Effect of daidzein on fermentation parameters and bacterial community of finishing Xianan cattle

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ABSTRACT
An experiment was conducted to determine the effects of adding different amounts of daidzein in diet on the fermentation parameters and bacterial community composition in the rumen of finishing cattle. A total of thirty head of 2-year-old finishing Xianan cattle were divided by weight into three groups and randomly allotted by group to one of three dietary treatments: (1) control; (2) 500 mg/kg daidzein and (3) 1000 mg/kg daidzein. All cattle were slaughtered to obtain rumen samples after a 80-d feeding trial. Results showed that adding 500 mg/kg daidzein reduced the operational taxonomic units (OTU) numbers, Shannon index and Chao1 index compared with control and 1000 mg/kg daidzein groups. Compared with control group at phylum level, Planctomycetos and Tenericutes were significantly more abundant in 500 mg/kg daidzein group, Verrucomicrobia and Fusobacteria were significantly rarer in 1000 mg/kg daidzein group. Compared with control group at genus level, Prevotella and Ruminococcus were significantly more abundant in 500 mg/kg daidzein group, Prevotella was significantly more abundant and Dethiosulfatibacter was significantly rarer in 1000 mg/kg daidzein group. Adding 1000 mg/kg daidzein significantly improved the total volatile fatty acids (TVFA) concentration and acetate content and significantly reduced the butyrate content compared with control group. Daidzein changed the fermentation parameters mainly by impacting the abundance of Prevotella and Ruminococcus. Current results suggest that adding 1000 mg/kg daidzein in diets can improve the rumen fermentation function for finishing Xianan cattle.

Introduction
Rumen is the primary site of digestion in ruminants due to a complex microbial community including anaerobic bacteria, archaea, ciliate protozoa and fungi (Wright and Klieve 2011). Rumen bacteria play a central role in regulating rumen fermentation and convert dietary polysaccharides to volatile fatty acids (VFAs), such as acetate, propionate, butyrate and valerate (Kamra et al. 2012). These VFAs provide the host with main metabolic energy and essential nutrients (Ahmed et al. 2013). The population of key rumen bacteria with respect to fatty acid metabolism, fibre degradation, methanogenesis and bacterial predation have a major impact on the rumen fermentation function and animal performance (Denman and McSweeney 2010). The population of key rumen bacteria with respect to fatty acid metabolism, fibre degradation, methanogenesis and bacterial predation have a major impact on the rumen fermentation function and animal performance (Denman and McSweeney 2010). Therefore, modulating rumen bacteria population to improve feed utilisation and enhance animal performance is a major research focus (Patra et al. 2012).

Various feed additives, such as isoflavones, have been evaluated to improve rumen fermentation function and boost animal productivity. Isoflavones which are commonly found in soybeans, alfalfa and certain other plants are structurally similar to oestrogen and have oestrogenic activity (Rowland et al. 1999). As the major metabolites from isoflavones, daidzein is known as a phytoestrogen and have possible beneficial biological activities which has been studied extensively (Cassidy 2003). The results show that daidzein possess various characteristics such as antioxidant, differentiation-inducing abilities and anti-proliferative (Zhang et al. 1997). Besides, daidzein can affect the growth of gastrointestinal microorganisms. During in vitro fermentation with piglet faeces as inoculum, the treatment of 50 mg/L daidzein group increased the numbers of Lactobacilli (Yao et al. 2004). The diet supplementation of daidzein at 10 mg/kg increased the growth of some rumen bacterial species.
apparently in goats (Yao et al. 2004). Infusion of daidzein into duodenum of two male water buffalo (500 mg/d, 12 d) induced increases of rumen bacterial protein, TVFA and ammonia concentration (NH₃-N), which suggested that daidzein improved rumen bacterial digestion and metabolism in ruminates (Chen 1999). Daidzein can be metabolised to equol by rumen microorganisms in cows (Qin et al. 2014). But in cattle, information on use of daidzein is limited.

Xianan cattle (Bos taurus) is the main meat specially in China (Ru et al. 2006). Xianan cattle is a natural hybrid of Charolais cattle (male parent) and Nanyang cattle (female parent). It has the characters of fast growth, high meat quality, coarse feedstuff tolerance and strong adaptability (Wang et al. 2008, 2010). However, few investigations has been focused on the rumen fermentation of Xianan cattle. Moreover, the effect of daidzein on the fermentation parameters and bacterial community in the rumen of finishing Xianan cattle have not been reported. Therefore, the purpose of this article was to study the effects of daidzein on fermentation parameters, bacterial community composition and the co-occurrence relationship between fermentation parameters and bacteria community of finishing Xianan cattle.

**Material and methods**

**Animals, diets and experimental design**

All procedures were specially approved by the ethics committee of Jiangxi Agricultural University. Daidzein was purchased from Ci Yuan Biotechnology Co., Ltd. (purity >98%, Shaanxi, China). A total of thirty head of 2-year-old finishing Xianan cattle (685.93 ± 50.85 kg initial body weight) were used in a 80-d feeding study during the finishing stage. These cattle were divided by weight into groups and randomly allotted by group to one of three dietary treatments: (i) control; (ii) 500 mg/kg daidzein (500 mg/kg concentrate) and (iii) 1000 mg/kg daidzein (1000 mg/kg concentrate). The cattle were tethered in individual stalls and fed a 80% concentrate diet in quantities sufficient to provide ad libitum consumption (Table 1), the design of feed formulation was in combination with actual production experiences. For daidzein treatments, daidzein was mixed into the mineral premix and added to the concentrate. Diets were supplied to the cattle twice daily at 07:00 and 14:00 h. Adding daidzein had no difference in forage and concentrate intake among three groups. Fresh water was available for ad libitum consumption throughout the study.

After 80-day feeding experiment, all cattle were slaughtered at a commercial slaughterhouse following the normal procedures (Editor-in-Chief of China Standard Press 2005). The rumen samples including solid and liquid fractions were obtained and stored at −80°C for follow-up analysis.

**Ruminal fermentation parameters**

All rumen samples were filtered through four layers of cheesecloth into tubes for analysis of pH, ammonia nitrogen (N) and VFAs. The pH of rumen fluid was determined immediately using a pH metre (pHS-3C; INESA Scientific Instrument, Shanghai, China). Concentration of ammonia nitrogen was determined (Model 721/721-100, Shanghai, China) colorimetrically using a spectrometer with ammonium chloride solution as a standard (Searle 1984). The VFA were determined by high-performance liquid chromatography (HPLC) (LC-10A; Shimadzu, Tokyo, Japan) and the analytical conditions were as follows: column, Shodex RSpak KC-811S-DVB gel C (8.0 mm × 30 cm, Shimadzu); oven temperature, 30°C; mobile phase, 3 mmol L⁻¹ HClO₄; flow rate, 1.0 mL min⁻¹; injection volume 5 μL; detector, SPD-M10AVP (Tian et al. 2014).

**DNA extraction, PCR amplification and sequencing**

Five rumen samples from each group were selected randomly for DNA extraction. Total genomic DNA was extracted from each rumen sample using the Stool Genomic DNA kit (CWBio, Tianjin, China) according to the manufacturer’s instructions. HPLC-purified primers targeting the V3 and V4 regions of the ribosomal RNA gene originally designed for pyrosequencing (Herlemann et al. 2011) were adapted to Illumina sequencing by complementing both 338F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACH...
VGGTWTCTAAT) with sample-specific barcodes. This region of the rRNA gene appears optimal for interrogating bacterial communities (Mizrahiman et al. 2013). The following protocol was used to amplify bacterial 16S rRNA gene sequences: 95°C for 3 min; followed by 25 cycles for 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final extension at 72°C for 5 min. The resulting PCR products were pooled and purified using QIAquick Gel Extraction Kit (Qiagen, Germany). A mixture of the amplicons was then used for sequencing on Illumina MiSeq platform (San Diego, CA).

Data analysis

After sequencing, the primers and spacers were trimmed. The paired-end (PE) reads were overlapped to assemble the V3 and V4-tag sequences using the Flash programme (Magoc and Salzberg 2011). To minimise the effects of random sequencing error, both the low-quality fragments and the sequences <240 bp were removed. The PCR chimaeras were checked and filtered out by UCHIME (Tiburon, CA) (Edgar et al. 2011). Each sample was randomly re-sampled and normalised at 1,270,455 sequences. The sequences were classified into OTU by setting a 0.03 distance limit using the CD-HIT programme (La Jolla, CA) (Li and Godzik 2006). The taxonomic assignment of OTUs was performed by Ribosomal Database Project (RDP) classifier at 80% threshold (Wang et al. 2007). The Shannon diversity index, Chao1 index, Simpson diversity index and rarefaction curves were generated using Mothur programme (Ann Arbor, MI) (Schloss et al. 2009). The Metastats programme from Mothur was used to identify significantly different phylotypes among the groups. The data analysis of ruminal fermentation parameters was determined by analysis of variance (ANOVA) with the SPSS statistical software (version 20.0, SPSS Inc., Chicago, IL). The results were expressed as the mean values and the standard error of mean (SEM). All statements of significance are based on a p value <0.05. The co-occurrence pattern was constructed between the OTUs and the rumen fermentation profiles using the method as described by Zhang et al. (2014). The co-occurrence networks were then visualised using Cytoscape 2.8.2 with a force-directed algorithm (San Diego, CA) (Smoot et al. 2011).

Results

Summary of sample sequences, OTU and bacterial α-diversity index

As shown in Table 2, a total of 1,077,218 raw high-quality 16S rRNA gene sequences were obtained from 15 rumen samples. Using these sequences, 32,021 core OTUs were identified based on 97% sequence identity (equal to species level). Coverage reached 0.99 for all sequences in the three groups, indicating good sequencing depth for investigation of rumen bacteria. The OTUs per sample, ACE, Chao1 and Shannon index in the 500 mg/kg daidzein group were lower than those in the Control group and 1000 mg/kg daidzein group.

Comparison of bacterial community composition in the three groups

As shown in Figure 1, the OTUs were assigned to 20 phyla and 50 genera. The top three phyla were Bacteroidetes, Firmicutes and Proteobacteria (Figure 1(a)). Prevotella, unclassified bacteria, and Succinivibrio were the top three genera in the three groups (Figure 1(b)). The Venn diagram and PCoA plots showed that the bacterial community in the 500 mg/kg daidzein group was different from those in the Control and 1000 mg/kg daidzein group (Figure 2).

Altered bacterial community composition between control and 500 mg/kg daidzein group

Metastats was used to investigate the associations between community composition of rumen bacteria and 500 mg/kg daidzein group. The 500 mg/kg daidzein and control groups exhibited statistically significant differences with regard to Planctomycetes and Tenericutes (p < .05) (Figure 3(a)).

Rumen bacterial communities were also compared at the genus level. The abundances of 18 genera differed between the 500 mg/kg daidzein and control groups. Among the different genera, Prevotella, Ruminococcus, Alloprevotella, Vampirovibrio and Thauera were relatively more abundant in the 500 mg/kg daidzein versus control group (p < .05). However, Dethiosulfatibacter, Subdivision3 genera incertae sedis,
Figure 1. Rumen bacterial compositions at phylum (a) and genus (b) levels across three groups.

Figure 2. Comparison of bacterial community in the rumen of Xianan cattle from the three groups based on the Venn (a) and PCoA (b) plotting.

Figure 3. Taxonomic differences of rumen bacteria at the bacterial phylum levels. Comparison of relative abundance in control versus 500 mg/kg daidzein (a) and control versus 1000 mg/kg daidzein (b). Hash (#) indicates $p < .05$. 
Anaerofilum, Blastopirellula, Thermogutta, Anaerohabidus, Parvibacter, Syntrophosethermus, Pseudosphingobacterium, Helioabillus, Haemophilus, Clostridium and Soonwooa were relatively more abundant in the control versus 500 mg/kg daidzein group ($p < .05$) (Figure 4(a)).

**Altered bacterial community composition between control and 1000 mg/kg daidzein group**

Metastats was used to investigate the associations between community composition of rumen bacteria and 1000 mg/kg daidzein group. The 1000 mg/kg daidzein and control groups exhibited statistically significant differences with regard to Verrucomicrobia and Fusobacteria ($p < .05$) (Figure 3(b)).

Rumen bacterial communities were also compared at the genus level. The abundances of 17 genera differed between the 1000 mg/kg daidzein and control groups. Among the different genera, Alloprevotella, Selenomonas, Succinimonas, Centipeda, Candidatus Endomicrobium, Akkermansia and Desemzia were relatively more abundant in the 1000 mg/kg daidzein versus control group ($p < .05$). However, Dethiosulfatibacter, Oscillibacter, Subdivision3 genera incertae sedis, Eubacteri, Parvibacter, Enterorhabdus, Fusobacterium and Snodgrassella were relatively more abundant in the control versus 1000 mg/kg daidzein group ($p < .05$) (Figure 4(b)).

**Ruminal fermentation parameters**

As shown in Table 3, the acetate content in the 1000 mg/kg group was significantly higher than that in control group ($p < .05$). The butyrate content was significantly lower in the 1000 mg/kg group than that in control group ($p < .05$). The TVFA concentration in control group was lower than that in the 500 mg/kg and 1000 mg/kg groups, but it was not significant in statistic ($p > .05$). There were no differences in the pH, NH$_3$-N, propionate, isobutyrate, valerate, isovalerate content and acetate-to-propionate ratio (A/P) among the three groups ($p > .05$).

Table 3. Effects of daidzein on pH, VFA profiles and NH3-N contents of Xianan cattle.

| Daidzein supplementation levels, mg/kg | 0 | 500 | 1000 | SEM | $p$ value |
|---------------------------------------|---|-----|------|-----|----------|
| pH                                   | 6.85 | 6.51 | 6.49 | 0.11 | .359     |
| NH$_3$-N, mg/100 mL                  | 19.90 | 16.17 | 19.41 | 1.58 | .973     |
| TVFA, mmol/L                         | 114.36 | 125.39 | 123.80 | 3.48 | .034     |
| Acetate, %                            | 50.24$^b$ | 56.07$^{ab}$ | 58.49$^a$ | 1.48 | .020     |
| Propionate, %                        | 19.83 | 20.14 | 22.35 | 1.37 | .490     |
| Butyrate, %                          | 22.26$^b$ | 16.89$^{ab}$ | 12.66$^a$ | 1.90 | .038     |
| Isobutyrate, %                       | 4.88 | 3.38 | 2.53 | 0.49 | .128     |
| Valerate, %                          | 1.77 | 2.57 | 2.90 | 0.42 | .521     |
| Isovalerate, %                       | 1.01 | 0.94 | 1.07 | 0.07 | .881     |
| Acetate to propionate                | 2.82 | 2.91 | 2.64 | 0.20 | .914     |

VFA: volatile fatty acid; TVFA: total volatile fatty acid. $n = 10$ cattles per treatment.

$^a$Means within a row with no common superscript differ significantly ($p < .05$).

Figure 4. Taxonomic differences of rumen bacteria at the bacterial genus levels. Comparison of relative abundance in control versus 500 mg/kg daidzein (a) and control versus 1000 mg/kg daidzein (b). Hash (#) indicates $p < .05$. 

Table 3. Effects of daidzein on pH, VFA profiles and NH3-N contents of Xianan cattle.
The interplay patterns between rumen fermentation and bacterial community

Total VFAs were interactive with four OTUs, positively correlated with *Prevotella* and negatively correlated with *Lachnospiraceae incertae sedis*, *Butyrivibrio* and *Succiniciasticum* \((p < .05)\) (Figure 5(a)). NH\(_3\)-N was negatively correlated with *Succiniciasticum* and *Sporobacter* \((p < .05)\) (Figure 5(b)). Acetate was interactive with 21 OTUs, positively correlated with *Ruminococcus*, *Coprococcus* and *Eubacterium* \((p < .05)\) (Figure 5(c)). Propionate was interactive with 5 OTUs, negatively correlated with *Anaerobacterium*, *Coprococcus* and *Eubacterium* \((p < .05)\) (Figure 5(d)). Butyrate was interactive with 11 OTUs, positively correlated with *Bradyrhizobium* and negatively correlated with *Gracilibacter*, *Prevotella* and *Treponema*, etc. \((p < .05)\) (Figure 5(e)). Isobutyrate was interactive with 13 OTUs, positively correlated with *Sporobacter*, *Vampirovibrio*, *Anaerovorax* and *Lachnospiraceae incertae sedis* and negatively correlated with *Prevotella*, *Sphaerochaeta* and *Asterolesplasma* \((p < .05)\) (Figure 5(f)). Across this network, *Prevotella* were positively associated with various metabolic phenotypes \((p < .05)\).

**Discussion**

The present study used high-throughput sequencing of Illumina MiSeq platform to provide a comprehensive view on understanding the alteration of rumen bacterial compositions and explore the association between rumen bacterial community and fermentation parameters in the rumen of finishing Xianan cattle fed with different amounts of daidzein.

It was well-known that daidzein could modulate the metabolism of animals, but little information was available regarding its effects on the diversity in rumen microflora of beef cattle. Current results showed that supplemental daidzein changed the \(\alpha\)-diversity and levels of specific bacterial taxa in rumen bacteria, particularly in 500 mg/kg daidzein group. This result was consistent with the study by Bao et al. (2016), in which the effect of daidzein on the rumen bacterial community of crossbred Xiangzhong black cattle was studied. In the present study, we found adding 1000 mg/kg daidzein had a better effect on improving rumen bacteria diversity than 500 mg/kg daidzein. However, the reason of improved bacterial diversity for 1000 mg/kg daidzein remains unclear. Yu et al. (2007) discovered that daidzein could increase the diversity of the piglets intestinal bacterial flora, which was not consistent.
with the present study. It should be noted that the diversity of bacteria was influenced by several factors, including breed, age, sex and diet (Lozupone et al. 2012).

Compared with control group at phylum level, *Planctomycetes* and *Tenericutes* were significantly more abundant in 500 mg/kg daidzein group, *Verrucomicrobia* and *Fusobacteria* were significantly rarer in 1000 mg/kg daidzein group. The alteration of *Tenericutes* and *Verrucomicrobia* was consisted with the results by Bao (2015), in which supplemental daidzein amount was 500 mg/kg only. *Planctomycetes* and *Fusobacteria* were identified for the first time in rumen of finishing cattle that exhibit significant differences after fed daidzein.

Compared with control group at genus level, genus *Prevotella* and *Ruminococcus* were significantly more abundant in 500 mg/kg daidzein group, genus *Prevotella* was significantly more abundant and *Dethiosulfatibacter* was significantly rarer in 1000 mg/kg daidzein group. The alteration of *Ruminococcus* was consisted with the results obtained from mice by Tamura et al. (2008), in which daidzein exhibited a positive effect on the growth of *Ruminococcus* in the caecum of mice. Yao et al. (2004) observed that daidzein significantly enriched some *Lactobacillus* species *in vitro* fermentation of piglets digesta. Kamra et al. (2012) stated that the numbers of *Clostridium* was significantly increased and *Lactobacillus* was significantly decreased in the faeces of mice after fed daidzein. Lu et al. (2010) concluded that daidzein could increased the number of *Clostridium* in the caecum of mice. These results were not consisted with the current study. The causes of the difference may be experimental animals, dietary composition, daidzein levels and sampling position from animals.

A symbiotic relationship existed between ruminant bacteria and their hosts. Ruminal fermentation triggered by rumen microorganisms ultimately determined the health, growth, and performance of ruminant hosts. VFA was an important indicator to reflect the rumen fermentation capacity. The primary function of VFA in rumen was to provide energy, 70–80% energy requirement of ruminants was provided by VFA (Feng 2004). As a precursor of adipose, acetate was conducive to lipogenesis in body. Butyrate could be converted into acetyl-CoA and synthesised to lactose and milk fat, but excessive butyrate will induce apoptosis of rumen epithelial cells and slower the absorption rate of VFA (Kristensen and Harmon 2004). Daidzein supplementation in our study led to higher total VFA concentration, but it was not significant in statistic (p > .05). This result was consistent with the study by Bao et al. (2016), in which daidzein significantly increased the TVFA and acetate content in rumen of Jingjiang cattle. The acetate content increased significantly and the butyrate content decreased significantly as the amount of daidzein increased, which meant that daidzein changed the rumen fermentation pattern. The rumen fermentation pattern in 1000 mg/kg daidzein group was acetate fermentation, which was in favour of lipogenesis. The lower butyrate was benefit for maintaining healthy ruminal fermentation. These result were similar with the finding of Wang et al. (2008).

The co-occurrence patterns further revealed the interaction between bacteria community and fermentation parameters. A few studies had provided evidence for the relationship between rumen bacterial community and fermentation parameters in the rumen. For example, the abundances of phylum *Bacteroidetes* were positively correlated with propionate concentration in rumen (Koenig et al. 2011), while *Firmicutes* were related positively with butyrate concentration (Moran and Shanahan 2014). The present study showed that *Prevotella* and *Ruminococcus* were positively related to TVFA and acetate, respectively. The genus *Prevotella* belong to the family *Prevotellaceae*, whose main fermentation products are acetate, propionate and succinate from carbohydrates (Jacobs et al. 2009). *Prevotella* contained highly active proteolytic and hemicellulolytic enzymes (Kabel et al. 2011), which could effectively degrade starch, pectin and xylan (Cotta 1992; Gardner et al. 1995; Krause et al. 2003), and comprised a large part of the genetic and metabolic diversity in rumen bacterial community (Ramsak et al. 2000; Bekele et al. 2010). As one of the most numerous groups of cellulolytic bacteria in the rumen (Aurilia et al. 2000), *Ruminococcus* can release phenolic monomers from plant material and play an important role in plant cell wall degradation in the rumen (Giraud et al. 1997).

**Conclusions**

In summary, adding daidzein significantly changed the diversity and community composition in rumen bacteria of finishing Xianan cattle, adding 500 mg/kg daidzein reduced the OTU numbers and bacteria diversity, adding 1000 mg/kg daidzein had a better effect on improving rumen bacteria diversity than 500 mg/kg daidzein. Adding 1000 mg/kg daidzein significantly improved the acetate content and significantly reduced butyrate content. Daidzein change the fermentation parameters mainly by impacting the amount of *Prevotella* and *Ruminococcus*. These results
indicated that adding 1000 mg/kg daidzein in diets can improve the rumen fermentation function for finishing Xianan cattle.

Disclosure statement
No potential conflict of interest was reported by the authors.

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