Response of rumen bacterial diversity and fermentation parameters in beef cattle to diets containing supplemental daidzein

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ABSTRACT
An experiment was conducted to determine the effects of soy isoflavone daidzein on ruminal fermentation, microbial protein synthesis and rumen bacterial community in beef cattle. Six rumen-cannulated adult beef cattle were assigned to three blocks according to similar body weight in a randomised block design, with two cattle each block. Each block randomly received one of the two dietary treatments: control (basal concentrate) and daidzein (supplementation with 500 mg daidzein/kg basal concentrate). High-throughput sequencing data showed that supplemental daidzein increased the relative abundance of bacteria belonging to phylum Bacteroidetes (*p*= 0.050), but reduced and tended to reduce the relative abundance of phylum Spirochaetae (*p*= 0.030) and Firmicutes (*p*= 0.080). At the genus level, the relative abundance of *Prevotella* (*p*= 0.036), *RC9_gut_group* (*p*= 0.019), *Succinivibrio* (*p*= 0.093) and *Ruminobacter* (*p*= 0.085) were increased or tended to be increased by daidzein supplementation. However, supplemental daidzein reduced or tended to reduce some uncultured and unclassified bacteria genus belonging to *Ruminococcaceae* (*p*= 0.028), *Prevotellaceae* (*p*= 0.088) and *Lachnospiraceae* (*p*= 0.068). The ruminal pH (*p*= 0.023) and ammonia-N (*p*= 0.031) concentration were lower in the daidzein group than in the control group, but the daidzein group tended to have greater ruminal total VFA (*p*= 0.063) concentration. Supplemental daidzein increased urinary excretion of allantoin (*p*= 0.005) and total purine derivatives (*p*= 0.007). Current results suggest that supplemental daidzein can affect ruminal fermentation by changing ruminal microbial community, increase the production of VFA and enhance microbial protein synthesis in the rumen, showing the potential of daidzein for improving ruminal fermentation in beef cattle.

Introduction
Daidzein is a natural isoflavone widely existed in plants, mostly legumes such as soybeans (Franke et al. 1994). Therefore, daidzein is present in virtually all natural-ingredient animal diets that use legumes as feedstuff. Because of its strikingly similar chemical structure to the mammalian oestrogen and weak oestrogenic activity in animals, daidzein is known as a phytoestrogen and has been studied extensively for possible beneficial biological activities (Cassidy 2003). The results show that daidzein possess a variety of characteristics such as anti-oxidant, anti-inflammatory, anti-proliferative, differentiation-inducing abilities and can thus affect many biological and physiological processes in animals including livestock (Zhang et al. 1997). As a feed additive, supplemental daidzein in diets increased the productive performance (growth, egg-laying, lactation), enhanced immune function and improved meat quality in pigs, poultry and dairy cows (Greiner et al. 2001; Zhao et al. 2004, 2005; Zhengkang et al. 2006; Rehfeldt et al. 2007; Liu et al. 2014). However, though daidzein has been studied extensively in animals, few data are available regarding its effects on beef cattle, especially on ruminal fermentation and rumen microbial community in beef cattle.

The rumen is inhabited by a diverse consortium of microorganisms, including bacteria, archaea, protozoa, fungi and viruses. A symbiotic relationship existed between these microorganisms and their ruminant hosts. Feed nutrients are degraded to volatile fatty acid.
acid (VFA, mainly acetic, propionate and butyric acids), conserved energy (as ATP), carbon dioxide, methane and ammonia with various extents by the microbial population in the rumen (Nagaraja et al. 1997). The ATP is used mainly for microbial growth including the maintenance of microbial cell functions and increase of microbial mass (Nagaraja et al. 1997). Meanwhile, VFA produced and microbial mass increased are as an energy and protein supply for the ruminants, respectively (Calsamiglia et al. 2007). Therefore, ruminal fermentation triggered by rumen microorganisms ultimately determines the health, growth and performance of ruminant hosts.

A few studies have provided evidence for the relationship between the particular bacterial groups and fermentation products in the rumen. For example, the phylum Bacteroidetes abundances were positively correlated strongly with propionate levels (Koenig et al. 2011; Moran and Shanahan 2014), while Firmicutes were related positively to the production of butyrate (Moran and Shanahan 2014). Carberry et al. (2012) reported a negative association between Prevotella spp. and branched chain fatty acid (isobutyrate and isovalerate). These results suggest that microbial community in the rumen affects the ruminal fermentation pattern. Therefore, it is necessary to understand the variation of these complex microbial populations when investigating the effects of dietary composition on rumen fermentation.

Based on the importance of understanding ruminal metabolism and few available information regarding its variation with dietary daidzein supplementation in beef cattle, the objective of this study was to evaluate the effects of supplemental daidzein on ruminal fermentation, microbial protein synthesis and rumen microbial community in beef cattle.

Materials and methods

This study was approved by the Animal Care and Use Committee of the College of Animal Science and Technology of the Jiangxi Agricultural University.

Animals and sampling

Daidzein was purchased from Ci Yuan Biotechnology Co., Ltd. (purity > 98%, Shaanxi, China). Six rumen-cannulated beef cattle with body weight 408.2, 433.8, 480.9, 497.8, 522.0 and 550.1 kg, respectively, were used in this study. These cattle were assigned to three blocks according to similar body weight in a randomised block design, with two cattle each block. Each block randomly received one of the two dietary treatments: control (basal concentrate) and daidzein (supplementation with 500 mg daidzein/kg basal concentrate). The cattle were tethered in individual stalls and fed an experimental diet in quantities sufficient to provide ad libitum consumption. The experimental diet consisted of rice straw and concentrate mixed in a 40:60 proportion (as-fed basis; Table 1). For daidzein treatment, daidzein was mixed into the mineral premix and added to the concentrate. Diets were supplied to the cattle twice daily at 08:00 and 18:00 h. Fresh water was available for ad libitum consumption throughout the study.

Experimental periods consisted of 14 d of diet adaptation and 5 d of experimental measurements. Samples of ruminal fluid were collected from multiple sites in the rumen at 0, 2, 4, 6, 8 and 10 h after feeding on d 15 and determined the ruminal pH immediately. One millilitre of ruminal fluid was preserved by adding 1 mL of deproteinising solution (100 g/L metaphosphoric acid and 0.6 g/L crotonic acid) to determine VFA. Ten millilitre of ruminal fluid was preserved by adding 1 mL of 1% H2SO4 to determine ammonia-N concentration. The samples were frozen at −20°C until analysed. On day 16, samples of ruminal contents were collected from multiple sites in the rumen before the morning feeding and stored at −80°C for DNA extraction.

To investigate the ruminal microbial protein synthesis, urine output was totally collected using urine collection apparatus from days 17 to 19 of the period. Urine was collected daily into containers with adding 10% H2SO4 (sufficient to maintain pH < 3), weighed, mixed well and 1% daily aliquot was pooled over the 3-day period per cattle. At last, 20 mL urine samples was diluted to 200 mL with distilled water and stored at −20°C for the analysis of allantoin and uric acid.

### Table 1. Basic diet and nutrient composition (daidzein was included according to treatment group).

| Diet ingredient                  | Inclusion levels (% as fed) |
|----------------------------------|-----------------------------|
| Rice straw                       | 40.0                        |
| Corn                             | 39.6                        |
| Wheat bran                       | 11.1                        |
| Soybean meal                     | 4.2                         |
| Cottonseed meal                  | 3.6                         |
| Limestone                        | 0.5                         |
| Mineral–vitamin premix<sup>a</sup> | 1.0                         |
| Chemical composition             |                             |
| Dry matter                       | 91.3                        |
| Ash                              | 10.4                        |
| Crude protein                    | 11.7                        |
| Neutral detergent fibre          | 41.7                        |
| Acid detergent fibre             | 25.9                        |
| Crude fat                        | 1.2                         |

<sup>a</sup> Mineral–vitamin premix (per kg): Vitamin A, 250,000 IU; Vitamin D3, 30,000 IU; Vitamin E, 800 IU; Cu, 1 g; Fe, 5 g; Mn, 4 g; Zn, 3 g; Se, 10 mg; I, 50 mg; Co, 10 mg.
Total urinary purine derivatives (PD) excreted were estimated as the sum of uric acid and allantoin.

**Measurement of metabolic phenotypes**

Ammonia-N concentration in the samples was analysed according to the method described by Weatherburn (1967). To determine total and individual VFA, acidified samples were centrifuged at 11,000×g for 10 min, with the supernatant fraction filtered through a 0.45-μm filter. The VFA concentrations in the filtered samples were determined by a gas chromatograph (Agilent Technologies 7820A, USA) equipped with a free fatty acid phase capillary column (30 m × 0.25 mm × 0.33 μm, Lanzhou Atech, Lanzhou, China) (Zhao et al. 2015). Crotonic acid was used as an internal standard. The uric acid concentration in urine samples was determined using an uric acid assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The allantoin concentration in urine samples was determined using the procedures of Chen and Gomes (1995).

**DNA extraction, PCR amplification of 16S rRNA and sequencing**

Microbial genomic DNA was extracted from rumen contents samples using the QIAamp DNA Stool Mini Kit (Qiagen, China) according to the manufacturer’s instructions. The V3–V4 region of the 16S rRNA gene was amplified using bacterial primers 338F (5′-ACTCCTACG GGA GGC AGC A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′) for all DNA samples (Li et al. 2014). PCRs were carried out in triplicate 20 μL mixture containing 4 μL of 5× FastPfu buffer, 10 ng of template DNA, 0.2 μM of each primer, 0.25 mM of each dNTP, and 1 U FastPfu polymerase (TransGen, China). Thermocycling parameters involved an initial denaturation step 95°C for 2 min, followed by 28 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min. The PCR products were electrophoresed on a 2% (w/v) agarose gel and recovered using an AxyPrep DNA Gel Extraction Kit (Axygen, China). The purified PCR products were quantified by QuantiFlour™-ST fluorimeter (Promega, China). Then, a composite sequencing library was generated by pooling in equimolar ratios of amplicons and sent for paired-end sequencing (2 × 250 bp) on an Illumina Miseq platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

**Bioinformatics analysis**

The raw Fastq data were processed and analysed as described by Sun et al. (2015). Operational Taxonomic Units (OTUs) were clustered with a 97% similarity cut-off from the clean Fastq data and chimeric sequences were identified and removed using Usearch 7.1 (http://drive5.com/uparse/). These OTUs were used for diversity (Shannon and Simpson), richness (Ace and Chao) and rarefaction curve analysis using Mothur v.1.30.1 (http://www.mothur.org) (Schloss et al. 2011). Representative sequences of OTUs were aligned to the Silva database (Release 119, http://www.arb-silva.de) for bacteria taxonomic assignments using Qiime (http://qiime.org/scripts/assign_taxonomy.html) (Quast et al. 2013).

**Statistical analysis**

Data for urinary purine derivatives and bacterial relative abundance were analysed using a Univariate General Linear Model (GLM) in IBM SPSS statistics version 20 (IBM, Chicago, IL), with treatment as a fixed effect and block as a random effect. Data for ruminal VFA, pH, and ammonia-N were analysed using the Mixed Models for repeated measures. For each variable analysed, data were subjected to two covariance structures: compound symmetry and autoregressive of order 1. The desirable covariance structure was determined according to Schwarz’s Bayesian criterion (Littell et al. 1998). Significance was declared at $p < 0.05$, and trends were discussed at $p < 0.10$.

**Results and discussion**

A total of 162,866 high-quality sequences were generated from the six samples from six cattle. The average length of the quality sequences was 441 bp. These sequences were assigned to 575 OTUs according to the 97% sequence identity. These sequences were assigned to 575 OTUs according to the 97% sequence identity. Based on the OTUs, bacterial richness indices were estimated by the method of Ace and Chao, and bacterial diversity indices were determined using the method of Simpson and Shannon (Table 2). The statistical results indicated...
there were no significant differences in the Ace, Chao and Simpson index, but the Shannon index was greater for control group than for daidzein group (p = .003). The results of Good’s coverage showed that 99.6% of the microbial species were sampled for the microorganisms of two treatments, indicating that the sampling effort had sufficient sequence coverage for each group.

Based on the Silva taxonomic database and using the analysis programme Qiime, 575 bacterial OTUs were classified and assigned to 16 phylum and 97 genus in the present study. On average, the top three bacterial phylum in the two diet groups were Bacteroidetes (66.68%), Firmicutes (22.73%) and Proteobacteria (5.12%) (S1 Table). However, some differences were observed in the phylum compositions between the two groups (Table 3). The distribution of Bacteroidetes in the daidzein group was greater than in control group (72.29% and 61.06%; p = .050). However, supplemental daidzein reduced and tended to reduce the relative abundance of bacteria belonging to the phylum Spirochaetae (p = .030) and Firmicutes (18.22% and 27.24%; p = .080), respectively.

At the genus level, Prevotella, RC9_gut_group and BS11_gut_group_norank were the dominant bacteria in the two diet groups, averaging 35.56%, 12.63% and 12.19%, respectively (S2 Table). Supplemental daidzein increased or tended to increase the relative abundance of Prevotella (37.41% and 33.72%; p = .036), RC9_gut_group (15.65% and 9.61%; p = .019), Succinivibrio (p = .093) and Ruminobacter (p = .085). Owing to less relative abundance (<0.1%), some genus with different distribution between the two groups were not shown. In addition, many uncultured and unclassified bacteria genus belonging to Ruminococcaceae (p = .028), Prevotellaceae (p = .088) and Lachnospiraceae (p = .068) were lower or tended to be lower in daidzein group than in control group.

Supplemental daidzein reduced ruminal pH (p = .023) and ammonia-N concentration (p = .031) compared with control cattle (Table 4). The total VFA concentration tended to be greater in cattle fed daidzein diets than those fed control diets (p = .063). No treatment differences were noted for the DM intake (p = .724) and molar proportion of butyrate (p = .225), whereas acetate (p = .022) and propionate (p = .069) proportions increased and tended to reduce, respectively, with supplemental daidzein, resulting in a significant increase in the acetate:propionate ratio (p = .024).

There are no differences in excretion of uric acid between the two groups (p = .194), but daidzein supplementation increased excretion of allantoin by 26 mmol/d (p = .005), which resulted in an increase in the excretion of total PD (p = .007) (Table 5).

It is well known that daidzein can modulate the metabolism of animals, but little information is available regarding its effects on ruminal fermentation of beef cattle. The present study showed the responses of ruminal fermentation characteristics and bacterial community of beef cattle fed diets with daidzein supplementation by high-throughput sequencing technology. These results will give helpful information on the effects and application of daidzein in ruminants farming.

The microorganisms present in the rumen play an important role in helping the ruminant host to digest various plant materials. In the present study, the microbial community of beef cattle was dominated by Bacteroidetes and Firmicutes on the phylum level regardless of group. This is consistent with the study by de Oliveira et al. (2013), in which Bacteroidetes and Firmicutes are found predominantly in both liquid and solid fractions of rumen from steers.

| Table 3. Effects of daidzein supplementation on the ruminal bacterial abundance at phylum and genus levels (%) in beef cattle. |
|---------------------------------------------------------------|
| **Item** | **Phylum level** | **Control** | **Daidzein** | **SEM** | **p** |
|--------|-----------------|-------------|-------------|--------|------|
| Bacteroidetes | 61.06 | 72.29 | 3.835 | .050 |
| Firmicutes | 27.24 | 18.22 | 2.963 | .080 |
| Spirochaetae | 0.83 | 0.38 | 0.132 | .030 |
| **Genus level** | | | | | |
| Prevotella | 33.72 | 37.41 | 4.065 | .036 |
| RC9_gut_group | 9.61 | 15.65 | 1.422 | .019 |
| Ruminobacter | 0.36 | 0.55 | 0.205 | .085 |
| Succinivibrio | 0.87 | 1.74 | 0.533 | .019 |
| Ruminococcaceae_uncultured | 7.62 | 3.50 | 1.014 | .028 |
| Prevotellaceae_uncultured | 4.66 | 2.50 | 0.758 | .088 |
| Lachnospiraceae_unclassified | 0.74 | 0.49 | 0.105 | .068 |

| Table 4. Effects of daidzein supplementation on DM intake, ruminal pH and fermentation in beef cattle. |
|---------------------------------------------------------------|
| **Item** | **Control** | **Daidzein** | **SEM** | **p** |
|--------|-------------|-------------|--------|------|
| DM intake, kg/d | 7.91 | 8.28 | 0.582 | .724 |
| pH | 6.66 | 6.42 | 0.052 | .023 |
| Ammonia-N, mg/dL | 13.17 | 8.18 | 0.699 | .031 |
| TVFA, mM | 95.27 | 117.73 | 4.387 | .063 |
| Acetate:Propionate | 3.39 | 3.69 | 0.060 | .024 |

| Table 5. Effects of daidzein supplementation on urinary excretion of purine derivatives (PD) in beef cattle. |
|---------------------------------------------------------------|
| **Item** | **Control** | **Daidzein** | **SEM** | **p** |
|--------|-------------|-------------|--------|------|
| Allantoin, mmol/d | 58.50 | 84.49 | 10.024 | .005 |
| Uric acid, mmol/d | 10.94 | 12.25 | 1.111 | .194 |
| Total PD, mmol/d | 69.44 | 96.74 | 11.046 | .007 |
Jami et al. (2013) also found that Bacteroidetes and Firmicutes were detected as the dominant phyla in the rumen samples from 6-month-old and 2-year-old Holstein cows. Current results showed that supplemental daidzein changed the ruminal bacterial community, increased the phylum Bacteroidetes and reduced the phylum Firmicutes and Spirochaetae. The increase in the phylum Bacteroidetes are largely the result of increased genus *Prevotella*, *RC9_gut_group* (Rikenellaceae family), *Succinivibrio* and *Ruminobacter*, while the repressed phylum Firmicutes was attributed mainly to reduced genus *Ruminococcaceae_uncultured* and *Lachnospiraceae_unclassified* with supplemental daidzein. Considering few references about the relation between daidzein and ruminal bacterial community, this paper only compared our results with those obtained from mice. Similar to our results, Zhang et al. (2012) reported increased Bacteroidetes and reduced Firmicutes in mice with ovariectomy in response to daidzein supplementation. The reasons responsible for these results are still not well-known, but it is recognised that intake of some polyphenols, including daidzein and equol (one of ruminal metabolites of daidzein), can modify the rumen and other gut microbial composition of host (Lundh 1995; Mao et al. 2007; Selma et al. 2009; Cardona et al. 2013). Rastmanesh (2011) pointed out that polyphenols may modulate microbiota balance through their more growth promoting effects on Bacteroidetes and more growth suppressing effects on Firmicutes. The selective effects can be attributed to a complex fact that both the phenolic substrates supplied to the gut bacteria and the metabolites produced may in turn modulate and cause fluctuations in the composition of the microflora populations through selective prebiotic effects and antimicrobial activities against gut pathogenic bacteria, namely the two-way phenolic–microbiota interaction (Selma et al. 2009; Cardona et al. 2013). In the present study, increased genus (*Prevotella*, *RC9_gut_group*, *Succinivibrio* and *Ruminobacter*) by daidzein all belonged to Gram-negative bacteria (Fonty and Chaucheyras-Durand 2006), while repressed genus *Ruminococcaceae_uncultured* and *Lachnospiraceae_unclassified* belonged to Gram-positive bacteria (Clarke et al. 2013). These results are consistent with previous studies in which Gram-positive bacteria are more sensitive to polyphenols than Gram-negative bacteria, due to the differences found in their wall composition (Puupponen-Pimiä et al. 2005; Patra 2012). In addition, while these Gram-positive bacteria groups were inhibited, others maybe thrived in the available niche of the ecosystem (Cardona et al. 2013). However, on the whole, the exact mechanism that daidzein modulates the rumen microbial community need be further investigated.

An important activity of the gut microbiota is formation of VFA, which contribute to ruminants’ health by sustaining rumen ecosystem and acting as an energy source (Ralphs et al. 1995). Production of total VFA during ruminal fermentation is indicative of availability of fermentable energy, especially complex carbohydrates polysaccharides, from the diet substrate. Microbial community and diversity greatly influences the production of VFA (Mohan et al. 2010). Supplemental daidzein tended to increase the total VFA production in the present study, which may be related to changed relative abundance of Bacteroidetes and Firmicutes. Accordingly, a positive relationship between the Bacteroidetes abundance and total VFA production was observed in some studies (Koenig et al. 2011), which attributed to more glycan-degrading enzymes possessed by Bacteroidetes than Firmicutes during fermentation (Mahowald et al. 2009). In addition, it has been suggested that Firmicutes is a phylum with fibrolytic metabolic pattern, which mainly digest cellulose, hemicellulose, and some simple sugars widely existing dietary forage (Sandri et al. 2014; Klang et al. 2015), while the phylum Bacteroidetes is mainly responsible for the digestion of starch from dietary grain (Cersosimo et al. 2015; Klang et al. 2015). Starch is digested faster and produces more VFA than fibre in rumen (Beauchemin et al. 2006). Therefore, increased phylum Bacteroidetes with supplemental daidzein may improve the production of VFA in the present study. In addition, based on more PD (an index of microbial protein synthesis) produced in daidzein group, supplemental daidzein may increase total microbial numbers in rumen, consequently resulting in more feed fermentation and VFA production.

Supplemental daidzein increased the molar proportion of acetate in the present study, which was inconsistent with previous report by Mao et al. (2007). Similarly, some studies also found that Bacteroidetes abundances were positively correlated strongly with propionate levels (Koenig et al. 2011; Moran and Shanahan 2014). The increase in acetate proportion in current study may be attributed to increased abundances of *Prevotella*, *RC9_gut_group*, *Succinivibrio*, and *Ruminobacter* by daidzein. The genus *Prevotella* and *RC9_gut_group* belong to the family *Prevotellaceae* and *Rikenellaceae*, respectively, whose main fermentation products are acetate, propionate and succinate from carbohydrates (Jacobs et al. 2009). The genus *Succinivibrio* and *Ruminobacter* belong to the family *Succinivibrionaceae*, which ferments carbohydrates to
acetate and succinate rather than propionate (Stackebrandt and Hespell 2006). Therefore, the combined action from these geni increased may be responsible for the increase of acetate proportion in the present study. However, the speculation may be inaccurate because it has been unclear that which is the predominate fermentation acid between acetate and propionate produced by the family Prevotellaceae and Rikenellaceae.

Low ruminal pH can be caused by an accumulation of ruminal VFA (Lana et al. 1998). In the present study, lower ruminal pH was observed in the daidzein group, which was in accordance with greater total VFA production in this group. The increased urinary excretion of PD suggested that the ruminal microbial protein synthesis would be increased with daidzein supplementation. Ruminal fermentable carbohydrate and available nitrogen source were the major dietary factors affecting microbial protein synthesis (Krause et al. 2002). The increased VFA production and reduced ammonia-N concentration by daidzein supplementation would support an increase in ruminal microbial protein synthesis. The reduction in ruminal ammonia-N concentration could be due to an enhanced growth of ruminal microbial populations that would increase the ammonia-N consumption. Similar to our results, Mao et al. (2007) also observed the increased microbial protein synthesis and reduced ammonia-N concentration by supplemental daidzein in an in vitro fermentation study using mixed rumen microorganisms.

Conclusions

Supplemental daidzein significantly affected the ruminal fermentation in beef cattle, which may be attributed to the effects of daidzein on ruminal microbial community. Increased phylum Bacteroidetes and genus Succinivibrio and Ruminobacter with supplemental daidzein maybe resulted in the increase in production of ruminal VFA and molar proportion of acetate, respectively. The addition of daidzein increased the production of VFA, reduced ammonia-N concentration, and enhanced microbial protein synthesis in the rumen, showing the potential of daidzein for improving ruminal fermentation in beef cattle.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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