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

Ahmed A. Ismail[1*], Marwa A. Ibrahim[2], Mahmoud A. Abdl-Rahman[1], Francois A. Sawiress[1]

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 (CH_4) 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 CO_2 molecule; however, methane has a relatively short lifespan in the atmosphere, ranging from 7 to 12 years, whereas CO_2 can persist for several centuries or longer [2].

Both natural sources and human activities contribute to atmospheric

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

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◦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,

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

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% meta-phosphoric 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

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

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 – (gas volume x 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]:

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

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 byRreal-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%).

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

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 µmol) was 34% lower than the control (230.9 µmol). Bentonite (205.8 µmol) and moringa (214.3 µmol) 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 alkalinization (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 µmol) and moringa (1040.5 µmol) groups recorded the highest TVFA values. In contrast, bentonite (1033.5 µmol) caused a modest reduction, and seaweed showed a significant decrease (918.9 µmol).

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

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

(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 YATP (7.23 mg/mmol ATP), while seaweed showed the lowest YATP (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

Routine monitoring of physical water quality parameters, combined with an assessment of farm topography, operational protocols, and management practices, alongside biweekly shrimp sampling, constitutes a sound aquaculture approach. This integrated strategy supports adaptive management, enabling timely responses to environmental stressors and biological performance indicators.

The investigated shrimp farm is in the Deeba Triangle, Port Said, Egypt. The Deeba Triangle is a significant marine aquaculture zone in Egypt characterized by flat, low-lying terrain with clay-silt soils of moderate permeability, typical of reclaimed deltaic lands. Such soil properties are advantageous for shrimp farming, offering good water retention while allowing sufficient drainage, and are frequently recommended for earthen pond aquaculture systems [18]**.** The selection of this region aligns with broader trends in aquaculture development, which often targets coastal or deltaic areas due to their accessibility to water sources and suitability for infrastructure [19]**.** The farm operates semi-intensive earthen ponds, a production model widely adopted in developing countries to balance productivity and environmental impact [19]. Pond preparation included bottom drying, a critical step in reducing organic matter accumulation and disrupting pathogen cycles, consistent with established best practices [21]. However, the absence of tiling, liming, and fertilization could limit improvements in pond sediment quality and reduce natural primary productivity, which may necessitate greater reliance on formulated feeds [22]. Disinfection was achieved using Virocid®, a widely used commercial product with proven efficacy against a broad spectrum of pathogens when applied as recommended by the manufacturer [23]. This measure helps minimize microbial load before stocking, thereby enhancing biosecurity. Post-larvae (PLs) were sourced from a nearby hatchery in Damietta, ensuring short transport times and minimizing stress factors known to influence early-stage survival and performance [24]. Although stocking was conducted directly into

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

grow-out ponds without a nursery phase, PLs were acclimated to pond conditions upon arrival, which mitigates osmotic shock and improves survival chances [25]. Group 2 (Mediterranean Sea, S2) exhibited a slightly higher initial mean weight (0.020 ± 0.000 g) compared to Group 1 (Lake Manzala, S1: 0.010 ± 0.010 g), potentially influencing early growth dynamics under comparable management conditions. Uniformity in stocking density, feeding regime, and aeration across all ponds was maintained to ensure valid comparisons between the two water sources. Commercial tilapia feed (30% crude protein) is commonly observed in Egyptian shrimp aquaculture. However, it should be continuously evaluated for species-specific adequacy [20]. Aeration, achieved using 8-horsepower paddlewheel aerators per pond, is a standard approach in semi-intensive systems and is essential for maintaining dissolved oxygen levels, especially under warm climatic conditions [26].

Our comparative study of the source water quality revealed that the Mediterranean Sea (S2) had higher salinity levels, nitrite, and total iron. In contrast, Lake Manzala (S1) had greater total hardness (Table 2). For ponds' water quality, total hardness was significantly higher in group 1 ponds, which received water from Lake Manzala (Table 3). Water temperatures for sources (S1: 29.78 ± 2.87, S2: 29.66 ± 3.34 ◦C) and ponds (Group 1: 30.22 ± 1.43, Group 2: 30.27 ± 1.52 ◦C) were within the optimum range for *L. vannamei* growth, 25-32 °C [27,28]**.** This finding was also reported by several studies [6,29,30]. *L. Vannamei* is known as an Euryhaline species [31]. However, the optimum salinity range for growth and production is 15-25 ppt [17]. In our study, the lowest salinity observed was (41.13 ± 3.24 ppt) in S1, and the highest was (50.38 ± 3.78 ppt) in Group 1. El-Mezayen et al. [8] reported that the average salinity measured during 2014 to 2015 in the same study area was (20.12 ± 8.57 ppt). This increase in salinity could be attributed to the rising temperature as a prominent climate change impact on the northern coast [6]. Also, it may be due to saltwater intrusion from the Mediterranean Sea due to rising sea levels [28]. Mahmoud et al. [11] confirmed the salinity fluctuation in Lake Manzala in the last decades. In our results, higher ammonia levels (> 0.1 mg/L NH_3-N) likely resulted from afternoon sampling, when increased temperature and pH raise toxic unionized ammonia (NH_3) concentrations due to natural daily fluctuations [18]. S2 (the Mediterranean Sea) showed significantly higher levels of nitrite, but it was within the normal range <0.005 ppm [32]. Furthermore, it showed significantly higher levels of total iron (S1: 0.29 ± 0.18, S2: 0.79 ± 0.34 ppm, $p < .0001$). It seems possible that this result is due to heavy metal pollution of the Mediterranean Sea along the Egyptian coast due to agricultural, industrial, domestic wastes, harbor activities, as well as the Nile River's Rosetta and Damietta drainage into the Mediterranean coast [33]. No research had mentioned a reference range for total iron concentrations in the culture water of *L. vannamei*; existing studies focus on iron supplementation through diet rather than iron levels in water [34,35]. Total hardness was higher in S1 (Lake Manzala) and the corresponding ponds (Group 1) (S1: 6,646.64 ± 1,707.99, Group 1: 8,680.98 ± 596.72 mg CaCO3/L). This finding is consistent with [36, 37]. While far above those Colón et al. [38] observed, the total hardness was about 340 mg $CaCO_3$/L in low-salinity cultured shrimp. This difference can be attributed to soil characteristics among different geographical areas. Another

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

possible explanation is that the salinity level may be associated with total hardness. The total hardness of seawater is about 6500 mg/L $CaCO_3$, while freshwater's is up to 400 mg/L $CaCO_3$ [18]. Other measured water quality parameters were within the acceptable level for *L. vannamei* culture.

The comparison of the growth and production performance of *L. vannamei* cultured in these water quality profiles revealed that group 2 ponds (n=3, water source the Mediterranean Sea) had gained target body weight in a shorter culture period, with higher weight gain g/wk and survival percentage (Table 5). The average initial body weight of the seedstock (PL12) used in this study was not statistically different (Group 1: 0.010 ± 0.010, Group 2: 0.020 ± 0.000 g, $p <$ .0906). This weight was also reported by Mirzaei et al. [39]. But not consistent with other studies [30,40]. This inconsistency may be due to the quality of the broodstocks and different hatchery management practices [39]. The final body weight was (Group 1: 15.37 ± 2.03, Group 2: 16.93 ± 0.80 g, p < .2839). Like the findings of Ghosh et al. [41]. These results contradict Eid et al. [30], who reported a final body weight of 40 g after a 120-day culture period of *L. vannamei* in a Deeba Triangle farm. Spatial water quality variance could explain this difference [8]. The most important finding of this study is the notably low survival percentage observed (Group 1: 30.00 ± 2.00, Group 2: 43.33 ± 2.52 %, $p <$ .0020). Our data contrasts with the earlier findings of Eid et al [30], who reported a survival percentage of 70%. Survival rates reported in the literature vary widely. Sadek and Nabawi [42] reported a survival percentage above 90%. It could be explained by the initial cultivation of juvenile *L. vannamei* rather than postlarvae and lower salinity levels than those observed in our study. The postlarval stages of *L. vannamei* exhibit reduced immunity, which leads to high mortality rates [43]. In our study, all recorded hardness levels were above the acceptable level for L. vannamei, which may explain the reduced survival percentage [36,37].

The main strength of our study is its focus on *L. vannamei*, a species increasingly important for aquaculture in the Egyptian Mediterranean coastal zone, providing valuable baseline data on growth and survival. However, limitations include not measuring key water quality parameters like dissolved oxygen, heavy metals, and pollution indicators. Additionally, sampling frequency was restricted. Future research should include a more thorough assessment of water quality, such as hardness, to understand better how these factors influence the reduced survival rates. Exploring nursery rearing and polyculture systems could also offer practical strategies to enhance shrimp production and sustainability [41,44,45].

6. Conclusions

Consistent pond preparation, feeding, and management confirmed that variations in *L. vannamei* performance were driven by differences in water quality between Lake Manzala and the Mediterranean Sea. Superior growth in seawater underscores its favorable physicochemical profile for shrimp health and production. Water quality emerges as a critical determinant of aquaculture success; thus, in contexts where seawater access is limited, strategies should focus on enhancing current water sources and applying site-specific management to maximize sustainability and productivity.

Conflict of interest: Nothing to declare

7. References

1. IPCC. Climate Change 2014: Synthesis Report. Geneva: IPCC; 2014.

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

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. Saunois 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, Khattab 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

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

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 Ruminoccus 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.

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

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

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

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	$36.2^{(b)} \pm 0.58$	$230.9^{(a)} \pm 0.769$
Bentonite	$33^{(c)} \pm 0.71$	$205.8^{(c)} \pm 0.377$
Moringa	$39.1^{(a)} \pm 0.40$	$214.3^{(b)} \pm 0.508$
Seaweeds	$34.5^{(b)} \pm 0.50$	$152.6^{(d)} \pm 0.469$

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

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

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

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

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	$8.68^{(a)} \pm 0.101$	$97.64^{(a)} \pm 0.89$
Bentonite	$7.60^{(c)} \pm 0.070$	$91.9^{(b)} \pm 1.25$
Moringa	$8.29^{(b)} \pm 0.058$	$96.52^{(a)} \pm 0.67$
Seaweeds	$5.7^{(d)} \pm 0.034$	$80.3^{(c)} \pm 0.64$

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

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047

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	$18^{(a)} \pm 0.69$	$0.184^{(b)} \pm 0.0078$	$7.23^{(b)} \pm 1.07$
Bentonite	$19.3^{(a)} \pm 1.90$	$0.211^{(a)} \pm 0.0212$	$7.98^{(a)} \pm 0.98$
Moringa	$10.5^{(b)} \pm 1.12$	$0.108^{(c)} \pm 0.0109$	$4.08^{(c)} \pm 1.16$
Seaweeds	$4.48^{(c)} \pm 1.40$	$0.037^{(d)} \pm 0.0148$	$1.82^{(d)} \pm 0.84$

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	$1.22^{(b)} \pm 0.036$	$1.64^{(b)} \pm 0.041$	$0.79^{(a)} \pm 0.012$
Moringa	$1.17^{(c)} \pm 0.023$	$1.73^{(b)} \pm 0.082$	$0.73^{(b)} \pm 0.032$
Seaweeds	$1.27^{(a)} \pm 0.028$	$2.90^{(a)} \pm 0.052$	$0.38^{(c)} \pm 0.026$

The previous Data represent the mean ± SE, n = 5, p < 0.05.
The significance between different means is calculated from LSD.

Online ISSN: 2537-1045
Print ISSN: 1110-1423
DOI: 10.21608/vmjg.2025.400474.1047