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Methane emissions and ^{13}C composition from beef steers consuming binary C_3–C_4 diets

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Abstract

Improvements in forage nutritive value can reduce methane emission intensity in grazing ruminants. This study was designed to evaluate how the legume rhizoma peanut (*Arachis glabrata*; **RP**) inclusion into bahiagrass (*Paspalum notatum*) hay diets would affect intake and CH_4 production in beef steers. We also assessed the potential to estimate the proportion of RP contribution to CH_4 emissions using $\delta^{13}C$ from enteric CH_4. Twenty-five Angus-crossbred steers were randomly allocated to one of five treatments (five steers per treatment blocked by bodyweight): 1) 100% bahiagrass hay (0%RP); 2) 25% RP hay + 75% bahiagrass hay (25%RP); 3) 50% RP hay + 50% bahiagrass hay (50%RP); 4) 75% RP hay + 25% bahiagrass hay (75%RP); 5) 100% RP hay (100%RP). The study was laid out using a randomized complete block design, and the statistical model included fixed effect of treatment, and random effect of block. Methane emissions were collected using sulfur hexafluoride (SF_6) technique, and apparent total tract digestibility was estimated utilizing indigestible neutral detergent fiber as an internal marker. A two-pool mixing model was used to predict diet source utilizing CH_4 $\delta^{13}C$. Inclusion of RP did not affect intake or CH_4 production ($P > 0.05$). Methane production per animal averaged 250 g CH_4/d and 33 g CH_4/kg dry matter intake, across treatments. The CH_4 $\delta^{13}C$ were −55.5, −60.3, −63.25, −63.35, and −68.7 for 0%RP, 25%RP, 50%RP, 75%RP, and 100%RP, respectively, falling within the reported ranges for C_3 or C_4 forage diets. Moreover, there was a quadratic effect ($P = 0.04$) on the CH_4 $\delta^{13}C$, becoming more depleted (e.g., more negative) as the diet proportion of RP hay increased, appearing to plateau at 75%RP. Regression between predicted and observed proportions of RP in bahiagrass hay diets based on $\delta^{13}C$ from CH_4 indicate $\delta^{13}C$ to be useful (Adj. $R^2 = 0.89$) for predicting the contribution of RP in C_3–C_4 binary diets. Data from this study indicate that, while CH_4 production may not always be reduced with legume inclusion into C_4 hay diets, the $\delta^{13}C$ technique is indeed useful for tracking the effect of dietary sources on CH_4 emissions.

Lay Summary

Investigating methods for reducing enteric methane emissions from ruminant livestock are important to reduce environmental impacts and improving production efficiency through reduced energy losses. This experiment evaluated the effects of increasing proportion of rhizoma peanut hay (a C_3 legume) into bahiagrass hay (a C_4 grass) on intake and methane production in beef steers. In addition, carbon stable isotopes (^{13}C) of the methane emitted were used to back-calculate the diet components consumed. Angus-crossbred steers were randomly allocated to one of five hay diets (treatments): 1) 100% bahiagrass; 2) 25% rhizoma peanut + 75% bahiagrass; 3) 50% rhizoma peanut + 50% bahiagrass; 4) 75% rhizoma peanut + 25% bahiagrass; 5) 100% rhizoma peanut. Inclusion of rhizoma peanut did not affect intake or methane production, but apparent total tract digestibility increased as proportion of rhizoma peanut increased in the diet. The carbon stable isotope composition observed from enteric methane production was within the expected ranges for C_3–C_4 forage diets. Furthermore, the carbon stable isotope composition from enteric methane production was useful in predicting contributions from each diet source in C_3–C_4 binary diets.

Key words: digestibility, forage, greenhouse gas emissions, intake, ruminant

Abbreviations: BW, body weight; DM, dry matter; DMI, dry matter intake; iNDF, indigestible neutral detergent fiber; MBW, metabolic body weight; OM, organic matter; RP, rhizoma peanut

Introduction

Improved forage nutritive value is the main factor for reducing emissions per unit of feed intake and animal product (Hristov et al., 2013). Methane emissions from livestock consuming legumes is often less (McCaughey et al., 1999; Waghorn et al., 2002), though not always (van Dorland et al., 2007), than when grasses make up the majority of the diet (Beauchemin et al., 2008). Reduction in enteric CH_4 emissions when legumes are included in the diet are often a result

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of presence of secondary metabolites (i.e., tannins, saponins, etc.), reduced fiber concentrations, or faster rate of passage out of the rumen (Beauchemin et al., 2008). Moreover, CH_4 emissions generally increase as the rate of feed degradation, and extent of fermentation increases in the rumen, leading to greater forage intake and improved animal performance (Beauchemin et al., 2008). As a result, considering absolute emissions (e.g., g CH_4/animal/d) along with emissions intensities (e.g., CH_4 production on basis of intake or animal product) are important for understanding pathways for reducing overall emissions and greenhouse gas release into the atmosphere.

Carbon stable isotope techniques have been utilized for tracking diet composition and selection in grazing livestock (Jaramillo et al., 2022). These techniques are contingent upon differences in accumulation of C isotope in plants of contrasting photosynthetic pathways, where ^{13}C is the heavy and ^{12}C the light C isotope (Farquhar et al., 1989; Norman et al., 2009). Carbon isotope ratios ($^{13}C/^{12}C$) are expressed as $\delta^{13}C$‰ vs. Pee Dee Belemnite standard (Fry, 2006). Legumes and temperate, cool-season grasses are C_3 (Calvin pathway) plants, while warm season, tropical and sub-tropical grasses are C_4 (dicarboxylic acid pathway) plants. The C_3 plant species contain more depleted (more negative) $\delta^{13}C$ composition compared to C_4 species, for which $\delta^{13}C$ composition of C_3 plants ranges from −25‰ to −20‰ and C_4 plants from −16‰ to −9‰ (Farquhar et al., 1989).

Few studies have evaluated C stable isotope (^{13}C) composition from enteric CH_4 production in ruminants. The reported in vivo studies have indicated $\delta^{13}C$ from enteric CH_4 is dependent on diet and sampling technique, but generally ranges between −80‰ and −50‰ (Schulze et al., 1998). Cattle consuming C_3 diets typically have CH_4-$\delta^{13}C$ ranging from −62‰ to −72‰, while C_4 diets will range from −48‰ to −58‰ (Tyler et al., 1988; van Cleef et al., 2021), indicating the contrasting composition according to diet consumed. Methanogenic archaea in the rumen generally discriminate against C-containing compounds of greater isotopic mass, resulting in fractionation within the rumen, and the substrate pool being more enriched in the heavier ^{13}C (Schulze and Giese, 1993). This fractionation occurs as pyruvate undergoes a carboxylation reaction, transferring C-atoms of the carbonyl- and methyl-group and forming acetate (Schulze and Giese, 1993). This is why methanogenic archaea are capable of sourcing C directly from the rumen substrate pool or from dietary substrates (Schulze and Giese, 1993).

Perennial, warm season, C_4 grasses are the main forage source of most beef cattle operations in the southeastern US, and bahiagrass (*Paspalum notatum*) has been among the most widely utilized forage grasses in the region. Similarly, rhizoma peanut (*Arachis glabrata*), a legume, has demonstrated persistence under grazing in the region (Ortega-S et al., 1992; Mullenix et al., 2016; Jaramillo et al., 2021). Thus far, the effects of CH_4 emissions from beef cattle consuming bahiagrass–rhizoma peanut mixed diets has not been reported. This study was developed to test the two hypotheses that 1) high inclusion levels of rhizoma peanut hay into bahiagrass hay diets would decrease methane production per unit of intake, and 2) ^{13}C composition of CH_4 would be useful for estimating diet source contribution to overall enteric CH_4 emissions from beef steers.

Material and Methods

All procedures involving animals were approved by the Animal Care and Use Committee of the Institute of Food and Agricultural Sciences at the University of Florida (Protocol # 201709925).

The experiment was carried out during the summer of 2017 at the Feed Efficiency Facility of University of Florida North Florida Research and Education Center, in Marianna, FL. Twenty-five Angus crossbred steers (341 ± 17 kg body weight [BW]) were used in a randomized complete block design, with bodyweight as the criterion for blocking. The steers were housed in 108-m^2 pens, for a total of 33 d. Methane emissions were evaluated from days 27 to 33. There was an adaptation period of 14 d after diets were introduced. Prior to the experimental period, steers were grazing common bermudagrass (*Cynodon dactylon*) pastures.

Individual animal intake was recorded with the GrowSafe system (GrowSafe Systems Ltd, Calgary, AB, Canada), which utilize radio frequency identification to record feed intake by weight change measured to the nearest gram. Water was available ad libitum. No mineral supplements were provided. Forage treatments were offered ad libitum by providing sufficient hay to maintain full feed troughs throughout each day of the experiment. Treatments were considered five proportions of "Florigraze" RP hay into "Argentine" bahiagrass hay: 1) 100% bahiagrass (0%RP); 2) 25% rhizoma peanut + 75% bahiagrass (25%RP); 3) 50% rhizoma peanut + 50% bahiagrass (50%RP); 4) 75% rhizoma peanut + 25% bahiagrass

Table 1. Chemical composition of rhizoma peanut (RP) inclusion into bahiagrass hay. Means indicate average of 10 samples ± standard deviation

Item[2]	% Rhizoma peanut in diet[1]				
	0% RP[1]	25% RP	50% RP	75% RP	100% RP
DM, % as fed	91 ± 0.02	92 ± 0.02	91 ± 0.01	91 ± 0.02	91 ± 0.02
OM, % DM	94 ± 0.01	93 ± 0.06	93 ± 0.06	92 ± 0.07	91 ± 0.07
CP[a], % DM	8.4 ± 0.02	9.0 ± 0.001	9.2 ± 0.003	11.1 ± 0.02	12.0 ± 0.007
IVDOM[b], %	46 ± 1.2	54 ± 1.4	57 ± 1.1	64 ± 1.8	68 ± 1.0
NDF, % DM	73 ± 3.4	71 ± 3.3	72 ± 3.8	70 ± 3.8	69 ± 4.4
$\delta^{13}C$, ‰	−16.04 ± 0.36	−19.39 ± 0.75	−22.14 ± 0.83	−26.22 ± 0.55	−29.41 ± 0.50

[a] 100% bahiagrass (0%RP); 25% rhizoma peanut + 75% bahiagrass (25%RP); 3) 50% rhizoma peanut + 50% bahiagrass (50%RP); 4) 75% rhizoma peanut + 25% bahiagrass (75%RP); 5) 100% rhizoma peanut (100%RP); RP, rhizoma peanut.

[b] DM, dry matter; OM, organic matter; CP, crude protein; IVDOM, in vitro digestible organic matter; NDF, neutral detergent fiber.

(75%RP); 5) 100% rhizoma peanut (100%RP). Diet chemical composition is presented in Table 1. All treatment proportions were weighed and mixed on as-fed basis. Mixing of diets was performed manually using rakes. Hay mixers or choppers were not used to minimize leaf breakage and shatter. Per Jaramillo et al. (2022), $\delta^{13}C$ from dry combustion analysis of feces was utilized to back-calculate diet proportions of RP or bahiagrass, indicating selection was not evident during this study.

Enteric CH_4 emissions were measured using the sulfur hexafluoride (SF_6) tracer technique (Johnson et al., 1994) during a 6-d collection period. Permeation tubes were filled with approximately 2.3 g of SF_6. Average release rate of the permeation tubes was 2.46 ± 0.54 mg/d. Permeation tubes were dosed via balling gun prior to the start of the experimental period. Gas collection canisters were constructed of polyvinyl chloride pipe to have a final volume of 2 L. Samples were collected by evacuating the collection canisters to 68.8 cm Hg and connecting the canister to a halter, equipped with a crimped capillary tube positioned to collect a sample from the nostrils. Three collection canisters and capillary tubes, placed 1 m above ground level, were used to determine environmental methane and SF_6 concentrations.

Methane and SF_6 analyses and calculations

Following field collection, methane and SF_6 concentrations in the canisters were transferred to evacuated serum bottles and analyzed through gas chromatography (Agilent 7820A GC; Agilent Technologies, Palo Alto, CA, USA). A flame ionization detector and an electron capture detector were used for CH_4 and SF_6 analysis, respectively, with a capillary column (Plot Fused Silica 25 m by 0.32 mm, Coating Molsieve 5A, Varian CP7536; Varian Inc., Lake Forest, CA, USA). Injector, column, and detector temperatures for CH_4 analysis were 80, 160, and 200 °C, respectively. For SF_6, temperatures were 50, 30, and 300 °C for the injector, column, and detector, respectively. The carrier gas for both CH_4 and SF_6 was N_2.

Methane emissions from individual steers were determined in relation to the SF_6 tracer gas captured in the collection canisters, according to equation 1:

$$Q_{CH_4} = Q_{SF_6} \times \frac{[CH_4]_y - [CH_4]_\beta}{[SF_6]_y - [SF_6]_\beta}, \qquad [1]$$

where Q_{CH_4} is the CH_4 emission per animal (g/d), Q_{SF_6} is the SF_6 release rate from the permeation tube (mg/d), $[CH_4]_y$ is the concentration of CH_4 in the collection canister, $[CH_4]_\beta$ is the concentration of CH_4 in the environmental canisters, $[SF_6]_y$ is the concentration of SF_6 in the animal collection canister, and $[SF_6]_\beta$ is the concentration of SF_6 in the environmental canister.

Methane $\delta^{13}C$ was analyzed using an IsoPrime Trace Gas (Elementar, Hanau, Germany) coupled to an isotope ratio mass spectrometer, by injecting 30 mL of gas sample into evacuated vacutainers. The specific Trace Gas instrumentation method is provided by Fisher et al. (2006). Briefly, the injected CH_4 sample goes through perchlorate and other traps to remove water and CO_2 that could contaminate the samples, leaving pure CH_4. The sample continues through combustion furnace where CH_4 is oxidized into CO_2. This CO_2 is then trapped and cryofocused in liquid N. The sample is then injected to the isotope ratio mass spectrometer. The CH_3 ^{13}C is obtained based on calibration of reference gas against a certified material. In this instance, ultrapure CO_2 (Linde, Danbury, CT) served as the Reference gas. The CO_2 Reference gas was validated against L-glutamic acid (USGS40 and USGS41a, U.S. Geological Survey, Reston, VA) as the reference material ($\delta^{13}C$ = −26.3‰ and +36.55‰, respectively).

The proportion of RP hay in the diet was back-calculated using $\delta^{13}C$ from CH_4 on each 0%RP and 100%RP (Jones et al., 1979). The proportions were estimated using equation 2 described by Jones et al. (1979):

$$\%RP = 100 - \left\{100 \times \frac{A - C}{B - C}\right\}, \qquad [2]$$

where %RP is the proportion of CH_4 production from RP intake, A is the $\delta^{13}C$ of the sample obtained from the collection canister, B is the $\delta^{13}C$ of CH_4 from 0%RP, and C is the $\delta^{13}C$ of CH_4 from 100%RP.

Apparent total tract digestibility

Feed and orts were collected daily from days 23 to 26 and fecal samples were collected at 0700 and 1500 h from days 24 to 27 by rectal grab. Orts were collected prior to the morning feeding, and all feed bunks were cleaned of any orts prior to feeding each day. All feed and orts samples were dried in a forced air oven at 55 °C to a constant weight, and weight was recorded. Fecal samples were stored at −20 °C until processing. Feces were also dried at 55 °C until reaching constant weight. Feed, ort, and fecal samples were ground to pass a 2-mm screen in a Wiley Mill (Model 4, Thomas-Wiley Laboratory Mill, Thomas Scientific, Swedesboro, NJ, USA), and pooled in similar proportions within a steer.

Apparent total tract digestibility was estimated using indigestible neutral detergent fiber (**iNDF**) as an internal marker. In short, 0.5 g of sample was weighed into Ankom F57 filter bags (Ankom Technology Corp. Macedon, NY, USA) and then incubated within the rumen of a cannulated steer grazing bahiagrass pastures for 288 h to ensure complete digestion of potentially digestible NDF. After incubation, samples were rinsed twice with tap water, followed by four rinses with distilled water, dried, and processed for NDF analysis.

The apparent total tract digestibility of organic matter (**OM**) was calculated using equation 3:

$$\text{OM digestibility (g/kg)} = 100 - 100 \times \left(\frac{\text{iNDF in feed}}{\text{iNDF in feces}}\right) \times \left(\frac{\text{OM in feces}}{\text{OM in feed}}\right). \qquad [3]$$

Statistical analysis

All response variables were analyzed using linear mixed model procedures as implemented in SAS PROC GLIMMIX (SAS/STAT 15.1, SAS Institute). The model included diet type as the fixed effect and block as the random effect. Differences were considered significant at $P \leq 0.05$, and tendencies at $0.05 < P < 0.10$. Polynomial contrasts (linear and quadratic effects) were used to test the effects of RP inclusion on a given response variable, using the contrast statement. The regression between predicted and observed proportions of RP in the CH_4 was conducted using PROC REG from SAS (SAS/STAT 15.1, SAS Institute).

Results and Discussion

The chemical composition of the diets was within the reported ranges for bahiagrass and RP (Foster et al., 2009a; Dubeux et al., 2017; Table 1), and both species fall within previously reported ranges for nutritive value parameters (Jaramillo et al., 2021). As expected, the $\delta^{13}C$ of the diets became more depleted as the proportion of RP increased. The diet $\delta^{13}C$ of 0%RP and 100%RP were −16.04‰ and −29.41‰, respectively, which are within the ranges for C_3 and C_4 species, respectively (Farquhar et al., 1989).

No differences among treatments ($P > 0.05$) were observed for intake on an as fed, dry matter (**DM**), or OM basis (Table 2). Dry matter intake (**DMI**) as a percent of BW did not differ ($P = 0.33$) and averaged 2.3% across treatments. The average intake on as fed, DM, and OM basis averaged 8.9, 8.3, and 7.7 kg/d, across treatments. The intake values are similar to an average DMI of 1.9% BW when beef steers consumed bahiagrass hay (Arthington and Brown, 2005). A quadratic effect was observed ($P = 0.001$) on apparent OM digestibility ($P = 0.001$) with digestibility increasing at a decreasing rate with increasing proportion of RP in the diet. The least digestible diet was 0%RP (45%), while 75%RP and 100%RP were the greatest, at 55%, each. Increases in ruminal liquid passage rates have been reported with increasing legume proportion in the diet (Poppi, 1997). This may explain the positive influence on digestibility from increasing RP proportion in the diet, in contrast with bahiagrass, which may have required greater retention time compared with RP for further degradation (Staples et al., 1984). Evidence of positive synergistic effects due to level of legume inclusion has been found previously (Dal Pizzol et al., 2017), including in temperate (C_3) grass–legume mixed diets (Niderkorn et al., 2015) and alfalfa (*Medicago sativa*)–“Tifton 85” bermudagrass (*Cynodon* sp.) diets (Pereira Neto et al., 2019).

The inclusion of RP in bahiagrass hay diets did not affect CH_4 emissions in grams per day ($P = 0.44$), grams per kilogram DMI ($P = 0.47$), grams per metabolic body weight (**MBW**; $P = 0.90$), or grams per digestible OM intake (**DOMI**; $P = 0.29$). Methane production was 250 g/d, averaged across treatments (Table 3), which is within the average range reported for beef cattle consuming bahiagrass hay (DeRamus et al., 2003). Variabilities with SF_6 tracer method likely contributed failing to observe treatment differences in the present study, especially with the elevated SEM reported (Table 3), even despite adhering to recommendations based on Hristov et al. (2018) for reducing variabilities when implementing the SF_6 tracer method. The fact that no differences were detected for CH_4 emissions based on production parameters in this study (e.g., DMI, MBW, or DOMI) warrants further evaluation. Perhaps a performance study allowing for adequate average daily gain measurements for steers consuming these same diets would provide insights into probable reductions in CH_4 intensity based on CH_4 per unit of bodyweight gain. For such study, it would be hypothesized that diets including RP would improve animal performance, providing a basis for reducing CH_4 emissions.

Methane production is greater when ruminants are fed C_4 compared with C_3 species, often as a result of greater proportions of structural carbohydrates in C_4 species (Archimède et al., 2011). It is generally accepted that ruminants consuming legumes produce less methane than when they consume grasses per unit of fermented OM (Archimède et al., 2011); however, variability among legumes exists in terms of composition and presence of plant secondary metabolites (Waghorn et al., 2002; Archimède et al., 2011). Specifically, condensed tannins may be present in greater proportions in certain tropical legumes, which can reduce methane emissions when fed (Archimède et al., 2011). Although condensed tannin concentrations were not evaluated in this study, RP hay does not contain high concentrations of condensed tannins or any other plant secondary compound (Foster et al., 2009b). Provided there is an inverse relationship between DMI and enteric methane production (Hristov et al., 2018), it is likely that methane production did not differ in this study since intake did not differ and RP hay did not contain any plant secondary metabolites.

Cattle consuming C_3 diets typically have $\delta^{13}C$ ranging from −62‰ to −72‰, while C_4 diets will range from −48‰ to −58‰ (Tyler et al., 1988; Schulze et al., 1998). The CH_4 $\delta^{13}C$ composition observed in this study is within the accepted range for microbial methane fermentation (Howarth, 2019), and values for 0%RP and 100%RP were −55.5‰ and −68.7‰, respectively, corroborating the ranges for enteric CH_4 produced from C_4 or C_3 species. In respiratory chambers, Klevenhusen et al. (2008) fed barley (C_3; *Hordeum vulgare*) and maize (C_4; *Zea mays*) diets to dairy cattle and reported methane $\delta^{13}C$ of −67.1‰ and −58.2‰. In the current study, CH_4 $\delta^{13}C$ became more depleted (quadratic effect; $P = 0.04$)

Table 2. Effects on intake and apparent total tract OM digestibility from increasing levels of rhizoma peanut (RP) in RP–bahiagrass hay diets

	% Rhizoma peanut in diet[1]					SEM[3]	*P*-value[2]		
	0% RP	25% RP	50% RP	75% RP	100% RP		Trt	L	Q
Intake, kg/d									
As fed	7.0	8.9	9.7	10.3	8.6	1.11	0.32	0.35	0.59
DM[4]	6.5	8.2	9.0	9.4	7.9	1.03	0.33	0.36	0.59
OM	6.1	7.7	8.4	8.8	7.4	0.96	0.36	0.34	0.61
Intake, %BW[5]	2.1	2.6	2.9	3.0	2.6	0.34	0.24	0.43	0.62
Digestibility, % OM	45.0	46.0	48.0	55.0	55.0	2.6	0.02	0.71	0.01

[1] 100% bahiagrass (0%RP); 25% rhizoma peanut + 75% bahiagrass (25%RP); 3) 50% rhizoma peanut + 50% bahiagrass (50%RP); 4) 75% rhizoma peanut + 25% bahiagrass (75%RP); 5) 100% rhizoma peanut (100%RP); RP, rhizoma peanut.

[2] *P* -values of treatment (trt.), linear (L), and quadratic (Q) effects.

[3] SEM, standard error of the mean.

[4] DM, dry matter.

[5] BW, bodyweight.

Table 3. Effect of increasing levels of rhizoma peanut hay (RP) in RP–bahiagrass hays on in vivo methane emissions and the $\delta^{13}C$ of methane emissions

	% Rhizoma peanut in diet					SEM	*P*-value[1]		
	0% RP	25% RP	50% RP	75% RP	100% RP		Trt.	L	Q
CH_4 emissions									
g/d	205	255	282	227	277	35.9	0.44	0.31	0.64
g/kg DMI[2]	37	37	31	27	34	5.2	0.47	0.88	0.26
g/MBW[3]	2	3	3	4	4	0.97	0.90	0.67	0.54
g/DOMI[4]	77	72	67	50	70	10.3	0.29	0.69	0.67
$\delta^{13}C$, ‰	−55.5	−60.3	−63.25	−63.35	−68.7	0.99	0.001	0.01	0.04

[1] *P*-values of treatment (Trt.), linear (L), and quadratic (Q) effects.
[2] Dry matter intake.
[3] Metabolic bodyweight = $BW^{0.75}$.
[4] Digestible organic matter intake.

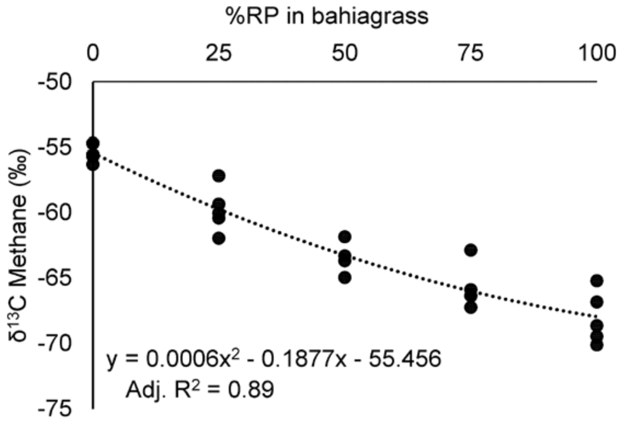

Figure 1. Regression analysis (quadratic effect; $P = 0.04$) of $\delta^{13}C$ from methane emitted by beef steers in response to increasing levels of rhizoma peanut (RP) proportion in bahiagrass hay diets.

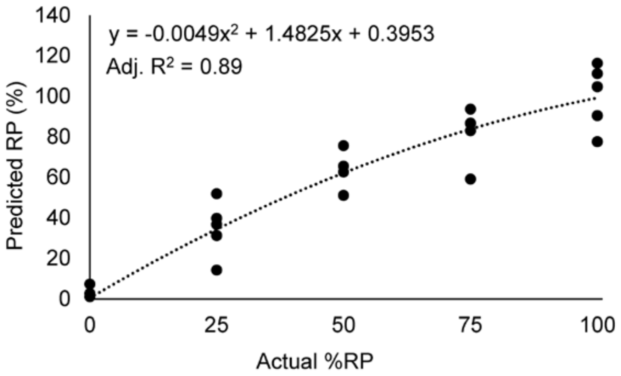

Figure 2. Regression analysis of predicted vs. observed rhizoma peanut (RP) proportion in bahiagrass hay diets, utilizing $\delta^{13}C$ from CH_4.

as the proportion of RP hay increased in the diet (Fig. 1; adj. R^2 of 0.89) likely because RP was contributing more to total CH_4 emitted. The similar pattern observed with the total tract apparent digestibility of these hay diets may explain the response of CH_4 $\delta^{13}C$, where the increasing digestibility of RP component within these mixed diets also resulted in greater contribution to CH_4 production.

Estimating the proportion of the individual diet components in these C_3–C_4 binary diets was successful utilizing the $\delta^{13}C$ from the collected CH_4 and the SF_6 technique (Fig. 2). Regressions from CH_4 $\delta^{13}C$ of predicted vs. observed proportions of RP in the diet indicated that a quadratic model had a greater adjusted R^2. These models may become useful in future studies to predict the dietary contribution from individual diet components to the overall methane production in grazing livestock.

In conclusion, increasing proportions of RP hay into bahiagrass hay-based diets did not affect intake or enteric CH_4 emissions from beef steers. The quadratic response of CH_4 $\delta^{13}C$ composition was likely driven by the synergistic effects from inclusion of RP into bahiagrass hay diets, where the digestibility of the RP component in the diets increased when fed in mixtures with bahiagrass. The $\delta^{13}C$ composition of CH_4 collected can be used as an indicator of the diet consumed by the steers, indicating an important tool for estimating the contributions of diet components to the overall emissions when diets consist of C_3–C_4 forages. Limitations to this technique related to the application of CH_4 $\delta^{13}C$ warrant further studies to evaluate the predictive ability of the ingredient contribution to enteric CH_4 when ruminants consume non-binary diets (i.e., consisting of more than two ingredients of similar photosynthetic pathways, or other diet types such as grain-based or total mixed rations).

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of Interest Statement

The authors report no conflicts of interest.

Data and Model Availability Statement

Data and models have not been deposited in an official repository. Data are available upon request.

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