0.504	12.6 ^(d) ± 0.070
Moringa	7.12 ^(a) ± 0.028	1040.5 ^(b) ± 0.374	14.11 ^(c) ± 0.068
Seaweeds	6.88 ^(c) ± 0.012	918.9 ^(d) ± 0.475	15.16 ^(b) ± 0.163

The previous Data represent the mean ± SE, n = 5, p < 0.05. (TVFA: total volatile fatty acids). Means with different superscripts are significantly different.

Table 5: Effect of three natural feed additives on Fermentation Efficiency (%), A/P Ratio, and molar proportions of IVFAs (%)

Treatment	Fermentation (%)	A/P Ratio	Acetate (%)	Propionate (%)	Butyrate (%)
Control	77.63 ± 0.056	2.04 ± 0.0039	54.3 ± 0.04	26.6 ± 0.05	12.5 ± 0.06
Bentonite	79.60 ± 0.032	1.63 ± 0.0051	49.6 ± 0.07	30.3 ± 0.06	10.4 ± 0.04
Moringa	77.34 ± 0.024	2.14 ± 0.0051	57.6 ± 0.11	26.8 ± 0.02	13.4 ± 0.03
Seaweeds	78.94 ± 0.033	1.75 ± 0.0081	50.5 ± 0.13	28.9 ± 0.12	14.3 ± 0.02

The previous Data represent the mean ± SE, n = 5, p < 0.05. Means with different superscripts are significantly different.

Table 6: Effect of Three Natural Feed Additives on Cellulolytic Activity (mmol/min) and True Substrate Degradability (mg)

Treatment	Cellulase (mmol/min)	TSD (mg)
Control	8.68 ^(a) ± 0.101	97.64 ^(a) ± 0.89
Bentonite	7.60 ^(c) ± 0.070	91.9 ^(b) ± 1.25
Moringa	8.29 ^(b) ± 0.058	96.52 ^(a) ± 0.67
Seaweeds	5.7 ^(d) ± 0.034	80.3 ^(c) ± 0.64

The previous Data represent mean ± SE, n=5, p<0.05. (TSD: true substrate degradability). Means with different superscripts are significantly different.





Table 7: Effect of three natural feed additives on MBP (mg), EMP and YATP (mg/mmol ATP)

Treatment	MBP (mg)	EMP	YATP (mg/mmol ATP)
Control	18 ^(a) ± 0.69	0.184 ^(b) ± 0.0078	7.23 ^(b) ± 1.07
Bentonite	19.3 ^(a) ± 1.90	0.211 ^(a) ± 0.0212	7.98 ^(a) ± 0.98
Moringa	10.5 ^(b) ± 1.12	0.108 ^(c) ± 0.0109	4.08 ^(c) ± 1.16
Seaweeds	4.48 ^(c) ± 1.40	0.037 ^(d) ± 0.0148	1.82 ^(d) ± 0.84

The previous Data represent the mean ± SE, n = 5, p < 0.05.

Means with different superscripts are significantly different.

(MBP: microbial biomass production, EMP: efficiency of microbial protein, YATP: microbial yield).

Table (8): Effect of three natural feed additives on total bacteria, cellulolytic, and methanogens.

Treatment	Total Bacteria	Cellulolytic Bacteria	Methanogens
Control	1.00	1.00	1.00
Bentonite	1.22 ^(b) ± 0.036	1.64 ^(b) ± 0.041	0.79 ^(a) ± 0.012
Moringa	1.17 ^(c) ± 0.023	1.73 ^(b) ± 0.082	0.73 ^(b) ± 0.032
Seaweeds	1.27 ^(a) ± 0.028	2.90 ^(a) ± 0.052	0.38 ^(c) ± 0.026

The previous Data represent the mean ± SE, n = 5, p < 0.05.

The significance between different means is calculated from LSD.

