EFFECT OF ADDING DIETARY ROSEMARY LEAVES ON IN VITRO METHANE PRODUCTION AND SOME RUMEN FERMENTATION TRAITS

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ABSTRACT

This study aimed to examine how including dietary dried rosemary leaves (DRL) of Holstein bulls affects methane production and certain rumen fluid characteristics *in vitro*. Rumen fluid was obtained from newly slaughtered calves and used in two separate trials: one for gas production and another for digestion. In the trials, two diets were implemented. The first was the standard diet for bulls, consisting of a concentrate diet and alfalfa hay (control group). The second diet included the addition of 250 g DRL to the essential diet. There was a significant decrease (P≤0.01) in methane and N-NH3 production, as well as in the ratio between unsaturated fatty acids (USF) and saturated fatty acids in the rosemary group compared to the control. In the first trial, the concentrations of both volatile and non-volatile fatty acids as well as the total USF increased significantly (P≤0.01) in the rosemary group compared to the control group. In the second trial, there was a significant increase (P≤0.01) in the digestibility of dry and organic matters and in metabolizable energy in the rosemary group. There was a decrease in the population of protozoa in the rosemary group compared to the control group. The addition of DRL can be seen as beneficial in modifying rumen fermentation. This can lead to a reduction in methane production and an improvement in rumen fermentation traits. As a result, there is an increase in the undegradable protein in the rumen that enters the small intestine. This ultimately means that more amino acids are available for production, and methane emissions from bulls can be reduced, contributing to sustainable agricultural development goals.

Keywords: Ruminants, methane, feed additives, volatile fatty acid, global warmer, sustainable agriculture

تأثير إضافة اوراق اكليل الجبل لعليقة ثيران الهولشتاين في انتاج غاز الميثان وبعض صفات تخمرات الكرش مختبريا

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المستخلص

هدفت هذه الدراسة إلى بيان تأثير إضافة أوراق إكليل الجبل المجففة إلى عليقة ثيران هولشتاين في إنتاج الميثان وبعض خصائص سائل الكرش مختبرياً. تم الحصول على سائل الكرش من العجول المذبوحة حديثًا واستخدامه في تجربتين منفصلتين: واحدة لإنتاج الغازات والأخرى للهضم. تم استخدام عليقتين، الأولى هي العليقة القياسية للثيران والتي تكونت من العليقة المركزة ودريس الجت (مجموعة السيطرة) والثانية التي تضمنت إضافة 250 جرام من أوراق إكليل الجبل المجففة للعليقة الاساسية. انخفض (P≤0.01) انتاج الميثان و $N-NH_3$ والنسبة بين الاحماض الدهنية غير مشبعة(USF) : المشبعة في مجموعة اكليل الجبل مقارنة مع مجموعة السيطرة. ازدادت تراكيز الاحماض الطيارة وغير الطيارة ومجموع USF لدى مجموعة الاكليل مقارنة مع مجموعة السيطرة (تجربة 1). ازداد معامل هضم المادة الجافة والعضوية والطاقة المتأيضة (P≤0.01) مع انخفاض اعداد البروتوزوا لدى مجموعة اكليل الجبل مقارنة مع السيطرة (تجربة2). أن إضافة أوراق إكليل الجبل المجففة استطاعت تعديل تخمر الكرش بشكل إيجابي من خلال تقليل إنتاج غاز الميثان وتحسين تخمرات الكرش، وهذا يؤدي إلى زيادة البروتين غير المتحلل في الكرش الذي يدخل الأمعاء الدقيقة، مما يعني توفر المزيد من الأحماض الأمينية للإنتاج وتقليل انبعاث الميثان من الثيران لتحقيق أهداف التنمية الزراعية المستدامة.

الكلمات المفتاحية: المجترات، الميثان، الإضافات الغذائية، الأحماض الدهنية الطيارة، الاحتباس الحراري العالمي، الزراعة المستدامة

Received:15 /4/2024, Accepted:14/8/2024, Published:30 June.

INTRODUCTION

In recent years, there has been a growing interest in the phenomenon of global warming, which is associated with rising global temperatures. Methane, the second most significant greenhouse gas, is considered to be important in global warming after CO_2. It absorbs the sun's heat, warms the atmosphere by absorbing energy, and slows the rate of its escape into space (26). The rising levels of methane gas have significant consequences for humans, animals, and the environment. Methane has a warming potential 28 times greater than CO_2. It is primarily produced through enteric fermentation (EF) and, to a lesser extent, from manure. The primary sources of EF are the rumen (90-87%) and the large intestine (13-10%). The estimated methane emission rate from cows is 150 g per animal per day. There is a global trend to reduce methane emissions from ruminant animals in order to mitigate global warming and redirect the energy lost by the animal to improve its productivity. This can be achieved by altering the path of hydrogen formed in the rumen to produce volatile fatty acids and microbial mass (47). Dairy cattle were the largest contributors to methane emissions in 2020, accounting for 72% of the total agricultural sector emissions (26). Methane gas has a shorter lifespan in the atmosphere (8.6 years) compared to CO_2. Hence, it is important to focus on reducing methane gas emissions and developing strategies to decrease its release, all while maintaining or increasing livestock production (26). Additionally, research has shown that 47% of methane emissions from ruminants are linked to poor animal performance (31). The reduction of gas emissions is closely tied to achieving the United Nations' sustainable development goals, which include addressing climate change, promoting responsible consumption and production, eradicating poverty and hunger, and ensuring good health and well-being. As a result, it is essential to implement researches or strategies aimed at enhancing animal reproductive efficiency and productivity (1,2,31,32,34,35) and reducing methane emissions (21,25,26) in order to support the UN sustainable development goals. One potential strategy involves using natural plant-based additives containing active compounds to enhance the quality of animal products (4, 5). These additives have also been utilized to mitigate methane emissions (21). Rosemary plants have numerous medical benefits (8). They are naturally available, easy to propagate at a high speed, and do not require complex environmental conditions for cultivation and propagation. Additionally, they are tolerant to drought. Rosemary plants contain flavones, rosmarinic acid, carnosol, carnosic acid, and phenolic acids, all of which act as antioxidants (9, 18). Several studies have demonstrated that rosemary powder extract and its oil can reduce methane gas (14, 15,17,25,33). Rosemary has also been found to decrease oxidative stress in high-producing bulls and cows (5,7,25), and enhance nutrient digestibility, rumen fermentation, growth performance, and milk production in lambs and dairy cows (11,17,25). Hence, the study aimed to investigate how adding dried rosemary leaves (DRL) to bulls' diets impacts in vitro methane production and some rumen fermentation characteristics.

MATERIALS AND METHODS

This study was conducted in the Nutrition Laboratory of the Department of Animal Production, College of Agricultural Engineering Sciences, University of Baghdad, from October 30, 2021, to November 30, 2022. The study aimed to examine the impact of adding DRL to the diet of Holstein bulls on *in vitro* methane gas production and the digestibility of certain rumen fluid characteristics. The experiment used two diets. The first was the basic diet typically fed to Holstein bulls for artificial insemination, consisting of 8 kg of concentrate per bull (Table 1) and 40 kg of alfalfa hay per bull (Control group). In the second group's diet, 250 g of DRL were added to the essential diet (rosemary group). Proximate analysis was conducted on the concentrate diet, alfalfa hay, and DRL (Table 2; 6). Additionally, mycotoxins screening was performed (Table 3) and some of the active constituents in DRL were assessed (Figure 1). This experiment utilized rumen fluid from a bull that had been slaughtered recently. The rumen fluid, along with all its components (including the consumed feed), was collected and placed in a clean, sterile, tightly sealed container to prevent air and water contamination. Subsequently, the container was put in a large thermal container filled with water at 39°C and promptly transported to the laboratory within 20 minutes The rumen contents were filtered using four layers of medical gauze in a CO_2 incubator set at 39°C. The rumen fluid was divided for two trials. In the first trial, the rumen fluid was used for *in vitro* gas production at 24, 48, 72, and 96 hours per diet with blank (6 replicates per incubation period per diet and blank) to determine total gas, methane production, and metabolic energy. In the second trial, the rumen fluid was used to determine some of the traits in vitro digestion for two diets with blank (6 replicates per diet and blank).

Table 1. The components of the concentrate diet

Components	%
Barley	35
Wheat bran	33
yellow corn	10
Soybean meal	20
Limestone	0.5
Salt	0.5
Vitamins and minerals	1
Total	100

Table 2. Proximate analysis of concentrate diet, dried rosemary leaves (DRL), and alfalfa hay.

Proximate analysis (%)	Con. Diet	Alfalfa hay	DRL
Crude protein	18.7	17	4
Fat	10.5	0.25	4.1
Fiber	--	30	26
Ash	1	12	8
Energy	3167	3300	2820
Humidity	7.04	7.10	6.60

Con= Concentrate,

Table 3. Screening for mycotoxins in Holstein bull diet and dried rosemary leaves

Screening (ppb)	Basic diet	Dried rosemary leaves
Aflatoxin	11.5	0.2
T-2 /HT-2	0.0	0.0
Ochratoxin	0.2	0.0

***In vitro* fermentation:**

Gas production and other rumen characteristics: Total gas and methane production were estimated using the Fievez et al. (19) method. The pH value was measured immediately during the incubation period without adding sodium hydroxide. The concentration of ammonia nitrogen was measured according to Umbreit et al. (41). The in vitro organic matter digestibility (IVOMD%) and metabolic energy (ME) were calculated using the total gas production volume (ml), crude protein (CP%), crude fiber (CF%)and ash (A) after an incubation period of 24 hours (30).

Volatile fatty acid determination: The determination of volatile fatty acids (acetic, butyric, and propionic;40) is done after a 24-hour incubation period using gas chromatography (GS-2010) with a DB-1 capillary column (30mm x 0.25um x 0.25mm). The injection temperature is 280ºC, the detector (FID) temperature is 330ºC, and the column oven (ZB-5) temperature ranges from 90-150ºC (increasing at 5ºC per minute) with a pressure of 105 kPa using inert nitrogen

Determination of fatty acids: The palmitic, stearic, oleic, linoleic, linolenic acids were determined (46)

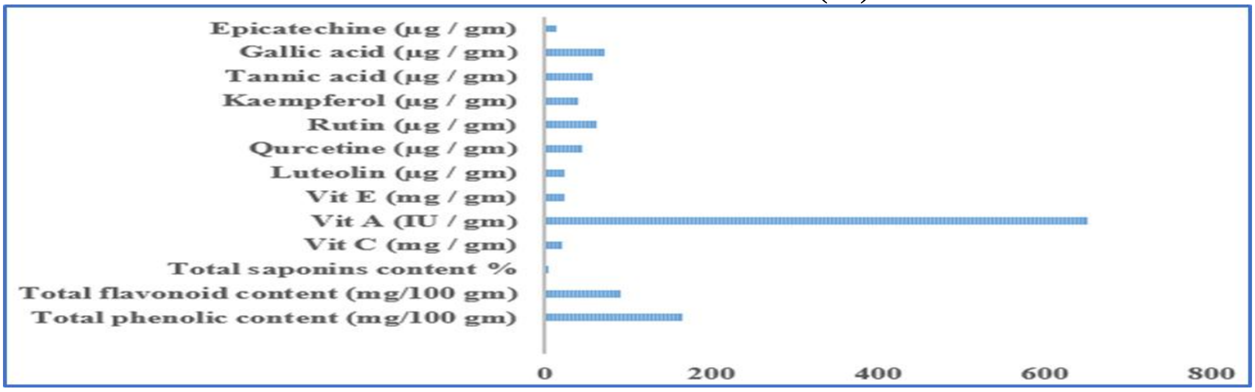

Figure 1. Assessment some of active constituent in dried rosemary leaves

by using gas chromatography [SE-30 capillary column (30mm x 0.25um x 0.25mm), temperature injection=280ºC, temperature detector (FID) =310ºC, column oven (ZB-1) =120-290 (10ºC/min.), pressure =100 kpa).

***In vitro* digestibility of some rumen characteristics :**The digestibility of dry and organic matter (IVOMD and IVDMD%) was estimated using the method of Tilley and Terry (39), which involved two successive stages of 48 hours each. This was necessary because the bulls' feed consisted of a concentrated diet and alfalfa hay, requiring microbial and enzymatic digestion. Metabolic energy (ME) is calculated according to MAFF (29), and the number of protozoa is determined following the method of Williams and Coleman (44).

Statistical analysis: All data statistically analysis by using the general linear model procedure in the SAS program, using CRD to examine the effect of groups (control and rosemary) on in vitro total gas and methane production, dry and organic matter digestibility, volatile fatty acid, some of fatty acid and metabolic energy. Duncan multiple range was used for compering means with significant differences.

RESULTS AND DISCUSSION

***In vitro* gas production and rumen characteristics:** The group that received rosemary showed lower ($P<0.01$) concentrations of total gas, methane production, and N-NH3 at different incubation periods compared to the control group (Table 4). Within each group (control and rosemary), the total gas and methane production had significantly lower ($P<0.01$) concentrations at 24 and 48 hours compared to the 72 and 96-hour incubation periods. The N-NH3 concentration (mg/dl) decreased as the incubation period progressed within each group. The highest concentration was recorded at the 24-hour incubation period, while the lowest concentration was observed at the 96-hour incubation period (Table 4). The pH of the rumen fluid did not differ between the two groups during all incubation periods except at 48 hrs., where it declined ($P\leq0.05$) in the rosemary group compared to the control groups (Table 4). The current findings support Daş (14), who found that adding rosemary powder to the silage reduced methane production. Our results are also aligned with Molho-Ortiz et al. (33), who showed that using essential oils or aqueous extracts of rosemary with a basic diet decreased total gas production, methane production, and dry matter digestibility in sheep. The current findings differed from those of Guneya et al. (20), who found that adding rosemary oil (250 and 500 mg/kg DM) to the lamb diet did not affect the N-NH4 inside the rumen. However, the DRL had a positive effect on *in vitro* rumen fermentation (Table 4). This may be due to the presence of active constituents in the DRL such as tannic acid, saponins, etc. (Figure 1). Tannic acid can reduce fiber digestion, decrease methane emission, bind to feed protein in the rumen through hydrogen bonds, and protect protein from degradation by ruminal microorganisms. As a result, more undegradable protein enters the small intestine, allowing for more accessible amino acids for production, such as semen in the testis (1, 43, 48). Saponins have impaired protein digestion in the rumen and interact with cholesterol in the cell membrane, causing cell rupture and selective ruminal protozoa elimination. Thus, they improve N-use efficiency and result in a probable increase in ruminant animal performance (23). Flavonoids reduced the total populations of protozoa and methanogens without adversely affecting ruminal fermentation characteristics in vitro incubation (24, 37). Many studies have noted that rosemary extract is an antioxidant with the potential to reduce methane emissions and change microbial fermentation (13,25). The decrease in total gas and methane production in the rosemary group at different incubation periods compared to the control group may be related to a decrease in the number of protozoa, which are largely responsible for methane production. This was found in our current study: the number of protozoa decreased significantly ($P < 0.06$) in the rosemary group compared to the control (Table 7). The N-NH3 levels, IVOMD, and metabolic energy (Table 4) showed significant declines ($P\leq0.01$) in the rosemary group compared to the control group based on gas production. This decrease is attributed to a significant decrease in total gas production in the rosemary group (Table 4). Active components present in rosemary, such as phenol, flavonoids, saponins, tannic acid, rutin, quercetin, etc. (Figure 1) may contribute to these changes. The decrease in IVOMD and ME may be due to the presence of tannins in the rosemary group. Tannins reduce protein degradation in the rumen, which leads to an increase in amino acid flow to the small intestine due to the complexes formed between tannins and protein. These effects on nutrition are reflected in animal performance (10).

Tannins can improve nitrogen use and decrease CH4 production. They can form complexes with proteins and carbohydrates in the rumen environment. The tannin-protein complex creates a protective layer on proteins, preventing degradation by bacteria. This complex is favored under pH 6.0 and 7.0, as in the rumen environment, and only dissociates under pH conditions of 2.5–3.0 (10).

Volatile fatty acids (VFAs): The concentrations of acetic, butyric, propionic, and total volatile fatty acids were higher in the rosemary group compared to the control group. Additionally, the ratio of acetic acid to propionic acid was higher in the control group than in the rosemary group (refer to Table 5). Volatile fatty acids (VFA) serve as the primary energy source for ruminants and are estimated to provide up to 75% of the total metabolizable energy. The main factors influencing the ruminal acetate-to-propionate ratio are the degradation rate of the diet and the microbial structure of the rumen (27). Reducing the ratio of acetate to propionate in the rumen can improve the efficiency of dietary energy utilization (27). May studies have demonstrated that, with the same diet, the acetate-to-propionate ratio in the rumen can be altered by dietary composition, the rate of dietary degradation, dietary metabolizable energy, rumen microbial and etc. (27,28,42). The DRL improve energy utilization by increasing the production of volatile fatty acids. This relationship may be explained by the active constituents in dried rosemary leaves, such as tannins, phenols, flavonoids, and saponins (Figure 1), which play a role in these results. Microorganisms can metabolize flavonoids to produce propionic acid and butyric acid (12). These acids help maintain the acid-base balance, regulate pH, and have various effects on rumen fermentation. Phenolic and tannins have been found to increase volatile fatty acid (VFA) production, shift the VFA profile from acetate to propionate, reduce the acetate- to- propionate ratio, stabilize rumen pH, and decrease protozoa and methane production (16, 36). These findings align with the results of our current studies (Tables 4 and 5).

Table 4. The effect of adding dried rosemary leaves to the diet of Holstein bulls on *in vitro* of the total gas, methane production, and some rumen fatty acids characteristics following different incubation periods (Mean ± SE).

Incubation periods / Trait: group	24 hrs.	48 hrs.	72 hrs.	96 hrs.	Significance level
Total gas (ml):					
Control	$33.00^{Ac}\pm0.41$	$34.50^{Ac}\pm0.29$	$36.25^{Ab}\pm0.23$	$39.00^{Aa}\pm0.58$	$P\leq0.01$
Rosemary	$19.75^{Bc}\pm0.63$	$21.25^{Bc}\pm0.48$	$23.00^{Bb}\pm0.58$	$25.00^{Ba}\pm0.41$	$P\leq0.01$
Significant	$P\leq0.01$	$P\leq0.01$	$P\leq0.01$	$P\leq0.01$	
Methanol(ml):					
Control	$14.75^{Ab}\pm0.48$	$16.00^{Ab}\pm0.41$	$15.00^{Aa}\pm0.41$	$19.25^{Aa}\pm0.48$	$P\leq0.01$
Rosemary	$8.25^{Bc}\pm0.48$	$8.75^{Bc}\pm0.75$	$12.00^{Bb}\pm0.41$	$14.00^{Ba}\pm0.71$	$P\leq0.01$
Significant	$P\leq0.01$	$P\leq0.01$	$P\leq0.01$	$P\leq0.01$	
pH:					
Control	$6.85^{Aa}\pm0.05$	$6.95^{Aa}\pm0.06$	$6.95^{Aa}\pm0.09$	$6.85^{Aa}\pm0.06$	NS
Rosemary	$6.95^{Aa}\pm0.06$	$6.78^{Bb}\pm0.03$	$6.85^{Aab}\pm0.05$	$6.98^{Aa}\pm0.03$	$P\leq0.05$
Significant	NS	$P\leq0.05$	NS	NS	
N-NH3 (mg/dl):					
Control	$35.19^{Aa}\pm0.43$	$33.11^{Ab}\pm0.3$	$29.98^{Ac}\pm0.51$	$27.03^{Ad}\pm0.33$	$P\leq0.01$
Rosemary	$29.09^{Ba}\pm0.40$	$24.17^{Bb}\pm0.38$	$21.14^{Bc}\pm0.53$	$18.89^{Bd}\pm0.03$	$P\leq0.01$
Significant	$p\leq0.01$	$p\leq0.01$	$p\leq0.01$	$p\leq0.01$	
IVOMD (%)			+		
Control	$61.97^{A}\pm0.36$	--	--	--	--
Rosemary	$50.93^{B}\pm0.18$	--	--	--	--
Significant	$P\leq0.01$	--	--	--	--
ME(MJ/kgDM):		--	--	--	--
Control	$8.55^{A}\pm0.07$	--		--	--
Rosemary	$6.47^{B}\pm0.1$	--	--	--	--
Significant	$P\leq0.01$	--	--	--	--

NS: Non-significant. Means with different capital superscripts within each column differed significantly between treatments. Means with different small superscripts within each row differed significantly between treatments

IVOMD (%) = 14.88 + 0.889 gas volume + 0.45 Crud protein + 0.651 ash ME (MJ/kg DM) = 1.06 + 0.157 gas volume + 0.084 Crud protein + 0.22 crude fiber − 0.081ash

In comparison to the control group (Table 6), the rosemary group showed significantly higher levels (P≤0.01) of palmitic, stearic, oleic, linoleic, and linolenic acid (%), as well as total unsaturated fatty acids (TU). There was no significant difference in the total saturated fatty acids (TS) and total fatty acids between the two groups. The ratio of TU to TS was significantly higher (P≤0.01) in the control group compared to the rosemary group. Fatty acids play a crucial role in cell membranes and various biological processes. In the rumen, lipids mainly undergo fat decomposition and biohydrogenation processes (45). Rumen fat decomposition is mainly fulfilled by lipase released by bacteria. Fatty acids released by fat decomposition can be quickly and completely hydrogenated by bacterial enzymes (22). Sun et al. (38) reported that increased unsaturated fatty acids in the in vitro substrate decreased the production of methane. Yang et al. (44) also indicated that the microbial community in the incubation system could be affected by elevating proportions of unsaturated fatty acids, affecting the yield of volatile fatty acids, whereas the CH4 concentration was reduced. So, we believe that the active component in dehydrated rosemary leaves might contribute to fat breakdown and biohydrogenation, as well as decrease methane production.

***In vitro* digestibility of some rumen characteristics:** The results of *in vitro* dry matter (IVDMD%), organic (IVOMD%) digestibility, and metabolic energy were higher (P ≤0.01) in the rosemary group than in the control group. At the same time, the number of protozoa decreased significantly (P≤ 0.06) in the rosemary group compared to the control group (Table 7). The current results agreed with Daş (14), who observed increases in vitro organic matter digestion (IVOMD) and metabolizable energy (ME) values in addition to the rosemary powder to silage. Organic matter digestibility can be used to measure the available energy and estimate protein microbial synthesis in the rumen (3). Therefore, we believe that rosemary reduces the number of protozoa and plays a role in increasing the production of microbial protein. In other words, it activates other types of microorganisms in the rumen fluid, and this may be due to the presence of other antioxidants such as vitamins and quercetin.

Table 5. Effect of adding dietary dried rosemary leaves on *in vitro* volatile fatty acid production of Holstein bulls (Mean ± SE).

Treatment / Volatile fatty acid (%)	Control	Rosemary	Significance level
Acetic(A)	$49.85^{B}\pm0.07$	$53.18^{A}\pm0.09$	p≤0.01
Butyric	$20.58^{B}\pm0.22$	$23.43^{A}\pm0.23$	p≤0.01
Propionic(P)	$19.94^{B}\pm0.12$	$22.60^{A}\pm0.15$	p≤0.01
Total Volatile fatty acid	$90.36^{B}\pm0.27$	$99.20^{A}\pm0.34$	p≤0.01
A/P	$2.5^{A}\pm0.01$	$2.35^{B}\pm0.01$	p≤0.01

*Means with different capital superscripts within each column differed significantly between treatments

Table 6. Effect of adding dietary dried rosemary leaves on *in vitro* fatty acid production of Holstein bulls (Mean ± SE)

Treatment / Fatty acid (%)	Control	Rosemary	Significance level
Palmitic	$3.05^{B}\pm0.09$	$4.36^{A}\pm0.23$	p≤0.01
Stearic	$5.16^{B}\pm0.06$	$6.82^{A}\pm0.13$	p≤0.01
Oleic	$15.01^{B}\pm0.04$	$16.47^{A}\pm0.17$	p≤0.01
Linoleic	$18.92^{B}\pm0.09$	$20.33^{A}\pm0.11$	p≤0.01
Linolenic	$0.45^{A}\pm0.01$	$0.60^{A}\pm0.02$	p≤0.01
Total saturated fatty acid (TS)	$11.19^{A}\pm2.92$	$11.18^{A}\pm0.28$	NS
Total unsaturated fatty acid (TU)	$34.39^{B}\pm0.10$	$37.40^{A}\pm0.27$	p≤0.01
Total fatty acid	$45.57^{A}\pm2.92$	$48.58^{A}\pm0.44$	NS
TU/TS	$1.71^{A}\pm0.01$	$1.61^{B}\pm0.01$	p≤0.01

*Means with different capital superscripts within each column differed significantly between treatments.

Table 7. Effect of adding dietary dried rosemary leaves on *in vitro* rumen characteristics of Holstein bulls (Mean ± SE).

Treatment / Analysis	CONTROL	Rosemary	Significance levels
IVDMD(%)	$72.69^{B}\pm0.36$	$77.64^{A}\pm0.34$	$p\leq0.01$
IVOMD (%)	$74.67^{B}\pm0.47$	$79.72^{A}\pm0.37$	$p\leq0.01$
ME (MJ/kg DM)	$11.2^{B}\pm0.003$	$11.96^{A}\pm0.002$	$p\leq0.05$
Protozoa ($\times 10^{6}$ cell/ml)	$7.73^{A}\pm0.26$	$6.93^{B}\pm0.30$	$p\leq0.06$

***Means with different capital superscripts within each row differed significantly.**

IVDMD (%) = [Sample DM- (residual DM- blank)/ Sample DM] ×100

IVOMD (%)= [Sample OM- (residual OM- blank)/ Sample OM] ×100

ME=0.015× IVOMD

CONCLUSION

The DRL positively influenced in vitro rumen fermentation by reducing methane gas production and decreasing N-NH3 levels. This was accompanied by an increase in volatile fatty acids in rumen fluids, IVDMD, and IVOMD. As a result, more undegradable protein entered the small intestine, leading to a greater availability of amino acids for production and a reduction in methane emissions from bulls.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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