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Effects of mixing red clover with alfalfa at different ratios on dynamics of proteolysis and protease activities during ensiling

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ABSTRACT

This study was conducted to study the effects of ensiled alfalfa (*Medicago sativa*) and red clover (*Trifolium pretense*) at different ratios on dynamics of fermentation parameters, N distribution, protein fractions, and protease activities during ensiling. Alfalfa and red clover were harvested and wilted to 35 and 25% dry matter, respectively, chopped to 1 cm, mixed, weighed into 1.0-L buckets at a density of 700 g/L, and ensiled for 1, 3, 7, 15, and 30 d at 30°C. The treatments were mixing ratio of alfalfa to red clover at 100:0, 70:30, 50:50, 30:70, and 0:100 (R0, R30, R50, R70, and R100, respectively; fresh weight). For each ensiling duration, 3 replicates of each treatment were prepared. With increasing proportion of red clover in silage, total N content and proportions of nonprotein N, peptide N, free amino acid N, and NH_3-N decreased linearly, and PC (indigestible true protein, acid detergent insoluble N) proportion increased linearly after ensiling. Moreover, the final pH was lower in R50 and R100 than R0 (4.29, 4.20 vs. 4.48, respectively) on d 30. Also, lactic acid concentration on d 30 was higher in R50, R70, and R100 silage compared with R0 (7.77, 7.66, and 8.76 vs. 6.34, % of dry matter, respectively). The proportion of NH_3-N in R50 was lower than in R0 but closer to R100 after ensiling. During ensiling, proteases including carboxypeptidase, aminopeptidase, and acid proteinase activities decreased as red clover proportion increased. However, no differences were detected in aminopeptidase and acid proteinase activities among R50, R70, and R100 during ensiling. Overall, 50:50 was the optimal mixing ratio of alfalfa with red clover, showing good fermentation quality with lower pH and higher lactic acid concentration, reduced protease activities and proteolysis compared with pure alfalfa silage, and also more total N content than pure red clover silage.
Key words: proteolysis, silage, alfalfa, red clover

INTRODUCTION

Extensive proteolysis occurs during the ensiling of alfalfa (*Medicago sativa*). Part of the true protein is degraded to NPN including mainly peptides, free amino acids (**FAA**), and NH_3-N (McDonald et al., 1991). In ensiled alfalfa, NPN can account for 44 to 87% of total protein (Papadopoulos and Mckersie, 1983; Muck, 1987). In addition, the availability of true protein also decreases in alfalfa silage because of the increasing amount of bound true protein (Guo et al., 2008). When alfalfa silage or alfalfa hay were fed to lactating dairy cows, although higher DM digestibility was observed in cows fed the silage, the higher concentration of ruminal NH_3 and milk urea-N indicated poor N utilization of alfalfa silage (Broderick, 1995). The economic loss and potential environmental pollution call for a better approach to minimize proteolysis in alfalfa during ensiling.

Compared with alfalfa, red clover (*Trifolium pretense*) shows a lower proteolysis rate as reflected in lower soluble NPN (6.8 to 40.8 vs. 18.9 to 66.8, % of total N, **TN**) during ensiling (Papadopoulos and Mckersie, 1983). Various studies have attributed inhibited proteolysis to polyphenol oxidase (**PPO**; EC 1.10.3.1; Mayer, 1986), a copper-containing enzyme naturally present in red clover (Jones et al., 1995b; Sullivan and Hatfield, 2006). After cutting or crushing, PPO enzymes in red clover are released from the cell and catalyze the oxidation of endogenous *o*-diphenols to quinones in the presence of oxygen (Macheix et al., 1991). Quinones then are polymerized to cross-linked protein complexes, resulting in inhibition of protein degradation (Bittner, 2006).

Protein protection was not found in ensiled transgenic alfalfa expressing PPO due to the lack of endogenous phenolic substrate (Getachew et al., 2009). Sullivan and Zeller (2013) found reduced proteolysis in alfalfa

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genetically altered to express PPO when they added *o*-diphenols and ensiled that alfalfa. Both PPO and *o*-diphenol substrate may be added to alfalfa by mixing alfalfa with red clover. A few studies attempted to ensile alfalfa with red clover to reduce proteolysis. Jones et al. (1995b) reported that mixing red clover extract and alfalfa extract at 1:1 ratio reduced alfalfa proteolysis by 70% compared with untreated alfalfa extract in laboratory scale. Marley et al. (2003) ensiled alfalfa/red clover bi-crops in 5 sowing ratios and reported that after ensiling, soluble N and FAA concentrations decreased with increasing red clover percentage and 1:1 was the optimal sowing ratio. However, in their study, sowing ratios do not reflect the final ratios of alfalfa and red clover at ensiling due to different growth of plants. Other indicators of proteolysis, protein fractions, and protease activities were not measured in their study. Furthermore, Broderick et al. (2001) and Broderick (2018) reported that lactating dairy cows can use N from red clover more efficiently than that from alfalfa as reflected in lower urea in milk and blood, and less N excretion without reducing milk yield and BW. These studies indicate that ensiling red clover with alfalfa has the potential to inhibit proteolysis and improved protein utilization by animals.

The objectives of this study were to evaluate the effects of ensiling alfalfa mixed with red clover at different ratios on dynamics of protein degradation and protease activities during the ensiling and determine the optimal mixing ratio for good fermentation quality and protein preservation. We hypothesize that ensiling red clover with alfalfa can improve fermentation quality, inhibit protease activities, and reduce proteolysis of the silage without negative effects on fermentation quality.

MATERIALS AND METHODS

Forage and Treatments

Alfalfa (WL525, Forage Genetics International, Nampa, ID) and red clover (Badong, China) were planted at the experimental farm of the Institute of Animal Sciences of Hubei Academy of Agricultural Sciences (Wuhan, China) and applied with no herbicides and fertilizers. Legumes were harvested at full bloom in third cutting, using a sickle by hand and leaving a 5 cm stubble. After chopping to approximately 1 cm with a forage cutter (Lingong Machinery, Shandong, China), alfalfa was wilted in the field for 4 h to reach a DM concentration of 35%; meanwhile, red clover was wilted to 25% DM. Microwave (M1-L213B, Midea Group Co., Ltd., Foshan, China) was used for rapid determination of DM (Farmer and Brusewitz, 1980). Alfalfa and red clover were mixed at ratios of 100:0, 70:30, 50:50, 30:70, and 0:100 (R0, R30, R50, R70, and R100, respectively, fresh weight, **FW**). All treatments received *Lactobacillus plantarum* (1×10^6 cfu/g of FW) plus sucrose (4 g/kg of FW) to facilitate fermentation. The lactic acid bacteria strain (GenBank accession number: KX870021) was isolated from cucumber pickle by the silage laboratory of China Agricultural University and identified as *L. plantarum* by 16S rRNA gene sequencing (Cai, 1999). Additive was dissolved in distilled water and sprayed at 10 mL/kg of FW on ensiling materials. After mixing, approximately 700 g of material was packed into 1.0-L plastic buckets (Hewanglan Paper and Plastic Products Factory, Beijing, China) to achieve a packing density of 700 g/L. A total of 15 silos were prepared for each treatment and stored in an incubator (SPX-250, Beijing Luxi Tech. Co. Ltd., Beijing, China) with temperature set at 30°C. Three silos from each treatment were opened on 1, 3, 7, 15, and 30 d, respectively.

Chemical Composition Analysis

Subsample of fresh forage before ensiling and treated silage after silo opening was dried in an oven (GZX-9140MBE, Shanghai Boxun Co. Ltd., Shanghai, China) at 65°C for 48 h for DM measurement. The dry samples were ground to pass through a 1-mm screen using a mill (FZ102, Test Instrument, Tianjin, China) and stored for further chemical analysis. Concentrations of NDF and ADF were determined as described by Van Soest et al. (1991) in an Ankom Fiber Analyzer (A2000I, Ankom Technology, Macedon, NY). Thermostable α-amylase and sodium sulfite were used in the NDF analysis, and the results were expressed on a DM basis without residual ash.

Twenty grams of each silage sample was mixed with 180 mL of distilled water and homogenized in a blender for 60 s, and filtered through 4 layers of cheesecloth and filter paper successively (Tian et al., 2017). The pH value was measured immediately using a pH meter (PHS-3C, INESA Scientific Instrument, Shanghai, China). The concentrations of organic acids including lactic, acetic, propionic, and butyric acid were measured by HPLC (Shimadzu, Tokyo, Japan; Xu et al., 2007).

Protein Degradation Indicator Analysis

Contents of FAA-N and NH_3-N were determined according to Broderick and Kang (1980). Total N was measured using the Kjeldahl nitrogen analyzer (Kjeltec 2300 Auto-Analyzer, FOSS Analytical AB, Hoganas, Sweden) according to AOAC (1990). Proportion of

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