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Effects of sucrose, formic acid and lactic acid bacteria inoculant on quality, *in vitro* rumen digestibility and fermentability of drooping wild ryegrass (*Elymus nutans* Griseb.) silage

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ABSTRACT. The aim of the study was to evaluate the effect of sucrose, formic acid and lactic acid bacteria (LAB) inoculant on chemical composition and *in vitro* rumen digestibility of drooping wild ryegrass (*Elymus nutans* Griseb.) silage. Fresh drooping wild ryegrass was harvested at boot stage and ensiled with: 1. control (untreated), 2. 20 g · kg^{-1} sucrose (S), 3. 3 g · kg^{-1} formic acid (FA) and 4. 0.005 g · kg^{-1} commercial LAB inoculant (LP). The addition of all three additives increased the Flieg point, and decreased pH and butyric acid content ($P < 0.05$). There was lower ammonia N content in silages treated with S and FA. On the other hand, LP addition elevated lactic acid content with simultaneous increase of the lactic to acetic acid ratio ($P < 0.001$). The silages were further anaerobically incubated at 39 °C for 48 h with buffered rumen fluid of lactating cows. All three additives increased volatile fatty acid production ($P = 0.008$). Addition of LP increased average gas production rate and ammonia N content ($P < 0.001$). In brief, well fermented drooping wild ryegrass silages were obtained by adding LP or S in comparison to FA. LP can be recommended as a silage additive for preparing well fermented silage, and FA could be applied as a regulator additive to control the level of lactic acid produced during drooping wild ryegrass ensiling.

Introduction

Drooping wild ryegrass (*Elymus nutans* Griseb.), an important alpine forage grass, is a perennial, caespitose and self-pollinating species in the genus *Elymus* L. (*Poaceae*: *Triticeae*). It is native to temperate and tropical Asia – from western and central Asia in the west to China and Mongolia in the east, from Russia in the north to India and the Himalayas area in the south (Chen et al., 2009). It grows in grassland, along river banks, among bushes, on mountain slopes and in swales, at altitudes from 1000 m up to 5000 m (Lu, 1993). In China, it is distributed in north, northwest and southwest part of the country, particularly on Qinghai Tibet Plateau (Miao et al., 2011). Drooping wild ryegrass is the valuable forage grass in the alpine regions due to its high adaptability, good tolerance to cold, drought

and biotic stress, good nutritional value and high yield (Fu et al., 2014). This species has been widely used in cultivated pastures because of favourable forage yield, improved quality and good adaptability to the local environment. It also plays an important role in animal husbandry and environmental sustenance in China.

In China, drooping wild ryegrass is commonly used for hay or pasture for grazing animals. Ensiling might be a better way for its preservation, especially when the weather is unreliable for wilting (McDonald et al., 1991). Appropriate additives can increase nutritional value and so have positive effect on silage quality (McEniry et al., 2014). For example, sucrose improved the silage quality by decreasing pH, dry matter (DM) loss and increasing lactic acid content, as it could provide fermentable substrates for lactic acid bacteria (LAB) (Heinritz et al., 2012). LAB play an important role in silage fermentation and its inoculants are often used to establish a desirable microbial flora in silage and to improve the fermentation quality. The silage with LAB inoculants addition may increase DM intake and DM and organic matter digestibilities (Bolsen et al., 1996). On the other hand, formic acid addition during ensiling induces antibacterial activity and reduces lactic acid production. Thus, a balance must have been maintained between sufficient lactic acid production for the effective silage preservation, and optimal concentration of carbohydrates in the form of soluble sugars which are better source of energy for rumen microbes than lactic acid.

In vitro gas production (GP) technique, which was developed to predict fermentation of ruminant feedstuffs, has been widely used to determine effects of different additives on *in vitro* digestibility and degradation rates of silages (Kozelov et al., 2008; Contreras-Govea et al., 2013). To our knowledge, little information is available about the fermentation characteristics and digestibility of drooping wild ryegrass silage with or without additives. The objective of this study was to determine the chemical composition of drooping wild ryegrass harvested in Northern China and to examine the influence of sucrose, formic acid and LAB inoculants addition on chemical composition and *in vitro* rumen fermentation characteristics of examined silage.

Material and methods

Forage harvest

First cut of drooping wild ryegrass was harvested at boot stage in experimental plot of Grassland Research Station of China Agricultural University (Hebei, China: 41°42'–41°57'N, 115°32'–115°59'E, altitude 1430 m above sea level, annual mean temperature 1.4 °C, average annual precipitation 400 mm) in July 2014. After the harvest, about 5 kg of fresh matter of drooping wild ryegrass was collected randomly from different sites in each of three plots. After mixing, the grass was sampled in triplicate and stored at −20 °C prior to chemical analyses.

Experimental design and silage preparation

The chopped grass (1 cm length) was assigned to the following four treatments: 1. untreated (control), 2. 20 g · kg^{-1} sucrose (S), 3. 3 g · kg^{-1} formic acid (FA) and 4. 0.005 g · kg^{-1} commercial bacteria inoculants (LP) containing *Lactobacillus plantarum* CNCM MA18/5U > 1.75 × 10^{10} CFU · g^{-1} and *Pediococcus acidilactici* CNCM MA18/5M > 7.5 × 10^{9} CFU · g^{-1} (Lallemand Inc., Montreal, Canada). All additives were diluted with the same volume of distilled water. All untreated or treated materials (650 g) were individually packed into 1 l plastic buckets (9 cm in diameter and 18 cm in height; Hewanglan paper and plastic products factory, Beijing, China). Three silos per treatment were arranged and stored indoors at ambient temperature around 29 °C. After 60-day ensiling process, the silos were opened, the samples were taken and stored at −20 °C for later chemical analysis and *in vitro* batch cultures.

In vitro batch culture

Following the experiment, silage samples from each treatment were oven-dried at 65 °C and ground to pass through 1-mm screen. The dried silages (500 mg) were weighed into 120 ml glass bottle with butyl rubber stoppers and Hungate's screw caps. Five bottles per silage sample were arranged. A volume of 50 ml of a freshly prepared buffer solution (pH 6.85; Menke and Steingass, 1988) and 25 ml filtrated rumen fluid collected from three rumen fistulated dairy cows 1 h before the morning feeding were added to the bottles. The bottles were purged with anaerobic N_2 for 5 s, sealed with butyl rubber stopper and Hungate's screw caps, and individually connected with medical plastic infusion pipes to gas inlets of an automated trace gas recording system (Yang et al., 2014) to continuously record cumulative gas production (GP). All bottles were incubated at 39 °C for 48 h. The whole experiment was repeated three times.

After the incubation, culture fluids were sampled and stored at −20 °C for later analysis of ammonia N and volatile fatty acid (VFA) concentrations. Remaining material in each bottle was individually filtrated with pre-weighed nylon bags (8 × 12 cm,

42 μm pore size) and dried at 65 °C for 48 h to a constant weight. Difference between initial material DM and residual DM, corrected by the blanks after the incubation, was calculated to determine the *in vitro* dry matter disappearance (IVDMD).

Chemical analyses

Twenty grams of each fresh silage sample were homogenized in a blender with 180 ml of distilled water for 1 min and then filtered through 4 layers of cheesecloth. Afterwards, pH value in the filtrate was measured immediately using a glass electrode pH meter (PHS-3C, INESA Scientific Instrument Co., Ltd., Shanghai, China).

Dry matter content was determined by oven-drying at 65 °C for 48 h. Neutral detergent fibre (NDF), acid detergent fibre (ADF), water soluble carbohydrates (WSC), nitrogen (N), phosphorus and potassium contents and buffering capacity were measured according to the methods previously described by Zhang et al. (2014a). The ammonia N concentration in silage and in *in vitro* culture fluid samples were determined by method of Broderick and Kang (1980). The organic acids, including lactic acid, acetic acid, propionic acid and butyric acid, concentrations in silages and *in vitro* culture fluid samples were determined by high performance liquid chromatography (HPLC) (LC-20A; Shimadzu, Tokyo, Japan). The analytical conditions were as follows: Shodex RSpak KC-811S-DVB gel C column (8 mm × 30 cm; Shimadzu, Tokyo, Japan), oven temperature 50 °C, mobile phase 3 mmol · l^{-1} $HClO_4$, flow rate 1.0 ml · min^{-1}, injection volume 5 μl, SPD-M20AVP detector.

Biometric an alysis

The Flieg points of silages were calculated based on the pH value and DM content of the silages using the following equation (Zhang et al., 2014b):

$$\text{Flieg point} = 220 + (2 \times \%\text{DM} - 15) - 40 \times \text{pH}$$

The cumulative GP at time (t) [GP(t), ml · g^{-1} DM] for each fermentation bottle was fitted to an exponential model:

$$GP_t = A'[1 - e^{-c'(t - Lag)}] \quad (1)$$

where: e – base of natural logarithm, A – asymptotic GP generated at constant fractional rate (c) per unit time, t – gas recording time, Lag – lag time phase before GP commenced.

The average GP rate (AGPR, ml · h^{-1}) was characterised as the average GP rate between the start of the incubation and the time when the GP was half of its asymptotic value according to the following equation:

$$\text{AGPR} = \frac{A \times c}{2 \times (Ln2 + c \times Lag)} \quad (2)$$

Data of silage fermentation and *in vitro* degradation characteristics were analysed by one-way analysis of variance to evaluate the additives effect. The means were then compared for significance by Duncan's multiple range method. All statistical analyses were performed using the general linear model procedure of SAS 9.0 (2002; SAS Institute, Cary, NC, USA). Significance was declared at $P < 0.05$ unless otherwise noted.

Results

Chemical composition

The DM content of drooping wild ryegrass before ensiling was 245 g · kg^{-1} (Table 1). The WSC content was 49.8 g · kg^{-1} DM and crude protein (CP) content was 180 g · kg^{-1} DM.

Table 1. Chemical composition (mean ± standard deviation; n = 3) of drooping wild ryegrass prior to ensiling

Indices	Content
Dry matter (DM) content, g · kg^{-1}	245 ± 5.1
Content, g · kg^{-1} DM	
crude protein	180 ± 2.7
water soluble carbohydrates	49.8 ± 2.4
neutral detergent fibre	539 ± 8.0
acid detergent fibre	253 ± 5.0
hemicellulose	286 ± 2.2
K	35.4 ± 0.9
P	3.79 ± 0.11
Buffering capacity, meq · kg^{-1}	306 ± 5.9

All used additives decreased the pH and increased Flieg point value of drooping wild ryegrass silage in comparison to control silage ($P < 0.001$; Table 2). As compared to the control, S and FA addition decreased ammonia N content, while lactic acid content was substantially increased by the S and LP addition ($P < 0.001$). In comparison to silages with S and FA addition, the acetic acid content in silage treated with LP was not increased in relation to the control one. In LP treated silage the increased lactic to acetic acid ratio was observed ($P < 0.001$). No butyric acid was detected after addition of each additive. NDF, ADF and phosphorus contents were decreased by the S addition. The content of WSC was greater in FA treated silage than in untreated and S or LP treated ones ($P < 0.001$).

Table 2. Effects of different additives on drooping wild ryegrass silage characteristics

Indices	Silage[1]				SEM	*P*-value
	control	S	FA	LP		
Dry matter (DM) content, g · kg^{-1}	259^{ab}	269^{a}	252^{b}	259^{ab}	2.1	<0.001
pH	5.86^{a}	4.03^{d}	4.68^{b}	4.39^{c}	0.214	<0.001
Content, g · kg^{-1} DM						
lactic acid	15^{c}	85^{a}	9^{c}	67^{b}	10.2	<0.001
acetic acid	13^{b}	31^{a}	35^{a}	8^{b}	4.0	0.002
butyric acid	5.7	ND	ND	ND	0.74	-
NDF	459^{b}	433^{c}	494^{a}	469^{b}	7.4	0.002
ADF	259^{a}	240^{b}	249^{ab}	260^{a}	3.0	0.017
K	36.8	33.5	35.1	35.6	0.46	0.062
P	6.1^{a}	5.2^{b}	5.8^{a}	5.0^{b}	0.14	<0.001
crude protein	176	173	173	165	3.6	0.761
WSC	20.7^{b}	17.0^{b}	37.3^{a}	19.9^{b}	2.57	<0.001
Lactic : acetic acid ratio	2.6^{b}	2.9^{b}	0.2^{c}	8.3^{a}	0.96	<0.001
Ammonia N, % total N	5.6^{a}	2.4^{b}	1.0^{c}	5.4^{a}	0.60	<0.001
Flieg point	23^{d}	97^{a}	68^{c}	81^{b}	8.4	<0.001

[1] control – untreated drooping wild ryegrass, S – drooping wild ryegrass treated with 20 g · kg^{-1} fresh matter (FM) sucrose, FA – drooping wild ryegrass treated with 3 g · kg^{-1} FM formic acid, LP – drooping wild ryegrass treated with 0.005 g · kg^{-1} FM lactic acid bacteria inoculants (*Lactobacillus plantarum* and *Pediococcus acidilactici*); ND – not detected; NDF – neutral detergent fibre; ADF – acid detergent fibre; WSC – water soluble carbohydrates; ^{abc} – means with different superscripts within the same row are significantly different at $P < 0.05$; SEM – standard error of mean

Table 3. Influence of additives on *in vitro* gas production (GP) kinetics and fermentation characteristics in culture fluids after 48-h incubation of drooping wild ryegrass silage together with mixed rumen microorganisms

Indices	Silage[1]				SEM	*P*-value
	control	S	FA	LP		
IVDMD	0.802	0.815	0.800	0.817	0.0046	0.440
GP_{48}, ml · g^{-1} DM	73.5	74.6	83.2	82.2	2.17	0.280
A, ml · g^{-1} DM	72.3	72.3	82.5	81.2	2.27	0.235
c, h	0.12^{ab}	0.14^{a}	0.10^{b}	0.13^{ab}	0.013	0.042
Halftime, h	2.8	2.6	3.0	3.0	0.08	0.284
AGPR, ml · h^{-1}	12.5^{b}	14.8^{a}	11.3^{b}	15.7^{a}	0.52	<0.001
Ammonia N content, mM	16.4^{b}	17.3^{b}	17.8^{b}	23.4^{a}	0.79	<0.001
Total VFA content, mM	139^{b}	179^{a}	169^{a}	176^{a}	5.1	0.008
VFA pattern, mol · 100 mol^{-1}						
acetic acid	62.8	66.5	66.6	65.3	0.75	0.262
propionic acid	28.3	25.8	26.2	26.6	0.57	0.444
butyric acid	8.5	7.5	7.2	7.5	0.23	0.225
Acetic : propionic acid ratio	2.3	2.6	2.5	2.5	0.07	0.406

[1] see Table 2; IVDMD – *in vitro* dry matter disappearance; GP_{48} – cumulative gas production at 48 h; DM – dry matter; A – asymptotic gas production; c – fractional rate for gas production of 'A'; AGPR – average gas production rate; VFA – volatile fatty acid; ^{ab} – means with different superscripts within the same row are significantly different at $P < 0.05$; SEM – standard error of mean

In vitro rumen fermentation

Cumulative GP and the GP rate constants for the 60-day silages are shown in Table 3 and the GP curves – in Figure 1. The S treated silage had higher fractional GP rate (c) than silage treated with FA (Table 3), however did not differ from untreated and LP treated ones. All three additives increased total VFA concentration in silages in comparison to untreated one ($P < 0.01$). Ammonia N content was only increased after LP treatment ($P < 0.001$). AGPR were greater in S and LP treated silages than in untreated and FA treated ones ($P < 0.001$). No significant difference was observed in GP_{48}, IVDMD and VFA pattern among the treatments.

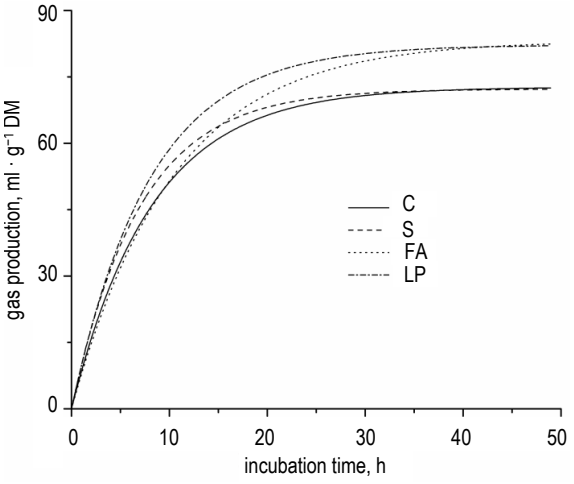

Figure 1. Cumulative gas production profiles of *in vitro* ruminal fermentation of drooping wild ryegrass silage untreated (C) or treated with 20 g · kg^{-1} sucrose (S), 3 g · kg^{-1} formic acid (FA), 5 g · kg^{-1} commercial lactic acid bacteria inoculant (LP)

Discussion

Silage quality. Fermentation quality depends on the properties of the ensiled crop (Weinberg and Muck, 1996). Buffering capacity of the examined silage (306 meq · kg^{-1} DM) was higher than that of typical Gramineae silage, the WSC concentration (49.8 g · kg^{-1} DM) was much lower than 60–70 g · kg^{-1} DM, the recommended theoretical requirement to achieve well preserved fermentation. A low pH ensures that the forage is retained in a stable form. pH values in all treatments, except S, were above 4.2, which is the critical value at or below which silages can be considered to be well preserved. It may suggest that silage fermentation was limited by low WSC content – similar results were previously reported by Lima et al. (2010) and Heinritz et al. (2012). Addition of LAB inoculant at

ensiling ensured vigorous fermentation, which resulted in increased accumulation of lactic acid, lower pH and improved forage conservation. It indicates that the inoculant has positive effect on stabilization of drooping wild ryegrass silage. Higher intake and improved animal performance are associated with well fermented, highly digestible silages containing high concentrations of lactic acid, and low content of acetic acid and cell wall. The obtained results suggest that S and LP treated silages were likely to achieve higher intake and better animal performance.

The lactic : acetic acid ratio was used to indicate the extent of homolactic fermentation in relation to heterolactic fermentation of sugars to lactic acid during ensiling. Homofermentative bacterial inoculants ferment WSC into organic acids, in particular lactic acid, which rapidly acidifies the silage and inhibits the growth of undesirable bacteria. Homofermentative LAB inoculants even in the 1970s have been found to be useful in promoting favourable fermentation in many forage species (Ohyama et al., 1975; Filya et al., 2007; Chen et al., 2013). In the present study, the addition of LP inoculant, containing such homofermentative LAB strains as *Lactobacillus plantarum* and *Pediococcus acidilactici*, increased the lactic acid content, lactic : acetic acid ratio and did not increased acetic acid content as expected. In contrast with sugars and bacterial inoculants that stimulate silage fermentation, formic acid restricts fermentation and decreases silage pH through direct acidification (McEniry et al., 2014). So the FA addition during drooping wild ryegrass ensiling could have caused the microorganisms inhibition from the beginning of silage fermentation, which may explain lower contents of end products fermentation such as lactic acid and ammonia N but higher of WSC. Similar results were also reported by Nadeau et al. (2000) and Contreras-Govea et al. (2013). The absence of butyric acid content in treated silages might be due to the lowered pH inhibiting the growth and proteolytic activity of microorganisms such as *Clostridia* bacteria (Heinritz et al., 2012). Silage applied with sucrose in comparison to the control decreased NDF and ADF contents, and this was in agreement with the results obtained in previous studies (Guo et al., 2014). The authors speculated that the sucrose addition might have stimulated the growth of fibrolytic bacteria naturally present in the environment when the silage was prepared.

***In vitro* rumen fermentation.** *In vitro* GP of feed samples incubated in ruminal fluid buffered with HCO_3^-/CO_2. GP technique, developed to predict fermentation of ruminant feedstuffs, was also applied to measure the degradation rates of feedstuffs (Krishnamoorthy et al., 1991). GP, measured manually or automatically, can be treated as a very good indirect indicator of fermentation kinetics (Rymer et al., 2005). Kozelov et al. (2008) noted that GP was significantly increased when lucerne was treated with $4\ l \cdot t^{-1}$ formic acid before ensiling. In the present study, GP of formic acid treated silage was increased by 13.2%. Addition of LAB inoculant showed no effect on GP of the silage, likely due to low content of WSC in the silage. The similar results were also reported by Muck et al. (2007) and Contreras-Govea et al. (2013). On the other hand, Kozelov et al. (2008) and Denek et al. (2011) found that bacterial inoculant increased GP and decreased the GP rate of lucerne silage. *In vitro* GP, degradation rate and VFA production were higher for high-WSC vs normal-WSC cultivars of sorghum (Amer et al., 2012). Therefore, the lowered WSC contents in examined silage might explain the differences in GP among the treatments.

In ruminants, it have been found that microorganisms present in the rumen can easily ferment a wide range of endogenous and exogenous substrates to produce VFA and such fermentation gases as CH_4, CO_2 or H_2. Some researchers (Cao et al., 2011; Fang et al., 2012) found that LAB inoculants showed little effects on IVDMD, ruminal VFA concentration though they improved fermentation quality of napier grass (*Pennisetum purpureum*), rice straw and vegetable residue silages. Although IVDMD was not affected, in the present study, VFA production was increased in the treated silages in comparison to the untreated control, and such result implicate that nutrients of the drooping wild ryegrass were partially utilized during ensiling, and the fermentable organic matter left in the silages could be further metabolized by rumen microbes due to the application of additives during ensiling. On the other hand, addition of sucrose or LAB inoculants slightly increased the average GP rate. It indicates that addition of sucrose or LAB inoculant might increase the degradation rate of drooping wild ryegrass silage in rumen. In the rumen, the feed protein is usually hydrolysed and deaminated to form peptides and free ammonia N by rumen microorganisms (Reynal et al., 2007). The amount of free ammonia N is the result of balance between protein hydrolysis and utilization (Reynal et al., 2007), and so the increase in ammonia N concentration in LP treatment in the present study may implicate that the hydrolysis and deamination of protein was increased.

Conclusions

Sucrose, formic acid and lactic acid producing bacterial inoculant improved the quality of drooping wild ryegrass silage, however sucrose and lactic acid producing bacterial inoculant addition gave better results as they also increased fermentation average gas production rate when exposed to rumen microbes. The results obtained in the present study implicate that sucrose and lactic acid producing bacterial inoculant can be recommended as a silage additive for preparing well fermented and rapidly degradable drooping wild ryegrass silage, while formic acid could be applied as a regulator additive to control the level of lactic acid produced during ensiling this forage.

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Conflict of Interest

We have no conflict of interest to declare.

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