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Effects of Tea Saponin on Rumen Protozoal Community in Dairy Cows: Postprint

Authors: Zhao Shiping, Yan Shuhong, Jiang Qihui, Chang Xiaoxiao, Wang Bing, Fang Luoyun, Jiang Linshu, Xiong Benhai

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Abstract

This study aimed to investigate the effects of tea saponin on the rumen protozoal community in dairy cows. Twelve healthy Holstein dairy cows [body weight (550$±$30) kg, milk yield 35 kg/(head · d), parity 2–4] were selected and randomly divided into 4 groups with 3 cows per group. All cows were fed a basal diet and orally administered 0 (control), 20, 30, or 40 g/(head · d) of tea saponin before morning feeding. The experimental period lasted 49 d, including a 14-d preliminary period and a 35-d formal experimental period. During the formal period, rumen fluid was collected using an oral sampler 1 h before morning feeding every 7 d. Denaturing gradient gel electrophoresis (DGGE) combined with 18S rDNA sequence analysis was used to study changes in the rumen protozoal community. The results showed that: 1) Tea saponin supplementation could selectively inhibit the growth of protozoa in the rumen. 2) Compared with the control group, the richness index and Shannon diversity index were significantly decreased ($P<0.05$), while the dominance index was significantly increased ($P<0.05$) in the 30 and 40 g/(head · d) tea saponin groups; however, the evenness index showed no significant change ($P>0.05$). Tea saponin significantly reduced the number of protozoa belonging to the order Entodiniomorphida within the subclass Vestibuliferia in the rumen. In conclusion, dietary supplementation with 30 or 40 g/(head · d) of tea saponin can inhibit the growth of rumen protozoa and reduce protozoal diversity in dairy cows.

Full Text

Effects of Tea Saponin on Rumen Protozoa Flora of Dairy Cows

ZHAO Shiping[1], YAN Shuhong[1], JIANG Qihui[1], CHANG Xiaoxiao[1], WANG Bing[1], FANG Luoyun[1], JIANG Linshu[1], *XIONG Benhai*[2]

[1]Key Laboratory for Dairy Cow Nutrition of Beijing, College of Animal Science and Technology, Beijing University of Agriculture, Beijing 102206, China
[2]Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China

Abstract

This study investigated the effects of tea saponin on rumen protozoa flora in dairy cows. Twelve healthy Holstein dairy cows [body weight (550$±$30) kg, milk yield 35 kg/(head · d), parity 2–4] were randomly divided into 4 groups with 3 cows each. All cows were fed a basal diet and received oral administration of 0 (control), 20, 30, or 40 g/(head · d) of tea saponin before morning feeding. The experiment lasted 49 days, including a 14-day preliminary period and a 35-day formal experimental period. During the formal period, rumen fluid was collected using an oral sampler 1 hour before morning feeding every 7 days. Denaturing gradient gel electrophoresis (DGGE) combined with 18S rDNA sequence analysis was used to examine changes in rumen protozoa flora. The results showed that: (1) tea saponin supplementation selectively inhibited protozoa growth in the rumen; (2) compared with the control group, both the richness index and Shannon diversity index were significantly reduced in the 30 and 40 g/(head · d) tea saponin groups ($P<0.05$), while the dominance index was significantly increased ($P<0.05$), though the evenness index showed no significant change ($P>0.05$); and (3) tea saponin significantly decreased the population of Entodiniomorphida protozoa within the Vestibuliferia subclass. In conclusion, dietary supplementation with 30 or 40 g/(head · d) tea saponin can inhibit protozoa growth and reduce protozoa diversity in the rumen of dairy cows.

Keywords: dairy cow; protozoa flora; tea saponin

Introduction

The rumen of ruminant animals contains a large population of protozoa that play important roles in fermentation. Protozoa assimilate starch and soluble sugars, storing them as amylopectin and thereby reducing cellulose content in the rumen to stabilize pH. They also inhibit the production of hydrogen and ammonia nitrogen. Therefore, selecting scientific and effective feed additives to modify rumen protozoa flora and regulate rumen fermentation represents an important approach to improving feed conversion efficiency and milk quality in dairy cows.

Tea saponin, also known as tea saponin glycoside, is a pentacyclic triterpenoid glycoside compound extracted from tea seeds (tea seed and tea fruit seed), composed of 7 aglycones, 4 sugars, and 2 organic carboxylic acids. As a natural surfactant and rumen fermentation regulator, tea saponin has been shown to improve ruminant production performance. Wang et al. reported that dietary

supplementation with a mixture of tea saponin and yucca saponin in goats reduced rumen protozoa numbers, pH, and the acetate/propionate ratio. Zhou demonstrated that while tea saponin supplementation in ruminant diets did not significantly affect total volatile fatty acid (TVFA) production, it significantly reduced methane output. Lai et al. found that tea saponin supplementation in dairy cow diets decreased protozoa survival rates. Hu et al. showed through in vitro experiments that adding 8 mg of tea saponin to 30 mL of fermentation fluid reduced protozoa numbers by 79%. Yan et al. reported that supplementation with 20, 30, or 40 g/(head · d) of tea saponin significantly reduced rumen protozoa numbers in a linear and quadratic manner as dosage increased.

While numerous studies have reported the inhibitory effects of tea saponin on protozoa proliferation, few have identified the specific protozoa species affected. This study employed DGGE technology combined with 18S rDNA sequence analysis to investigate the effects of tea saponin on rumen protozoa flora in dairy cows, elucidate its mechanism of action in rumen fermentation, and provide a theoretical basis for tea saponin application in dairy production.

Materials and Methods

1.1 Experimental Material

Tea saponin was purchased from Changshan Branch of Zhejiang Oriental Tea Co., Ltd. The main active component was pentacyclic triterpenoid saponin with a saponin content of 60.2%. Other components included: crude protein 5.5%, crude fiber 26.2%, moisture 4.1%, crude ash 4.0%, and pH 5.0–6.5.

1.2 Experimental Animals and Management

The study was conducted from August to September 2014 at the Nankou Second Farm of Beijing Sanyuan Lvhe Dairy Center. Twelve healthy Holstein dairy cows [body weight (550$±$30) kg, milk yield 35 kg/(head · d), parity 2–4] were randomly allocated to 4 groups (n=3) based on similar milk yield, parity, and lactation stage. During the trial, cows were fed a basal diet following the farm' s total mixed ration (TMR) feeding protocol. Feeding and milking occurred three times daily (07:30, 14:30, 21:30) with free access to water and exercise. The composition and nutrient levels of the basal diet are presented in Table 1 .

1.3 Experimental Design

All groups received the basal diet. Due to the poor palatability of tea saponin, which prevented consistent voluntary intake, oral administration was performed before morning feeding each day. Tea saponin doses of 20, 30, or 40 g were dissolved in 200 mL water and administered orally before morning feeding at levels of 0 (control), 20, 30, or 40 g/(head · d). The total experimental period was 49 days, comprising a 14-day preliminary period and a 35-day formal experimental period. Milk yield was recorded daily, and samples were collected every 7

days during the formal period, with rumen fluid collected 1 hour before morning feeding.

1.4 Sample Collection and Analysis

1.4.1 Milk Sample Collection and Analysis Milk samples were collected every 7 days during the formal period. Samples from morning, afternoon, and evening milkings were mixed in a 4:3:3 ratio and sent to Beijing Sanyuan Dairy Center for composition analysis using a LACTOSCAN automatic ultrasonic milk composition analyzer.

1.4.2 Rumen Fluid Collection Rumen fluid was collected every 7 days during the formal period using an oral sampler 1 hour before morning feeding. The fluid was filtered through four layers of cheesecloth and stored in liquid nitrogen for subsequent analysis of rumen fermentation parameters and protozoa flora.

1.4.3 Extraction of Total Rumen Microbial DNA Total rumen microbial DNA was extracted using the bead-beating cetyltrimethylammonium bromide (CTAB) method. Briefly, 1.5 mL of rumen fluid was centrifuged at 1,000×g and the supernatant discarded. Eight hundred microliters of CTAB and sterilized zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm) were added, and the mixture was homogenized in a bead beater for 2 minutes. After incubation at 70°C for 20 minutes, the sample was centrifuged at 13,000×g for 10 minutes. Five hundred microliters of supernatant was mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 13,000×g for 10 minutes. Three hundred microliters of supernatant was then mixed with 280 L of isopropanol, incubated at room temperature for 5 minutes to precipitate DNA, and dissolved in TE buffer. DNA concentration and purity were measured using a micro-ultraviolet spectrophotometer, and samples were stored at -20°C.

1.4.4 Rumen Protozoa PCR Protozoa were amplified from total rumen microbial DNA using universal protozoan primers GC-1617R (with GC clamp) and 1320F. The primer sequences were: GC-1617R: 5' -CGCCCGCCGCGCCCCGCGCCCGGCCCGCCCCCGCCCGGGGCCAATTGCAAAGATCTATCC-3' (GC clamp underlined) and 1320F: 5' -GGTGGTGCATGGCCG-3' [10]. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The 50 L PCR reaction mixture contained 25 L Premix Taq™ (Ex Taq™ Version 2.0), 1 L each of GC-1617R and 1320F, 1 L template DNA, and 22 L sterile double-distilled water. PCR conditions were: initial denaturation at 94°C for 4 min; 40 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s; final extension at 72°C for 5 min [11]. PCR products were detected by 1.5% agarose gel electrophoresis.

1.4.5 DGGE Analysis of Rumen Protozoa DGGE analysis was performed on PCR products using 8% polyacrylamide gels with a denaturing gradient of 30–50% (formamide and urea). Electrophoresis was conducted at 80 V for 15 hours. Gels were stained with GelGreen and photographed using a gel imaging system.

1.4.6 DNA Recovery and Cloning Sequencing Specific bands were excised from DGGE gels and DNA was recovered by boiling [12]. PCR amplification was performed using primers 1617R and 1320F without GC clamp. Products were purified using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit, ligated into PMD-18T vector, and transformed into *E. coli* JM109. Positive clones were sequenced by Sangon Biotech (Beijing) Co., Ltd., and sequences were compared against KGHL, KI, and RDP databases in GenBank.

1.5 Statistical Analysis

DGGE profiles were analyzed using Quantity One software, and cluster analysis was performed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm. Diversity indices were calculated as follows:

$H = -\Sigma[(ni/n)\ln(ni/n)]$
$E = H/\ln S$
$C = \Sigma(ni/n)^2$

where H is the Shannon index, ni is the peak density of band i, n is the sum of peak densities of all bands in the lane, R is the richness index, S is the total number of bands, E is the evenness index, and C is the dominance index.

A phylogenetic tree was constructed using MEGA 5.10 software. Experimental data were organized in Excel and analyzed using one-way ANOVA in SPSS 17.0 software. Duncan' s multiple range test was used for mean comparisons, with $P<0.05$ considered statistically significant.

Results

2.1 Effects of Tea Saponin on Milk Yield and Composition

As shown in Table 2 , there were no significant differences in milk yield or fat-corrected milk (FCM) yield between the 20 and 30 g/(head · d) tea saponin groups and the control group ($P>0.05$). However, the 40 g/(head · d) tea saponin group showed significantly reduced milk yield and FCM yield compared with the control ($P<0.05$). Milk yield and FCM yield exhibited quadratic changes with increasing tea saponin levels, showing an initial increase followed by a decrease. Specifically, the 30 g/(head · d) group increased milk yield and FCM yield by 4.47% and 9.63%, respectively, compared with the control.

No significant differences were observed in milk protein content, milk fat content, milk urea nitrogen (MUN) content, or somatic cell count (SCC) among the

20, 30, and 40 g/(head · d) tea saponin groups and the control (P>0.05). However, milk fat content increased linearly (P=0.041) and SCC decreased linearly (P=0.045) with increasing tea saponin levels. Compared with the control, milk fat content increased by 2.69%, 9.43%, and 12.46% in the 20, 30, and 40 g/(head · d) groups, respectively, while MUN content decreased by 10.65%, 5.35%, and 2.41%, respectively. The 30 g/(head · d) group reduced SCC by 7.99%. Lactose content was significantly reduced in all tea saponin groups compared with the control (P>0.05), with greater reductions observed at higher supplementation levels.

2.2.1 Electrophoresis Profile of Rumen Protozoa 18S rDNA PCR Products

The electrophoresis profile of rumen protozoa 18S rDNA PCR products is shown in Figure 1 [Figure 1: see original paper]. The amplification produced a single band approximately 290 bp in size, consistent with the expected fragment length based on primer design.

2.2.2 DGGE Profile of Rumen Protozoa

The DGGE profile of rumen protozoa 18S rDNA is presented in Figure 2 [Figure 2: see original paper]. The number of bands in the 20, 30, and 40 g/(head · d) tea saponin groups was lower than in the control group. Dominant bands were numbered from top to bottom (1–11), with some showing reduced intensity or disappearance in the tea saponin treatment groups. The weakening or disappearance of these dominant bands indicates that tea saponin selectively inhibited the growth of certain rumen protozoa.

2.2.3 Effects on Rumen Protozoa Diversity Indices

Analysis of the DGGE profiles revealed significant effects on protozoa diversity indices (Table 3). The richness index and Shannon diversity index were significantly lower in the 30 and 40 g/(head · d) tea saponin groups compared with the control (P<0.05). The 20 g/(head · d) group also showed lower values, but differences were not significant (P>0.05). Both Shannon index (P=0.003) and richness index (P=0.012) decreased linearly with increasing tea saponin levels, while richness index also showed a quadratic decrease (P=0.016).

The dominance index was significantly higher in the 30 and 40 g/(head · d) groups compared with the control (P<0.05), and increased linearly with supplementation level (P=0.042). No significant differences in evenness index were observed among any treatment groups (P>0.05).

2.2.4 Similarity Index of Rumen Protozoa

The similarity index of rumen protozoa DGGE profiles ranged from 0.28 to 0.89 across different tea saponin levels, indicating relatively large individual

variations (Figure 3 [Figure 3: see original paper]).

2.2.5 DNA Sequence Comparison and Phylogenetic Analysis

Sequences obtained from bands 1–11 excised from DGGE gels were submitted to GenBank (accession numbers KU355836–KU355846). Phylogenetic analysis using MEGA 5.10 software revealed that tea saponin supplementation primarily affected Entodiniomorphida protozoa within the Vestibuliferia subclass (Table 4 and Figure 4 [Figure 4: see original paper]).

Discussion

Yan et al. [7] reported that different levels of tea saponin supplementation significantly reduced rumen pH and ammonia nitrogen concentration while increasing microbial protein, propionate, and butyrate concentrations, though TVFA and acetate concentrations remained unaffected. This study utilized DGGE combined with 18S rDNA sequence analysis to investigate changes in rumen protozoa diversity and community structure following tea saponin supplementation. This combined approach was necessary because DGGE alone has limitations, but when integrated with gene cloning and sequence analysis, it provides more comprehensive information.

Previous studies have demonstrated that tea saponin reduces rumen protozoa numbers [13–15]. Our results confirm that tea saponin selectively inhibits the growth of certain protozoa species. Sequence comparison revealed that tea saponin primarily affects Entodiniomorphida protozoa within the Vestibuliferia subclass. Because DGGE only detects dominant protozoa, many species were not represented in the profiles. Analysis of diversity indices showed significant effects of tea saponin, indicating that it influences not only protozoa quantity but also community diversity. This may occur because tea saponin affects bacterial diversity, and bacteria interact with protozoa [16], ultimately altering protozoa diversity. Wallace et al. [14] suggested that tea saponin kills rumen protozoa by complexing with cholesterol on their cell surfaces. Variations in surface cholesterol composition among protozoa species may explain why tea saponin only binds to certain species, thereby altering community diversity. These findings align with Chen [17], who reported that tea saponin inhibited rumen protozoa growth in vitro and changed species proportions, with Dasytricha increasing and Entodinium decreasing significantly at 0.6% supplementation. Similar results were reported by Zhou [18].

Conclusion

Dietary supplementation with 30 or 40 g/(head · d) tea saponin effectively inhibited rumen protozoa growth and reduced protozoa diversity in dairy cows.

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