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Estimated enteric methane production from volatile fatty acids in the ruminal fluid of dairy cows supplemented with *Lithothamnium Calcareum*

Produção de metano entérico estimado a partir dos ácidos graxos voláteis do fluido ruminal de vacas leiteiras suplementadas com Lithothamnium Calcareum

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RESUMO

O objetivo do estudo foi estimar a produção de metano (CH_4) a partir das proporções de ácidos graxos voláteis (AGV) no fluido ruminal e determinar a sua relação com o consumo de matéria seca (CMS) em vacas leiteiras suplementadas com *Lithothamnium calcareum*. Seis vacas lactantes multíparas da raça Holandesa, foram distribuídas em dois grupos experimentais, grupo controle (CON; n=3) suplementada com 1,1% de MS de bicarbonato de sódio; e grupo *Lithothamnium calcareum* (LITHO; n=3), com 0,5% da MS de *Lithothamnium calcareum*. Coletas de líquido ruminal foram realizadas uma vez por semana para avaliação de pH e AGV. Diariamente foram registrados o CMS dos animais. A produção de CH_4 foi estimada através de equações baseadas nas proporções de AGV ruminais. Não houve diferença entre o CMS (kg/dia), concentrações de AGV e relação acetato/propionato entre os grupos (P>0,05). O rendimento de metano (g CH_4/kg CMS) não teve diferença (P>0,05) entre os grupos nos modelos de equação testados. Não houve uma correlação satisfatória entre o CMS e o rendimento de CH_4 para animais do o grupo LITHO e CON. *Lithothamnium calcareum* como aditivo alimentar não foi capaz de reduzir a produção de CH_4, estimada a partir equações baseadas nas proporções de AGV ruminais.

PALAVRAS-CHAVE: Gás de efeito estufa; mitigação de metano; algas marinhas; ruminantes.

ABSTRACT

The aim of this study was to estimate methane (CH4) production from the proportions of volatile fatty acids (VFA) in ruminal fluid and to determine its relationship with dry matter intake (DMI) in dairy cows supplemented with Lithothamnium calcareum. Six multiparous lactating Holstein cows were divided into two experimental groups: control group (CON; n=3) supplemented with 1.1% DM of sodium bicarbonate; and Lithothamnium calcareum group (LITHO; n=3) supplemented with 0.5% DM of Lithothamnium calcareum. Ruminal fluid was collected once a week to evaluate pH and VFA. DMI of the animals was recorded daily. CH4 production was estimated using equations based on ruminal VFA proportions. There was no difference in DMI (kg/day), VFA concentrations and acetate/propionate ratio between the groups (P>0.05). The methane yield (g CH4/kg DMI) did not differ (P>0.05) between the groups in the equation models tested. There was no satisfactory correlation between DMI and CH4 yield for animals in the LITHO and CON groups. Lithothamnium calcareum as a feed additive was not able to reduce CH4 production, estimated from equations based on ruminal VFA proportions.

KEYWORDS: Greenhouse gas; methane mitigation; seaweed; ruminants.

Livestock farming has become central to environmental debates in recent years, due to deforestation for pasture establishment and its direct contribution to greenhouse gas (GHG) emissions, particularly carbon dioxide (CO_2) and methane (CH_4) (SIMANUNGKALIT et al. 2023). The largest contribution from the livestock sector comes from cattle and sheep production systems which account for up to 18% of global GHG emissions mainly in the form of enteric methane and the decomposition of straw (KINLEY et al. 2020).

Enteric methane, a natural byproduct of ruminal fermentation, not only contributes to environmental impacts but also represents a dietary energy efficiency loss, directly affecting animal productivity and accounting for up to 11% of gross dietary energy consumed by ruminants (ROQUE et al. 2019).

Dietary manipulation is considered the most direct and effective strategy for reducing CH_4 emissions from ruminant production systems (MAIA et al. 2016). Among them, the use of food additives and supplementation with high concentrate content are effective in reducing CH_4 (EUGÉNE et al. 2011). Recent advances in feed additives capable of mitigating enteric methane emissions by directly or indirectly inhibiting methanogenic archaea have been investigated, with red seaweed supplementation showing promising results due to its halogenated compounds known to inhibit cobamide-dependent methanogenesis (KINLEY et al. 2020). *Lithothamnium calcareum* is a red sea algae widely used in the feeding of ruminants as a tampon of the ruminal pH (NEVILLE et al*.* 2022). However, no studies have investigated the antimethanogenic properties of *Lithothamnium calcareum* as a feed additive.

The CH_4 emissions from cattle are difficult to measure, depend on expensive equipment and qualified people, and are impractical for large-scale routine use in dairy farms (HAMMOND et al. 2015). Thus, some alternatives have been used to predict the production of CH_4, with the consumption of dry matter (CDM), milk fatty acid profile (CHARMLEY et al. 2016) and proportions of AGV in ruminal fluid (WILLIAMS et al. 2019). Model equations based on CDM, diet composition, body weight, milk production and composition were used by NIU et al. (2018) to predict methane production (g/day) in dairy cows, and WILLIAMS et al. (2019) presented equations to predict methane production in dairy cows based on AGV proportions in ruminal fluid, including acetate, propionate, and butyrate.

Therefore, this study aimed to estimate methane production in Holstein dairy cows supplemented with *Lithothamnium calcareum* as an antimethanogenic feed ingredient compared to sodium bicarbonate in a high-starch diet, predicted from AGV proportions in ruminal fluid, and to determine the relationship between CH_4 production and CDM.

Six multiparous lactating Holstein cows (63.74 ± 18.63 days in milk, 694 ± 20 kg BW, 3 ± 0.59 parity, and pre-experimental milk yield of 38.83 ± 9.29 kg/d) were used in the study. The cows were housed in a compost-bedded pack barn with wood shavings and rice hulls at a commercial dairy farm in Rio Grande, Rio Grande do Sul, Brazil, with free access to individual automated feeders and water. The study protocol was approved by the Committee of Ethics and Animal Experimentation of the Federal University of Pelotas, registered under code 13784-2021.

The cows were randomly divided into two groups: Control (CON) and *Lithothamnium calcareum* (LITHO) groups, each consisting of three animals, were managed together during the 56-day experimental period. Both treatments received the same basal diet as total mixed ration (TMR) with a concentrate: forage ratio of 54:46% and 29.28% starch in TMR. The differences between groups were attributed to the inclusion of 0.5% dietary DM of *Lithothamnium calcareum* for the LITHO group and 1.1% dietary DM of sodium bicarbonate for the CON group, as recommended by the farm nutritionist, which were previously mixed in the feed bunk with TMR immediately before feeding.

Daily feed intake data were collected using individual automatic feeders. To assess the correlation between CDM and other parameters, weekly CDM averages were calculated considering only weeks with at least five days of recorded intake.

Ruminal fluid samples were collected via rumenocentesis 4 h after morning feeding on day 0 (before algae and sodium bicarbonate supplementation) and on days 7, 14, 21, 28, 35, 42, 49, and 56 of the experimental period. Ruminal pH was measured immediately using a benchtop pH meter. Rumen fluid samples were stored at -80 °C and subsequently analyzed for AGV concentrations (acetate, propionate, and total butyrate) using proton nuclear magnetic resonance spectroscopy as described by WANG et al. (2021).

Methane yield (MY, g CH_4/kg of DMI) was calculated using equations validated by WILLIAMS et al. (2019), using independent published data from dairy and slaughter cattle consuming a wide variety of diets, which can be used in research to estimate methane production from AGV data. The following equations proposed by WILLIAMS et al. (2019) were used:

$RM = 4{,}08 \times (acetate / propionate) + 7{,}05$ Equation 1

RM = 3,28 × (acetate + butyrate) / propionate + 7,6 Equation 2

RM = 316 / propionate + 4,4 Equation 3

Equation 1 is based on a well-established relationship between methane production and the acetate-to-propionate ratio. Equation 2 is based on a well-established relationship between methane production and (acetate + butyrate)/propionate ratio, while equation 3 represents a simplified version of equations 1 and 2 (WILLIAMS et al. 2019).

The results were analyzed using the JMP® Pro 14 program (SAS Institute inc. 2018-2024) through the PROC MIXED, for DMI, AGV (acetate, propionate and butyrate), ralation acetate/propionate and yield of CH_4, having as fixed effects the group, the collection and their interactions and the animal as random effect. The data collected was subjected to analysis of variance (ANOVA) and when the "F" test was significant, the treatment means were compared using the Tukey test ($P<0.05$). The covariance structures (Residual, Unequal Variances, Unstructured, AR(1), Antedependent Equal Variances, Antedependent) were evaluated, and variance components yielded the lowest Bayesian information criterion value, thus being selected for repeated measures analyses. The correlation between CH_4 yield, DMI, and the proportions of acetate, propionate, and butyrate were analyzed using Pearson's correlation in JMP® Pro 14 software. If significant correlation is observed ($r > 0.84$, $p < 0.05$), simple linear regression analysis will be performed.

The inclusion of *Lithothamnium calcareum* in the TMR of dairy cows in this study had no effect (P = 0.15) on DMI during the treatment period. There was also no difference in DMI in weekly average (*P*= 0.31), ruminal pH (*P*=0.57), total concentrations of acetate, propionate and butyrate (P=0.88; P=0.65 and p=0.62, respectively), as well as, the A/P ratio were not significantly different between the LITHO and CON diets (2.64 and 2.59 respectively, *P*= 0.77), as described in Table 1.

Table 1. DMI, ruminal pH and individual VFA (acetic, propionic, butyric; mol/100 mol VFA) of Holstein cows fed diets supplemented with sodium bicarbonate (CON) or *Lithothamnium calcareum* (LITHO).

	Group[1] mean±EPM[2]		P Value
Parameters	CON	LITHO	Group
CMS/kg/day	24,43±1,47	25,89 ± 1,47	0,15
CMS/kg/week	25,63±0,68	26.61 ± 0.66	0,31
pH ruminal	6,79±0,11	6,71±0,11	0,57
Acetate	60,62±1,42	60,33±1,42	0,88
Propionate	24,11±0,74	23,62±0,74	0,65
Butirato	15,14±0,84	15,92±0,84	0,52
Acetate/Propionate (Mm)	2,59±0,12	2,64±0,12	0,77

[1]Group: CON = Sodium bicarbonate; LITHO = *Lithothamnium calcareum*. [2] Mean ± standard error of average (SEA).

Methane yield (g CH_4/kg DM) did not differ among groups in any of the three tested equation models (Eq. 1 (*P* = 0,77), Eq. 2 (*P* = 0,60), Eq. 3 (*P* = 0.61), showing similar performance between groups, as shown in Table 2.

The results in Table 3 show that CH_4 yield did not exhibit a satisfactory positive correlation (above 0.84) with weekly DMI across all tested equation models (Eq.1: r 0,47), (Eq.2: r 0,49) and (Eq.3: r 0,49) for the LITHO group, despite significant P-values ($P<0.05$). No significant correlation was found between CH_4 yield and weekly DMI in the CON group. There was also no satisfactory positive correlation between weekly DMI with acetate ratios (r 0,40, p<0,05) and a satisfactory negative correlation with propionate ratios (r -0,47, p<0,05) in the LITHO group (Table 4).

Table 2. CH_4 yield of Holstein cows fed diets supplemented with sodium bicarbonate (CON) or *Lithothamnium calcareum* (LITHO).

	Group[1] mean±EPM[2]		*P* Value
Parameters	CON	LITHO	Group
CH_4 1 (g of CH4/kg MS)[3]	17,64±0,50	17,85±0,50	0,77
CH_4 2 (g of CH4/kg MS)[4]	18,19±0,40	18,49±0,40	0,60
CH_4 3 (g of CH4/kg MS)[5]	17,78±0,38	18,06±0,38	0,61

[1]Group: CON = Sodium bicarbonate; LITHO = *Lithothamnium calcareum*. [2] Mean ± standard error of the average (SEA); [3]RM = 4,08 × (acetate/propionate) + 7,05; [4]RM = 3,28 × (acetate + butyrate) / propionate + 7,6; [5]RM = 316 / propionate + 4,4.

Table 3. Correlation between weekly DMI and CH_4 yield of Holstein cows fed diets supplemented with sodium bicarbonate (CON) or *Lithothamnium calcareum* (LITHO).

	Group CON[1]		
	r Value	R^2 value	P Value
DMI (weekly) x CH_4 1[2]	-0,11	0,01	0,58
DMI (weekly)x CH_4 2[3]	-0,12	0,01	0,53
DMI (weekly) x CH_4 3[4]	-0,12	0,01	0,55
	Group LITHO[1]		
	r Value	R^2 value	P Value
DMI (weekly) x CH_4 1[2]	0,47	0,22	0,01
DMI (weekly) x CH_4 2[3]	0,49	0,24	<0,01
DMI (weekly) x CH_4 3[4]	0,49	0,23	<0,01

[1]Group: CON = Sodium bicarbonate; LITHO = *Lithothamnium calcareum*. [2]RM = 4,08 × (acetate/propionate) + 7,05; [3]RM = 3,28 × (acetate + butyrate) / propionate + 7,6; [4]RM = 316 / propionate + 4,4.

Table 4. Correlation between acetate, propionate and butyrate proportions with weekly DMI(s) of Holstein cows fed diets supplemented with sodium bicarbonate (CON) or *Lithothamnium calcareum* (LITHO).

	Group CON[1]		
	r Value	R^2 value	P Value
Acetic x DMI (weekly)	-0,12	0,01	0,54
Propionic x DMI (weekly)	0,09	0,01	0,65
Butírico x DMI (weekly)	0,10	0,01	0,60
	Group LITHO[1]		
	r Value	R^2 value	P Value
Acetic x DMI (weekly)	0,40	0,16	0,03
Propionic x DMI (weekly)	-0,47	0,22	0,01
Butírico x DMI (weekly)	-0,28	0,08	0,14

[1]Group: CON = Sodium bicarbonate; LITHO = *Lithothamnium calcareum*.

The supplementation of red algae and fast fermentable starch-rich diets, favor the production of propionate, due to the deviation of hydrogen to propionate formation, resulting in less hydrogen available for methanogenesis, therefore, the A/P ratio in the rum influence the production of CH_4 (MORGAVI et al. 2010, KINLEY et al. 2020), being these rich in concentrate diets efficient methods to decrease enteric methane production (EUGÈNE et al. 2011). SAUVANT & GIGER-REVERDIN (2009) observed a decrease in CH_4 production when the concentrate ratio was >40% of DM consumption and EUGÉNE et al. (2011) observed a reduction in enteric CH_4 emissions in feedlot bulls supplemented with extruded linseed lipids combined with dietary starch.

In vitro studies showed a 99% reduction in CH_4, decrease in acetate and a higher concentration of propionate with supplementation of red algae *Asparagopsis taxiformis* included in 2% of dietary organic matter (MACHADO et al. 2016) and studies *in vivo* observed a decrease in CH_4 production in dairy cows by 26.4% and 67.2% with the supplementation of 0.5% and 1% of the organic matter of *Asparagopsis armata*, respectively (ROQUE et al. 2019). However, to our knowledge, the effects of the red algae *Lithothamnium calcareum* on CH_4 emissions have been scarcely investigated in dairy cows fed high-starch diets.

The production of CH_4 of dairy cows can be predicted from the AGV ratios in the rum using equation models, being an inexpensive and practical approach to estimating methane emissions (NIU et al. 2018). In this study, predicted CH_4 yield did not differ among treatments across the three tested equation models. However, the predicted CH_4 production in both groups was consistent with measurements obtained through traditional methods used to quantify CH_4 production (WILLIAMS et al. 2019), i.e., the AGV parameters in the ruminal fluid combined with measured or estimated DMI, offer a potential tool to predict the production of CH_4 in ruminants.

The lack of difference in CH_4 production between LITHO and CON diets aligns with the VFA profile concentrations and A/P ratio, which were similar between groups, likely maintaining consistent net H_2 production. While acetate production during ruminal fermentation increases H_2 and promotes methanogenesis, propionate formation competitively utilizes H_2, reducing its availability for methanogenesis (CAREGA & DANTAS 2017). Thus, CH_4 production is influenced by ruminal acetate and propionate production rates 2020).

Similarly, *Lithothamnium calcareum* supplementation did not affect DMI during the 56-day trial period, nor did it influence VFA production and CH_4 yield. Furthermore, no significant correlation was observed between CH_4 yield and DMI or the proportions of acetate, propionate, and butyrate, contradicting Williams et al. (2019) which demonstrated in their study that methane yield was positively and linearly correlated with acetate and butyrate proportions, and inversely with propionate.

In most studies that evaluated the effects of red algae on methane measurement, CH_4 production was reduced, being related to increased ruminal propionate production and a beneficial decrease in A/P ratio (MACHADO et al. 2016). KINLEY et al. (2020) demonstrated that including *Asparagopsis taxiformis* at just 0.20% of organic matter intake in high-grain total mixed rations altered ruminal fermentation patterns, decreasing acetate and increasing propionate, potentially eliminating enteric CH_4 production compared to the control diet in beef cattle. ROQUE et al. (2019) in a study with lactating Holstein dairy cows, using a TMR containing seaweed at 1% of intake, reported a reduction of CH_4 by 67% and LI et al. (2018) reported an 80% reduction in enteric CH_4 production when *Asparagopsis* was offered at 3% of OM intake in Merino sheep, along with decreased acetate and increased propionate in the rumen. These authors attributed such results to bromoform, a potent antimethanogenic compound present in these red algae.

Although *Lithothamnium calcareum* is a species of red algae, it does not contain bromoform in its composition, it is basically composed of calcium carbonate and magnesium in a polymorphic structure similar to honey beet that favoured an increase in the specific surface of action prolonging the period of action (DIAS 2000, NEVILLE et al. 2019), being widely used as a tampon agent of the ruminal pH in highly energetic diets as well as sodium bicarbonate. In the present study, ruminal pH values for LITHO and CON diets remained at 6.71 and 6.79, respectively, which favored methanogenic bacteria, as these microorganisms are highly sensitive to pH changes and require near-neutral conditions (6.0 to 6.5) (BERCHIELLI et al. 2011), and thus, even if a diet high in starch was provided, supplementation with *Lithothamnium calcareum* did not reduce methane mitigation, corroborating the study of SIMANUNGKALIT et al. (2023) where CH_4 yield (g/kg DMI) of steers supplemented with *Lithothamnium calcareum* was higher than those of the monensin group.

Therefore, *Lithothamnium calcareum* supplementation in high-starch diets did not reduce estimated enteric CH_4 production compared to a standard diet with sodium bicarbonate, based on equations using ruminal volatile fatty acid proportions in lactating Holstein cows, and there was no relationship between dry matter intake and gas production. However, further research is needed to fully understand the potential of *Lithothamnium calcareum* as an antimethanogenic feed additive for dairy cattle, exploring not only energy-rich diets but also other dietary compositions.

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