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Enteric methane emissions and protozoa populations in Holstein steers fed spent mushroom (*Flammulina velutipes*) substrate silage-based diets

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Highlights

- Ensiling spent mushroom substrate (SMS) releases phenolic compounds.
- Ensiling SMS with urea and whole crop corn enhances its nutrition value.
- Feeding ruminants SMS decreases rumen protozoa populations and methane emission.

Abstract

Direct modification of rumen microbial fermentation could provide universal and cost-effective solutions to reduce methane emissions from ruminant livestock. In this study, the effect

of feeding spent mushroom (golden needle mushroom, *Flammulina velutipes*) substrate (SMS)-based silage supplemented with or without urea on the enteric methane emission and total rumen protozoa populations in Holstein steers was investigated. Spent mushroom substrate and whole crop corn were ensiled for 60 days with or without urea as follows: Silage 1 (SMS 900 g/kg and whole crop corn 100 g/kg); Silage 2 (SMS 900 g/kg, urea 10 g/kg and whole crop corn 90 g/kg); Silage 3 (SMS 800 g/kg and whole crop corn 200 g/kg); Silage 4 (SMS 800 g/kg, urea 10 g/kg and whole crop corn 190 g/kg) on dry matter (DM) basis. Five dietary treatments were prepared as follows: 1) a control diet made-up of 500 g/kg of concentrate and 500 g/kg of bermuda hay (*Cynodon dectylon*), and 2) four diets formulated by replacing 400 g/kg of the bermuda hay in the control diet with the four SMS-based silages described above. Five Holstein steers (mean BW 542 ± 72 kg) were assigned to a 5 × 5 Latin square design in which the five dietary treatments were offered across 5 periods, with 14 days of adaptation plus 7 days of samples collection in each period. Holstein steers fed diets containing SMS-based silages had lower total protozoa population (3.37×10^5/mL vs. 6.09×10^5/mL), rumen acetate (55.43 mM/L vs. 57.08 mM/L) and methane emission (211 g/day vs. 252 g/day) ($P < 0.05$) than Holstein steers fed control diet. When comparing the inclusion levels of SMS-based silages in the diets, cattle fed diets of lower levels of SMS-based silages (800 g/kg of SMS) had higher acetate contents (56.61 mM/L vs. 54.25), protozoa population (3.92×10^5/mL vs. 2.84×10^5/mL) and methane emission (226 g/day vs. 196 g/day) than heifers fed diets of higher levels of SMS-based silage (900 g/kg of SMS). The study demonstrates that feeding Holstein steers with SMS-based silage significantly decreases protozoa populations in the rumen and enteric methane emission. Although the mechanisms are not fully understood, the phytochemicals in SMS could be responsible for the reduction in rumen protozoa populations and the inhibition of rumen methanogenesis.

Keywords: enteric methane; spent mushroom substrate; silage; protozoa; steer

Abbreviations: SMS, Spent mushroom substrate; Silage (1-4), Spent mushroom substrate silage made by different formulas; Diet (1-4), Bermuda hay with different resources of Silage1-4; DM, Dry matter; OM, Organic matter; CP, Crude protein; EE, Ether extract; aNDF, Neutral detergent fiber; ADF, Acid detergent fiber; ADL, Acid detergent lignin (sa); NFC, Non-fiber carbohydrate; GE, Gross energy.

1. Introduction

Enteric methane production in ruminants is one of the most important contributors to global greenhouse gas emission (Hook et al., 2011). This greenhouse gas is mainly produced by rumen methanogens from carbon dioxide and hydrogen released during feed fermentation (Janssen and Kris, 2008; Ushida, 2010). Morgavi et al. (2010) and Newbold et al. (2015) recently reported a strong positive association between protozoa numbers and methane production, suggesting that both protozoa and rumen methanogens play important roles during methane production. One methane mitigation strategy would be to reduce protozoa concentration in the rumen, which are important hydrogen producers that closely interact with methanogens in the rumen (Kumar et al., 2009). The chemical composition of feed affects rumen fermentation and ecology, hence enteric methane emission.

The agro-industrial by-products, such as spent mushroom substrate (SMS), are commonly used as feed resources for ruminants worldwide (Kwak et al., 2009; Kaur et al., 2010; Oh et al., 2010; Kaur et al., 2012). Mushroom is grown on compost usually comprised of ingredients such as straw, sawdust, wood shavings or wood crust, seed shells and those materials rich in cellulose and lignin (Milan et al., 2007; Medina et al., 2009). High fiber contained in SMS makes the polysaccharides less accessible to ruminal microbial digestion through blocking cellulose and hemicellulose in rumen bacteria (Silvana et al., 2006). Akinfemi et al. (2008) and Kim et al. (2011) suggested ensiling as an appropriate preservation method for SMS because of the decrease in cell wall contents during the fermentation process. Rangubhet et al. (2014) recently reported that ensiling SMS improves its nutritive value by increasing crude protein and decreasing cell wall

contents. In addition, Xu et al. (2010) reported that fermentation during the ensiling process can remove the odor associated with SMS and increase its acceptance by livestock.

Mushrooms have the ability to secret ligninolytic enzymes that help to degrade several lignocellulosic substrates and generate the phenolic compounds including tannins during their cultivation process (Park et al., 2014; Janusz et al., 2015). Aslam and Saifullah (2013) reported that the spent substrates of *Agaricus biporus* and *Pleurotus florida* contain 8.75 µg/mg and 10.75 µg/mg phenolic compounds, respectively. Research has shown that condensed and hydrolysable tannins and phenolic polymers of relatively high molecular weight modulate rumen fermentation by reducing protein degradation in the rumen and inhibition of methanogenesis (Mueller-Harvey, 2006; Wanghorn, 2008). Tannins have antimicrobial properties and their presence in feeds affect *in vitro* ruminal gas production, volatile fatty acids profile, and have the potential to decrease methane emissions from ruminants (Patra and Saxena, 2011; Cieslak et al., 2013; Mangwe et al., 2016). Indeed, Sika deer fed tannin rich plants had lower protozoa populations, which were observed to be positively correlated to methanogens in the rumen (Li et al., 2015). However, there is limited information on rumen fermentation microbiota and fermentation patterns in the ruminants fed SMS-based silage. The inhibitory effects of phenolic compound on methanogenesis in rumen have been ascribed to both direct effects on methanogenic archaea and protozoa (Patra et al., 2012) and indirect effects on H2 production as a result of decreased fiber digestion (Patra et al., 2017). In a pilot study, our lab discovered that spent substrate from *Flammulina velutipes* (golden needle mushroom) contained phenolic compounds and an *in vitro* ruminal fermentation trial revealed that inclusion of SMS in diets of ruminants reduced methane production (Rangubhet, unpublished data). The SMS may also contain other bioactive compounds, apart from phenolic compounds, that may also modify rumen fermentation and microbial ecology (Mizuno, 1995; Beelman et al., 2003). Therefore, the purpose of this study was to investigate the effects of feeding silage made of spent mushroom substrate with or without urea on protozoa population and enteric

methane production in Holstein steers. It was hypothesized that including SMS-based silage in diets would reduce protozoa population and enteric methane emission in Holstein steers.

2. Materials and Methods

2.1 Mushroom cultivation

The golden needle mushroom (*Flammulina velutipes*) spent substrate used in making silage was obtained from a golden needle mushroom farm in Wufeng district, Taichung province in Taiwan (24°4'N, 120°42'E). The golden needle mushroom was cultivated in bottled substrate consisting of sawdust, rice bran, wheat bran and corn flour. *Flammulina velutipes* was cultivated according to Peng (2010) and Harith et al. (2014). The spent substrate was obtained after the fruit body of the golden needle mushroom was harvested.

2.2 Silage preparation

For the ensiling process, the spent mushroom substrate (SMS) was mixed with whole crop corn and urea to create four silage treatments as follows: Silage 1 (SMS 900 g/kg and whole crop corn 100 g/kg); Silage 2 (SMS 900 g/kg, urea 10 g/kg and whole crop corn 90 g/kg); Silage 3 (SMS 800 g/kg and whole crop corn 200 g/kg); Silage 4 (SMS 800 g/kg, urea 10 g/kg and whole crop corn 190 g/kg), all on a dry matter basis. The SMS was divided into four 900 kg piles (fresh weight basis) with each pile being assigned to one of the four treatments (Silage 1, Silage 2, Silage 3 and Silage 4) as described above. The ingredients were then mixed and packed into 300 kg per plastic bag, sealed tightly, and stored under shade at ambient temperatures (25-31°C) to allow ensiling for 60 days. In a pilot study, ensiling SMS-based silage for 60 days showed superior fermentation results (i.e. higher flieg's score and low pH) than ensiling for 30 and 45 days. Samples were taken in triplicates at different locations from each silo immediately after opening, and processed for laboratory analysis. The remainder of the silage was divided into daily portions and placed in a closed barn for use in the feeding experiment.

2.3 Chemical analysis

All samples for chemical analysis were dried in a forced draught oven at 65°C until they reached constant weight (48 h) and ground to pass a 1 mm screen with a Wiley mill. Dry matter, ash, crude fat or ether extract (EE), and crude protein (CP) were analyzed according to methods of AOAC (2000). Briefly, DM was determined by drying samples in an oven at 105°C for at least 12 h (AOAC 2000; method 930.15). Ash was determined by burning the DM sample in a muffle furnace at 550°C for at least 6 h (AOAC 2000; method 923.03). Organic matter (OM) was calculated as the weight loss after ashing. Crude fat was extracted by ethyl ether extraction to get crude fat content (AOAC 2000; method 920.39). Crude protein was analyzed by the Kjeldahl procedure (AOAC 2000, method 990.03). Neutral detergent fiber (aNDF) and acid detergent fiber (ADF) were determined according to Van Soest et al. (1991) using Fiber Analyzer (ANKOM 200/220, ANKOM Technology, New York, US). Both NDF and ADF were expressed inclusive of residual ash. Neutral detergent fiber was analyzed with α-amylase, but without the use of sodium sulfite. Acid detergent lignin (ADL) was determined according to Van Soest et al. (1991). The difference between aNDF and ADF and the difference between ADF and ADL were used to represent the hemicellulose and cellulose, respectively. Gross energy (GE) was analyzed using an automatic oxygen bomb calorimeter (Parr® model 6200; Parr Instrument Co., Illinois, US). Total phenol and tannin were determined by Folin-Ciocalteu's method as described by Tamilselvi et al. (2012).

2.4 Experimental design and animal management

The feeding experiment was conducted at the National Chung Hsing University Dairy Farm Unit, in accordance with the protocol approved by the Institutional Animal Care and Use Committee of National Chung Hsing University, Taiwan (IACUC No. 104-013). Five Holstein steers (542 ± 72 kg body weight) were offered each of the four SMS silage-based diets plus one

control diet (without SMS silage) as shown in Table 1. The control diet was made-up 50% bermuda hay + 50% commercial concentrate for beef cattle (No. 1015, Great Wall Feedtech Co., Ltd., Taiwan). The other four diets were formulated by replacing 40% of the bermuda hay in the control diet with the four SMS silages described in Section 2.2. The animals were arranged into a 5 × 5 Latin square design (five periods and five animals), with 14 days of adaptation plus 7 days of samples collection in each period. Animals were housed in an open-barn with free stalls. However, the animals were confined in individual pens at the time of feeding.

The diets were offered twice daily *ad libitum* at 0800 hours and 1600 hours with freely accessible water and mineral blocks. Voluntary feed intake of each steer was measured by subtracting the quantity of refusals from the amount of feed offered (on DM basis). Three hundred grams (on fresh weight basis) of feed and fecal samples were collected twice daily (1 h after feeding). All samples (feed and feces) were pooled immediately per period and subsampled for analyses to determine digestibility coefficient. The samples were stored at −20°C pending further analyses. The digestibility coefficient of nutrients was calculated by subtracting the fecal nutrient concentration from nutrient intake and expressing the outcome as a proportion of nutrient intake.

2.5 Rumen fermentation

Approximately 250 mL of rumen fluid was collected 4 h post feeding on day 15 of each period in order to determine rumen fermentation products and pH levels. Ruminal fluid was collected through a hand-operated suction pump (No.34960.400, H. Hauptner and Richard Herberholz GmbH& Co. KG Solingen esophageal tube, Solingen, Germany) as described by Pounder (1952). To avoid dilution of the rumen fluid by saliva, the first batch of rumen fluid (300 mL) sucked through esophagus was discarded. After collection, pH was measured immediately using a glass electrode pH meter (Sartoius Professional Meter PP-50, Gottingen, Germany). Thereafter rumen fluid was filtered through four layers of cheese cloth. A subsample of 5 mL rumen fluid was acidified with 25% metaphosphoric acid at the ratio of 5:1 and centrifuged (1,800

× g) at 4°C for 10 min. The supernatant was filtered through a micro filter (0.45μm pore size), decanted into 20 mL polypropylene tube and directly frozen at -20°C for later analysis of rumen fermentation parameters. Short chain fatty acids (SCFAs) concentrations were determined by gas chromatograph (Gas Chromatography Thermo Scientific trace-1300, Milan, Italy) with flame ionization detectors to detect acetic acid, propionic acid, and butyric acid. Briefly, the clear supernatant (1 μL) was injected onto a gas chromatograph via a split injector. The temperatures of column, injector, and detector were set at 125°C, 180°C, and 200°C, respectively. Hydrogen, nitrogen, and air were used as carrier gas at pressures of 3 kg/cm^2, 4 kg/cm^2, and 4 kg/cm^2, respectively. Identification and quantification was conducted with the aid of a Supelco volatile fatty acid standard mix (Sigma-Aldrich No.46975-U).

2.6 Protozoa quantification

For protozoa quantification, 5 mL of rumen fluid were mixed with equal volume of methyl green formalin saline (MFS) solution for 30 min in order to fix and stain the nucleus of protozoa (Onodera et al., 1977). Protozoa samples were stored at 4°C in darkness pending counting. Protozoa populations were counted directly using a counting chamber (0.1 mm^2) under an optical microscope following the procedure described by Dehority (1993).

2.7 Methane emission

After two weeks of the adaptation period to treatment diets, methane emissions were measured twice daily, two days per period. For each measurement, the beef cattle were confined in individual respiration chambers, 1 h after morning (0800 – 1200 h) and afternoon (1400 – 1800 h) feeding. Two open circuit respiration chambers, whose outside dimensions were 5.49 m (length) × 2.28 m (width) × 2.11 m (height), were used for methane production measurement. When the chamber doors were closed, air entered through the air-flow controlled chambers via a ventilation duct and exited through an exhaust. Air samples at the air entrance and the exhaust of the open

circuit chambers were collected hourly for four hours when cattle were confined in the chamber in order to quantify the average of methane emission. Methane concentration was measured using gas chromatography (Gas Chromatography Thermo Scientific Trace-1300, Rodano-Milan, Italy) with flame ionization detectors (Steele et al., 1987). Temperature of column, injector, and detector was set at 80°C, 100°C, and 150°C, respectively. Hydrogen, nitrogen, and air were used as carrier gas at pressure 3 kg/cm^2, 4 kg/cm^2, and 5 kg/cm^2, respectively. The following working curve was constructed using methane standard with gas chromatography:

$$CH_4 \text{ (ppm)} = 0.0003 \times CH_4 \text{ peak area} + 1.0342$$

The daily CH_4 production was calculated as follow:

$$CH_4 \text{ (g/day)} = (A \times \Delta CH_4 \times 10^{-3}) \times (1 \times 0.082 \times K/P) \times 16$$

where A is chamber air volume (m^3/day), ΔCH_4 is $CH_{4\ \text{out - in}}$ concentration (ppm), 10^{-3} is the conversion for L/m^3, 0.082 is gas constant, K is temperature of air-flow controlled chamber (ºK), and P is pressure of air-flow controlled chamber, 16 is the molecular weight of CH_4. The model of daily CH_4 production is adapted from McGinn et al. (2004).

2.8 Statistical analyses

All data were analyzed using the General Linear Model procedure of SAS (2006) including diet, period and animal as main effects in the model. Significance of differences between treatment diets was tested by Tukey's test (SAS, 2006) and was declared at $P < 0.05$. A mixed model ANOVA was conducted to determine the effect of SMS and urea content in the diets on the nutrient intake, apparent digestibility, rumen fermentation parameters, protozoa population and methane emission. Data were analyzed for a 5 × 5 Latin square design according to the following statistical model:

$$Y_{ijk} = \mu + H_i + P_j + T_k + e_{ijk},$$

where μ is the overall mean, H_i is the random effect of Holstein beef steer, P_j and T_k are the fixed effects of periods and treatment rations, respectively, and e_{ijk} is the error term. All reported values

are least square means, which were separated using the PDIFF option in SAS. The effects of SMS content (800 g/kg or 900 g/kg in silage), urea content (0 g/kg or 10 g/kg in silage), and their interactions on the nutrients intake, apparent digestibility, rumen fermentation parameters, protozoa population and methane emission were analyzed with the model:

$$Y_{ijk} = \mu + M_i + U_j + (M_i \times U_j) + e_{ijk}$$

where μ is the overall mean, M_i is the fixed effect of SMS content in silage, U_j is the fixed effect of urea content in silage, $M_i \times U_j$ is the fixed effect of the interaction between SMS and urea, and e_{ijk} is the error term. Person correlation analysis (SAS, 2006) was used to examine the association between protozoa populations and methane emission. Differences were considered significant at $P < 0.05$.

3. Results

3.1 Chemical composition

All SMS silage-based diets had higher fiber fractions (aNDF, ADF and ADL) compared to the control diet. Increasing the proportion of whole crop corn in the SMS-based silages numerically decreased the fiber contents (aNDF, ADF and ADL) of the silage. Treatment diets that contained SMS silage had higher content of total phenol and tannin than the control diet (Table 1).

3.2 Nutrient intake and digestibility

Diets did not affect ($P > 0.05$) the intake of all chemical components with the exception of ADL (Table 2). Inclusion of SMS-based silage in the diets of Holstein steers increased ADL intake ($P < 0.05$). Dry matter intake was not significantly different between treatment diets. Levels of SMS (80% and 90%) and urea supplementation did not have the significant interaction effect on DM and nutrients intake.

The results revealed that dry matter digestibility coefficients in Holstein steers fed SMS silage-based diets ranged between 702-725 g/kg of DM, and did not differ from that of the control

diet (Table 3). Digestibility coefficients of all nutrients in SMS silage-based diets did not differ from the control diet. Even though results in Table 2 revealed significantly higher intakes in ADL and numerically higher intakes in aNDF and ADF in SMS silage-containing rations, this did not affect the digestibility coefficient of all nutrients as anticipated.

3.3 Rumen fermentation parameters

Rumen fermentation parameters of Holstein steers presented in Table 4 revealed that inclusion of SMS-based silage in the treatment diets did not affect ($P > 0.05$) rumen pH, NH_3 and butyrate concentration. Inclusion of SMS-based silage in the treatment diets significantly increased total short chain fatty acid (SCFA) and propionate (P) content, but decreased acetate (A) content and A:P ratio, when compared to the control diet. The results also showed significant effects on SCFA when SMS and urea were included in the rations. Acetate concentration was higher when 800 g/kg DM of SMS was mixed with 10 g/kg DM of urea (Diet 4), than when no urea was added (Diet 1) (Table 4). On the other hand, inclusion of urea in SMS-based silage (Diet 2 and Diet 4) decreased ($P < 0.05$) butyric acid content.

3.4 Protozoa populations and methane emission

Average protozoa populations and methane emission results shown in Table 5 revealed that total protozoa population in rumen fluid of Holstein steers differed ($P < 0.05$) across the dietary treatments. Inclusion of SMS-based silage in diets of steers resulted in lower total protozoa population when compared to the control diet (Table 5). Increasing the percentage of SMS silage in the diets resulted in progressive reduction ($P < 0.05$) in total rumen protozoa population in Holstein steers.

The population of *Spirotrichs* was higher ($P < 0.05$) in diets containing SMS-based silage than in the control diet, whereas the population of *Holotricha* did not differ between treatment diets ($P > 0.05$). Holstein steers fed SMS-based silage diets had lower ($P < 0.05$) methane emission

compared to those fed control diet (Table 5). The lowest methane yield was observed at the highest SMS-based silage inclusion level in the experimental diets ($P < 0.05$).

4. Discussion

4.1 Chemical composition

Mushrooms are grown on culture substrates usually comprised of ingredients such as sawdust, wood crust, and wood shavings, materials that are rich in cellulose and lignin. Lignin makes the polysaccharides less accessible to ruminal microbial digestion by blocking access of rumen microbes to cellulose and hemicellulose (Silvana et al., 2006). However, as they grow, *Flammulina velutipes* secrete extracellular enzymes that include phenol oxidase and peroxidase, which can efficiently decompose lignocellulose (Janusz et al., 2015), making it more easily digested by ruminants (Adamovic et al., 1998; Streeter et al., 1982). Accordingly, ash, crude protein, and fat content of the spent mushroom substrates increase with time but not the cell wall compounds (Streeter et al., 1982; Zadrazil and Puniya, 1995). Even though, the results from the current study revealed that the fiber fractions in SMS silage-based diets were higher than in the control diets, the values are lower than the normally reported fiber content values of sawdust (the main component of the SMS) before mushroom cultivation (Horisawa et al., 1999), indicating that decomposition reduced the fiber contents of the spent mushroom substrates used in the current study. The concentration of other chemical component did not differ between SMS-based silage and control diets, indicating the potential use of SMS silage-based diet as a roughage source for ruminants.

Inclusion of SMS silage in treatment diets resulted in increased total phenol and tannin content, in agreement with earlier findings where the spent residues of *Pleurotus florida* and *Agaricus spp.* were found to contain higher levels of phenolic compounds (Aslam and Saifullah, 2013). Chang and Miles (2004) reported that phenol compounds are liberated from the lignin during decomposing. The authors further stated that the process of breakdown of lignin is not well-

understood, but there is an apparent correlation between the ability to degrade lignin and the production of extracellular phenolases, such as laccase, peroxidase, and tyrosinase, which oxidize phenolic compounds, e.g. gallic and tannic acids. These oxidative reactions have long been considered to be involved in the conversion of complex phenols to simple aromatic compounds that can be absorbed by mushroom mycelium and used for its growth. (Chang and Miles et al., 2004; Sanchez, 2010). This explains the higher contents of total phenols and tannins in diets containing SMS silage observed in this study.

4.2 Nutrients intake and digestibility

The higher daily intakes of the fiber fractions in diets containing SMS-based silage observed in this study were expected since the silage-based diet had higher fiber contents than the control diet (Table1). The main components of SMS-based silage were cell wall fractions but the digestibility coefficient was not affected by high CF and ADL intake. The results from the current study suggest that SMS silage can replace up to 400 g/kg (DM basis) of bermuda grass hay in diets of Holstein steers without significantly altering the apparent digestibility of most nutrients. Nutrient digestibility coefficients obtained from the current study are similar to those obtained by Khattaba et al. (2013), who reported that adding urea to date palm (*Phoenix dactylifera*) diets increased the digestibility of DM, OM and CP in sheep. Okano et al. (2005) and Kutlu et al. (2000) reported that SMS treated with urea had a positive effect on the digestibility coefficients of CP and fiber content. The improvements in digestibility after urea addition are due to increased N supply to fibrolytic microbes (Abdullah et al., 2004; Sarwar et al., 2006) as well as possible breakdown of lignocellulosic bonds via ammonia, an alkaline product of urea hydrolysis (Islam et al., 2001).

4.3 Rumen fermentation parameters

According to Eugene et al. (2004), rumen ciliate protozoa influence fermentation dynamics of feeds and, consequently, rumen microbial ecology. This also results in changes in the amount

and proportion of the end products from rumen fermentation including methane. When protozoa engulf organic matter and hydrolyzed fiber during fermentation, the main SCFAs produced are acetate and butyrate (Hillman et al., 1995) and relatively low quantities of propionic acid. Increasing fiber content in diets usually results in increased ruminal pH, A:P ratio, and a decrease in total SCFAs concentration (Xu et al., 2010). However, results from the current study showed that acetate was lower in the rumen of animals fed with SMS silage-based diets than those on the control diet. The reduction in acetate concentration in rumen fluid of steers fed diets containing SMS silage is probably due to the particle size of SMS, which was smaller than that of bermuda hay. Smaller particle size may have had a higher rate of digestion and out flow through the hind gut in steers fed SMS silage-based diets (Zebeli et al., 2008).

4.4 Methane emission and protozoa populations

There are two primary protozoa groups in rumen known as *Spirotrichs* and *Holotricha* that have different dietary niches (Newbold et al., 2015). The former primarily ingests and ferments particulate materials whereas the latter utilizes soluble carbohydrates (Samanta et al., 2003). The population size of rumen protozoa tends to be positively correlated with that of cellulolytic bacteria and methanogens (Newbold et al., 2015). Many researchers have reported a positive relationship between methane emission and protozoa populations in the rumen (Carberry et al., 2014; Wallace et al., 2014; Morgavi et al., 2010). Hegarty (1999) and Nguyen et al. (2016) revealed that the removal of protozoa from the rumen resulted in a decrease in methane production. An updated analysis by Morgavi et al. (2010) on this subject showed that removal of protozoa from rumen resulted in a 10.5% decrease in methane emissions. The results from the current study also revealed a positive correlation between methane emission and protozoa population ($r = 0.433$, $P < 0.05$). The results from the current study confirm the significant impact that rumen protozoa have on methane production under certain conditions.

Zhang et al. (2007) reported that the optimal pH range for methanogens is 6.8–7.2. The pH values (6.62-6.72) in the rumen of animals feeding on SMS silage-based diets were below the optimal pH range whereas the rumen pH (6.81) in animals feeding on the control diet was within this optimal range. This is in keeping with the methane emission results obtained from the current study as methane emissions were high in animals fed the control diet than in those fed SMS silage diets. Further studies are required to investigate the reasons that led to the drop-in pH in SMS-based silage diets because the differences in SCFAs, although significance was minimal, and thus cannot fully explain the differences in pH.

Phenolic compounds, including tannins have been recognized as modulators of rumen fermentation with potential to inhibit methanogenesis (Bhatta et al., 2014; Mangwe et al., 2016). Phenolic compounds play an important role as antimicrobials by interacting with the extracellular microbial enzymes, as well as depriving microbe substrates required for growth (Patra and Saxana, 2011). In addition to direct inhibition on methanogenic archaea and protozoa (Patra et al., 2012), it is possible that phenolic compounds could indirectly affect on H_2 production (Patra et al., 2017) or form insoluble complexes with protein resulting in suppression of overall microbial degradation and methane emissions (Patra and Saxena, 2011). In the current study, there was a significant reduction in rumen fermentation and protozoa population as the level of total phenol and tannin increased in the SMS silage-based diet when compared to the control diet. The reduction in rumen fermentation and protozoa population as the level of total phenols and tannins increased in the SMS-based silage diets is similar to the observations made by Bhatta et al. (2013). Feeding tropical plants containing total phenols and tannins has been demonstrated to reduce methane and total gas production (Pal et al., 2015).

5. Conclusions

In conclusion, the spent mushroom substrate from golden needle mushroom (*Flammulina velutipes*) can be mixed with urea and whole crop corn to enhance its nutritive value. The present

study revealed that spent golden needle mushroom substrate contains phenolic compounds. It further demonstrated that feeding ruminants SMS significantly decreases protozoa populations in the rumen and enteric methane emission. Although the mechanisms are not fully understood, this work supports methane mitigating strategies based on reduction of rumen protozoa populations, and the inhibition of methanogenesis in the rumen probably through the presence of phytochemicals in SMS. As proved in Holstein steers, feeding SMS-based silage is a possible way of reducing the loss of dietary energy as ruminal methane. It is also a possible strategy to reduce the contribution of ruminants to greenhouse gas production.

Conflict of interest

The authors have no conflict of interest to declare.

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Table 1

Ingredients and chemical composition of treatment diets

SMS[a] silage preparation (g of DM)		Silage 1[a]	Silage 2[a]	Silage 3[a]	Silage 4[a]
SMS[a]		900	900	800	800
Whole crop corn		100	90	200	190
Urea		0	10	0	10
Treatment rations (g/kg of DM)	Control	Diet 1[a]	Diet 2[a]	Diet 3[a]	Diet 4[a]
Silage	00	200	200	200	200
Bermuda hay	500	300	300	300	300
Concentrate	500	500	500	500	500
Chemical composition[b]					
DM, g/kg	908	813	811	813	809
			g/kg of DM		
OM, g/kg	924	927	927	926	927
CP, g/kg	151	142	146	144	148
EE, g/kg	52	55	53	52	56
aNDF, g/kg	609	627	622	622	619
ADF, g/kg	219	256	253	247	246
ADL, g/kg	69	99	98	92	92
Hemicellulose, g/kg	390	370	369	375	373
Cellulose, g/kg	148	156	152	153	153
GE, MJ/kg	177	176	177	178	179
Total phenol, mg of GAE[c]/g	5.35	9.63	9.89	9.02	9.22
Total tannin, mg of GAE[c]/g	0.79	1.96	2.03	1.81	1.89

[a] SMS, Spent mushroom substrate (DM = 523 g/kg; OM = 895 g/kg; CP = 77.6 g/kg; NDF = 794 g/kg; ADF = 635 g/kg; ADL = 271 g/kg; GE = 163 MJ/kg); Silage (1-4), Spent mushroom substrate silage of different formulations; Diet (1-4), Diets in which 40% of bermuda hay in control diet was replaced with SMS silage 1-4.

[b] DM, Dry matter; OM, Organic matter; CP, Crude protein; EE, Ether extract; aNDF, Neutral detergent fiber; ADF, Acid detergent fiber; ADL, Acid detergent lignin; GE, Gross energy.

[c] GAE, Gallic acid equivalent.

Table 2

Daily intake of feed components (on DM basis, unless otherwise stated) in Holstein steers fed with spent mushroom substrate silage-based treatment diets

Components[a]	Control[b]	Diet 1[b]	Diet 2[b]	Diet 3[b]	Diet 4[b]	SEM[c]	Significance (*P*-value)		
							Diet[d]	SMS[e]	Urea[f]
Total DM intake, kg/day, as is	13.3	13.0	13.4	13.6	13.8	0.82	0.996	0.818	0.919
OM, kg/day	12.2	12.1	12.4	12.6	12.8	0.76	0.996	0.815	0.903
CP, kg/day	2.02	1.85	1.97	1.97	2.06	0.12	0.930	0.713	0.682
EE, kg/day	0.70	0.71	0.72	0.71	0.79	0.04	0.749	0.631	0.614
aNDF, kg/day	8.12	8.20	8.38	8.48	8.64	0.52	0.965	0.795	0.849
ADF, kg/day	2.92	3.34	3.47	3.41	3.42	0.21	0.226	0.077	0.072
ADL, kg/day	0.94[b]	1.30[a]	1.33[a]	1.26[a]	1.28[a]	0.06	0.004	0.525	0.688
Hemicellulose, kg/day	5.20	4.83	4.97	5.13	5.18	0.31	0.916	0.670	0.862
Cellulose, kg/day	1.97	2.04	2.06	2.10	2.13	0.13	0.777	0.589	0.498
GE, MJ/day	235	229	238	243	248	3.52	0.984	0.706	0.923

[a] DM, Dry matter; OM, Organic matter; CP, Crude protein; EE, Ether extract; aNDF, Neutral detergent fiber; ADF, Acid detergent fiber; ADL, Acid detergent lignin; GE, Gross energy.

[b] Diets consisted of control (500 g/kg of bermuda hay and 50 g/kg of concentrate), and four treatment rations with 200 g/kg of SMS inclusion rate in place of bermuda hay, i.e., Diet 1 (200 g/kg of SMS 1, 300 g/kg of bermuda hay and 50 g/kg of concentrate), Diet 2 (200 g/kg of SMS 2, 300 g/kg of bermuda hay and 500 g/kg of concentrate), Diet 3 (200 g/kg of SMS 3, 300 g/kg of bermuda hay and 500 g/kg concentrate), and Diet 4 (200 g/kg SMS 4, 300 g/kg bermuda hay and 500 g/kg concentrate).

[c] SEM, standard error of mean.

[d] Means (n = 5) in the same row with different superscripts are significantly different ($P < 0.05$).

[e] Probability of significant effect due to SMS content in diets (excluding the control diet).

[f] Probability of significant effect due to urea content in diets (excluding the control diet).

Table 3

Digestibility (on DM basis, unless otherwise stated) of nutrients in Holstein steers fed with spent mushroom substrate silage-based treatment diets

Components[a]	Control[b]	Diet 1[b]	Diet 2[b]	Diet 3[b]	Diet 4[b]	SEM[c]	Significance (*P*-value)		
							Diet[d]	SMS[e]	Urea[f]
DM, g/kg, as is	727	702	717	716	725	246	0.954	0.821	0.626
OM, g/kg	745	722	735	736	745	235	0.951	0.803	0.633
CP, g/kg	778	754	782	759	781	244	0.881	0.943	0.321
EE,g/kg	717	740	731	768	770	486	0.919	0.645	0.951
aNDF, g/kg	744	712	723	720	736	226	0.856	0.631	0.555
ADF, g/kg	603	592	615	595	617	371	0.983	0.996	0.552
ADL, g/kg	454	550	557	630	571	543	0.282	0.114	0.636
Hemicellulose, g/kg	824	793	800	804	814	162	0.684	0.377	0.623
Cellulose, g/kg	673	618	648	571	645	341	0.299	0.476	0.140
GE, MJ/kg	174	163	170	175	181	28.5	0.956	0.753	0.811

[a] DM, Dry matter; OM, Organic matter; CP, Crude protein; EE, Ether extract; aNDF, Neutral detergent fiber; ADF, Acid detergent fiber; ADL, Acid detergent lignin; GE, Gross energy.

[b] Diets consisted of control (500 g/kg of bermuda hay and 50 g/kg of concentrate), and four treatment rations with 200 g/kg of SMS inclusion rate in place of bermuda hay, i.e., Diet 1 (200 g/kg of SMS 1, 300 g/kg of bermuda hay and 50 g/kg of concentrate), Diet 2 (200 g/kg of SMS 2, 300 g/kg of bermuda hay and 500 g/kg of concentrate), Diet 3 (200 g/kg of SMS 3, 300 g/kg of bermuda hay and 500 g/kg concentrate), and Diet 4 (200 g/kg SMS 4, 300 g/kg bermuda hay and 500 g/kg concentrate).

[c] SEM, standard error of mean.

[d] Means (n = 5) in the same row with different superscripts are significantly different ($P < 0.05$).

[e] Probability of significant effect due to SMS content in diets (excluding the control diet).

[f] Probability of significant effect due to urea content in diets (excluding the control diet).

Table 4

Rumen fermentation parameters in Holstein steers fed with spent mushroom substrate silage-based treatment diets (on DM basis)

Parameters	Control[a]	Diet 1[a]	Diet 2[a]	Diet 3[a]	Diet 4[a]	SEM[b]	Significance (*P*-value)		
							Diet[c]	SMS[d]	Urea[e]
pH	6.81	6.64	6.56	6.72	6.62	0.06	0.358	0.600	0.059
NH_3, mM/L	10.2	8.36	9.49	9.28	9.53	0.81	0.593	0.092	<0.001
Molar of SCFA, mM/L									
Total SCFA[f]	95.5[c]	97.3[b]	97.8[b]	98.0[a,b]	98.3[a]	0.44	<0.001	0.015	0.103
Acetate	57.1[a]	53.4[c]	54.5[c]	56.3[b]	56.8[b]	0.36	0.004	<0.001	0.399
Propionate	21.9[c]	24.2[a,b]	23.6[b]	24.1[a,b]	24.2[a]	0.33	0.001	<0.001	0.042
Butyrate	16.4	19.6	19.5	17.5	17.2	0.45	0.413	0.061	0.663
A:P[g]	2.59[a]	2.22[c]	2.30[b,c]	2.33[b]	2.36[b]	0.03	<0.001	<0.001	0.036

[a] Diets consisted of control (500 g/kg of bermuda hay and 50 g/kg of concentrate), and four treatment rations with 200 g/kg of SMS inclusion rate in place of bermuda hay, i.e., Diet 1 (200 g/kg of SMS 1, 300 g/kg of bermuda hay and 50 g/kg of concentrate), Diet 2 (200 g/kg of SMS 2, 300 g/kg of bermuda hay and 500 g/kg of concentrate), Diet 3 (200 g/kg of SMS 3, 300 g/kg of bermuda hay and 500 g/kg concentrate), and Diet 4 (200 g/kg SMS 4, 300 g/kg bermuda hay and 500 g/kg concentrate).

[b] SEM, standard error of mean.

[c] Means (n = 5) in the same row with different superscripts are significantly different ($P < 0.05$).

[d] Probability of significant effect due to SMS content in diets (excluding the control diet).

[e] Probability of significant effect due to urea content in diets (excluding the control diet).

[f] Short chain fatty acids.

[g] Acetate to propionate.

Table 5

Methane emission and protozoa population in Holstein steers fed with spent mushroom substrate silage-based treatment diets (on DM basis)

Componens	Control[a]	Diet 1[a]	Diet 2[a]	Diet 3[a]	Diet 4[a]	SEM[b]	Significance (*P*-value)			
							Diet[c]	SMS[d]	Urea[e]	SMS × Urea[f]
Protozoa population, 10^5cell/mL										
Total	6.09[a]	2.64[d]	3.04[d]	3.75[c]	4.05[b]	0.20	<0.001	<0.001	0.001	0.197
Holotrichs	0.68	0.31	0.49	0.46	0.62	0.11	0.155	0.208	0.156	0.829
Spirotrichs	5.40[a]	2.34[e]	2.58[d]	3.31[c]	4.05[b]	0.18	<0.001	<0.001	0.225	0.220
Methane emission										
g/day	252[a]	194[b]	198[b]	224[b]	228[b]	10.9	0.021	<0.001	0.001	0.001
g/kg DMI	18.5[a]	14.1[c]	14.2[c]	17.1[b]	16.9[b]	0.80	0.022	<0.001	0.002	0.001
Energy loss from methane										
MJ/day	18.3[a]	15.0[c]	14.3[c]	15.9[b]	16.1[b]	0.79	0.023	<0.001	0.002	0.001

[a] Diets consisted of control (500 g/kg of bermuda hay and 50 g/kg of concentrate), and four treatment rations with 200 g/kg of SMS inclusion rate in place of bermuda hay, i.e., Diet 1 (200 g/kg of SMS 1, 300 g/kg of bermuda hay and 50 g/kg of concentrate), Diet 2 (200 g/kg of SMS 2, 300 g/kg of bermuda hay and 500 g/kg of concentrate), Diet 3 (200 g/kg of SMS 3, 300 g/kg of bermuda hay and 500 g/kg concentrate), and Diet 4 (200 g/kg SMS 4, 300 g/kg bermuda hay and 500 g/kg concentrate).

[b] SEM, standard error of mean.

[c] Means (n = 5) in the same row with different superscripts are significantly different (P < 0.05).

[d] Probability of significant effect due to SMS content in diets (excluding the control diet).

[e] Probability of significant effect due to urea content in diets (excluding the control diet).

[f] Probability of significant effect due to the interaction of SMS × urea content in diets (excluding the control diet).