Effects of Tea Saponin on Rumen Fermentation and Rumen Microbiota in Dairy Cows (Post-print)

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Abstract

This study aimed to investigate the effects of tea saponin on rumen micro-fermentation and rumen microbiota in dairy cows. Twelve healthy Holstein dairy cows with similar body weight were selected and randomly allocated into four groups. All cows were fed a basal diet and administered 0 (control), 20, 30, or 40 g/d of tea saponin via gavage, with the tea saponin mixed with water. A feeding trial was conducted comprising a 14-day preliminary period and a 35-day formal experimental period. During the formal experimental period, rumen fluid was collected using an oral sampler at 1 hour before morning feeding every 7 days to determine rumen fermentation parameters, and rumen microbial abundance was quantified using qRT-PCR. The results showed: 1) Compared with the control group, tea saponin significantly decreased rumen fluid pH (30, 40 g/d groups) and ammonia nitrogen concentration (20, 30, 40 g/d groups) ($P<0.05$), though all values remained within the normal range. It significantly increased microbial protein (30, 40 g/d groups), propionate (20, 30, 40 g/d groups), and butyrate concentrations (20, 30, 40 g/d groups) ($P<0.05$). The microbial protein concentration in the 30 g/d group increased by 20.20%. However, tea saponin had no significant effect on total volatile fatty acids and acetate concentrations ($P>0.05$). 2) Compared with the control group, the abundances of protozoa and Butyrivibrio fibrisolvens in rumen fluid were significantly decreased in all tea saponin groups ($P<0.05$), while the abundances of methanogens, Ruminococcus albus, Fibrobacter succinogenes, Ruminococcus flavefaciens, and fungi showed no significant changes ($P>0.05$). In conclusion, dietary supplementation of tea saponin improved the rumen fermentation pattern and significantly affected the rumen microbiota in dairy cows, with a dosage of 30 g/d being most suitable for dairy cows.

Full Text

Effects of Tea Saponin on Rumen Fermentation and Rumen Microflora in Dairy Cows

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Abstract: This experiment was conducted to investigate the effects of tea saponin on rumen fermentation and rumen microflora in dairy cows. Twelve healthy Holstein cows with similar body weight were randomly divided into four groups. All cows were fed a basal diet and drenched with 0 (control), 20, 30, or 40 g/d of tea saponin mixed with water. The experiment consisted of a 14-day preliminary period followed by a 35-day formal trial period. During the formal trial period, rumen fluid was collected using an oral sampler at 1 hour before morning feeding every 7 days to determine rumen fermentation parameters. Rumen microbial content was measured using qRT-PCR. The results showed that: (1) Compared with the control group, tea saponin significantly decreased rumen fluid pH (in the 30 and 40 g/d groups) and ammonia nitrogen concentration (in the 20, 30, and 40 g/d groups) ($P<0.05$), but all values remained within the normal physiological range. Tea saponin significantly increased microbial protein (in the 30 and 40 g/d groups), propionic acid (in the 20, 30, and 40 g/d groups), and butyric acid concentrations (in the 20, 30, and 40 g/d groups) ($P<0.05$). The microbial protein concentration in the 30 g/d group increased by 20.20% compared with the control. However, tea saponin had no significant effect on total volatile fatty acids and acetic acid concentrations ($P>0.05$). (2) Compared with the control group, tea saponin significantly reduced the contents of rumen protozoa and *Butyrivibrio fibrisolvens* ($P<0.05$), but caused no significant changes in the contents of methanogens, *Ruminococcus albus*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and fungi ($P>0.05$). In conclusion, supplementation with tea saponin improved the pattern of rumen fermentation and significantly affected rumen microflora in dairy cows, with 30 g/d being the most appropriate dosage.

Keywords: tea saponin; dairy cow; rumen fermentation; microbial flora

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Compared with monogastric animals, the physiological characteristic of dairy cows is the presence of a rumen that provides habitat for diverse microorganisms. The essence of rumen fermentation is the fermentation and degradation of dietary cellulose, protein, and other substances by rumen microorganisms to achieve more efficient utilization [1]. However, rumen fermentation also causes losses of energy and ammonia nitrogen (NH -N) and produces methane, leading to environmental pollution [2]. Therefore, selecting scientific and effective

feed additives to regulate rumen fermentation is a critical step for unlocking the nutritional potential of dairy cows, improving feed conversion efficiency, and enhancing milk quality.

Tea saponin, also known as tea saponin glycoside, is a pentacyclic triterpenoid saponin compound extracted from tea seeds (tea seed and tea fruit seed) [3], composed of seven aglycones, four sugar moieties, and two organic carboxylic acids [4]. Tea saponin has been widely applied in pesticide production [5], chemical industry [6], and animal husbandry [7]. Research indicates that tea saponin is not only a natural surfactant but also possesses extensive biological activities and can be used as a rumen fermentation regulator in ruminants to improve animal performance [8]. Lai et al. [9] reported that dietary supplementation with tea saponin could inhibit protozoa growth and improve ruminant production performance. Hu et al. [10] reported in an in vitro gas production experiment that adding 8 mg of tea saponin to 30 mL of fermentation fluid reduced protozoa numbers by 79%. Wang et al. [11] reported that adding a mixture of tea saponin and yucca saponin to goat diets decreased rumen fluid pH and the acetate/propionate ratio. Zhou [12] found that adding tea saponin to ruminant diets did not significantly change total volatile fatty acid (TVFA) production but reduced methane production and altered rumen fermentation patterns. Overall, current research on tea saponin' s regulation of rumen fermentation patterns in dairy cows is rarely reported, particularly regarding its effects on rumen fermentation and microflora. Therefore, this experiment investigated the effects of drenching tea saponin on rumen fermentation and microflora in lactating dairy cows to provide experimental evidence for tea saponin as a rumen fermentation additive.

Experimental Animals and Management

The experiment was conducted from August to September 2014 at the Nankou Second Farm of Beijing Sanyuan Lvhe Dairy Center. Twelve healthy Holstein cows with good body condition, body weight of (550±30) kg, daily milk yield of approximately 35 kg/d, and parity of 2–4 were selected and randomly divided into four groups (n=3) based on similar milk yield, parity, and lactation stage. During the trial period, cows were fed according to the farm's total mixed ration (TMR) feeding program. Cows were fed and milked at 07:30, 14:30, and 21:30 daily. After TMR feeding, cows had free access to exercise and water. The composition and nutrient levels of the TMR are shown in Table 1 .

Experimental Design

Both the control and treatment groups were fed the basal diet. Due to the poor palatability of tea saponin, cows could not consume it consistently through feeding; therefore, tea saponin was drenched before morning feeding daily. Tea saponin at doses of 20, 30, and 40 g was dissolved in 200 mL water respectively. The treatment groups received 20, 30, or 40 g/d of tea saponin via drenching before morning feeding. The entire experimental period lasted 49 days, including

a 14-day preliminary period. Rumen fluid was collected at 1 hour before morning feeding every 7 days during the formal trial period.

Sample Collection and Analysis

Rumen Fluid Collection Rumen fluid was collected at 1 hour before morning feeding using an oral sampler, filtered through four layers of gauze, and pH was measured immediately using a portable pH meter. Other samples were stored in liquid nitrogen for subsequent determination of rumen fermentation parameters and microflora.

Determination of NH -N, Volatile Fatty Acids (VFA), and Microbial Protein (MCP) Concentrations After thawing, rumen fluid was centrifuged at 5,000×g for 10 min, and the supernatant was collected for determination of NH -N, VFA, and MCP concentrations. NH -N concentration was determined by spectrophotometry according to Broderick et al. [13]. MCP concentration was determined by colorimetry according to Zhou [14]. VFA concentration was determined by gas chromatography using 2-ethylbutyric acid (2-EB) as an internal standard according to Hu et al. [15].

Determination of Rumen Microbial Content Extraction of Total Rumen Microbial DNA. Total DNA was extracted using the bead-beating cetyltrimethylammonium bromide (CTAB) method [16]. Briefly, 1.5 mL of rumen fluid was centrifuged at 1,000×g to remove the supernatant. Then 800 L of CTAB buffer and sterilized zirconia beads (0.3 g of 0.1 mm beads and 0.1 g of 0.5 mm beads) were added. The mixture was homogenized in a bead-beater for 2 min, incubated in a 70°C water bath for 20 min, and centrifuged at 13,000×g for 10 min. Then 500 L of the supernatant was mixed with 500 L of saturated phenol/chloroform/isoamyl alcohol (25:24:1), centrifuged at 13,000×g for 10 min, and 300 L of the supernatant was mixed with 280 L of isopropanol. After standing at room temperature for 5 min to precipitate DNA, the pellet was dissolved in TE buffer. The concentration and purity of extracted DNA were determined using a micro UV-Vis spectrophotometer and stored at -20°C.

qRT-PCR Reaction Conditions and Primer Design. The qRT-PCR reaction conditions were established using Sybr Premix Ex TaqTM reagent in a 20 L reaction system [17]. Primers for total bacteria, fungi, methanogens, protozoa, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Butyrivibrio fibrisolvens*, and *Ruminococcus albus* were referenced from literature [18-20] (Table 2). Primer specificity was verified by amplifying bacterial 16S rDNA from total rumen microbial DNA. The qRT-PCR reaction system contained: Premix Taq™ (Ex Taq™ Version 2.0) 10 L, forward and reverse primers 0.5 L each, template DNA 0.5 L, and sterile double-distilled water 8.5 L, for a total volume of 20 L. The qRT-PCR parameters were: denaturation at 95°C for 7 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 7 min.

Statistical Analysis Experimental data were organized using Excel and analyzed using one-way ANOVA in SAS 9.2 software. Differences were considered significant at $P<0.05$.

Results

Effects of Tea Saponin on Rumen Fermentation

The rumen fermentation parameters after tea saponin administration are shown in Table 3 . Compared with the control group, supplementation with 30 and 40 g/d of tea saponin significantly decreased rumen fluid pH ($P<0.05$), while 20, 30, and 40 g/d of tea saponin significantly decreased NH -N concentration ($P<0.05$). Supplementation with 40 g/d of tea saponin significantly decreased the acetate/propionate ratio ($P<0.05$), and 30 and 40 g/d of tea saponin significantly increased MCP concentration ($P<0.05$). The MCP concentration in the 30 g/d group increased by 20.20% compared with the control. Additionally, 20, 30, and 40 g/d of tea saponin significantly increased propionic acid and butyric acid concentrations ($P<0.05$). No significant differences were observed among groups in TVFA and acetic acid concentrations ($P>0.05$).

As shown in Figures 1 [Figure 1: see original paper] through 3 [Figure 3: see original paper], tea saponin decreased rumen fluid pH and NH -N concentration while increasing MCP concentration at all time points, but pH and NH -N values remained within the normal physiological range. The patterns of change over time were inconsistent, with no clear trends for pH and NH -N. However, MCP production was highest in the 30 g/d group.

Figures 4 [Figure 4: see original paper] through 8 [Figure 8: see original paper] show that tea saponin increased propionic acid and butyric acid concentrations at all time points while generally decreasing the acetate/propionate ratio, with no obvious changes in TVFA and acetic acid concentrations. Over time, propionic acid and butyric acid concentrations showed a trend of initial increase followed by decrease and then increase again, while the acetate/propionate ratio showed no clear pattern but was relatively lower in the 40 g/d tea saponin group.

Electrophoresis Detection of Reverse Transcription PCR Products of Rumen Microbial DNA

The results of 1.5% agarose gel electrophoresis detection of reverse transcription PCR products are shown in Figure 9 [Figure 9: see original paper]. Each amplification product matched the expected fragment size, and all target fragments appeared as single bright bands without non-specific bands or tailing, indicating that the designed primers and extracted genomic DNA were suitable for qRT-PCR.

Effects of Tea Saponin on Rumen Microbial Content

The effects of tea saponin administration on rumen microbial content are shown in Table 4 . Compared with the control group, supplementation with 20, 30, and 40 g/d of tea saponin significantly decreased the contents of rumen protozoa and *Butyrivibrio fibrisolvens* ($P<0.05$), but had no significant effects on the contents of fungi, methanogens, *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Fibrobacter succinogenes* ($P>0.05$).

As shown in Figures 10 [Figure 10: see original paper] through 16 [Figure 16: see original paper], tea saponin exhibited obvious inhibitory effects on protozoa and *Butyrivibrio fibrisolvens* at all time points, while showing no significant changes in fungi, methanogens, *Ruminococcus albus*, and *Fibrobacter succinogenes* contents. Over time, fungi and *Ruminococcus albus* contents showed a trend of initial increase followed by decrease, while other microbes showed no clear patterns. However, with increasing tea saponin dosage, protozoa and *Butyrivibrio fibrisolvens* contents decreased markedly, whereas *Ruminococcus flavefaciens*, *Ruminococcus albus*, methanogens, and *Fibrobacter succinogenes* showed no clear trends.

Discussion

Effects of Tea Saponin on Rumen Fermentation Parameters in Dairy Cows

Rumen fluid pH is a fundamental indicator for evaluating rumen fermentation status and is primarily influenced by dietary concentrate-to-forage ratio [21]. The rumen is the direct site of microbial fermentation, and pH that is too high or too low adversely affects normal growth, development, and fermentation of rumen microorganisms [22]. The optimal pH for protozoa, anaerobic fungi, and bacteria are 5.8, 7.5, and 6–7, respectively, while normal dairy cow rumen fluid pH ranges from 5.5 to 6.8, with an optimal range of 6.0–6.3 [23]. Thus, a neutral to slightly acidic environment is optimal for dairy cow rumen microorganisms. Studies have demonstrated that dietary supplementation with tea saponin significantly affects rumen fluid pH. Zhou [14] reported that tea saponin significantly decreased rumen fluid pH in Hu sheep, consistent with findings by Ye et al. [24] and Hu et al. [25]. This experiment found that 30 and 40 g/d of tea saponin significantly decreased dairy cow rumen fluid pH, but pH values in all treatment groups fluctuated between 6.32 and 6.42, remaining within the normal physiological range and thus not adversely affecting normal rumen microbial metabolism. The pH reduction may be attributed to the effect of removing rumen protozoa on the explosive fermentation of starch and soluble sugars, resulting in increased butyric acid and lactic acid production with slower absorption by the rumen wall [26], thereby decreasing rumen fluid pH.

Approximately 55–95% of carbohydrates in ruminants are fermented in the rumen, decomposed into pyruvate, and further broken down into volatile fatty acids (VFAs). VFAs are an important energy source for ruminants, providing

70–80% of their energy, and rumen metabolic activity is measured by VFA concentration and composition [11]. Research has shown that dietary tea saponin supplementation can alter rumen fermentation patterns. Ye [27] reported that tea saponin significantly increased propionic acid concentration in Hu sheep in vitro experiments. Lin et al. [28] reported that tea saponin regulated rumen fermentation patterns by altering VFA composition, with the positive effect primarily due to decreased acetate/propionate ratio, consistent with findings by Zhang [29]. This study found that drenching dairy cows with tea saponin significantly increased propionic acid and butyric acid concentrations while decreasing the acetate/propionate ratio, with no significant change in TVFA concentration. These results indicate that tea saponin can provide more energy and improve feed conversion efficiency by altering rumen fermentation patterns in dairy cows.

NH -N is an important product of rumen metabolism in dairy cows, and its concentration is a key indicator of rumen internal environment quality. Approximately 80% of rumen bacteria use NH -N as their sole nitrogen source for growth, whereas rumen protozoa cannot utilize NH -N to synthesize protein but produce large amounts of NH -N [30]. Studies have shown that tea saponin can reduce rumen NH -N concentration by inhibiting protozoal activity [31]. Ye [27] reported that adding 0.25%, 0.50%, and 1.00% tea saponin to in vitro cultures decreased NH -N concentration in culture substrates to varying degrees. Yuan [32] reported that adding tea saponin to Hu sheep rumen culture fluid significantly decreased NH -N concentration. This experiment found that drenching dairy cows with different concentrations of tea saponin significantly decreased NH -N concentration, but all values remained within the normal range. Han and Chen [33] suggested that when rumen fluid NH -N concentration reaches 8.5 mg/dL, microbial protein synthesis capacity becomes saturated, and exceeding this concentration does not increase MCP production. Excessively high NH -N concentration is absorbed by the rumen wall, leading to high plasma urea nitrogen concentration and increasing nitrogen metabolic burden [34]. Tea saponin supplementation can reduce NH -N concentration and alleviate its negative effects on rumen microorganisms [35].

MCP is the primary nitrogen source provider for ruminants [36], supplying 40–80% of the animal' s nitrogen requirement. Therefore, MCP metabolism determines the nutritional metabolic level of rumen microflora [37]. Rumen bacteria can synthesize MCP using fermentation products, which enters the abomasum with digesta to provide more than half of the protein required by ruminants. Protozoa cannot synthesize protein themselves and rely on engulfing bacteria as a nitrogen source, with only a small proportion reaching the abomasum before autolysis [38]. Thus, removing protozoa undoubtedly reduces ruminal protein fermentation and increases protein utilization efficiency. Bird et al. [39] confirmed that defaunation significantly increased the flow rates of total nitrogen, non-NH -N, various amino acids, and total amino acids to the duodenum, increasing rumen MCP output by 20%. Yuan et al. [40] reported that dietary tea saponin supplementation inhibited protozoal activity and increased MCP production in Hu sheep rumen culture fluid, with 0.8% dosage showing the

best effect, consistent with findings by Hu [41] in Boer goats. This experiment found that drenching dairy cows with tea saponin during feeding significantly increased MCP concentration, though the underlying mechanism requires further investigation.

Effects of Tea Saponin on Rumen Microflora in Dairy Cows

The rumen of ruminants harbors large and diverse microbial populations, mainly including protozoa, bacteria, and fungi [42], and the essence of rumen fermentation is the digestion of cellulose, hemicellulose, and other plant materials by rumen microorganisms. Therefore, rumen microorganisms and their interactions have become an important focus of rumen fermentation regulation research.

Currently, there are two viewpoints regarding protozoal retention. One suggests that rumen protozoa can degrade cellulose and stabilize pH, thus warranting retention. The other argues that protozoal engulfment of bacteria dominates rumen microbial relationships, and since protozoa die through autolysis without providing substantial MCP to the host, their removal is more beneficial for animal production. Studies have found that tea saponin significantly inhibits rumen protozoa in ruminants. Ye et al. [43] found that adding 0.25%, 0.50%, and 1.00% tea saponin to in vitro culture substrates inhibited protozoal growth to varying degrees and improved rumen fermentation status. Guo et al. [44] confirmed that tea saponin significantly reduced rumen fluid protozoa numbers, with additions of 10, 20, 30, and 40 g tea saponin per kilogram of culture medium reducing protozoa numbers by 19%, 25%, 45%, and 79%, respectively. Daiz et al. [45] added 25 g and 50 g of plant saponin to sheep diets (315 g/head) and found that protozoa numbers decreased by 57% and 84%, respectively, compared with the control. This experiment found that drenching dairy cows with tea saponin significantly reduced rumen fluid protozoa content. The antiprotozoal mechanism of tea saponin may involve binding to cholesterol in protozoal cell membranes, preventing repair or shedding, leading to membrane disruption and cell content leakage [46].

Bacteria and fungi play an 80% role in the degradation of dietary fiber in ruminants. Studies have reported that in vitro, rumen fungal cellulolytic enzyme activity is higher than that produced by major rumen cellulolytic bacteria [47]. However, due to slower reproduction compared with bacteria, fungi do not dominate rumen fermentation, with rumen bacteria being primarily responsible for fiber degradation. The main fiber-degrading bacteria in the rumen are *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens* [48]. Mao et al. [37] found that tea saponin supplementation had no effect on *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* numbers. Zhang et al. [49] reported that tea saponin supplementation had almost no effect on fungal numbers. This experiment found that tea saponin had selective effects on rumen bacteria and fungi. Drenching dairy cows with tea saponin significantly reduced *Butyrivibrio fibrisolvens* content, while causing no significant changes in *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus*

flavefaciens contents. Fungal content showed a decreasing trend but the difference was not significant. This selective effect may be because *Fibrobacter succinogenes* is a Gram-negative bacterium with a double-membrane cell wall. Wang et al. [50] reported that Gram-negative bacteria have higher tolerance to external substances than Gram-positive bacteria like *Ruminococcus*. Although *Ruminococcus flavefaciens* and *Ruminococcus albus* are Gram-positive, these two bacteria have lipopolysaccharide layers outside their cell membranes similar to Gram-negative bacteria [51], which can prevent entry of exogenous substances like tea saponin.

Methane production in ruminant rumen fermentation has two sources: protozoal metabolism and methanogenic archaea. Studies have found that approximately 10–20% of methanogens in the rumen are symbiotic with protozoa [52], with these methanogens adhering to protozoal surfaces in a mutually beneficial relationship that facilitates methane production and protozoal growth. Vogels et al. [53] showed that reduced protozoal numbers and decreased hydrogen production (a substrate for methanogens) both inhibited methane production. This experiment found no significant difference in methanogen content in dairy cow rumen with tea saponin supplementation. However, Guo et al. [54] found that tea saponin inhibited expression of the methane synthesis key enzyme gene *mcrA* in methanogens, reducing their activity. Analysis suggests that tea saponin may affect methanogens through two mechanisms: indirectly by inhibiting symbiotic rumen protozoa, or directly by reducing methanogen activity without significantly affecting their numbers. The mechanism of tea saponin' s effect on methane production requires further investigation.

Conclusions

1. Tea saponin significantly decreased rumen fluid pH and NH -N concentration in dairy cows, but all values remained within the normal physiological range. Tea saponin significantly increased propionic acid, butyric acid, and MCP concentrations while decreasing the acetate/propionate ratio, with no significant effect on TVFA concentration.
2. Tea saponin significantly reduced the contents of rumen protozoa and *Butyrivibrio fibrisolvens*, but had no significant effects on the contents of fungi, *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and methanogens.
3. Based on comprehensive evaluation, a dosage of 30 g/d tea saponin is most appropriate for dairy cows.

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Note: Figure translations are in progress. See original paper for figures.