Journal of Animal and Veterinary Advances 11 (13): 2181-2186, 2012
ISSN: 1680-5593

Effect of Epiphytic Microorganisms and Exogenous Lactic Acid Bacteria on the Formation of Non-protein Nitrogen During the Ensiling of Alfalfa

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Abstract: The effect of epiphytic microorganisms and exogenous Lactic Acid Bacteria (LAB) on the formation of Non-Protein Nitrogen (NPN) components during the ensiling process was investigated. All silages were prepared in mini-silos of 100 mL polypropylene centrifuge tubes and kept in an incubator at 30°C and tubes of each treatment were opened after 1, 3, 7, 14 and 35 days of ensiling. Treatments included three variations: nothing added (control); epiphytic microorganisms killed (treated with Ultraviolet (UV) rays) and xogenous lactic acid bacteria (inoculants of lactic acid bacteria after treatment with UV rays) added.

Key words: Alfalfa silage, epiphytic microorganisms, exogenous lactic acid bacteria, proteolysis, silages, butyric acid

INTRODUCTION

Extensive proteolysis in ensiled legume forages is a main factor that restricts legume silage from serving as a high-quality feed for ruminants. During the process of ensiling, most herbage proteins are degraded into peptide nitrogen (peptide-N), free Amino Acids Nitrogen (AA-N), ammonia Nitrogen (NH_3-N) and other forms of Non-Protein Nitrogen (NPN) by plant protease and microbial protease (Ohshima and McDonald, 1977).

Previous studies have shown that 4 classes of endopeptidases and 5 classes of exopeptidase are present in alfalfa leaves, each playing a different role in alfalfa protein degradation (Guo *et al*., 2011; Tao *et al*., 2011). Winters *et al*. (2002) indicated that the extent of protein hydrolysis during ensilage is influenced by factors other than the rate of pH decline and plant protease activity and that microbial proteases play a role. Moreover, Heron *et al*. (1986) pointed out that NH_3-N and amines are largely end products of microbial protease activity rather than of plant enzymes. However, few studies have addressed the effects of epiphytic microorganisms on the proteolysis in ensiled alfalfa forage. Many studies have shown that addition of Lactic Acid Bacteria inoculants (LAB) at ensiling ensures rapid and vigorous fermentation which results in faster accumulation of lactic acid, lower pH at earlier stages of ensiling and improved forage conservation (Tengerdy *et al*., 1991; Jones *et al*., 1992; Wang *et al*., 2009) but they did not sufficiently consider the impact of epiphytic microorganisms. The aim of this study was therefore to compare the effect of epiphytic microorganisms and exogenous lactic acid bacteria on the formation of NPN composition during the alfalfa ensiling process.

MATERIALS AND METHODS

Determination of the UV irradiation time and dosage of exogenous lactic acid bacteria

Plant material: Alfalfa (*Medicago sativa*. L. cv. Algonquin) was planted in the experimental plots at the China Agricultural University, Beijing, China (N39°57', E116°18'). Fresh forage from second-cut and early-bloom field-grown plants was cut from the petioles on July 23, 2010 and immediately taken to the laboratory.

Determination of the UV irradiation time: The fresh forage samples were chopped to about 1-2 cm lengths using a paper-cutter and tiled as thinly as possible in the purified clean bench then the ultraviolet light was turned up to kill microorganisms attached to the surface of the alfalfa plant. After an extensive survey of the published literature, 5 time gradients were prepared for the cut alfalfa forage (Table 1) and then the irradiation time was determined by measuring the remaining bacteria number and DM content.

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Table 1: The lactic acid bacteria number and the DM content of alfalfa forge at different irradiation time

Irradiation time (h)	The lactic acid bacteria number (cfu g^{-1} FW)	DM (g kg^{-1} FW)
0	1.8×10^{9a}	180.3^{e}
6	4.3×10^{5b}	229.6^{d}
12	1.6×10^{3c}	258.1^{c}
24	3.5×10^{3d}	300.9^{b}
48	500^{e}	339.9^{a}
60	350^{e}	380.0^{a}
SEM	26.7	11.46

Determination of dosage of exogenous lactic acid bacteria: According to the irradiation time, researchers wilted the alfalfa forage for the same long time to make the DM content consistent across treatments and then determined the number of the lactic acid bacteria per gram in the wilted forage. After determining the actual lactic acid bacteria number of the commercial lactic acid bacteria preparation and the remaining bacteria number in the UV ray treated forage, researchers calculated the dosage to ensure that the forage treated with UV ray+LAB had the same number of lactic acid bacteria as the wilted alfalfa forage.

Preparation of experimental samples

Plant material: A second cut of alfalfa was taken on July 30, 2010 from the same experimental plots described before, leaving a stubble of about 10 cm. Fresh forage samples were immediately taken to the laboratory, chopped into lengths of about 1.2 cm using a paper-cutter and then treated.

Design of experiments: Treatments included 3 variations: nothing added (control); treated with UV rays and inoculants of LAB (LaLsIL Dry: Lactobacillus >3,00. The 10^{10} cfu g^{-1}, Pediococcus >2,00, 10^{10} cfu g^{-1}, enzyme (cellulose/hemicellulase) activity >20 000 IE g^{-1}, manufactured by Lallemand (Montreal, Quebec, Canada). Some chopped alfalfa was wilted for 48 h at room temperature (27°C) to a DM content of approximately 35 g kg^{-1} and nothing was added as control. Other chopped alfalfa was treated with UV rays for 48 h at room temperature (27°C) and then divided into two portions. To one portion, nothing was added as the UV ray treatment and to the other portion, inoculants of LAB were added (0.05 g kg^{-1} Fresh Weight (FW).

Both portions were prepared on the day of ensiling. A 45 g LAB was added in 1 L distilled water and applied in solution (mixed fully) at a rate of 1 mL/50 g of herbage. An equal volume of distilled water alone was added to the other treatments. Mini-silos in triplicate were made for each of the treatments following the method described (Owens *et al*., 1999) and all the samples were prepared in the clean workbench (except control). Briefly, 50 g forage was placed into 100 mL polypropylene centrifuge tubes with intermittent packing with 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) and 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 weeks) in order to prevent backflow of water into the tubes.

Analytical procedures: Triplicate silos from each treatment were opened at 1, 3, 7, 14 and 35 days of ensiling and immediately frozen (-80°C) in sealed plastic bags until further chemical analysis the 0 day (untreated) samples were taken immediately after forage was chopped). Silage Dry Matter (DM) was determined on a 20 g sub-sample of each frozen sample of ensiled forage by drying at 60°C for 48-72 h in a forced-air oven. 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, USA) and analyzed for Crude Protein (CP) by the Association of Official Analytical Chemists method 990-03 (AOAC, 1990).

Water Soluble Carbohydrate (WSC) in fresh forage and silage was determined using the method of McDonald and Henderson (1964). A 10 g sample from each silo was placed in a blender jar, diluted with distilled water to 100 g and macerated for 30 sec in a high-speed blender and then filtered through four layers of medical gauze. The filtrate pH was measured immediately.

After the filtrate was centrifuged for 15 min at 10,000×g and filtered with the dialyzer of 0.45 μm, Lactic Acid (LA), Acetic Acid (AA), Propionic Acid (PA) and Butyric Acid (BA) in the ensiled forages were analyzed by High Performance Liquid Chromatography (HPLC) (KC-811 column, Shodex; Shimadzu; Japan; oven temperatures were 25°C; flow was 1 mL min^{-1}; SPD 210 nm) as described by Xu (2006) and the number of lactic acid bacteria in the filtrate was counted by using GYP-$CaCO_3$ agar plates (Masuko *et al*., 1992) which were incubated for 5 days at 35°C.

About 10 mL of 250 g L^{-1} (w/v) trichloroacetic acid was added to 40 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×g for 15 min and the supernatant was analyzed for ammonia nitrogen (NH_3-N) and Amino Acid Nitrogen (AA-N) (Broderick and Kang, 1980) and for NPN by the Kjeldahl method noted before. Peptide nitrogen (peptide-N) concentration was determined by the increase in AA-N in

the trichloroacetic acid supernatant after digesting with 6 N HCl for 21 h at 105°C, under an N_2 atmosphere (Ohshima and McDonald, 1977).

Statistical analysis: Analysis of variance was used to test the statistical significance of treatment, time of ensiling and the additive x time interaction using the univariate procedure of the Statistical Package for the Social Science (SPSS 17.0, SPSS, Inc., Chicago, IL). When the F-test indicated a significant (i.e., $p<0.05$) additive effect, means separations were conducted using a least significant difference test. Polynomial contrasts were used to test the linear, quadratic and cubic effects of increasing fermentation time. A probability of $p<0.05$ was used to denote significance unless otherwise indicated. Parameters were plotted when additive x time interactions were significant ($p<0.05$) to aid in interpretation of results. The effect of the treatments on silage fermentation of day 35 was analyzed using the Duncan test.

RESULTS

UV irradiation time and the dosage of exogenous lactic acid bacteria: Lactic acid bacteria number and dry matter content decreased with the extension of radiation time (Table 1). No differences ($p>0.05$) in lactic acid bacteria number were observed between 48 and 60 h which demonstrated that 48 h was enough (Table 1). After illumination for 48 h, the dry matter content was 34% which was suitable for making silage (Guo *et al*., 2007). Therefore, researchers chose 48 h as the forage processing time.

Lactic acid bacteria number per gram in wilted alfalfa forage, forage treated with UV rays and commercial LAB inoculums was 9.0×10^8 (not listed), 500 and 1.04×10^{12} (not listed), respectively so researchers added about 0.045 g commercial LAB inoculums in every 50 g forage.

Fermentation characteristics: After 35 days fermentation, UV ray+LAB treated silages had lower pH values, lower propionic acid and butyric acid concentrations and higher lactic concentrations than the control ($p<0.05$) which indicated that they fermented better than the control (Table 2). Silages treated with UV rays and with a small number epiphytic microorganisms had the highest pH value (Table 2); silages treated with UV ray+LAB had the lowest ($p<0.05$) pH value throughout the entire fermentation (Table 3). The results indicated the effects of exogenous microorganisms on fermentation characteristics of ensilaged alfalfa.

Table 2: Fermentation characteristics of alfalfa silage on day 35 of fermentation

	DM[e] g kg^{-1}		g kg^{-1} DM			
Treatment	FM	pH	LA[f]	AA	PA	BA
Control	362.07^{a}	5.41^{b}	23.89^{b}	7.72^{ab}	28.46^{a}	6.19^{a}
UV ray[a]	345.02^{c}	5.57^{a}	19.33^{c}	6.90^{ab}	14.69^{ab}	3.61^{b}
UV ray+LAB[b]	353.86^{b}	4.67^{c}	31.13^{a}	10.60^{a}	3.77^{b}	3.34^{b}
SEM[c]	14.90	0.81	5.75	1.97	6.67	2.53
Sig[d]	**	*	**	*	*	NS

Means within columns not sharing a common letter differ ($p<0.05$), $^{*}p<0.05$, $^{**}p<0.01$; NS: Not Setectable; [a]UV ray: Ultraviolet ray; [b]LAB: Lactic Acid Bacteria; [c]SEM: Standard Error of Means; [d]Sig: Significance; [e]DM: Dry Matter; FW: Fresh Weight; [f]LA: Lactic Acid; AA: Acetic Acid; PA: Propionic Acid; BA: Butyric Acid

Table 3: Effects of treatment and time of ensiling on fermentation characteristics and the protein fractions of alfalfa silage

	DMg mg g^{-1}		CP[h] mg g^{-1}	Composition of total N (mg g^{-1} total N)			
Parameters	FM	pH	DM	NPN[i]	AA-N	NH_3-N	Peptide-N
Treatments							
Control	371.23^{a}	5.84^{ab}	272.13	566.74^{a}	72.20^{a}	7.58^{a}	486.96^{a}
UV ray[a]	342.41^{b}	6.10^{a}	269.01	511.19^{b}	59.72^{b}	2.98^{b}	448.49^{b}
UV ray+LAB[b]	349.50^{b}	5.61^{b}	273.31	497.41^{b}	58.47^{b}	1.91^{c}	437.03^{b}
SEM[c]	5.03	0.21	3.25	9.54	4.35	0.18	10.91
Time (days of ensiling)							
0	354.72^{a}	6.18^{a}	286.88^{a}	200.52^{c}	29.76^{c}	0.00^{d}	170.76^{b}
1	355.27^{ab}	5.68^{ab}	284.83^{a}	512.93^{b}	45.92^{b}	0.00^{d}	467.01^{a}
3	352.99^{ab}	5.76^{ab}	271.97^{b}	511.61^{b}	68.65^{a}	0.23^{d}	442.72^{a}
7	354.32^{ab}	5.71^{ab}	263.25^{c}	507.29^{b}	67.12^{a}	1.17^{c}	439.00^{a}
14	344.39^{b}	5.44^{b}	256.91^{c}	521.54^{ab}	58.77^{a}	4.93^{b}	457.84^{a}
35	348.01^{b}	5.29^{b}	248.01^{d}	540.10^{a}	64.13^{a}	13.47^{a}	462.51^{a}
SEM	6.16	0.27	3.99	11.69	5.33	0.22	13.36
Sig[d]							
Treatment	**	**	**	**	**	**	**
L[e]	**	*	NS	**	**	**	**
Q	**	NS	**	**	**	**	**
C	**	**	**	**	**	NS	**
Time (T)	*	*	**	**	**	**	**
L	*	**	**	**	**	**	**
Q	NS	NS	NS	**	*	**	**
C	NS	**	NS	**	NS	**	**
A*T[f]	**	NS	**	**	**	**	**

Means within columns not sharing a common letter differ ($p<0.05$), $^{*}p<0.05$, $^{**}p<0.01$; NS: Not Significant; [a]UV ray: Ultraviolet ray; [b]LAB: Lactic Acid Bacteria; [c]SEM: Standard Error of Means; [d]Sig: Significance; [e]L: Linear; Q: Quadratic; C: Cubic; [f]A×T: Interaction between treatment and time; [g]DM: Dry Matter; [h]CP: Crude Protein; [i]NPN: Non-Protein Nitrogen; AA-N: Free Amino Acid Nitrogen; NH_3-N: Ammonia Nitrogen; Peptide-N: Peptide Nitrogen

Nitrogen distribution: The contents of NPN, AA-N, NH_3-N and peptide-N were affected ($p<0.05$) by treatment and ensiling time and there was an additive x ensiling time interaction (Table 3). Control silages had higher NPN, AA-N, NH_3-N and peptide-N content than silages treated with UV rays throughout the entire ensiling (Table 3-7). A decrease in the content of CP occurred after ensiling for 3 days ($p<0.05$).

The rate of NPN increase during the initial days was much faster than during the subsequent days and the control silage had a higher NPN content than treated silages during the ensiling process ($p<0.05$, Table 3 and 4). The NH_3-N concentration in the TN increased after

Table 4: Changes of NPN in alfalfa silages (mg g^{-1} total N)

	Days					
Treatment	0	1	3	7	14	35
Control	200.52	631.04[a]	628.38[a]	625.40[a]	633.82[a]	681.30[a]
UV ray[a]	200.52	559.72[b]	572.54[b]	563.35[b]	589.19[ab]	581.80[b]
UV ray+LAB[b]	200.52	576.71[b]	558.10[b]	544.21[b]	559.63[b]	545.30[c]
SEM[c]	4.53	33.59	11.01	37.55	20.14	13.89
Sig[d]	NS	**	**	**	**	**

Table 5: Changes of AA-N in alfalfa silages (mg g^{-1} total N)

	Days					
Treatment	0	1	3	7	14	35
Control	29.76	65.39[a]	92.52[a]	78.82[ab]	82.01[a]	84.71[a]
UV ray[a]	29.76	36.85[b]	91.52[a]	86.81[a]	48.87[c]	64.49[c]
UV ray+LAB[b]	29.76	46.93[b]	48.81[b]	68.95[b]	77.66[b]	78.71[b]
SEM[c]	0.56	15.64	19.60	13.72	8.50	8.88
Sig[d]	NS	NS	NS	*	**	**

Table 6: Changes of NH_3-N in alfalfa silages (mg g^{-1} total N)

	Days					
Treatment	0	1	3	7	14	35
Control	0.00	0.00	0.41[a]	2.80[a]	13.98[a]	28.31[a]
UV ray[a]	0.00	0.00	0.03[c]	0.75[b]	3.30[b]	13.78[b]
UV ray+LAB[b]	0.00	0.00	0.30[ab]	0.59[b]	1.58[c]	9.01[c]
SEM[c]	-	-	0.07	0.22	0.32	1.00
Sig[d]	-	-	**	**	**	**

Table 7: Changes of Peptide-N in alfalfa silages (mg g^{-1} total N)

	Days					
Treatment	0	1	3	7	14	35
Control	170.76	565.65[a]	535.45[a]	543.78[a]	537.83[a]	568.28[a]
UV ray[a]	170.76	522.87[b]	480.99[b]	475.78[b]	537.02[a]	503.52[b]
UV ray+LAB[b]	170.76	529.78[b]	508.99[ab]	474.67[b]	480.39[b]	457.58[c]
SEM[c]	4.90	32.55	21.63	45.11	20.53	16.66
Sig[d]	NS	**	**	**	**	**

Means within columns not sharing a common letter differ ($p<0.05$), $^{*}p<0.05$, $^{**}p<0.01$; [a]UV ray: Ultraviolet ray; [b]LAB: Lactic Acid Bacteria; [c]SEM: Standard Error of Means; [d]Sig: Significance; NS: Not Significant

ensiling for 3 days (Table 3). The changes in the peptide-N concentration (Table 2) during the period of ensiling were similar to those of the AA-N displaying a rapid increase in the early days of ensiling and a sustained increase in later fermentation.

DISCUSSION

Effect of epiphytic microorganisms on the formation of NPN constituents: UV rays have been used extensively for water and food sterilizing (Gibbs, 2000; McCarty and Scanion, 1993; Stother, 1999; Honer, 1988) and the use of UV rays is a disinfection method that can be applied to inactivate microbes (Tran and Farid, 2004). The peak ultraviolet absorption efficiency for DNA lies between 250 and 280 nm. The UV rays at this germicidal wavelength alter the genetic material in cells so that bacteria, viruses, moulds and other microorganisms can no longer reproduce and may be considered inactive (Billmeyer, 1997; Giese, 1997).

In the current study, after 48 h of treatment with UV rays, the lactic acid bacteria number decreased ($p<0.05$) with the extension of illumination time (Table 1). The reason for this may be that the membrane function of lactic acid bacteria was damaged by near UV radiation (Ito and Ito, 1983; Arami *et al*., 1997). The LA content of UV ray treated silage on day 35 was lower ($p<0.05$, Table 1) and the pH value was higher than in the control silage which could verify this viewpoint. However, the contents of acetic acid and propionic acid showed no difference compared with the control which may be because some other microorganisms have resistance against UV radiation (Takeshitaa *et al*., 2003).

Control silages had higher NPN, AA-N, NH_3-N and peptide-N content than silages treated with UV rays throughout the entire ensiling (Table 3) and the contents of NPN, AA-N, NH_3-N and peptide-N in the silage treated with UV radiation were 85, 76, 49 and 89%, respectively of those in the control silage at day 35 which showed that epiphytic microorganisms could affect the formation of protein (Table 3-6). The reason for this may be that most herbage proteins are degraded by plant protease and microbial protease during the process of ensiling (Ohshima and McDonald, 1977) but after 48 h of treatment with UV rays, most of the microbial membrane function was damaged by near UV radiation (Ito and Ito, 1983; Arami *et al*., 1997). NH_3-N content in silages treated with UV rays was 51% of that in control which suggested that NH_3-N was largely an end product of microbial activity. A similar result in ensiled ryegrass was reported by Heron *et al*. (1988).

Effect of exogenous LAB on the formation of NPN constituents: Extensive studies have been conducted to use the inoculants of LAB to create the acidic environment as quickly as possible to improve the fermentation quality of legumes (Harrison *et al*., 1989; McAllister *et al*., 1998; Schmidt *et al*., 2009). Because the additive LaLsIL Dry used in the study contained exogenous lactobacillus, inoculation with these strains probably resulted in propagation of lactic acid bacteria to inhibit the growth of clostridia and aerobic bacteria which improved the silage quality (Wang *et al*., 2009).

In the study, researchers added almost the same amount of lactobacillus as the death quantity after illumination by UV rays. However, UV ray+LAB treated silage still had lower pH and lactic acid content, as well as propionic acid and butyric acid content, at day 35 of ensiling ($p<0.05$, Table 1). In addition, exogenous lactobacillus had a lower pH value throughout the entire fermentation compared with the control (Table 3) which is consistent with Wang *et al*. (2009). The reasons may be:

Exogenous lactobacillus contained in the LaLsIL Dry had a better effect on improving silage quality than epiphytic lactobacillus and the UV rays produced some microorganisms which had an adverse effect on fermentation quality. Silages treated with UV ray+LAB showed reduced concentrations of NPN, NH_3-N and AA-N compared to the control alfalfa silage at every phase of ensiling (Table 3-5) and contents of NPN, AA-N, NH_3-N and peptide-N in the silage treated with UV ray+LAB were decreased by 20, 7, 68 and 19%, respectively compared with the control silage on day 35 (Table 3-6). The results may suggest that exogenous lactobacillus had a better effect than epiphytic lactobacillus on saving protein components. The proportion of peptide-N increased rapidly at the beginning of ensiling, followed by a decline (Table 6). This change was similar to that observed by Muck (1987) and Fairbairn suggests that extensive proteolysis exceeded the degradation of AA-N at early stages and that the later decrease may be due to utilization of peptides by the epiphytic lactobacillus. However because the components of LaLsIL Dry were not single lactobacillus because the other components may have affected the formation of alfalfa silage and because the UV rays could not kill all the microorganisms, more accurate experiments were necessary to clarify the effect of microbes on protein degradation of alfalfa silage.

CONCLUSION

Results showed that after 35 days fermentation, UV ray+LAB treated silages had lower pH values, lower propionic acid and butyric acid concentrations and higher lactic concentrations than the control; Control silages had higher NPN, AA-N, NH_3-N and peptide-N content than silages treated with UV rays throughout the entire ensiling which showed that epiphytic microorganisms could affect the formation of protein; silages treated with UV ray+LAB showed reduced concentrations of NPN, NH_3-N and AA-N compared to the control alfalfa silage at every phase of ensiling which may suggest that exogenous lactobacillus had a better effect than epiphytic lactobacillus on saving protein components.

ACKNOWLEDGEMENTS

Researchers Lian Tao and Menglu Li have equally contributed to this study. Financial supports from the National Natural Science Foundation of China (30800800) and the Ph.D. Programs Foundation of Ministry of Education of China (200807301008) are gratefully acknowledged.

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