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Research Article

The Effect of Black Tea Lees on *In vitro* Rumen Fermentation Parameters and Methane Emissions

[1]S. Kurono, [1,2]J.J.D. Manlapig, [1,2]J.C.A. Crisostomo, [1]M. Kondo, [1]T. Ban-Tokuda and [1]H. Matsui

[1]Graduate School of Bioresources, Mie University, TSU, Japan
[2]Department of Animal Science, College of Agriculture, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines

Abstract

Background and Objectives: Global efforts to mitigate methane emissions from ruminant livestock are critical for addressing climate change and enhancing feed efficiency. Black Tea Lees (BTL), a by-product rich in polyphenolic tannins, may serve as a functional feed ingredient to reduce enteric methane emissions. This study evaluated the effects of substituting oat hay with BTL at varying inclusion levels (0-30%) on *in vitro* rumen fermentation characteristics and methane production. **Materials and Methods:** Proximate composition, neutral detergent fiber, acid detergent fiber and acid detergent lignin of BTL was measured. Total extractable phenols, total extractable tannins and condensed tannins in the BTL were also analyzed. *In vitro* rumen fermentation was carried out to assess dry matter digestibility, methane production and organic acid production. **Results:** Chemical analysis revealed that BTL contained high levels of crude protein and tannins. Total gas production and methane yield (CH_4 per gram of digested dry matter) decreased significantly with increasing levels of BTL substitution. Notably, CH_4 production was significantly suppressed at 25 and 30% inclusion levels, though digestibility declined at these higher concentrations. Substitution at 5 and 10% reduced CH_4/DDM without negatively affecting dry matter digestibility. Organic acid profiles were altered, with reduced acetate and increased lactate observed in a dose-dependent manner. These findings suggest that low-level incorporation of BTL in ruminant diets may effectively reduce methane emissions while maintaining ruminal fermentation efficiency. **Conclusion:** The valorization of BTL thus offers a promising strategy to mitigate greenhouse gas emissions and support sustainable livestock production.

Key words: Black tea lees, by-product, *in vitro* rumen fermentation, methane mitigation, sustainability

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Corresponding Author: Hiroki Matsui, Graduate School of Bioresources, Mie University, TSU, 514-8507, Japan Tel: +81 59-231-9593

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The global population is projected to reach approximately 9.7 billion by 2050[1]. In parallel with this rapid population growth, the demand for livestock-derived products is expected to double by 2050[2]. As is already well recognized, enteric methane (CH_4) emissions from ruminants account for approximately 15% of anthropogenic CH_4 emissions[3]. Methane possesses a global warming potential approximately 25 times greater than that of carbon dioxide[4]. Moreover, depending on the type of feed provided, ruminants lose an estimated 2-12% of the gross energy intake as methane[5]. As a result, the mitigation of CH_4 emissions from ruminant livestock represents an urgent challenge.

Black tea is a beverage derived from the leaves of *Camellia sinensis*, a species of the Theaceae family. The leaves undergo fermentation and are then steeped in hot water. Excluding water, black tea is the most widely consumed beverage worldwide[6]. Tannins, a class of polyphenolic compounds, are secondary metabolites produced by higher plants[7]. Spent tea lees, the residue following black tea extraction, are rich in tannins, a type of polyphenol[8]. Tannins can have both beneficial and adverse effects on livestock performance and product quality[7]. Reported negative impacts include reduced feed intake, decreased fiber digestibility and overall declines in animal performance. Conversely, several studies have demonstrated that tannins can enhance protein utilization, combat internal parasites and contribute to improved productivity[9,10]. One notable beneficial effect of tannins is their potential to suppress methanogenesis in the rumen[11]. Hydrolysable tannins, in particular, exhibit a potent methane-inhibitory effect, which is further amplified hydrolysis by microbial tannase in the rumen. Nutritive values of black tea by-product and anti-nutritive activity of tannins was evaluated in an *in vitro* rumen fermentation using various molecular weights of Polyethylene Glycols (PEG), Polyvinyl Pyrrolidone (PVP) and polyvinyl polypyrrolidone as tannin-binding agents[12]. Significant improvement in gas production by addition of PEG4000, 6000 and 20000 and PVP was observed only from black tea by-product. The PEG6000 and 20000 also improved *in vitro* organic matter digestibility and metabolizable energy contents of black tea by-product. In this study, only black tea by-product was used for sole substrate for *in vitro* fermentation. Consequently, the effect of tannins was pronounced. If Black Tea Lees (BTL) can be employed as a partial substitute for forage, the tannins they contain may serve to mitigate CH_4 emissions. This approach would simultaneously promote the valorization of food industry by-products and contribute to the reduction of greenhouse gas emissions.

The present study aimed to evaluate the effects of substituting a portion of forage with spent black tea leaves on rumen fermentation parameters and methane production in an *in vitro* rumen fermentation system.

MATERIALS AND METHODS

BTL sample: Representative wet BTL were obtained from a tea drink factory. The BTL were dried for 48 hrs at 80°C in forced-air dryer.

Chemical analysis: The proximate composition of BTL was carried out following the AOAC procedure[13]. Sample was oven dried at 135°C for 2 hrs to determine Dry Matter (DM) content. N was determined by organic elemental analyzer (Vario EL, DKSH, Elementor Japan Co., Ltd., Yokohama, Japan). Crude Protein (CP) content was calculated as N×6.25. Using petroleum ether as the extracting agent, crude fat (EE) was analyzed using the Soxhlet extract method (60-80°C). Crude ash content was analyzed by incinerating the samples in a muffle furnace at 550°C.

Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and Acid Detergent Lignin (ADL) were analyzed following the method described by Van Soest *et al.*[14].

Total Extractable Phenols (TEP), Total Extractable Tannins (TET) and Condensed Tannins (CT) in the BTL were analyzed according to Kondo *et al.*[8]. Chemical analyses for oat hay and BTL were carried out in duplicate and quintuplicates, respectively.

Experimental animals: The animal care and sampling procedures were approved by the Institutional Animal Care and Use Committee of Mie University (Approval No. 2024-38) and were performed in accordance with the guidelines for laboratory animals at Mie University. Three wether (Corriedale) housed in individual pens were used as rumen fluid donors. Sheep were fed with 60% oat hay and 40% concentrate containing corn, soybean, wheat bran and alfalfa pellets (=1:1:1:0.5). The animals were fed twice daily and clean water and mineral blocks were available *ad libitum*.

***In vitro* rumen fermentation:** The experiment was performed as described by Manlapig *et al.*[15]. Approximately 270 mL of rumen fluid were collected from each donor before the morning feeding, kept at 39°C and immediately transported

Table 1: Composition of substrates and inoculum in *in vitro* batch cultures

		Substitution level (%)					
Items	Control	5	10	15	20	25	30
Oat hay (g)	0.150	0.135	0.120	0.105	0.090	0.075	0.060
Black tea leaf (g)	0.000	0.015	0.030	0.045	0.060	0.075	0.090
Corn starch (g)	0.150	0.150	0.150	0.150	0.150	0.150	0.115
Buffered rumen fluid (mL)	30	30	30	30	30	30	30

to the laboratory. The rumen fluid was then filtered through four layers of surgical gauze and equal portions were pooled. The pooled rumen fluid was diluted four times with prewarmed McDougall buffer and flushed with nitrogen (N_2) gas[16]. Ground oat hay and cornstarch were used as substrates in all treatment conditions (Table 1). The oat hay was substituted with dried BTL at 5, 10, 15, 20, 25 and 30% in treatments (Table 1). These substrates were weighed into serum bottles (50 mL volume). Each treatment was replicated three times. The diluted rumen fluid was dispensed into the serum bottle under nitrogen gas. The serum bottles were sealed with butyl rubber stoppers then crimped with aluminum caps and incubated for 48 hrs at 39°C in a shaking water bath at 170 rpm.

Gas production and ruminal fermentation profiles: After incubation, gas production and ruminal fermentation profiles were determined as described by Manlapig *et al.*[15]. Briefly, the total gas produced in the cultures was quantified using a glass syringe equipped with a Lure-lock three-way stopcock fitted with a 21-gauge needle. Headspace gas composition and concentration (%) was analyzed using a gas chromatograph (GC-8AIT, Shimadzu Corporation, Kyoto, Japan) equipped with a Shincarbon ST column (3 mm diameter, 2 m long) and a Thermal Conductivity Detector (TCD). High-purity argon gas (99.999%) was used as the carrier gas. Supernatant from cultures was collected for the analysis of organic acids and stored at -80°C until analysis.

The Dry Matter Digestibility (DMD) was assessed using the method outlined by Manlapig *et al.*[15]. This involved centrifugation at 1000×g for 10 min at 4°C to collect the residual substrate. After centrifugation, the pellet was moved to pre-weighed aluminum containers and dried in an oven at 80°C for 48 hrs. The residues' weights were recorded before (0 hr) and after (24 hrs) incubation, allowing us to estimate mass loss in relation to the pre-digestion mass using the provided formula:

$$\text{DMD}(\%)=\left[\frac{0\,\text{hr}-24\,\text{hrs}}{0\,\text{hr}}\right]\times 100$$

To calculate the amount of CH_4 produced per gram of digested dry matter (DDM), we divided the mass of DDM by the total CH_4 produced:

$$\frac{CH_4}{\text{DDM}}=\frac{CH_{4\ \text{produced}}}{\text{DDM}_{\{0\,\text{hr}\}}-\text{DDM}_{\{24\,\text{hrs}\}}}$$

The concentration of organic acid was determined using a high-performance liquid chromatography system following a method described by Kondo *et al.*[17].

Statistical analysis: Statistical analysis was carried out using SAS version 9.4[18]. Data on headspace gas and composition, DMD and organic acid production were subjected to a One-way Analysis of Variance (ANOVA). Comparisons between means were performed using the Tukey method. The differences between means with values of $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

In this study, the potential utilization of spent BTL as a feed resource and their capacity to mitigate methane emissions from ruminants were evaluated using an *in vitro* rumen fermentation system.

Table 2 illustrates the chemical composition of the BTL employed in this experiment. DM and CP of BTL were higher than those of oat hay. The contents of CP, NDF, ADF and ADL in the BTL used herein were comparable to the values reported by Kondo *et al.*[12]. In contrast, the CP and Ether Extract (EE) levels in BTL reported by Sezmis *et al.*[19] were lower than those observed in this study, whereas the NDF, ADF and ADL contents were higher. Additionally, TEP, TET and CT in the current BTL samples were lower than the levels reported by Kondo *et al.*[8,12]. These discrepancies are likely attributable to variations in processing conditions among different tea brewers.

Table 3 summarizes the fermentation characteristics of BTL after 48 hrs of *in vitro* rumen incubation. DMD was significantly decreased at substitution levels of 15, 25 and 30% relative to the control. Paya *et al.*[20] investigated the effects of

Table 2: Chemical composition of oat hay, corn starch and black tea lees

Items	Oat hay	Corn starch[21]	Black tea lees
DM (g kg^{-1})	864	855	955
OM (g kg^{-1} DM)	917	999	924
CP (g kg^{-1} DM)	90	1	272
EE (g kg^{-1} DM)	26	1	19
NDF (g kg^{-1} DM)	566	Not detected	489
ADF (g kg^{-1} DM)	556	Not detected	251
ADL (g kg^{-1} DM)	109	Not detected	102
TEP (g kg^{-1} DM)	Not determined	Not determined	47.1
TET (g kg^{-1} DM)	Not determined	Not determined	45.9
CT (g kg^{-1} DM)	Not determined	Not determined	3.7

DM: Dry matter, OM: Organic matter, CP: Crude protein, EE: Ether extract, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, ADL: Acid detergent lignin, TEP: Total extractable phenolics, TET: Total extractable tannins and CT: Condensed tannins

Table 3: Dry matter digestibility, gas production, CH_4 concentration, CH_4 volume, CH_4 per Digested Dry Matter (DDM), total organic acid concentration and organic acid composition after 48 hrs *in vitro* rumen fermentation

		Substitution level (%)						
Items	Control	5	10	15	20	25	30	Standard error
Dry matter digestibility (%)	54.24^{a}	54.53^{a}	53.03^{ab}	49.25^{cd}	53.31^{ab}	50.62^{bc}	46.50^{d}	0.26
Gas production (mL)	58.00^{a}	50.67^{b}	50.67^{b}	48.00^{bc}	48.00^{bc}	45.33^{cd}	44.00^{d}	1.74
CH_4 (%)	11.35^{a}	11.22^{a}	11.10^{ab}	10.81^{abc}	10.70^{abc}	10.47^{bc}	10.22^{c}	0.16
CH_4 (mL)	6.58^{a}	5.68^{b}	5.62^{bc}	5.19^{cd}	5.14^{d}	4.75^{de}	4.50^{e}	0.26
CH_4 m (DDM g^{-1})	48.55^{a}	40.89^{b}	40.81^{b}	38.38^{bc}	35.12^{c}	33.95^{c}	34.59^{c}	1.94
Total organic acid concentration (mmol L^{-1})	55.77^{a}	53.73^{b}	51.59^{c}	49.51^{d}	47.43^{e}	44.09^{f}	41.18^{g}	1.97
Lactate (%)	1.07^{d}	2.85^{cd}	5.82^{bc}	8.07^{ab}	10.02^{a}	10.31^{a}	7.75^{ab}	1.33
Acetate (%)	55.93^{ab}	55.87^{abc}	54.47^{c}	52.47^{d}	52.02^{d}	54.63^{bc}	56.62^{a}	0.67
Propionate (%)	26.11^{a}	24.73^{ab}	22.91^{abc}	22.80^{abc}	21.71^{bc}	19.81^{c}	20.98^{c}	0.82
Butyrate (%)	16.90^{a}	16.55^{ab}	16.80^{a}	16.66^{a}	16.26^{ab}	15.25^{bc}	14.65^{c}	0.33

$^{a-g}$Means within a row a without a common superscript letter differ significantly (p<0.05)

black tea extract on *in vitro* digestibility and found no significant impact upon its inclusion. Gas production was significantly reduced in all treatment groups compared to the control. The proportion of methane (CH_4, %) in the gas phase was significantly lower at the 25 and 30% substitution levels. The total volume of CH_4 (mL) generated during fermentation was also significantly reduced in all treatment groups in a substitution level-dependent manner. Methane yield per unit of digested dry matter (CH_4/DDM) and total concentrations of organic acids were significantly diminished across all treatment groups, also in a substitution level-dependent trend. Ramdani *et al.*[21] incorporated BTL into a total mixed ration consisting of ryegrass hay and rice straw at inclusion rates of 10 and 20% and reported a significant reduction in CH_4 production (p<0.01) during *in vitro* rumen fermentation without negatively impacting organic matter degradability. Similarly, methane output was markedly decreased in diets supplemented with black tea waste extracts compared to the control following a 24 hrs incubation period[17]. The present findings corroborate these results.

Lactate proportions were elevated in all treatment groups relative to the control, with a substitution level-dependent pattern. Acetate proportions were significantly reduced at substitution levels of 10, 15 and 20%, while propionate proportions were significantly lower at 20, 25 and 30% substitution levels. Paya *et al.*[20] reported a reduction in propionate concentrations with the inclusion of black tea waste extract, whereas Ramdani *et al.*[21] observed no significant changes, suggesting inconsistent effects of black tea on propionate production. Butyrate proportions were significantly decreased at substitution levels of 25 and 30% compared to the control. Because microbial population was not determined in this study, it cannot be concluded, however, the propionate producers such as *Selenomonas ruminantium* or *Megasphaera elsdenii* may be inhibited by tannins in the BTL.

CONCLUSION

In conclusion, at substitution levels of 5 and 10%, there was no significant reduction in DMD, while CH_4/DDM was significantly decreased compared to the control. These findings indicate that such levels of BTL incorporation may be feasible without adversely impacting ruminal digestion.

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