

Article

The Effect of Adding Green and Black Tea Waste Extracts on Rumen Fermentation Parameters by In Vitro Techniques

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Abstract: The increase in global temperatures over the past few decades due to greenhouse gas emissions has raised concerns and necessitated further research in climate change mitigation and adaptation. Methane is a prominent greenhouse gas that significantly contributes to climate change, with a substantial amount generated through fermentation processes occurring in the rumen of ruminant animals. The potential of plant secondary metabolites, especially those derived from tannin-rich plants, warrants investigation to modify rumen fermentation and mitigate methane emissions in livestock diets. The objective of this study was to assess the impact of extracts obtained from green and black tea waste on rumen fermentation dynamics and gas (methane) production, utilizing in vitro methods. For this purpose, rumen fluid was collected from two fistulated sheep and subjected to three treatments: (1) a basal diet (control), (2) a basal diet + green tea waste extract (5% of dry matter), (3) a basal diet + black tea waste extract (5% of dry matter). The study assessed the effects of incorporating extracts from green and black tea waste on various parameters, including digestibility, protozoa population, ammonia nitrogen levels, volatile fatty acids, and methane gas production following a 24-h incubation period. Statistical analysis of the data was conducted using SAS software within a completely randomized design framework. The findings indicated that the addition of green and black tea waste extracts significantly decreased methane gas production ($p < 0.05$), protozoa count ($p < 0.05$), and ammonia nitrogen concentrations in rumen fluid ($p < 0.05$) when compared to the control group. The addition of green and black tea waste extracts has significantly altered the concentration of VFAs in rumen fluid ($p < 0.05$). Specifically, the addition of green tea waste extract has led to a highly significant reduction in acetic acid, ($p < 0.01$) and the addition of both extracts has resulted in a significant increase in propionic acid ($p < 0.05$). Consequently, the results suggest that the inclusion of green and black tea waste extracts in livestock diets may effectively mitigate methane emissions in the rumen, thereby reducing feed costs and reducing environmental pollution.

Keywords: digestibility; environmental pollution; methane emission; secondary metabolites; tannin; tea waste



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1. Introduction

Methane is the second most significant anthropogenic contributor to global radiative forcing, following carbon dioxide, and possesses a global warming potential that is 28 times greater than that of carbon dioxide [1]. Projections based on the Representative Concentration Pathways (RCP) indicate that methane will continue to hold this position in the future, underscoring its importance in mitigation strategies aimed at achieving objectives such as the reduction of global warming [2]. The predominant source of methane emissions

resulting from human activities is livestock agriculture, primarily attributed to enteric fermentation in ruminants and the management of animal manure. Currently, livestock production accounts for one-third of global anthropogenic methane emissions, comparable to methane emissions from fossil fuels [3]. According to reports by FAOSTAT [4], methane emissions from livestock increased by 51.4% between 1961 and 2018, following the rise in the number of ruminants and the increased amount of animal manure from various livestock groups. With the projected surge in demand for livestock products, especially in developing countries, this upward trend is likely to continue in the future. The highest methane emissions are from the agricultural sector, accounting for approximately 50.6%. Within the agricultural sector, livestock represents about 18% of global greenhouse gas emissions. Among livestock, ruminants produce approximately 81% of greenhouse gases due to the extensive presence of methanogenic microbes in the rumen, responsible for 90% of methane production [5]. Methane formation in ruminants indicates a loss of 2 to 14% of the energy in consumed feed [6]. Therefore, reducing methane emissions in animals will conserve energy and increase efficiency, or to put it in other words, feed costs can be lowered.

Mitigating methane emissions is recognized as a significant challenge within the field. Numerous methane inhibitors have undergone extensive testing for their potential to diminish methane production. Nevertheless, many of these inhibitors have demonstrated adverse effects on rumen fermentation characteristics when incorporated into the diet at elevated dosages necessary for effective methane suppression. Furthermore, certain inhibitors have exhibited toxicity to livestock. In this context, consumer demand has significantly shifted towards the use of phytochemicals, which are natural products that can alter rumen fermentation [7]. Plants synthesize a variety of secondary metabolites that do not play a direct role in primary biochemical functions, including growth, development, and reproduction. According to Naumann et al. [8], these secondary compounds can be classified into three major groups: terpenes (including essential oils), alkaloids (widely used in the pharmaceutical industry), and phenolic compounds (including flavonoids, lignin, tannins, etc.). Tannins, which are prevalent among bioactive compounds, are categorized into two distinct groups based on their structural characteristics: hydrolyzable tannins (HT) and condensed tannins (CT). Tannins are found in various crops and forage plants, as well as their products like fruits, vegetables, grains, and beverages (tea, cocoa, and wine) [9]. Historically, tannins have been known for their anti-nutritional properties, but recently, they have attracted attention as substances capable of altering and modulating rumen fermentation [10].

Tea, derived from the leaves of the *Camellia sinensis* plant, ranks among the most widely consumed non-alcoholic beverages globally. As reported by the International Tea Committee, worldwide tea consumption exceeded 5.8 million tons in the year 2019. Along with the growing consumption of tea, the amount of tea waste has also increased rapidly [11,12]. Black and green tea leaves and their consumed residues contain significant amounts of protein, minerals, and plant secondary metabolites, including tannins and saponins [13]. Given the large volume of agricultural waste production worldwide, including tea waste, circular solutions are sought. Said tea waste has some nutritional value, and it can be used in the diet of ruminants. This not only reduces costs but can also prevent environmental pollution.

Tannins represent a heterogeneous category of water-soluble phenolic compounds that interact with proteins, resulting in the formation of both soluble and insoluble complexes within the skin, leaves, and roots of a majority of plant species. These compounds exert both direct and indirect influences on rumen microbial populations, leading to a decrease in the degradability of rumen protein, a reduction in methanogenesis, and an alteration in the biohydrogenation processes of unsaturated fatty acids. However, these effects may partly result from the antimicrobial properties of tannins, which reduce fiber digestion, leading to incomplete digestion of the diet by the rumen microbial population [14]. Ramdani et al. [15] concluded that including tea tannins in the diet of fattening lambs could increase their

weight without affecting feed intake and protein digestibility. This indicates that the protein bound within the rumen is not efficiently digested; however, it can be absorbed in the small intestine as a bypass protein. Furthermore, dietary tannins have the potential to diminish methane production in the rumen. Similarly, tea saponins can reduce methane production by decreasing protozoa and methanogenic bacterial activity [16]. In the study by Niderkorn et al. [17], including sainfoin (*Onobrychis viciifolia* Scop.) or hazelnut (*Corylus avellana* L.) shells in ruminant diets reduced rumen fermentation, methane production, and protein degradability. In the study by Ramdani et al. [18], the use of both types of tea leaves (black and green) in sheep diets was investigated, and it was reported that adding green and black tea leaves to the diet reduced ammonia nitrogen and methane without affecting rumen degradability. These researchers reported that reducing ammonia nitrogen and methane production in the rumen could be beneficial for protein and energy utilization efficiency. The incorporation of tea powders into ruminant diets has been shown to decrease the production of ammonia and methane in the rumen while not significantly impacting the profiles of volatile fatty acids or the degradability of the diet. Tea powders contain varying amounts of alkaloids, catechins, and theaflavins, which can potentially reduce ammonia and methane production in the rumen without any detrimental effects on rumen function in vitro and possibly on the production efficiency of ruminants [19]. The incorporation of extracts from green and black tea waste resulted in a significant decrease in gas production during the incubation period, as well as a reduction in dry matter degradability within the diet. Additionally, a significant reduction in rumen dry matter disappearance and a strong increase in post-rumen dry matter disappearance were observed with the addition of green and black tea waste extracts [12].

The mitigation of methane emissions from ruminant livestock is crucial not only for environmental sustainability but also for the optimal utilization of nutrients. In similar studies, the addition of raw tea waste to the diet has been considered, which, due to its low nutritional value, can lead to a reduction in the energy available to the animal. However, in the present study, the addition of extract derived from tea waste has been considered, which can provide the same efficiency without reducing the energy available to the animal. Consequently, the aim of this research was to examine the impact of extracts derived from green and black tea waste on protozoal populations, ammonia nitrogen levels, and methane gas production.

2. Materials and Methods

2.1. Preparation of Tea Waste and Extraction

Tea waste was sourced from tea production facilities located in Northern Iran. For the extraction process, 50 g of the ground sample, which had been sieved through a 2-mm mesh, was placed in an Erlenmeyer flask. Subsequently, 500 mL of an ethanol solution, composed of 475 mL of 95% ethanol and 25 mL of distilled water, was added to the flask [12]. The mixture was then incubated at a temperature of 25 °C for a duration of 48 h, after which it was filtered using Whatman No. 1 filter paper (pore size 11 µm) to yield the extract [20]. Total phenolic compounds, total tannins, and condensed tannins were measured using the method described by Makkar [21].

2.2. Chemical Composition of the Basal Diet

The ingredients and chemical composition of diets are reported in Table 1. The experiment included three treatments: 1. a basal diet with green tea waste extract (5% of dry matter), 2. a basal diet with black tea waste extract (5% of dry matter), and 3. a basal diet (control). It should be noted that tea extract (green and black) at a concentration of 5% dry matter has been added to the diet and has not replaced other feed components.

Table 1. Ingredients and chemical composition of diets (%DM).

alfalfa	21.49
corn silage	20.56
straw	4.08
wheat bran	0.81
rice bran	0.16
sugar beet molasses	1.32
cottonseed	5.06
sugar beet pulp	3.73
barley grain	13.39
corn grain	12.56
corn gluten	5.03
soybean meal	8.23
soybean seed	0.82
urea	0.29
dicalcium phosphate	0.31
calcium carbonate	0.41
magnesium oxide	0.17
sodium chloride	0.17
sodium bicarbonate	0.86
vitamin premix	0.57
Nutrient composition	
ME (Mcal/kg DM)	2.42
CP (%DM)	15.2
ASH (%DM)	6.6
EE (%DM)	4.1
NDF (%DM)	33.1
ADF (%DM)	24.3

ME: metabolizable energy, CP: crude protein, EE: ether extract, NDF: neutral detergent fiber, ADF: acid detergent fiber.

Rumen fluid required for the experiment was obtained from two fistulated sheep that were maintained on a diet consisting of 55% forage and 45% concentrate. The collected rumen fluid was subsequently filtered using a multilayer cloth and promptly transported to the laboratory in a flask.

2.3. Methane Emission

Methane production was measured using gas syringes. In this method, 300 milligrams of feed material were placed inside the syringes. To examine the effect of black and green tea waste extracts, the extracted tea waste was added to the syringes at 5% dry matter. Each syringe was then filled with 20 milliliters of a mixture of rumen fluid and McDougall's buffer (at a ratio of 2:1). The syringes were incubated at 39 °C. After 24 h of incubation, the produced gas was recorded, and samples of the gas were taken in 10-milliliter vacuum tubes. Methane production was determined using gas chromatography (GC) [22]. The column, injector, and detector temperatures were set at 100 °C, 200 °C, and 250 °C, respectively. Helium was used as the carrier gas at a flow rate of 24 milliliters per minute. The methane concentration was determined using a regression equation derived from pure methane gas (as a standard) at various concentrations.

2.4. Protozoa Population

To count protozoa, rumen fluid samples were taken at zero and 24 h after incubation. The rumen fluid samples were fixed with formalin solution (100 milliliters of 40% formaldehyde and 8.5 g of pure NaCl salt in 1 L of distilled water) at a 1:4 ratio (rumen fluid to formalin). For counting, one drop of each sample was placed on a graded slide, covered with a cover slip, and observed under a light microscope at 10× magnification [23].

2.5. Determination of Ammonia Nitrogen

Ammonia nitrogen was determined using three solutions: phenol, hypochlorite, and standard ammonium chloride, according to the method described by Broderick and Kang [24]. For the control sample, 40 µL of distilled water, 40 µL of standard solution, 2 mL of hypochlorite solution, and 2.5 mL of phenol solution were added. After 24 h of incubation, the experimental samples were centrifuged at 1500 rpm for 10 min. Then, 40 µL of the supernatant was taken and mixed with 40 µL of distilled water, 2.5 mL of phenol solution, and 2 mL of hypochlorite solution. The samples were vortexed and incubated at 37 °C for 10 min. The optical density of the samples was read using a spectrophotometer at 550 nanometers.

2.6. In Vitro Disappearance Technique

To assess the impact of adding green and black tea waste extracts on the in vitro disappearance of feed, Holden's [25] method was employed. Rumen fluid was collected approximately 2 h after the morning feeding from two fistulated sheep that had been fed a diet consisting of 45% concentrate and 55% forage for 21 days. The Daisy incubator (Ankom Technology, Macedon, NY 14502, USA), containing six 4 L jars, was used in this method. Initially, the test feed was dried in an oven at 55 °C for 48 h and then ground using a 2 mm screen. The feed samples were placed in nitrogen-free and ash-free bags, which were then sewn shut. For the buffer, two solutions were prepared: Solution A (10 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g NaCl , 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 1 g urea, brought to a volume of 1 L) and Solution B (15 g Na_2CO_3 and 1 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, brought to a volume of 100 mL). Twenty milliliters of Solution B were added to 1 L of Solution A, and the mixture was heated to 39 °C. For each treatment, two 4 L jars were prepared, with three replicates per treatment in each jar, totaling six replicates per treatment. The bags containing the feed samples were placed in the jars along with 1400 mL of buffer and 400 mL of rumen fluid and incubated at 38 °C for 48 h. Then, 40 mL of 6N hydrochloric acid and 8 g of pepsin were introduced into the jars, which were subsequently incubated for an additional 24 h at the same temperature. Ultimately, the samples were dried in an oven at 100 °C, and the requisite calculations were conducted to assess in vitro disappearance.

2.7. Statistical Analysis

All experimental data were subjected to a one-way analysis of variance using the analysis of variation model (ANOVA) by SAS software (version 9.2). The statistical model used was $Y_{ij} = \mu + T_i + e_{ij}$, in which the dependent variable Y_{ij} was the total mean, T_i was the treatment effect, and e_{ij} the residual effect.

3. Results and Discussion

3.1. Phenolic Compounds

The findings indicate that there are notable differences in the phenolic compounds present in green and black tea waste, as illustrated in Table 2. Specifically, the total phenolic compounds, tannins, and condensed tannins were found to be significantly more abundant in green tea waste than in black tea waste ($p < 0.05$). This disparity may be attributed to the degradation of these compounds that occurs in black tea waste as a result of the fermentation (oxidation) process that the tea leaves undergo in tea production facilities. This process is carried out to improve the quality and appearance of tea, such as its color and flavor, which has similarly been reported by other researchers [26,27]. The tea waste utilized in the current research was derived from by-products obtained during the processing of tea plants intended for human consumption. Therefore, their chemical composition varies depending on the composition of the plant material, processing methods, and types of extracted components, similar to other agricultural by-products [28].

Table 2. Phenolic compounds of green and black tea waste (g/kg of DM).

	Total Phenolic Compounds	Total Tannins	Condensed Tannins
Green tea waste extract	20.3 ^a	14.8 ^a	5.9 ^a
Black tea waste extract	18.7 ^b	12.7 ^b	5.0 ^b
SEM	0.034	0.368	0.062
<i>p</i> -value	<0.0001	0.016	<0.0001

Means with non-common letters have a significant difference ($p < 0.05$). SEM: standard error of means.

3.2. Methane Production and Rumen Ecosystem

Table 3 illustrates the impact of incorporating extracts from green and black tea waste on methane gas production. After a 24-h incubation period, methane production was significantly reduced in the diets supplemented with green and black tea waste extracts when compared to the control diet ($p < 0.05$). Furthermore, the diet containing green tea waste extract exhibited lower methane production than the diet with black tea waste extract. This difference can be attributed to the higher concentrations of phenolic compounds, including tannins, present in green tea relative to black tea.

Table 3. The effect of adding green and black tea waste extract on the amount of methane production, protozoa population, and ammonia nitrogen.

	Treatment			SEM	<i>p</i> -Value
	Treat 1	Treat 2	Control		
Methane (% of total gas produced)	5.63 ^c	13.37 ^b	18.50 ^a	1.28	0.001
protozoa population (10^6 per mL of rumen fluid)	1.58 ^b	2.08 ^b	3.21 ^a	0.18	0.0002
ammonia nitrogen (mg/deciliter)	2.78 ^c	4.64 ^b	7.1 ^a	0.02	0.0001

Treat 1: diet containing green tea extract, treat 2: diet containing black tea extract. Means with non-common letters have a significant difference ($p < 0.05$). SEM: standard error of means.

The results of the protozoa count are reported in Table 3. The lowest number of protozoa was found in the diet containing green tea waste extract, while the highest number was observed in the control diet, indicating a significant reduction in protozoa numbers ($p < 0.05$).

According to the results presented in Table 3, the highest reduction in ammonia concentration was significantly influenced by the extracts of green and black tea waste compared to the control diet.

Cieslak et al. [29] reported that tannins from sources such as *Vaccinium vitis-idaea* L., at a rate of 2 g per kilogram of dry matter, directly affect methane production and indirectly influence hydrogen production by reducing feed degradability, thereby decreasing energy loss through methane emissions (resulting in a 24–30% reduction in methane production). They also noted that the reduction in protozoa leads to decreased methane production without negatively affecting the digestibility of organic matter and volatile fatty acid production. Given the symbiotic relationship that exists between rumen protozoa and methanogens, the inhibition of protozoa results in a corresponding inhibition of methanogens, which ultimately leads to a decrease in methane production. Ramdani [30] found that adding green and black tea leaves at levels of 0, 50, and 100 g per kilogram of dry matter to the diet significantly reduced methane gas production after 28 h of incubation compared to the control. Similarly, Nasehi et al. [31] reported that methane production by ruminants was significantly reduced when green and black tea wastes were included in the diet. Comparable results were observed in studies by Sinz et al. [32] and Kolling et al. [33].

Kim et al. [34] investigated the addition of extracts from five plants containing flavonoids (5% of dry matter), including green tea, and reported that these extracts reduced methane production without negatively affecting rumen fermentation characteristics after 24 h of incubation in vitro. This indicates that these plants have positive effects as bioactive modulators for ruminants. Studies have shown that condensed tannins negatively impact

methanogens and protozoa without adversely affecting volatile fatty acid production and organic matter digestibility [35]. Methanogens are inhibited by secondary metabolites, leading to reduced methane production by ruminants, suggesting that secondary metabolites can be used as feed additives to manipulate the rumen and decrease methane production.

The protozoa count at time zero only included rumen fluid as a baseline. There is limited information on the effects of these additives and their active compounds on protozoa populations and the impact of metabolites on these microorganisms. The reduction in protozoa numbers is likely due to the phenolic compounds present in the secondary metabolites. Similar results have been observed in other studies where the use of secondary metabolites led to a decrease in protozoa numbers [29,36].

Protozoa produce hydrogen in the rumen comparable to gram-positive bacteria and increase methane production through symbiosis with methanogenic bacteria. Although protozoa have beneficial effects, their reduction positively impacts ruminant productivity [34]. Demirtas et al. [37] examined the effects of flavonoid-rich plant extracts on rumen methanogen population, microbial population, and fermentation characteristics in vitro. They reported a decline in the population of methanogens, which subsequently led to a reduction in methane production. Additionally, there was a notable decrease in the population of rumen protozoa, with reductions observed of up to 60% as a result of the presence of these plants.

The markedly reduced levels of ammonia observed in the tests with both green and black tea waste extracts, in comparison to the control diet, can be ascribed to the presence of tannins in these teas, which play an inhibitory role in the process of proteolysis. Tannins form hydrogen bonds with proteins, creating hydrophobic interactions and coating protein surfaces, thus preventing enzyme-substrate interactions, which inhibits protein degradation [38].

Soroor and Moeini [39] found that using jujube (*Ziziphus jujuba*) in animal diets improved fermentation and reduced ammonia nitrogen by 43% without negatively affecting digestibility. Abarghoyi and Rouzbehan [40] reported that phenolic compounds in grape pomace extract reduced ammonia, protozoa population, and protein degradation while increasing bypass protein digestibility. Sinz et al. [32] found that extracts from acacia, grape seeds, and green tea reduced ammonia concentration by 23%, consistent with this study's results.

3.3. In Vitro Disappearance

Figure 1 presents the impact of green and black tea waste extracts on feed in vitro disappearance. The incorporation of these extracts did not result in any significant changes in the ruminal and total tract disappearance of dry matter, crude protein, or neutral detergent fiber. Given the limited data on the effects of green and black tea waste extracts on nutrient digestibility, the focus was on the impact of tannins, the main component of the extracts. Benchaar et al. [41] reported no effect on dry matter and other nutrient digestibility, such as organic matter, crude protein, and acid detergent fiber, with 0.64% dry matter extract from quebracho tannins. Conversely, Beauchemin et al. [42] found reduced crude protein digestibility in beef cattle with 1–2% quebracho tannin extract. Carulla et al. [43] reported decreased organic matter, crude protein, and fiber digestibility with 2.5% acacia tannin extract in sheep. While tannins improve nitrogen utilization in ruminants [44], their effect on crude protein digestibility varies with concentration and type. For example, 1.8% condensed tannins from big trefoil reduced crude protein digestibility, while the same concentration from birdsfoot trefoil had a lesser effect [45]. Similar to this study, Dschaak et al. [46] and Baah et al. [47] found no change in dry matter, organic matter, and neutral detergent fiber digestibility with 0.6% quebracho tannin extract in low-forage diets (<50% forage) for Holstein dairy cows and Jersey heifers, respectively.



Figure 1. The effect of adding green and black tea waste extract on rumen and total tract disappearance of DM (A), CP (B), and NDF (C).

3.4. Volatile Fatty Acids

The effects of green and black tea waste extracts on volatile fatty acids are shown in Table 4. Adding black tea waste extract increased acetic and butyric acid compared to green tea waste extract and control diets ($p < 0.05$). Both green and black tea waste extracts reduced propionic acid ($p < 0.05$). Green tea waste extract increased isobutyric acid compared to black tea waste extract and control diets ($p < 0.05$).

Table 4. The effect of adding green and black tea waste extract on volatile fatty acids concentration (millimol per liter).

	Treatment			SEM	p-Value
	Treat 1	Treat 2	Control		
Acetic acid	77.82 ^b	84.36 ^a	72.76 ^b	1.62	0.006
Propionic acid	16.01 ^b	16.19 ^b	16.61 ^a	0.10	0.02
Butyric acid	17.15 ^b	18.22 ^a	17.43 ^b	0.20	0.02
Isobutyric acid	1.20 ^a	0.28 ^b	0.50 ^b	0.10	0.002
Valeric acid	1.45 ^c	1.53 ^b	1.72 ^a	0.01	0.0001
Isovaleric acid	2.29	2.38	2.48	0.07	0.29

Treat 1: diet containing green tea extract, treat 2: diet containing black tea extract. Means with non-common letters have a significant difference ($p < 0.05$). SEM: standard error of means.

Rumen protozoa population is a key factor in volatile fatty acid ratios, particularly shifting toward acetic and butyric acids [48]. The reduction in acetic and butyric acids in this study is attributed to the reduced protozoa population (Table 3). The decrease in valeric and isovaleric acids, derived from amino acid deamination, also indicates reduced protein degradation in the rumen and increased protein bypass to the intestine [49], consistent with this study. Increased propionic acid concentration from pistachio waste extract (high in tannins) reported by Taghavi et al. [49] contrasts with this study, likely due to in vivo versus in vitro differences.

4. Conclusions

This study concludes that adding green and black tea waste extracts to ruminant diets can reduce rumen methane production without affecting in vitro digestibility. Considering the large volume of tea waste produced globally, utilizing this agricultural by-product in the diet of ruminants can save on feed costs and waste disposal expenses while also helping

to reduce climate change in the livestock sector. All of these factors necessitate further animal in vivo studies.

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