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Effects of Caffeine Doses on Rumen Fluid Fermentation Profile and Nutrient Digestibility when Fed Lactating Cow Diet on Continuous Cultures Fermentation

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EFFECTS OF CAFFEINE DOSES ON RUMEN FLUID FERMENTATION PROFILE AND NUTRIENT DIGESTIBILITY WHEN FED LACTATING COW DIET ON CONTINUOUS CULTURES FERMENTATION

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ABSTRACT

Caffeine is commonly known as a potent bioactive compound. This molecule plays an essential role in plants' defense. Because of its antimicrobial activity, caffeine has been cataloged as a toxic component for microbes by targeting DNA repair mechanisms and suppressing microbial proliferation. Nowadays, the caffeine action to enhance mixed microbial cultures digestion has captured the attention of researchers.

In ruminants, the inhibition of ruminal methanogenesis has been deeply studied to enhance rumen fermentation to increment metabolizable energy. In dairy cows, methane production represents a loss of 6.0 ± 1.18% of the energy intake (Niu et al., 2018), reducing the metabolizable energy produced from the total feed energy intake. However, the action of other plant secondary metabolites have been shown to enhance rumen fermentation, nitrogen metabolism, decrease methane production, and improve animal productivity and health (Kamra et al., 2006; Rochfort et al., 2008). But the diversity of the composition of these molecules in nature has challenged the determination of which component would be better as an additive to enhance rumen fermentation. However, the same caffeine molecule has been found in a diversity of plants due to convergent evolution. Caffeine may be a potential alternative additive in ruminants due to its natural bioactivity and composition.

The present thesis determined the effects of caffeine doses on rumen fermentation profile and nutrient digestibility when dual continuous culture fermenters were fed with lactating cows' diet. Caffeine has been shown to decrease DM, OM, and starch digestibility linearly, while A:P ratios tended to decrease linearly.

DEDICATION

I dedicate this thesis to my parents Rubén Toledo and Luz Villafañe-Morales, and my husband, Vladimir Villanueva-López. This achievement is a result of the effort of my parents to bring me the best education they could and the unlimited support of my husband.

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CHAPTER ONE

CAFFEINE AS A POTENTIAL ADDITIVE TO ENHANCE RUMEN FERMENTATION

Introduction

Caffeine (1,3,7-trimethylxanthine) is a purine alkaloid molecule that occurs naturally in some plant seeds, nuts, or leaves, which acts as a natural pest repellent and insecticide (Nathanson, 1984; Mathavan et al., 1985; Hollingsworth et al., 2003), bactericide (Kim and Sano, 2008; Sledz et al., 2015) and even attractant for pollination (Wright et al., 2013). Due to convergent evolution, the same caffeine molecule is produced in several plants, such as cacao, citrus, guarana, coffee, and tea lineages (Huang et al., 2016). Caffeine is considered a secondary plant metabolite. Plant secondary metabolites are compounds that are not part of the biochemical metabolism of plant growth and reproduction (Patra and Saxena, 2010).

By-products from the coffee and cocoa industry (with caffeine contents) have been tested on batch co-digestion techniques for the microbial activity enhancement of rumen fluid and cow manure. Increments in bacterial growth, feed digestion, and biogas production has been reported in the co-digestion of rumen fluid or cow manure with by-products from the coffee and cocoa industry (Corro et al., 2013; Rico et al., 2014). The co-digestion of the soluble fraction of spent coffee ground and cow manure showed a reduction of bacterial lag time in co-digestion (9 to 10 days) in comparison to control (10 to 12 days) (Luz et al., 2017). The easier hydrolysis of the soluble fraction of spent coffee ground in co-digestion leads to an increment of volatile fatty acid concentration, lower pH, and, consequently, a faster digestion process (Luz et al., 2017). On the other hand, cocoa

shells have shown limited anaerobic digestion due to the low hydrolysis rate (Rico et al., 2014). However, co-digestion with 200 g of a substrate of cocoa shells (at 5% and 10% of the total substrate) and cow manure produced higher amounts of cumulated methane than cow manure (200 g) digestion alone (Rico et al., 2014). It is known that coca by-products are rich in fiber, cocoa shells have 60.54% of dietary fiber on dry matter basis, and 10.09% (dry matter basis) consist of soluble fiber (Lecumberri et al., 2007). It is known that high fiber content on substrates increases methane production on fermentation process.

The caffeine activity in these studies has been briefly described as a toxic component that may increase the microbial lag phase (Corro et al., 2013; Widjaja et al., 2017). Previous research has cataloged caffeine, tannins, and other polyphenols in coffee pulp as anti-nutritional components that may cause low feed intake, protein digestibility, and nitrogen retention when the coffee pulp is used as animal feed (Pandey et al., 2000). Despite the previous caffeine description as a toxic component of by-products used as animal feed, Prabhudessai et al. (2009) suggested that caffeine may increase biogas production by potentially increasing microbial activity; this is the first time that pure caffeine has been tested in anaerobic fermentation.

In ruminants, the inhibition of rumen methanogenesis has been deeply studied to enhance rumen fermentation and increment metabolizable energy, and, at the same time, decrease greenhouse gas emissions (Patra and Saxena, 2010). In dairy cows, methane production represents a loss of 6.0 ± 1.18% of the energy intake (Niu et al., 2018), reducing the metabolizable energy produced from the total feed energy intake. In terms of environmental concerns, methane production from enteric fermentation represents about 12% of the total methane released into the atmosphere (Moss et al., 2000).

Chemical feed additives have been developed to improve cattle feed efficiency while at the same time decreasing methane production. Ionophores are commercial feed additives that promote growth and feed efficiency in cattle (Novilla, 2012). Ionophores directly affect gram-positive bacteria that produce lactate, acetate, and butyrate in the rumen, consequently, bacteria that produce propionate increase (Novilla, 2012). Ionophores promote the decrease of methanogenesis by shifting the bacterial population and shifting fermentation pathways to propionate production, but not by a direct effect on methanogenic bacteria (Moss et al., 2000). However, due to a possible microbial adaptation to ionophores, inconsistency in methane reduction data and the possible development of animal toxicity have driven the research on natural products as feed additives (Russell and Houlihan, 2003; Guan et al., 2006; Novilla, 2012).

Plants produce diverse secondary metabolites as part of their defense, acting as antimicrobial and insecticides (Patra and Saxena, 2010). Plant secondary metabolites such as saponins and essential oils have been shown to change rumen microbial profile (Wallace, 2004; Patra and Saxena, 2009a). Enhancing rumen fermentation, nitrogen metabolism, and decreasing methane production improve animal productivity and health (Wallace, 2004; Kamra et al., 2006; Rochfort et al., 2008). However, the high diversity of essential oils and saponins composition challenges determining which component would be better as an additive to enhance rumen fermentation.

Caffeine is commonly known as a metabolic stimulant. In anaerobic fermentation seems to increase microbial activity, increasing biogas production from anaerobic fermentation (Prabhudessai et al., 2009). Caffeine may inhibit bacterial proliferation by interacting with DNA repair mechanisms, such as the SOS response pathway (Whitney and

Weir, 2015). More specifically, caffeine may interact with the UmuC gene which is regulated by the DNA repair pathway (SOS), producing persistent damage in sensitive bacterial cells (Whitney and Weir, 2015).

This literature review aims to report the caffeine interaction with microorganisms, effects of anaerobic fermentation, mode of action, and potential in-vivo impacts to evaluate caffeine's potential use as an additive to enhance rumen fermentation.

Caffeine

a. Description

Caffeine (1,3,7-trimethylxanthine) is an alkaloid compound derived from adenine and guanine purines in plants (Ashihara and Crozier, 1999; Ashihara et al., 2017) and is considered a secondary plant metabolite. Plant secondary metabolites are compounds that are not part of the biochemical metabolism of plant growth and reproduction and play an essential role in plants' defense (Patra and Saxena, 2010). Nearly 98% of caffeine is consumed in caffeinated beverages (Lieberman et al., 2019); while it is also found in many foods, dietary supplements, and drugs, commonly known for its capacity to act as an adenosine receptor antagonist with psychotropic and anti-inflammatory activities (Daly, 2007). Sources of habitual caffeine intake fluctuate culturally. Caffeine-containing beverages preferred in African and Asian countries are tea and soda, while in Europe, North America, Latin America, and the Caribbean are coffee and soda (Reyes and Cornelis, 2018). Daily caffeine consumption per capita in

adults from the United States is close to 186 mg/d and has been stable during the last ten years (Lieberman et al., 2019). However, normal caffeine consumption levels in the United States differ on ethnicity, age, sex, smoking status, caloric intake, and alcohol intake (Lieberman et al., 2019).

Caffeine consumption increases by its popularity as a psychostimulant to improve cognitive performance and alertness (Hewlett and Smith, 2007; Hogervorst et al., 2008). The increment in job demand and early morning drivers has increased coffee consumption, one of the most popular caffeinated beverages globally (Steptoe and Wardle, 1999; Reyner and Horne, 2000). Previous research also demonstrated that early morning coffee consumption improves encoded information's memory ability and increases blood pressure and pulse rate (Smith et al., 1999). However, a recent report evidenced that while caffeine intake is positively associated with weekly work hours and is most likely to be consumed during morning hours (from 6:00 am to 9:00 am), employment status and occupation category were not associated (Lieberman et al., 2019). Nowadays, it is known that the caffeine chemical structure is similar to adenosine (Huang et al., 2005), acting as an antagonist to adenosine receptors (A_1 and A_{2A} receptors) in the brain, inhibiting the action of adenosine (Fisone et al., 2004) and promoting alertness (Huang et al., 2005). Due to its action as an adenosine receptor antagonist, caffeine enhances locomotor activity in experimental animals like mice (Yacoubi et al., 2000) and delays fatigue during exercise (Davis et al., 2003). Behavioral effects in humans have been reported, where caffeine in some individuals can induce anxiety, develop dependence, or improve memory and cognition (Smith, 2002).

The unique properties of caffeine as a metabolic stimulant have captured some researchers' attention, who have studied for the first time, the action of this molecule as an additive to enhance the anaerobic fermentation of food waste (Prabhudessai et al., 2009). Prabhudessai et al. (2009) reported a 16% increment of biogas production after adding 100 ppm of caffeine in batch anaerobic digestion with a mixture of food waste and cow manure inoculum (80:20 mix ratio; 8% of total solids contents). Finally, they suggested that caffeine may increase biogas production by potentially increasing microbial activity (Prabhudessai et al., 2009).

Caffeine can be utilized by aerobic and anaerobic microorganisms as a carbon and nitrogen source, present in its heterocyclic structure (Gummadi et al., 2009, 2012; Ceja-Navarro et al., 2015; Chen et al., 2018). However, caffeine has shown antibacterial properties (Ramanaviciene et al., 2003; Ibrahim et al., 2006; Kim and Sano, 2008; Sledz et al., 2015; Chakraborty et al., 2020). Despite the antibacterial effects, methylxanthines like caffeine are degraded by some groups of bacteria and fungi in simpler molecules that act as nucleotide precursors (Gummadi et al., 2012). Initial studies on microbial caffeine degradation were reported in fungal strains like *Penicillium roqueforti* and Stemphylium sp., bacterial strains of *Bacillus coagulans* (Schwimmer et al., 1971), others Penicillium sp. and Aspergillus sp. (Roussos et al., 1995; Hakil et al., 1998; Brand et al., 2002).

Recent research has successfully demonstrated that degradation of the coffee ground by different fermentation techniques increases biogas production (Qiao et al., 2013; Li et al., 2015; Kim et al., 2017). However, the caffeine behavior during fermentation was not considered until 2018, when the complete caffeine degradation

by methanogenesis in-vitro was done to evaluate its methane potential and kinetic behavior (Chen et al., 2018). The volume of methane produced during anaerobic digestion from an initial substrate is generally defined as a methane potential. Anaerobic microorganisms from a sludge inoculum in a bioreactor could completely degrade caffeine to produce methane in concentrations of 500 mg/L, 1000 mg/L, and 2000 mg/L, while methanogenesis was inhibited in concentrations above 4000 mg/L may be due to antibacterial effects (Chen et al., 2018). Caffeine concentrations of 500 mg/L, 1000 mg/L, and 2000 mg/L resulted in a methane conversion potential of 99.9%, 99.6% and 100% respectively (Chen et al., 2018).

After the caffeine degradation behavior described by Chen et al. (2018) and the significant findings as a stimulant molecule to enhance fermentation by Prabhudessai et al. (2009), caffeine could represent a potential additive to enhance ruminant fermentation.

b. Mode of action

The major caffeine biosynthesis pathway is a four-step sequence with the initial substrate xanthosine followed by three methylations and one nucleosidase reaction (Ashihara et al., 2017). However, caffeine is not part of the biochemical process needed for plant growth and reproduction; it is considered a secondary metabolite produced by plant metabolism (Patra and Saxena, 2010). Most of the secondary metabolites present in plants play an important role in plant defense (Patra and Saxena, 2010). The presence of caffeine in plants also has been reported as part of plant defense, acting as a natural

pesticide, pest repellent, and antibacterial action (Hollingsworth et al., 2003; Kim and Sano, 2008; Kim et al., 2011; Ceja-Navarro et al., 2015; Sledz et al., 2015).

Leaves from wild tobacco type (WT) and transgenic lines for caffeine production were inoculated with *Pseudomonas syringae* pv. *Glycinea,* resulting in severe necrosis from 24 h to 48 h after inoculation in WT leaves, while transgenic lines inhibit the infection (Kim and Sano, 2008). During the experiment, caffeine also shows a direct impact against *Pseudomonas syringae* pv. *Glycinea* in cultures on medium with caffeine concentrations higher than two mM (Kim and Sano, 2008). Sledz et al. (2015) also demonstrated that at a higher caffeine concentration (5 mM), plants pathogens were affected after the solution exposure, suppressing the DNA replication for *Dickeya solani, Ralstonia solanacearum,* and *Pectobacterium atrosepticum* and reducing RNA synthesis for *Pectobacterium carotovorum* subsp. *carotovorum, Pseudomonas syringae* pv. *Tomato and Xanthomonas campestris* subsp. *campestris.* The antibacterial property of caffeine has not been shown just in plant-bacteria; it has also been demonstrated against environmental bacteria such as *E*scherichia *coli, Pseudomonas fluorescens, and Pseudomonas aeruginosa* (Ramanaviciene et al., 2003; Ibrahim et al., 2006; Chakraborty et al., 2020). Previous data suggest that caffeine's antibacterial effects on *E. coli* are due to its inhibition of DNA synthesis resulting from 47% of inhibition of thymidine kinase, avoiding incorporating the nucleoside thymidine into the DNA (Sandlie et al., 1980). Also, it inhibits uridine and leucine uptake, damaging the RNA synthesis and protein synthesis (Putrament et al., 1972). Additional data demonstrate that an increment of caffeine concentration (from 0 µg/mL to 80 µg/mL) inhibits swarming motility, quorum sensing between the organism, and secretion of

virulence factors (protease and pyocyanin) of *Pseudomonas aeruginosa,* resulting in a reduced biofilm formation ability (Chakraborty et al., 2020). Treated soil with 1% or 2% of caffeine solution caused poisoning death in almost 100% of slugs (*Veronicella cubensis*), while in a growing medium of orchids with 2% caffeine solution killed 95% of orchid snails (*Zonitoides arboreus*) (Hollingsworth et al., 2003). A caffeine solution of ≥ 0.1% in plant material reduces slug feeding, acting as a repellent (Hollingsworth et al., 2003). Furthermore, other studies reported that the use of transgenic tobacco lines, which produce different levels of caffeine in the leaf tissue (5 µg/g and 0.4 µg/g fresh weight of caffeine), triggers the repellence of cutworm caterpillars (*Spodoptera litura*) (Kim et al., 2006).

Even though it has been shown that caffeine is effective as an insect repellent, pesticide, and antibacterial, there is another pest that is resistant to caffeine, the coffee berry borer (*Hypothenemus hampei*) (Ceja-Navarro et al., 2015). Previous research has concluded that resistance cannot be associated with caffeine action and suggested that the insect has evolved to degrade or excrete the caffeine to avoid its toxic effects (Guerreiro Filho & Mazzafera, 2003). Recently, it has been demonstrated that caffeine degradation in *Hypothenemus hampei* is mediated by the action of its gut microbiota (Ceja-Navarro et al., 2015). The reproductive ability of coffee berry borer with removed gut microorganisms was highly impacted, representing a decrease of 95% in eggs and larvae compared with non-antibiotic treated insects, and no progression to pupa or adult stage during the 44-day experiment (Ceja-Navarro et al., 2015). In this experiment, Ceja-Navarro et al. (2015) identified the ndmA gene expression (coding for the alpha subunit of a caffeine demethylase) in *Pseudomona fulva*; they suggested

that *Pseudomona fulva* is directly involved with gut caffeine degradation in wild populations of *Hypothenemus hampei*.

Additional evidence that some microorganisms are capable of using caffeine as a source of energy, increasing bacterial activity during gut fermentation, and modifying the genus ratios from the total gut microbiome has been reported (Jaquet et al., 2009; Mills et al., 2015; Kleber Silveira et al., 2018; Nishitsuji et al., 2018). Previous data reported that rumen microbial activity, measured by in-vitro gas production, did not decrease after adding a substrate with a concentration of up to 35% of coffee grounds (Campbell et al., 1976). However, when coffee ground concentrations levels were incremented from 35% to 50%, the gas production linearly decreased ($P < 0.05$) (Campbell et al., 1976). Campbell et al. (1976) extracted the coffee grounds by different procedures, where the caffeine concentration was lightly or not affected by extractions. The caffeine concentration reported by each coffee ground extraction was: untreated 0.13 %, hot-water extracted 0.14%, defatted 0.14%, defatted-hot-water extracted 0.15%, defatted with 0.2% NaOH extracted .15% (Campbell et al., 1976). However, this study did not measure the caffeine left after fermentation to determine microbial utilization. The prebiotic action in human fecal microorganisms from coffee by-products has been shown on the in-vitro technique (Borrelli et al., 2004). Prebiotic efficiency from coffee silverskin was determined in batch cultures inoculated with fecal material from three different healthy individuals (Borrelli et al., 2004). In the same study, Borrelli et al. (2004) determined that *bifidobacteria* had a predominant growth, while coliforms' growth was limited and inhibited for *Bacteroides* spp. and clostridia. Later on, studies with caffeine-containing beverages reported alterations in the human

gut microbiome with an increment of bacterial metabolic activity. Jaquet et al., (2009) experimented on sixteen adult human volunteers and received 3 cups of coffee daily for three weeks; fecal samples were collected and analyzed before and after treatments. They reported an increment of *Bifidobacterium* spp. ratio and metabolic activity after 3 three weeks of coffee consumption. The largest increment of *Bifidobacterium* spp. was observed in the individuals with low counts of this group of bacteria in the total microbiome composition before treatment, without a significant effect on the total of microorganism (Jaquet et al., 2009). The other four individuals showed an increment of butyrate-producing bacteria, such as *Lachnospiraceae* and *Roseburia* (Jaquet et al., 2009). Mills et al. (2015) developed an experiment in 24 hours batch culture fermentation. The microbial inoculum was obtained from three human individuals' fecal samples and treated with three different coffee commercial brands with different levels of chlorogenic acid (Mills et al., 2015). They concluded that the intake of coffee increases the *Bifidobacterium* spp. due to chlorogenic acid and other components present in the coffee beverage, such as caffeine (Mills et al., 2015).

Recently, caffeine treatments have impacted the gut microbiome ratio in rat models without changes in diversity. Wistar rats presented an altered microbiota ratio without loss of diversity after 21 days of low caffeine dose (0.0007 g/kg), treatment administrated once a day (Kleber Silveira et al., 2018). In the caffeine-treated group, *Lactobacillus* ratios decreased compared to the control (Kleber Silveira et al., 2018). Also, Tsumara Suzuki obese diabetes (TSOD) mice treated with caffeine presented a decrease in *Prevotella* percentage in the gut microbiome (Nishitsuji et al., 2018).

Changes in bacterial species ratios without losing the total microbiome by adding caffeine to the diet may represent a positive shift in the production of volatile fatty acid ratios and other metabolites in rumen fermentation. Consequently, more energy is available for ruminant animals.

c. By-products with high caffeine content

Coffee and cocoa production are among the most significant industries worldwide that produce high amounts of by-products rich in metabolites such as caffeine and theobromine. During the coffee crop year of 2019 to 2020, approximately 169.34 million bags of 60 kg were produced (International Coffee Organization - What's New), close to 112 million tons of coffee beans. On the other hand, around 4,697 million tons of cocoa beans were produced from 2019 to 2020 (Statistics - International Cocoa Organization). Meanwhile, about 90% of the coffee beans are considered waste (Campos-Vega et al., 2015)**,** and around 70% of the cocoa production is considered farm waste (Oddoye et al., 2013). The major coffee by-products are coffee pulp, coffee husk, silver skin, and coffee spent ground, with a range of caffeine content (dry-weight basis) of 1.5 ± 1.0 %, 1.0 ± 0.5 %, 0.03 ± 0.6 %, and 0.02 ± 0.1 % respectively (Murthy and Madhava Naidu, 2012). The cocoa industry's more significant amounts of waste are the cocoa husk (Oddoye et al., 2013), with a range of caffeine content of 1.59 – 4.21 mg/g^{-1} dry weight (Barbosa-Pereira et al., 2018).

By-products from the coffee and cocoa industries have been studied for ruminant feeding. Researchers have concluded that up to 10.5% of coffee hulls can be

included in the total dry matter of lactating cows' diet (Souza et al., 2005). The same group of researchers determined that coffee hulls could replace up to 12% of the corn silage dry matter in lactating cows' diets (Rocha et al., 2006). A significant increment of acetic acid, butyric acid, and total volatile fatty acids ($P < 0.05$) has been observed when the coffee pulp is added at a 16% level in the sheeps' diet (Salinas-Rios et al., 2015). In Latxa dairy ewes, presented an increment of acetic and butyric acid contents in the rumen, and consequently, an increment of milk yield and composition when spent coffee ground were added at 100 g/kg level in the concentrate (de Otálora et al., 2020). On the other hand, from the cocoa industry, cocoa shells have been shown to be a valuable ingredient for crossbred Anglo-Nubian goats' diets (Aregheore, 2002). While the addition of urea or fungal treated Cocoa pod shell (to decrease lignin content) in Holstein steers diet, represented in an increment of average daily gain ($P < 0.01$), nitrogen utilization ($P < 0.05$) and total volatile fatty acids ($P < 0.01$) (Laconi and Jayanegara, 2015).

The utilization of coffee and cocoa by-products for the microbial activity enhancement of rumen fluid and cow manure has been reported in batch co-digestion techniques. A summary of cumulated biogas and methane production levels of the co-digestions is shown in Table 1.1**Error! Reference source not found.**. Rapid bacterial growth occurred after a month of microbial adaptation in co-digestion of coffee pulp and cow manure, resulting in faster digestion of nutrients and a higher rate of CH_4 production in comparison with controls (Corro et al., 2013). The low rate of digestion of total solids and volatile solids during the first 30 days of co-digestions of coffee pulp with cow manure and rumen fluid has also been reported in other research (Widjaja et

al., 2017). However, the co-digestion of the soluble fraction of spent coffee ground and cow manure showed a reduction of bacterial lag time in co-digestion (9 to 10 days) in comparison to control (10 to 12 days) (Luz et al., 2017). The easier hydrolysis of the soluble fraction of spent coffee ground in co-digestion leads to an increment of volatile fatty acid concentration, lower pH, and, consequently, a faster digestion process (Luz et al., 2017). Interestingly, the major VFA generated as the primary substrate for CH_4 formation was acetate for co-digestions of coffee pulp with cow manure, rumen fluid, and both. (Widjaja et al., 2017). On the other hand, cocoa shells have shown limited anaerobic digestion due to the low hydrolysis rate (Rico et al., 2014). However, cocoa shells and cow manure co-digestion produced higher amounts of cumulated methane than cow manure digestion alone, as shown in **Error! Reference source not found.** (Rico et al., 2014). Recently, biogas production of cocoa pod husk in semi-continuous digestion with an inoculum of pre-digested cow manure was not inhibited under the organic loading rate (300 ml/day) and hydraulic retention time (10 days) conditions (Darwin et al., 2018).

Two of the reviewed studies of the caffeine-containing by-products in co-digestion with ruminant microorganisms have mentioned caffeine and described it as a toxic component that may increase the microbial lag phase (Corro et al., 2013; Widjaja et al., 2017). Accordingly, with Corro et al. (2013), caffeine, free phenols, and tannins present in coffee pulp could cause a low conversion of total solids and volatile solid and no methane production during the first month of coffee pulp with cow-manure digestion. However, biogas production and methane volume were higher after the adaptation period than in controls, as shown in **Error! Reference source not found.**

(Corro et al., 2013). Widjaja et al. (2017) described similar results and conclusions when digestion rate was limited during the first 30 days for co-digestions of coffee pulp with cow manure, rumen fluid, and both.

Previous research has cataloged caffeine, tannins, and other polyphenols in coffee pulp as anti-nutritional components that may cause low feed intake, protein digestibility, and nitrogen retention when the coffee pulp is used as animal feed (Pandey et al., 2000). However, despite the previous caffeine description as a toxic component of by-products used as animal feed, Prabhudessai et al. (2009) have cataloged caffeine as a microbial stimulant, suggesting its potential as a microbial fermentation enhancer. Recently, the caffeine microbial degradation kinetic has been described (Chen et al., 2018). The results reported by Prabhudessai et al. (2009) and Chen et al. (2018) are the first evidence available about caffeine's effects on anaerobic fermentation.

d. Extraction and purification of caffeine

Polar organic solvents are the most common method to extract caffeine from coffee beans and their by-products (Belščak-Cvitanović and Komes, 2017). Caffeine is soluble in different solvents such as chloroform, dichloromethane, acetone, ethyl acetate, water, methanol, ethanol, and carbon tetrachloride, where caffeine has shown to be more soluble with chloroform (Shalmashi and Golmohammad, 2010). Caffeine can be obtained in polyphenolic-containing extracts using solid-liquid solvent extraction assisted with water or ethanol as solvents (Belščak-Cvitanović and Komes, 2017). Occasionally, to extract higher amounts of caffeine, solid-liquid solvent extraction needs to be followed by liquid-liquid extraction. The resulting liquid mixture from the solid-liquid solvent extraction using spent coffee ground with water was filtered and extracted with the second step of liquid-liquid extraction of chloroform: isopropanol (3:1) (Sousa et al., 2015). Another study used the filtrated liquid from green coffee beans and spent coffee ground extracted with distilled water and magnesium oxide to perform a subsequent extraction with chloroform (Ramalakshmi et al., 2009).

Other nontoxic extractions alternatives are needed due to the residuals of the organic solvents in the habitually solid-liquid and liquid-liquid extractions (Belščak-Cvitanović and Komes, 2017). Supercritical fluid extraction using carbon dioxide as a solvent is one of the recommended methods (Belščak-Cvitanović and Komes, 2017).

The caffeine solubility in this technique strongly depends on the extraction conditions. Variation of the temperature and pressure parameters affects the caffeine solubility using Supercritical carbon dioxide for caffeine recovery (Azevedo et al., 2008).

Microwave-assisted extraction is a relatively new technique that allows extracting higher and faster caffeine concentrations from solids than conventional methods with nontoxic solvents (water, citric acid-water or ethanol-water) (Serdar et al., 2017). Microwave-assisted extraction is a method that applies microwave energy, and a solvent recovers specific compounds from different sources (Belščak-Cvitanović and Komes, 2017). Caffeine can be successfully extracted at 180 °C in 10 minutes of microwave-assisted extraction (Lopes et al., 2020).

Caffeine as a fermentation additive

Caffeine has received different description categories in the literature depending on its action and study focus. The most common function of secondary plant metabolite is plant defense (antimicrobial, pesticide, insect repellent, etc.). Despite this description, researchers decided to investigate how some microorganisms are resistant or capable of degrading caffeine. Research in the gut microbiome using the insect (*Hypothenemus hampei*) model shows that some bacteria have evolved to degrade caffeine (Ceja-Navarro et al., 2015). In contrast, caffeine in human and rat models has modified gut microbiome ratios and subsequent metabolites produced from fermentation (increasing acetate and butyric acid) (Borrelli et al., 2004; Jaquet et al., 2009; Kleber Silveira et al., 2018).

The interest in reducing biomass waste and increasing biogas production motivated researchers to investigate the effects of caffeine-containing by-products in fermentation. As previously described, caffeine has been cataloged as a toxic component, which may delay (up to a month) the bacterial lag time from rumen fluid and cow manure (Corro et al., 2013; Widjaja et al., 2017).

Interestingly, caffeine has been cataloged as a microbial stimulant, suggesting its potential as a microbial fermentation enhancer in previous research conducted by Prabhudessai et al. (2009). Recently, the caffeine microbial degradation kinetic has been described (Chen et al., 2018). Understanding the bioactivity background and the biogas production pathway from caffeine degradation increases interest in studying this compound as a potential additive for rumen microbial fermentation.

a. Effect on anaerobic fermentation

Biogas product

The major steps of the biogas generation pathway from biomass degradation are hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Schnürer, 2016). Each successive metabolic reaction involves different groups of microorganisms: hydrolytic-acidogenic bacteria and fungi, syntrophic acetogenic bacteria, and methanogenic archaea (Schnürer, 2016). During the first two steps, polymers of lipids, proteins, carbohydrates, and others are converted into monomers like long-chain fatty acids, glycerol, amino acids, and sugars. Further, in fermentation, monomers are converted

into short-chain fatty acids, alcohols, hydrogen, and carbon dioxide. Syntrophic acetogens use the acids and alcohols to form hydrogen, carbon dioxide and acetate. Finally, Methanogens produce the methane from hydrogen, carbon dioxide and acetate.

Researchers are actively studying compounds that enhance the biogas generation process to decrease biomass waste and increment methane production for biofuels (Paritosh et al., 2020). The production of biogas and methane from coffee and cocoa by-products has been reported; see **Error! Reference source not found.**. However, the effect of caffeine by itself in these studies was not considered. Some researchers just mentioned the compound, concluding that caffeine and other molecules like polyphenols present in the by-products may increase the adaptation period for microbial degradation (Corro et al., 2013; Widjaja et al., 2017).

Prabhudessai et al. (2009) did the first study on the caffeine effect as a stimulant in anaerobic environments and suggested that caffeine may increase biogas production by potentially increasing microbial activity. Adding 100 ppm of caffeine in batch anaerobic digestion with a mixture of food waste and cow manure as inoculum (80:20 mix ratio; 8% of total solids contents) incremented biogas production up to 16% in comparison with the control (Prabhudessai et al., 2009). Later, Chen et al. (2018) measured the caffeine biochemical methane potential and determined the kinetic behavior of caffeine. The complete conversion of caffeine to methane occurs under concentrations of 500, 1000, and 2000 mg caffeine/L requiring just 2.7, 9.1, and 16.8 days respectively (Chen et al., 2018). The short lag phase in concentrations up to 2000 mg caffeine/L reported by Chen et al. (2018) agrees with the data reported by

Prabhudessai et al. (2009) when biogas production started on day two with the addition of caffeine (50, 100, 150 ppm caffeine) while the control (0 ppm caffeine) started at day four. However, concentrations above 4000 mg caffeine/L inhibited methanogenesis; Chen et al. (2018) suggested that this inhibition resulted from the caffeine's anti-bacterial action.

According to Widjaja et al. (2017), acetate was the major VFA generated as the primary substrate for CH_4 formation in co-digestions of coffee pulp with cow manure, rumen fluid, and both. Coffee pulp has an NDF composition of 49.34 ± 2.64 and ADF of 41.91 ± 1.62 (as % of dry matter) (Ameca et al., 2018). A feed with high fiber content increases acetate production during the fermentation process. However, during caffein degradation, volatile fatty acids reached their peak approximately at day eight when an amount of ≤3 mg/L of acetate was found by Chen et al. (2018). In addition, ammonium nitrogen reached its peak approximately on day twelve during the trial of caffeine degradation by Chen et al. (2018). Based on this previous information, Chen et al. (2018) suggested that caffeine degradation occurs in two major steps; first, caffeine is transformed into intermediates, and then the intermediates are converted to methane.

Microorganisms have been shown to degrade caffeine mainly by demethylation. Fungi strains can demethylase caffeine at position seven, leading the theophylline route (Hakil et al., 1998). While bacterias mainly convert caffeine to methylxanthines to xanthine by the action of the demethylase enzyme (Asano et al., 1993). Based on the caffeine degradation under aerobic conditions, Chen et al. (2018) have suggested a

pathway of caffeine catabolism under an anaerobic environment. Three demethylations occur from caffeine to xanthine, where each methyl released is hydrolyzed into methanol, which methanogens will utilize to form methane (see **Error! Reference source not found.**).

Rumen fermentation

Ruminants have a unique digestive system compared to other mammals with monogastric digestion, where microbial fermentation in the rumen compartment is vital to produce animal energy. According to Hungate (1984), ruminants are the most well-known model of the cooperative mammal-microbe relationship. The animal host provides the substrate to ruminal microbes, where polymers of carbohydrates, lipids, and proteins are first hydrolyzed to monomers (Owens and Goetsch, 1993). The first step of rumen digestion of polymers is the extracellular hydrolysis, which is mainly mediated by bacterial enzymes; fungi and protozoa contribute to this process (Wu, 2017).

The two major steps in the rumen regarding extracellular lipid degradation are the first hydrolysis of ester linkages of lipids followed by biohydrogenation of unsaturated fatty acids (Drackley and Bauman, 2006). The metabolic hydrogen used for biohydrogenation is around 1 to 2% (Jenkins, 1993). After microbes hydrogenate free fatty acids, C18:0 and C18:1 isomers are the major fatty acids produced in the rumen (National Research Council, 2001). However, the proportion of the hydrogenation of

trans-11 C18:1 to produce stearic acid (C18:0) depends on the rumen's condition (Jenkins, 1993).

Digestion of proteins is also first mediated by extracellular microbial enzymes. During extracellular proteolysis (proteases, peptidases, and deaminases), proteins from feed, endogenous protein (saliva and epithelial cells), and lysed microbes in the rumen are cleaved into monomers (peptides and amino acids) (National Research Council, 2001). The primary proteolytic bacteria are *Ruminobacter amylophilus*, *Butyrivibrio fibrisolvens, Streptococcus bovis, Selenomonas ruminantium, Megasphaera elsdenii* (Nagaraja, 2016). Amino Acids (a.a.) resulting from extracellular proteolysis are degraded to ammonia (Wu, 2017).

Microbial enzymes also mediate the extracellular hydrolysis of carbohydrates. The most known rumen cellulolytic bacteria are *Ruminococcus albus, Ruminococcus flavefaciens, and Fibrobacter succinogenes* (Weimer et al., 2009). The ruminococcin group has fibrolytic enzymes organized in the cellulosome complex, while Fibrobacter succinogenes contains polysaccharide hydrolases in its cell surface (Ding et al., 2001; Weimer et al., 2009). The cellulolytic enzyme complexes are present on the bacterial surface, therefore is required bacterial adherence to the substrate (Lynd et al., 2002). Consequently, cellulose degradation depends on the surface available to attach (Fields et al., 2000). These enzymes are essential to break down the plant cell wall to release soluble carbohydrates and starch (Van Soest, 1994). All the soluble forms of starch are hydrolyzed to monosaccharides by amylose and amylopectin enzymes (Wu, 2017).

Protozoa may mediate intracellular hydrolysis of polymers to their monomers. Protozoa play an important predatory role in the rumen to control the number of its bacteria (Van Soest, 1994). According to Van Soest (1994), when protozoa predate bacteria, feed particles such as starch granules, chloroplast, or lignified particles are also engulfed; bacteria continuously metabolize polymers after being engulfed.

Most of the monomers cleaved from the dietary feed by hydrolysis are available for microbial utilization and, therefore, energy for the ruminant. The protein fermentation products are ammonia, volatile fatty acids (acetate, propionate, and butyrate), and long-chain fatty acids (iso-butyrate, valerate, isovalerate, and 2-methyl butyrate) (Weimer et al., 2009). Ratios of acetate, butyrate, and propionate in the rumen do not change dramatically with different feed protein levels (Weimer et al., 2009). Lipid degradation occurs first by hydrolysis of triglycerides, and the glycerol released from hydrolysis is rapidly fermented; the long-chain free fatty acids may be reduced or isomerized by rumen microbes (Jenkins, 1993). The major sources of energy for dairy cattle are carbohydrates, consisting of 60% to 70% of the diet's total (National Research Council, 2001). Carbohydrate monomers provide energy for rumen microbes, and products from microbial metabolism serve as energy for the animal host. Rumen fermentation of sugars starts when the monomers of carbohydrates, glucose, and fructose, are utilized by microorganisms to form acids, methane, and carbon dioxide (Lin et al., 1985). The fermentation products are the primary source of carbon and energy for the animal host, and the products are absorbed through the rumen wall to the blood for tissue utilization (Lin et al., 1985).

Anaerobic fermentation is a metabolic process that transforms carbohydrates into organic acids, biogases, and alcohols under anaerobic conditions. The fermentation that occurs in the rumen is an incomplete oxidation in which the final electron acceptors are organic compounds (Ungerfeld, 2020). First, rumen bacteria may convert carbohydrate monomers to pyruvate through glycolysis in the cytosol. Glucose, a six-carbon sugar, is converted to pyruvate mainly via the Embden-Meyerhof pathway and occasionally via the Entner-Duodoroff route, while pentoses and hexoses can enter the Pentose Phosphate pathway (Lin et al., 1985). Depending on the organisms and growth conditions, pyruvate is then fermented to form different end products, with the predominant fermentation products being acetate, propionate, and butyrate (Lin et al., 1985). Acetyl-CoA produced from pyruvate becomes available mainly for the formation of acetate and also enters the synthesis pathways of butyrate and propionate (Wolin, 1979; Lin et al., 1985). Propionate is mainly formed by the Succinate (randomizing) and Acrylate (non-randomizing) pathways from pyruvate fermentation (Lin et al., 1985; Gonzalez-Garcia et al., 2017). The propionate formation via the randomizing pathway happens when the carbon in the second position of pyruvate appears randomly in positions 2 and 3 of succinate, which is then metabolized to propionate in the rumen or is excreted and taken up by succinate consumers (Ungerfeld, 2020).

On the other hand, when propionate is produced via the non-randomized pathway, the carbon from the second position of pyruvate appears in the second position of propionate (Ungerfeld, 2020). Lactate is an intermediate molecule from the non-

randomized pathway but also can be secreted and utilized by other cells to form acetate, propionate, and butyrate (Chen et al., 2019). Production of butyrate involves the reduction of two molecules of acetyl-CoA to acetoacetyl-CoA followed by β-hydroxybutyric-CoA and crotonyl-CoA to butyryl- CoA (Lin et al., 1985; Ungerfeld, 2020).

Rumen fermentation is not 100% efficient when methane, a final product of fermentation, is produced and released into the atmosphere, representing an environmental problem since this molecule is a potent greenhouse gas (Martin et al., 2010; Castillo-González et al., 2014). Hungate (1967) found that H_2 is the main metabolic hydrogen donor for methane formation in rumen fermentation. Nowadays is known that bacterial and archaeal genomes encode enzymes that catalyze H_2, being H_2 transferred and incorporated between the rumen microbes (Greening et al., 2019). Pathways of acetate and butyrate formation release hydrogen that can be up-taken by methanogens to reduce carbon dioxide to methane, while propionate competes with methane as a metabolic hydrogen sink in rumen fermentation (Ungerfeld, 2020). However, alternative pathways that incorporate hydrogen have been described in rumen fermentation. Acetogens (*Blautia*, *Acetitomaculum*), fumarate and nitrate reducers (*Selenomonas*, *Wolinella*), and sulfate reducers (*Desulfovibrio*) have been shown to work as a hydrogen sink in rumen fermentation (Greening et al., 2019).

Methane production and the profile of volatile fatty acids are determined by the kinetics of feed fermentation and passage rate, which impact the hydrogen concentration and consecutively influence the balance between pathways that produce

and incorporate metabolic hydrogen (Ungerfeld, 2020). Traditionally, the inclusion of readily fermentative carbohydrates in the ruminant diet, the increment of feed intake, the processed forage, and the inclusion of unsaturated fat in the diet may improve feed digestibility and reduce the loss of energy released as methane. Ruminants' diets are usually based on forages and grains. Forages have more structural carbohydrates, such as cellulose and hemicellulose, which have a relatively low rate of digestion (Janssen, 2010). On the other hand, grains contain more storage polysaccharides such as sugars, starch, and fructans, which are more rapidly digested in the rumen (Janssen, 2010). Methane production decrease when ruminants are fed with diets containing more proportion of concentrate in the diet than forage (Beauchemin and Mcginn, 2005). The increment of concentrate in the barely based and corn-based diets resulted in a reduction of methane losses by 38% in the barely fed animals and by 64% in the corn fed animals compared to the initial high forage diets fed cattle in an experiment (Beauchemin and Mcginn, 2005). Interestingly, in the same experiment, while methane production decreased, the ratio of volatile fatty acids was changed; decreased acetate and increased propionate proportion (Beauchemin and Mcginn, 2005). Acetate production in the rumen may promote methane formation, while propionate production competes with methanogenesis for the use of hydrogen in the rumen (Moss et al., 2000; Ungerfeld, 2020). Furthermore, methane production decrease when the quality of forages increase (Boadi and Wittenberg, 2002; Warner et al., 2017). Previously has been described that high-quality forages have less proportion of structural carbohydrates and more digestible organic matter content, which may increase the

passage of rumen feed, decreasing the retention time needed for methane formation (Moe and Tyrrell, 1979). The rumen fermentation rate and passage rate increase when the proportion of readily fermentative carbohydrates in the diet increase; therefore, the acetate to propionate ratio changes, decreasing methanogenesis (Getabalew et al., 2019).

Methane production per kg of digestible organic matter intake decrease under high feed intakes conditions, it happens by the increment of passage rate, decreasing the complete oxidation of the main volatile fatty acids (Boadi and Wittenberg, 2002). High rumen passage rates limit the establishment of slow growth microorganisms, such as acetate degrading methanogens and propionate and butyrate degrading syntrophic bacteria (Van Soest, 1994). On the other side, limit-fed ruminants had higher feedstuff digestibility and lower loss of energy excreted as methane or other gases (Zanton and Heinrichs, 2009). When ruminants are limit-fed, the feedstuff's passage rate through the rumen decreases and increases the retention time of nutrients in the rumen; in consequence, microbes have more time to degrade a higher amount of food particles (Zanton and Heinrichs, 2009). However, in another experiment, volatile fatty acids ratios are affected mainly by forage quality and not by the feeding mode (ad-libitum or precision feeding) (Pino et al., 2018).

Treated gains that are easier to digest and the reduction of the particle size of forages promote the increase of feed digestibility rate resulting in the shift of the proportion of volatile fatty acids (Kazemi-Bonchenari et al., 2017). Holstein's calves increased propionate production when barely was finely ground ($P = 0.03$) and

increased acetate production when barely was treated with citric acid (P = 0.04) (Kazemi-Bonchenari et al., 2017). Smaller particle size may allow more microbial access, increase enzymatic activity, increase ruminal fermentation rate, and decrease ruminal pH (Owens et al., 1998). It is well known that the particle size, dry matter, and neutral detergent fiber contents affect the salivary secretion during eating, as more time spent eating will induce more production of saliva, reducing ruminal pH (Beauchemin et al., 2008; Maulfair and Heinrichs, 2013). Ruminal pH will affect the microbiome environment, changing microbial profile, and consequently, ratios of fermentation products will be produced (Van Soest, 1994). Under high concentrate diets, fibrolytic bacteria are negatively affected by the low ruminal pH, and amylolytic bacteria groups may predominate (Tajima et al., 2001). Studies have shown that treating grains with organic acids, such as citric acid, lactic acid, or tannic acid, aids in maintaining a higher pH in the rumen, increasing fiber digestibility, and improving feed efficiency (Deckardt et al., 2016; Kazemi-Bonchenari et al., 2017). In addition, processing forages before ingestion, processes like the forage preservation method, result in less methane than untreated forages. Total methane production decreases more under the alfalfa silage diet compared to alfalfa hay (Benchaar et al., 2001). Also, methane was reduced when alfalfa hay was ground and pelleted (Benchaar et al., 2001)

The intense research in the search to improve ruminal fermentation has led to looking for alternative additives.

Comparison with other additives

The inhibition of rumen methanogenesis has been deeply studied to enhance rumen fermentation and increment metabolizable energy, but also in the context of greenhouse gas emissions (Patra and Saxena, 2010). Chemical feed additives have been developed to improve cattle feed efficiency while decreasing methane production. Ionophores are commercial feed additives that promote growth and feed efficiency in cattle (Novilla, 2012). Normally, propionate increases when microbial fermentation pathways change and methane production decreases (Moss et al., 2000). It is well known that propionate competes with methane as a metabolic hydrogen sink in rumen fermentation (Ungerfeld, 2020). Ionophores directly affect gram-positive bacteria that produce lactate, acetate, and butyrate in the rumen, consequently, gram bacteria that produce propionate increase (Novilla, 2012). Ionophores promote the decrease of methanogenesis by shifting the bacterial population and shifting fermentation pathways to propionate production, but not by a direct effect on methanogenic bacteria (Moss et al., 2000). The mode of action of ionophores is by facilitating the ionic transport across microorganisms' lipid membrane with the formation of cationic complexes (Na^{+}, K^{+}, Ca^{++}) (Novilla, 2012). When increase the osmotic gradient of the cells by the ion imbalance results in a disruption of cell membranes (Novilla, 2012).

Studies have shown that rumen Gram-positive bacteria, which are sensitive to ionophores, can adapt and resist the action of ionophores after a long time of exposure; also, the reduction of methane production was not persistent (Russell and Houlihan, 2003;

Guan et al., 2006). Furthermore, error of ionophore dosages can result in high toxicity for the animal, leading to muscles necrosis (Novilla, 2012). The inconsistent effects of ionophores in bacterial fermentation and the possible development of animal toxicity have driven the research on natural products as feed additives. Plants produce diverse secondary metabolites as part of their defense, acting as antimicrobial and insecticides (Patra and Saxena, 2010). Plant secondary metabolites such as saponins and essential oils have been shown to change rumen microbial profile (Wallace, 2004; Patra and Saxena, 2009a), enhancing rumen fermentation, nitrogen metabolism, and decreasing methane production, while improving animal productivity and health (Wallace, 2004; Kamra et al., 2006; Rochfort et al., 2008).

Essential oils are organic-solvent extracts present in different parts of plants (flowers, leaves, stems, bark, fruit pulps, roots, and seeds) that protect plants against bacterial, fungal, or insect invasion (Kamra et al., 2012). Due to their antimicrobial effects, essential oils are used as a food preservative and in traditional medicine (Kamra et al., 2012). In addition, they acts as stimulant of digestive systems (Hernández et al., 2004).

Essential oils have been shown to change the diversity of archaea in Canadian Arcott ewes rumen without changing the total numbers of these microorganisms and without affecting the rumen's methanogenic capacity (Ohene-Adjei et al., 2008). There is evidence in the literature that essential oils alter the protozoal populations (Cardozo et al., 2006). Also, it is well known that rumen methanogens have a symbiotic relationship with rumen protozoa; consequently, the methanogens population will decrease when rumen protozoa abundance decrease (Ohene-Adjei et al., 2008). There are essential oils such as

thyme, oregano, cinnamon, and garlic or their principal components (thymol, carvacrol, cinnamaldehyde, and allicin, respectively) that have been reported to reduce methane production (Macheboeuf et al., 2008; Patra and Yu, 2012). On the other side, some studies reported in a review paper have shown that essential oils reduced methane production and methanogenic activity by reducing feed digestion (Cobellis et al., 2016). Despite that, most of the studies reported a decrease in rumen protozoa and methanogens, essential oils also reduce cellulolytic bacteria such as *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* (Cobellis et al., 2016). For this reason, finding an essential oil that mitigates methane production without affecting feed digestion represents a challenge (Cobellis et al., 2016). Furthermore, essential oils have been shown to inhibit *Ruminobacter amylophilus* and *Prevotella spp*. (Wallace et al., 2002) which are ammonia producers bacteria in the rumen. When ammonia producers bacteria are inhibited, decrease rumen ammonia and increase undegraded protein available for animal utilization (Patra, 2012).

More research into which essential oil may improve rumen fermentation better without affecting feed digestibility and rumen fermentation is needed. This represents a challenge with the high diversity of essential oils. Factors that impact its composition include plant species, the growth environment of the plants, stage of plant growth, parts of the plants utilized to extract the essential oil, and the extraction method (Cobellis et al., 2016).

Saponins are other secondary plant metabolites that protect plants against bacterial and fungal diseases (Cobellis et al., 2016). Saponins can reduce ammonia and methane

production and increase propionate (Patra and Saxena, 2009a). Previously has been suggested that the increment of rumen propionate production after saponins treatment may be due to rechanneling hydrogen from methane to propionate pathways (Patra and Saxena, 2010). As well as essential oils, saponins may reduce methane production by lowering the protozoal abundance and, consequently, the symbiotic archaea (Patra, 2012). Also, a direct effect of the archaea activity rate of methane production or the activities of methane-producing genes can be affected directly by saponins (Patra and Saxena, 2010; Patra, 2012). Most of the research on saponins has been done to reduce ciliate protozoa, increasing microbial protein synthesis (Patra and Saxena, 2010). However, the saponin's activity results have been inconsistent, and others report a decrease in activity in long-term experimental studies (Jouany and Morgavi, 2007). These inconsistencies may involve the type and dose of saponins used in the experiments, experimental diet, and microbial adaptation to saponins (Patra and Saxena, 2010; Cobellis et al., 2016).

In contrast, caffeine, another secondary plant metabolite, has been studied as a stimulant in an anaerobic environment. Prabhudessai et al. (2009) suggested that caffeine may increase biogas production by potentially increasing microbial activity. Adding 100 ppm of caffeine in batch anaerobic digestion with a mixture of food waste and cow manure as inoculum (80:20 mix ratio; 8% of total solids contents) incremented biogas production up to 16% in comparison with the control (Prabhudessai et al., 2009). In a second study, the caffeine biochemical methane potential and determine the kinetic behavior of caffeine was measured (Chen et al., 2018). The complete conversion of caffeine to methane occurs under concentrations of 500, 1000, and 2000 mg caffeine/L requiring just 2.7, 9.1, and 16.8

days respectively (Chen et al., 2018). The short lag phase in concentrations up to 2000 mg caffeine/L reported by Chen et al. (2018) agrees with the data reported by Prabhudessai et al. (2009) when biogas production started on day two with the addition of caffeine (50, 100, 150 ppm caffeine) while the control (0 ppm caffeine) started at day four. However, concentrations above 4000 mg caffeine/L inhibited methanogenesis; Chen et al. (2018) suggested that this inhibition resulted from the caffeine's anti-bacterial action. Both studies have been completed in batch cultures to measure caffeine's biogas potential and degradation kinetic. Batch cultures can be used as an initial phase of rumen microbial research, but fermentation results in an in-vivo trial may change due to the lack of absorption and outflow simulation. Caffeine has not been studied in in-vivo trials to observe fermentation effects, however, spent coffee ground, a coffee by-product that contains caffeine, has been tested in ruminants. Doses of spent coffee ground (0, 30, 50, and 100 g SCG / kg) were added to the dairy ewes diet to test the effect on rumen microbiota (Goiri et al., 2020). Goiri et al. (2020) reported an increase in phylum *Firmicutes* and *Bacteroidetes* while decreasing the *Proteobacteria* phylum. Also, Goiri et al. (2020) reported an increment of *Butyrivibrio*, *Blautia*, *Fibrobacter*, and *Treponema* genera, accompanied by an increment of acetic and butyric acid in the rumen. After spent coffee ground doses in ewe diets, bacteria from the genus *Prevotela* were inhibited (Goiri et al., 2020). These results are in accordance with Widjaja et al. (2017), who report that the major VFA generated as the primary substrate for CH_4 formation was acetate for co-digestions of coffee pulp (coffee by-product) with cow manure, rumen fluid, and both. However, it is

known that due to the rumen passage rate and absorption of acids, primary volatile fatty acids will not be completely oxidized to methane (Boadi and Wittenberg, 2002).

Additional research about the effects on rumen fermentation when doses of caffeine are added to the diet is needed. Most of the literature available is about co-digestions with coffee by-products with rumen fluid or cow manure (see **Error! Reference source not found.**). Effects of coffee by-products in-vivo have been done by Goiri et al. (2020). Even though caffeine is present in these by-products, further research on pure caffeine effects in rumen fermentation is necessary to determine its potential as a natural additive.

Caffeine as a Bioactive compound

As previously described, caffeine is commonly known as a metabolic stimulant. In anaerobic fermentation seems to increase microbial activity, increasing biogas production from anaerobic fermentation (Prabhudessai et al., 2009). Also, caffeine can be utilized by both aerobic and anaerobic microorganisms as a source of carbon and nitrogen, present in its heterocyclic structure (Gummadi et al., 2009, 2012; Ceja-Navarro et al., 2015; Chen et al., 2018). However, like other plant secondary metabolites, caffeine has shown antibacterial properties (Ramanaviciene et al., 2003; Ibrahim et al., 2006; Kim and Sano, 2008; Sledz et al., 2015; Chakraborty et al., 2020). Caffeine may inhibit bacterial proliferation by interacting with DNA repair mechanisms, such as the SOS response pathway (Whitney and Weir, 2015). SOS response is a regulatory mechanism produced when bacterial cells are exposed to stress conditions and have DNA damage (Pavlin et al.,

2021). This response induces cell mutations and antibiotic resistance (Pavlin et al., 2021). Caffeine interacts with the UmuC gene, regulated by the DNA repair pathway (SOS) (Whitney and Weir, 2015). Whitney and Weir (2015) described that caffeine exposure to *Escherichia coli* caused persistent damage. Also, Whitney and Weir (2015) observed filamentous growth in bacteria cells; this structure appears when cells have DNA damage; they continue proliferating at the same rate but do not fully divide.

The caffeine interaction with cell DNA has mainly been tested in *Escherichia coli* (Sandlie et al., 1983; Selby and Sancar, 1990; Whitney and Weir, 2015). *Escherichia coli* is a Gram-negative bacterium and facultatively anaerobic. Ruminal Gram-negative bacteria are mainly acetate producers, and some are butyrate producers (Van Soest, 1994). Pathways for acetate and butyrate formation release hydrogen that can be up-taken by methanogens to reduce carbon dioxide to methane (Ungerfeld, 2020). If rumen Gram-negative are more sensitive to caffeine exposures than Gram-positive bacteria, it would be possible that caffeine induces a shift in fermentation products, decreasing acetate and butyrate while increasing propionate. In contrast, literature on co-digestion of caffeine-containing by-products with cow manure and rumen fluid reported that the major volatile fatty acid generated was acetate (Widjaja et al., 2017). Additional research about pure caffeine's effects on rumen bacteria and rumen fermentation products is necessary.

Continuous culture fermenters

The literature available about the effects of caffeine in anaerobic fermentation is limited. Literature about the effects of by-products with caffeine content in anaerobic fermentation is available (**Error! Reference source not found.**). However, there are two studies that have tested pure caffeine effects in anaerobic fermentation: first, Prabhudessai et al. (2009), who suggested that caffeine may increase gas production by the potential increment of microbial activity, and second Chen et al. (2018) who measured the caffeine biochemical methane potential and determined the kinetic behavior of caffeine. All this literature available has used batch cultures fermentation techniques. Batch culture is one of the techniques used to grow microorganisms. It is a closed system in which the substrate is added at the beginning of the process. Usually, the operation stops when nutrients become limited, and fermentation products are formed.

When a researcher intends to test a new component's effect on rumen fermentation, continuous cultures systems are an inexpensive alternative technique (compared to in-vivo trials) that simulates the rumen fermentation process better and provides preliminary data (Koch, 2017). In comparison with batch cultures, continuous cultures are fermentation systems that continuously receive a supply of nutrients. The principal advantage of continuous culture systems over batch culture fermentation is the remotion of fermentation end products and maintaining the fermentation process for more time (Hristov et al., 2012). Continuous cultures fermenters also allow the stratification of feed particles, producing

different solid and liquid turnover rates, which mimics the passage rate of the rumen (Teather and Sauer, 1988).

Continuous culture systems might be the fermentation technique that better simulates rumen fermentation, however, there are some weak areas. Under continuous fermentation, bacterial and protozoa communities decrease (Muetzel et al., 2009). Due to these microbial changes, this system has lower volatile fatty acid and acetate concentrations and lower neutral detergent fiber and organic matter digestibility (Hristov et al., 2012). Compared with in-vivo trials, continuous cultures have a lack of absorption, differences in fluid and particle passage rates, and feed intake (per rumen volume) (Hristov et al., 2012). As previously explained, all these factors impact fermentation and feed digestion. However, continuous cultures allow the researchers to obtain preliminary data on nutrient digestibility and the profile of fermentation end products.

Despite the differences between continuous culture and rumen fermentation, it is still a quick, economic, and safe technique to have initial measures for potential new additives before in-vivo trials. It is important to note that additional research on caffeine effects in anaerobic fermentation using continuous cultures is necessary. Caffeine methane potential and caffeine digestion kinetics have been measured. Anaerobic microorganisms from a sludge inoculum in a bioreactor could completely degrade caffeine to produce methane in concentrations of 500 mg/L, 1000 mg/L, and 2000 mg/L, while methanogenesis was inhibited in concentrations above 4000 mg/L may be due to antibacterial effects (Chen et al., 2018). Caffeine concentrations of 500 mg/L, 1000 mg/L, and 2000 mg/L resulted in a methane conversion potential of 99.9%, 99.6% and 100% respectively (Chen et al., 2018).

Analysis of caffeine in continuous cultures with rumen fluid inoculum will bring a better idea of what would happen in rumen fermentation. Data from nutrient digestibility and fermentation products profile will be available for further research.

Potential effects of Caffeine In-vivo

The effects of caffeine in vivo have been little studied in ruminants. The first study was published in 1970. To better understand milk fat depression in cattle, Hawkins and Davis evaluated the effects of intraruminal infusions of caffeine and coffee on blood plasma levels of free fatty acids and triglycerides. However, because the animals decreased their diet intake, caffeine effects on plasma levels of free fatty acids and triglycerides were not detectable. Later on, Brown and Harris, (1988) wanted to know how caprine milk composition and production may change by inducing adipose tissue mobilization with intravenous caffeine doses. They found that caffeine does not affect in situ adipose tissue mobilization in ruminants. In 1996 caffeine pharmacokinetics was studied in cattle and sheep. Intravenous doses of caffeine (5mg/kg) are metabolized via hepatic activity in sheep and cattle (Danielson and Golsteyn, 1996). The total plasma clearance of caffeine differs between male and female animals from 8 to 12 months of age, where females can metabolize caffeine at a higher rate than males (Janus and Antoszek, 2000). A more recent study suggests that the effect of intravenous administration of 4.2 g of caffeine in the circulatory and nervous system was stimulated to an appropriate degree, indicating that cows tolerated caffeine (Link et al., 2005).

Most of these studies from 1988 to 2005 administrated caffeine intravenously, and Hawkins and Davis (1970) infused caffeine intraruminaly. Recently, Robertson et al. (2018) evaluated how oral supplementation of grazing ewes with caffeine may affect lamb survival. Ewes received a daily caffeine dose of 1.6 g for the first 14 days of life, resulting in a significantly reduced lambs mortality on the 1rst day of age ($P = 0.029$) in the caffeine group compared to the control (Robertson et al., 2018). However, it is necessary to continue researching how caffeine may affect ruminant performance if caffeine doses are administrated as a diet additive. Nevertheless, more literature is available on how the inclusion of by-products with caffeine contents in ruminants' diets affects physical aspects, growing performance, lactation, health, and ruminal fermentation products. The literature available helps us understand what effects expect if caffeine is used as a ruminant diet additive; however, we cannot ignore that these by-products have other components that affect in-vivo trials.

a. Physiological effects

Campbell et al. (1976) studied the diuretic effect on six Holstein steers after 2 weeks of consuming a diet with 15% of coffee ground. Steers have shown an increase in urinary output ($P<.005$), urinary nitrogen ($P<.01$), and sodium ($P<.05$) after the treatment. On the other side, urinary solids ($P<.005$) and urinary minerals ($P<.005$) decreased after two weeks of treatment (Campbell et al., 1976). The ratio of water to dry matter intake was higher ($P<.05$) for the animals under 15% coffee ground

treatment than in the control group (Campbell et al., 1976). This may explain the increase in urinary output; however, the increase in water intake was not significant for the treated animals (Campbell et al., 1976). Salinas-Rios et al. (2015) suggested that the increment of water intake is due to caffeine in coffee by-products such as coffee pulp; urine excretion increases with high doses of coffee pulp. Tavares et al. (2005) do not found any significant effect on the animals' behavior after forty-two crossbred lactating Holstein–Zebu cows receiving continuous (for 56 days) concentrates with 25% coffee hulls as partial replacement of corn in the concentrate. These findings agree with the study completed by Link et al. (2005), who measured the caffeine effect on the ruminant nervous system.

b. Body weight and voluntary dry matter intake

Tavares et al. (2005) reported body weight reduction in animals under 25% of coffee hulls treatment, however, it was not significant to change body conditions scores. Lower dry matter intake was reported in animals consuming concentrate with 25% of coffee hulls (Tavares et al., 2005). Groups of goats with a 50% cocoa shell diet or 50% cocoa dust diet lowered their voluntary dray matter intake; the author attributed this change in consumption to the presence of low palatable components (alkaloid theobromine) (Aregheore, 2002). Theobromine is also present in coffee by-products (Hoseini et al., 2021). However, Pedraza-Beltrán et al. (2012) reported that the average body weight did not change in dairy cows with 0%, 10%, 15%, and 20% of coffee pulp

in their concentrates. Salinas-Rios et al. (2015) also reported no effect on weight gain, feed intake, feed conversion, and rumen pH in sheep with 16% of coffee pulp in their diets. Also, the included spent coffee ground in the concentrate (0, 30, 50, and 100 g/kg DM) did not affect the average daily gain and forage total dry matter intake of ewe (de Otálora et al., 2020).

c. Lactation effects

Tavares et al. (2005) reported that crossbred lactating Holstein–Zebu cows receiving concentrates with 25% coffee hulls as partial replacement of corn in the concentrate resulted in a reduction of milk and solid yield. The milk yield reduction was not followed by a simultaneous decrease in body condition in animals with 15% in the coffee hulls treatment, as compared to the control treatments (Tavares et al., 2005) The inclusion of coffee hulls as a non-forage fiber source in concentrate diets increased the neutral detergent fiber (NDF) in the diet contents; this increment of NDF did not result in an increment in milk fat percentage (Tavares et al., 2005). The author explained that the high amount of NDF and the low milk yield potential from these crossbreed animals might affect the results obtained in milk, solids, and fat yield. Interestingly, even though the inclusion of coffee hulls lowered concentrate intake in animals, coffee hulls concentrate had the highest feed conversion based on the ratio of milk yield to concentrate intake calculation (Tavares et al., 2005). In contrast, Souza et al. (2005) found that the inclusion of coffee hulls (at 0%, 8.75%, 17.5%, and 26.25%

levels) as a replacement for ground corn in concentrates did not affect milk yield and contents (fat, protein, and total solids) in lactating cows. According to Souza et al. (2005), coffee hulls could be added up to 10.5% of the total diet dry matter. As well, Pedraza-Beltrán et al. (2012) found no difference ($P>0.05$) between the average daily milk yield crossed Holstein–Brown Swiss–Zebu cows and concentrates with 0%, 10%, 15%, and 20% levels of coffee pulp. The addition of coffee pulp does not show differences ($P>0.05$) between milk fat, protein, and total solids contents (Pedraza-Beltrán et al., 2012). The coffee pulp can be included up to 20% in the concentrate to replace corn and canola cake without affecting milk yield (Pedraza-Beltrán et al., 2012). Finally, a more recent study reported that the low inclusion of spent coffee ground (0, 30, 50, and 100 g/kg levels) in the concentrate resulted in a milk yield quadratic effect ($P < 0.001$), while fat and protein corrected milk increased linearly ($P = 0.099$) in ewes (de Otálora et al., 2020). Also, a linear increase in fat yield ($P = 0.002$) and concentration ($P < 0.001$) has been evidenced after spent coffee ground treatment (de Otálora et al., 2020). Yields and concentrations of protein ($P = 0.043$ and $P = 0.038$ respectively) and lactose ($P = 0.007$ and $P = 0.012$ respectively) were affected quadratically in experimental animals in the same study. According to de Otálora et al. (2020), coffee by-products' low inclusion may serve as a functional ingredient for ruminal fermentation due to the high amount of phenolic compounds available in these by-products.

d. Health effects

The addition of 16% of coffee pulp decreased the levels of a by-product of lipid metabolism, malondialdehyde ($P < 0.05$), in 6-month-old Blackbelly male cross-breed sheep, evidencing a reduction of plasma oxidation and preventing a state of oxidative stress in the animals (Salinas-Rios et al., 2015). Components such as chlorogenic acid, caffeic acid, and gallic acid have been described as antioxidants phenolic acids that reduce levels of free radicals (Yen et al., 2002; Marinova et al., 2009). Salinas-Rios et al. (2015) found that chlorogenic acid, caffeic acid, and gallic acid were present in the coffee pulp at levels of 5.61%, 1.47%, and 0.26%, respectively.

Another study evidenced that the inclusion of coffee pulp (0%, 7%, 14%, 21%, and 29% levels) in Pelibuey crossbred male lambs' diet reduced ($P < 0.05$) the amount of neutrophils cells (Hernández-Bautista et al., 2018). Caffeine is commonly known for its activity on adenosine receptors; accordingly, Hernández-Bautista et al. (2018) said that caffeine might prevent inflammatory responses, indirectly decreasing neutrophils count in the blood.

e. Microbiome and Fermentation effects

Inclusion of 35% of fermented cocoa pod in the concentrate diet increased total volatile fatty acids ($P < 0.01$) compared to the control in Holstein steers, as a consequence of acetate ($P < 0.05$) and propionate ($P < 0.01$) increment (Laconi and

Jayanegara, 2015). Laconi and Jayanegara (2015) also reported the amount of rumen microbial protein yield ($P < 0.01$), ruminal ammonia concentration ($P < 0.01$), and allantoin urine ($P < 0.01$) increased in comparison to the control animals.

Changes in volatile fatty acids amount could be related to the bioactivity of caffeine, which has been cataloged as a microbial stimulant in in-vitro batch cultures (Prabhudessai et al., 2009). In non-ruminant animals (mice) the oral administration of caffeine (0.0007 g/kg) for 21 days affects bacterial groups without any loss of microbial diversity compared with the control animals (Kleber Silveira et al., 2018). Also, the caffeine oral administration results in a change in volatile fatty acids amount in mice. Plasma propionate and butyrate concentrations increased ($P < 0.05$ for each concentration) in comparison with the non-treated mice (Nishitsuji et al., 2018). The metabolic intermediate, lactate, decreased in caffein-treated animals ($P < 0.01$) compared to control in the same study. Recently, doses of spent coffee ground (0, 30, 50, and 100 g SCG / kg) were added to the dairy ewes diet to test the effect on rumen microbiota (Goiri et al., 2020). Goiri et al. (2020) reported an increase in phylum *Firmicutes* and *Bacteroidetes* while decreasing the *Proteobacteria* phylum. Also, Goiri et al. (2020) reported an increment of *Butyrivibrio*, *Blautia*, *Fibrobacter*, and *Treponema* genera, accompanied by an increment of acetic and butyric acid in the rumen. After spent coffee ground doses in ewe diets, bacteria from the genus *Prevotela* were inhibited (Goiri et al., 2020).

Conclusion

Caffeine is commonly known as a metabolic stimulant. Prabhudessai et al. (2009) suggested that caffeine may increase biogas production by potentially increasing microbial activity. This is the first time that pure caffeine has been tested in anaerobic fermentation. In anaerobic fermentation seems to increase microbial activity, increasing biogas production in anaerobic fermentation of food waste and cow manure mixture (80:20) (Prabhudessai et al., 2009). Caffeine bioactivity has been studied. Caffeine may inhibit bacterial proliferation by interacting with DNA repair mechanisms, such as the SOS response pathway (Whitney and Weir, 2015). More specifically, caffeine may interact with the UmuC gene which is regulated by the DNA repair pathway (SOS), producing persistent damage in sensitive bacterial cells (Whitney and Weir, 2015). Unlike other plant secondary metabolites such as essential oils or saponins, the same caffeine molecule is produced in several plants, such as cacao, citrus, guarana, coffee, and tea lineages, due to convergent evolution (Huang et al., 2016). This can be an advantage in researching a natural additive; due to the nature of the molecule, results may be more consistent. However, all the caffeine trials to test its effect on fermentation have been done on batch cultures. Also, the literature available on caffeine effects in ruminants is limited. To support the hypothesis of the potential use of caffeine as an additive to enhance rumen fermentation, other fermentation techniques, such as continuous cultures fermentation, are necessary to produce preliminary data on rumen fermentation effects.

CHAPTER TWO

EFFECTS OF CAFFEINE DOSES ON RUMEN FERMENTATION PROFILE AND NUTRIENT DIGESTIBILITY WHEN FED A LACTATING COW DIET UNDER CONTINUOUS CULTURES CONDITIONS

Abstract

Caffeine is a secondary plant metabolite commonly known for its bioactivity properties. This molecule increases microbial activity during anaerobic digestion. This study aimed to determine the effects of caffeine doses on rumen fermentation profile and nutrient digestibility when dual continuous culture fermenters were fed a lactating cow's diet. We hypothesize that adding caffeine doses into continuous culture fermenters with rumen fluid inoculum will not affect anaerobic fermentation or nutrient utilization. Fermenters were fed twice a day (at 0800 and 2000 h) with an experimental diet of high producing dairy cows (53.55 g/DM day; Forage:Concentrate ratio, F:C of 40:60). Four levels of caffeine (0 ppm, 50 ppm, 100 ppm, and 150 ppm) were added to the diets as a treatment. The experiment was arranged in a randomized complete block design. Two blocks of four fermenters were run in two replicated periods of ten days. Statistical analyses were performed in SAS version 9.4 for Windows (SAS Institute Inc., Cary, NC) using a mixed procedure. Linear and quadratic contrasts were performed to evaluate the effects of caffeine doses. Results do not support our initial hypothesis stating that caffeine will not affect rumen fermentation or nutrient utilization. The addition of caffeine doses at 50, 100, and

150 ppm on continuous culture fermentation decreases protozoal counts (*Diplodinium* spp. and *Ophryosoloex* spp.) linearly. Consequently, ammonia concentrations had linear and quadratic decrease effects. DM, OM, and starch digestibility had a significant linear decrease effect after added caffeine doses. The pH values and maximum pH reached on the cultures linearly decreased when caffeine was present. Total VFAs were unaffected; however, the A:P ratio decreases linearly when caffeine doses increase.

Introduction

Caffeine (1,3,7-trimethylxanthine) is a secondary plant metabolite that occurs naturally in some plant seeds, nuts, or leaves. Plant secondary metabolites are compounds that are not part of the biochemical metabolism of plant growth and reproduction (Patra and Saxena, 2010). Caffeine, as a secondary plant metabolite, plays an essential role in plant defense, which acts as a natural pest repellent and insecticide (Nathanson, 1984; Mathavan et al., 1985; Hollingsworth et al., 2003), and bactericide (Kim and Sano, 2008; Sledz et al., 2015).

By-products from the coffee and cocoa industry (with caffeine contents) have been studied to increase microbial activity on batch cultures with rumen fluid and cow manure. Incremental bacterial growth and biogas production have been reported on co-digestion of rumen fluid or cow manure with by-products from the coffee and cocoa industry (Corro et al., 2013; Rico et al., 2014). The co-digestion of the soluble fraction of spent coffee ground and cow manure showed a reduction of bacterial lag time in co-digestion (9 to 10 days) in comparison to control (10 to 12 days) (Luz et al., 2017). Rapid bacterial growth occurred after a month of microbial adaptation in co-digestion of coffee pulp and cow manure, resulting in faster digestion of nutrients and a higher rate of CH_4 production in comparison with controls (Corro et al., 2013). In addition, co-digestion of cocoa shells and cow manure produced higher amounts of cumulated methane than cow manure digestion alone (Rico et al., 2014).

Previous research has cataloged caffeine, tannins, and other polyphenols in coffee pulp as anti-nutritional components that may cause low feed intake, protein digestibility, and nitrogen retention when the coffee pulp is used as animal feed (Pandey et al., 2000). Despite the previous caffeine description as a toxic component of by-products used as animal feed, Prabhudessai et al. (2009) suggested that caffeine may increase biogas production by potentially increasing microbial activity during the anaerobic digestion of food waste and cow manure mixture (80:20). Prabhudessai et al. (2009) evaluated the effect of pure caffeine in anaerobic fermentation for the first time.

In ruminants, the inhibition of rumen methanogenesis has been deeply studied to enhance rumen fermentation, improve metabolizable energy and decrease greenhouse gas emissions (Patra and Saxena, 2010). In dairy cows, methane production represents a loss of 6.0 ± 1.18% of the energy intake (Niu et al., 2018), reducing the metabolizable energy produced from the total feed energy intake. In terms of environmental concerns, methane production from enteric fermentation represents about 12% of the total methane released into the atmosphere (Moss et al., 2000).

Plants produce diverse secondary metabolites as part of their defense, acting as antimicrobial and insecticides (Patra and Saxena, 2010). Plant secondary metabolites such as saponins and essential oils have been shown to change rumen microbial profile (Wallace, 2004; Patra and Saxena, 2009a). Enhancing rumen fermentation, nitrogen metabolism, and decreasing methane production, improving animal productivity and health (Wallace, 2004; Kamra et al., 2006; Rochfort et al., 2008). However, the high diversity of essential oils and saponins composition challenges determining which component would

be better as an additive to enhance rumen fermentation. In contrast, the same caffeine molecule is produced in several plants, such as cacao, citrus, guarana, coffee, and tea lineages, due to convergent evolution (Huang et al., 2016). Moreover, caffeine has been reported as a microbial metabolic stimulant. In anaerobic fermentation of food waste and cow manure seems to increase microbial activity, increasing biogas production (Prabhudessai et al., 2009).

These studies suggest that caffeine-containing by-products increase microbial activity and consequently enhance biomass digestion. Thus, the aim of this study was to determine the effects of caffeine doses on rumen fluid fermentation profile and nutrient digestibility when dual continuous culture fermenters were fed with lactating cows' diet. We hypothesize that the addition of caffeine doses into continuous culture fermenters with rumen fluid inoculum and fed with lactating cows' diet will not affect the anaerobic fermentation or nutrient utilization.

Material and methods

a. Experimental design

Eighth dual-flow continuous culture fermenters in a randomized complete block design were inoculated with rumen fluid and fed with four treatments levels. Two blocks of four fermenters were run in two replicated periods of ten days. Each period consists of ten days: seven for microbial adaptation to the diet and data and

sample collection during the last three days. At the beginning of each period, the fresh inoculum was obtained from three fistulated Holstein dairy cows. Treatments were randomly assigned to a different fermenter at the beginning of each period to avoid differences between fermenters.

b. Substrate: diet composition and treatment

Fermenters were fed twice a day (at 0800 and 2000 h) with a total substrate of 53.55 g/DM day. Corn silage was the forage source for the experimental lactating cow diet. The ratio of Forage:Concentrate (F:C) was 40:60 on a dry matter basis (**Error! Reference source not found.**). The particle size of the substrate was 2 mm. Four treatment levels at 0 ppm, 50 ppm, 100 ppm, and 150 ppm of CAFFEINE BAKER 500GM (Avantor Performance Materials U.S., Catalog # JTE268-7) were added to the diets as a treatment and were randomly assigned to the fermenters at the start of each period. Diets with treatment levels were prepared before starting each period.

c. Inoculum and culture conditions

Rumen fluid was collected from three rumen fistulated cows and strained through two cheesecloth layers into a sealed container. All surgical and animal care procedures were approved by the Clemson University Institutional Animal Care and

Use Committee. The fermenters' design and operation were based on a previous design outlined by Teather and Sauer, (1988), with some modifications, including the use of an overflow sidearm that angled downward at approximately 45^{o} to facilitate emptying. Around 1800 h, the rumen contents were collected from three rumen cannulated Holstein cows fed a 50% forage:50% concentrate diet and strained through two layers of cheesecloth into a prewarmed sealed container. The filtered rumen fluid was combined from all cows, mixed with a buffer in a 1:1 ratio according to the methods of Slyter et al., (1966), and purged with CO_2 until inoculation into the continuous culture fermenters. Moreover, the time from inoculum collection to fermenter inoculation did not exceed 60 min. Peristaltic pumps were calibrated and used to continuously influx 90 ml/h of buffer into the culture to maintain a liquid dilution rate from 10% to 12%. To maintain anaerobic conditions, carbon dioxide was continuously purged at a rate of 20 ml/min. Temperature was held at 39°C by using a water bath that circulated warm water between the fermenters. Continuous pH meters measured culture values every 20 minutes throughout the ten-day periods and were reordered with data acquisition software (LabVIEW; National Instruments, Austin, TX). The culture pH was allowed to fluctuate during the day. The stirring rate was 45 rpm, allowing the stratification of particles (gases, middle layer of liquid and small particles, and lower layer of dense feed particles).

d. Sample collection and analysis

Samples collection started from day 8 to day 10 on each ten-day period. The overflow was collected in a 2 L Erlenmeyer flask placed on ice, and 10 mL H2SO4 (50% solution) were added to each flask to stop fermentation for accurate measures of feed fermentation products. Overflow volume was measured from each fermenter every 12 hours to calculate the total daily volume. Containers designated for each fermenter were used to collect 20% of the overflow sample and frozen at -20°C from day 8 to day 10. Composite overflow samples were later thawed, homogenized, and 20% of a subsample was collected to analyze dry matter, neutral detergent fiber, acid detergent fiber, and starch. On day 10, culture samples collection was performed every two hours, from 0 hours (before feeding) to 12 hours (after feeding) for volatile fatty acid, ammonia, and protozoa. The pH values from the culture were also measured manually with VWR® SympHony® pH Meter every two hours on day 10. Samples from culture and overflow were collected on day 10 to measure dry matter. Fermenters' impellers revolutions were increased to 100 rpm to mix the cultures and ensure the uniformity of the sample on the last day of collection times.

Diet (input) and overflow (output) samples were analyzed for dry matter; samples were oven-dried at 102° C. Samples for NDF and ADF analysis were oven dry at 102 °C and ground at 2.2 mm. NDF and ADF analysis was based on protocols resources from Ankom Technology, Macedon N.Y. (Method 6, 2011 and Method 5, 2011, respectively). Diet and overflow samples were placed in a muffle furnace at 600° C for six hours to analyze ash to burn all organic material

and measure the total inorganic components, the mineral values. Ash samples analyses were performed as described by the Association Official Analytical Chemist (AOAC). Overflow samples were centrifuged at 40,000 G for 20 minutes at 4 °C; sample supernatant was removed for DM analysis. Diet and overflow organic matter (OM) values are obtained from the total amount of the sample minus ash. Dry matter from the overflow was corrected by subtracting DM from the buffer that was pumped into the cultures. Organic matter, NDF, and ADF from the overflow were corrected for DM. The digestibility of DM, OM, NDF, and ADF was calculated for each nutrient as follows: $\frac{(Input-output)}{Input} \times 100$. Where input is the nutrients contents in the diet and output is the nutrients remaining in the overflow.

Four ml of culture sample were pipetted into a 15 ml polycarbonate centrifuge tubes containing 1-mL of 25% (w/v) metaphosphoric acid vortex and then centrifuged at 9,000 rpm for 30 minutes at 4°C (Erwin et al., 1961). The supernatant was used for VFA, ammonia, and D and L lactate analysis. For sample preparation before VFA analysis, 0.5 mL of supernatant was combined (in duplicates) with 0.5 of ultra-pure water and 0.1 ml of internal standard (100 mM 2-Ethyl-butyric acid solution) in a 1.5 ml microcentrifuge tube. Samples were slowly vortexed, and after standing in the refrigerator for 30 minutes, samples were centrifuged at 12,000 rpm for 12 minutes and filtered with a 0.45 µm syringe filter into a gas chromatography (GC) vial. VFA profiles were analyzed by GC

according to the methods of Yang and Varga, (1989). The retention time of known standards was used to calibrate GC methods for VFA sample identification.

Modifications of ammonia analysis methods described in Chaney and Marbach, (1962) were performed to accommodate samples and reagents amounts in a 96-well microplate reader and read the solution absorbance at 625 nm of wavelength. Analysis to calculate starch concentration was based on the methods described by Bach Knudsen, (1997). Incubated samples were pipetted in a 96-well microplate, and absorbance was read at 500 nm of wavelength.

For protozoa samples preparation, 4 ml of culture were transferred into a 10 ml vial containing 4 mL of methyl green-formaldehyde solution and stored at 4° C for further analysis. Preserved protozoa sample was pipetted in a counting chamber (Fuchs-Rosenthal Bright-Line counting cell, 0.2 mm depth; Hausser Scientific, Horshamm, PA) and placed in a microscope for quantification. Genera identification was conducted as described by Dehority (1993).

e. Statistics

Statistical analyses were conducted in SAS version 9.4 for Windows (SAS Institute Inc., Cary, NC) using mixed procedure. The experiment was a randomized complete block design, and response variables were analyzed using the following model: $Y_{ijk} = \mu + C_i + P_j + CP_{ij} + F_k + e_{ijk}$. Where Yijk is the dependent variable, μ is the overall mean, C_i is the fixed effect for caffeine levels (i = 1 to 4), P_j is the fixed effect of the period (j = 1 to 2), CP is the interaction of C and P, F_k is the

random effect of the fermenters within blocks (k = 1 to 8), and eijk is the residual error. The fixed effect of time, the interactions of time with the period, time with treatment, and the time with period and treatment were added to the model for repeated measures. The first order of autoregressive structure, AR (1), was used for repeated measures analysis. Linear and quadratic contrasts were performed to evaluate the effects of caffeine doses. The least square means are presented in tables, and evidence for statistical significance was declared at $P \leq 0.05$, while trends for main effects and interactions are discussed at $0.10 \geq P > 0.05$.

Results and Discussion

a. Caffeine effects on nutrients digestibility

Nutrient digestibility coefficients are presented in **Error! Reference source not found.**. DM and OM digestibility had a significant linear decrease effect (P = 0.01). When caffeine doses increased further than 50 ppm, OM and DM digestibility tended to decrease (Figure 2.1**)**. As observed in the case of OM and DM, fermenters receiving 50 ppm of caffeine dose had the highest digestibility values for NDF and ADF (Figure 2.2). Despite that observation, NDF and ADF digestibility were numerically similar among treatments (Table 2.2). Starch digestibility also resulted in a significant linear decrease effect (Table 2.2; P = 0.01).

In our study, we used pure caffeine, a compound present in coffee and coffee by-products. The decrease in OM and DM digestibility has also been observed in a previous study that includes coffee by-products in ruminants. The replacement of timothy and alfalfa hay with 10% and 20% of wet coffee grounds on a DM basis has been shown to linearly decrease the digestibility of OM ($P < 0.01$) and DM ($P<0.01$) in sheep animals (Xu et al., 2007). High-quality forages have less proportion of structural carbohydrates and more digestible organic matter content, which may increase DM and OM digestibility (Moe and Tyrrell, 1979). The content of NDF and ADF in wet coffee grounds is approximately 68.8% and 54.8%, respectively, which is higher than the amount found in alfalfa (NDF of 41.1% and ADF of 31.1%) and timothy (NDF of 60.8% and ADF of 39.6%) (Xu et al., 2007). When high-quality forage is replaced with an ingredient with a higher amount of structural carbohydrates and less readily fermentative carbohydrates, the digestibility of DM, OM, and starch is expected to decrease.

b. Caffeine effects on Volatile fatty acids, pH, and ammonia

Total volatile fatty acid concentration, volatile fatty acids proportions, and ammonia concentration are presented in Table 2.3. Total volatile fatty acids were not affected by caffeine doses (Table 2.3 and Figure2.3). Acetate to propionate ratio (A:P) tended to decrease among treatments (Table 2.3; $P = 0.07$). Interestingly

acetate slightly decreased at 6 hours after treatment (figure 2.4), and propionate increased (Figure 2.5). A:P ratio was lower on fermenters receiving 100 ppm of caffeine, but this was not significant between treatments (Figure 2.6). Some secondary plant metabolites also have been shown to decrease A:P without impacting the total VFA production (Wang et al., 2009). These results agree with the findings shown by Prabhudessai et al. (2009) when they reported that the addition of 100 ppm caffeine dose to the anaerobic fermentation of food waste with 8% of total solids (TS) resulted in the highest biogas production (408.5 ml/g TS) in comparison with control (0 ppm) which produced 182.5 ml/g TS. Prabhudessai et al. (2009) concluded caffeine potentially increases microbial activity when added to anaerobic fermentation. In addition, other secondary plants metabolites, such as saponins, have been shown to increase rumen propionate production after treatment by rechanneling hydrogen from methane to propionate pathways (Patra and Saxena, 2010).

The addition of caffeine (50, 100, and 150 ppm doses) resulted in a significant linear and quadratic decrease in ammonia (Table 2.3; $P < 0.0001$). However, the addition of 50 ppm of caffeine had the highest impact on the reduction of ammonia production over the 12 h of sampling compared to the fermenters with no caffeine addition ($P < 0.05$; see Figure 2.7). Other plant secondary metabolites have been shown to decrease ruminal hyper-ammonia-producing bacteria (HAB), responsible for the deamination of amino acids to ammonia in the protein degradation pathway (Wallace et al., 2002). The addition of essential oils (10.5 mL

of eucalyptus oil at 80% of purity with 7.35 g of mulethi root powder) daily reduced the production of ruminal ammonia by up to 50% in buffalos (Chanu et al., 2020). The increment of saponin doses (2.0 mL and 4.0 mL) on batch cultures with rumen fluid resulted in a significant decrease ($P < 0.01$) in ammonia levels (Singh et al., 2020). Reducing ruminal ammonia is correlated with improved protein efficiency and feed efficiency utilization by utilizing ammonia for de novo synthesis of amino acids. Buffalos supplemented with essential oils tend ($P < 0.10$) to improve feed conversion ratio and conversion of dietary protein for body weight gain compared to control animals; however, no differences in nutrient digestibility were observed (Chanu et al., 2020). In contrast, our in-vitro trial has shown a linear effect for DM, OM, and starch digestibility in fermenters receiving caffeine doses.

Rumen fluid pH values are presented in Table 2.4. Means of pH measurements and maximum pH had a significant linear decrease effect ($P < 0.0001$ and $P = 0.004$, respectively). However, the minimum pH had a quadratic tendency ($P = 0.09$). A linear effect was also observed with the time in which pH values were below six ($P = 0.001$). The addition of coffee by-products decreased pH values due to the increment of acids produced by the increment of microbial fermentation. The addition of spent coffee water in co-digestions with cow manure resulted in a pH reduction (pH = 5) during the first days of the fermentation period and incremented the cumulated biogas (2,558 mL) in comparison with cow manure digestions (pH = 7 during the first days of fermentation; 993 mL of cumulated biogas) (Luz et al., 2017). Co-digestion of coffee pulp with cow manure resulted in

a lower initial pH (4.5) than the pH (6.5) of cow manure digestion alone (Corro et al., 2013). A summary of references that evidenced the increment of biogas from caffeine-containing by-products in anaerobic digestions is presented in Table 1.1.

It is important to note that coffee or cocoa by-products have other components that may impact the anaerobic fermentation, possibly by the synergistic action of caffeine and other components. Mills et al. (2015) conducted 24 hours of batch culture fermentation inoculated with human fecal samples and fed with three different brands of coffee (with different levels of chlorogenic acid) and chlorogenic acid alone as a control. Mills et al. (2015) concluded that coffee intake selectively modulates the colonic microbiota populations and increases acetate and butyrate production due to the presence of chlorogenic acid and other components such as caffeine. However, different outcomes can be obtained when caffeine-containing by-products are used in anaerobic fermentation due to their complex composition. Widjaja et al. (2017) reported a low rate of digestion of total solids and volatile solids during the first 30 days of co-digestions of coffee pulp with cow manure and rumen fluid. While Luz et al., (2017) reported a reduction of bacterial lag time on co-digestion of the soluble fraction of spent coffee ground and cow manure (9 to 10 days) in comparison to control (10 to 12 days). The easier hydrolysis of the soluble fraction of spent coffee ground in co-digestion leads to an increment of volatile fatty acid concentration, lower pH, and, consequently, a faster digestion process (Luz et al., 2017)

c. Caffeine effects on protozoa populations

Total protozoa counts and identification are shown in Table 2.5. The addition of caffeine doses of 50 ppm, 100 ppm, and 150 ppm resulted in a significant linear increment effect for *Diplodinium* spp. (P = 0.03) However, the addition of caffeine doses resulted in a numerical decrease of *Diplodinium* spp. Compared to 0 ppm of caffeine dose (Figure 2.8). Also, *Ophryosoloex* spp. resulted in a linear decrease tendency (P = 0.09). Interestingly, the presence of *Ophryosoloex* spp. resulted in a mean of 0 when caffeine doses were increased to 150 ppm (Figure 2.8). These results suggest that the increment of caffeine doses in dairy cows' experimental diet may inhibit the growth of *Ophryosoloex* spp. in rumen fluid fermentation. The effect of plant secondary metabolites on the ruminal population has been studied in search of natural methods to decrease methane production by ruminal defaunation. The addition of 2 g/d of anise extract (an essential oil) to four Holstein heifers for 21 days resulted in a decrease in protozoal counts (*Entodinium* spp. and Holotrichs) and leading the reduction of ruminal ammonia production (Cardozo et al., 2006). The effects of saponins and essential oils have been reported to decrease ruminal protozoal counts and ammonia concentrations (Patra and Saxena, 2009b; Wang et al., 2009). Ruminal protozoa play an important role in ammonia production by proteolysis and deamination of bacterial and dietary nitrogen (Van Soest, 1994; Szumacher-Strabel and Cieślak, 2010). Because protozoa predate bacteria, the ruminal bacterial protein decrease,

ammonia production increases, and as a consequence, N efficiency decrease (Benchaar et al., 2008). When ruminal defaunation occurs, it decreases ammonia production, and more bacteria can utilize ruminal nitrogen for de novo synthesis of ammino acids (Szumacher-Strabel and Cieślak, 2010). The decrease in protozoa counts may be a reason for the reduction of ammonia concentration in the present study and the numerical increase in total VFAs due to a possible reduction in bacteria predation.

Conclusion

In conclusion, these results do not support our initial hypothesis stating that caffeine will not affect rumen fermentation or nutrient utilization. The addition of caffeine doses at 50, 100, and 150 ppm on continuous culture fermentation resulted in a significant linear decrease in protozoal counts of *Diplodinium* spp. and tends to decrease numbers of *Ophryosoloex* spp. linearly. At the same time, ammonia concentrations had linear and quadratic decrease effects. DM, OM, and starch digestibility had a significant linear decrease effect after added caffeine doses. However, NDF and ADF digestibility had no significant effect. The pH values and maximum pH reached on the cultures linearly decreased when caffeine was present. On the other side, the amount of time the fermenters had pH values below six increased linearly when caffeine doses increased. The pH values below 6 inhibit fiber digestibility, evidencing a decrease of DM when caffeine doses are incremented in the present study. Total VFAs were unaffected; however, the A:P ratio

tends to decrease linearly when caffeine doses increase. Further in-vivo trials may be necessary to evaluate the caffeine effects and potential as a natural ruminant additive.

TABLES

Table 1. 1 Biogas and methane produced from caffeine-containing by-products in anaerobic co-digestions.

Reference	Batch Cultures	Control	Co-digestion	Cumulated Methane		Cumulated Biogas	
				Control	Co-digestion	Control	Co-digestion
(Luz et al., 2017)	4	100 g of cow manure and 1.5 L of water	4.8 g vs. of soluble fraction of spent coffee ground and 100 g of cow manure	475[d]	1336[d]	993[d]	2558[d]
(Corro et al., 2013)	3	A= 80% wt cow-manure and 20% wt water B= 80% wt coffee pulp and 20% wt water	40% wt coffee pulp, 40% wt cow-manure, and 20% wt water	A= [a] 11.27[e] B= [a] 22.16[e]	[a] 52.48[e]	A= ± 0.04[f] B= ± 0.02[f]	± 0.06[f]
(Widjaja et al., 2017)	3	NA	Coffee pulp and water (CPW) (1:2) with variable inoculums: A= CPW and cow manure B= CPW and rumen fluid C= CPW, cow manure, and rumen fluid	NA	A= 85.1[g] B= 11.3[g] C= 60.0[g]	NA	NA
(Rico et al., 2014)	12	Mono-digestions of interest: A= 246.7 g inoculum and 3.3 g cocoa shell B= 222.49 g inoculum and 27.51 g cow manure	Co-digestions of interest: A= 5% of cocoa shell and 20% of manure B= 10% of cocoa shell and 20% of manure	A= [b]195[h] B= [b]18.0[h]	A= [b]25.1[h] B= [b]30.5[h]	NA	NA
(Darwin et al., 2018)	[c]2	300 mL/day cow manure	30 g/day cocoa husk with 300 mL/day digested cow manure.	NA	NA	[b]21[i]	[b]55 .64[i]
(Ward-Doria et al., 2016)	9	Rumen fluid and pig manure (2:1)	Inoculum (rumen fluid and pig manure) and cocoa pod husk: A= 1:1, B=2:1, C= 3:1	487.1[d]	A and B were not reported; C= 538.84[d]	0.12[h]	A= 0.034[h] B= 0.098[h] C= 0.169[h]

[a] Percent (%) of the volume of the total cumulated biogas produced; [b] Methane or biogas yield; [c] semi-continue anaerobic system; [d] mL; [e] volume %; [f] yield/m^3; [g] $Ndm^3/Kg_{CODremoval}$; [h] L CH4 kg^{-1}; [i] mL/g VS; [j] $m^3CH_4/KgVS_{substrate}$

Table 2.1 Diet and nutrient composition used to feed dual continuous culture fermenters

Diet ingredient (%DM)	
Corn Silage	42.8
Ground Corn	17.7
Soybean Meal	11.2
Citrus pulp	0.2
Beet pulp	0.2
Soyplus	9.3
Soy hulls	17.0
Mineral mix	1.6
Nutrient Composition	
% DM[a]	89.3
CP, % DM[a]	17.1
RDP, % CP[a]	57.7
RUP, % CP[a]	42.3
NDF, % DM[a]	31.5
ADF, % DM[a]	20.1
Starch, % DM[b]	28.0
Sugar, % DM[b]	3.6
Soluble Fiber, % DM[b]	7.9
Fat, % DM[a]	3.1

[a] Analysis of individual ingredients was conducted by Cumberland Valley Analytical.

[b] Estimated composition from diet simulation using individual ingredients

Table 2.2 Nutrients' digestibility values in response to caffeine doses (0, 50, 100, and 150 ppm) in dual continuous cultures fermenters.

Digestibility (% DM)	Caffeine concentrations				SE	*P*-value	
	0 ppm	50 ppm	100 ppm	150 ppm		Linear	Quadratic
DM	65.53	66.34	63.97	62.80	0.46	0.01	0.14
OM	72.30	73.19	71.27	70.33	0.37	0.01	0.12
NDF	54.83	57.48	55.65	53.98	1.20	0.51	0.12
ADF	51.10	52.46	51.74	49.35	0.92	0.28	0.13
Starch	97.93	97.97	97.83	97.76	0.03	0.01	0.13

Table 2.3 Volatile fatty acids and ammonia produced from dual continuous cultures fermenters in response to caffeine doses (0, 50, 100, and 150 ppm).

	Caffeine concentrations					*P*-value	
Culture fermentation	0 ppm	50 ppm	100 ppm	150 ppm	SE	Linear	Quadratic
Total VFA, mM	76.52	75.79	88.46	78.76	6.66	0.46	0.54
VFA concentrations, mol/100 mol							
Acetic acid	39.36	41.45	40.10	42.84	2.17	0.32	0.87
Propionic acid	23.23	26.71	30.46	27.04	2.86	0.16	0.16
Butyric acid	21.65	18.84	18.26	18.64	2.35	0.39	0.52
Isovaleric acid	0.98	1.00	1.25	1.21	0.33	0.53	0.93
Valeric acid	11.95	10.28	8.09	8.39	1.52	0.09	0.53
Caproic acid	2.75	1.64	1.77	1.86	0.41	0.20	0.18
Isoacids	0.10	0.08	0.08	0.01	0.05	0.27	0.63
A:P	1.92	1.73	1.45	1.68	0.20	0.07	0.36
Ammonia, µM	3.98	2.67	2.90	3.48	0.08	<.0001	<.0001

Table 2.4 Postprandial pH profile in dual continuous cultures fermenters in response to caffeine doses (0, 50, 100, and 150 ppm).

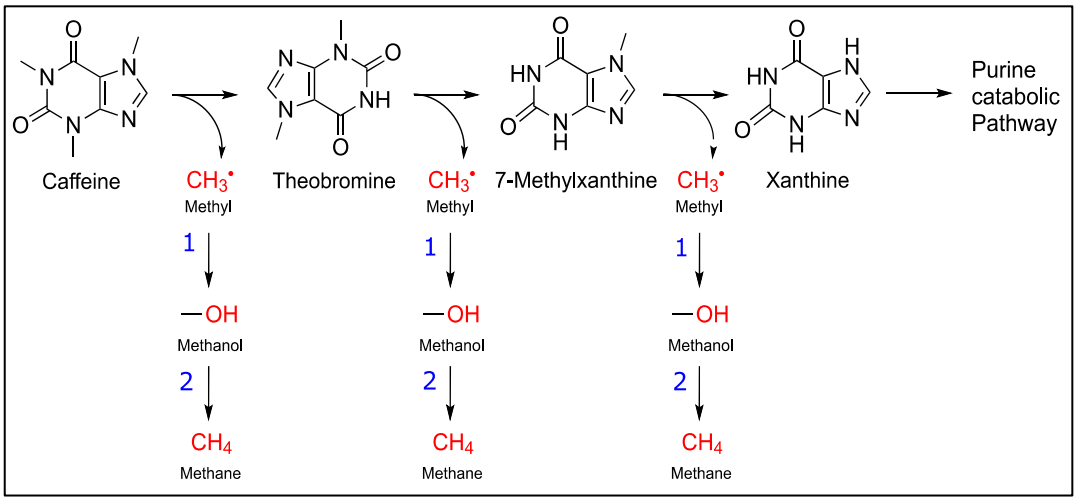
	Caffeine concentrations					*P*-value	
Culture pH	0 ppm	50 ppm	100 ppm	150 ppm	SE	Linear	Quadratic
pH	6.07	6.11	6.00	5.99	0.02	<.0001	0.15
Maximum pH	6.37	6.40	6.30	6.28	0.02	0.004	0.16
Minimum pH	5.74	5.78	5.73	5.67	0.04	0.18	0.09
pH, h < 6.0	5.00	4.50	8.00	7.00	0.35	0.001	0.39

Table 2.5 Protozoal population in dual continuous cultures fermenters in response to caffeine doses (0, 50, 100, and 150 ppm).

	Caffeine concentrations					*P*-value	
Protozoa 10^2/mL	0 ppm	50 ppm	100 ppm	150 ppm	SE	Linear	Quadratic
Total Protozoa	67.30	58.48	67.30	63.84	5.47	0.95	0.62
Entodinium spp.	24.44	25.89	26.90	27.68	3.43	0.28	0.88
Epidinium spp.	17.30	16.41	16.29	16.52	1.01	0.56	0.63
Daysitricha spp.	7.03	4.80	8.82	6.36	1.55	0.78	0.94
Isotricha spp.	1.12	0.78	2.12	1.23	0.40	0.34	0.48
Diplodinium spp.	16.96	9.93	12.83	12.05	1.32	0.03	0.10
Ophryosoloex spp.	0.45	0.67	0.33	0.00	0.23	0.09	0.19

FIGURES

Figure 1.1 Caffeine catabolism pathway and methanogenesis suggested by Chen et al. (2018).



(1): Hydrolysis step (2): Methanol consuming methanogens

Figure 2.1 Dry matter and organic matter digestibility values in response to caffeine doses (0, 50, 100, and 150 ppm) in dual continuous cultures fermenters.

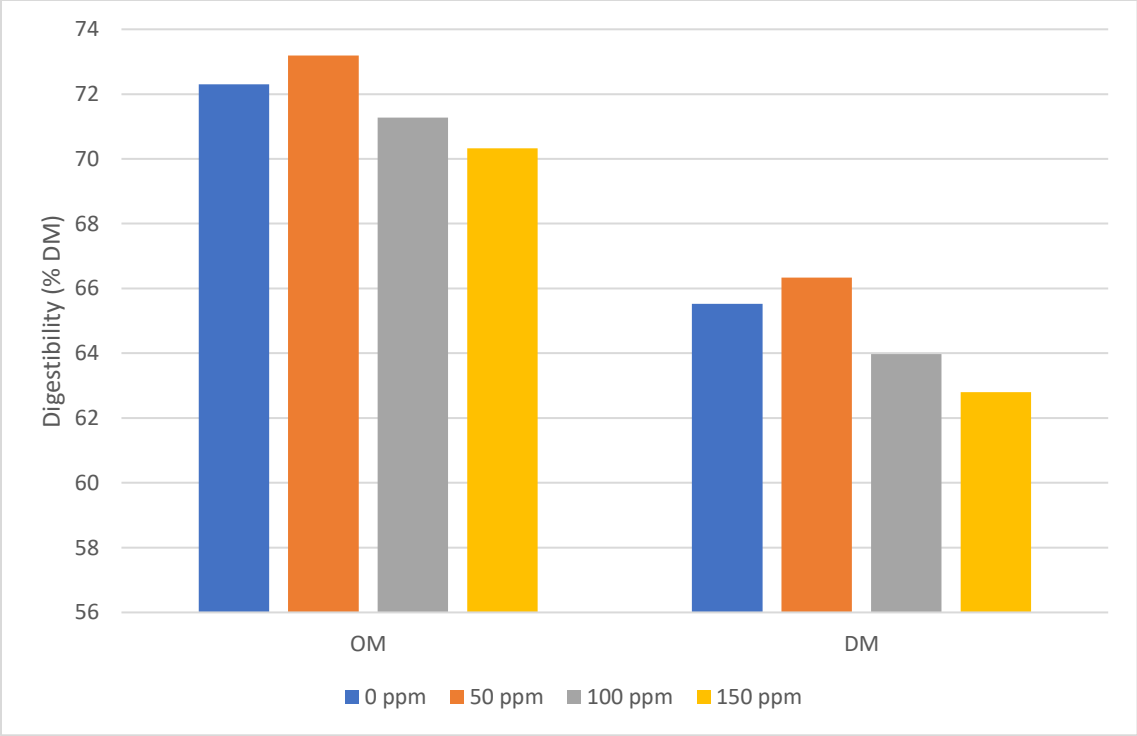


Figure 2.2 NDF and ADF digestibility values in response to caffeine doses (0, 50, 100, and 150 ppm) in dual continuous cultures fermenters.

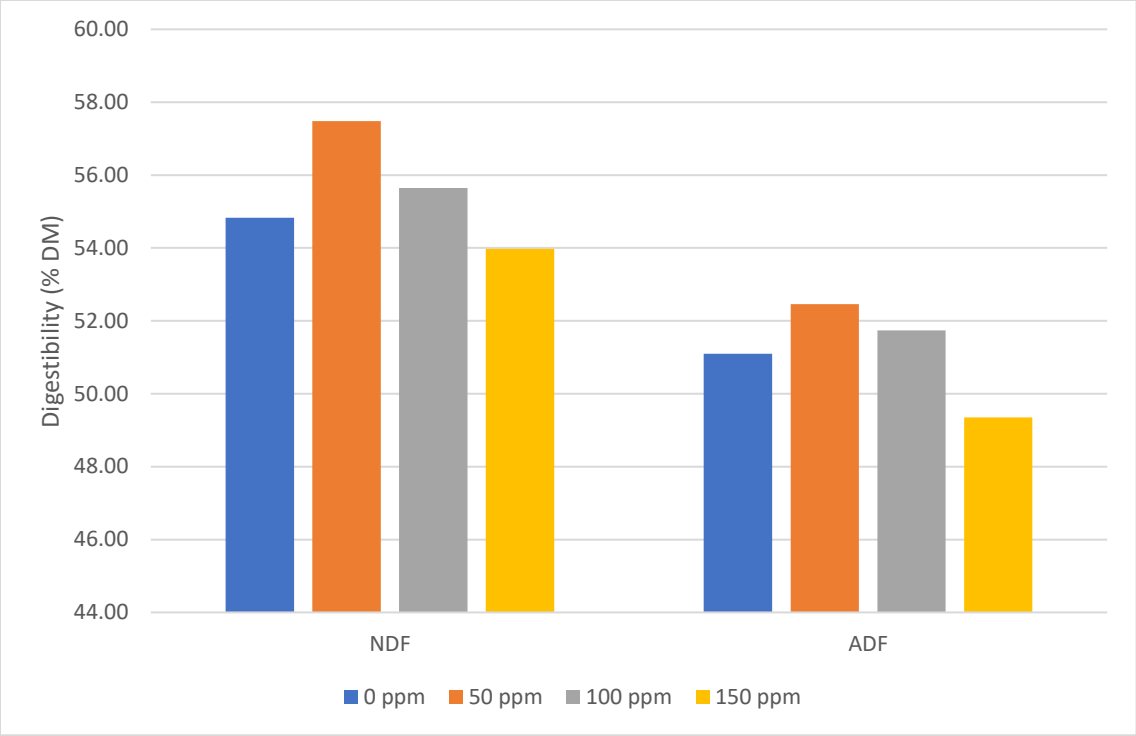


Figure 2.3 Postprandial total volatile fatty acids values on dual continuous cultures fermenters in response to caffeine doses (0, 50, 100, and 150 ppm).

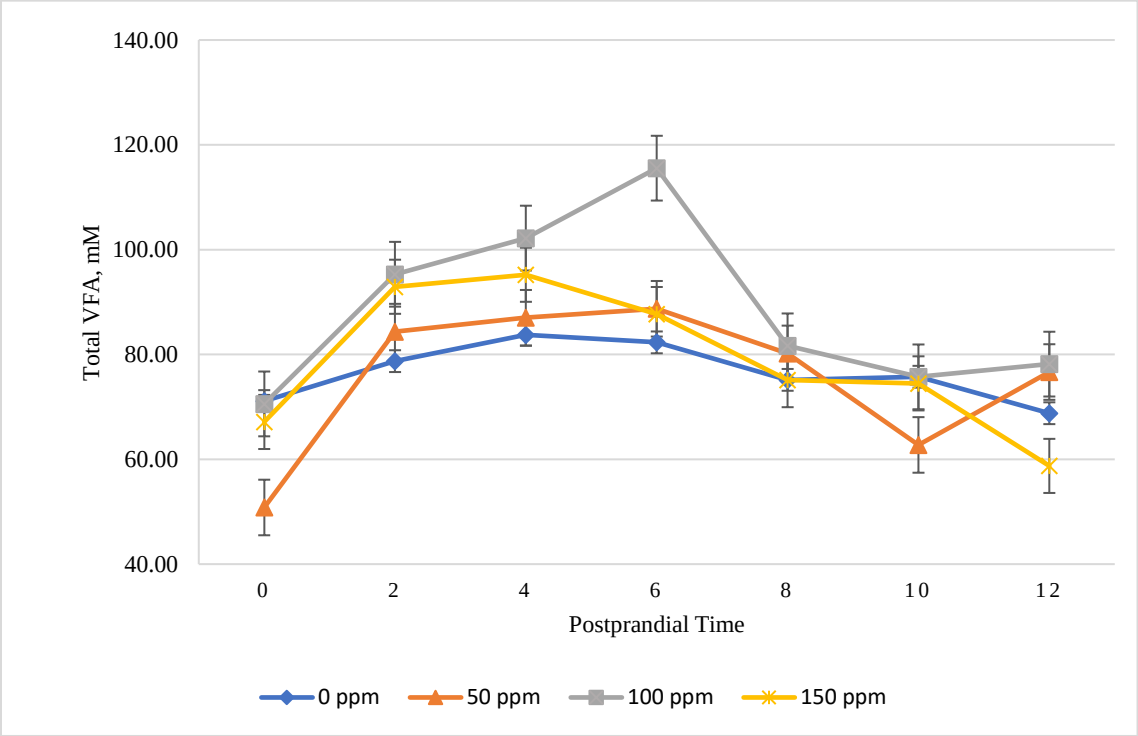


Figure 2.4 Postprandial acetic acid concentration in proportion to total VFA (mol/100 mol) on dual continuous cultures fermenters in response to caffeine doses (0, 50, 100, and 150 ppm).

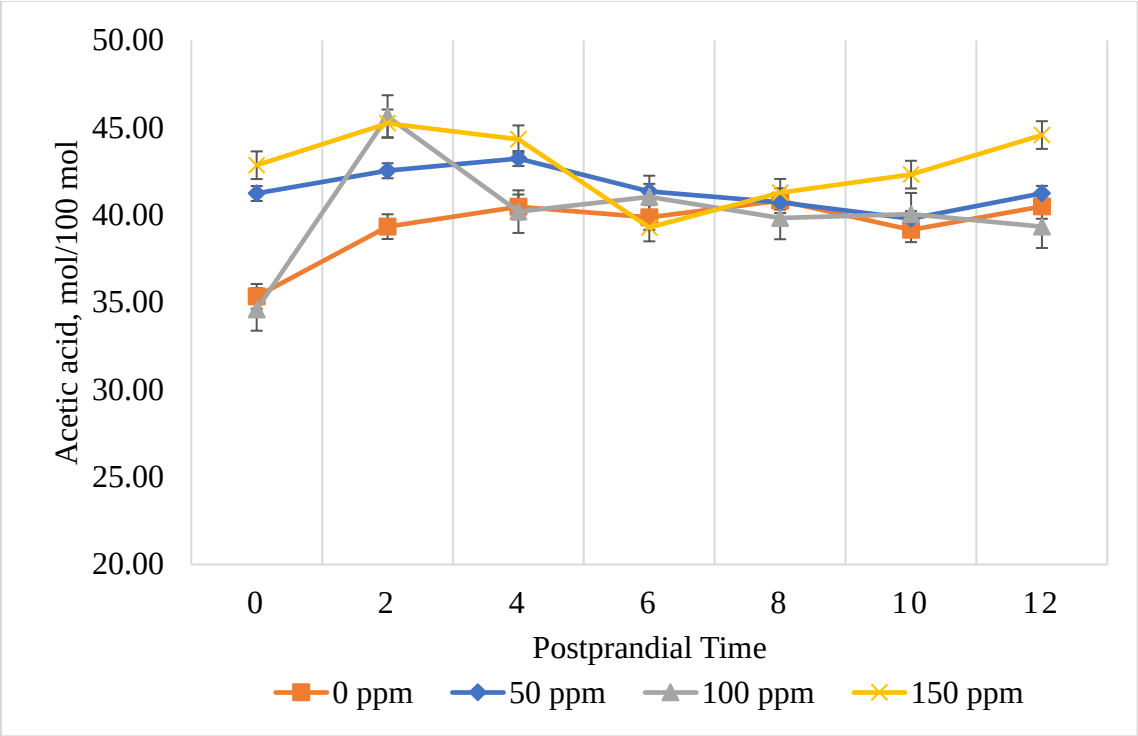


Figure 2.5 Postprandial propionic acid concentration in proportion to total VFA (mol/100 mol) on dual continuous cultures fermenters in response to caffeine doses (0, 50, 100, and 150 ppm).

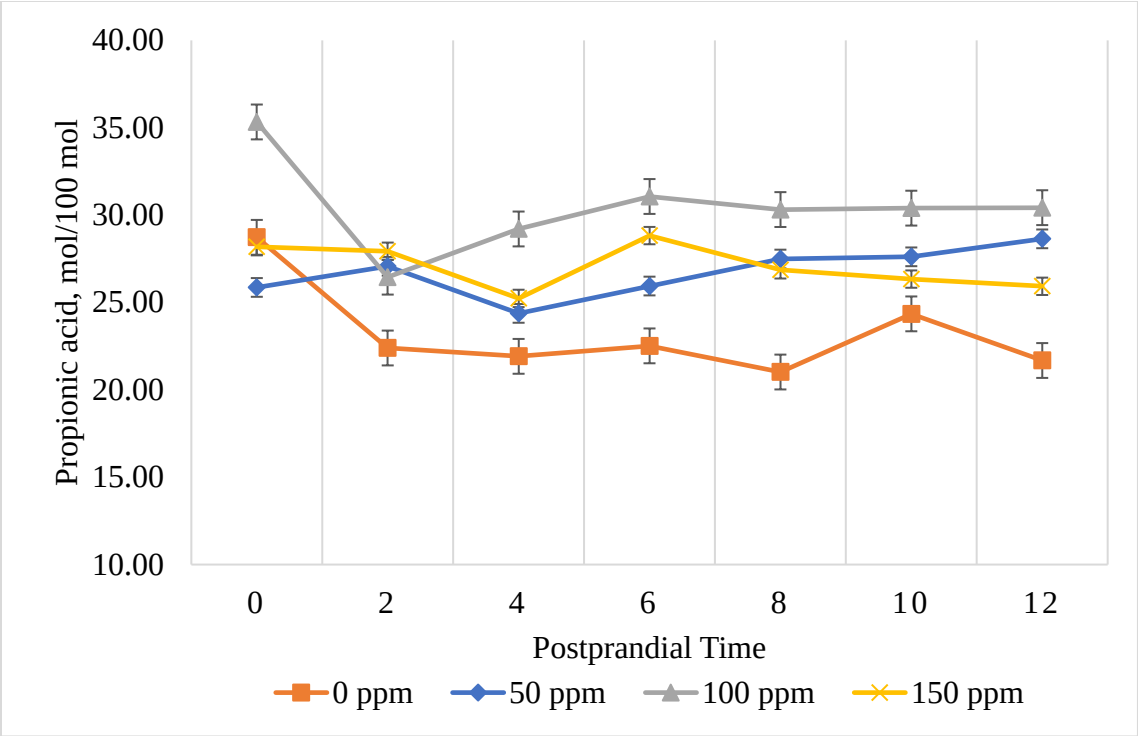


Figure 2.6 Postprandial acetate:propionate ratios concentration on dual continuous cultures fermenters in response to caffeine doses (0, 50, 100, and 150 ppm).

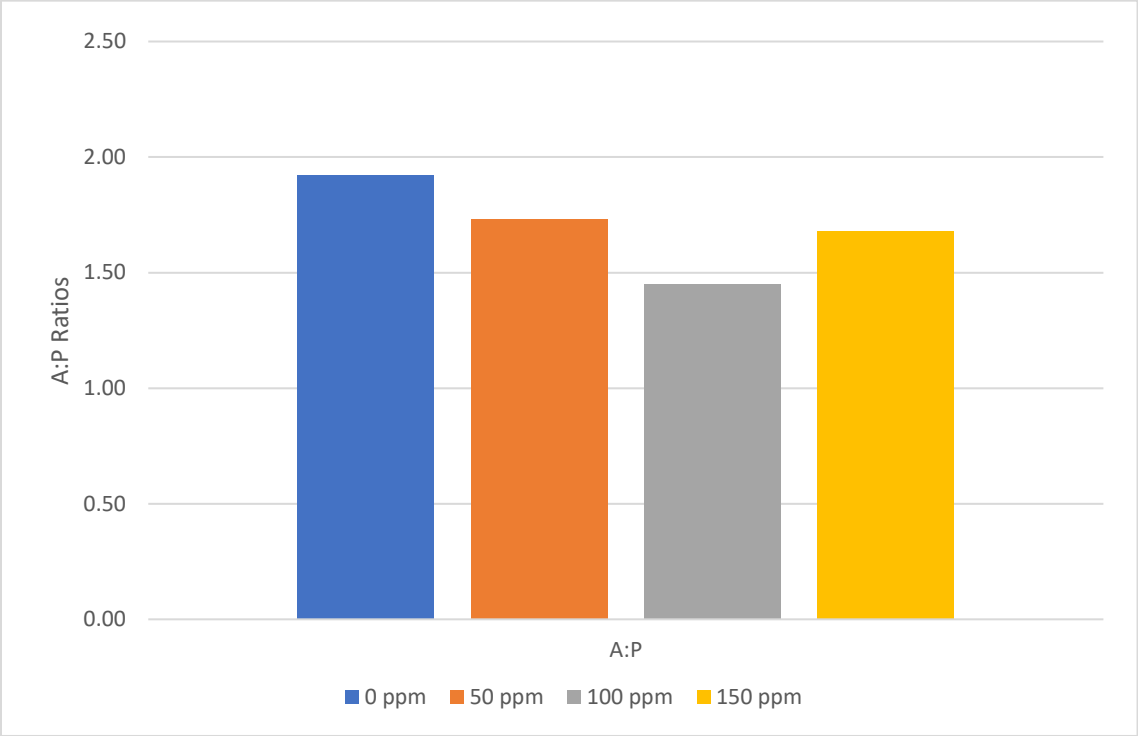


Figure 2.7 Ammonia concentration on dual continuous cultures fermenters in response to caffeine doses (0, 50, 100, and 150 ppm).

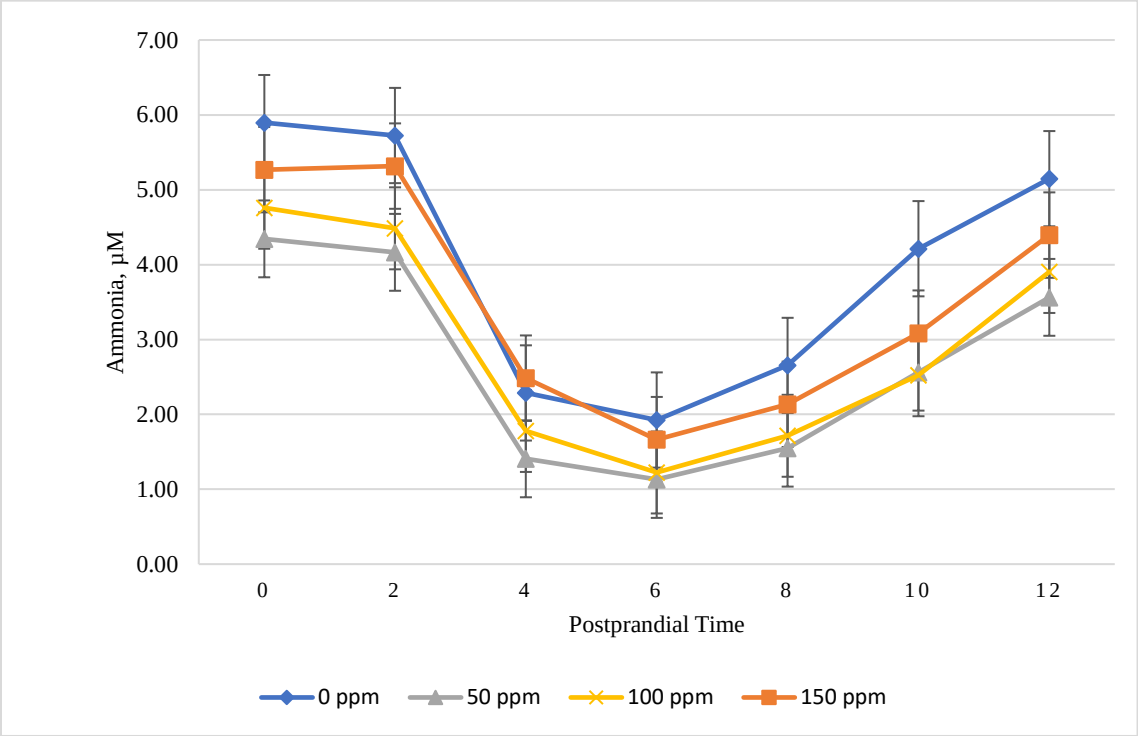
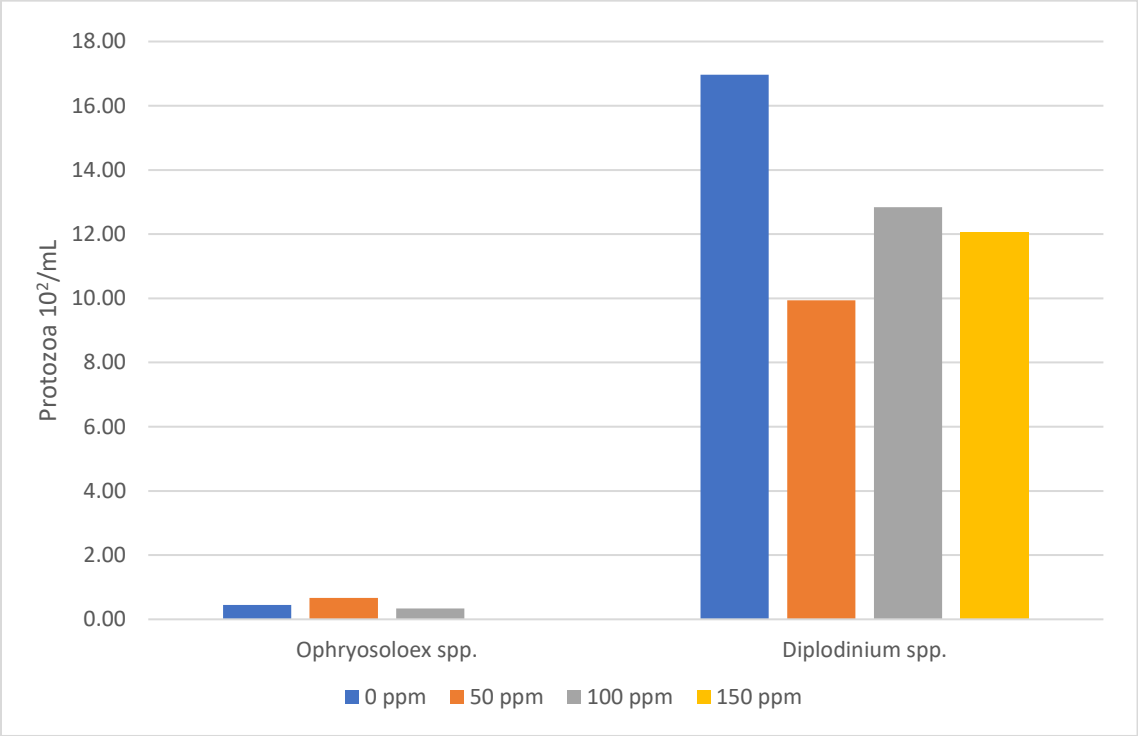


Figure 2.8 *Ophryosoloex* spp. and *Diplodinium* spp. amounts on dual continuous cultures fermenters in response to caffeine doses (0, 50, 100, and 150 ppm).



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