Effects of plant enzyme inactivation or sterilization on lipolysis and proteolysis in alfalfa silage

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ABSTRACT

This experiment studied the contribution of plant enzymes and microbial activity on lipolysis and proteolysis in ensiled alfalfa. Before ensiling, the wilted alfalfa was treated with plant enzyme inactivation by autoclaving or with sterilization by γ -ray irradiation. The treated alfalfa was then inoculated with commercial lactic acid bacteria inoculants and ensiled for 40 d. Alfalfa without treatment was ensiled as the control. The content of total fatty acid (FA) after ensiling decreased 43% in the control silage and 28% in the γ -ray-treated silage, but did not change in the autoclave-treated silage. Among the major FA (C16:0, C18:2n-6, C18:3n-3), a considerable increase was observed in proportion of C16:0 in the control silage as compared with fresh alfalfa; conversely, decreases in proportions of C18:2n-6 and C18:3n-3 occurred during ensilage. Silage treated with γ -ray radiation at ensiling had a smaller proportion of C16:0 and greater proportions of C18:2n-6 and C18:3n-3 than control silage. Autoclave treatment further decreased proportions of C16:0 and most of the other FA, and increased C18:2n-6 and C18:3n-3 proportions in comparison with γ -ray treatment. Proportions of C16:0, C18:2n-6, C18:3n-3 and other detected FA (except for the proportion of C15:0) did not differ between fresh forage and autoclave-treated silage. Remarkably, smaller nonprotein nitrogen content was observed in the autoclave-treated silage compared with the γ ray-treated silage or the control silage. These results indicated that an extensive lipolysis occurred during ensiling of alfalfa, and plant enzymes played a major role in lipolysis and proteolysis.

Key words: lipolysis, fatty acid composition, proteolysis, alfalfa silage

INTRODUCTION

Interest in the changes of FA composition in herbages and forages after ensiling is increasing. The FA composition of forage lipids is dominated by a high proportion of PUFA of linoleic acid (C18:2n-6) and α -linolenic acid (C18:3n-3; Clapham et al., 2005). High intake of PUFA has been proven to increase the concentration of PUFA in ruminant products (Wood and Enser, 1997), and consequently be beneficial to human health (Simopoulos, 2001). Therefore, most of the studies on the FA composition of silages are concerned with losses of C18:2n-6 and C18:3n-3 during ensiling (Alves, et al., 2011).

Based on previous studies, the effects of ensiling on total FA content and the FA composition of forages are not consistent. Some studies indicated that ensiling increased total FA content and the proportions of C18:2n-6 or C18:3n-3 (Boufaied et al., 2003; Arvidsson et al., 2009; Van Ranst et al., 2009; Alves et al., 2011), whereas a contrary result was obtained in other research (Makoni et al., 1993; Dewhurst and King, 1998; Elgersma et al., 2003; Vanhatalo et al., 2007). The differences were thought to be mainly due to the DM losses during ensiling. However, as with extensive proteolysis in ensiled forage, especially in alfalfa silage, numerous studies have reported that lipolysis in silages is also a very extensive process (Steele and Noble, 1984; Elgersma et al., 2003; Van Ranst et al., 2009). So far, limited information is available on lipolysis in ensiled alfalfa. Several studies found that red clover had a lower lipolytic activity, as compared with grasses, because polyphenol oxidase has been shown to reduce proteolysis and lipolysis in red clover through denaturing of proteases and lipases (Lee et al., 2004; Lee et al., 2008; Van Ranst et al., 2011). It was proposed that lipolysis of forages during ensiling may be caused by both microbial and plant enzyme activation (Elgersma et al., 2003), and the lactic acid bacteria (LAB) commonly found in forages have the ability to biohydrogenate C18:2n-6 and C18:3n-3 (Ogawa et al., 2005). However, whether the lipolysis during ensiling is mainly caused by microbial or plant lipases is still not

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clear. Hence, the objectives of the present study were to evaluate changes in the FA composition of alfalfa forage after ensiling, and to clarify the importance of microbial and plant enzyme activation on the lipolysis of forages during ensiling.

MATERIALS AND METHODS

Plant Material

Alfalfa (*Medicago sativa* L. 'Forerunner') was grown in experimental plots of the Beijing Academy of Agro-Forestry Sciences at Xiaotangshan, Beijing, China (N39°34', E116°28'). Triplicate experimental plots were used, each plot with an area of 3 × 10 m. The experimental plots had similar fertilizer management, and the average soil OM in the experimental plots was 11.1 g/kg; effective N, P, and K in the soil were 82.9, 19.0, and 115.0 mg/kg, respectively. Third-cut fresh forage was randomly harvested at late bud to early bloom on July 23, 2011, from each of the 3 experimental plots, leaving a stubble of 10 cm. The fresh forage samples were then immediately taken into the laboratory for processing.

Determining the Radiation Dose and Time

The fresh forage samples were chopped to about 1 to 2 cm using a paper cutter, and wilted to a DM content of approximately 280 g/kg of fresh weight (FW). Some of the wilted alfalfa forages were then divided into 2 portions, each about 600 g, with one portion of the wilted forage equally subdivided into 10 subsamples. After evenly spreading each subsample on a paper sheet, all the subsamples were placed at the ⁶⁰Co irradiation laboratory (Peking University, Beijing, China) and irradiated for 2 h with γ -ray radiation doses of 15 or 25 kGy (5 subsamples for each radiation dose, with radiation doses determined after an extensive survey of published literature) to sterilize the epiphytic bacteria. The other portion of the wilted forage was also equally subdivided into 10 sterilized, triangular flasks with middle ventilated screw caps, and then put into an autoclave and treated at 121°C, 115 MPa for 15 and 30 min (5 subsamples for each autoclave time), respectively, to inactivate the plant enzyme and eliminate epiphytic bacteria (Sharma, 2007). After treatments of sterilization and plant enzyme inactivation, 10 g of forage from each subsample was used to enumerate LAB, yeast, and mold, as described by Schmidt and Kung (2010), and to determine γ -ray radiation dose and time of autoclave treatment.

Experimental Treatments and Mini-Silo Preparation

After determination of γ -ray radiation dose and treatment time in autoclave required for sterilization and

plant enzyme inactivation, the third-cut fresh forage samples were randomly harvested again at early bloom on July 28, 2011, from the same experimental plots as previously described. Fresh forage samples were immediately taken into the laboratory and chopped to about 1 to 2 cm in length using a paper cutter. Chopped forages from each experimental plot were then assigned to one of the following treatments: (1) untreated (sterilized water); (2) commercial inoculants (LaLSil Dry; contains *Lactobacillus buchneri* NCIMB 40788, *Pediococcus acidilactici* CNCM MA18/5M, β -glucanase, and xylanase; manufactured by Lallemand, Montréal, Québec, Canada) after treatment of γ -ray irradiation at a dose of 25 kGy for 2 h; or, (3) LaLSil Dry after autoclaving treatment at 121°C, 115 MPa for 15 min. For conducting the experimental treatments, some chopped alfalfa from each of the 3 experimental plots was wilted for 24 h at room temperature (26–27°C) to a DM content of approximately 300 g/kg of FW and directly used for ensiling as control. The rest of the chopped alfalfa, after wilting for 22 h at room temperature to a DM content of approximately 280 g/kg of FW, was divided into 2 portions. One portion was treated with γ rays for 2 h at room temperature (26°C) as described above, and then thoroughly mixed with the inoculant solution at ensiling; the application rate of the inoculant was administered according to guidelines from the manufacturer. The other portion was also thoroughly mixed with the same inoculant solution at ensiling after treatment in an autoclave. Before ensiling, DM contents of all treatments were adjusted to about 320 g/kg of FW. Five mini-silos were made for each treatment by using the forage from each of the 3 experimental plots, following the method described previously (Owens et al., 1999), and all mini-silos were prepared in an aseptic operating board. Briefly, 60 g of forage was placed into 100-mL sterilized polypropylene centrifuge tubes with intermittent packing using a porcelain pestle. Tubes were capped with rubber stoppers fitted with gas traps. A 20-gauge needle connected with a Tygon tube (150 mm long and 3.97 mm outside diameter) was inserted into each tube of the silo. The free ends of the Tygon tubes were placed into a 30°C water bath to allow gas to escape. Gas traps were removed after active fermentation had ceased (within 2 wk) to prevent backflow of water into the tubes.

Chemical and FA Analyses

Mini-silos from each treatment were opened at 40 d of ensiling and immediately frozen (−80°C) in sealed plastic bags until further chemical analysis (the fresh and wilted forage samples were taken before experiment). A 10-g sample from each silo was placed in a blender

jar, diluted with distilled water to 100 g, macerated for 30 s in a high-speed blender, and then filtered through 4 layers of medical gauze; filtrate pH was measured immediately. After the filtrate was centrifuged at 4°C for 15 min at $10,000 \times g$ and filtered with the dialyzer of 0.45 μm , lactic acid, acetic acid, propionic acid, and butyric acid in the ensiled forages was analyzed by HPLC (KC-811 column, Shodex, Shimadzu, Japan; oven temperature, 50°C; flow rate, 1 mL/min; detector SPD-10Avp, Shimadzu). One milliliter of 250 g/L (wt/vol) of TCA was added to 4 mL of the filtrate from each silo and the solution was allowed to stand at room temperature for 1 h or was held overnight at 4°C to precipitate the protein. The solution was then centrifuged at 4°C, $18,000 \times g$ for 15 min, and the supernatant was analyzed for ammonia nitrogen and amino acid nitrogen (AA-N; Broderick and Kang, 1980). Peptide-N concentration was determined by the increase in AA-N in the TCA supernatant after digesting with 6 *N* HCl for 21 h at 105°C, under an N_2 atmosphere (Ohshima and McDonald, 1978). Nonprotein nitrogen of forage samples were analyzed by using TCA to precipitate the true protein as described by Licitra et al. (1996).

The fresh forage and the remaining ensiled forage from each silo were freeze-dried in a freeze dryer (TFD5503, Skadi Co., Ede, the Netherlands) for 96 h to measure DM content. After weighing, the dried sample was ground through a 1 mm screen in a mill (Perten Laboratory Mill 3100, Seedburo Equipment Co., Des Plaines, IL) and analyzed for total N by the Association of Official Analytical Chemists method 976.06 (AOAC, 1990). Water-soluble carbohydrate (WSC) in fresh forage was determined using the method of Thomas (1977).

For analysis of lipids in the forages, the lyophilized forage samples were milled through a 1-mm screen. The ground forage (5 g) was placed in filter paper, saturated with ether, placed in a Soxhlet extractor, and extracted with 100 mL of ether at 65 to 70°C. Methyl esters for forage lipids were prepared according to methods of Ichihara et al. (1996) using a base catalyst. Extracted lipids (10 mg) were dissolved in 2 mL of hexane followed by addition of 0.2 mL of 2 *M* methanolic KOH. The sample was vortexed for 2 min at 23°C. After centrifugation at $500 \times g$ for 5 min at 4°C, an aliquot of the hexane layer was collected for GC-MS analysis.

Methyl esters of FA were separated and quantified by GC-MS (model 6890N/5973N, Agilent Technologies, Wilmington, DE) fitted with a fused-silica capillary column (HP-20M 60 m $\times$ 0.32 mm $\times$ 0.3 μm , Hewlett-Packard, Palo Alto, CA) with He as the carrier gas. The injector temperature was 230°C. The oven temperature was programmed at an initial temperature of 80°C maintained for 1.0 min, then increased to 180°C

Table 1. Microbial (wet basis) and chemical compositions (g/kg of DM, unless otherwise stated) of fresh alfalfa

Item	Value
LAB, ¹ log cfu/g of fresh forage	8.56 $\pm$ 0.02
Yeasts, log cfu/g of fresh forage	5.59 $\pm$ 0.06
Molds, log cfu/g of fresh forage	4.74 $\pm$ 0.04
DM	220 $\pm$ 4.57
CP	225.3 $\pm$ 2.4
NDF	418 $\pm$ 9.01
ADF	342 $\pm$ 8.22
WSC ²	49.03 $\pm$ 0.85

¹Lactic acid bacteria.

²Water-soluble carbohydrate.

at 10°C/min, held at 180°C for 2 min, raised to 220°C at 2°C/min, and maintained at the final temperature for 48 min; total run time was 80 min. Fatty acids were identified by comparison with retention times of FA in standard samples (Sigma, St. Louis, MO; Larodan Fine Chemicals, Malmö, Sweden). Fatty acids were quantitatively analyzed by a 5-level standard curve.

Statistical Analysis

Five mini-silos were made for each treatment by using the forage from each of the 3 experimental plots; in total, 15 observations in each treatment were obtained. After averaging these observations, 5 observations of each treatment were subjected to one-way ANOVA, and means were compared using Duncan test (SPSS 17.0, SPSS Inc., Chicago, IL).

RESULTS

Chemical and Microbial Composition

The nutrient and microbial contents of fresh alfalfa before ensiling are given in Table 1. No LAB, yeasts, or molds were detected in alfalfa forages after γ -ray radiation at 25 kGy for 2 h or autoclave at 121°C, 115 MPa for 15 min (Table 2). Therefore, the forage can be sterilized by the above treatments. Treatments of γ -ray irradiation and autoclaving decreased pH ($P < 0.05$), WSC ($P < 0.05$), and increased the concentrations of lactic acid and propionic acid ($P < 0.05$) in silages when compared with the control silage (Table 3). Butyric acid in silage treated by autoclaving was greater ($P < 0.05$) than control silage or γ ray-treated silage. No difference was found in acetic acid concentration between control silage and silage treated with γ -ray irradiation, whereas a pronounced amount of acetic acid was observed in autoclave-treated silage ($P < 0.05$) compared with the control silage or silage treated with γ -ray irradiation. After 60 d of ensiling, total numbers of viable LAB were smaller in autoclave-treated silage

Table 2. Counts of lactic acid bacteria, yeasts, and molds¹ in alfalfa forages after treatments with different γ -ray radiation doses or different times in autoclave

Measurement	Lactic acid bacteria	Yeasts	Molds
Radiation dose, kGy			
0 ²	2.7×10^8	1.3×10^6	3.2×10^5
15	5×10^3	No growth	No growth
25	No growth	No growth	No growth
Autoclave time, min			
0 ³	2.7×10^8	1.3×10^6	3.2×10^5
15	No growth	No growth	No growth
30	No growth	No growth	No growth

¹Results stated as colony forming units per gram of fresh weight.²Wilted alfalfa without γ -ray radiation.³Wilted alfalfa without autoclave treatment.

than in control or γ ray-treated silages. Numbers of yeasts were not detectable in all silages, and all silages had similar numbers of molds.

FA Composition in Fresh and Ensiled Alfalfa

The concentration of total FA after ensiling decreased 43% in the control silage and 28% in the γ ray-treated silage, but did not change in the autoclave-treated silage (Table 4). Compared to fresh alfalfa, the control silage had greater proportions ($P < 0.05$) of C12:0, C14:0, C15:0, C16:1, C18:0, *cis*-9 C18:1, C20:0, C22:0, and C24:0. Among the major FA (C16:0, C18:2n-6, C18:3n-3), a considerable increase ($P < 0.05$) was observed in proportion of C16:0 in the control silage as compared with fresh alfalfa; conversely, decreases in proportions of C18:2n-6 and C18:3n-3 occurred ($P < 0.05$). However, similar concentrations of C16:0 in fresh forage, control silage, and the silage treated with γ -ray was observed (5.51, 5.31, and 5.76 g/kg of DM, respectively). Fatty acid C18:2 *trans*-9,*trans*-15 was

not originally present in fresh alfalfa but was produced during ensiling. Besides C12:0, C14:0, C15:0, C18:0, *cis*-9 C18:1, C20:0, and C24:0, silage treated with γ -ray radiation at ensiling had smaller proportion of C16:0 and greater proportions of C18:2n-6 and C18:3n-3 ($P < 0.05$) than the control silage. Autoclave treatment further decreased proportions of C16:0 and most of the other FA, and increased C18:2n-6 and C18:3n-3 proportions in comparison with γ -ray treatment ($P < 0.05$). However, proportions of C16:0, C18:2n-6, C18:3n-3 and the other detected FA (except for proportion of C15:0) did not differ between fresh forage and autoclave-treated silage ($P > 0.05$).

Nitrogen Distribution in Alfalfa Silage

As expected, more than 70% of total N in control silage was NPN, which was almost 3 times of that in the wilted alfalfa forage (Table 5). Silages treated with γ -ray irradiation and autoclave had smaller NPN, AA-N, NH₃-N, and peptide-N ($P < 0.001$) than the control

Table 3. Dry matter content, pH, concentrations of water-soluble carbohydrates (WSC), and organic acids in ensiled alfalfa forages

Item	Silage			SEM	P-value
	Control	γ -Ray treated	Autoclaved		
DM	319 ^a	327 ^b	329 ^b	1.6	0.025
pH	4.97 ^c	4.56 ^b	4.19 ^a	0.005	<0.001
WSC, g/kg of DM	17.4 ^c	13.9 ^b	10.3 ^a	0.46	<0.001
Lactic acid, g/kg of DM	29.9 ^a	56.7 ^b	63.1 ^c	2.22	<0.001
Acetic acid, g/kg of DM	7.9 ^a	7.5 ^a	30.9 ^b	1.67	<0.001
Propionic acid, g/kg of DM	0.0 ^a	10.3 ^b	9.7 ^b	0.72	<0.001
Butyric acid, g/kg of DM	0.9 ^b	0.1 ^a	4.6 ^c	0.30	<0.001
LAB, ¹ log cfu/g of silage	9.08 ^b	9.75 ^c	8.45 ^a	0.19	<0.001
Yeasts, log cfu/g of silage	ND ²	ND	ND	—	—
Molds, log cfu/g of silage	5.26	5.13	5.47	0.19	0.79

^{a-c}Within a row, means without a common superscript letter differ ($P < 0.05$).¹Lactic acid bacteria.²ND = not detectable.

Table 4. Total FA content (mg/g of DM), and FA composition (g/100 g of total FA) of fresh alfalfa, wilted alfalfa, and alfalfa silages after 40 d of ensiling without physical treatment and treated by γ -ray radiation or autoclave before ensiling

Item	Forage before ensiling		Silage			SEM
	Fresh	Wilted	Control	γ -Ray treated	Autoclaved	
Total FA	29.57 ^c	27.56 ^c	16.82 ^a	21.29 ^b	29.20 ^c	1.214
C12:0	0.07 ^a	0.09 ^{ab}	0.17 ^c	0.12 ^b	0.09 ^{ab}	0.006
C14:0	0.19 ^a	0.25 ^b	0.40 ^d	0.31 ^c	0.21 ^{ab}	0.012
C15:0	0.17 ^{ab}	0.13 ^a	0.37 ^c	0.33 ^c	0.23 ^b	0.013
C16:0	18.65 ^a	18.77 ^a	31.57 ^c	27.06 ^b	20.28 ^a	0.737
C16:1	1.04 ^a	0.94 ^a	1.55 ^b	1.48 ^b	1.06 ^a	0.037
C18:0	2.91 ^a	3.16 ^a	5.08 ^c	4.30 ^b	3.22 ^a	0.127
C18:1 <i>cis</i> -9	1.73 ^b	1.10 ^a	1.82 ^b	1.64 ^b	1.35 ^a	0.044
C18:1 <i>trans</i> -11	0.06 ^{ab}	0.00 ^a	0.05 ^{ab}	0.31 ^c	0.14 ^b	0.020
C18:2n-6	19.66 ^c	17.71 ^b	15.34 ^a	16.56 ^{ab}	17.34 ^b	0.219
C18:2 <i>trans</i> -9, <i>trans</i> -15	0.00 ^a	0.00 ^a	2.41 ^c	1.02 ^b	0.75 ^{ab}	0.154
C18:3n-3	54.53 ^c	56.93 ^d	37.85 ^a	45.36 ^b	53.92 ^c	1.016
C20:0	0.29 ^a	0.28 ^a	0.63 ^b	0.51 ^b	0.33 ^a	0.024
C22:0	0.50 ^a	0.48 ^a	0.86 ^b	0.80 ^b	0.46 ^a	0.034
C24:0	0.20 ^a	0.21 ^a	1.88 ^c	1.10 ^b	0.57 ^{ab}	0.109

^{a-d}Within a row, means without a common superscript letter differ ($P < 0.05$).

silage. Remarkably, less NPN ($P < 0.001$) was observed in the autoclave-treated silage compared with the γ -ray-treated silage or the control silage. The NPN content in the autoclave-treated silage only increased about 50 g/kg of total N in comparison with that in the wilted forage at ensiling, but it increased about 320 g/kg of total N in the γ -ray-treated silage, and 480 g/kg total of N in the control silage. Similar to the fresh forage, most of the NPN in alfalfa silages was peptide-N, however, proportions of peptide-N to total NPN decreased from about 90% in fresh alfalfa to about 70% in the control silage and about 80% in autoclave-treated silage.

DISCUSSION

Treatment with γ -irradiation is often used to sterilize forage at ensiling for research on the role of plant and microbial enzymes in changes of the major nitrogenous components in silage (Heron et al., 1986; Winters et al., 2000). The doses of γ -irradiation in these studies ranged from 20 to 30 kGy; generally, very little γ -irradiation is required to inactivate microorganisms (Goldblith,

1971). In the present experiment, with a small amount of forage, doses of 15 and 25 kGy were given over a period of 2 h. After microbiological examination using plate count agar, the irradiated alfalfa samples were found to be sterilized. Although autoclaving is used as a conventional method for sterilization, it is also used to inactivate plant or food enzymes (Bollag and Dec, 1998; Svihus et al., 2000). Master et al. (2004) pointed out that high-pressure sterilization is a combined process where both pressure and temperature contribute to sterilization by the inactivation of spores and enzymes. Therefore, in the present study, the alfalfa forage was autoclaved to destroy plant enzymes. To stay close to fermentation in silage, we inoculated LAB to the alfalfa at ensiling after γ -ray irradiation or autoclaving treatment. In this case, the 2 treatments mentioned represented ensiled alfalfa forage with and without plant enzyme activities, respectively.

In agreement with previous research on ensiled ryegrass (Merry et al., 1995; Winters et al., 2000), exogenous LAB inoculation led to a rapid decrease in pH and, remarkably, increased lactic acid in ensiled alfalfa

Table 5. Total N and N composition (g/kg of total N, unless otherwise noted) of wilted alfalfa and alfalfa silages after 60 d of ensiling without physical treatment and treated by γ -ray radiation or autoclave before ensiling

Item	Fresh forage before ensiling		Silage			SEM
	Fresh alfalfa	Wilted alfalfa	Control	γ -Ray treated	Autoclaved	
Total N, g/kg of DM	36.0 ^a	38.5 ^b	39.6 ^b	40.2 ^b	38.6 ^b	0.29
NPN	155.6 ^a	256.4 ^b	732.9 ^e	577.1 ^d	297.7 ^c	28.39
NH ₃ -N	<0.01 ^a	1.1 ^a	101.9 ^d	44.2 ^c	16.7 ^b	5.34
FAA-N ¹	16.7 ^a	47.5 ^b	128.6 ^d	85.0 ^c	41.5 ^b	5.41
Peptide-N	140.0 ^a	208.1 ^b	502.4 ^e	446.5 ^d	238.9 ^c	18.29

^{a-e}Within a row, means without a common superscript letter differ ($P < 0.05$).

¹Free amino acid N.

after sterilizing the epiphytic microorganism by γ -ray irradiation or autoclaving. A large amount of acetic acid was observed in autoclave-treated silage as compared with silage treated with γ -ray irradiation, which suggested that a great extent of heterofermentation occurred in autoclave-treated silage as a result of *Lactobacillus buchneri* fermentation (Arriola et al., 2011). The notable difference between the 2 treatments was that plant enzymes were inactivated in autoclave-treated silage. High pressure and temperature may generate more fermentable substrate for the growth of *Lactobacillus buchneri*; however, no change was found in WSC of the fresh forages after γ -ray irradiation and autoclaving treatments (data not shown). Therefore, the reason for the notable amount of acetic acid concentration in autoclave-treated silage is not clear. A greater amount of butyric acid was found in autoclave-treated silage compared with the control silage or γ -ray-treated silage. The presence of this acid in silage is usually associated with the activity of saccharolytic *Clostridia* spp. (Heron et al., 1986). Although autoclaving is known to remove *Bacillus* and *Clostridium* spores (Sharma, 2007), the greater amount of butyric acid in autoclave-treated silage indicated the contamination of clostridia in the present study.

The present study showed a decrease in the total FA content in control silage compared with fresh alfalfa. Similar results were reported by Whiting et al. (2004) in alfalfa, and in other reports on grasses (Makoni et al., 1993; Dewhurst and King, 1998; Elgersma et al., 2003; Vanhatalo et al., 2007). However, contrary results were also reported, in which an increase of total FA or lipid was obtained in silages compared with fresh forage (Boufaied et al., 2003; Arvidsson et al., 2009; Van Ranst et al., 2009; Elves et al., 2011). The reason for the contrasting reports is still not completely clear. The proposed reason for the discrepancy is that the greater total FA concentration in silages than in fresh materials is due to DM losses during ensiling associated with aerobic and anaerobic fermentation or losses in effluent (Van Ranst et al., 2009; Elves et al., 2011). We did not measure the DM losses in the present study, but high amounts of acetic acid in autoclave-treated silage suggest more DM losses in autoclave-treated silage than control silage. However, the total FA in autoclave-treated silage was comparable to the fresh forage. Moreover, it was hypothesized that the changes in total FA during ensiling was most likely due to degradation of DM other than FA (Van Ranst et al., 2009), but our results in alfalfa silage did not support this hypothesis.

The major FA in both fresh alfalfa and alfalfa silage were C16:0, C18:2n-6 and C18:3n-3, which was in consistent with previous reports on fresh forages and

silages (Boufaied et al., 2003; Whiting et al., 2004; Van Ranst et al., 2009; Khan et al., 2012). Unlike previous reports (Van Ranst et al., 2009; Khan et al., 2012), changes in the composition of FA in alfalfa occurred after ensiling, especially at C16:0, C18:2n-6, and C18:3n-3. Both proportions and concentrations of C18:2n-6 and C18:3n-3 in the control alfalfa silage decreased compared with fresh alfalfa. Although the proportion of C16:0 increased in silage, the concentration was comparable to the fresh forage (concentrations of C16:0 in fresh alfalfa, control, γ -ray irradiation, and autoclave-treated silages were 5.5, 5.3, 5.7, and 5.9 mg/g of DM, respectively). Therefore, the decrease in total FA in alfalfa silage compared with the fresh forage was mainly due to degradation of both C18:2n-6 and C18:3n-3, and as it was proposed that plant lipases are recognized to convert C18:2n-6 and C18:3n-3 into hydroperoxy PUFA that are further catabolized to yield a range of volatile compounds (Elves et al., 2011).

As indicated in a previous study, LAB commonly found in forages have the ability to biohydrogenate C18:2n-6 and C18:3n-3 (Ogawa et al., 2005). Therefore, the changes in FA composition of grass during ensiling may be due to microbial intervention during the process of ensiling, but plant enzymes might also be active during ensiling (Dawson et al., 1977). The present study showed that the total FA content and the C18:3n-3 concentration in silage treated with plant enzyme inactivation plus inoculation of LAB was similar to that in fresh forage. Plant enzyme inactivation also inhibited the reduction of C18:2n-6 and the increase of C16:0 proportions during ensiling. Based on comparison of the total FA content in fresh alfalfa and in silages treated with γ -ray irradiation and autoclaving, it can be deduced that plant enzyme was the major contributor to the lipolysis during ensiling. This deduction can also be reflected by the changes in proportions of C18:3n-3 in the 3 treatments previously mentioned, or by the C18:3n-3 concentrations in these treatments (16.1, 9.6, and 15.7 mg/g or DM in fresh alfalfa, γ -ray irradiation, and autoclave-treated silages, respectively). Elgersma et al. (2003) proposed that the changes in FA composition in silage compared with fresh grass could be caused by plant enzymatic processes after cutting, and not the subsequent ensiling process. However, the current results in alfalfa did not support this hypothesis. A smaller proportion of C18:3n-3 and total FA content in the control silage as compared with the γ -ray irradiation-treated silage indicates that the diversely epiphytic microbes in alfalfa could degrade more FA during ensiling than the simply inoculated LAB. Anyhow, contribution of microbes to the lipolysis during ensiling was not as consequential as that of plant lipase

in consideration of the data on either total FA contents or C18:3n-3 concentrations in the fresh alfalfa and those of in silages.

Proteolysis during ensilage and its relevance to ruminant N utilization is a conventional topic in silage research. Numerous studies have been conducted to investigate contribution of microbe-originated enzymes and plant enzymes to formation of NPN in silage. The dominant role that plant enzymes play in proteolysis during ensiling has been proven by a classical method of sterilizing forage at ensiling (Heron et al., 1986) or by using specific inhibitors of endo- and exopeptidases (Guo et al., 2011; Tao et al., 2011). Plant enzyme inactivation before ensiling further validated the leading role of plant enzymes in proteolysis and could effectively reduce both the extensive proteolysis and lipolysis which occur in ensiled alfalfa.

CONCLUSIONS

The results in total FA content of fresh and ensiled alfalfa indicate that an extensive lipolysis occurred during ensiling, which was mainly due to the degradation of C18:2n-6 and C18:3n-3. Plant enzymes played a major role in lipolysis during ensiling of alfalfa, as shown by comparison of the lipolysis extent in silages treated with sterilization or plant enzyme inactivation. The diversely epiphytic microbes in alfalfa contributed much more to lipolysis during ensiling than the purely inoculated LAB. Plant enzyme inactivation could effectively inhibit both proteolysis and lipolysis in ensiled alfalfa.

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