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SF_6 Tracer Technique to Estimate Methane Emission in a Dual-Flow Continuous Culture System: Test and Application

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Abstract: This study aimed to evaluate the sulfur hexafluoride (SF_6) tracer technique for estimating methane (CH_4) emissions in dual-flow continuous culture systems (DFCCS). In experiment 1 (Exp1), fermenters were filled with water, and known CH_4 concentrations (0, 1.35, 2.93, or 4.43 g/d) were injected using permeation tubes with SF_6 release rates (3.30 or 9.65 mg/d). Headspace gas was collected using canisters, and the SF_6 technique estimated CH_4 recovery. Experiment 2 (Exp2) involved a DFCCS fermentation trial with ruminal fluid from three Holstein cows, testing diets with soybean meal or its partial replacement (50%) by Chlorella or Spirulina. Headspace gas was collected at intervals post-feeding. Standard curves for SF_6 and CH_4 quantification were inadequate for DFCCS samples, with the CH_4:SF_6 ratio differing from standards, indicating the data needs further SF_6 release rate evaluation. In Exp1, a high correlation (r = 0.97) was found between infused and calculated CH_4, indicating good repeatability. Low and high SF_6 rates performed similarly at low CH_4 infusion, but high SF_6 overestimated CH_4 at high infusion. Exp2 showed CH_4 emissions irrespective of SF_6 rate and indicated reduced CH_4 emissions and increased NDF degradation with algae-containing diets. Further evaluation of the SF_6 tracer technique is warranted for DFCCS.

Keywords: *Chlorella*; methanogenesis; SF_6 release rate; *Spirulina*

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1. Introduction

The 6th assessment from the Intergovernmental Panel on Climate Change reported that global greenhouse gas emission continues to rise [1]. Agriculture, forestry, and the land use sector is the second greatest contributor, accounting for 22% of total emissions. Among its sub-sectors, livestock production, particularly ruminant production, is one of the largest anthropogenic greenhouse gas contributors, responsible for 22.7% of the total greenhouse gas in this category. Given these numbers and increased societal negative perception of ruminant production, efforts have been made with the goal of reducing the enteric CH_4 emissions from ruminant production [2]. Nonetheless, ruminants may lose 2–12% of the overall gross energy consumed because of the synthesis of ruminal CH_4 [3,4].

To develop strategies to mitigate enteric CH_4 production, a reliable technique is required to quantify CH_4 emissions. Several techniques are available to estimate CH_4 emission from ruminants, including enclosed techniques (e.g., respiration calorimetry), prediction equations, isotopic tracer techniques, and the use of inert tracer gas (e.g., sulfur hexafluoride [SF_6]) techniques [5]. Some of these techniques are costly, labor-intensive, and may limit the number of animals feasible to be used [6]. To address these issues, the SF_6 tracer technique was developed [7], proving to be a reliable method for in vivo measurement of CH_4 production [8,9].

Another challenge in animal production research is related to the use of animals in research. According to the National Research Council [10], laboratories and research institutes must apply the 3Rs alternatives—replacement, reduction, and refinement—which guide researchers to optimize animal use in research. Consequently, several alternative methods have been developed to minimize animal use, notably in vitro methodologies. Although not capable of fully replacing the use of animals, it offers additional capabilities to maximize data collection that are complimentary to in vivo assessments. In the context of evaluating ruminal metabolism and enteric CH_4 production, numerous in vitro techniques have been developed [11].

These in vitro techniques can be classified into two main classes: batch culture and continuous culture (CC), and both are focused on evaluating the endpoint degradation of nutrients and fermentation kinetics. While batch culture is a technique that does not permit media renewal, CC does, supporting microbial adaptation to the system and offering a more robust approach. In a meta-analysis carried out by our research group [12], it has been demonstrated that the dual-flow continuous culture system (DFCCS) is effective in depicting the treatment effects on ruminal nutrient degradability and microbial protein synthesis efficiency when compared to in vivo omasal sampling, and is considered a reliable method to evaluate ruminal fermentation. A critical factor in the DFCCS is measuring gas production, specifically CH_4, due to the non-hermetic nature of most of the DFCCS. Wenner et al. [13] reported a method to estimate CH_4 production from DFCCS; however, such a technique requires the utilization of sensors that are costly and require the DFCCS to be hermetically closed.

Because measuring CH_4 is a pressing global issue, most DFCCS, which are robust in vitro systems, are not equipped for reliably measuring CH_4, and the SF_6 tracer technique is widely used in vivo. The adaptation of the SF_6 tracer technique to DFCCS would expand the ability of researchers to reliably measure CH_4 in ways that better represent in vivo conditions.

To the best of our knowledge, this study is the first attempt to evaluate the SF_6 tracer technique to estimate CH_4 production within in vitro systems. Thus, the objective of the current assessment was to evaluate the SF_6 tracer technique to estimate enteric CH_4 production using a DFCCS and to investigate the effects of levels of SF_6 release rates and CH_4 recovery/production. To achieve such goals, two experiments were conducted. In experiment 1, known quantities of CH_4 were injected into the fermenters, and two levels of SF_6 release were used to estimate CH_4 recovery. In experiment 2, CH_4 emissions were determined using SF_6 when a typical dairy diet was provided. It was hypothesized that the SF_6 tracer technique is a suitable method to estimate CH_4 production in DFCCS, regardless of the SF_6 release rate.

2. Materials and Methods

The estimation of enteric CH_4 was carried out using the SF_6 tracer technique, described by Johnson et al. [7] and Jonker and Waghorn [9], then adapted to a DFCCS. To test the utilization of SF_6 to estimate CH_4 in a DFCCS, a pretrial and a fermentation trial were performed. For the scope of this article, we are going to refer to the experiments as experiment 1 (Exp1) and 2 (Exp2), respectively. The collection of ruminal inoculum from ruminally cannulated cows used in Exp2 was performed in accordance with guidelines from the Institutional Animal Care and Use Committee of the University of Florida.

2.1. Experiment 1—Experimental Design

A DFCCS with eight fermenters (experimental units) containing only deionized water instead of ruminal content was used. In addition, artificial saliva injection was replaced with deionized water to avoid any confounding effects. The system was set up with only deionized water to prevent any fermentation and intrinsic CH_4 production, which enabled us to carry out a recovery test with known CH_4 concentrations. The experimental design was a split-plot, where the whole plot was considered a group of 4 fermenters,

and the subplot was an individual fermenter within each plot. The first experimental factor (randomly applied to the whole plot) was the SF_6 release rate, composed of a low (3.30 ± 0.73 mg/d) or a high (9.65 ± 0.59 mg/d) release rate. The second experimental factor (randomly applied to the subplot) was the level of CH_4 injection (0, 1.35 ± 0.13, 2.93 ± 0.35, or 4.43 ± 0.25 g/d) to simulate an increasing level of methanogenesis. Levels of CH_4 injection were based on recent literature values [14–16] that reported CH_4 emissions of up to 49 mL/g of DMI. In order to cover a wide range of possible CH_4 emissions, the highest levels were designed to mimic a CH_4 emission of 60 mL of CH_4/g of DMI. The experiment was conducted over two 5 d periods, including a 2 d of adaptation followed by 3 d of gas collection. Crossover of the first treatment factor was applied to the whole plot in the second experimental period, and the treatments applied to the subplot were randomly assigned within each main plot.

The gas injected into the fermenters was acquired from Gasco®, providing a 99.999% CH_4 purity level (MEUHPA16, Oldsmar, FL, USA). Regulation of gas release was achieved through the utilization of a central gas regulator (Model 90005520, Portagas®, Pasadena, TX, USA) connected to a central pipe, which enables individual connecting pipes equipped with 2-way flow control valves to supply the correct amount of CH_4 into each individual fermenter (Figure 1). To ensure that the CH_4 infusion rate was adequate, CH_4 flow checking was carried out daily for each fermenter at 0800, 1100, 1300, and 1600 h using a manual bubble flowmeter (20562, MilliporeSigma, St. Louis, MO, USA), resulting in continuous release rates of approximately 0, 1.5, 3.0, and 4.5 mL/min according to the experimental treatments. Any necessary CH_4 flow adjustments to assure accuracy were made accordingly.

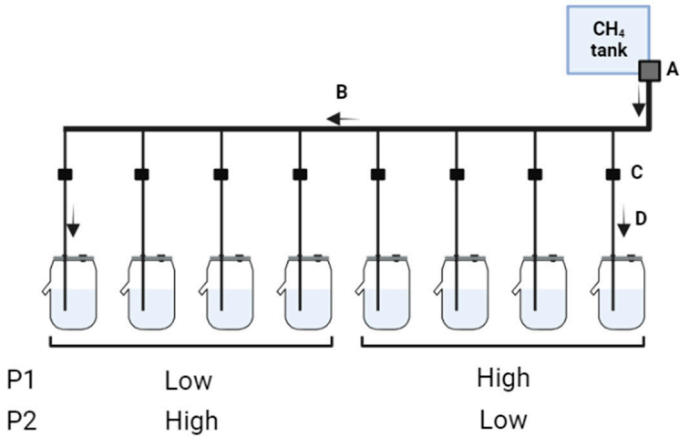

Figure 1. Schematic representation of CH_4 infusion system. A—the central gas regulator that is responsible for the regulation of CH_4 infusion from the gas cylinder to the central pipe, B—the central pipe connects the gas regulator to each connecting pipe, C—2-way flow control valves responsible for the fine adjustments of CH_4 flow into each fermenter, and D—individual connecting pipes that conduct CH_4 flow from the central pipes into each fermenter. Fermenters were divided into two groups (main plots) before the first period (P1), and each group of fermenters received contrasting SF_6 release rates. In the second period (P2) a treatment crossover of contrasting SF_6 release rates was carried out.

2.2. Experiment 2—Experimental Design

Implementation of the SF_6 tracer technique to estimate CH_4 emissions in a DFCCS was applied in an experimental setting, in which a control diet with soybean meal (SBM) as the main source of protein and two other diets containing the partial replacement of the SBM with algal biomass were tested. Algal biomass was derived from either *Chlorella pyrenoidosa* (CHL) or *Spirulina platensis* (SPI) species, and the experiment was carried out in a split-plot design, where the main plot was the SF_6 release rate (3.30 ± 0.73 or 9.65 ± 0.59 mg/d) and the split-plot was arranged into a 3 × 3 Latin square design, with 3 treatments and 3 fermentation periods, comprised of 7 d of adaptation followed by 3 d of

gas collection. Dietary treatments contained (all in DM basis) 16.0 ± 0.10% crude protein, 2.50 ± 0.11% ether extract, 34.9 ± 0.65% NDF, 18.6 ± 0.10% ADF, 31.0 ± 0.17% starch, and 1.78 ± 0.01 Mcal/kg net energy for lactation, detailed information about the ingredient and diets composition and algae sources can be found in the companion manuscript [17]. The algae inclusion rates were based on our previous studies [17,18]. The SF_6 permeation tubes utilized for Exp2 were identical to the ones used on Exp1; however, each permeation tube was randomly assigned to each fermenter, while the same permeation tube was kept on the selected fermenter across the experimental periods.

Three ruminally cannulated lactating Holstein dairy cows were used to serve as ruminal content donors. The cows were situated at the Dairy Research Unit of the University of Florida in a free-stall barn alongside the rest of the herd, receiving a total mixed ratio containing 50% corn silage. On the first day of each experimental period, the ruminal content was collected from the cows 2 h after the morning feeding, filtered through 4 layers of cheesecloth, and stored in a prewarmed thermos. The inoculum was transported within 30 min of the collection to the laboratory and incubated into the prewarmed DFCCS. All procedures and analytical methods used for Experiment 2 are described in our companion paper [17]. Briefly, for the analysis of nutrient degradability, 24 h effluents from day 8 to 10 were collected, and a subsample was freeze-dried. Feed and dried effluent samples were analyzed for DM, OM, and NDF, and the degradability of these nutrients was calculated from the difference in input and output from the fermenters.

2.3. SF_6 Permeation Tube Preparation and Collection Canister

The permeation tubes and the canisters used for the collection during Exp1 were previously described by Henry et al. [19]. Briefly, permeation tubes consisted of brass tube bodies (volume = 1.86 mL), nylon washers, and a Teflon membrane, secured with a porous (2-µm porosity) stainless steel frit and a brass nut. Permeation tubes were filled with ~2.3 g of SF_6, kept at 39 °C, and weighed 12 times within 38 d for determination of SF_6 release rate. To select contrasting release rates of SF6, a batch of 50 permeation tubes was prepared, and their release rate was characterized at 38 d, as described earlier. From this batch, 8 permeation tubes with high or low release rates were selected for the current study.

During Exp1 (deionized water only), gas collection canisters were used. The equipment was constructed of polyvinyl chloride pipe to have a final volume of 2 L. Samples were collected by evacuating the collection canisters to 68.6 cm of Hg and connecting the canister to the lid of the fermenters, which were equipped with a crimped capillary tube that was positioned to sample directly from the top of the headspace of the fermenter. The volume of the collection canisters and the crimped capillary tubes were designed to allow half of the vacuum to remain after 24 h. Following detachment from the fermenters, the canister was pressurized with nitrogen to one atmosphere, and then, a 50 mL gas sample was collected and stored in hermetically sealed and previously evacuated 125 mL glass bottles [20]. Among the 48 observations during Exp1, more than 60% had the canisters' relative final pressure ranging between 50 and 70% of the initial pressure, which is in line with previous observations in the DiLorenzo lab (Figure 2A). Approximately 18% of the observations exceeded 80% of the initial pressure, which could indicate a clog in the collection system during the day.

During Exp2, the rate of clogged collection systems (relative pressure greater than 80%) was much greater, almost 50% (Figure 2B), during collection d 1 and 2 of the first period. Aiming to minimize such issues, starting from collection d 3 onwards, a gas spot sampling directly from the headspace of the fermenters was performed. The lid of the fermenters had two open holes that were closed with a rubber stopper, one of which was used to introduce substrate into the fermenter and the second for gas spot sampling. The rubber stopper had a 0.5 cm diameter hole in the center, which allowed access to the headspace without completely opening the fermenter or removing the rubber stopper. Prior to any ruminal fluid collection from the fermenter or opening the feeding system, a needle connected to a 50 mL syringe was placed into the rubber stopper's hole, and a sample of the headspace gas

was collected after gently mixing the headspace gas by hermetically pumping out and in the headspace gas three times. Six 10 mL spot gas samples were collected daily and pooled into hermetically closed and previously evacuated 125 mL glass bottles. This change in collection protocol due to clogging issues indicates that the use of canisters similar to the ones used in in vivo experiments might not be adequate for our application, and further research on this matter is warranted.

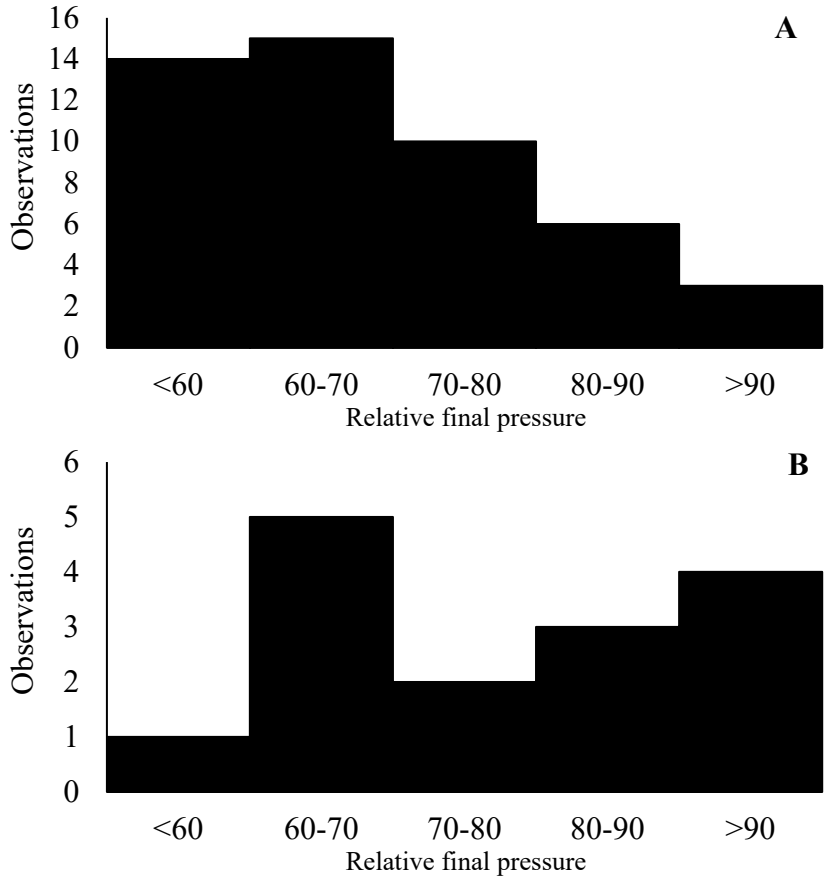

Figure 2. Distribution of the relative final pressure of the canisters during experiments 1 (n = 48) and 2 (n = 16). The X-axis represents the distribution of the relative final pressure of canisters. The Y-axis represents the number of observations.

2.4. Dual-Flow Continuous Culture System

The experiment was carried out in a DFCCS, similar to the one developed by Hoover et al. [21], and used in recent studies in our current laboratory [22–24], in which a schematic representation is shown in Figure 3. The fermenter bases were manufactured at the Ohio State University and described in Wenner et al. [25]. The fermenter setup consisted of a glass jar measuring 15 cm in width, 22 cm in height, and 0.5 cm in glass thickness (approximately 1800 mL volume). The circular steel lid of the jars has a diameter of 15 cm with multiple apertures to accommodate the central mixer, liquid outflow filter, temperature sensor, heater, artificial saliva infuser, and two nitrogen infusers, one releasing nitrogen gas into the liquid phase at the bottom of the jar, while the other releases the nitrogen into the headspace of the jar.

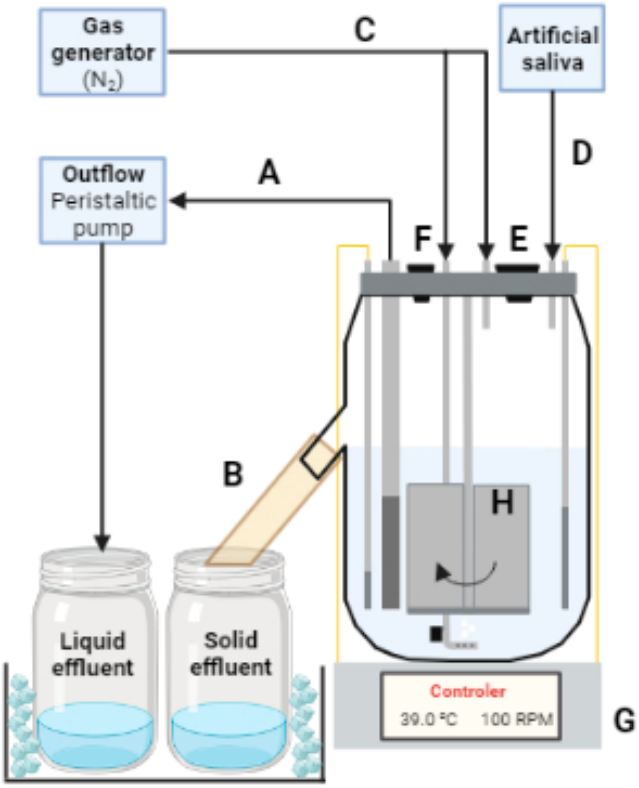

Figure 3. Schematic representation of the Department of Animal Sciences, University of Florida, dual-flow continuous culture system. A—liquid outflow effluent that passes through the 1 mm pore-size stainless steel mesh is pumped out of the fermenter by a peristaltic pump and stored into a container immersed in a cold-water bath; B—liquid and solid particles that are not pumped out by the peristaltic pump can leave the fermenters by flowing out through the solid outflow; C—gas generator pumps nitrogen gas into the fermenters (headspace and liquid phase) to keep the anaerobiosis within the fermenters; D—artificial saliva is stored in a container and is pumped into the fermenters at a rate of 3 mL/min; E—opening for feeding of the fermenters; F—opening for additional collections, for instance, this opening was used for collection of headspace gas sample during experiment 2; G—base of the fermenter that contains the temperature controller and is responsible for the temperature maintenance, in additional, a magnetic stirrer device is located in the base of the fermenter, which propels the central mixer (H) at a set rotation.

The central mixer consists of a 20 cm central steel bar equipped with 4 flat acrylic blades attached to the bottom, measuring 10 cm in height and 3 cm in width each, and a magnet that is responsible for the stirring action of the equipment. At the base of the fermenters, a magnetic stirrer device is located, which propels the central mixer, and a rotation controller is also present, which allows agitation of the fermenter's content at 100 rotations per minute. In addition, in the base of the fermenters, a temperature controller is present, which is attached to an immersible temperature sensor and heater. The temperature sensor is responsible for detecting the current temperature of the liquid fraction inside the jar; the controller located in the base of the fermenter will then turn on or off the heater to increase or decrease the temperature of the liquid according to a pre-determined setting (39 °C).

To keep the anaerobic environment within the jar, two nitrogen gas apparatuses were attached to the lid of the fermenters, maintaining pressure inside of the jar to inhibit atmospheric oxygen from entering the jar. Each nitrogen infuser continuously releases 300 mL of nitrogen gas into the headspace and liquid phase. The artificial saliva injector is an apparatus also located on the lid of the fermenter. The saliva is stored in a 50 L tank and individually transferred to each fermenter at a rate of 3 mL/min by a peristaltic pump.

The lid also has a liquid outflow apparatus composed of an 18 cm long and 1 cm diameter polyvinyl chloride pipe. In the 1/3 bottom (6 cm) of the pipe, 4 transversal lines of 0.5 cm wholes were placed and covered with a 1 mm pore-size stainless steel mesh. This design allows the retention of solid particles (that cannot pass through the mesh) within the jar, enabling the liquid fraction (that can pass through the mesh) to flow into the liquid effluent container at a rate of 1.7 mL/min. Additionally, a 2 cm diameter opening is positioned 11 cm from the bottom of the jar, facilitating the collection of solid fractions into a separated solid effluent container. Both effluent containers are maintained in a cold-water bath at 4 °C. The solid passage rate is regulated by the difference between the inlet of artificial saliva and feed, subtracted from the liquid outflow.

2.5. CH_4 and SF_6 Analyses

To address the high concentration of CH_4 and SF_6 from the collected samples, a serial dilution with N_2 gas using a 50 mL syringe to achieve a dilution of 1:150 and 1:2000 (*v*:*v*) was carried out for Exp1 and Exp2, respectively. Subsequently, CH_4 and SF_6 concentrations were analyzed by gas chromatography (Trace 1310 Gas Chromatograph, Thermo Scientific, Waltham, MA, USA). The analysis involved different detection techniques utilizing a flame ionization detector for CH_4 and an electron capture detector for SF_6 analysis. A capillary column (HayeSep Q and Porapak Q 0.5 m × 2.0 mm, 80/100, SilcoSteel, Restek Corp., Centre County, Bellefonte, PA, USA) facilitated the separation process. Injector, column, and detector temperatures for CH_4 analysis were 80, 160, and 200 °C, respectively. For SF_6, temperatures were 80, 60, and 250 °C for the injector, column, and detector, respectively. The carrier gas used for both CH_4 and SF_6 was N_2. Three gas mix standards were used for the analyses. The concentration of CH_4 and SF_6 in the standards was 4.27, 50.7, and 101.4 ppm and 30.2, 110, and 518 ppt, respectively.

The emission of CH_4 from fermenters in relation to the SF_6 tracer gas captured in the samples was calculated according to Johnson et al. [7] following the equation:

$$Q_{CH_4} = Q_{SF_6} \times \left(\frac{CH_4}{SF_6} \right) \quad (1)$$

where Q_{CH_4} is considered the CH_4 emission per fermenter (mg/d), Q_{SF_6} is the SF_6 release rate (mg/d), CH_4 is the concentration of CH_4 in the sample (ppm), and SF_6 is the concentration of SF_6 in the sample (ppt).

2.6. Statistical Analysis

Exp1 was conducted in a split-plot design where groups of fermenters (n = 2) were considered whole plots, and individual fermenters (n = 4) were considered subplots. In the first experimental period, the level of SF_6 release was randomly assigned to the whole plot, and a crossover of treatments was carried out during the second period. Within each fermenters group (whole plot), the level of CH_4 infusion was randomly assigned to individual fermenters within each experimental period. Normality of residuals and homogeneity of variance were examined after model fitting for each continuous dependent variable using the Shapiro–Wilk test from the UNIVARIATE procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA), and maximum studentized residue of ±4 was allowed.

Pearson correlation between CH_4 infusion and recovery was carried out using the Corr procedure of SAS 9.4. Statistical analysis of CH_4 recovery was performed using a multiple regression approach from the MIXED procedure of SAS 9.4 using the backward elimination process. The final model was composited of the SF_6 release as a categorical variable and CH_4 infusion as a continuous predictor, using the model:

$$Y_{ijkl} = \mu + D_i + S_j + C_k + C_k \times C_k + S_j \times C_k + P_l + e_{ijkl}$$

where Y_{ijkl} was the observation *ijkl*, μ was the overall mean, D_i was the fixed effect of day of collection (i = 1–3), S_j was the fixed effect of SF_6 release rate (j = 1–2), C_k was the continuous

fixed effect of CH_4 infusion (k = 1–4), $C_k \times C_k$ was the quadratic continuous fixed effect of CH_4 infusion, $S_j \times C_k$ was the interaction fixed effect of SF_6 release rate and the continuous fixed effect of CH_4 infusion, P_l was the random effect of the experimental period (l = 1–2), and e_{ijkl} is the random residual.

In addition, CH_4 production and SF_6 concentration were analyzed using both SF_6 release and CH_4 infusion as categorical variables using the model:

$$Y_{ijkl} = \mu + D_i + S_j + C_k + S_j \times C_k + P_l + e_{ijkl}$$

where Y_{ijkl} was the observation $ijkl$, μ was the overall mean, D_i was the fixed effect of day of collection (i = 1–3), S_j was the fixed effect of SF_6 release rate (j = 1–2), C_k was the categorical fixed effect of CH_4 infusion (k = 1–4), $S_j \times C_k$ was the interaction fixed effect of SF_6 release rate (j = 1–2) and the categorical fixed effect of CH_4 infusion (k = 1–4), P_l was the random effect of the experimental period (l = 1–2), and e_{ijkl} was the random residual. Orthogonal contrasts were used to depict the effect of CH_4 and SF_6 release rate, where we conducted a contrast to evaluate the effects of SF_6 release rate (Low vs. High) and the polynomial contrast to evaluate the linear and quadratic effect of CH_4 release.

Exp2 was conducted in a split-plot design, where the main plot was the SF_6 release rate and the split-plot was arranged into a 3 × 3 Latin Square design, where the fermenter within the period was the experimental unit. Statistical analysis of the CH_4 emission, yield per unit of degraded nutrient, and SF_6 concentration were performed using the MIXED procedure of SAS 9.4, using the model:

$$Y_{ijkl} = \mu + T_i + S_j + T_i \times S_j + D_k + P_l + e_{ijkl}$$

where Y_{ijkl} was the observation $ijkl$, μ was the overall mean, T_i was the fixed effect of treatment (i = 1–3), S_j was the fixed effect of SF_6 release rate (j = 1–2), $T_i \times S_j$ was the fixed effect of the interaction between treatment (i = 1–3) and SF_6 release rate (j = 1–2), D_k was the random effect of day of collection (k = 1–3), P_l was the random effect of the period (l = 1–3), and e_{ijkl} was the random residual. Orthogonal contrasts were used to depict the treatment and SF_6 release rate, where we conducted a contrast between low vs. high SF_6 release rate, control vs. algae-containing feed, and CHL vs. SPI. To depict the interaction effect, contrasts between control vs. algae-containing feed and CHL vs. SPI were carried out at each level of the SF_6 release rate. Significance was declared at $p \leq 0.05$, and the tendency was declared at $0.05 < p \leq 0.10$ or $0.05 < p \leq 0.20$ for main and interaction effects, respectively.

3. Results

3.1. CH_4 and SF_6 Analyses

The standard curve for the predicted CH_4 and SF_6 concentrations for Exp1 and Exp2 are presented in Figures 4 and 5, respectively. With the standard curves commonly used for the estimation of CH_4 emissions in in vivo settings, the concentration of CH_4 and SF_6 was underestimated and overestimated, respectively. This means that during the analysis of the samples, the concentration of CH_4 in the samples was below the analytical limits of the standards (Figures 4A,C and 5A,C), while the concentration of SF_6 in the samples was above the analytical limits of the standards (Figures 4B,D and 5B,D). Those results indicate that an adjustment of SF_6 released inside the fermenters is required. For instance, the ratio between CH_4:SF_6, in mg units, for the standards was on average 266, while the CH_4:SF_6 ratio in Exp1 samples was 1146 ± 390 and 386 ± 180, and in Exp2 it was 709 ± 74.3 and 262 ± 31.3 for the low and high SF_6 release rate, respectively.

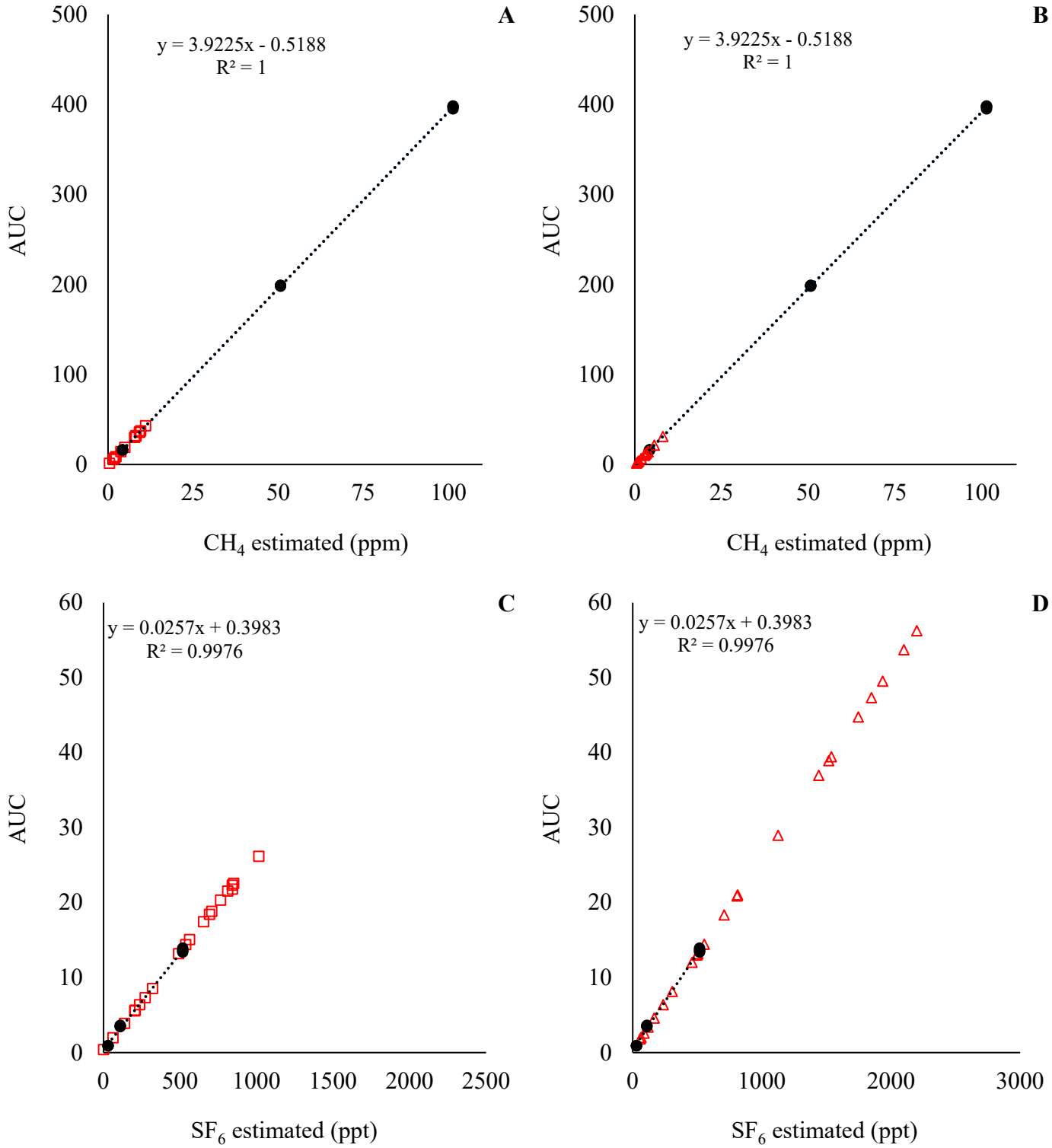

Figure 4. CH_4 and SF_6 analyses for experiment 1. At the top left of the graphics is presented equation and R^2 for the standard curve; in addition, the Y-axis is the area under the curve (AUC) used to estimate CH_4 and SF_6 concentrations. Solid circles (●) represent the observations for the standard curve, and the dashed line is the fitted standard curve. Open squares (□) represent the samples from the low SF_6 release rate, and open triangles (Δ) represent the samples from the high SF_6 release rate.

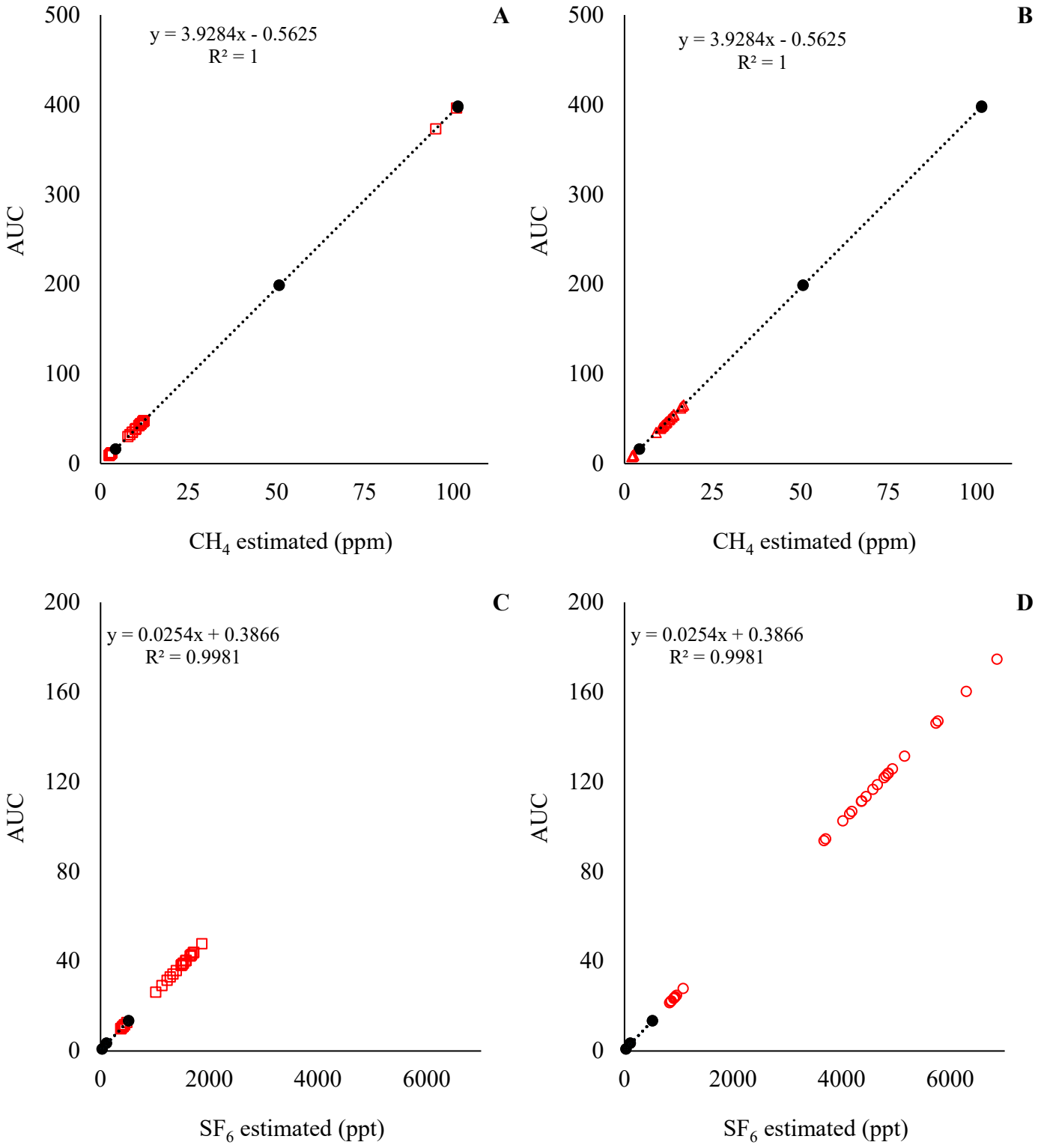

Figure 5. CH_4 and SF_6 analyses for experiment 2. At the top left of the graphics is presented equation and R^2 for the standard curve; in addition, the Y-axis is the area under the curve (AUC) used to estimate CH_4 and SF_6 concentrations. Solid circles (•) represent the observations for the standard curve, and the dashed line is the fitted standard curve. Open squares (□) represent the samples from the low SF_6 release rate, and open triangles (Δ) represent the samples from the high SF_6 release rate.

3.1.1. Experiment 1

Correlation analysis between the CH_4 infusion rate within the fermenters and the estimated CH_4 emissions (in g/d) using the SF_6 tracer technique is presented in Figure 6. The correlation coefficient (r) from the Pearson analysis using the whole dataset (n = 36 observations) demonstrated that those two variables are positively correlated (r = 0.97 and $p < 0.001$). To check if the correlation was influenced by all observations, the data corresponding to the treatment zero CH_4 infusion was removed, while a similar pattern (r = 0.92 and $p < 0.001$) for the remaining 24 observations was observed.

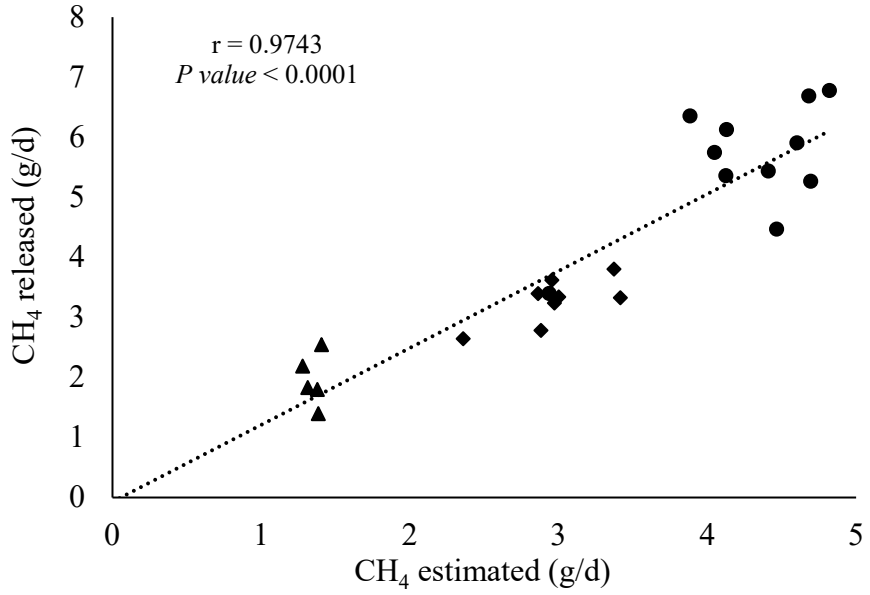

Figure 6. Pearson correlation analysis between CH_4 infused and estimated by the SF_6 tracer technique (n = 36 observations). Triangle, diamond, and circles represent CH_4 infused between 0.93–1.61, 2.06–3.41, and 3.88–4.82 g/d, respectively.

Multiple regression analysis, where CH_4 and SF_6 release were used as a predictor of CH_4 recovery, was evaluated. A tendency for an interaction effect between the SF_6 release and the quadratic term of CH_4 ($p = 0.13$) suggests that the CH4 recovery predicted was dependent on the SF_6 released within the fermenter. By assessing the regression lines, at low levels of CH_4 infusion, a similar CH_4 recovery was predicted; however, with increasing levels of CH_4 infusion, a discrepancy between CH_4 recovery predicted from low and high SF_6 release can be noticed. This suggests that a high SF_6 release rate overestimated the CH_4 compared to the actual CH_4 recovery in scenarios with high CH_4 infusion. In addition, there was a linear ($p < 0.001$) and a quadratic ($p < 0.01$) effect for CH_4 infusion. The coefficients for the linear and quadratic effects were 0.9256 ± 0.1109 and 0.000073 ± 0.000024 (coefficient $\pm$ standard error). The regression analysis indicates that the SF_6 tracer technique overestimates the CH_4 recovery; for instance, CH_4 infusion between 0.93 to 1.61, 2.06 to 3.41, and 3.88 to 4.82 g/d overestimated the CH_4 recovery by 5.85, 20.0, and 31.4%, respectively. Those results indicate that the SF_6 tracer technique would not be efficient for measuring CH_4 emission in scenarios with high CH_4 emission. In addition, the effect of the days of collection was evaluated, and no statistical differences were observed, which indicates consistency across collection days.

The effects of CH_4 and SF_6 released as categorical data on CH_4 recovery and SF_6 concentration are presented in Figure 7. In both response variables, the day was not affected ($p > 0.17$). No interaction effect ($p = 0.71$) between CH_4 and SF_6 released, nor was the effect of SF6 released ($p = 0.86$) observed for CH_4 recovery. The main effect of CH_4 infusion ($p < 0.001$) was observed. Orthogonal contrast indicated a positive linear effect ($p < 0.001$) on CH_4 recovery. Results indicated that, independently of the SF_6 released, the technique is

adequate to detect different levels of CH_4 recovery in in vitro continuous culture systems. When SF_6 concentration was evaluated, an interaction effect ($p = 0.07$) between CH_4 and SF_6 release was observed. Low SF_6 release enabled a similar SF_6 concentration of the samples regardless of the level of CH_4 infusion. However, high SF_6 release had a quadratic response (contrast $p < 0.001$), which suggests that the concentration of the sample SF_6 reduces with an increase in CH_4 infusion. This may indicate that the high SF_6 release rate is not adequate in scenarios of high CH_4 recovery.

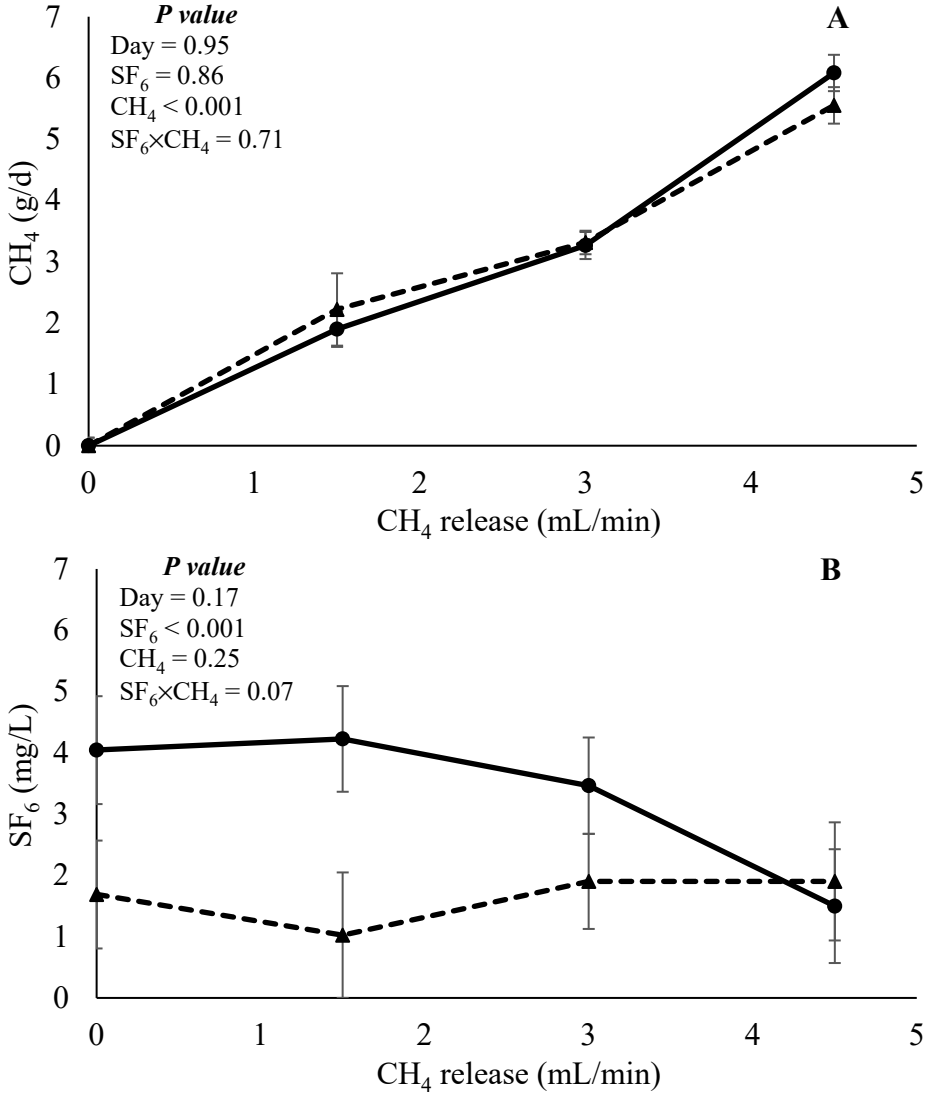

Figure 7. Effects of interaction between CH_4 and SF_6 release on the estimation of CH_4 recovery (g/d) and concentration of SF_6 (mg/L). Solid triangles (▲) and circles (●) represent low and high SF6 release, respectively, in experiment 1.

3.1.2. Experiment 2

The CH_4 emission (in g/d) and SF_6 concentration obtained from the Exp2 evaluating the effects of partial replacement of SBM with CHL or SPI on a DFCCS is presented in Figure 8. There was no interaction effect between the treatment and SF_6 release for both CH_4 emission ($p = 0.27$) and SF_6 concentration ($p = 0.46$), neither a main effect for treatment ($p = 0.31$ and 0.79, respectively), except by a tendency ($p = 0.06$) for a main effect of SF_6 release on SF_6 concentration, in which the concentration of SF_6 was lower for the low SF_6 release in comparison to the high SF_6 release (0.22 and 0.41 mg/L, respectively). When contrasts were applied, regardless of the SF_6 release rate, a tendency (contrast control vs.

algae, $p = 0.10$) was observed for the algae-containing diets to have a reduction in daily CH_4 yield when compared to the control diets (2.28 vs. 2.53 g/d).

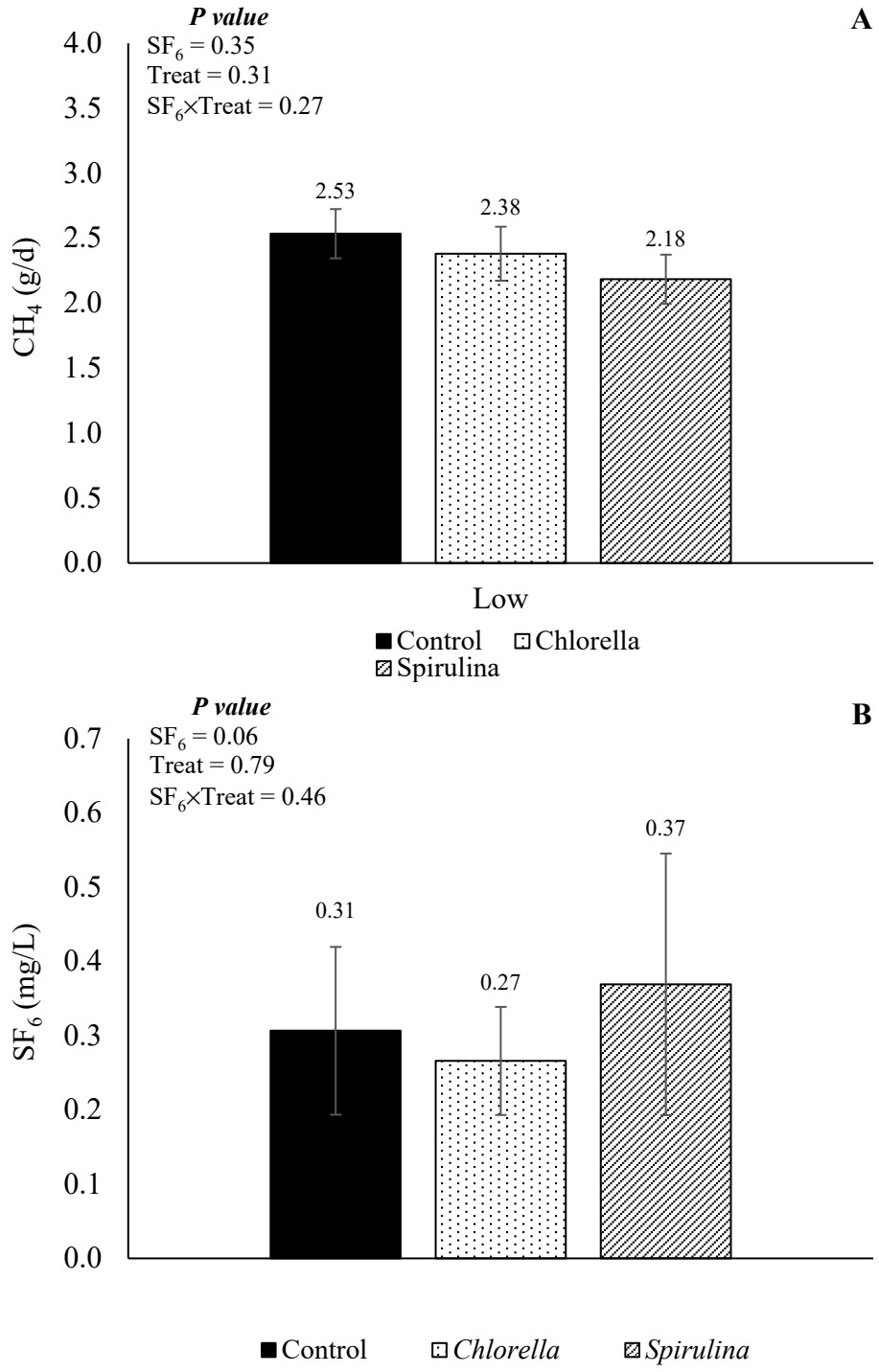

Figure 8. Effects of SF_6 release and partial replacement of soybean meal (control) with *Chlorella* or *Spirulina* in CH_4 production (**A**) and concentration of SF_6 (**B**) in the sample.

Besides CH_4 yield, the nutrient degradability in grams and CH_4 production per unit of nutrient degraded is presented in Table 1. Replacing SBM with algae in the diet did not affect the total DM (contrasts $p = 0.62$) and OM (contrasts $p = 0.35$) degraded, nor did CH_4 per unit of digestible DM (contrasts $p = 0.17$) and digestible OM (contrasts $p = 0.20$). However, algae-containing diets enabled an increase in NDF degradation (contrasts $p < 0.01$),

which resulted in a reduction in the CH_4 per unit of NDF degraded (contrasts $p < 0.001$). No effect ($p \geq 0.20$) of algae species was observed.

Table 1. Effects of partial replacement of soybean meal with *Chlorella* or *Spirulina* on CH_4 production and true degradability of dry matter (DMD), organic matter (OMD), and neutral detergent fiber (NDFD).

	Treatments [1]			SEM	Contrast *p*-Value [2]	
	Control	*Chlorella*	*Spirulina*		CRT vs. Algae	CHL vs. SPI
CH_4 production						
mg/g DMD	35.0	31.5	31.2	3.42	0.17	0.93
mg/g OMD	41.8	39.4	37.2	4.08	0.20	0.56
mg/g NDFD	138	107	94.2	14.6	<0.001	0.20
True degradability of nutrients, g/d						
DMD	72.1	73.0	68.7	2.96	0.62	0.23
OMD	60.5	59.3	57.8	2.63	0.35	0.57
NDFD	19.3	22.4	22.8	1.76	<0.01	0.78

[1] Control diet contained 17.8% soybean meal (SBM), and *Chlorella* and *Spirulina* diets contained 7.71 and 7.12% SBM and the same concentration of their respective algae, respectively. All diets contained 16% crude protein and approximately 35% NDF. Detailed diet composition is listed in Lobo et al. [17]. [2] CRT vs. Algae (contrast between CRT against *Chlorella* and *Spirulina*), CHL vs. SPI (contrast between *Chlorella* against *Spirulina*).

4. Discussion

4.1. SF_6 Marker to Estimate CH_4 Emissions in DFCCS

The main goal of this study was to evaluate the SF_6 tracer technique to estimate CH_4 recovery and yield in a DFCCS. To the best of our knowledge, this is the first attempt to use the SF_6 tracer technique to estimate CH_4 production within in vitro systems, which would allow the system to measure enteric CH_4 emissions from different diets. We have demonstrated that this technique may be a useful tool to estimate CH_4 emission and yield from DFCCS; however, some adjustments during gas collection or CH_4 analysis are required to improve the methodology.

For instance, a greater level of SF_6 release enabled a better CH_4:SF_6 ratio, which was close to the ratio obtained from the standards. A greater CH_4:SF_6 ratio could generate variability in the data due to issues related to the standard curve fitting and the analytical process. In addition, due to the fact that the collection of gas was carried out directly from the headspace of the fermenters, the sample was concentrated, and serial dilutions were required to adequately fit the samples to the capability of the GC standard curves. This process of serial dilution may be another factor that could contribute to potential analytical bias in the data.

Pinares-Patino et al. [26] have conducted a study to evaluate the effects of the rate of SF_6 release (1.90, 3.62, 5.28, or 11.1 mg/d) for the estimation of CH_4 emission in vivo. Authors reported no effects of SF_6 release rate on CH_4 concentration (48.0 ppm); however, there was a positive linear effect of SF_6 release rate on the SF_6 concentration (119, 238, 279, and 524 ppt, respectively) and a negative linear effect of SF_6 release on CH_4:SF_6 ratio (455, 266, 225, and 105, respectively). When evaluating the effects of the SF_6 release rate upon CH_4 emission and yield by unit of dry matter intake, the authors reported a positive linear effect for both variables. Those results suggest that CH_4 emission and yield per unit of degraded nutrients are dependent on the SF_6 release rate, where a greater SF_6 release rate may estimate greater values of CH_4 yield. In another study, Martin et al. [27] evaluated the interaction effects of the site of collection (breath gas or ruminal gas) and the rate of SF_6 release on the estimation of CH_4 emission in non-lactating cannulated Holstein cows. Authors reported no interaction effects between those two factors; in addition, a high SF_6 release rate (3.15 mg/d) enabled a greater estimation of CH_4 emission (105.3 vs. 118.1 L/8-h period) when compared to a low SF_6 release rate (1.58 mg/d). Current study

results partially corroborate the findings of Pinares-Patino et al. [26] and Martin et al. [27]. It was demonstrated that at low levels of CH_4 production, the effects of SF_6 release rate are small; however, when the technique is used on scenarios with a high CH_4 production, high levels of SF_6 release rate would estimate a greater CH_4 emission than low SF_6 release rate.

The CH_4 recovery estimated by the SF_6 tracer demonstrated that the technique overestimates CH_4 recovery in scenarios with high production of CH_4. This may be due to the frequency of CH_4 flow used and the accuracy of the method. We evaluated the flow of CH_4 infusion four times daily, and adjustments were made accordingly; however, the flow between analyses could have oscillated, increasing the release rate within the fermenters, which could explain the greater CH_4 estimations from the SF_6 tracer technique. Also, we could attribute those effects to noise during the collection or analyses. For instance, a better collection system, scaling off SF_6 release, and sample dilution process should be considered and further modified to improve the accuracy of the technique to estimate CH_4 emission.

Another important point observed from the results of SF6 concentration is that an interaction effect between CH4 and SF6 release was observed, indicating that when high CH_4 was released into the fermenter, a low SF_6 was recovered. This is an interesting result with an unclear explanation, and further studies are required to investigate that response. One explanation that we could propose to explain this result is the high dilution rate of the SF_6 gas within the fermenter jar; since a greater CH_4 was released within the jar, a more diluted sample was obtained.

4.2. Effects of the Partial Replacement of SBM with Algae Biomass

Current results demonstrated that algae-containing diets tended to reduce CH_4 yield and improved NDF degradation, consequently reducing CH_4 production by unit of NDF. In a previous study [18] conducted by the current research team, the effects of the replacement of SBM with algae biomass on carbohydrate-contrasting diets were evaluated. It was demonstrated that a partial replacement of SBM with algae biomass can have a linear reduction in the degradability of nutrients such as DM, OM, and NDF. However, when CH_4 was evaluated, a linear reduction in CH_4 yield and production by unit of NDF and a tendency for a negative linear effect in CH_4 production per unit of DM and OM were observed. Such a linear effect observed in the before mentioned study could be mainly attributed to the effect of the replacement of SBM with SPI, which enabled the greatest reduction in CH_4 yield. Those results reported in our companion study in a batch culture trial corroborated our findings, which further indicate the reliability of the SF_6 technique to measure CH_4 in DFCCS.

In the current companion paper [17], which contains the kinetics and daily metabolite production, as well as the degradability of nutrients and nitrogen metabolism, it was demonstrated that partial replacement of SBM with algae had the potential to reduce the degradability of the CP and improve the efficiency of nitrogen utilization, along with an increase in the degradability of NDF. In another experiment, the co-digestion of switchgrass and *Spirulina* was carried out with the objective of evaluating the kinetics of CH_4 production [28]. The author reported a positive linear correlation between the level of *Spirulina* inclusion and CH_4 yield, which was attributed to the fact that the biomass of the switchgrass used was mostly undegradable carbohydrates, while the *Spirulina* was composed of more digestible nutrients.

Regarding the utilization of the SF_6 tracer technique to estimate CH_4 production in DFCCS, it is important to note that the collection scheme for the experiment using ruminal content (experiment 2) had to be modified. That modification in the collection protocol was carried out because of the high incidence of collection system clogs due to the humidity in the headspace of the fermentation jar. Evaluation of a system to reduce the humidity of the collected gas should be carried out in future studies. Despite clogging issues in the collection system that prevented the collection using the canisters, the utilization of spot sampling overcame these issues and did not affect the validity and effectiveness of the technique to estimate CH_4 production.

5. Conclusions

This study revealed novel insights into the estimation of CH_4 using the SF_6 tracer technique in a DFCCS. Notably, samples generated from DFCCS may not be appropriate for the traditional standard curves used for in vivo settings, which could lead to an underestimation of CH_4 and an overestimation of SF_6 concentrations. This highlights the need for an adjustment in the SF_6 release within fermenters, which would allow the use of those standard curves. Moreover, while the SF_6 tracer technique can efficiently detect varying levels of CH_4 yield to a certain extent, it tends to overestimate CH_4 yield in scenarios with higher CH_4 emissions and when permeation tubes with a high rate of SF_6 release are used. Future studies should focus on evaluating lower levels of SF_6 release into the fermenters individually or developing a system attached to the nitrogen infusion apparatus that could be used to deliver the tracer gas marker efficiently into the fermenters. In addition, further investigation is required on the collection system during a fermentation trial, in which a greater number of collections may induce the clogging of the system due to the humidity of the gas in the headspace of the fermenters. A water trap device would be needed to improve the viability of gas collection directly from the headspace of the fermenters. These findings underscore the importance of refining and calibrating measurement techniques for accurate CH_4 yield estimations in various scenarios.

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