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In Vitro Evaluation of Green Tea Extract's Influence on Local Sheep

Ririn Siti Rahmatillah*[1], Diky Ramdani[2], Iman Hernaman[3] , Anuraga Jayanegara[4]

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[1,2]Department of Animal Production, Faculty of Animal Husbandry, Universitas Padjadjaran, ,Jatinangor Campus, Jl. Raya Bandung Sumedang KM.21 Sumedang, West Java, Indonesia 45363

[3]Department of Animal Nutrition and Feed Technology, Faculty of Animal Husbandry, Universitas Padjadjaran, Jatinangor Campus, Jl. Raya Bandung Sumedang KM.21 Sumedang 45363, Indonesia

[4]Department of Animal Nutrition and Feed Technology, Faculty of Animal Husbandry, IPB University, Jl. Raya Darmaga Bogor, 16680 Indonesia

e-mail:
*[1]*ririn17003@mail.unpad.ac.id,*
[2]diky.ramdani@unpad.ac.id,
[3]iman.hernaman@unpad.ac.id,
[4]anuragaja@apps.ipb.ac.id.

Corresponding author

Abstract. *In recent years, exploring natural supplements such as green tea extract and their potential benefits in livestock nutrition has gained considerable attention among researchers and agriculturalists. Green tea extract contains a high concentration of bioactive substances, such as polyphenols and catechins. This study aimed to explore the effects of varying doses of green tea extract on rumen fermentation parameters through an in vitro trial using sheep rumen liquids. A completely randomized experimental design was utilized to test the effect of 4 different doses of green tea extract treatments (0, 140, 280, and 560 mg/kg) on in vitro Hohenheim gas test method. The current study shows no significant effect of administering green tea extract at various doses on total gas production, total gas production per in vitro dry matter and organic matter degradability, volatile fatty acid per in vitro dry matter and organic matter degradability, methane production per inv vitro dry matter and organic matter degradability of green tea extract. Although no significant effects were observed on the investigated parameters, this study provides important insights in the understanding of the potential use of green tea extract in modulating fermentation processes in the ruminant's stomach. In conclusion, the findings of this study underscore the impact of GTE on gas production regulation, highlighting its potential implications for gastrointestinal health.*

Keywords: *degradability, gas production, hohenheim gas test*

Citation

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INTRODUCTION

Soil Researchers and agriculturalists have been paying close attention to the investigation of natural supplements and their possible advantages in ruminants nutrition in recent years (Kolling et al., 2022; Ramdani et al., 2023). Green tea extract is unique among these supplements because it contains a high concentration of bioactive substances, such as polyphenols and catechins, which have been linked to a number of health benefits. Green tea extract has potential benefits for animal performance and health when used as a dietary additive in livestock production (Li et al., 2019; Ma et al., 2021). However, its effects on lamb performance remain underexplored, warranting further investigation in this study. To effectively incorporate it into animal diets, however, requires a detailed assessment and knowledge of its effects, particularly with regard to rumen fermentation and gas production.

Understanding the possible impacts of green tea extract supplementation is particularly important in the particular context of lamb production (Zhong et al., 2015; Zhong et al., 2014; Rahmatillah et al., 2024). In Indonesia, local lamb breeds sometimes struggle with nutrition (Ramdani et al., 2024), so adding natural supplements to their meals could be a long-term way to improve wellbeing and production. Thus, in order to shed light on green tea's potential as a nutritional strategy to enhance the efficiency of rumen fermentation and ultimately contribute to the sustainable improvement of lamb production systems, this study aims to evaluate the use of green tea extract in vitro using the Hohenheim gas test method specifically in local lamb.

MATERIALS AND METHODS

Ethics Approval

The Padjadjaran University Research Ethics Commission has accepted the use of local lambs (Garut lamb) that have been slaughtered as rumen fluid donors in this experiment (number: 1337/UN6.KEP/EC/2023)

Study period and location

The maceration process for the original tea leaves was conducted on October 27, 2023, at the Biotechnology Research and Testing Laboratory, Faculty of Animal Husbandry, Universitas Padjadjaran, Indonesia, followed by evaporation at the Central Laboratory of the same university on October 30, 2023. The in vitro incubation, as well as dry matter and organic matter degradability assessments, were carried out from December 25, 2023, to January 15, 2024, at the Biotechnology Research and Testing Laboratory. The VFA measurement was completed at the Central Laboratory on January 16, 2024.

Green Tea Extract (GTE) and Diet Preparation

The Gambung Tea and Quinine Research Centre, located near Bandung in West Java, Indonesia, provided the steam-brewed green tea leaves used in this study.. Subsequently, a 50% concentration ratio of ethanol to water solvent was used to extract the original tea leaves, which were then macerated for 24 hours and twice rinsed. A rotating evaporator set to 45 °C and 50 rpm was used to extract the GTE solution after maceration. Thick GTE was created by this method and applied as a treatment.

A totally randomized experimental design with six repetitions divided into two in vitro runs was utilized to investigate the effects of four distinct GTE treatment dosages (0, 140, 280, and 560 mg/kg) on fermentation profiles, tGP (total gas production), and in vitro degradability. The basal diet consisted of 60:40 commercial concentrate and paddy straw. 21 percent palm kernel meal, 19 percent dried cassava, 15 percent rice bran, 11 percent coffee skin, 9 percent copra meal, 8 percent molasses, 6 percent cocoa shell, 3.9 percent cassava, 2 percent soybean meal, 2 percent premix (LagantorF1 Costumix, Kalbe

Animal Health), 2 percent lime, 1 percent rapeseed meal, 1 percent distillers dried grains, 1% corn germ feed, 5% wheat pollard, 0.3 percent sodium bicarbonate, and 2 percent salt made up the concentrate concentration.

Buffered Innoculum

Each in vitro experiment employed the rumen fluid (RF) of three adjacent lambs that were obtained from a local butcher in Parongpong, Bandung, West Java at 4 P.M. Since the lambs came from the same company, their feeding history and final weight were fairly similar. As soon as the lambs Were slaughtered, the rumen was removed, and the RF was swiftly filtered through two layers of muslin fabric in a big funnel that was fastened to three insulated, warm flasks for the lamb RF. After that, the flasks were filled and carefully sealed to maintain anaerobic conditions for future storage. They were taken to the laboratory for immediate use after being collected. After that, each RF was measured exactly and quickly transferred by filtering between two layers of muslin cloth into a preheated 2.5-liter dark bottle that held a buffer solution in a 1:2 (RF:buffer) ratio (McDougall, 1948). The bottle was then maintained at 39°C in a water bath. After flushing the RF-buffered inoculum vial with CO2 to exclude oxygen, it was securely sealed using a dispenser. Drop by drop, HCl was used to bring each RF-buffered inoculum's pH down to about 7 ± 0.2.

In vitro Incubation

Vaseline was applied thinly to a 100 mL glass syringe (Fortuna Optima), which was then equipped with a straight stopcock and about 200 ± 6 mg of each sample (40:60 Paddy straw silage: concentrate). About 30 mL of RF-buffered inoculum were poured into each glass syringe, and it was then placed in a shaking water bath that was kept at 39°C. Every two hours throughout the entire day, the total gas production (tGP) from each syringe was measured per gram of sample. tGP was also calculated based on dry matter, organic matter degradability and total VFA. To stop the syringes from fermenting further, enough ice was added to the water bath after incubation to replace most of the warm water (Ramdani et al., 2017). Then, to assess the pH and in vitro dry matter degradability (IVDMD), the complete contents of each syringe including the inoculum and residue were transferred into a 50 ml polyethylene tube that had been previously weighed. Samples were prepared for volatile fatty acid (VFA) analysis by centrifuging each tube at 4000 rpm for 20 minutes to separate the liquid phase for further examination. After being thoroughly cleansed with water, every last particle of residue left in the syringe is moved into the proper tube. This undigested residue was dried in a drying oven at 60°C in accordance with Khan and Chaudhry's (2010) guidelines in order to calculate IVDMD

Degradability and fermentation profile Analyses

All of the DM samples were placed in a furnace (Thermo Scientific) and lit in order to evaluate organic matter (OM). Over the course of five hours, the temperature rose to 550 degrees Celsius. It was removed once more and allowed to cool in a desiccator prior to being weighted. The IVOMD was computed and displayed as a percentage of degradability by calculating the difference between OM in the diet and OM in the residue. The IVDMD was computed as a percentage based on the weight loss of the feed sample. The material was cooked in an oven to 60 degrees Celsius for 48 hours in order to perform DM analysis.

After the incubation period, each sample was preserved using H3PO4, following the method for VFA analysis outlined by Ramdani et al. (2022). A GCMS (AGILENT 7890A), detector (MS AGILENT 5977B GC/MS), and

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column (Agilent, 123-3262 DB-FFAP) measuring 60 meters in length, 0.325 mm in diameter, and 0.25 um in film thickness were used to analyze each sample. The mobile phase, helium gas, is used and moves at a milliliter per minute. 160°C is the injector temperature. The temperature rises by 15°C/min to 115°C, where it remains for three minutes after the column is held at 80°C for one minute. It then increases by 3°C/min to 130°C and then increases by 15°C/min to 230°C, a temperature it maintains for three minutes. The ratio split injection method is 15: 1. The sample peak area value will be compared to the standard single peak area in order to calculate the of the volatile free acid mix (Volatile Free Acid Mix; Supelco. CRM46975). Volatile fatty acids (VFA) were calculated by comparing the peak area results with dry matter degradability.

Chemical Analysis

Each paddy straw silage or concentrate sample was randomly sampled in three or five separate parts and pooled. The samples were collected in many blue plastic barrels or sacks, respectively. Every sample was dried for approximately 48 hours at 60 degrees Celsius in an oven. Then, using standard protocols of the Association of Official Analytical Collaboration (AOAC, 2005), each dried sample was ground in a sample disc mill until it passed through a 1-mm sieve. From there, it was subjected to various nutrient analyses to determine crude protein (CP, AOAC 990.03), ash (AOAC 942.05), and ether extract (EE, AOAC 920.39). While acid detergent fiber (ADFom) was measured using Van Soest's (1973) method, the neutral detergent fiber (NDFom) contents were examined using Van Soest et al. (1991) approach without the use of amylase and decalin. According to Ramdani et al. (2022), the approach of Menke and Steingass (1988) was used to analyze metabolic energy (ME). The Folin–Ciocalteu technique was used to evaluate total phenols (TP) and total tannins (TT) (Makkar, 2003; Ramdani et al., 2020) With the exception of DM and ME, which were expressed as percentages of fresh samples and MJ/kg, respectively, other nutrient levels were expressed as percentages of DM (Table 1.)

Stastitical Analysis

One-way ANOVA and Tukey's test were used to compare means in the statistical analysis of the data conducted using MINITAB 16 statistical software. At $P < 0.05$, statistical significance was established. The Anderson-Darling normality test was used to determine whether the residual data were normal, and it was passed at $P > 0.05$.

RESULTS AND DISCUSSION

Green Tea Extract on Ruminal Fermentation

The results of tea extraction revealed a total phenol content of 79.6% and a total tannin content of 47.1% (Table 1). Tea can yield 21% extract containing polyphenolic compounds (Bindes et al., 2019). Polyphenolic compounds, even at low to moderate doses in feed, have been shown to exert positive effects on livestock performance and health (Deepika et al., 2022).

Numerous investigations have examined the possible impacts of green tea extract on several physiological parameters, encompassing its influence on metabolism and digestion in both humans and animals. Although some studies (Li et al., 2019; Ma et al., 2021) have shown encouraging results, indicating that green tea extract may impact metabolic processes and enhance general health, the precise effects on ruminal fermentation and nutrient utilization, as indicated by the parameters analyzed in this study, are still unclear. The current study's lack of substantial effects runs counter to earlier findingst that suggested green tea extract might have a role in regulating ruminal fermentation and nutrient utilization. It is crucial to consider any potential causes of these inconsistent results, such as modifications to the animal models employed, quantities of green tea extract given,

Table 1. Mean proximate contents (% DM or otherwise stated, n = 2) of feed materials.

Nutrients	Concentrate	Paddy straw silage	Tea Extract
Dry Matter	88,70%	35,94%	-
Organic Matter	88,82%	88,85%	-
Crude Protein	14.7%	7.33%	-
EE	4.88%	3.04%	-
ADF	28.0%	38.2%	-
NDF	56,2%	44.4%	-
ME	6.70 MJ/Kg	3.48 MJ/Kg	-
Total phenol	-	-	79.6%
Toral tanins	1.75%	4.80%	47.1%

Ether extract; ADF: Acid Detergen Fiber; NDF: Neutral Detergen Fiber; ME: Metabolisable Energy

Moreover, more research is necessary to determine the mechanism by which green tea extract may influence ruminal fermentation and nutrient utilization. Although the health advantages of green tea's bioactive ingredients, like polyphenols and catechins, have been linked to several studies (Yang et al., 2020), little is known about how these chemicals interact specifically with the rumen environment and how they affect microbial populations. These results are in line with other studies on the bioactive substances found in green tea, especially polyphenols like catechins, which have been linked to several health advantages, including the management of the gastrointestinal tract (Makkar, 2003). Moreover, the lack of substantial difference that was seen at later time periods may be a symptom of a saturation effect, in which the body's reaction to GTE reaches a maximum that is beyond which additional dosages have declining effects. Pharmacokinetic investigations of a variety of bioactive substances, including polyphenols, have reported this saturation occurrence (Cerbin-Koczorowska et al., 2021).

Gas Production and Degradability

The current findings (Table 2) presented indicate that administering green tea extract at various doses did not yield significant effects on several tGP, tGP/IVDMD, tGP/IVOMD, and tGP/VFA of GTE. While these results highlight the lack of statistically significant changes, understanding the relevance of each parameter in rumen fermentation processes remains crucial. Gas production, degradability and volatile fatty acids (VFA) in the sheep rumen are closely related because they are part of the complex rumen fermentation process. Research by Jiao et al. (2016) showed that gas production in the rumen of sheep is mainly influenced by microbial fermentation which produces CH_4 and VFA as by-products. CH_4 production in the rumen of sheep is related to methanogenic microbial activity, while VFA production, such as acetate, propionate, and butyrate, is influenced by the type of feed consumed and the composition of rumen microbes.

Table 2. Means *in vitro* tGP, tGP/IVDMD, tGP/IVOMD, and tGP/VFA of GTE at 24 h incubation

Measurement	Control	T1	T2	T3	SEM	P-Value
tGP (ml/g)	127	120	120	124	4.35	0.624
tGP/IVDMD (mL/%)	0.627	0.586	0.588	0.593	0.039	0.867
tGP/IVOMD (mL/%)	0.461	0.450	0.439	0.471	0.017	0.618
tGP/VFA (ml/mM)	0.046	0.042	0.049	0.045	0.007	0.936

tGP: total gas production; IVDMD: in vitro dry matter degradability; IVOMD: in vitro organic matter

Measuring total gas production (tGP) in in vitro studies offers several key advantages, particularly in evaluating feed fermentation capacity and understanding the fermentation mechanisms within the rumen. The gas produced during fermentation reflects microbial activity in degrading feed components, especially fiber, starch, and protein (Owens & Basalan, 2016). The measurement of tGP provides a comprehensive overview of fermentation efficiency and the potential energy production from specific feeds (Maccarana et al., 2016). Additionally, tGP can be used to estimate volatile fatty acid (VFA) production, which is the primary energy source for ruminants (Wang et al., 2020). In this study, the highest tGP was observed in the control group, indicating a potential inhibitory effect of tea extract on microbial activity. Based on the current findings, although not significant, the highest tGP was observed in the control, indicating that the administration of tea extract reduces gas production. Similarly, in tGP/IVDMD, the gas production per degradable dry matter in untreated samples was higher compared to those treated with tea extract. However, current findings require further exploration to determine their practical implications.

In relation to tGP, the degradability of dry matter (DMD) and organic matter (OMD) in in vitro studies is crucial for evaluating the nutritional quality of feed provided to ruminants. A higher DMD value indicates that the feed is more efficiently digested by rumen microorganisms. Meanwhile, OMD reflects the ability of rumen microbes to metabolize organic matter components, such as proteins, fats, and carbohydrates, which are essential for supporting the physiological functions of livestock (Genzebu & Tesfay, 2015). This relationship is key to improving feed efficiency and ensuring optimal energy production in ruminant. In this study, the lack of significant differences in DMD and OMD further underscores the need to refine GTE dosage and application methods to maximize its impact on nutrient utilization.

Volatile Fatty Acids (VFA)

The measurement of volatile fatty acids (VFA) in in vitro studies provides valuable insights into how feedstuffs are fermented by rumen microbes and the potential energy available to livestock (Vastolo, 2022). Although the current study (Table 3) did not observe significant differences in VFA/IVDMD ratios, these results suggest that GTE supplementation does not significantly alter carbohydrate fermentation pathways in the rumen. A deeper understanding of VFA production helps in formulating more efficient feed strategies to enhance livestock performance, including growth and milk production, while also reducing methane emissions, a greenhouse gas with negative environmental impacts (Pourbayramian et al., 2021).

Similarly, the ratios of acetate (C2)/IVDMD, propionate (C3)/IVDMD, and butyrate (C4)/IVDMD did not show significant differences in this experiment. The insignificant reduction in VFA/IVDMD suggests that the fermentation of feed into volatile fatty acids, particularly acetate (C2), propionate (C3), and butyrate (C4), remains largely unaffected. These VFAs are the final products of carbohydrate fermentation by rumen microbes and play a crucial role in the energy metabolism of ruminants. Interestingly, while

Table 3. Means in vitro VFA/IVDMD, C2/IVDMD, C3/IVDMD, and C4/IVDMD of GTE at 24 h incubation time (n = 6)

Measurement	Control	T1	T2	T3	SEM	P-Value
VFA/IVDMD (mM/%)	0.374	0.382	0.324	0.342	0.057	0.882
C2/IVDMD (mM/%)	0.224	0.225	0.201	0.201	0.033	0.904
C3/IVDMD (mM/%)	0.007	0.007	0.006	0.007	0.006	0.777
C4/IVDMD (mM/%)	0.010	0.011	0.009	0.010	0.009	0.600

IVDMD: in vitro dry matter degradability; IVOMD: in vitro organic matter degradability; VFA: volatile fatty acid. C2: acetate; C3: propionate; C4: butyrate.

Methane Production

In addition, methane (CH_4) is a byproduct of microbial fermentation in the rumen, contributing to energy loss in ruminants. Although treatment demonstrated a reduction in CH_4/IVDMD and CH_4/IVOMD (Table 4), the differences were not statistically significant, suggesting that the observed effects may not solely result from GTE supplementation. It is estimated that around 2-12% of the energy consumed by ruminants is lost in the form of methane (Broucek, 2014; Getabalew et al., 2019; Piñeiro-Vázquez et al., 2015). Measuring methane production in in vitro studies allows for the assessment of feed fermentation efficiency and helps in identifying strategies to reduce methane emissions. VFA production in the rumen, specifically the proportions of acetate (C2), propionate (C3), and butyrate (C4), correlates with methane production. Acetate and butyrate produce hydrogen (H_2) during fermentation, which can be used by methanogens to produce methane (Moss et al., 2000). These strategies not only improve energy utilization efficiency but also mitigate negative environmental impacts. Research on methane production is critical for developing mitigation strategies, such as altering feed composition, using feed additives, or manipulating rumen microbes to reduce emissions (Ribeiro et al., 2015).

Table 4. Means in vitro CH4, CH4/IVDMD and CH4/IVOMD of GTE at 24 h incubation time (n = 6)

Measurement	Control	T1	T2	T3	SEM	P-Value
CH4 (mM)*	4.13	4.17	3.02	3.81	0.518	0.389
CH4/IVDMD (mM/%)	0.102	0.102	0.076	0.091	0.015	0.608
CH4/IVOMD (mM/%)	0.075	0.078	0.053	0.072	0.009	0.302

IVDMD: in vitro dry matter degradability; IVOMD: in vitro organic matter degradability; CH4 calculated by using the formula: 0.45(C2) – 0.275(C3) + 0.4(C4) (Moss et al., 2000).

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In vitro methane measurements are also useful in evaluating the effects of different feeds on methane production before applying them in the field. The observed reduction in methane production (CH_4/IVDMD and CH_4/IVOMD) in the treatment of this study suggests that tea extract has potential to reduce greenhouse gas emissions from ruminant digestive systems. Although a reduction was observed, the difference was not statistically significant, suggesting that the observed changes may not be attributed solely to the supplementation.

Although no significant effects were observed on the investigated parameters, this study provides important insights in the understanding of the potential use of green tea extract in modulating fermentation processes in the ruminant's stomach. As the results did not show significant changes in certain parameters, this study provides a basis for further research on the dosage and formulation of green tea extract that can provide the desired effects in reducing CH4 emissions and increasing feed utilization efficiency in livestock.

CONCLUSION

In conclusion, the findings of this study underscore the significant impact of GTE on gas production regulation, highlighting its potential implications for gastrointestinal health. However, the investigation into administering green tea extract at various doses did not yield significant alterations in ruminal fermentation parameters.

AUTHOR CONTRIBUTION

The contribution of each author in this researR.S.R. Conceptualization, Methodology, Software, Writing – original draft & editing. D.R. Conceptualization, Methodology, Writing – review, Validation, Supervision. I.H. Conceptualization, Methodology, Validation, Supervision. A.J. Conceptualization, Methodology, Validation, Supervision

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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