Investigating Enteric Methane Mitigation: Assessing the Impact of Various Materials on Ruminants' Fermentation, Intake, and Digestibility

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消化管からのメタン発生抑制:

種々の素材が反芻動物の胃内発酵、飼料摂取量および消化率に及ぼす影響の検討

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General Introduction

Farming animals, particularly within the ruminant sector, stands as a linchpin in the landscape of regenerative agriculture and global food security (Adegbeye et al., 2020). Ruminants, exemplified by cows, play an irreplaceable role in providing substantial quantities of milk and meat globally, thereby fulfilling a fundamental role in addressing the world's dietary needs (Sekaran et al., 2021). These animals underscore their significance by contributing a staggering 51% of the total protein derived from the global livestock industry. Within this notable contribution, 67% is attributed to milk, while the remaining 33% is allocated to meat, emphasizing the crucial role of ruminants in shaping the nutritional landscape (*OECD-FAO Agricultural Outlook 2023-2032*, 2023).

However, amidst this indispensable contribution, the global population is undergoing rapid expansion. Projections indicate that the world's populace will reach around 9.7 billion by 2050, with further estimates soaring to an estimated 10.9 billion by 2100. This burgeoning population poses a consequential increase in the demand for meat and milk, projected to surge by 14% for meat and over 30% for milk by 2030 (*OECD-FAO Agricultural Outlook 2023-2032*, 2023). This surge in demand places additional pressure on the livestock sector, particularly ruminant farming, to meet the nutritional needs of the growing global population.

Yet, as ruminants strive to meet this escalating demand, they present a substantial challenge concerning environmental sustainability. These animals emerge as significant emitters of greenhouse gases (GHGs), with 44% composed of methane (CH_4) and the remaining part almost evenly divided between 27% carbon dioxide (CO_2) and 29% Nitrous oxide (N_2O). In 2019, the agriculture sector contributed 22% of the total anthropogenic GHG emissions produced (*FAO Publications Catalogue 2023*, 2023;

Intergovernmental Panel on Climate Change (IPCC), 2023). The notable contribution of ruminants to GHG emissions makes them primary drivers of climate change, a phenomenon that poses severe threats to the well-being of both current and future generations (Althor et al., 2016).

The rapid escalation of GHG levels raises profound concerns, prompting warnings from institutions like the World Bank (*Reality Check: Lessons from 25 Policies Advancing a Low-Carbon Future*, 2023). If current trends persist, the Earth is on a trajectory to become 4 °C warmer, surpassing a critical threshold. This alarming temperature rise is associated with various devastating consequences, including recurrent and severe heatwaves, rampant wildfires, prolonged droughts, catastrophic floods, and an array of social disruptions such as famines and mass migrations (*Reality Check: Lessons from 25 Policies Advancing a Low-Carbon Future*, 2023; UN Climate Change, 2021).

In the complex interplay of addressing the rising global demand for nutrition while mitigating the environmental impacts of GHG emissions, the livestock industry, particularly in the ruminant sector, faces a pivotal challenge. Ruminant animals, exemplified by cows, produce GHGs such as CO_2 and CH_4 as natural by-products of digestion, a process called enteric fermentation. This intricate process involves methanogenic archaea, small organisms in their stomachs, producing CH_4, which is then released mostly when the animal burps. Unfortunately, this release signifies a substantial loss, amounting to up to 12% of the energy they consume, making it a notable inefficiency in the industry (Pickering et al., 2015).

In light of increasing public apprehension regarding the detrimental impacts of enteric CH_4 emissions on the efficacy and sustainability of livestock production, the scientific community, particularly in the realm of animal husbandry, is actively exploring strategies

for effective management. This quest involves a multifaceted approach, integrating scientific research, technological innovations, and conscientious agricultural practices. The primary goal is to mitigate GHG emissions from ruminants without compromising the environment, farmers' profits, or the animals' welfare (Adegbeye et al., 2020; Honan et al., 2021).

By fostering innovation and adopting sustainable practices, the industry strives to transition toward more efficient production systems. This approach not only ensures the fulfillment of essential nutritional needs for a growing global population but also contributes significantly to safeguarding the future of our planet. Embracing these solutions not only meets the immediate demands for food but also establishes a harmonious balance, ensuring the well-being of both humanity and the environment in the long run.

The efforts of research groups worldwide to combat CH_4 emissions and optimize production methods are noteworthy (Beauchemin et al., 2020; Hopkins et al., 2016). Among the most effective methods is dietary intervention through feed additives and by-products. Historically focused on improving animal health, dietary changes are now seen as promising for mitigating CH_4 emissions due to their impact on rumen microbes (Beauchemin et al., 2020; Hopkins et al., 2016). This approach offers a dual advantage, enhancing livestock production efficiency while preserving the environment (Beauchemin et al., 2020).

Numerous feed additives have undergone extensive research to mitigate CH_4 emissions. However, current studies, such as those available at Beauchemin et al. (2022) and Ahmed et al. (2022), indicate that there are currently no fully effective and sustainable options. A significant challenge faced by animal husbandry researchers is achieving 'clean,

green, and ethical' production. This necessitates that livestock products be free from chemical or synthetic compounds in ruminants' feed, aligning with animal welfare guidelines to minimize environmental impact and ensure sustainable and responsible livestock farming (Guyomard et al., 2021).

Due to the prohibition of synthetic compounds, such as antibiotics, in livestock feed across several countries, there's a growing global interest in utilizing bioactive components from plants and their by-products as alternative feed additives (Guyomard et al., 2021; Sandström et al., 2022). These include natural compounds like polyphenols, flavonoids, saponins, tannins, and organic acids, which have the potential to influence animal behavior, alter rumen fermentation, and inhibit various rumen microorganisms. While there are promising results in inhibiting rumen methanogens, challenges emerge when transitioning from *in vitro* studies to *in vivo* applications (Beauchemin et al., 2020; Kobayashi et al., 2016).

Utilizing human consumption by-products as food for ruminants is considered a potential way to replace conventional feed in the agriculture sector. Coffee, the second-most consumed beverage globally after water, generates various by-products during its processing stages. Spent coffee waste (SCW) is a major by-product containing significant amounts of organic compounds, lipids, phenolic compounds, and polysaccharides (Batbekh et al., 2023; Otálora et al., 2020). SCW, with its bioactive compounds, finds application in various industries, including biofuel, beauty products, construction, and animal feed (Carta et al., 2022; Kang et al., 2022; Wong et al., 2022; Xu et al., 2007). However, for SCW to serve as an alternative feed source for the livestock sector, consideration must be given to dosages, methods, and effects on animal health and

performance (Batbekh et al., 2023; Xu et al., 2007). The chemical composition of coffee by-products from previous studies described in Table 1.

Previous research extensively explored SCW as a potential ruminant feed, revealing various advantages and disadvantages, dependent on the applied dosage (Badarina et al., 2013; Batbekh et al., 2023; Campos-Vega et al., 2015a; Seo et al., 2015; Xu et al., 2007). High doses of SCW might impact nutrient digestibility, potentially affecting animal health and production (Batbekh et al., 2023; Xu et al., 2007). Understanding optimal dosage effects is crucial when considering SCW in ruminant diets, considering factors like cost, availability, processing, and compatibility with other dietary components (Xu et al., 2007). Implementing appropriate processing methods, such as ensiling, is vital to mitigating negative effects. Technologies for creating superior animal feed using SCW are crucial, especially in countries like Japan, enabling long-term storage. Silage production, combining wet and dry by-products, gains interest due to reduced effluent risk, improved rumen function, and longer storage potential. The lactic acid and soybean curd in silage enhance fermentation quality and digestibility and reduce CH_4 production.

Table1

Chemical composition of coffee by-products.

Parameters (%)	Coffee pulp	Coffee husk	Silver skin	Coffee spent
Cellulose	63.0 ± 2.5	43.0 ± 8.0	17.8 ± 6.0	8.6 ± 1.8
Hemicellulose	2.3 ± 1.0	7.0 ± 3.0	13.1 ± 9.0	36.7 ± 5.0
Protein	11.5 ± 2.0	8.0 ± 5.0	18.6 ± 4.0	13.6 ± 3.8
Fat	2.0 ± 2.6	0.5 ± 5.0	2.2 ± 1.9	ND
Total fiber	60.5 ± 2.9	24 ± 5.9	62.4 ± 2.5	ND
Total polyphenols	1.5 ± 1.5	0.8 ± 5.0	1.0 ± 2.0	1.5 ± 1.0
Total sugars	14.4 ± 09	58.0 ± 20.0	6.65 ± 10	8.5 ± 1.2
Pectic substance	6.5 ± 1.0	1.6 ± 1.2	0.02 ± 1.0	0.01 ± 0.005
Lignin	17.5 ± 2.2	9.0 ± 1.6	1.0 ± 2.0	0.05 ± 0.05
Tannins	3.0 ± 5.0	5.0 ± 2.0	0.02 ± 0.1	0.02 ± 0.1
Chlorogenic acid	2.4 ± 1.0	2.5 ± 0.6	3.0 ± 0.5	2.3 ± 1.0
Caffeine	1.5 ± 1.0	1.0 ± 0.5	0.03 ± 0.6	0.02 ± 0.1

From Mussatto et al. (2011b), Franca et al. (2009) and Murthy and Madhava Naidu (2010).
ND: not determined.

To address these challenges, researchers explore anti-methanogenic materials. In past studies, organic acids such as fumarate demonstrated the capability to reduce CH_4 in both *in vitro* and *in vivo* (Bayaru et al., 2001; Carro & Ranilla, 2003; Gheller et al., 2020; Islam & Lee, 2019; Palangi & Macit, 2021). Decreasing fumarate in the rumen alters how hydrogen is processed, leading to more propionate production and less CH_4 (Islam & Lee, 2019). Other organic acids, like citrate and itaconate, common in the swine and poultry industries, may also regulate rumen microbiota and metabolic activity, influencing CH_4 production. Additionally, acrylate, a substance in rumen metabolic pathways, is being explored as a potential additive to reduce CH_4 production in the rumen (Bampidis & Robinson, 2006; Oryza,S. et al., 2021; Oryza,S. et al., 2021b).

This growing interest in natural feed additives aims to enhance rumen fermentation efficiency, reduce GHGs, and ensure the sustainability and ethical practices of livestock production (Ku-Vera et al., 2020; Ponnampalam et al., 2023; Thornton et al., 2010). Exploring these innovative solutions seeks to strike a balance between effective CH_4 reduction and maintaining optimal animal performance and welfare. The availability of multiple additives is key to the widespread and sustainable implementation of methane-reducing strategies globally.

This integrated study comprises two parts. The first part explores the use of by-products from human consumption as feed and additives for ruminants, examining both raw and ensiled forms under *in vitro* conditions. The latter part concentrates on *in vivo* conditions, exploring the application of novel CH_4 suppressors.

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Chapter I

Assessment of the Impact of Coffee Waste as an Alternative Feed Supplementation on Rumen Fermentation and Methane Emissions in an *In Vitro* Study

Abstract

Spent coffee waste (SCW), a common by-product of the coffee industry, has shown potential as a dietary source for ruminants. This study aimed to determine the optimal inclusion dosage and application method of SCW in ruminant feed, investigating its impact on the rumen ecosystem and gas emission production. The *in vitro* study consisted of three trials with distinct experimental designs. In the first trial and experimental design (TRIAL. 1), a control diet with 500 mg of fresh matter basal diet (60% hay/40% concentrate) was used, and SCW was used as a feed additive at 1%, 10%, and 20% of the substrate. The second experimental design involved the same control diet, with SCW replacing either some of the hay (TRIAL. 2) or some of the concentrate mixture (TRIAL. 3) at four different dosages (30:70, 50:50, 70:30, and 100). When SCW was included as a feed additive, an increase in the production of volatile fatty acids (VFA) and gas was observed, but no suppressive effects on CH_4 production were noted. Conversely, when SCW replaced hay or concentrate, there were significant reductions in CH_4 production with increasing SCW inclusion levels. However, these reductions in CH_4 production were associated with negative effects on nutrient digestibility and total VFA production. These findings suggest that SCW could serve as a potential prebiotic feed additive. Moreover, the use of SCW as a substitute for silage at dosages of 70:30 and 50:50 appears feasible in animal feed (hay and concentrate).

I.1. Introduction

The world population is expected to reach approximately 9.7 billion people by 2050 and 10.4 billion people by 2100 (United Nations, n.d.). This growing population needs to be fed, so the prevalence of animal products, such as meat and milk, in human diets needs to be considered (Billen et al., 2015). Ruminants play an important role in animal production and contribute significantly to the overall quantity of animal products on the market. In the future, there will be an increased demand for ruminants in order to meet the food requirements of the growing global population. However, ruminants are also the most significant contributors to greenhouse gas (GHG) emissions, particularly CH_4. When their numbers increase, this has a significant effect on global warming (JIRCAS, 2010). In addition, the production of CH_4 during the fermentation process in the rumen is correlated with the loss of energy from the consumed feed. Moreover, CH_4 is 25 times more potent than CO_2 in terms of trapping heat from the sun (Hook et al., 2010). Therefore, reducing CH_4 emissions from ruminants would significantly decrease the associated environmental impacts, as long as energy utilization efficiency is not affected (Hristov et al., 2015). Thus, there is a need to find strategies that improve feed efficiency, balance the supply of nutrients to meet animal requirements, reduce environmental impacts and achieve economic benefits (JIRCAS, 2010). So, it is important for animal nutrition researchers to focus on finding alternative options to replace conventional resources and feed additives (Kobayashi et al., 2010).

Accordingly, using by-products from human food as feed for livestock is considered a possible solution. Coffee is one of the most popular beverages in the world and several by-products are generated throughout its processing stages. One of the major by-products of coffee is SCW, which contains large amounts of organic compounds, particularly lipids, polyphenols and polysaccharides (Campos-Vega et al., 2015). Due to the presence of these bioactive compounds, SCW is used in several industries, including biodiesel, cosmetics, construction

and animal feed (Carta et al., 2022; Kang et al., 2022; Wong et al., 2022; Xu et al., 2007; Yun et al., 2020). Moreover, the utilization of SCW as an alternative feed source for ruminants could help to mitigate CH_4 emissions, reduce waste and improve the environmental sustainability of livestock production; however, the dosages, processing methods and effects on animal health and performance need to be carefully considered.

Previously, researchers (Badarina et al., 2013; Campos-Vega et al., 2015; Seo et al., 2015; Xu et al., 2007) have focused on the potential of raw or ensiled SCW as a feed source for ruminants and have observed that SCW has some disadvantages, depending on the dosage. High levels of certain compounds, such as polyphenols, can hinder nutrient digestibility and affect animal health and production. Additionally, the use of SCW as a feed source for ruminants depends on a number of factors, such as cost, availability, processing and compatibility with other dietary components. Furthermore, appropriate processing methods (such as ensiling) need to be determined to mitigate any negative effects of compounds on animal performance and nutrient utilization. Large quantities of high-moisture by-products are produced in many countries, including Japan; therefore, there is a need to develop technologies to design superior animal feed using SCW and enable the long-term storage of the resulting silage (Cao et al., 2011). In Japan, there is an increasing interest in making silage by mixing wet and dry by-products, which offers a number of advantages, such as reduced risk of effluent production, stabilized rumen function and extended storage periods (Cao et al., 2011). The addition of lactic acid and soybean curd to silage when ensiling it with fresh grass or certain vegetable residues can improve fermentation quality. Moreover, when mixed with silage, these additives can also increase dry matter digestibility and reduce ruminal CH_4 production (Cao et al., 2011; Wang et al., 2022). Therefore, the objective of this *in vitro* study was to assess the impact of using raw or ensiled SCW as a feed additive or a partial replacement for the basal components (hay or concentrate) in ruminant diets on rumen fermentation profiles and CH_4

production. Moreover, it is also important to establish the optimal level of SCW in animal diets (Seo et al., 2015; Xu et al., 2007). However, there are still limitations to the potential use of SCW as a feed additive or replacement for conventional feed and the exact optimal dosages and methods remain unclear.

I.2. Materials and Method

I.2.1. Basal diet and SCW

The basal diet consisted of ground Kleingrass (*Panicum coloratum*) hay with a particle size of 1mm and a concentrate mixture. The SCW, both raw and ensiled, was provided in powder form by Sanyu Group Co., Ltd., Kanagawa, Japan. The chemical compositions of the SCW and basal diet components are detailed in Table 1.

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Table 1. Chemical composition of the feed used in this study

(Dry Matter g/kg)	Klein grass	Concentrate	Coffee (Raw)	Coffee (Silage)
Dry Matter (g/kg in fresh matter)	908.6	884.4	961.3	956.6
Organic Matter	909.1	943.6	978.2	961.8
Crude Protein	140.8	180.0	126.0	154.9
Ether Extract	20.6	35.0	140.0	146.3
NDF[1]	696.2	524.4	708.0	792.9
ADF[2]	366.7	97.0	442.4	422.8
ADL[3]	89.6	20.0	232.2	234.0

[1]NDF = neutral detergent fiber.

[2]ADF = acid detergent fiber.

[3]ADL = acid detergent lignin.

I.2.2. Preparation of the silage

The silage preparation was conducted at Sanyu Group Co., Ltd., Kanagawa, Japan. SCW was obtained from Starbucks coffee stores across Japan. After being stored at the Customer Futures Distribution Center by Starbucks' chilled logistics, samples were collected at the factory by Sanyu Group logistics. After being drained at the stores, the SCW was packed in plastic bags, sprayed with vinegar spray and sealed for storage. The collected substrates were mixed with dried bean curd, bran, soy sauce dregs, vinegar and lactic acid bacteria. Then, the mixture was put in the polyethylene bags and placed into a stainless steel container for incubation. The entire ensiling process lasted for 14 days and was performed in the Sanyu Group factory.

I.2.3. Collection of Rumen fluid

In this experiment, the used animals were kept and cared for by the Field Science Center, Obihiro University of Agriculture and Veterinary Medicine, Japan. The animal management and sampling procedures were approved by the Obihiro University of Agriculture and Veterinary Medicine, Animal Care and Use Committee (approval number, 20-119).

In this study, two rumen-fistulated, non-lactating Holstein cows, which were about 9 years old, were used as rumen fluid donor animals. The cows were fed at maintenance level on a diet of orchard grass hay (organic matter, 980 g/kg; crude protein, 132 g/kg; neutral detergent fiber, 701 g/kg; acid detergent fiber, 354 g/kg; acid detergent lignin, 40 g/kg; dry matter base), with access to free drinking water and mineral blocks. Approximately 650 milliliters of rumen fluid were collected from each cow using a vacuum tube, strained through four layers of surgical gauze. Subsequently, approximately 1.3 liters of this strained fluid were placed into a pre-warmed Thermos flask with hot water. The collected rumen fluid was immediately transferred to the laboratory.

I.2.4. Experimental Design and Incubation Procedure

In the present study, 500 mg of the substrate was added to pre-weighed and nylon bags that have a fixed size and a pore size of 53 ± 10 μm (BG1020, Sanshin Industrial Co., Ltd., Kanagawa, Japan), which were sealed using a heat-sealer before being placed into fermentation bottles.

This study was conducted using three experimental designs. The first experimental design (TRIAL. 1) was performed using a control diet of 500 mg of fresh matter basal diet (60% hay/40% concentrate), with SCW (both raw and ensiled) being used as a feed additive at 1%, 10%, and 20% of the substrate. In TRIAL. 1, the raw and ensiled SCW were added directly to the bottle and the experiments were conducted separately. The second experimental design was conducted using the same control diet as TRIAL. 1 but the SCW was added to the feed as a substrate in a nylon bag. The TRIAL. 2 and TRIAL. 3 were carried out on different days to assess the effects of replacing a portion of the hay or concentrate with SCW. TRIAL. 2 focused on replacing part of the hay with SCW, while TRIAL. 3 examined the replacement of a proportion of the concentrate with SCW. In the TRIAL. 2, four different dosages of SCW (raw and ensiled) were added to the basal diet to replace the hay 70:30 (42% hay/18% SCW/40% concentrate); 50:50 (30% hay/30% SCW/40% concentrate); 30:70 (18% hay/42% SCW/40% concentrate); and 100 (60% SCW/40% concentrate). In the TRIAL. 3, another four dosages of SCW (raw and ensiled) were used to replace a proportion of the concentrate 70:30 (60% hay/28% concentrate/12% SCW); 50:50 (60% hay/20% concentrate/20% SCW); 30:70 (60% hay/12% concentrate/28% SCW); 100 (60% hay/40% SCW). In TRIAL. 1, each group had four replicates and the experiment was repeated on four separate days. In TRIAL. 2 and TRIAL. 3, each group had three replicates and the experiments were repeated on three different days. In all of the trials, each run included two bottles of blank.

Via continuous CO_2 flushing, 40 mL of artificial saliva (McDougall, 1948) and 20 mL of rumen fluid were added to each fermentation bottle. The bottles were then reinjected with CO_2

before being sealed with rubber and aluminum caps (Maruemu Co., Ltd., Osaka, Japan). The incubation procedure was as described by (Ahmed et al., 2022).

I.2.5. In Vitro Incubation Procedure

After 24 h of incubation, total gas production was measured using a gas-tight syringe and headspace gas was collected from each bottle and stored in a vacuum tube (BD Vacutainer, Becton Drive, NJ, USA). Then, the gas composition was analyzed via gas chromatography (GC-8A, Shimadzu Corp., Kyoto, Japan), as described previously by Ahmed et al. (2022). Next, the bottles were opened, the pH was measured immediately using a pH meter (LAQUA F-72, HORIBA Scientific, Kyoto, Japan) and 1 mL of the culture medium was collected in an Eppendorf tube (Eppendorf AG, Hamburg, Germany) and centrifuged at 16,000×g at 4°C for 5 min. Following the centrifugation, the supernatant was gathered for further VFA analysis, which was measured via high-performance liquid chromatography (Shimadzu LC-20 HPLC, Shimadzu Corp., Kyoto, Japan). To determine the *in vitro* dry matter digestibility (IVDMD), the nylon bags containing the substrate were rinsed with tap water until the effluent became clear. They were then dried at 60°C for 48 h to enable us to measure the IVDMD, which was calculated as the percentage of DM that disappeared from the initial DM weight that was input into the bags.

I.2.6. Chemical analysis

The chemical composition analyses of the SCW, hay and concentrate mixture were performed according to the Association of Official Analytical Chemists procedures (AOAC, 1995). The DM content was determined by drying the matter in an oven at 135°C for 2 h (930.15). The OM and ash contents were measured by placing the samples in a muffle furnace at 500°C for 3 h (942.05). Nitrogen (N) content was measured according to the method of Kjeldahl (984.13) using an electrical heating digester (DK 20, VELP Scientifica, Usmate (MB), Monza, Italy) and an automatic distillation apparatus (UDK 129 VELP Scientifica, Usmate

(MB), Monza, Italy), CP was then estimated as N × 6.25. The NDF and ADF contents were estimated and expressed as the inclusive residual ash values using an ANKOM200 fiber analyzer (Ankom Technology, Methods 6 and 5, respectively; ANKOM Technology Corp., Macedon, NY, USA). The NDF content was measured using sodium sulfite without heat-stable α-amylase (FSS, ANKOM Technology).

I.2.7. Statistical analysis

All data were analyzed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). For all experiments, the data were analyzed using PROC MIXED models, including the treatments as fixed effects, whereas the experimental runs were considered random effects. The values are presented as the means with the pooled standard errors of the means. Any differences in means between the experimental groups were estimated using Tukey's test. The statistical significance difference was declared at $p < 0.05$.

I.3 Results

I.3.1 TRIAL. 1

The inclusion of both raw and ensiled SCW as an additive at 1%, 10% and 20% levels resulted in increased total gas production (8.7%-11.8%) compared to the control diet (Tables 2 and 4). Similarly, CH_4 and CO_2 production per digestible DM also increased, except for CH_4 production per digestible DM at the 1% addition level of raw SCW by 4% (Table 2) and total gas production at the 1% addition level of ensiled SCW, which decreased by 3.5% (Table 4). The IVDMD was significantly lower with the addition of raw SCW at the 10% and 20% levels compared to the control diet (3.4%-12.06%; Table 3), while ensiled SCW did not show any significant effects on digestibility (Table 5). The results regarding rumen fermentation showed mostly increased parameters when both raw and ensiled SCW were used as an additive (Tables 3 and 5). The total VFA production was significantly increased in the raw SCW groups and there was also an observable increase in the ensiled SCW groups (Tables 3 and 5). Additionally, the raw SCW groups showed increases in propionate production, while the ensiled SCW groups demonstrated increased butyrate production (Tables 3 and 5). Notably, none of the groups showed any significant effects on pH or the acetate-to-propionate (A:P) ratio (Tables 3 and 5). However, more significant effects on rumen parameters were observed when raw SCW was used as a feed additive.

I.3.2 TRIAL. 2 and TRIAL. 3

The inclusion of both the raw and ensiled SCW resulted in decreases in CH_4 production and total gas production compared to the control group (Tables 6 and 8). When hay was replaced with SCW, there were significant reductions in total gas production (5.4%-27.2%) and CO_2 production (5.5%-26.7%) in both the raw and ensiled SCW groups (Table 8). However, the

reductions in CH_4 production (ml/day) were more pronounced when the concentrate was replaced by either raw or ensiled SCW, with reductions ranging from 6% to 59%, compared to the grass diet replacement groups, which showed reductions of 1.9%-38% (Tables 6 and 8). The IVDMD was also significantly reduced by the inclusion of SCW, with the hay replacement groups showing reductions of 3%-27% and the concentrate replacement groups showing reductions of 3.8%-33.5% (Tables 7 and 9). However, the lower dosage of SCW as a concentrate replacement did not show significant reductions in IVDMD in any groups (Table 6). The VFA production was significantly reduced in all tested groups when raw SCW was used to replace both hay and concentrate (Tables 7 and 9), but there were no significant effects on VFA production in some of the ensiled SCW groups (Tables 7 and 9).

Additionally, butyrate production was unchanged when SCW was used to replace hay, although the percentage of production increased (Table 9). In contrast, when SCW was used to replace the concentrate, all groups showed significant reductions in butyrate production (Table 7). The A:P increased in all groups when raw SCW was used to replace hay, except for the 70:30 raw SCW group (Table 9), while none of the ensiled SCW groups showed any significant effects. In contrast, when the concentrate was replaced with SCW, most groups showed significant increases in the A:P ratio, except for the 70:30 ensiled group (Table 7).

Table 2: Effect of raw SCW as a feed additive on rumen fermentation characteristics

Item	Control	1%	10%	20%	SEM	P-Value
pH	6.69	6.69	6.68	6.69	0.006	0.090
IVDMD%	48.83^{ab}	50.29^{a}	47.16^{a-c}	42.94^{c}	1.21	0.008
Acetate (*mM*)	164.62	166.82	166.77	168.19	3.51	0.080
Propionate (*mM*)	53.88^{b}	55.37^{ab}	56.01^{ab}	56.37^{a}	1.54	0.020
Butyrate (*mM*)	20.03	20.52	20.35	20.57	0.58	0.300
TVFA[1] (*mM*)	238.55^{b}	242.71^{ab}	243.13^{ab}	245.14^{a}	4.45	0.040
Acetate (%)	68.98	68.80	68.63	68.64	0.67	0.210
Propionate (%)	22.57	22.74	22.98	22.95	0.46	0.110
Butyrate (%)	8.44	8.46	8.39	8.41	0.21	0.820
A:P[2]	3.13	3.11	3.08	3.06	0.08	0.200

[1]TVFA: Total Volatile Fatty Acids. [2]A/P: Acetate/Propionate. SEM: Standard Error of the Mean. $^{a, b, c}$ in the same row with different superscript means differ significantly ($p < 0.05$).

Table 3: Effect of raw SCW as a feed additive on gas profile

Item	Control	1%	10%	20%	SEM	P-Value
Total gas (ml/day)	52.91^{c}	56.00^{ab}	57.50^{ab}	58.66^{a}	1.26	<.0001
Total gas /DDM[1] (ml/g)	108.05^{b}	111.51^{b}	124.97^{b}	153.80^{a}	6.08	0.0001
CH_4 (%)	5.16	4.81	4.79	4.94	0.07	0.0600
CO_2 (%)	94.84	95.19	95.21	95.06	0.07	0.0600
CH_4 /DDM (ml/g)	5.54^{bc}	5.32^{bc}	5.92^{b}	7.46^{a}	0.26	0.0004
CH_4 (ml/day)	2.72	2.69	2.75	2.91	0.07	0.1500
CO_2 /DDM (ml/g)	102.51^{bc}	106.19^{bc}	119.04^{b}	146.34 ^{a}	5.83	0.0001
CO_2 (ml/day)	50.19^{c}	53.30^{ab}	54.75^{ab}	55.75^{a}	1.21	<.0001

[1] DDM, Digestible Dry Matter. SEM: Standard Error of the Mean. $^{a, b, c}$ in the same row with different superscript means differ significantly ($p < 0.05$).

Table 4: Effect of silage SCW as a feed additive on gas profile

Item	Control	1%	10%	20%	SEM	P-Value
Total gas (ml/day)	56.50^{bc}	54.50^{c}	61.70^{ab}	63.20^{a}	1.14	0.003
Total gas /DDM[1] (ml/g)	103.23^{ab}	97.30^{b}	112.12^{ab}	118.28^{a}	2.80	0.010
CH_4 (%)	3.95^{c}	6.97^{a}	5.73^{a-c}	6.04^{ab}	0.35	0.005
CO_2 (%)	96.04^{a}	93.02^{bc}	94.26^{ab}	93.95^{bc}	0.35	0.005
CH_4 /DDM (ml/g)	4.09^{c}	6.81^{ab}	6.42^{a-c}	7.15^{a}	0.40	0.011
CH_4 (ml/day)	2.24^{b}	3.82^{a}	3.53^{ab}	3.82^{a}	0.21	0.009
CO_2 /DDM (ml/g)	99.14^{ab}	90.48^{b}	105.69^{ab}	111.13^{a}	2.65	0.010
CO_2 (ml/day)	54.25^{a-c}	50.67^{c}	58.21^{ab}	59.42^{a}	1.06	0.002

[1] DDM, Digestible Dry Matter. SEM: Standard Error of the Mean. $^{a, b, c}$ in the same row with different superscript means differ significantly ($p < 0.05$).

Table 5: Effect of silage SCW as a feed additive on rumen fermentation characteristics

Item	Control	1%	10%	20%	SEM	P-Value
pH	6.66	6.64	6.66	6.65	0.005	0.460
IVDMD%	54.72	56.01	55.34	53.81	0.84	0.850
Acetate (*mM*)	151.50	148.07	152.28	156.86	1.42	0.180
Propionate (*mM*)	66.04	64.72	63.67	67.68	1.28	0.750
Butyrate (*mM*)	25.23^{ab}	24.70^{b}	27.31^{a}	27.37^{a}	0.39	0.009
TVFA[1] (*mM*)	242.77	237.51	243.28	251.92	2.72	0.320
Acetate (%)	62.41	62.34	62.65	62.27	0.17	0.890
Propionate (%)	27.19	27.25	26.07	26.84	0.31	0.550
Butyrate (%)	10.38	10.40	11.26	10.87	0.16	0.150
A:P[2]	2.29	2.28	2.41	2.32	0.03	0.600

[1]TVFA: Total Volatile Fatty Acids. [2]A/P: Acetate/Propionate. SEM: Standard Error of the Mean. $^{a, b}$ means in the same row with different superscript differ significantly ($p < 0.05$).

Table 6: Effect of SCW as a replacing concentrate on gas profile

Item	Control	Raw				Silage				SEM	P-Value
		30:70	50:50	70:30	100	30:70	50:50	70:30	100		
Total gas (ml/day)	48.22^{a}	45.67^{bc}	42.11^{d}	38.44^{d}	31.00^{e}	47.56^{b}	45.72^{bc}	41.11^{d}	36.56^{d}	0.65	<.0001
Total gas /DDM[1] (ml/g)	204.98^{ab}	201.71^{bc}	198.31^{bcd}	197.23^{bcde}	183.86^{befg}	206.99^{a}	204.77^{ab}	194.16^{bcef}	180.05^{g}	1.54	0.004
CH_4 (%)	4.78^{a}	4.40^{a}	4.19^{a}	3.93^{b}	3.07^{c}	4.57^{ab}	3.85^{b}	4.08^{b}	3.60^{b}	0.09	<.0001
CO_2 (%)	95.22^{a}	95.60^{a}	95.80^{a}	96.07^{b}	96.93^{b}	95.43^{b}	96.15^{ab}	95.92^{ab}	96.40^{a}	0.09	<.0001
CH_4 /DDM (ml/g)	9.69^{a}	8.70^{ab}	8.20^{bc}	7.63^{bc}	5.72^{d}	9.33^{ab}	8.70^{abc}	7.94^{c}	6.66^{d}	0.19	<.0001
CH_4 (ml/day)	2.32^{a}	2.02^{ab}	1.77^{bc}	1.52^{c}	0.95^{d}	2.18^{ab}	1.98^{b}	1.68^{c}	1.32^{d}	0.05	<.0001
CO_2 /DDM (ml/g)	##	187.8	187.02	186.31	181.79	194.38	192.74	186.19	178.09	1.42	0.15
CO_2 (ml/day)	45.91^{a}	43.64^{a}	40.34^{b}	36.93^{c}	30.05^{d}	45.37^{ab}	43.74^{ab}	39.43^{c}	35.23^{d}	0.61	<.0001

[1] DDM: Digestible Dry Matter. SEM: Standard error of the mean. $^{a, b, c, d, e, f, g}$ means in the same row with different superscript differ significantly ($p < 0.05$).

Table 7: Effect of SCW as a replacing concentrate on rumen fermentation characteristics

Item	Control	Raw				Silage				SEM	P-Value
		30:70	50:50	70:30	100	30:70	50:50	70:30	100		
pH	6.64^{d}	6.68^{c}	6.71^{ab}	6.72^{ab}	6.73^{a}	6.66^{a-d}	6.66^{abc}	6.68^{ab}	6.70^{a}	0.01	<.0001
IVDMD%	53.53^{a}	51.19^{a}	47.24^{b}	43.17^{c}	35.60^{d}	51.52^{ab}	49.81^{b}	46.19^{c}	42.69^{d}	0.65	<.0001
Acetate (*mM*)	39.27^{a}	38.03^{b}	52.24^{c}	49.58^{d}	45.36^{e}	55.75^{ab}	54.28^{bc}	52.59^{c}	50.32^{d}	0.94	<.0001
Propionate (*mM*)	14.24^{a}	13.18^{b}	36.95^{b}	35.65^{c}	33.46^{d}	38.73^{b}	37.97^{bc}	37.24^{cd}	36.14^{d}	0.27	<.0001
Butyrate (*mM*)	3.74^{a}	3.35^{b}	12.20^{c}	11.17^{d}	9.69^{e}	13.66^{ab}	13.14^{bc}	12.50^{c}	11.66^{d}	0.18	<.0001
TVFA[1] (*mM*)	57.24^{a}	54.56^{b}	3.09^{bc}	2.76^{c}	2.21^{d}	3.38^{b}	3.16^{bc}	2.85^{cd}	2.53^{d}	1.13	<.0001
Acetate (%)	68.14^{e}	69.22^{d}	70.24^{c}	71.36^{b}	73.23^{a}	68.92^{c}	69.46^{b}	70.30^{b}	71.27^{a}	0.41	<.0001
Propionate (%)	25.04^{a}	24.31^{a}	23.47^{b}	22.64^{c}	21.38^{d}	24.67^{ab}	24.36^{abc}	23.85^{cd}	23.17^{d}	0.16	<.0001
Butyrate (%)	6.82^{a}	6.47^{ab}	6.29^{bc}	6.00^{cd}	5.40^{e}	6.41^{ab}	6.19^{bc}	5.85^{cd}	5.56^{d}	0.36	<.0001
A:P[2]	2.73^{e}	2.86^{d}	3.00^{c}	3.16^{b}	3.43^{a}	2.80^{cd}	2.86^{bc}	2.95^{b}	3.08^{a}	0.03	<.0001

[1]TVFA: Total Volatile Fatty acids. [2]A/P: Acetate/Propionate. SEM: Standard error of the mean. $^{a, b, c, d, e}$ means in the same row with different superscript differ significantly ($p < 0.05$).

Table 8: Effect of SCW as a replacing grass on gas profile

Item	Control	Raw				Silage				SEM	P-Value
		30:70	50:50	70:30	100	30:70	50:50	70:30	100		
Total gas (ml/day)	53.89^{a}	50.44^{b}	47.44^{c}	46.06^{c}	39.22^{d}	50.89^{b}	51^{bc}	49^{bc}	44.89^{d}	0.5	<.0001
Total gas /DDM[1] (ml/g)	207.58^{ab}	211.89^{bc}	215.41^{bcd}	208.43^{bcde}	199.53^{befg}	199.41^{abc}	199.96^{ab}	201.20^{bcef}	188.91^{g}	1.4	<.0001
CH_4 (%)	4.83	4.61	4.75	4.72	4.11	4.73	4.99	4.57	4.28	0.1	0.3
CO_2 (%)	95.2	95.4	95.25	95.28	95.9	95.3	95	95.43	95.7	0.1	0.3
CH_4 /DDM (ml/g)	10	9.76	10.2	9.82	8.21	9.4	10.02	9.16	8.14	0.2	0.03
CH_4 (ml/day)	2.60^{a}	2.32^{a}	2.25^{a}	2.17^{a}	1.61^{b}	2.40^{abc}	2.55^{ab}	2.23^{abc}	1.92^{c}	0.1	<.0001
CO_2 /DDM (ml/g)	197.58^{ab}	202.13^{ab}	205.20^{a}	198.61^{ab}	191.32^{b}	190.00^{abc}	189.94^{abc}	192.04^{ab}	180.77^{c}	1.3	<.0001
CO_2 (ml/day)	51.29^{a}	48.12^{b}	45.20^{c}	43.89^{c}	37.61^{d}	48.49^{b}	48.45^{bc}	46.77^{bc}	42.96^{d}	0.5	<.0001

[1] DDM: Digestible Dry Matter. SEM: Standard error of the mean. $^{a, b, c, d, e, f, g}$ means in the same row with different superscript differ significantly ($p < 0.05$).

Table 9: Effect of SCW as a replacing grass on rumen fermentation characteristics

Item	Control	Raw				Silage				SEM	P-Value
		30:70	50:50	70:30	100	30:70	50:50	70:30	100		
pH	6.59^{b}	6.61^{ab}	6.61^{ab}	6.61^{ab}	6.63^{a}	6.60^{b}	6.58^{b}	6.60^{b}	6.59^{b}	0.004	0.0001
IVDMD%	58.14^{a}	52.60^{b}	48.27^{c}	48.05^{c}	42.30^{d}	56.41^{ab}	56.12^{abc}	53.19^{cd}	51.57^{d}	0.57	<.0001
Acetate (*mM*)	37.15^{a}	35.67^{b}	52.04^{c}	51.36^{c}	47.67^{d}	55.80^{abc}	55.99^{ab}	54.75^{bcd}	34.16^{d}	0.68	<.0001
Propionate (*mM*)	13.95^{a}	12.99^{b}	34.21^{c}	33.79^{c}	31.50^{d}	36.15^{abc}	36.21^{ab}	35.26^{bc}	12.84^{d}	0.24	<.0001
Butyrate (*mM*)	5.98^{abcde}	5.95^{abcde}	12.06^{c}	11.63^{c}	10.43^{d}	13.61^{ab}	13.62^{abc}	13.42^{abcd}	6.19^{a}	0.07	<.0001
TVFA[1] (mM)	57.08^{a}	54.62^{b}	5.77^{de}	5.94^{abcde}	5.75^{e}	6.04^{abcd}	6.17^{ab}	6.07^{abc}	53.07^{d}	0.86	<.0001
Acetate (%)	64.78^{d}	64.98^{bcd}	65.41^{abc}	65.46^{ab}	65.71^{a}	64.45^{ab}	64.33^{ab}	64.07^{b}	63.93^{b}	0.30	<.0001
Propionate (%)	24.36^{a}	23.73^{ab}	23.14^{bc}	22.61^{c}	21.87^{d}	24.34^{a}	24.27^{a}	24.47	24.2	0.11	<.0001
Butyrate (%)	10.87^{d}	11.29^{c}	11.44^{b}	11.93^{b}	12.43^{a}	11.21^{bc}	11.40^{bc}	11.46^{ab}	11.88^{a}	0.32	<.0001
A:P[2]	2.66^{d}	2.74^{cd}	2.83^{bc}	2.9^{b}	3.01^{a}	2.7	2.65	2.62	2.65	0.02	<.0001

[1]TVFA: Total Volatile Fatty acids. [2]A/P: Acetate/Propionate. SEM: Standard error of the mean. $^{a, b, c, d, e}$ means in the same row with different superscript differ significantly ($p < 0.05$).

I.4 Discussion

I.4.1 Nutritive value

In the present study, the proximate analysis showed that SCW has a high nutritive value (NDF > 650g/kg; ADF > 350g/kg; CP > 120g/kg). Previously, coffee grounds have been shown to contain high protein, fat and fiber levels, meaning that they can be considered a feed source for ruminant (Campbell et al., 1976). Several studies have used coffee pulp and husk as feed for ruminants (Badarina et al., 2013; Maxiselly et al., 2022; Mazzafera et al., 2002; Murthy & Madhava Naidu, 2012). Additionally, some previous studies have reported that coffee residue contains high levels of organic compounds and is an appropriate substrate for fermentation processes (Badarina et al., 2013; Seo et al., 2015; Xu et al., 2007).

I.4.2 SCW a feed additive at the 1%, 10%, 20% levels of DM

In the present study, the addition of raw SCW resulted in increased gas production and improved rumen fermentation parameters. The addition of raw SCW also led to increases in total VFA production and propionate production, with no changes in acetate production. These findings could be attributed to the presence of polyphenols and fatty acids in SCW, which have been shown to exhibit anti-methanogenic effects in the rumen (Beauchemin & McGinn, 2006; Bodas et al., 2012; Cieslak et al., 2012). However, the decrease in IVDMD with higher dosages of raw SCW was consistent with previous studies, which have indicated that plant secondary metabolites, including phenolic compounds and fatty acids, can slow intake degradation while improving ruminant production. These products can reduce the nutritive value of SCW at increased dosages, but they can also exhibit other beneficial rumen modulation effects, such as reducing protein and starch degradation and inhibiting amino acid degradation, via selective actions on certain rumen microorganisms. Some *in vivo* studies have reported that these

compounds can improve live weight, milk production, and ovulation rate in ruminants (A. K. Patra et al., 2013; Patra & Saxena, 2010; Waghorn & McNabb, 2003).

The addition of ensiled SCW resulted in increased gas production without any significant effects on the IVDMD or rumen fermentation parameters. However, there were increases in butyrate production with higher dosages of ensiled SCW. This could be attributed to the presence of certain compounds in the silage, which can affect the diversity of ruminal bacteria and the ability of certain bacterial taxa to degrade lignocellulosic material, ultimately leading to increased butyrate concentrations (Otálora et al., 2020; Goiri et al., 2020). The results from both the raw and ensiled SCW groups were almost the same, but the addition of raw SCW had a stronger impact on rumen parameters. In addition, both raw and ensiled SCW can be considered good sources of energy and protein, as previously described by Senevirathne et al. (2012). Therefore, they could potentially be used as prebiotic feed additives to enhance the health status of animals.

I.4.3 SCW as a feed replacement

In the present study, significant reductions in production were observed in almost all groups in TRAIL. 3 ($p < 0.05$). Previous studies have reported that coffee grounds contain significant amounts of lipids, particularly palmitic acid (C16:0) and linoleic acid (C18:2), which can contribute to reductions in CH_4 production (Jenkins et al., 2014; Somnuk et al., 2017). Dietary lipids, especially medium-chain fatty acids (MCFAs) and long-chain unsaturated fatty acids (UFAs), have been shown to decrease CH_4 production in ruminants (Beauchemin & McGinn, 2006; Castagnino et al., 2015; Dong et al., 1997; Machmüller et al., 1998). The addition of 1% fat, the most common source of MCFAs, can reduce CH_4 production by 3.1% to 9.1% (Aemiro et al., 2016; Grainger et al., 2008). Similarly, McGinn et al. (2004) found that the addition of sunflower oil decreased CH_4 production by 22% per 5% of DM. The results of the present study

confirmed that SCW, with its high fat content (up to 140 g fat/kg DM), increased dietary fat concentrations by 1.8% and suppressed CH_4 emissions by 12.9%.

In our findings, the use of SCW to replace hay or concentrate in the diet of ruminants resulted in significant decreases in IVDMD in almost all groups ($p < 0.05$). High lipid contents in ruminant diets have been shown to reduce DM, OM and fiber digestibility (Beauchemin & McGinn, 2006; Castagnino et al., 2015; McGinn et al., 2004; Patra et al., 2013). Previous studies have also reported that SCW contains a maximum of 14.7 wt% oil and high concentrations of tri- and monoglycerides (wt%), which can hinder feed particle adhesion and reduce nutrient availability for ruminal bacteria (Somnuk et al., 2017). Furthermore, the decreases in total gas and VFA production could be attributed to the interference of glycerol in SCW, which leads to slow feed particle adhesion and reduced degradability (Abo El-Nor et al., 2010; Castagnino et al., 2015).

Contrary to expectations, the addition of SCW resulted in reduced VFA production and increased pH compared to control, particularly in the concentrate replacement groups. This finding contradicted the results of some previous studies that showed little or no impact of SCW on ruminal pH or VFA production (Senevirathne et al., 2012; Seo et al., 2015). The reductions in VFA production and increases in pH could be attributed to certain polyphenolic compounds, such as tannins, lignans and caffeic acids, that are present in SCW. These compounds have been shown to reduce total VFA concentrations and alter ruminal microbial diversity (Badarina et al., 2013; Hassanat & Benchaar, 2013). These reductions in total VFA could also be related to the structures of tannin carbohydrate and protein compounds that cannot be degraded by rumen microbes or are toxic to ruminal microbes (Hassanat & Benchaar, 2013). The changes in the rumen microbial community caused by the SCW could also have led to altered rumen fermentation.

In previous studies, researchers have explored the use of coffee grounds as silage as a strategy to increase the nutritional value of animal diets and address environmental concerns (Badarina et al., 2013; Seo et al., 2015; Xu et al., 2007). The ensiling process involves fermenting and preserving the coffee grounds, potentially enhancing their digestibility, nutrient profile and storage time (Xu et al., 2007). However, in the present study, no significant effects of ensiled SCW were observed on rumen fermentation parameters. This suggested that ensiled SCW did not have any notable impacts on rumen microbial activity, gas production or volatile fatty acid production under the specific experimental conditions of this study.

Additionally, the use of SCW (raw or ensiled) as a replacement for hay or concentrate led to significant decreases in IVDMD and total VFA production, mostly at higher dosages. However, at lower dosages of ensiled SCW (specifically 70:30 and 50:50), there were no significant reductions in some ruminal fermentation parameters. Thus, these dosages could be feasible as a replacement for traditional animal feed.

I.5 Conclusions

The findings of this study indicated that SCW could serve as a feasible alternative to conventional ruminant feed ingredients due to its nutrient composition. The inclusion of SCW in ruminant diets has demonstrated promising results in terms of reducing CH_4 emissions, which is advantageous for minimizing the environmental impacts of ruminant agriculture. However, it is important to note that higher dosages of SCW may have adverse effects on animal production, although some lower dosages could be considered for use in animal feed. Therefore, further research is required to comprehensively evaluate the implications of using SCW in ruminant diets, as well as to determine the most suitable administration methods and optimal dosage levels. By conducting additional studies, we could gain deeper insights into the potential benefits and limitations of using SCW as a feed ingredient for ruminants. This knowledge could aid in the development of sustainable and efficient feeding strategies that could optimize animal health, productivity and environmental sustainability. It is through continuous investigation and scientific exploration that we can unlock the full potential of SCW as a valuable resource in ruminant nutrition and contribute to a more sustainable agricultural future.

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Chapter II

Citric acid: Impacts on Nutrient Feed Intake, Digestibility and Rumen Fermentation in Sheep

Abstract

The objective of this study was to explore the potential of citric acid as a feed additive for mitigating enteric methane (CH_4) emissions in sheep. In the current study, we measured nutrient intake, digestibility, ruminal fermentation (at 0, 3, 6, 9, and 12 hours after feeding), and CH_4 emissions with citric acid-supplemented Corriedale sheep. The experimental design involved a replicated 2 × 2 crossover with 15-day periods and included four Corriedale wether sheep (initial BW of 77.5 ± 2.75 kg). The treatments consisted of a control group (no additive) and citric acid (40 *mM*). The basal diet comprised Klein grass hay and a commercial concentrate mixture at a ratio of 2:1. Four respiration chambers for experimental treatments (animals fed the same diet two by two) were equipped to measure CH_4 emissions for two days in each period. The results of this study indicated that citric acid, when added as an additive to the sheep diet at high dosages, led to a reduction in animal feed intake and digestibility without suppressing CH_4 production.

II.1 Introduction

CH_4 emissions are an unavoidable consequence of the natural digestive process occurring in both wild and domesticated ruminant animals, including cattle, goats, sheep, and buffalos (Kelly & Kebreab, 2023). Within the intricate ecosystem of the ruminant's digestive tract, microbes break down food and fibers, providing essential energy and nutrients to the animal while simultaneously releasing CH_4 as a by-product of digestible energy loss. This biological phenomenon, known as enteric fermentation, stands as a significant contributor to CH_4 emissions arising from livestock. Approximately 12 percent of a ruminant's energy intake typically dissipates as CH_4 through this enteric fermentation process (Hill et al., 2016). However, the specific amount of CH_4 expelled by the animal is contingent upon several factors, such as the type and quality of the animal's diet, its health condition, reproductive status, and the surrounding environmental factors (Broucek, 2014).

Consequently, substantial efforts have been dedicated to mitigating CH_4 emissions from the rumen by regulating rumen fermentation through dietary manipulation. This approach primarily involves the utilization of feed or feed additives (Ahmed et al., 2022). Currently, feed additives are undergoing approval processes for practical application in numerous countries globally (Palangi & Lackner, 2022). Moreover, ongoing research is actively exploring additional functional additive candidates to broaden the array of options for alternative materials. The exploration of alternative additive candidates is crucial due to limited availability, particularly when locally or seasonally produced, and the persistent concern regarding potential diminishing effectiveness over time, as seen with antibiotic additives. Hence, having multiple readily available additives could significantly enhance the sustainable application of methane-reducing solutions on a

global scale. Therefore, some of the alternative feed additives, such as organic acids, are suppressing CH_4 production in the rumen indirectly. For example, fumaric acid, malic acid, formic acid, and other organic acids have been shown to reduce the amount of CH_4 in ruminants *in vitro* (Newbold & Rode, 2006; Palangi & Macit, 2021; Yamada et al., 2023) and *in vivo* conditions (Bayaru et al., 2001; Beauchemin & McGinn, 2006; Foley et al., 2009). However, not all studies have confirmed that organic acids suppress CH_4 in the field (Beauchemin & McGinn, 2006). Additionally, nowadays, animal nutritionists are interested in citrus-based products for ruminants or non-ruminants as a CH_4 suppressor or protein source (Yamada et al., 2023). Although Tanpong et al. (2021) used the by-product of citric acid as an energy source for quails, and their team also described citric acid by-products as an alternative energy source in animal feed (Tanpong et al., 2019). Thus, Suriyapha et al. (2022) described that citric acid waste fermented yeast waste did not have any significant effect on the ruminant's intake or digestibility. Furthermore, they described citric acid waste fermented yeast waste that could be used as an alternative source of additive without any negative impact on tropical lactating cows.

The aims of this study were to investigate the impact of citric acid, a feed additive approved for sheep, on enteric CH_4 production. Our investigation aimed to validate specific *in vitro* findings, as reported by Yamada et al. (2023), through *in vivo* analysis. This approach allowed us to bridge the gap between laboratory *in vitro* results and real-life *in vivo* conditions, providing a comprehensive understanding of the potential effects of citric acid as a CH_4 suppressor in the context of live sheep.

II.2 Material and method

II.2.1 Animals, treatments, and experimental procedures

The experimental design, animal handling, and sample collection processes were supervised by the Obihiro University of Agriculture and Veterinary Medicine, Animal Care, and Use Committee (approval number 22-182). The selected four sheep were matched in age and body weight, with an average weight of 77.5±2.75 kg, for a crossover design. The experiment spanned two periods of 15 days each, totaling 30 days, including 10 days for adaptation and 5 days for sampling. All animals were housed in a metabolic cage equipped with ventilated respiratory collection hoods individually, providing a sufficient place for standing or lying down comfortably.

The animals were fed twice a day at 8:30 and 16:30 h with a basal diet at a rate of 55 g dry matter (DM)/kg of BW $^{0.75}$/day, following the recommendation of Pen et al. (2007). The diet consisted of Kleingrass (*Panicum coloratum*) hay and a commercial concentrate mixture (Sky Dairy, Chubu Feed Co., Ltd., Aichi, Japan) at a ratio of 2:1. All four sheep had unrestricted access to clean fresh water and a lick mineral block containing iron oxide (1742 mg), ferric oxide (196 mg), copper sulfate (377 mg), cobalt sulfate (66 mg), zinc sulfate (1235 mg), manganese carbonate (1046 mg), calcium iodate (77 mg), sodium selenite (33 mg), and sodium chloride (971 g/kg) (KOEN® SELENICS TZ, Nippon Zenyaku Kogyo Co., Fukushima, Japan).

The treatment divided into two groups: a control (0 *mM* citric acid) and a treatment group (40 *mM* citric acid). The additional amount of citric acid per day is mixed through concentrates with the morning feed only. Animals were kept in the same room, keeping lights on, from 8 h to 17 h. Samples of the offered diet, refusal, fecal and urine samples

were gathered throughout the collection period and subjected to analysis for nutrient content using established protocols.

II.2.2. Chemical analyses and apparent total-tract digestibility assay

All the refused feed, feces, and urine were sampled over a 5-day period. The collection period was adjusted from day 10 to day 14, and the samples were kept frozen until the next analysis. When the collection period ended, the sampled feces were thawed at temperatures between 18-24°C for approximately 24 hours. After the thawing process, a 200g subsample from each treatment was dried at 60°C for 48 hours in the oven. The dried samples were then ground for subsequent laboratory analysis to determine apparent total tract digestibility. Daily urine collection was done using buckets with 100 mL of 10% (v/v) H_2SO_4 to maintain a pH below 3, with approximately 150 mL of the collected urine stored daily in the freezer for subsequent nitrogen (N) analysis. Following the AOAC International (1995) procedures offered and refused feed, feces, and urine samples were analyzed in triplicate for each treatment and period. Dry matter (DM) content was determined by drying samples in an oven at 135°C for 2 hours (method 930.15). Organic matter (OM) and ash were assessed by subjecting samples to a muffle furnace at 500°C for 3 hours (method 942.05). N content in samples and urine was determined using the Kjeldahl method (method 984.13), and crude protein (CP) was estimated as N × 6.25. An ANKOM200 fiber analyzer (Ankom Technology Methods 6, 5, and 8; ANKOM Technology Corp., Macedon, NY, USA) was used to measure neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin. The chemical composition of the basal diet is detailed in Table 1.

Table 1. Chemical composition of basal diet (g/kg Dry Matter)

Item[1]	Klein Grass	Concentrate
DM (g/kg in fresh matter)	908.6	884.4
OM	909.1	943.6
CP	140.8	180.0
EE	20.6	35.0
NDF	696.2	524.4
ADF	366.7	97.0
ADL	89.6	20.0

[1]DM: Dry Matter; OM: Organic Matter; CP: Crude Protein; EE: Either Extract; NDF: Neutral Detergent Fiber; ADF: Acid Detergent Fiber; ADL: Acid Detergent Lignin

II.2.3. Daily monitoring and health condition

The health status of the animals was monitored twice a day, checking for cough, diarrhea, nasal and ocular discharges. Body weight and average daily gain (ADG) were recorded weekly, and feed intake (FI) was documented daily.

II.2.4. Gas measurement and formula

The measurement of CH_4 and CO_2 occurred over a two-day period (days 13 and 14) using open-circuit respiration chambers. Each sheep was positioned in a gas collection chamber designed to maintain the sheep's head inside for the systematic collection of gas

emissions. CH_4 and CO_2 emissions from each chamber were recorded using an infrared gas analyzer. The machine of the gas analyzer was linked to a personal computer (PC) and programmed for data collection from each respiration cage, following a previously described protocol (Takahashi et al., 1999).

Before commencing gas measurements every single period on the 13th day morning, the analyzer underwent calibration using a calibrating gas containing 2.04% CO_2 and 907 ppm CH_4. The calculation of CH_4 energy involved determining the gas volume obtained from the respiratory chamber, following the formula: CH_4 energy (kJ) = CH_4 (L) × 39.57 (BROUWER E., 1965).

II.2.5. Rumen fluid sampling and rumen fermentation parameter analysis

On day 15, rumen fluid was gathered at five distinct intervals: prior to morning feeding (0 h), and then at 3, 6, 9, and 12 hours after morning feeding. The collection of rumen fluid samples involved inserting a stomach tube through the esophagus and utilizing a vacuum pump to extract the fluid. The initial portion of rumen fluid was not used as a sample, and the subsequent portion was promptly filtered through two layers of medical gauze. Around 45 mL of rumen fluid from each treatment was amassed in 50 mL tubes. A quick electronic pH meter measurement of the rumen fluid's pH took place. Upon reaching the laboratory, 3 mL from each tube was further transferred into two 2 mL Eppendorf tubes®. Volatile fatty acids (VFA) were analyzed following the procedures previously outlined by Ahmed et al. (2021).

II.2.6. Statistical analysis

In the analytical process, a replicated 2 × 2 design was deployed, utilizing the MIXED model procedure within SAS 9.4 (SAS Institute Inc., Cary, NC, USA). This model systematically factors in treatment and period as fixed effects while considering animals as random effects. For the examination of CH_4 and CO_2, a comprehensive analysis spanning 2 days was specifically integrated as fixed effects. Furthermore, the model applied to rumen fermentation data embraced treatment interactions as fixed effects. Significance was attributed to differences at $p < 0.05$, with tendencies acknowledged within the range of $0.05 < p < 0.10$. The elucidation of results adopted the presentation of least squares means, complemented by the standard error of the mean (SEM). To unravel the correlation coefficients among variables, the implementation of PROC CORR within SAS was executed, employing Pearson's method and a two-tailed test to gauge significance.

II.3 Results

II.3.1 Citric acid supplementation on nutrient intake in sheep

The treatments, whether supplemented with or without citric acid, did not display any signs of illness or disease throughout the entire duration of the experiment.

The intricate examination of nutrient intake in sheep, as delineated in Table 2, reveals the nuanced impact of including citric acid in the basal diet. Notably, the incorporation of citric acid resulted in a discernible decrease in fresh matter intake (FI), DM and CP ($p > 0.05$). Moreover, trends in reduced OM intake were observed among the sheep, albeit not reaching statistical significance ($p > 0.05$). The intake of NDF, while not demonstrating statistically significant outcomes in the current study ($p < 0.09$), reflects the complex interplay of dietary components and the potential influence of citric acid on the nutritional dynamics of the basal diet. This multifaceted exploration underscores the need for a comprehensive understanding of the intricate relationships between dietary additives and nutrient intake in ruminants.

Table 2. Citric acid supplementation on nutrient intake in sheep

Item[1] *(g/day)*	**Control**	**Citric acid**	**SEM**	***P*-value**
FI	1564.19	909.34	165.49	0.040
DM	1353.03	796.19	141.04	0.040
OM	1241.25	685.27	133.80	0.052
CP	189.02	101.77	22.00	0.040
NDF	292.54	192.30	57.26	0.090

[1] FI: Fresh matter intake; DM: Dry matter; OM: Organic matter; CP: Crude protein; NDF: Neutral detergent fiber

II.3.2 Citric acid on apparent total tract digestibility

Table 3 serves as a detailed repository, offering a thorough examination of apparent nutrient digestibility concerning various dietary interventions, with a particular emphasis on the incorporation of citric acid into the dietary regimen of sheep. This comprehensive analysis delves into the nuanced responses of the sheep's digestive system to the varying nutritional treatments explored in the study. Notably, the specific focus on citric acid inclusion in the sheep's diet unveils insightful observations.

Within this context, it is noteworthy to highlight that the augmentation of the diet with citric acid yielded results indicative of a lack of substantial influence on apparent ruminal digestibility. Despite the intricate interplay of dietary components and the physiological intricacies of the ruminal environment, the addition of citric acid emerged as a factor with no discernible impact on the sheep's digestive processes, particularly in the rumen.

This nuanced exploration not only contributes to the understanding of the dynamic relationship between dietary composition and nutrient assimilation but also sheds light on the specific role, or lack thereof, that citric acid plays in the intricate tapestry of the sheep's digestive physiology. The findings presented in Table 3 thus provide a valuable resource for researchers and practitioners seeking a deeper comprehension of the effects of citric acid supplementation on apparent nutrient digestibility in the context of sheep nutrition.

Table 3. Addition of citric acid on apparent total tract digestibility of nutrients in sheep

Item[1] ***(g/kg)***	**Control**	**Citric acid**	**SEM**	***P*-value**
DM	665.56	475.80	64.39	0.163
OM	648.34	402.27	76.10	0.167
CP	722.94	521.41	63.81	0.120
NDF	576.87	266.60	106.64	0.192

[1]DM: Dry matter; OM: Organic matter; CP: Crude protein; NDF: Neutral detergent fiber

II.3.3 Citric acid supplementation on CH_4 emissions

Table 5 provides a comprehensive overview of the influence of incorporating citric acid on CH_4 emissions. The various metrics used to measure CH_4 emission, including L/day, g/day, L/kg DM intake, g/kg DM intake, L/kg OM, g/kg OM, g/kg DDMI, and g/kg DOMI, did not show any effects that were statistically significant. Despite observing a numerical reduction in CH_4 production in ml/min/MBW, L/day and g/day, it is noteworthy that the intakes for CH_4 production displayed a numerical increase. These findings suggest a nuanced impact of citric acid on CH_4 emission metrics, warranting further exploration and consideration in the broader context of ruminant dietary management.

Table 5. Citric acid supplementation on CH_4 emissions in sheep.

CH_4 emission[1]	**Control**	**Citric acid**	**SEM**	***P*-value**
ml/min/MBW	0.51	0.48	0.02	0.579
L/day	19.81	18.53	0.99	0.064
g/day	14.15	13.24	0.71	0.064
L/kg DMI	14.14	28.65	4.48	0.155
g/kg DMI	10.15	20.46	3.19	0.157
L/kg OM	14.23	31.05	5.41	0.175
g/kg OM	10.16	22.18	3.86	0.175
g/kg DDMI	15.75	26.75	3.00	0.203
g/kg DOMI	17.67	36.14	5.07	0.200

[1]MBW: Metabolic Body Weight, DMI: Dry Matter Intake, OMI: Organic Matter Intake, DDMI: Digestible Dry Matter Intake, DOMI: Digestible Organic Matter Intake.

II.3.4 Citric acid supplementation on CO_2 emissions

Additionally, the result of CO_2 production is shown in Table 6. CO_2 production did not change with the inclusion of citric acid in any parameters, such as L/day, g/day, L/kg DM intake, and g/kg DM intake.

Table 6. Addition of citric acid on Carbon dioxide (CO_2) emissions in sheep.

CO_2 emissions[1]	**Control**	**Citric acid**	**SEM**	***P*-value**
ml/min/MBW	6.21	5.77	0.55	0.539
L/day	241.66	223.15	11.64	0.106
g/day	172.61	159.39	8.31	0.106
L/kg DMI	173.59	336.42	47.68	0.128
g/kg DMI	123.99	240.30	34.06	0.128

[1]MBW: Metabolic Body Weight, DMI: Dry Matter Intake

II.3.5 *Citric acid supplementation on Rumen Fermentation Profile*

The basal diet treated with citric acid exhibited no significant effect on TVFA, showing a reduction as a tendency ($p < 0.08$). Acetate production, when compared to the control, did not yield any significant results ($p > 0.1$). However, concentrations of propionate and butyrate (mmol/L) showed a notable decrease, with a significant reduction of 18.92% and 25.82%, respectively ($p < 0.05$), when compared to the control group. Furthermore, the addition of citric acid to the diet did not result in a significant effect on the A/P ratio ($p = 0.3$). Exploring the proportions of the three main VFAs, acetate percentage demonstrated an increase of 4.57% ($p = 0.004$), while propionate and butyrate percentage production experienced a decrease with the inclusion of citric acid, showing reductions of 7.96% and 17.83%, respectively ($p < 0.01$). These nuanced shifts in VFAs emphasize the impact of citric acid on rumen fermentation patterns and underline the intricate nature of dietary interventions in influencing microbial processes in the rumen. Although there was no significant increase in pH with the citric acid supplement.

Table 3. Citric acid supplementation on rumen fermentation profile in sheep.

Variable[1]	Control	Citric acid	SEM	*P*-value
TVFA (*mM*)	81.70	72.19	3.06	0.085
Acetate (*mM*)	58.77	54.20	1.80	0.164
Propionate (*mM*)	14.11	11.44	0.64	0.025
Butyrate (*mM*)	8.83	6.55	0.78	0.034
Acetate (%)	72.02	75.31	0.91	0.302
Propionate (%)	17.21	15.84	0.29	0.004
Butyrate (%)	10.77	8.85	0.74	0.005
A/P ratio	4.34	4.65	0.12	0.014
pH	6.63	6.78	0.09	0.112

[1] TVFA: Total Volatile Fatty Acids, A/P ratio: Acetate/Propionate ratio.

II.4 Discussion

Mitigating CH_4 emissions not only serves to reduce environmental impact but also holds the potential to increase ruminant feed efficiency. Methanogenesis, the process by which CH_4 is made in the rumen, needs energy. Finding ways to decrease CH_4 without negatively affecting rumen fermentative processes could result in both environmental and economic benefits for the cattle industry. Numerous synthesized and organic compounds have shown anti-methanogenic effects in *in vitro* conditions (Cattani et al., 2016; Chagas et al., 2019; Ku-Vera, Jiménez-Ocampo, et al., 2020). However, many of these compounds sometimes have negative impacts on animal intake, digestibility, and fermentation production (Patra et al., 2017).

In this study, citric acid was used as a feed additive to suppress products for ruminants, but it did not have any significant effect on CH_4 output. The high-forage diet used in this study consisted of Klein grass hay and a commercial concentrate mixture at a ratio of 2:1. Previously, Yamada et al. (2023) reported that citric acid, used as a feed additive for *in vitro* batch culture conditions, suppresses CH_4 production using the same ratio as the basal diet. Additionally, they also suggested that a high-forage basal diet treated with citric acid suppresses CH_4 production by 9%-10%. They reported that citric acid reduced CH_4 production without the accumulation of hydrogen and increased propionate but increased short-chain fatty acids (SCFA), especially acetate production in the rumen (Yamada et al., 2023). They also suggested that CH_4 production might occur through the acrylate pathway and discussed the necessity of testing this material on ruminant animals. However, *in vitro* and *in vivo* studies are incomparable, as *in vivo* processes are sometimes not accurately represented. Previous studies mostly describe that the supplementation of organic acids has a positive or no effect on feed intake and digestibility of ruminants (Bampidis &

Robinson, 2006; Clemmons et al., 2021; Gheller et al., 2020). However, in the current study, the addition of citric acid to feed significantly reduced nutrient intake, although the ruminal fermentation process, specifically total VFA and acetate, propionate, and butyrate, remained unaffected. In previous studies, feed intake decreased due to some feed additives, such as essential oils, ionophores, and other additives, and these materials also led to a lack of digestibility in the rumen (Azzaz et al., 2015; Beauchemin & McGinn, 2006; Hou et al., 2023). However, these materials decreased utilization and simultaneously suppressed CH_4 production too (Azzaz et al., 2015). In the current study, addition of citric acid did not affect the ruminal digestibility. Although, there was no significant reduction in CH_4 production or the fermentation process, but utilization was reduced, possibly due to the higher dosage of citric acid and potentially lower palatability for the animals.

II.5 Conclusions

This study demonstrates that citric acid reduces feed intake when added in high amounts. However, these materials did not affect the digestibility and fermentation process of the rumen. Although some *in vitro* studies have shown promising results in suppressing CH_4 emission, the translation to lower CH_4 emission *in vivo* is influenced by many other confounding factors. Further research is still required to identify optimal levels and usage methods for these products in commercial ruminant feed.

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Chapter III

Itaconic acid: Impacts on Feed Intake, Digestibility and Rumen Fermentation in Sheep

Abstract

Methane (CH_4) is a major contributor to GHG emissions globally, and its increasing production poses significant threats to humans, animals, and the environment. The livestock sector, particularly ruminant animals, is responsible for approximately 40% of agricultural CH_4 emissions.

The aim of this study was to identify organic acids that can effectively reduce enteric CH_4 emissions from ruminants. The research assessed the impact of an organic acid, namely itaconic acid, on feed intake, digestibility, CH_4 emissions, and ruminal fermentation in Corriedale sheep following a specific diet. The experimental design involved replicated 2 × 2 and 15-day periods. The present study utilized four Corriedale wether sheep (initial BW of 76.5±5.1 kg) as a treatment.

The treatments consisted of two animals for the control group (no additive) and another two animals for itaconic acid (40 *mM*). The basal diet included Klein grass and a commercial concentrate mixture at a ratio of 2:1. Four chambers were equipped to measure CH_4 emissions over a 2-day period in each cycle. Feces, urine, and refusal samples were collected throughout the collection period. Rumen fluid was collected at five different times, including before morning feed, at 3-hour intervals, with the final collection 12 hours after morning feed.

The present study did not show any significant results in CH_4 production for all collected data except CH_4 emissions (L/kg DMI). CH_4 emissions (L/kg DMI) increased significantly in the study, and there were also some significant changes in rumen fermentation results. All fermentation parameters, whether statistically or non-statistically, showed a reduction in this study, and due to this result, total VFA was also significantly

reduced ($p > 0.04$). Furthermore, itaconic acid is potentially a feed additive for the livestock sector, but there is still limited information on its advantages and disadvantages. Further research is needed to clarify the potential of this product.

III.1 Introduction

CH_4, the second GHG following carbon dioxide CO_2, possesses a higher energy-absorbing capacity than CO_2. Moreover, CH_4 boasts a 28-fold greater greenhouse potential than CO_2 (Intergovernmental Panel on Climate Change (IPCC), 2023). The surge in atmospheric CH_4 concentrations is attributed to burgeoning human populations, intensified feed production, and anthropogenic sources driving CH_4 production (Philip Thornton & Mario Herrero, 2010).

Ruminant animals, including cattle, sheep, and goats, inherently emit CH_4 during their physiological digestive processes (Soren et al., 2017). Notably, cattle and dairy cows emerged as the predominant contributors to CH_4 emissions in 2020, constituting 72% of the total sector emissions (Reisinger et al., 2021).

Throughout anaerobic bacterial fermentation, CH_4 emission arises in the rumen and the hindgut and is subsequently released into the environment (la Fuente et al., 2019). Microorganisms break down feed components into smaller molecules like amino acids, simple sugars, and volatile compounds (Terry et al., 2019). Bacteria and protozoa constitute 80% of the microbial mass inside the rumen, generating CH_4 (Newbold & Ramos-Morales, 2020). This process, however, results in a loss of 2–12% of the host's metabolic energy intake. Hence, a global endeavor is imperative to curtail ruminant emissions (Günal et al., 2019).

Feed additives assume the role of methanogen inhibitors, both directly and indirectly. In recent years, researchers have considered strategies for mitigating enteric CH_4, particularly focusing on organic acids such as malic and fumaric acids. These acids, intermediates of the citric acid cycle in the propionate-succinate pathway, provide an alternative electron-sink pathway, diverting hydrogen that might otherwise contribute to

methanogenesis. Studies by Bayaru et al. (2001) and Carro & Ranilla (2003) extensively explored the *in vitro* effects of fumaric and malic acids, reporting dose-dependent reductions in CH_4 production. Nonetheless, certain *in vitro* studies, exemplified by Czerkawski & Breckenridge (1972), suggested that specific organic acids might stimulate CH_4 production.

The methane-suppressing mechanism is indirect, involving the reduction of fumarate in the rumen, thereby altering the hydrogen-metabolizing pathway. This shift induces a transition from CH_4 to propionate production, a pivotal step in reducing CH_4 emissions. Furthermore, organic acids like citrate, gluconate, and itaconate, prevalent in the swine and poultry industries, await thorough evaluation for their impact on rumen metabolism. It is possible that these acids may regulate the rumen microbiota and metabolic activity, contributing to CH_4 suppression. Additionally, potential candidates for suppressing ruminal CH_4 production include known acids used in synthesizing fumarate and malate, potentially enhancing propionate production. For example, the addition of fumarate to rumen batch cultures has demonstrated a decrease in CH_4 production and an increase in propionate production (Newbold et al., 2005). Therefore, exploring precursors and intermediates of rumen metabolic pathways as candidate additives is essential for comprehending their potential to mitigate CH_4 emissions.

The aim of our present study was to investigate the impact of a novel feed additive, itaconic acid, registered for feeding to sheep, on enteric CH_4 production and utilization in ruminants. Intriguingly, several previous studies have highlighted itaconic acid's anti-inflammatory and anti-pathogenic activities in mammals (Cordes et al., 2015; O'Neill & Artyomov, 2019; Peace & O'Neill, 2022). However, there is limited information available

regarding the use of itaconic acid in ruminant feed. Only Yamada et al. (2023) have described its potential in mitigating CH_4 production from ruminants, albeit in an *in vitro* setting. Our study builds upon this, confirming these *in vitro* results through *in vivo* analysis.

The inclusion of itaconic acid as a registered feed supplement for sheep prompted our investigation into its effects on enteric CH_4 production. Notably, while previous research suggests anti-inflammatory and anti-pathogenic properties in mammals, its application in ruminant feed remains an understudied area. Previously, some studies provided a valuable *in vitro* perspective, demonstrating CH_4 mitigation potential. Our study bridges the gap by extending these findings to *in vivo* conditions, thus contributing to the understanding of itaconic acid's role in ruminant nutrition.

III.2 Material and method

III.2.1 *Animals, treatments, and experimental procedures*

The experimental design, animal care, and sampling process of the current study were all approved by the Obihiro University of Agriculture and Veterinary Medicine Animal Care and Use Committee (approval numbers 23-37). Each sheep, weighing 76.5±5.1 kg, was used in a 2 x 2 cross-over design. The experiment spanned a total of 30 days, divided into two 15-day periods. Each period consisted of an adaptation period of 10 days and a 5-day sampling period. The current study treatment comprised a control group (0 *mM* itaconic acid) and a treatment group (20 *mM* itaconic acid). The further methods used are all followed by a second chapter.

The chemical composition of the basal diet is described in Table 1.

Table 1. Chemical composition of basal diet (g/kg Dry Matter)

Item[1]	Klein Grass	Concentrate
DM (g/kg fresh matter)	908.6	884.4
OM	909.1	943.6
CP	140.8	180.0
EE	20.6	35.0
NDF	696.2	524.4
ADF	366.7	97.0
ADL	89.6	20.0

[1]DM: Dry matter; OM: Organic matter; CP: Crude protein; EE: Either extract; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; ADL: Acid detergent lignin

III.3 Results

III.3.1 *Addition of itaconic acid to nutrient intake*

There were no observable changes in DM and OM intake ($p > 0.05$) following the inoculation of itaconic acid in the sheep's diet, as indicated in Table 3. Furthermore, the intake of crude protein (g/d) did not exhibit significant variations ($p > 0.05$), and the intake of neutral detergent fiber (NDF) remained consistent ($p > 0.05$) between the groups supplemented with itaconic acid and those without supplementation.

Table 2. Addition of itaconic acid on nutrient intake in sheep (g/day).

Item[1]	Control	Itaconic acid	SEM	*P*-value
FI	1387.74	1326.07	54.64	0.27
DM	1367.14	1318.84	20.43	0.30
OM	1263.26	1218.09	19.11	0.30
CP	193.70	182.39	4.40	0.30
NDF	716.08	678.82	16.56	0.30

[1] FI: Fresh Matter Intake; DM: Dry Matter; OM: Organic Matter; CP: Crude Protein; NDF: Neutral Detergent Fiber

III.3.2 *Addition of itaconic acid to apparent nutrient digestibility*

Table 3 presents a comprehensive overview of the apparent nutrient digestibility in response to dietary treatments, specifically the inclusion of itaconic acid in the sheep's diet. The digestibility of DM, OM, NDF, and CP showed no discernible impact attributable to the addition of itaconic acid ($p > 0.05$).

Table 3. Addition of itaconic acid on apparent total tract digestibility of nutrients (g/kg) in sheep

Item[1]	Control	Itaconic acid	SEM	*P*-value
DM	694.20	702.21	10.12	0.68
OM	671.07	678.32	12.12	0.76
CP	754.37	739.46	5.37	0.13
NDF	592.17	589.72	18.48	0.93

[1]DM: Dry Matter; OM: Organic Matter; CP: Crude Protein; NDF: Neutral Detergent Fiber

III.3.3 *Addition of itaconic acid to Rumen Fermentation*

The impact of itaconic acid concentration on ruminal parameters was examined in this study. Surprisingly, ruminal pH remained unaffected by the inclusion of itaconic acid in the diet. However, a more detailed analysis revealed significant alterations in total volatile fatty acids (VFAs) and propionate concentration due to the supplementation of itaconic acid, as highlighted in Table 4. While other individual VFAs did not display significant changes, there was a distinct rise in the proportion of acetate by 2.75%. Intriguingly, propionate and butyrate production experienced a reduction of 14.19% and 5.41% respectively, when compared to the control group. These findings emphasize the nuanced effects of itaconic acid on ruminal fermentation dynamics.

Table 4. Effect of Itaconic acid on rumen fermentation profile at different times in sheep.

Variable[1]	Control	Itaconic acid	SEM	*P*-value
TVFA (*mM*)	79.69	74.47	2.92	0.043
Acetate (*mM*)	56.64	55.22	2.05	0.281
Propionate (*mM*)	14.32	11.48	0.79	0.013
Butyrate (*mM*)	8.42	8.18	0.31	0.611
Acetate (%)	71.57	73.54	0.56	0.008
Propionate (%)	17.90[a]	15.36	0.52	0.028
Butyrate (%)	10.53	11.10	0.45	0.011
A/P ratio	4.01	4.82	0.17	0.383
pH	6.66	6.64	0.05	0.919

[1] TVFA: Total Volatile Fatty Acids, A/P ratio: Acetate/Propionate ratio.

III.3.4 *Addition of itaconic acid to CH_4 emission*

In the *in vivo* assessment, the inclusion of itaconic acid in the sheep's diet did not show any significant changes in CH_4 emissions (L/d) or (g/d) ($p > 0.05$), as indicated in Table 5. However, CH_4 production parameters, such as g/kg OMI, DDM and DOMI, did not demonstrate statistically significant variations.

Table 5. Effect of Citric acid supplementation on CH_4 emissions in sheep.

CH_4 emission[1]	**Control**	**Itaconic acid**	**SEM**	***P*-value**
ml/min/MBW	0.62	0.64	0.02	0.512
L/day	23.13	24.03	0.54	0.486
g/day	16.52	17.16	0.39	0.486
L/kg DMI	16.96	18.21	0.35	0.174
g/kg DMI	12.12	12.95	0.25	0.174
L/kg OM	17.07	18.33	0.35	0.152
g/kg OM	12.19	13.09	0.25	0.152
g/kg DDMI	28.32	30.90	1.63	0.094
g/kg DOMI	18.29	20.80	0.88	0.194

[1]MBW: Metabolic Body Weight, DMI: Dry Matter Intake, OMI: Organic Matter Intake, DDMI: Digestible Dry Matter Intake, DOMI: Digestible Organic Matter Intake.

III.4 Discussion

CH_4 emissions were not affected when itaconic acid was included in a sheep diet at a dose of 20 *mM*. Several studies, including Yamada et al. (2023), have reported that the addition of 10 to 30 *mM* of organic acids in the ratio of ruminants mitigates CH_4 production significantly, by about 5 to 23% (Bayaru et al., 2001; Carro & Ranilla, 2003; Clemmons et al., 2021; Palangi & Macit, 2021). Carro & Ranilla (2003) reported that inoculating fumarate (from 0 to 10 *mM*) into substrate in batch culture increased the production of VFA, decreased the A:P ratio, and reduced CH_4 production by up to 5%. Similarly, studies (Islam & Lee, 2019) indicate that most organic acids reduce CH_4 production by acting as hydrogen sinks and stimulating the proliferation of ruminal microorganisms.

However, there is less information on the effects of organic acids used *in vivo* Bayaru et al. (2001) added 20 g/kg of DMI of fumaric acid (about 18 *mM*) into a cattle feed and found a 23% reduction in CH_4, along with a significant increase in propionate production. Similar results (Palangi & Macit, 2021) were observed with several organic acids, either separately or in a mixture, at varying levels on VFA and CH_4 mitigation in the Awassi Rams diet. They found a significant reduction in CH_4 production after 24 h of incubation, while some organic acids increased the propionic acid percentage, especially fumaric acid. Contrastingly, McGinn et al. (2004) and Beauchemin & McGinn (2006) fed approximately 15 *mM* and an estimated 50 *mM* of fumaric acids to cattle diets, respectively, reporting no effect on rumen fermentation or CH_4 emissions. Due to the demand for clarity on this topic, it is necessary to investigate recently used organics or suggest novel feed additives, such as new organic acids. Previously, Yamada et al. (2023) reported that itaconic acid suppressed CH_4 production by 60% without affecting rumen

fermentation. In the same study, the proportion of butyrate increased due to the increase of Megasphaera, an important bacterium in the rumen that metabolizes lactate to produce butyrate. According to Cordes et al. (2015), itaconic acid has antipathogenic activities, leading to changes in ruminal microbiome diversity when the sheep diet contains itaconic acid, as observed in our present study. Although our present study results showed a significant reduction in total VFA, propionate and butyrate ($p < 0.05$), the proportion of propionate in the rumen decreased, and the A/P ratio increased ($p < 0.05$). The propionate reduction result is in agreement with Yamada et al. (2023); contrastingly, they also described butyrate production as increased by itaconic acid, but our result did not follow this result.

Yamada et al. (2023) described that itaconate did not result in accumulating hydrogen as a feed in ruminants' diets; CH_4 suppression might have been due to the acrylate pathway via lactate production. These results disagree with our findings. In our study, CH_4 production was not suppressed by itaconic acid. Yamada et al. (2023) reported that rumen pH might also be reduced by itaconic acid, but in our findings, there was no effect on rumen pH ($p > 0.05$).

III.5 Conclusions

The inclusion of itaconic acid at 20 *mM* in sheep diets did not impact CH_4 emissions, contrasting previous findings with organic acids. While some *in vitro* studies have suggested CH_4 reduction potential with fumarate, translating these effects to live animals is less explored. Also, some *in vivo* studies showed promise in reducing cattle CH_4 by 23% with fumaric acid, but inconsistent results from other studies questioned its efficacy. Itaconic acid, whose previous *in vitro* study reported reducing CH_4 by 60%, yielded conflicting results in our study, indicating variability in its effectiveness. Overall, the search for reliable CH_4 suppressors continues, emphasizing the need for nuanced understanding and exploration of novel additives for consistent mitigation in ruminant diets.

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General Summary

The current thesis study contains two parts: the first one focuses on the use of by-products from human consumption as feed or feed additives for ruminants *in vitro*, while the other, the main objective of our study, involves the addition of organic acids *in vivo* conditions, exploring the application of novel CH_4 suppressors. Each part includes both *in vitro* and *in vivo* experiments with a specific emphasis on mitigating CH_4 emissions, enhancing animal productivity, and at the same time, addressing economic concerns related to ruminant feed.

Ruminant livestock, notably cattle, supply meat and milk on a global scale. Nevertheless, the expanding global population and its growing appetite for meat and milk present formidable challenges to the livestock sector, particularly in the realm of environmental sustainability. While fulfilling nutritional requirements, ruminants make a substantial contribution to GHG emissions, prominently featuring CH_4 as a major constituent.

The escalating levels of GHGs, especially CH_4, raise concerns about climate change, prompting efforts to find solutions in the livestock industry. Nowadays, researchers highlight the inefficiency of ruminant digestion, leading to CH_4 emissions and energy loss. Researchers are actively seeking strategies to mitigate these emissions without compromising animal welfare or farmer profits. In the last decades, increased attention has been given to CH_4 mitigation technologies, with promising findings from dietary interventions. However, most of these strategies have economic disadvantages, conflict with animal welfare regulations, adverse effects of some approaches on animal performance, and rumen fermentation. Due to this, there is a need to find new materials

that can mitigate CH_4 and be beneficial for farmers and animals. Dietary interventions, including feed additives and by-products, are being explored as potential solutions.

By-products, especially those derived from human food, are considered a potential idea for inclusion in animal diets. In the first chapter of our thesis, we explored a specific by-product, namely spent coffee waste (SCW), which comprises products deemed unconsumable by humans for ruminants' diets. SCW, rich in organic compounds, is examined for its potential as ruminant feed, with attention given to dosage, processing methods, and effects on animal health.

The current first chapter is conducted *in vitro* batch culture using two different experimental designs with three TRIALS. The first experimental design, TRIAL. 1, was performed with a control diet of 500 mg (fresh matter basal diet, 60% hay/40% concentrate), and coffee waste was used as a feed additive at 1%, 10%, and 20% of the substrate. The second experimental design, TRIAL. 2, and TRIAL. 3, were performed with the control group and coffee waste replaced part of the grass (TRIAL. 2) or the concentrate mixture (TRIAL. 3). The four different dosages (30:70, 50:50, 70:30, and 100) inclusion levels of SCW in the basal diet (raw or ensilage) were achieved by replacing the grass or concentrate.

During our *in vitro* batch culture study, TRIAL 1, SCW was supplemented as a feed additive, resulting in an increase in the production of volatile fatty acids by up to 2.7% and gas production by 10.86%, but it did not show a suppressive effect on CH_4 production. In contrast, in TRIAL 2 and 3, when SCW was included as a replacement for grass or concentrate for substrate, there was a significant reduction in CH_4 production with increasing levels of SCW inclusion. This reduction in CH_4 production was accompanied by negative effects on nutrient digestibility and total volatile fatty acid production. These

findings demonstrate that SCW could be used as a potential prebiotic feed additive. However, when SCW is used as a replacement at 70:30 and 50:50 for silage, these dosages appear to be feasible for use in the field as a substitute for animal feed (grass and concentrate).

Amidst the exploration of new strategies to mitigate CH_4 emissions, feed additives, particularly organic acids, emerge as a potential avenue. Previous studies have investigated various organic acids as CH_4 suppressors for ruminants; however, their results exhibit variability and inconsistency. Furthermore, the majority of the data stems from *in vitro* studies, with limited information from *in vivo* experiments. Given these challenges, there is a pressing need to identify a new supplement that can effectively suppress enteric CH_4.

In our second chapter, we explored the use of citric acid as a feed additive for sheep. Recent research has expressed interest in citrus-based products as CH_4 suppressors or protein sources for animal diets (Yamada et al., 2023). Notably, previous studies have utilized citric acid by-products and citric acid waste-fermented yeast waste as alternative energy sources, demonstrating no adverse effects on chicken farming or tropical lactating cows.

In this *in vivo* study, we measured nutrient intake, total tract digestibility, ruminal fermentation (at 0, 3, 6, 9, and 12 hours after feeding), and CH_4 emissions in Corriedale sheep fed a diet supplemented with citric acid. The experiment followed a replicated 2 × 2 crossover design with 15-day periods and involved 4 Corriedale wether sheep (initial BW of 77.5±2.75 kg). Treatments included a control group (no additive) and the addition of citric acid (40 mM). The basal diet comprised Kleingrass hay and a commercial concentrate mixture at a ratio of 2:1. The animals in the four respiration chambers were

equipped to measure CH_4 emissions for two days each period, with two 24 h measurement cycles. In the present study, the addition of citric acid reduced feed intake and digestibility, though it did not exhibit suppressing effects on CH_4 production.

Our third chapter investigated the effects of itaconic acid as a feed additive at a dosage of 20 mM. Given its potential anti-inflammatory and anti-pathogenic activities, itaconic acid was hypothesized to counteract methanogens in the rumen, presenting a possible avenue for mitigating CH_4 production from ruminants. However, information on the use of itaconic acid as a feed additive in ruminant diets is exceedingly limited. Only one study has reported its potential to reduce CH_4 in *in vitro* conditions. Therefore, the objectives of our current study mirror those of the second chapter, measuring the same parameters using the same methods.

Despite its potential, itaconic acid did not yield significant results in CH_4 production for all analyzed parameters, except for CH_4 emissions (L/kg DMI). Our study observed a significant increase in CH_4 emissions (L/kg DMI) in sheep whose diet contained itaconic acid. All rumen fermentation parameters, including statistically significant changes in propionate and non-statistically significant changes in acetate and butyrate, exhibited a reduction in our third chapter, leading to a significant decrease in total VFA. While itaconic acid demonstrates potential as a feed additive for the livestock sector, there is still limited information on its advantages and disadvantages. Further research is needed to clarify the full potential of this product.

要約

本論文の第 1 章は、反芻動物の飼料または飼料添加物としてのヒトの食品副産物の利用に関して *in vitro* 実験によって評価したものであり、第 2 章は、本研究の主な目的である、反芻動物に対する有機酸の添加によるメタン(CH_4)抑制の効果を検討した。第 1 章では *in vitro*、第 2 章では *in vivo* 実験を行い、CH_4 排出の抑制、家畜の生産性向上、同時に反芻家畜の飼料に関する経済的懸念への対応に重点を置いて検討した。

反芻家畜、特に牛は、世界規模で肉と牛乳を供給している。とはいえ、世界人口の増加と肉と牛乳に対する需要の増大は、畜産部門、特に環境の持続可能性の点において課題が突きつけられている。反芻家畜は栄養要求を満たす一方で、温室効果ガス排出に大きく寄与しており、特に CH_4 が主要な構成要素となっている。

大気中では温室効果ガス、特にCH_4のレベルが上昇していることから、気候変動に対する懸念が高まり、畜産業においてその解決策を見出す努力が続けられている。現在、研究者たちは、CH_4 の排出とエネルギーロスにつながる反芻動物の消化効率の悪さに着目している。研究者たちは、動物福祉や酪農家の利益を損なうことなく、これらの排出を軽減する戦略を積極的に模索している。ここ数十年、CH_4 緩和技術に注目が集まっており、飼養管理から有望な知見が得られている。しかし、これらの戦略の大半は経済的デメリット、動物福祉規制との抵触、畜産物の生産性やルーメン発酵への悪影響がある。このため、CH_4

を緩和し、酪農家と家畜にとって有益な新素材を見つける必要がある。飼料添加物や副産物を含む食餌への介入が、解決策として検討されている。

副産物、特に人間の食物に由来する副産物は、家畜の飼料に配合できる可能性があると考えられている。本論文の第 1 章では、特定の副産物、すなわち使用済みコーヒー廃棄物（SCW）について検討した。有機化合物を豊富に含む SCW について、反芻動物の飼料としての可能性を、投与量、加工方法、動物の健康への影響に注意を払いながら検討した。

第 1 章では、3 つの TRIAL による 2 つの異なる実験計画を用いて、試験管内バッチ培養を行った。最初の実験デザインである TRIAL.1 では、対照飼料を 500mg（乾草 60%/濃厚飼料 40%）とし、飼料添加物として SCW を基質の 1%、10%、20%として用いた。第二の実験計画である TRIAL.2、および TRIAL.3 は対照群とし、SCW を牧草の一部（TRIAL.2）または濃厚飼料の一部（TRIAL.3）に置き換えた。基礎飼料（生またはサイレージ）中の SCW の含有量は、牧草または濃厚飼料を置き換えることで 4 種類（30:70、50:50、70:30、100）とした。

試験管内バッチ培養試験である TRIAL 1 では、SCW を飼料添加物として投与した結果、揮発性脂肪酸の生産量が最大で 2.7%増加し、ガス生産量が 10.86%増加したが、CH_4 生産量の抑制効果は認められなかった。対照的に、TRIAL2 と 3 では、SCW を牧草や濃厚飼料に置き替わる基質として配合した場合、SCW 配合量の増加に伴い CH_4 生産量が有意に減少した。この CH_4 産生量の減少は、栄養消化率および総揮発性脂肪酸産生量への負の影響を伴っていた。これらの結果は、SCW がプレバイオティック飼料添加物として利用できる可能性を示して

いる。しかし、SCW をサイレージの代替物として 70：30 および 50：50 で使用する場合、これらの用量は動物飼料（牧草および濃厚飼料）の代替品として牧場で使用することが可能であると思われる。

CH_4 排出を抑制する新たな戦略が模索される中、飼料添加物、特に有機酸が手段として浮上してきた。これまでの研究では、反芻家畜の CH_4 抑制剤として様々な有機酸が研究されてきたが、その結果にはばらつきがあり、一貫性がない。さらに、データの大半は *in vitro* 試験によるもので、*in vivo* 実験による情報は限られている。これらの課題を考えると、消化管内 CH_4 を効果的に抑制できる新しいサプリメントを特定することが急務である。

第 2 章では、羊の飼料添加物としてのクエン酸の利用について検討した。最近の研究では、柑橘類をベースとした製品が CH_4 抑制剤や動物用飼料のタンパク質源として注目されている（Yamada ら、2023）。注目すべきは、これまでの研究で、クエン酸副産物やクエン酸廃棄物-発酵酵母廃棄物を代替エネルギー源として利用し、養鶏や熱帯泌乳牛に悪影響がないことを実証していることである。本 *in vivo* 試験では、クエン酸を添加した飼料を与えたコリデール種羊の栄養摂取量、消化率、ルーメン発酵（給餌後 0、3、6、9、12 時間）、CH_4 排出量を測定した。実験は 1 期 15 日間の 2×2 クロスオーバーデザインに従い、4 頭のコリデール種ヒツジ（初期体重 77.5 ± 2.75kg）を用いた。対照群（無添加）とクエン酸（40 mM）添加群に分けた。基礎飼料は、クレイングラス乾草と市販の濃厚飼料を 2：1 の割合で混合したものとした。4 つの呼吸試験チャンバー内の動物は、各期間 2 日間、24 時間の測定サイクルで 2 回、CH_4 排出量が測定された。

本研究では、クエン酸の添加は飼料摂取量と消化率を低下させたが、CH_4 産生を抑制する効果は認められなかった。

第 3 章では、飼料添加物としてのイタコン酸の効果を 20mM の用量で調査した。抗炎症作用および抗病原性作用が期待されることから、イタコン酸はルーメン内のメタン生成菌を抑制し、反芻動物からの CH_4 産生を低下させる可能性があると考えられた。しかし、反芻胃動物の飼料添加物としてのイタコン酸の利用に関する情報は極めて限られている。試験管内条件下で CH_4 削減の可能性を報告した研究は 1 件のみである。したがって、今回の研究の目的は第 2 章と同じであり、同じ方法で同じパラメーターを測定した。

上記の可能性にもかかわらず、イタコン酸は CH_4 排出量（L/kg DMI）を除くすべての分析パラメータにおいて、CH_4 産生に有意な結果をもたらさなかった。我々の研究では、飼料にイタコン酸を配合したヒツジにおいて、CH_4 排出量（L/kg DMI）の有意な増加が観察された。第 3 章では、プロピオン酸の統計的に有意な変化を含む全てのルーメン発酵パラメータが減少し、総揮発性脂肪酸が有意に減少した。イタコン酸は畜産部門の飼料添加物としての可能性を示しているが、その利点と欠点に関する情報はまだ限られている。この物質の可能性を完全に解明するためには、さらなる研究が必要である。

General Conclusion

The findings from this comprehensive study highlight the potential of alternative feed ingredients in addressing the challenges of ruminant agriculture, particularly in reducing CH_4 emissions. Spent coffee waste (SCW) emerges as a promising alternative due to its nutrient composition, showing potential to mitigate the environmental impact of ruminant farming. However, caution is warranted regarding higher dosages, as they may adversely affect animal production. Further research is essential to assess the implications, optimal dosages, and administration methods of SCW in ruminant diets for a more informed and sustainable approach.

Examining citric acid and itaconic acid as feed additives revealed insights into their effects on feed intake, digestibility, and CH_4 emissions. Citric acid, despite reducing feed intake and digestibility due to the higher dosage, did not impact rumen fermentation. However, the translation of *in vitro* CH_4 suppression to *in vivo* scenarios remains complex and requires additional research to determine optimal levels and usage methods in commercial ruminant feed.

Itaconic acid at 20 *mM* in sheep diets did not significantly impact CH_4 emissions, emphasizing the variability in the effectiveness of organic acids. The search for reliable CH_4 suppressors continues, underscoring the importance of nuanced understanding and exploration of novel additives for consistent mitigation in ruminant diets. Overall, these studies contribute valuable insights to the ongoing efforts to develop sustainable feeding strategies that optimize animal health, productivity, and environmental sustainability in the field of ruminant nutrition.

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