SHORT COMMUNICATION

# Effect of increasing levels of *Chlorella* spp. on the *in vitro* fermentation and methane production of a corn silage-based diet

## *Efecto de niveles incrementales de Chlorella spp. sobre la fermentación in vitro y la producción de metano de una dieta a base de ensilaje de maíz*

## *Efeito do aumento dos níveis de Chlorella spp. na fermentação in vitro e na produção de metano em uma dieta à base de silagem de milho*

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**Abstract**

**Background:** Generally, the forages used in cow-calf and backgrounding cattle operations have low crude protein and high fiber concentration, limiting animal performance and increasing greenhouse gas emissions. *Chlorella* spp., a green microalga, shows promising potential to provide nutrients, especially nitrogen, to low-protein diets. However, information is limited regarding the effects of *Chlorella* spp. on the *in vitro* fermentation and methane ($CH_4$) production of diets. **Objective:** To evaluate the effects of increasing inclusion levels of algae (*Chlorella* spp.) on ruminal *in vitro* fermentation profile and $CH_4$ production of a corn silage-based diet. **Methods:** Incubations were conducted on three separate days using corn silage and gin trash as substrate (70:30 ratio, respectively). Treatments were control (without algae) and 1, 5, and 10% of algae inclusion in the substrate replacing the basal diet. Ruminal fluid was collected from two ruminally cannulated Angus crossbred steers fed *ad libitum* a corn silage and gin trash diet. Final pH, concentration of volatile fatty acids (VFA) and ammonia nitrogen ($NH_3$-N), *in vitro* organic matter digestibility (IVOMD), total gas, and $CH_4$ production were determined after 24 h of incubation. Variables were evaluated

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using the MIXED procedure of SAS software, and means were compared using orthogonal polynomial contrasts. **Results:** Algae inclusion linearly increased ($p<0.01$) the IVOMD. However, the final pH and concentration of VFA and $NH_3$-N did not differ ($p>0.05$) among algae levels. Molar proportion of VFA and the acetate:propionate ratio was not affected ($p>0.05$) by increasing algae inclusion. Finally, total gas and $CH_4$ production were not different ($p>0.05$) among treatments. **Conclusion:** The inclusion of *Chlorella* spp. does not modify the ruminal *in vitro* fermentation profile nor the $CH_4$ production of a corn silage-based diet.

**Keywords**: *additives; backgrounding systems; cow-calf operations; ensiled forages; green micro-algae; low-protein diets; methanogenesis; protein supplementation.*

**Resumen**

**Antecedentes:** Generalmente, los forrajes en los sistemas de producción de cría y levante de ganado tienen baja cantidad de proteína cruda y alta de fibra, limitando la productividad animal e incrementando la emisión de gases de efecto invernadero. *Chlorella* spp., una microalga verde, presenta características promisorias para proveer nutrientes, especialmente nitrógeno, en dietas bajas en proteína. Sin embargo, existe información limitada relacionada con la inclusión de *Chlorella* spp. sobre la fermentación y la producción de metano ($CH_4$) *in vitro* de la dieta. **Objetivo:** Evaluar el efecto de incrementar la inclusión de alga (*Chlorella* spp.) sobre el perfil de fermentación *in vitro* y la producción de $CH_4$ de una dieta basada en ensilaje de maíz. **Métodos:** Las incubaciones fueron realizadas en tres días diferentes usando ensilaje de maíz y residuo de algodón como sustrato (en relación 70:30, respectivamente). Los tratamientos fueron: un tratamiento control (sin alga), e inclusiones de 1, 5 y 10% de alga en el sustrato. El fluido ruminal fue colectado de dos novillos mestizos Angus con cánula ruminal, alimentados con una dieta de ensilaje de maíz y residuo de algodón a voluntad. El pH final, la concentración de ácidos grasos volátiles (VFA) y nitrógeno amoniacal ($NH_3$-N), la digestibilidad *in vitro* de la materia orgánica (IVOMD), y la producción de gas total y $CH_4$ fueron determinadas después de 24 h de fermentación. Las variables fueron evaluadas usando el procedimiento MIXED del software SAS y las medias fueron comparadas usando contrastes de polinomios ortogonales. **Resultados:** Niveles crecientes de alga incrementaron ($p<0,01$) linealmente la IVOMD. Sin embargo, el pH final y la concentración de AGV y $NH_3$-N no fueron diferentes ($p>0,05$) entre los niveles de alga. Además, las proporciones molares de VFA y la relación acetato:propionato no se afectaron con el incremento ($p>0,05$) en la concentración de alga. Finalmente, la producción de gas y de $CH_4$ no fueron diferentes ($p>0,05$) entre tratamientos. **Conclusión:** La inclusión de *Chlorella* spp. no modifica la fermentación *in vitro* ni la producción de $CH_4$ en una dieta basada en ensilaje de maíz.

**Palabras clave:** *aditivos; dietas bajas en proteína; forrajes conservados; metanogénesis; microalgas verdes; sistemas de cría; sistemas de levante; suplementación proteica.*

**Resumo**

**Antecedentes:** Geralmente, as forrageiras utilizadas em sistemas de criação de bovinos de corte nas fases de cria e recria apresentam baixa concentração de proteína bruta e alta concentração de fibra, limitando a produtividade animal e aumentando o impacto ambiental. *Chlorella* spp. apresenta características promissoras para fornecer nutrientes, especialmente nitrogênio, em dietas com baixo teor de proteína. No entanto, informações sobre a inclusão de *Chlorella* spp. na fermentação e produção de metano ($CH_4$) *in vitro* ainda são escassas na literatura. **Objetivo:** Avaliar o efeito do aumento da inclusão de algas (*Chlorella* spp.) no perfil de fermentação *in vitro* e na produção de $CH_4$ em uma dieta à base de silagem de milho. **Métodos:** As incubações foram realizadas em três dias diferentes utilizando como substrato silagem de milho e resíduo de algodão (na proporção 70:30, respectivamente). Os tratamentos utilizados foram: controle (sem alga) e três diferentes níveis de inclusão de 1, 5 e 10% alga no substrato. O fluido ruminal foi coletado de dois novilhos Angus canulados no rúmen consumindo silagem de milho e resíduos de algodão ad libitum. O pH final, a concentração de ácidos graxos voláteis (VFA), nitrogênio amoniacal ($NH_3$-N), digestibilidade *in vitro* da matéria orgânica (IVOMD), produção total de gás e $CH_4$ foram determinados após 24 h de fermentação. As variáveis foram avaliadas utilizando o PROC MIXED do software SAS e as médias comparadas por meio de testes polinomiais ortogonais. **Resultados:** O aumento dos níveis de algas aumentou linearmente ($p<0,01$) a IVOMD. No entanto, pH final, concentração de VFA e NH3-N não diferiram ($p>0,05$) entre os níveis de algas. Além disso, as proporções molares de AGV e a relação acetato:propionato não foram afetadas pelo aumento ($p>0,05$) na concentração de algas. Adicionalmente, a produção total de gás e $CH_4$ também não apresentaram diferenças ($p>0,05$) em função dos níveis crescentes de algas. **Conclusão:** A inclusão de *Chlorella* spp. não modificou a fermentação *in vitro* ou a produção de $CH_4$ em dieta à base de silagem de milho.

**Palavras-chave:** *aditivos; conservação de forragem; dietas hipoproteicas; metanogênese; microalgas verdes; operações de bezerros; sistemas de criação; suplementação proteica.*

## Introduction

Improving animal performance is a valuable strategy to promote sustainable agricultural systems as it provides high-quality protein for a rapidly growing human population while reducing the environmental impact per unit of product (Gerber *et al.*, 2013; Hristov *et al.*, 2013b; Knapp *et al.*, 2014). In tropical and subtropical regions, cow-calf and backgrounding operations are typically maintained with low-quality forages and limited supplementation, resulting in reduced animal performance and, subsequently, a more significant, adverse environmental impact (Pardo *et al.*, 2009; Beauchemin *et al.*, 2010; Silveira *et al.*, 2011). Generally, low-quality forages have reduced crude protein and increased fiber content, which may impair ruminal fermentation and result in increased enteric methane emission (Kurihara *et al.*, 1999; Hess *et al.*, 2003; Tiemann *et al.*, 2008).

Feed additives have been implemented in ruminant diets to supply limited nutrients, improve animal performance, and reduce methane emissions of nitrogen excretion (Beauchemin *et al.*, 2008; Leng, 2008; Hristov *et al.*, 2013a). Diets limited in crude protein require supplemental nitrogen to promote microbial fermentation, enhancing energy and metabolizable protein production (Currier *et al.*, 2004; Leng, 2008). In the current economy, access to quality protein supplements is limited due to increased cost and scarce availability (Tarnonsky *et al.*, 2022); therefore, new, and local protein sources should be evaluated.

Green micro-algae, such as *Chlorella* spp., exhibit a promising chemical composition for inclusion in protein-deficient diets. Generally, the concentration of crude protein and ether extract of green micro-algae varies between 15 to 60 and 2 to 22%, respectively (Becker, 2007; McCauley *et al.*, 2020). Early research evaluating the effects of green micro-algae inclusion in high-quality diets on modulating lipid biohydrogenation and reducing $CH_4$ emissions produced contrasting results. Although green micro-algae supplementation increased the polyunsaturated lipid content in milk fat, it reduced dry matter intake affecting animal productivity and increasing $CH_4$ yield (Moate *et al.*, 2013). Conversely, green microalgae inclusion in protein-deficient diets increased nutrient digestibility, nitrogen flow through the intestine, and nitrogen retention (Drewery *et al.*, 2014). Research is limited regarding the supplementation of green microalgae in silage-based diets on fermentation parameters and $CH_4$ production; therefore, this experiment aimed to evaluate the effects of increasing inclusion levels of *Chlorella* spp. on the ruminal *in vitro* fermentation profile and $CH_4$ emissions of a corn silage-based diet. It was hypothesized that increasing the inclusion of *Chlorella* spp. in a protein-deficient diet would improve fermentation and reduce $CH_4$ emissions.

## Materials and Methods

### *Ethical considerations*

All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee (#202111460; 14-11-2021).

### *Location and animal adaptation*

This experiment was conducted at the North Florida Research and Education Center in Marianna, FL. Two ruminally-cannulated Angus-crossbred steers (808.8±36.3 Kg of BW) were used as ruminal fluid donors for the *in vitro* incubations. Steers were fed a corn silage, cotton gin trash, and premix of vitamins and minerals diet (70, 28, and 2% on a dry matter basis, respectively) at least 35 d before collecting ruminal fluid.

### *Experimental treatment*

The diet fed to the steers during the adaptation period was used as a substrate for the *in vitro* incubations. *Chlorella* spp. algae were provided dried and pelleted by a local company (Origo, LLC, Venus, Florida). Diet was dried for 48 h at 55 °C. Corn silage, gin trash, and algae were ground to pass a 2-mm screen in a Wiley mill (Thomas Scientific, Swedesboro, NJ) and analyzed for dry matter (DM), ash, crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) at a commercial laboratory (Dairy One Laboratory, Ithaca, New York; Table 1). In

addition, algae were analyzed for amino acid and fatty acid profiles at a commercial laboratory (Dairyland Laboratories, Arcadia, Wisconsin, USA) (Tables 2 and 3).

**Table 1.** Chemical composition (%DM) of ingredients used in the experiment.

| Item | Ingredient | | |
|---|---|---|---|
| | Corn silage | Gin trash | Algae[1] |
| Crude protein | 8.0 | 16.0 | 19.5 |
| Neutral detergent fiber | 27.4 | 61.7 | 13.6 |
| Acid detergent fiber | 16.6 | 58.8 | 7.9 |
| Ether extract | 3.4 | 3.2 | 1.4 |
| Ash | 2.8 | 10.3 | 54.2 |
| Organic matter[2] | 97.2 | 89.7 | 45.8 |

[1]*Chlorella* spp.. [2]Organic matter: 100 - % Ash.

**Table 2.** Amino acid and fatty acid profile of algae (*Chlorella* spp.) used in the *in vitro* incubations.

| Amino acid profile (%CP) | | Fatty acid profile (g/100 g of fatty acid) | |
|---|---|---|---|
| Lysine | 5.13 | Palmitic acid | 38.85 |
| Methionine | 1.80 | Stearic acid | 4.44 |
| Isoleucine | 3.60 | Oleic acid | 30.20 |
| Leucine | 8.15 | Linoleic acid | 16.14 |
| Threonine | 5.77 | Linolenic acid | 10.37 |
| Valine | 5.82 | | |
| Arginine | 8.36 | Saturated | 43.29 |
| Histidine | 1.48 | Monounsaturated | 30.20 |
| Phenylalanine | 5.66 | Polyunsaturated | 26.51 |
| Tryptophan | 1.64 | | |

**Table 3.** Calculated chemical composition of the substrate with increasing algae (*Chlorella* spp.) concentration used in the *in vitro* incubations.

| Chemical composition | Algae inclusion (%) | | | |
|---|---|---|---|---|
| | 0 | 1 | 5 | 10 |
| Crude protein | 10.4 | 10.5 | 10.8 | 11.2 |
| Neutral detergent fiber | 37.7 | 37.4 | 36.1 | 34.6 |
| Acid detergent fiber | 29.3 | 29.0 | 27.8 | 26.3 |
| Non-structural carbohydrates[1] | 43.5 | 43.3 | 42.4 | 41.3 |
| Ether extract | 3.3 | 3.3 | 3.2 | 3.2 |
| Ash | 5.1 | 5.5 | 7.4 | 9.8 |
| Organic matter[2] | 95.0 | 94.5 | 92.6 | 90.2 |

[1]Non-structural carbohydrates: 100 – (% Crude protein + % Neutral detergent fiber + % Ether extract + % Ash). [2]Organic matter: 100 - % Ash.

Treatments were designed with increasing proportions of algae in a corn silage and gin trash mixture (70:30 on a dry matter basis, respectively), with algae inclusion levels selected to describe a typical range of additive supplementation in beef cattle diets. Thus, treatments were as follows: control without algae and 1, 5, and 10% of algae inclusion, substituting the corn silage and cotton gin trash mixture (Table 3).

*Rumen fluid collection and in vitro incubations*

*In vitro* incubations were conducted on three separate days (replicates). Ruminal fluid, collected from a representative sample of digesta, was strained through four layers of cheesecloth, placed in pre-warmed thermos containers, and transported to the laboratory within 30 min of collection. In the laboratory, ruminal fluid was maintained under constant $CO_2$ flux, combined in equal proportions from the donor steers, and then mixed with McDougall buffer to a 1:4 ratio of rumen fluid to buffer (i.e. inoculum).

Treatments were weighed in duplicate into Ankom bags (0.70 g), heat-sealed, and placed in a 125-mL serum bottle following the procedure described by Amaro *et al.* (2021) with modifications. Thus, two bottles per treatment were incubated on each incubation day. Briefly, inoculum (50 mL) was added to each bottle, including two bottles without substrate (blanks) under constant $CO_2$ flux. Bottles were fitted with a butyl stopper, crimp sealed, and placed in an incubator for 24 h at 39 °C, set at 60 rpm. At the end of incubation, before removing the stopper, the final gas pressure was recorded using a manual transducer (Digital Test Gauge, Ashcroft Inc., Stratford, CT, USA). A subsample of gas was collected from the bottle headspace to determine methane concentration and was stored in vacuum vials for further $CH_4$ analysis. After removing the stopper, the final pH of the fermentation fluid was recorded. Two 10-mL subsamples were collected and acidified by adding 100 µL of a 20% (vol/vol) $H_2SO_4$ solution and frozen at -20 °C until further analyses. Ankom bags were removed from the bottles, washed with tap water until the effluent was clear, dried in a forced-air oven set at 60 °C for 48 h, and reserved until further analysis.

*Laboratory analysis*

The concentration of VFA in ruminal fluid samples was determined in a liquid-liquid solvent extraction using ethyl acetate (Ruiz-Moreno *et al.*, 2015). Samples were centrifuged for 15 min at 10,000×*g*. Ruminal fluid supernatant was mixed with a meta-phosphoric acid (25% wt/vol):crotonic acid (2 g/L, internal standard) solution at a 5:1 ratio, and samples were frozen overnight, thawed, and centrifuged for 10 min at 10,000×*g*. The supernatant was transferred into glass tubes (12×75 mm; Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA, USA) and mixed with ethyl acetate in a 2:1 ratio of ethyl acetate to the supernatant. After shaking tubes vigorously and allowing the fractions to separate, the ethyl acetate fraction (top layer) was transferred to 9 mm-vials (Fisherbrand; Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples were analyzed by gas chromatography (Agilent 7820A GC, Agilent Technologies, Palo Alto, CA, USA) using a flame ionization detector and a capillary column (CP-WAX 58 FFAP 25 m×0.53 mm, Varian CP7767, Varian Analytical Instruments, Walnut Creek, CA, USA). The column temperature was maintained at 110 °C, and injector and detector temperatures were 200 and 220 °C, respectively.

The concentration of ruminal ammonia nitrogen ($NH_3$-N) was analyzed after centrifuging ruminal fluid samples at 10,000×*g* for 15 min at 4 °C (Avanti J-E, Beckman Coulter Inc., Palo Alto, CA, USA) following the phenol-hypochlorite technique described by Broderick and Kang (1980) with the following modification: absorbance was read on 200 µL samples at $OD_{620}$ in flat-bottom 96-well plates (Corning Costar 3361, Thermo Fisher Scientific Inc., Waltham, MA, USA) using a plate reader (Fisherbrand UV/VIS AccuSkan GO Spectrophotometer, Thermo Fisher Scientific Inc., Hampton, NH, USA).

Dried Ankom bags were ashed at 550 °C for 6 h to determine the undigested organic matter on the remaining fermentation residue. Thus, the *in vitro* organic matter digestibility (IVOMD) was calculated as shown:

IVOMD (%)=[(incubated organic matter – residual organic matter)/incubated organic matter]×100

A gas subsample was analyzed to measure $CH_4$ concentration by gas chromatography (Agilent 7820A GC; Agilent Technologies, Palo Alto, CA, USA). A flame ionization detector was used with a capillary column (Plot Fused Silica 25m × 0.32mm, Coating Molsieve 5A, Varian CP7536; Varian Inc. Lake Forest, CA, USA). Injector, column, and detector temperatures were 80, 160, and 200 °C, respectively. Injector pressure was 20 psi with a total flow of 191.58 mL/min and a split flow of 185.52 mL/min with a 100:1 split ratio. Column pressure was 20 psi with a flow of 1.8552 mL/min. The detector makeup flow was 21.1 mL/min. The carrier gas was $N_2$, and the run time was 3 min.

*Statistical analysis*

Data were analyzed as a randomized complete block design with three replicates (blocks) using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Bottles were considered the experimental unit, and the model included the fixed effects of algae inclusion and the random effect of incubation day (replicate). Means were compared using orthogonal polynomial contrasts. Significance was declared at $p \leq 0.05$.

## Results

The inclusion of *Chlorella* spp. algae did not affect ($p>0.05$) final pH, concentration, or proportion of VFA, acetate to propionate ratio, the concentration of $NH_3$-N, or total gas and $CH_4$ production (Table 4). However, increasing the inclusion of algae linearly increased ($p<0.01$) the IVOMD.

## Discussion

The chemical composition of algae exhibited a reduced concentration of NDF and ADF (13.6 and 7.9%, respectively) and a greater concentration of ash (51%) compared with a forage as corn silage (Table 1). A decrease in dietary fiber content typically results in enhanced

ruminal fermentation. Additionally, previous studies have reported variable concentration of ash in *Chlorella* spp. related to growing condition and cultivation process modifying the organic matter concentration (Drewery *et al.*, 2014; Wild *et al.*, 2019a). In the present experiment, the decrease in organic matter concentration due to increasing algae inclusion may have limited the amount of potentially fermentable material available (Lodge-Ivey *et al.*, 2014) (Table 3), resulting in a similar content of non-structural carbohydrates among treatments (between 40 and 44%) (Table 3), thus potentially explaining the similar fermentation and lack of differences in final pH and concentration of VFA. Additionally, it is expected that comparable fermentation conditions should not alter the microbial population or diversity and subsequent proportion of VFA produced (Murphy *et al.*, 1982), as was observed in similar studies where green micro-algae inclusion resulted in little changes in the concentration of VFA and the microbial community on in vivo conditions (Drewery *et al.*, 2014; McCann *et al.*, 2014).

An increase in the concentration of $NH_3$-N with algae supplementation was observed in an *in vitro* fermentation study from Kianai *et al.* (2020); however, the concentration of $NH_3$-N was not affected by increasing algae inclusion in the present study (Table 4). A similar concentration of $NH_3$-N among treatments may be explained by decreased protein hydrolysis or increased $NH_3$-N utilization by rumen microorganisms (Sniffen *et al.*, 1992). Algae protein has displayed greater resistance to hydrolysis by rumen microorganisms than plant protein (Lodge-Ivey *et al.*, 2014). The structure and accessibility of algae protein or the interrelation with other chemical compounds as phenols may limit proteolysis during fermentation, resulting in decreased concentration of $NH_3$-N and possibly increased the amount of rumen undegradable protein (Lamminen *et al.*, 2019; Wild *et al.*, 2019b). In this regard, Drewery *et al.* (2014) suggested that algae inclusion may reduce the concentration of $NH_3$-N in rumen fluid, increase the non-ammonia nitrogen flow to the intestine, and reduce the excretion of N in the urine, suggesting lower protein fermentability in the rumen.

**Table 4.** Increasing proportion (%) of algae on fluid fermentation pH, the concentrations of volatile fatty acids (VFA) and ammonia-N, *in vitro* organic matter digestibility, and gas and methane production in a corn silage-base diet.

| Variable[1] | Algae inclusion (%) | | | | SEM | P–value[2] | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 5 | 10 | | Linear | Quadratic | Cubic |
| pH | 6.51 | 6.58 | 6.58 | 6.53 | 0.049 | 0.926 | 0.255 | 0.352 |
| Total fatty acids, m*M* | 28.36 | 25.89 | 28.96 | 26.82 | 2.844 | 0.919 | 0.509 | 0.201 |
| Acetate, m*M* | 14.75 | 13.87 | 15.10 | 14.25 | 1.364 | 0.996 | 0.524 | 0.255 |
| Propionate, m*M* | 8.89 | 7.85 | 8.78 | 8.12 | 0.920 | 0.774 | 0.644 | 0.176 |
| Butyrate, m*M* | 3.51 | 3.02 | 3.74 | 3.27 | 0.593 | 0.904 | 0.385 | 0.166 |
| Acetate, mol/100 mol | 52.01 | 53.61 | 52.69 | 53.18 | 1.038 | 0.617 | 0.885 | 0.164 |
| Propionate, mol/100 mol | 31.06 | 30.61 | 30.06 | 30.42 | 1.757 | 0.128 | 0.066 | 0.674 |
| Butyrate, mol/100 mol | 12.29 | 11.37 | 12.64 | 12.05 | 1.134 | 0.698 | 0.518 | 0.172 |
| Acetate:Propionate | 1.69 | 1.76 | 1.78 | 1.76 | 0.132 | 0.396 | 0.297 | 0.390 |
| $NH_3$-N, m*M* | 5.34 | 5.11 | 4.86 | 4.34 | 0.829 | 0.146 | 0.983 | 0.821 |
| IVOMD, % | 67.80 | 67.78 | 68.85 | 69.59 | 0.990 | 0.002 | 0.688 | 0.518 |
| Gas production, mL/g $OM_d$ | 116.4 | 100.7 | 123.9 | 98.3 | 13.63 | 0.339 | 0.202 | 0.101 |
| Methane production, m*M*/g $OM_d$ | 1.02 | 0.84 | 1.05 | 0.90 | 0.272 | 0.827 | 0.408 | 0.093 |

[1]IVOMD: *In vitro* organic matter digestibility; OMd: Organic matter degraded. [2]Probability of the linear, quadratic, or cubic effect of increasing the level of algae. SEM: Standard error of the mean; $NH_3$-N: Ammonia nitrogen; IVOMD: *In vitro* organic matter digestibility; $OM_d$: Organic matter degraded.

Conversely, rumen microbes can utilize $NH_3$-N or preformed amino acids to synthesize microbial protein (Sniffen *et al.*, 1992). The amino acid profile of algae demonstrated a greater concentration of limiting amino acids (Table 2); however, the amino acid profile shows a lesser biological value than other feed proteins (Becker, 2007), although it is necessary to determine the degradability and passage of this algae protein in further studies. Novel protein sources should provide an adequate amino acid profile according to diet characteristics and animal requirements. Thus, algae could increase the synthesis of rumen microorganisms providing limiting amino acids and maintaining a similar $NH_3$-N concentration after 24 h of fermentation. Nevertheless, this hypothesis should be tested in future experiments.

In this study, increasing inclusion levels of algae linearly increased organic matter digestibility (Table 4). Organic matter digestibility does not account for mineral concentration in the substrate and residue; therefore, increasing organic matter digestibility implies that the organic fraction of *Chlorella* spp. has greater digestibility than the corn-silage and gin trash mixture. Increasing digestibility should result in greater VFA concentration, gas production, or microbial synthesis (Dijkstra *et al.*, 2005). In this experiment, the algae inclusion did not affect the concentration and proportion of VFA and gas production (Table 4). Thus, increasing the synthesis of microbes could explain the greater IVOMD maintaining similar VFA concentration among different levels of algae supplementation.

Algae inclusion did not modify gas and $CH_4$ production in this experiment (Table 4). Greater gas production is associated with greater digestibility when algae from freshwater are incubated (Dubois *et al.*, 2013). However, reduced fermentation of the *Chlorella* spp. relative to other freshwater algae (Lamminen *et al.*, 2019; McCauley *et al.*, 2020) and the potential increase in non-ammonia nitrogen when *Chlorella* was supplemented (Drewery *et al.*, 2014) precluded the possibility of recognizing differences in gas production. In addition, $CH_4$ is produced according to the $H_2$ dynamic during fermentation (Janssen, 2010; Ungerfeld, 2015). In this experiment, the concentration and proportion of VFA did not differ among algae inclusion; therefore, it was not expected to observe differences in $CH_4$ production (Table 4). Production of $CH_4$ is reduced when $H_2$ is redirected to reduced products (e.g. propionate or microbial synthesis) instead of methanogenesis (Ungerfeld, 2015) or due to the presence of secondary compounds that may affect $CH_4$ synthesis as bromoform or phlorotannin (Machado *et al.*, 2016; Abbott *et al.*, 2020). Green microalgae show no secondary compounds that could affect methanogenesis (Abbott *et al.*, 2020); however, microalgae have shown contradictory results on $CH_4$ production (Fievez *et al.*, 2007; Kiani *et al.*, 2020). The greater concentration of polyunsaturated fatty acids (e.g. DHA) explained the $CH_4$ reduction reported by Fievez *et al.* (2007) but no by Kiani *et al.* (2020). Polyunsaturated fatty acids can capture $H_2$ during biohydrogenation in the rumen and show an antimicrobial effect on the rumen microbial population modifying the fermentation profile and reducing $CH_4$ production (Johnson and Johnson, 1995; Beauchemin *et al.*, 2009). Other algae strains or different cultivation, harvesting, and processing practices may increase the antimethanogenic compounds of green microalgae (Wild *et al.*, 2019a; Kiani *et al.*, 2020).

In conclusion, including green microalgae from *Chlorella* spp. in a corn silage-based diet does not significantly modify *in vitro* fermentation profile and $CH_4$ production. Future research could evaluate the effect of algae inclusion on rumen undegradable protein and microbial synthesis and their technical and economic inclusion for ruminants in cow-calf and backgrounding operations.

## Declarations

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This study was funded by the North Florida Research and Education Center of the University of Florida.

*Conflict of interest*

The authors declare they have no conflicts of interest regarding the work presented in this report.

*Author contributions*

JV, ND: conceptualization and experimental design. JV, FT, AM, IFM, FP: laboratory procedures and data collection. TMS, ND: project administration. JV, FT: wrote the document. All authors have read and agreed to the published version of the manuscript.

*Use of artificial intelligence (AI)*

No AI or AI-assisted technologies were used during the preparation of this work.

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