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Combined Effects of Sodium Bentonite, *Moringa oleifera*, and Red Seaweed on In Vitro Rumen Fermentation and Methane Mitigation

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Abstract

THIS research aimed to study the effect of a combination of three natural feed additives sodium bentonite, *Moringa oleifera*, and red seaweed (*Jania granifera*) mixtures on in vitro rumen fermentation, methane mitigation, enzyme activity, and microbial population dynamics in Baladi sheep. Rumen fluid was incubated in gas-tight syringes with feed supplemented with mixes of additives for 24 hours. Key parameters assessed included total gas and methane production, pH, ammonia levels, total and individual volatile fatty acids (VFAs), cellulase activity, microbial biomass, and microbial efficiency. The relative abundance of total bacteria, cellulolytic bacteria, and methanogenic archaea was quantified using qPCR. Moringa oleifera and bentonite mixture increased gas production, microbial yield, and acetate molar proportions. As well as maintaining high cellulase activity, indicating enhanced microbial growth, but showed a decent reduction in methane (nearly 15%). Red seaweed & bentonite mixed supplementation significantly reduced methane production by approximately 39% and methanogenic archaea abundance to 0.25-fold compared to the control, but it decreased total VFAs and cellulase activity. The seaweed & moringa mixed treatment showed a satisfactory methane reduction (30%) with high digestibility and fermentation efficiency, rendering it the best choice for balanced rumen fermentation among the previous groups. These findings highlight the potential of these additives to optimize rumen fermentation and reduce methane emissions.

Keywords: Baladi sheep, *Jania granifera*, methane, *Moringa oleifera*, sodium bentonite.

Introduction

Methane (CH_4), a potent greenhouse gas, is the second-largest contributor to global warming after carbon dioxide, with a significantly higher heat-trapping capacity despite its shorter atmospheric lifespan of 7–12 years compared to CO_2's centuries-long persistence [1;2]. Approximately 60% of methane emissions stem from human activities, with agriculture, particularly ruminant livestock, fossil fuel operations, and organic waste decomposition in landfills being major sources. Natural sources, such as wetlands, wildfires, and termites, account for the remaining 40% [3;4;5]. In ruminants, methane is produced during enteric fermentation by methanogenic archaea, which utilize hydrogen (H) and carbon dioxide (CO_2) generated by other rumen microbes, leading to energy losses of 2–12% of dietary intake [6;7]. Alternative substrates, such as formic acid and methylamines, also contribute to methanogenesis [8]. The rumen microbial ecosystem, comprising bacteria, protozoa, and fungi, maintains fermentation stability through interspecies hydrogen transfer, preventing hydrogen accumulation that could hinder carbohydrate fermentation [9;10]. Ruminant livestock contribute significantly to global food security but are also major sources of methane (CH_4), a potent greenhouse gas produced during rumen fermentation. Mitigating methane emissions while maintaining rumen fermentation efficiency is critical for sustainable livestock production. Feed additives, such as plant-based compounds (e.g., *Moringa oleifera*, seaweed) and clay minerals (e.g., bentonite), have shown promise in modulating rumen

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fermentation, reducing methane, and enhancing nutrient utilization, especially when used in mixtures [11;12]. This current study investigated the effect of mixes of three dietary treatments (*Moringa oleifera* with bentonite, seaweed with bentonite, and seaweed with *Moringa oleifera*) on rumen fermentation parameters in vitro.

Material and Methods

This experiment was conducted at the Department of Physiology, Faculty of Veterinary Medicine, Cairo University, Egypt.

Rumen Fluid Collection

Rumen contents were obtained from five slaughtered Baladi sheep fed a diet composed of Tipn and wheat grains (50:50 ratio), as outlined in Table 1. The collected rumen contents were placed in a prewarmed thermos flask and transported to the laboratory. The rumen fluid was filtered through four layers of cheesecloth into a separate flask flushed with oxygen-free $\mathbf{CO_2}$. The filtered fluid was then combined with McDougall's buffer at a 1:2 (v/v) ratio, as described by McDougall [13]. Incubation occurred in a CO_2 incubator maintained at 39°C with 5% CO_2 for 24 hours [14].

Preparation of Treatment Systems and In Vitro Fermentation

Feed samples (200 mg) were placed into 60 mL calibrated plastic syringes with Vaseline-lubricated pistons. Each syringe received 30 mL of buffered rumen fluid. Additives were added at a rate of 4% of DMI for sodium bentonite, 7% of DMI for *moringa oleifera*, 3% of DMI for *Jania granifera* [15;16;17]. Four systems were prepared in quintuplicate for each group, namely; the control, moringa and bentonite, *Jania granifera* and bentonite, and *Jania granifera* and moringa. Moringa leaves were sourced from Haraz, a local market in Cairo, Egypt, while sodium bentonite was obtained from El Aksa Company. Seaweed was collected from Alexandria shores. Both moringa leaves and seaweed were identified by Prof. Abo Shanab S, Botany Department, Faculty of Science, Cairo University.

pH Measurement

After 24 hours of incubation, fluid samples were transferred to plastic bottles, and pH was immediately measured using a JENWAY 3510 pH meter.

Analysis of Total and Individual Volatile Fatty Acids (VFAs)

To determine total VFA concentrations and individual VFA proportions, 5 mL of fermentation fluid was mixed with 1 mL of 25% meta-phosphoric acid, centrifuged at 7,000 × g for 10 minutes, and the supernatant was stored at -20°C until assayed. Total VFA concentrations were quantified using steam distillation [18].

Individual VFA proportions were analyzed via YL 9100 High Performance Liquid Chromatography [19].

Ammonia Nitrogen Concentration

For ammonia nitrogen measurement, 5 mL of fermentation fluid was mixed with 2 mL of 0.2 N HCl, centrifuged at 5,000 × g for 20 minutes, and the supernatant was stored at -20°C. Ammonia concentrations were determined spectrophotometrically [20].

Methane Measurement

Methane concentration in the fermentation gas was measured immediately after collecting from each syringe using a (Testo 317-2) portable gas analyzer equipped with a catalytic bead sensor. Methane oxidation on the heated catalytic surface caused a change in electrical resistance proportional to its concentration which is expressed in micromoles [21].

Extracellular Cellulase Activity

Fermentation fluid samples were centrifuged at 3,000 × g for 20 minutes to isolate the supernatant. Half mL aliquot of supernatant (crude enzyme) was mixed with 0.5 mL of 1% carboxymethyl cellulose (CMC) in 0.05 M sodium citrate buffer and incubated at 55°C for 1 hour without shaking. The reaction was terminated by boiling for 5 minutes, followed by centrifugation at 7,000 × g for 5 minutes. Reducing sugars in the supernatant were quantified calorimetrically, with one unit of enzyme activity defined as the amount producing 1 micromole of glucose equivalent per minute [22].

In Vitro True Substrate Degradability (TSD)

True substrate degradability was determined by transferring the contents of each syringe into a beaker and rinsing the syringe with neutral detergent solution (NDS). The contents were incubated in NDS for 1 hour to dissolve microbial biomass, leaving undigested feed. The residue was filtered, dried at 130°C for 2 hours, and weighed to calculate degradability [23]. TSD was calculated as:

TSD = (weight of feed before incubation) - (feed residues after NDS incubation).

Stoichiometric Calculations

1. Acetate/Propionate (A/P) Ratio: Calculated from measured acetate and propionate values.

2. Fermentation Efficiency (FE): Determined using the formula:

$$FE = \frac{(0.622 \times A + 1.092 \times P + 1.56 \times B) \times 100}{(A + P + 2 \times B)}$$

where A, P, and B represent the moles of acetic, propionic, and butyric acids, respectively, expressed as a percentage [23].

Microbial Biomass Production (MBP), Microbial Yield (YATP), and Efficiency of Microbial Protein Production (EMP):

3. MBP was calculated as:

MBP = TSD - (gas volume × stoichiometric factor 2.2) [24].

4. EMP was derived as:

EMP = (TSD - (gas volume × 2.2)) / TSD [25].

5. Microbial yield (YATP) was calculated as the mass of microbial cells (mg) per millimole of ATP generated, with ATP yields of 2 for acetate, 3 for propionate, 3 for butyrate, and 1 for methane [26].

Quantitative Analysis of Microbial Populations

Total DNA was extracted from rumen fluid using the QIAamp UCP Pathogen Mini Kit (Cat. No. 50214). DNA purity and concentration were assessed using a nano spectrophotometer at A260/A280 [27]. Real-time quantitative PCR (qPCR) was performed using the Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System with SYBR Green dye to quantify total bacteria, cellulolytic bacteria, and methanogenic archaea [28;29;30]. Amplification conditions included an initial denaturation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds and annealing/extension at 60°C for 1 minute. Fluorescent product detection occurred at the end of each cycle. Bacterial populations were expressed relative to the total bacterial abundance (set as 1.00) at 0 hours [31].

Statistical Analysis

Data was analyzed using one-way analysis of variance (ANOVA). Treatment means were compared using the least significant difference (LSD) test at a 5% significance level [32].

Results

Gas Production and methane Output

Total gas production was highest in the Sw+M group (40.4 mL), followed by M+B (39.0 mL), while the lowest value was observed in Sw+B (32.4 mL) compared with the control (36.5 mL). In contrast, methane output peaked in the control group (243.9 µmol) and was lowest in Sw+B (148.7 µmol), followed by Sw+M (169.5 µmol) and M+B (207.2 µmol).

pH, Ammonia N concentration, and TVFA

Rumen pH values varied significantly, with the highest recorded in M+B (7.02), while Sw+M (6.78) and Sw+B (6.80) showed the lowest value, compared with the control (6.91). Ammonia-N concentration followed an opposite trend, being lowest in M+B (13.16 mg/100 mL), followed by Sw+B (13.92 mg/100 mL) and Sw+M (14.6 mg/100 mL), while the control had the highest value (16.68 mg/100 mL). For total volatile fatty acids (TVFA), the control group yielded the greatest concentration (1047.3), while the lowest was in Sw+B (904.6), followed by Sw+M (948.8) and M+B (981.2).

IVFA and A/P ratio

Acetate proportion was greatest in the control (53.89 mol/100 mol), followed by M+B (52.9) and Sw+M (51.0), while the lowest was observed in Sw+B (50.3). Propionate proportion was highest in Sw+B (29.8), followed by Sw+M (27.6) and M+B (27.3), but the control group recorded the lowest value (26.43). For butyrate, the highest concentration was measured in Sw+M (13.6), followed by Sw+B (13.5), while M+B had the lowest (12.3) compared with the control (12.69). Consequently, the acetate-to-propionate ratio (A/P) was recorded the greatest in the control (2.27), followed by M+B (1.94), Sw+M (1.85), and lowest in Sw+B (1.68).

Cellulase Activity and TSD

Cellulase activity reached its peak in control (8.75 mmol/min), closely followed by Sw+M (8.7 mmol/min), whereas the lowest activity was observed in Sw+B (6.33 mmol/min), with M+B showing an intermediate value (8.24 mmol/min). True substrate degradability (TSD) followed a similar pattern, being highest in Sw+M (98.2 mg), followed by control (97.48 mg), while Sw+B recorded the lowest (89.9 mg), and M+B was intermediate (96.7 mg).

Fermentation efficiency, MBP, EMP

Fermentation efficiency was highest in Sw+B (79.28%), followed by Sw+M (78.50%) and M+B (78.10%), while the control had the lowest (77.45%). Microbial biomass production (MBP) was also greatest in Sw+B (18.68 mg), followed by control (17.96 mg), while the lowest values were found in Sw+M (9.3 mg) and M+B (11.0 mg). Similarly, the efficiency of microbial protein synthesis (EMP) was highest in Sw+B (0.210), followed by the control (0.192). The lowest value was recorded in Sw+M (0.094), with M+B being intermediate (0.114). Microbial yield reflected the same trend, being greatest in Sw+B (7.61 mg/mmol ATP), followed by the control (7.38), while Sw+M (3.83) and M+B (4.48) recorded the lowest value.

Microbial Populations

The abundance of total bacteria, compared to the control group, was greatest in Sw+M (1.32), followed by Sw+B (1.21) and M+B (1.12). Cellulolytic bacteria counts were highest in Sw+B (3.7), followed by M+B (3.2) and Sw+M (3.1), with the control being lowest (1.0). In contrast, methanogenic archaea were most abundant in control (1.0 reference), while Sw+B (0.25) showed the lowest population, followed by Sw+M (0.33) and M+B (0.47).

Discussion

Dynamics End products of ruminal fermentation include volatile fatty acids, ammonia, and gases [10]. The control group produced 36.5 mL of gas, serving as the baseline for comparison. This level of gas production reflects normal rumen fermentation under standard conditions, driven by the microbial degradation of carbohydrates and proteins, which produces CO_2, H_2, and CH_4 the moderate gas output indicates balanced microbial activity [33]. In the Moringa + Bentonite (M+B) group, gas production was significantly higher than in the control group. This increase suggests enhanced microbial fermentation, likely due to Moringa's high crude protein and fermentable energy content, which stimulates microbial activity. Bentonite may further enhance fermentation and gas production by stabilizing rumen conditions [34]. Gas production decreased in the Seaweed + Bentonite (Sw+B) group. Seaweed's bioactive compounds, such as phlorotannins and bromoform, likely suppress microbial fermentation, reducing gas output [35]. The Seaweed + Moringa (Sw+M) group showed the highest gas production. This reflects enhanced fermentation due to Moringa's nutrient-rich profile leads to stimulation of microbial activity despite seaweed anti-methanogenic properties [36;35]. These variations in gas production are intrinsically linked to methane dynamics, as methanogenesis represents a key sink for hydrogen produced during fermentation, influencing overall gas composition and volume. Methane production in the control group was the highest among the treatments. This reflects the typical activity of methanogenic archaea in the rumen environment, where hydrogen from fermentation is utilized for methanogenesis, contributing to energy loss [37]. In the M+B group, methane production decreased to 15% compared to the control. Moringa's phenolic compounds and tannins likely inhibit methanogenic archaea, and also protozoa (major source of hydrogen) diverting hydrogen away from methanogenic pathway [38]. The Sw+B group exhibited 39% reduction compared to the control, the largest among treatments. Seaweed's bromoform inhibits methyl-coenzyme M reductase in methanogenic archaea, reducing methane synthesis, this seems true as the current study prove not only reduction in methanogenic archea functional activity but also population density reduction since results of qpcr showed 70% decrease in methanogenic archea [39]. Additionally, bentonite may adsorb methane precursors [40]. Methane production in the Sw+M group showed 30% reduction compared to the control. The combination of Moringa's tannins and seaweed's bromoform likely synergistically inhibits methanogenic archaea [39;38]. The observed methane reductions align closely with shifts in methanogenic archaea populations, underscoring how additive mixtures modulate archaeal abundance to redirect hydrogen fluxes away from methanogenesis toward more productive fermentation pathways. In the control group, methanogenic archaea had a relative abundance of 1, serving as the reference for microbial shifts. The balanced microbial community supports typical fermentation patterns [41]. In the M+B group, methanogenic archaea decreased. Bentonite stimulation of protein synthesis and bacterial growth combined with moringa's tannins, causing inhibition of methanogens, aligns with its methane-reducing effects [38]. In the Sw+B group, methanogenic archaea decreased, suggesting that seaweed's selective inhibition of methanogens aligns with its methane-reducing effects [42]. In the Sw+M group, a similar pattern to the Sw+B group was obtained. The combination of Moringa and seaweed enhances bacterial growth while inhibiting methanogens, supporting methane reduction [38;42].

pH is critical for fermentation to proceed in rumen and any alteration in pH has a drastic effect on microbial activity. The control group had a pH of 6.91, within the optimal range (6.5–7.0) for rumen microbial activity. This stable pH supports efficient fermentation and microbial growth, as deviations can impair fiber digestion and volatile fatty acid (VFA) production [43]. In the M+B group, the pH was the highest among all groups regardless possessing the lowest value of ammonia and a very high level of TVFA which could be attributed to the synergism between moringa, adding alkaloid contents [44], and bentonite, which exchanges cations; together, this can increase the buffering capacity of bentonite. Also, bentonite favors the production of propionate, which is less acidic than acetate, leading to overall lesser free H+ ions and higher PH [45]. The Sw+B group showed a pH decrease, reflecting the decreased ammonia concentrations due to the adsorbing effect of bentonite [40]. This aligns with seaweed's impact on fermentation pathways [12]. The Sw+M group had a lower pH, also reflecting increased total VFA production due to the moringa effect, particularly acetate, driven by seaweed's effect on fermentation pathways [12]. These pH shifts are closely intertwined with ammonia nitrogen levels, as ammonia acts as a buffering agent while also serving as a key nitrogen source for microbial protein synthesis, influencing overall acid-base balance in the rumen. Ammonia concentration in the control group was 16.68 mg/100 mL, indicating substantial protein degradation by rumen microbes. High ammonia levels in the control reflect the standard efficiency of nitrogen utilization, as excess ammonia is not incorporated into microbial protein [46]. In the M+B group, ammonia concentration was the lowest among treatments. Moringa's tannins bind dietary proteins, reducing their degradation [47]. Also, bentonite adsorbs more ammonia nitrogen [48].

The Sw+B group had an ammonia concentration of 13.92 ± 0.086 mg/100 mL ($P < 0.05$). Seaweed's inhibition of protozoa, which contribute to high levels of ammonia production (due its proteolytic and deaminating capacity), likely improves nitrogen utilization to synthesize more microbial protein [49]. Ammonia concentration in the Sw+M group decreased due to moringa's tannins and seaweed's protozoal inhibition, likely reducing protein degradation, improving nitrogen efficiency [50;49]. Modulations in pH and ammonia collectively shape the production of total volatile fatty acids (TVFAs), which represent the primary energy currency for ruminants and are sensitive to shifts in microbial metabolism driven by these environmental factors. TVFAs are the primary energy source for ruminants, high value indicates active microbial metabolism [51]. The obtained value of TVFA in control is 1047.3 reflecting normal fermentation and energy availability for the host. In the M+B group, TVFA was decreased, and this reduction may reflect Moringa's tannins inhibiting certain microbial populations, leading to lesser cellulolytic activity [52]. The Sw+B group had the lowest TVFA among treatments. This reflects seaweed's suppression of protozoa (efficient cellulolytic microorganisms) and bentonite's adsorbing abilities, reducing overall fermentation [53]. TVFA in the Sw+M group is also reduced due to the combined inhibitory effects of Moringa and seaweed [52]. The composition of individual VFAs further refines our understanding of these dynamics, as their molar proportions reflect specific fermentation pathways that are modulated by pH, ammonia availability, and total VFA pools, ultimately influencing energy partitioning and host metabolism. The three main volatile fatty acids are acetic (the main source of milk fat), propionic (the main glucose precursor), and butyric (the main source of energy for rumen epithelium). In the control group, acetic acid was 53.89 mol/100 mol, propionic acid was 26.43 mol/100 mol, and butyric acid was 12.69 mol/100 mol. The high acetate proportion is typical in roughage-based diets, supporting methanogenesis, while propionate and butyrate molar proportions indicate balanced fermentation pathways [54]. In the M+B group, acetic acid decreased, propionic acid increased, and butyric acid decreased. The shift toward propionate suggests a redirection of hydrogen away from acetogenic-methanogenic pathways and improving energy efficiency [55]. The Sw+B group showed lesser acetic acid, more propionic acid, and more butyric acid as the shift toward propionate and butyrate supports energy efficiency and milk fat synthesis, respectively [56]. In the Sw+M group followed a similar pattern like Sw+B that enhances energy efficiency and supports milk fat synthesis [56]. This interplay culminates in the acetate-to-propionate (A/P) ratio, a pivotal indicator of hydrogen utilization efficiency that bridges VFA profiles with broader fermentation outcomes, where lower ratios signify reduced methane losses and enhanced glucogenic potential. The control group had an A/P ratio of 2.27, typical for diets favoring acetate production, which provides hydrogen for methane synthesis. A higher A/P ratio is associated with less efficient energy utilization due to methane losses [57]. In the M+B group, the A/P ratio decreased, indicating a shift toward propionate production. This is consistent with Moringa's ability to alter fermentation pathways, reducing methane production [58]. The Sw+B group had the lowest (A/P) ratio among treatments. This suggests a strong shift toward propionate, reducing hydrogen availability for methanogenesis [42]. The Sw+M group also had a moderate reduction, indicating a shift toward propionate production, reducing methane synthesis [58].

Cellulase activity in the control group was 8.75 mmol/min, indicating standard fiber degradation capacity by cellulolytic bacteria. This supports efficient breakdown of structural carbohydrates, contributing to VFA production [59]. In the M+B group, cellulase activity decreased slightly, suggesting minor inhibition of cellulolytic bacteria by Moringa's bioactive compounds and bentonite's inhibition of cellulolytic activity by binding to the substrates. However, the high TDS indicates that fiber digestion remains effective [60]. The Sw+B group had significantly reduced cellulase activity, indicating that inhibition of cellulolytic bacteria is carried out mainly by seaweed's bioactive compounds, potentially limiting fiber digestion [61]. Cellulase activity in the Sw+M group had a value similar to the control, suggesting that Moringa counteracts seaweed's inhibitory effect on cellulolytic bacteria, maintaining fiber digestion [60]. These enzymatic responses are directly tied to cellulolytic bacterial populations, whose abundance determines the rumen's capacity to hydrolyze complex polysaccharides, thereby influencing the rate and extent of fiber degradation. In the control group, cellulolytic bacteria had a relative abundance of 1, serving as the reference for microbial shifts. The balanced microbial community supports typical fermentation patterns [41]. In the M+B group, cellulolytic bacteria increased. Bentonite stimulation of protein synthesis and bacterial growth combined with moringa's tannins aligns with its effects [38]. In the Sw+B group, the rise in concentrations of cellulolytic bacteria suggests that seaweed's stimulation of cellulolytic bacteria aligns with its effects [42]. In the Sw+M group, a similar pattern to the Sw+B group was obtained. The combination of Moringa and seaweed enhances bacterial growth [38;42]. Ultimately, the synergy between cellulase activity and cellulolytic bacteria manifests in true substrate digestibility (TSD), a comprehensive metric of nutrient liberation that integrates microbial enzymatic prowess with substrate availability, highlighting the additives' role in optimizing feed utilization. In the control group, TSD was 97.48 mg, reflecting the standard digestibility of the control diet. This suggests effective microbial degradation of organic matter, maximizing nutrient availability [62]. In the M+B group, TSD was similar to the control (P > 0.05). Moringa's nutrient-rich profile supports microbial degradation, while bentonite stabilizes fermentation, maintaining high digestibility [36]. The Sw+B group showed a decrease in TSD, indicating reduced fiber digestion due to inhibited cellulolytic activity, this may be attributed to the synergistic effect between seaweed and bentonite which suggests a potential reduction in nutrient availability by bentonite and inhibition of cellulolytic activity by seaweed [67]. In the Sw+M group, TSD was measured the highest among treatments due to moringa's nutrient and fiber content counteracting seaweed's inhibitory effect on cellulolytic bacteria, and supporting higher digestibility [62].

Fermentation efficiency in the control group was 77.45 %, indicating effective energy conversion from substrates to VFAs. This baseline efficiency exhibits standard rumen microbial performance [61]. In the M+B group, fermentation efficiency increased, likely due to the shift toward propionate production, which improved energy utilization [46]. The Sw+B group had the highest fermentation efficiency, which suggests optimized energy utilization, also due to increased propionate production [68]. Fermentation efficiency in the Sw+M group had a similar pattern to the Sw+B group [68]. This enhanced efficiency naturally extends to microbial yield, as greater propionate formation implies more ATP availability per unit of substrate fermented, fostering higher biomass production per energy unit and underscoring the additives' impact on metabolic thriftiness. Microbial yield in the control group was 7.38 mg/mmol ATP, reflecting typical microbial biomass production in an ordinary diet [62]. In the Moringa + Bentonite (M+B) group, microbial yield decreased, consistent with reduced MBP and EMP, likely due to Moringa's inhibitory effects on microbial growth [63]. The Seaweed + Bentonite (Sw+B) group had a microbial yield similar to the control, indicating sustained microbial biomass production [62]. Microbial yield in the Seaweed + Moringa (Sw+M) group decreased, as confirmed by reduced MBP and EMP [63].

In the control group, MBP was 17.96 mg, and EMP was 0.192. These values indicate moderate microbial protein synthesis, supporting nutrient supply to the host [64]. In the M+B group, both MBP and EMP decreased due to bentonite's adsorbing abilities and moringa's tannins that inhibit microbial growth, and reduce biomass production [65]. The (Sw+B) group increased both due to Seaweed's support for microbial growth enhances protein synthesis, despite reduced TVFA [61]. Also, bentonite adsorbs more ammonia nitrogen, favoring more microbial protein synthesis [48]. (Sw+M) group had a similar pattern to the (M+B) group, as moringa's tannins are likely to limit microbial growth [65]. These metrics of microbial biomass and protein efficiency are underpinned by total bacterial abundance, which sets the foundational microbial density for protein synthesis and biomass accrual, revealing how additives selectively promote or constrain overall bacterial proliferation to balance growth with resource utilization. In the control group, total bacteria had a relative abundance of 1, serving as the reference for microbial shifts. The balanced microbial community supports typical fermentation patterns [41]. In the M+B group, total bacteria increased. Bentonite stimulation of protein synthesis and bacterial growth combined with Moringa's tannins aligns with its effects [38]. In the Sw+B group, the rise in concentrations of total bacteria suggests that seaweed's selective stimulation aligns with its effects [42]. In the Sw+M group, a similar pattern to the Sw+B group was obtained. The combination of Moringa and seaweed enhances bacterial growth [38;42].

Conclusion

The control group exhibited typical rumen fermentation with high methane production and balanced microbial activity. The M+B mixtures enhanced fermentation but reduced nitrogen efficiency while reducing methane by 15%, supported by Moringa's nutrients and bentonite's buffering. The Sw+B group had achieved the largest methane reduction (39%) but reduced TDS, cellulase activity, and indicating potential limitations in fiber digestion. The Sw+M group had balanced methane reduction (30%) with high digestibility and fermentation efficiency, making it a promising option for sustainable ruminant production. It could also be encapsulated (to ensure safety and enhance absorption) in further in vivo studies to extend the impact of these additives on rumen fermentation patterns and the long-term effects on animal health, productivity, and the overall economic value of their use in sheep livestock.

Conflict of interest.

No conflicts of interest have been declared.

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Author Contributions.

Francois A. Sawiress: Led investigation, visualization, supervision, review, and editing, as well as methodology development and conducted the final revision.

Marwa A. Ibrahim: Contributing to methodology and data curation.

Mahmoud A. Abdl-Rahman: Led investigation, visualization, and supervision,

Ahmed A. Ismail: Contributed to drafting the original manuscript and developing the methodology.

Ethical of approval

The study was carried out in Cairo University's Physiology Department and was authorized by the Faculty of Veterinary Medicine's Institutiona Animal Care and Use Committee (IACUC) (Approval Number: Vet Cu13102024984).

TABLE 1. Chemical analysis of the feed ration on a dry matter basis

Chemical analysis	% of dry matter
Crude fiber	**28.5**
Crude proteins	**12.4**
Ether extract	**3.7**
Nitrogen-free extract	**35.8**
Total ash	**13.8**

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. Ruminal gas production (mL) and methane output (µmol)

Group	Total Gas (mL)	Methane (µmol)
Control	$36.5^{b} \pm 0.52$	$243.9^{a} \pm 0.774$
M+B	$39.0^{a} \pm 0.73$	$207.2^{b} \pm 0.372$
Sw+B	$32.4^{c} \pm 0.46$	$148.7^{d} \pm 0.513$
Sw+M	$40.4^{a} \pm 0.58$	$169.5^{c} \pm 0.478$

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

TABLE 4. Ruminal pH, Ammonia-N (mg/100 mL), and TVFA (mg/L)

Group	pH	Ammonia-N (mg/100 mL)	TVFA (mg/L)
Control	$6.91^{b} \pm 0.033$	$16.68^{a} \pm 0.673$	$1047.3^{a} \pm 0.163$
M+B	$7.02^{a} \pm 0.030$	$13.16^{d} \pm 0.501$	$981.2^{b} \pm 0.079$
Sw+B	$6.80^{c} \pm 0.026$	$13.92^{c} \pm 0.383$	$904.6^{d} \pm 0.058$
Sw+M	$6.78^{c} \pm 0.014$	$14.60^{b} \pm 0.478$	$948.8^{c} \pm 0.169$

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

TABLE 5. Ruminal individual VFA proportions (mol/100 mol), and A/P ratio

Group	Acetate (mol/100 mol)	Propionate (mol/100 mol)	Butyrate (mol/100 mol)	A/P Ratio
Control	$53.89^{a} \pm 0.06$	$26.43^{c} \pm 0.09$	$12.69^{b} \pm 0.05$	$2.27^{a} \pm 0.0045$
M+B	$52.9^{b} \pm 0.08$	$27.3^{b} \pm 0.05$	$12.3^{c} \pm 0.04$	$1.94^{b} \pm 0.0057$
Sw+B	$50.3^{d} \pm 0.13$	$29.8^{a} \pm 0.03$	$13.5^{a} \pm 0.02$	$1.68^{d} \pm 0.0048$
Sw+M	$51.0^{c} \pm 0.17$	$27.6^{b} \pm 0.14$	$13.6^{a} \pm 0.03$	$1.85^{c} \pm 0.0076$

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

TABLE 6. Ruminal cellulase activity(mmol/min) and TSD (mg)

Group	Cellulase Activity (mmol/min)	TSD (mg)
Control	$8.75^{a} \pm 0.107$	$97.48^{b} \pm 0.92$
M+B	$8.24^{c} \pm 0.073$	$96.7^{b} \pm 1.23$
Sw+B	$6.33^{d} \pm 0.062$	$89.9^{c} \pm 0.74$
Sw+M	$8.7^{b} \pm 0.038$	$98.2^{a} \pm 0.69$

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

TABLE 7. Ruminal fermentation efficiency (FE) (%), MBP (mg), EMP, YATP (mg/mmol ATP)

Group	FE (%)	MBP (mg)	EMP	YATP (mg/mmol ATP)
Control	$77.45^{d} \pm 0.064$	$17.96^{b} \pm 0.58$	$0.192^{b} \pm 0.0097$	$7.38^{a} \pm 1.13$
M+B	$78.10^{c} \pm 0.041$	$11.0^{c} \pm 1.82$	$0.114^{c} \pm 0.0231$	$4.48^{b} \pm 1.05$
Sw+B	$79.28^{a} \pm 0.035$	$18.68^{a} \pm 1.26$	$0.210^{a} \pm 0.0073$	$7.61^{a} \pm 1.18$
Sw+M	$78.50^{b} \pm 0.028$	$9.3^{d} \pm 1.47$	$0.094^{d} \pm 0.0128$	$3.83^{c} \pm 0.96$

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

TABLE 8. Ruminal relative microbial populations (qPCR)

Group	Total Bacteria	Cellulolytic Bacteria	Methanogenic Archaea
Control	1.00^{d}	1.0^{c}	1.0^{a}
M+B	$1.12^{c} \pm 0.042$	$3.2^{b} \pm 0.086$	$0.47^{b} \pm 0.037$
Sw+B	$1.21^{b} \pm 0.027$	$3.7^{a} \pm 0.072$	$0.25^{d} \pm 0.019$
Sw+M	$1.32^{a} \pm 0.031$	$3.1^{b} \pm 0.035$	$0.33^{c} \pm 0.023$

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

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تأثير مزيج من بنتونيت الصوديوم، ومورينجا أوليفيرا ، ومستخلص الأعشاب البحرية الحمراء (جانيا جرانيفيرا) على تخمير الكرش في المختبر وتقليل الميثان

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الملخص

يهدف هذا البحث إلى دراسة تأثير خليط من ثلاثة مكونات طبيعية - بنتونيت الصوديوم، والمورينجا أوليفيرا، والأعشاب البحرية الحمراء (جانيا جرانيفيرا) – على تخمرات الكرش في المختبر، وانبعاثات الميثان، ونشاط الإنزيمات، وديناميكيات التجمعات الميكروبية في أغنام البلدي. حيث حُضّن سائل الكرش في محاقن محكمة الغلق مع علف مُضاف إليه مزيج من الإضافات لمدة 24 ساعة. وشملت المعايير الرئيسية التي تم تقييمها إجمالي إنتاج الغاز والميثان، ودرجة الحموضة (pH)، ومستويات الأمونيا، والأحماض الدهنية المتطايرة الكلية والفردية (VFAs)، ونشاط السليولاز، والكتلة الحيوية الميكروبية، والكفاءة الميكروبية. وحُددت الوفرة النسبية للبكتيريا الكلية، والبكتيريا المحللة للسليلوز، والعتائق الميثانوجينية باستخدام تفاعل البوليميراز المتسلسل الكمي (qPCR).النتائج اظهرت ان خليط المورينجا أوليفيرا والبنتونيت ادى إلى زيادة إنتاج الغازات، وزيادة العائد الميكروبي، وزيادة النسب المولية للأسيتات، مع الحفاظ على نشاط السليولاز المرتفع، مما يشير إلى تعزيز النمو الميكروبي، ولكنه أظهر انخفاضًا معنويا في الميثان (حوالي 15%). وقد أدى إضافة خليط الأعشاب البحرية الحمراء والبنتونيت إلى انخفاض ملحوظ في إنتاج الميثان بنسبة تقارب 39%، ووفرة العتائق الميثانوية إلى 0.25 ضعف مقارنةً بالمجموعة الضابطة، ولكنه خفض إجمالي الأحماض الدهنية المتطايرة ونشاط السليولاز. وقد أظهر خليط الأعشاب البحرية والمورينجا انخفاضًا مُرضيًا في الميثان (30%)، مع قابلية هضم وكفاءة تخمير عالية، مما يجعله الخيار الأمثل لتخمير متوازن للكرش بين المجموعات السابقة. وتُبرز هذه النتائج قدرة هذه الإضافات على تحسين تخمير الكرش وتقليل انبعاثات الميثان.

الكلمات الدالة :الأغنام البلدي، المورينجا الجرانيفيرية، الميثان، المورينجا أوليفيرا، بنتونيت الصوديوم.