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ANIMAL PRODUCTION

Dynamics of change in fermentation and fatty acid profiles in high moisture alfalfa silage during ensiling at different temperatures

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ABSTRACT: *The objective of present study was to investigate the dynamics of change in fermentation and fatty acid profiles in high moisture (DM=232g/kg FW) alfalfa silages during ensiling at 45°C, 30°C and 15°C. After ensiling for 1, 3, 7, 21, 39 and 65 days, silages was sampled and examined. Lactic fermentation changing into butyric fermentation in silage ensiled at 30°C and 45°C occurred on ensiling for 21 and 65 days, respectively, as accompanied with high ammonia-N content (>150g/kg N), which subsequently resulted in a sudden increase of pH (P < 0.05). In contrast, the increase of lactic acid content was observed in silage ensiled at 15°C during ensiling for 65 days (P<0.05). As the ensiling temperature rose, considerable losses in total FA, C18:2n6 and C18:3n3 increased after ensiling for 1 day as compared with alfalfa before ensiling (P < 0.05) mainly due to thermolability of polyunsaturated FA and multiplication of lactic acid bacteria. Contents of total FA, C18:2n-6 and C18:3n-3 fluctuated in silage stored at 30°C and 45°C during ensiling from 3 to 65 days (P < 0.05), but decreased at 15°C due to the intervention of aerobic bacteria, yeasts and lipoxygenase. Therefore, after ensiling for 65 days, high moisture alfalfa silage ensiling at 15°C had better fermentation quality than at 30°C and 45°C. Temperature had significant influence on dynamics of change in FA profile in alfalfa silage during ensiling.*
Key words: *silage, fermentation quality, fatty acid, temperature.*

A dinâmica de mudança na fermentação e perfis de ácidos graxos em silagem de alfafa de alta humanidade durante a ensilagem a diferentes temperaturas

RESUMO: *O objetivo do presente estudo foi investigar a dinâmica de mudança na fermentação e perfis de ácidos graxos em silagens de alfafa de alta humidade durante a ensilagem a 45°C, 30°C e 15°C. Após a ensilagem por 1, 3, 7, 21, 39 e 65 dias, as amostras foram amostradas e examinadas. A fermentação láctica mudou para a fermentação butírica em silagens ensiladas a 30°C e 45°C. Estas ocorreram em ensilamento durante 21 e 65 dias, respectivamente, acompanhado de alto teor de amônia-N (>150g/kg N), o que resultou em um aumento súbito de pH (P < 0,05). Em contraste, o aumento do teor de ácido láctico foi observado na silagem ensilada a 15°C durante a ensilagem durante 65 dias (P < 0,05). À medida que a temperatura de ensilagem aumentou, as perdas consideráveis na FA total, C18: 2n6 e C18: 3n3, aumentaram após a ensilagem durante 1 dia, em comparação com a alfafa antes da ensilagem (P < 0,05), principalmente devido à termolabilidade da FA poliinsaturada e à multiplicação de bactérias do ácido lático. Os conteúdos de FA total, C18: 2n-6 e C18: 3n-3, foram flutuação na silagem armazenada a 30°C e 45°C durante a ensilagem de 3 a 65 dias (P < 0,05), mas diminuiu a 15°C devido à intervenção de bactérias aeróbias, leveduras e lipoxigenase.*
Palavras-chave: *silagem, qualidade de fermentação, ácido gordo, temperatura.*

INTRODUCTION

Fatty acids (FA) in fresh forage is dominated by a high proportion of linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3) (CLAPHAM et al., 2005). High intake ration containing sufficient fresh forage can increase the concentration of C18:2n-6 and C18:3n-3 in ruminant products and consequently be beneficial to human health (SIMOPOULOS, 2001). Fresh forage silage is increasingly fed to ruminant in many regions of the world. FA in silages, mainly dynamics of change in C18:2n-6 and C18:3n-3, has gained high attention during ensiling (ALVES et al., 2011).

Previous studies have reported the increase or reduction in FA of silage as compared with fresh forage (BOUFAIED et al., 2003; ELGERSMA et al., 2003; ARVIDSSON et al., 2009; VAN RANST et al., 2009; ALVES et al., 2011). However, the real reason is not completely known. One explanation is that plant enzymes and microbes play role in FA change during ensiling (ELGERSMA et al., 2003; DING et al., 2013). Plant lipases and lipoxygenases are the

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main enzymes responsible for change of FA because plant lipases release FA from damaged tissues after cutting and followed ensiling (CHOW et al., 2004) and plant lipoxygenases oxidize FA (FEUSSNER & WASTERNACK, 2002). Activity of enzymes and microbes is influenced by temperature (MCDONALD et al., 1991; LIU et al., 2016). However, until now few publications are related to plant enzymes and microbes in alfalfa silage ensiled at different temperatures, which may provide important information for further regulation of fermentation and FA.

The purpose of this study was to investigate dynamics of change in fermentation and FA profiles in high moisture alfalfa silage during ensiling at 15°C, 30°C and 45°C for 65 days, and the activity of lipase and lipoxygenase at different pH values and temperatures in a simulative ensiling system.

MATERIALS AND METHODS

Silage material and silage making

Alfalfa (*Medicago sativa cv.* Jili) was planted on September 25, 2014, in a field of Nanjing Agricultural University (Nanjing, China). At the early flowering stage on April 15, 2015, alfalfa (DM=232 g/kg FW) was harvested for making silage. Alfalfa was chopped into 1 to 2 cm-long pieces by a forage chopper (Sh-2000, Shanghai Donxe Industrial Co., Ltd., Shanghai, China).

An experiment on alfalfa silage ensiled at 3 temperatures (15°C, 30°C and 45°C) × 7 ensiling times (0, 1, 3, 7, 21, 39 and 65 days) × 4 replicates was designed. Chopped fresh alfalfa was mixed well and subdivided into 72 smaller batches. The weight of each batch was 750g, with each batch corresponding to one plastic laboratory silo (1000mL capacity). The silo was filled with a batch and sealed with a screw top and plastic tape. Three incubators were used to achieve the ensiling temperatures of 15°C, 30°C and 45°C. Subsequently, randomly selected 24 silos were kept in the corresponding incubator. After ensiling for 1, 3, 7, 21, 39 and 65 days, randomly selected 4 silos in each incubator were opened.

Microbial and chemical analyses

The microorganism numbers in the fresh materials and silages were determined by the plate count method (DING et al., 2013). Ten grams of the fresh alfalfa was shaken well with 90mL of sterilized saline solution (8.50g/L NaCl) and serial dilutions (10^{-1} through 10^{-7}) were made in sterile saline solution. Lactic acid bacteria (LAB) was counted on deMan Rogosa and Sharp agar medium (Difco Laboratories, Detroit, MI, USA) after incubation in an anaerobic incubator (N_2: H_2: CO_2 = 85:5:10, YQX-II，CIMO Medical Instrument Manufacturing Co., Ltd, Shanghai, China) at 37°C for 2 days. Aerobic bacteria were cultured and counted on nutrient agar medium (Guangdong Huankai Microbial Science and Technology Co., Ltd., Guangzhou, China), yeasts were counted on potato dextrose agar (Guangdong Huankai Microbial Science and Technology Co., Ltd., Guangzhou, China) acidified with a sterilized tartaric acid solution to pH 3.5. The agar plates were incubated at 37°C for 2 days. All microbial data were transformed to $\log_{10}$ and presented on a wet weight basis.

Fifty grams of silage was taken after ensiling, mixed with 200mL of distilled water, and stored at 4°C for 18 hours. The mixture was then filtered, and the filtrate was used for measuring pH value using a glass electrode pH meter (HI221, Hanna Ltd., Rome, Italy). The buffering capacity, contents of dry matter (DM), water-soluble carbohydrates (WSC), crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF) of alfalfa material or silage was determined using the same methods in our previous study (LIU et al., 2016). After ensiling for 1, 3, 7, 21, 39 and 65 days, silos were opened and the silages were mixed thoroughly. Silages were sampled, and DM and microbes of silage were measured by the same method with the fresh alfalfa. The filtrate of silage was used for measuring pH value, contents of ammonia-N and organic acid. Contents of ammonia-N and organic acids (lactic acid, acetic acid, propionic acid and butyric acid) were determined by the same procedures in our previous study (LIU et al., 2016).

FA analysis

Lipids were extracted using a slightly modified version of the method described by Folch et al. (1957). Briefly, the 1 g frozen dried sample was added with 5mL preheated isopropanol in a glass tube, heated at 75°C for 15min, and then cooled to room temperature. Glass tube was added with 3mL chloroform and 1mL water and was incubated with shaking for 60 minutes. Liquid extract was transferred to a fresh tube, extract tissue was added with 4.5mL chloroform: methanol (2:1 v/v) two times until tissues are grayish white. All extracts were combined and added 2mL 1mol/L KCl. After mixing, the extracts were centrifuged at 1820g at 16°C, and 16mL lower phase was obtained. After concentrated by Termovap sample concentrator (MD200-2, Allsheng Instruments Co., Ltd., Hangzhou, China) at 45°C, the 2mL sample was added 2mg of nonadecanoic acid (C19:0; Sigma,

Shanghai, China) as internal standard and 5mL of 2.5% H2SO4 (v/v) in methanol, and then heated at 80°C for 60 minutes to methyl esterification. A 1.5mL of heptane was added and followed by 1mL 0.9% NaCl (w/v) to extract fatty acids methyl esters (FAME).

FAME was analyzed on Agilent 7890A gas chromatograph (Agilent Technologies Inc., München, Germany) with a capillary column HP-88 (100m × 0.25mm i.d. ×0.2μm, Agilent Technologies Inc., Shanghai, China). The temperature program was used: 150°C for 2min, followed by an increase at a rate of 0.8°C/min until 220°C. Temperatures of the injector and detector were 250°C, respectively. A FAME mixture obtained from Sigma (Supelco 37 component, Supelco Inc. Bellefonte, PA, USA) was used as a standard to quantify individual FA.

Activity of lipase and lipoxygenase analyses in simulative ensiling system

Reagents were prepared using lactic acid, acetic acid, butyric acid and ammonium oxalate for simulating ensiling system with diffent pHs according to the fermentation profiles of silage (Table 1). The activities of lipase and lipoxygenase in fresh leaves of alfalfa and at diffent reagents and temperatures were analyzed by Plant Lipase and Lipoxygenase Activity Kit (Shanghai Cablebridge Biotechnology Co., Ltd., Shanghai, China) according to the method in MALEKIAN et al. (2000).

Statistical analyses

The statistical analyses were performed using the IBM Statistical Packages for the Social Sciences (IBM SPSS 20.0 for Windows). Repeated measures analysis of variance (General Linear Models) (3 temperatures × 6 ensiling times × 4 replicates) were used to evaluate effects of temperatures, ensiling time and their interactions on the fermentation characteristics and microbial compositions in high moisture alfalfa silages. Repeated measures analysis of variance (General Linear Models) (3 temperatures × 7 ensiling times × 4 replicates) were used to evaluate effects of temperatures, ensiling time and their interactions on FA profile in high moisture alfalfa silages. The data were analyzed by two-way ANOVA (4 pHs ×3 temperatures ×3 replicates) to evaluate effects of pH, temperatures and their interactions on the activity of lipase and lipoxygenase, respectively. The data were analyzed by one-way ANOVA (3 temperatures ×4 replicates) to evaluate of temperatures on chemical composition of alfalfa silage after ensiling for 65 days. The means were then compared for significance using Tukey's test at $P < 0.05$.

RESULTS AND DISCUSSION

Characteristics of alfalfa before ensiling

The present study showed that alfalfa had low contents of DM (232g/kg FW) and WSC (50.8g/kg DM), and had high crude protein content (180g/kg DM), NDF (404g/kg DM) and ADF (317g/kg DM) content and buffering capacity (226mEq/kg DM). Epiphytic LAB (5.92$\log_{10}$ cfu/g FM) was less than aerobic bacteria (6.68$\log_{10}$ cfu/g FM) and yeasts (6.71$\log_{10}$ cfu/g FM). In addition, the activity of lipase (1.05U/100mg) in fresh alfalfa was lower than lipoxygenase (16.6U/100mg).

Effect of temperature on dynamic of change in fermentation characteristics

Lactic fermentation changing into butyric fermentation in silage stored at 30°C and 45°C occurred on ensiling for 21 and 65 days, respectively, as accompanied with high ammonia-N content (>150g/kg N) (Table 2), and resulted in a sudden increase in pH of silage ($P < 0.05$). This indicated protein degradation and poor fermentation quality. In contrast, the increase of lactic acid content was observed in silage ensiled at 15°C during ensiling for 65 days ($P < 0.05$). This can be attributed to the eco-physiological properties of variational microflora in alfalfa ensiling at different temperatures and stages, supported by the LAB number, pH, contents of lactic acid, butyric acid and ammonia-N were influenced

Table 1 - Reagents with different pH values.

Ingredients	pH			
	4.5	5.0	5.5	6.0
Lactic acid aqueous solution 85:100 (w/v, μL)	15	5	2.5	2.5
Acetic acid aqueous solution 99.5:100 (w/v, μL)	35	25	10	2.5
Butyric acid aqueous solution 98:100 (w/v, μL)	20	50	100	0
Ammonium oxalate aqueous solution 6.21:100 (w/v, mL)	5.5	8	9	1
Distilled water (mL)	14.43	11.92	10.90	19.00

Table 2 - Effects of temperature on dynamics of change in fermentation characteristics and microbal composition of alfalfa silage during ensiling for 65 days.

Items [a]	Tem	----------ET (d)----------						Means of Tem	SEM	----------Significance----------		
		1	3	7	21	39	65			Tem	ET	Tem×ET
pH	45°C	5.92	4.94	4.95	4.88	4.83	5.81	5.22	0.056	0.001	<0.001	<0.001
	30°C	6.05	5.57	5.29	5.70	5.73	5.45	5.63				
	15°C	6.28	6.24	5.87	5.75	5.56	5.39	5.85				
Means of ET		6.08	5.58	5.37	5.44	5.37	5.55					
Lactic acid (g/kg DM)	45°C	3.43	22. 7	16.5	23.8	25.6	8.35	15.5	0.603	<0.001	<0.001	<0.001
	30°C	13.1	22.3	25.0	0.76	0.52	2.20	10.7				
	15°C	0.56	9.87	13.7	10.5	12.7	14.2	10.3				
Means of ET		5.70	16.1	18.4	11.7	12.9	8.26					
Acetic acid (g/kg DM)	45°C	1.06	3.50	3.66	3.61	5.71	20.5	6.35 c	1.371	<0.001	<0.001	0.100
	30°C	11.2	17.8	24.7	32.1	25.6	53.9	27.6 a				
	15°C	2.87	10.1	13.7	17.8	28.9	36.3	18.3 b				
Means of ET		5.04D	10.5CD	14.0BC	17.8BC	20.1B	36.9A					
Propionic acid (g/kg DM)	45°C	0.13	0.40	0.27	0.30	0.40	4.33	0.97	0.611	0.002	<0.001	0.010
	30°C	0.43	0.23	1.50	6.50	10.6	15.1	5.73				
	15°C	0.13	0.27	0.33	0.07	0.40	1.70	0.48				
Means of ET		0.23	0.30	0.70	2.29	3.80	7.04					
Butyric acid (g/kg DM)	45°C	0.30	1.80	0.93	3.90	4.83	20.4	5.36	1.165	<0.001	<0.001	<0.001
	30°C	0.83	1.03	1.37	30.9	36.4	38.9	18.2				
	15°C	0.07	0.20	0.80	0.37	0.01	1.30	0.46				
Means of ET		0.40	1.01	1.03	11.7	13.8	20.2					
Ammonia-N (g/kg N)	45°C	63.4	86.9	72.3	79.8	106	240	108	8.056	0.006	<0.001	<0.001
	30°C	88.2	154	145	197	192	197	162				
	15°C	46.4	109	98.7	133	163	128	113				
Means of ET		66.0	117	105	137	154	188					
LAB (log_{10} cfu/FM)	45°C	8.37	6.66	7.03	5.22	4.89	5.48	6.28	0.050	<0.001	0.001	<0.001
	30°C	7.94	7.70	7.96	7.51	7.60	6.95	7.61				
	15°C	7.54	7.44	7.58	8.25	7.72	8.25	7.80				
Means of ET		7.95	7.27	7.52	6.99	6.74	6.89					
Aerobic bacteria (log_{10} cfu/FM)	45°C	6.20	5.10	4.30	3.32	3.11	2.11	4.02c	0.076	<0.001	<0.001	0.071
	30°C	7.68	6.52	5.58	5.22	4.11	4.15	5.54b				
	15°C	8.50	7.20	6.35	5.39	5.48	5.61	6.42a				
Means of ET		7.46A	6.27B	5.41C	4.64D	4.23DE	3.96E					
Yeasts (log_{10} cfu/FM)	45°C	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	2.00	0.086	<0.001	0.008	0.002
	30°C	4.38	2.00	2.91	<2.00	<2.00	<2.00	2.55				
	15°C	3.78	3.62	2.79	4.92	2.45	3.55	3.52				
Means of ET		3.39	2.54	2.57	2.97	2.15	2.52					

Means with different lowercase letters in the same column (a–c) or capital letters (A-E) in the same row indicated a significant difference according to Tukey's test at $P < 0.05$. [a]cfu, colony-forming units; DM, dry matter; ET, ensiling time; FM, fresh matter; LAB, lactic acid bacteria; N, nitrogen; SEM, standard error of the means; Tem, temperature; Tem×ET, the interaction of temperature and ensiling time.

by the interaction of temperature and ensiling time ($P < 0.05$) (Table 3). CAO et al. (2011) and WANG & NISHINO (2013) reported that prolonging storage time of silage tended to decrease pH and to increase lactic acid content and amount of LAB at low temperature. Most clostridia species are mesophilic bacteria and vigorous butyric fermentation is often found in the middle and later stages of ensiling (MCDONALD et al., 1991). LIU et al. (2011) reported that the vigorous butyric fermentation with high pH and ammonia-N content was at 30°C after ensiling for 45 days. Butyric fermentation is sometimes associated with high acetic acid production because of proteolytic clostridia producing ammonia-N, acetic acid and butyric acid from peptides and amino acids (PAHLOW et al., 2003). This was confirmed in the present study which

showed sudden increases in contents of acetic acid and ammonia-N ($P < 0.05$) when silage ensiled at 30°C and 45°C separately occurred vigorous butyric fermentation. However, the acetic acid content of silage ensiled at 15°C, with weak butyric fermentation, still increased as prolonging the ensiling time ($P < 0.05$). Especially, acetic acid content was 2.6 times higher than lactic acid content after ensiling for 65 days and is accompanied with high ammonia-N (>120g/kg N). Based on the present study, compared with at 45°C and 30°C, higher LAB and aerobic bacteria number might be responsible for acetic fermentation and ammonia-N formation at 15°C ($P < 0.05$). Some researchers proposed that: facultatively heterofermentative LAB and enterobacteria were contributors for acetic fermentation and ammonia-N formation, *e.g., Lactobacillus plantarum* can deaminate serine to produce acetic acid and ammonia-N (LIU et al., 2003; PARVIN & NISHINO, 2009); Alfalfa and ryegrass silage with many *Hafnia alvei* was vigorous acetic fermentation and had high ammonia-N content (>100g/kg N) (MCDONALD et al., 1991).

Effect of temperature on chemical composition of final silage

Silage ensiled at 15°C had higher CP content ($P < 0.05$) and lower contents of NDF ($P < 0.05$ or $0.05 < P < 0.1$) and ADF ($P < 0.05$) than at 30°C and 45°C, since fermentation qualities of silage at 30°C and 45°C were poorer than at 15°C (Figure 1). Similar with previous studies (MCDONALD et al., 1991; LIU et al., 2012), clostridia degraded most nutrients and leave cell wall residue in poor quality silage.

Effect of temperature on dynamic of change in FA profile

As shown in table 3, C16:0, C18:2n6 and C18:3n3 were the main FA composition in fresh alfalfa. Temperature influenced contents of total FA, C18:2n6 and C18:3n3 in silage during ensiling ($P < 0.05$). Compared with alfalfa before ensiling, there was a considerable loss of total FA content in silage after ensiling 65 days ($P < 0.05$). This was attributed to a fact that C18:2n-6 and C18:3n-3 lipolysis mainly caused the loss of FA despite there was an increase in C16:0 content during ensiling at any temperatures ($P<0.05$), which was similar to the results in previous studies (DING et al., 2013; KE et al., 2015). HAN & ZHOU (2013) found that the C18:2n-6 and C18:3n-3 losses in silages were mainly due to the activity of lipoxygenase. Further results showed that activity of lipoxygenase responsible for FA lipolysis was higher than lipase responsible for FA formation at each pH and temperature in the simulative ensiling system ($P < 0.05$) (Figure 2).

As the ensiling temperature rose, losses in contents of total FA, C18:2n6 and C18:3n3 increased

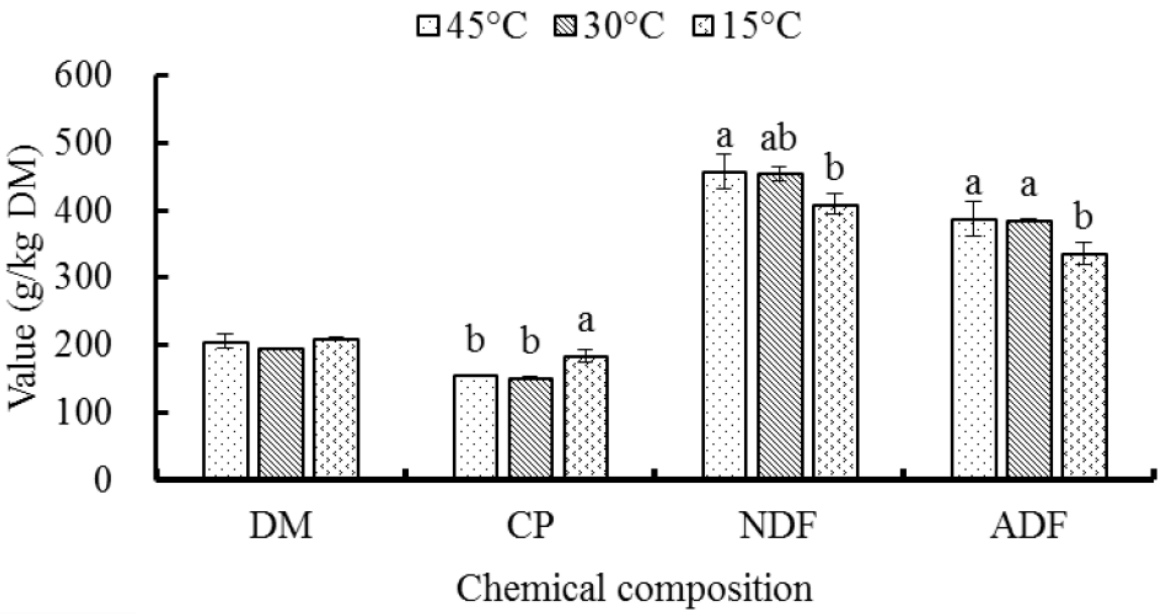

Figure 1 - Effect of temperatures on chemical composition of alfalfa silage after ensiling for 65 days. Means with different lowercase letters above the column (a-d) indicated a significant difference according to Tukey's test at $P < 0.05$.

Table 3 - Effects of temperature on dynamics of change in the contents (g/kg DM) of total fatty acid and detected fatty acids during ensiling for 65 days.

Items[a]	Tem	ET (d)							Means of Tem	SEM	Significance		
		0	1	3	7	21	39	65			Tem	ET	Tem×ET
Total FA	45°C	33.8	20.1	23.9	20.7	22.8	23.9	23.1	24.0 b	0.616	0.007	<0.001	0.090
	30°C	33.8	25.0	26.3	27.7	25.7	30.7	26.2	27.9 a				
	15°C	33.8	31.1	29.3	26.8	27.4	25.4	20.3	27.7 a				
Means of ET		33.8A	25.4B	26.5B	25.1B	25.3B	26.7B	23.2C					
C16:0	45°C	7.35	6.25	6.00	5.12	5.63	6.03	8.17	6.36	0.199	<0.001	0.028	<0.001
	30°C	7.35	9.30	9.73	9.30	11.10	10.13	8.77	9.38				
	15°C	7.35	8.33	9.20	9.60	8.87	10.67	8.20	8.89				
Means of ET		7.35	7.96	8.31	8.01	8.53	8.94	8.38					
C16:1	45°C	0.70	0.22	0.42	0.34	0.32	0.37	0.57	0.42 b	0.042	0.024	0.305	0.403
	30°C	0.70	0.63	0.63	0.70	0.50	0.73	0.53	0.63 a				
	15°C	0.70	0.63	0.67	0.37	0.80	0.53	0.53	0.60 a				
Means of ET		0.70	0.49	0.57	0.47	0.54	0.55	0.54					
C18:0	45°C	1.66	1.39	1.31	1.17	1.22	1.34	1.47	1.37	0.032	0.002	0.081	0.044
	30°C	1.66	1.47	1.50	1.70	1.47	1.80	1.37	1.57				
	15°C	1.66	1.60	1.67	1.57	1.87	1.77	1.40	1.65				
Means of ET		1.66	1.48	1.49	1.48	1.52	1.64	1.41					
C18:1	45°C	2.16	0.77	0.88	0.69	0.82	0.80	1.03	1.02 b	0.084	0.014	0.001	0.723
	30°C	2.16	2.00	1.43	1.47	1.43	1.30	1.00	1.54 a				
	15°C	2.16	1.00	1.17	1.20	0.87	1.43	0.90	1.25 ab				
Means of ET		2.16A	1.26B	1.16B	1.12B	1.04B	1.18B	0.98BC					
C18:2n6	45°C	5.99	4.87	5.66	5.11	5.50	5.65	4.67	5.35 b	0.125	0.007	0.057	0.186
	30°C	5.99	5.43	6.27	6.30	5.60	6.40	5.40	5.91 a				
	15°C	5.99	7.80	6.50	7.17	6.10	5.90	4.13	6.22 a				
Means of ET		5.99	6.04	6.14	6.19	5.73	5.98	4.73					
C18:3n3	45°C	15.9	6.57	9.65	8.22	9.36	9.68	7.13	9.50a	0.334	0.049	<0.001	0.056
	30°C	15.9	8.07	7.60	8.20	8.27	9.67	9.80	9.64a				
	15°C	15.9	9.77	9.30	6.90	6.10	5.70	4.13	8.26b				
Means of ET		15.9A	8.13B	8.85B	7.77B	7.91B	8.35B	7.02C					

Means with different lowercase letters in the same column (a–b) or capital letters (A-C) in the same row indicated a significant difference according to Tukey's test at $P < 0.05$. Total fatty acids content was summed by each detected fatty acid. [a] ET, ensiling time; FA, fatty acid; SEM, standard error of the means. Tem, temperature; Tem×ET, the interaction of temperature and ensiling time.

after ensiling for 1 day as compared with alfalfa before ensiling ($P < 0.05$) (Table 3). This could be attributed to the fact that multiplication of epiphytic LAB was promoted at a high ensiling temperature at the initial stage of ensiling, supported by higher LAB number (8.37log10 cfu/g FM) at 45°C than at 30°C and 15°C on ensiling for 1 day ($P < 0.05$). As the temperature rose in the simulative ensiling system, the activity of lipoxygenase decreased ($P < 0.05$) (Figure 2), but the total FA, C18:2n-6 and C18:3n-3 lipolysis in silage was not restrained after ensiling for one day. This indicated that intervention of lipoxygenase was few in FA lipolysis at 45°C. Actually, epiphytic LAB have the ability to bio-hydrogenate C18:2n-6 and C18:3n-3 (OGAWA et al., 2005; KISHINO et al., 2009), and their activity of biohydrogenation could be enhanced by increasing temperature (TAKEUCHI et al., 2015). KUMARATHASAN et al. (1992) and JUITA et al. (2012) reported that polyunsaturated acids, such as C18:2n-6 and C18:3n-3, undergo rapid oxidation at elevated temperature.

During ensiling from 3 to 65 days, contents of total FA, C18:2n-6 and C18:3n-3 fluctuated in silage ensiled at 30°C and 45°C, but decreased in silage ensiled at 15°C ($P < 0.05$) (Table 3). Therefore, after ensiling for 65 days, silages maintained at 15°C had lower contents of total FA and C18:2n-6 than silages maintained at 30°C ($P < 0.05$) and 45°C ($0.05 < P < 0.1$), and had lower content of C18:3n-3 than silages maintained at 30°C and 45°C ($P < 0.05$). The former

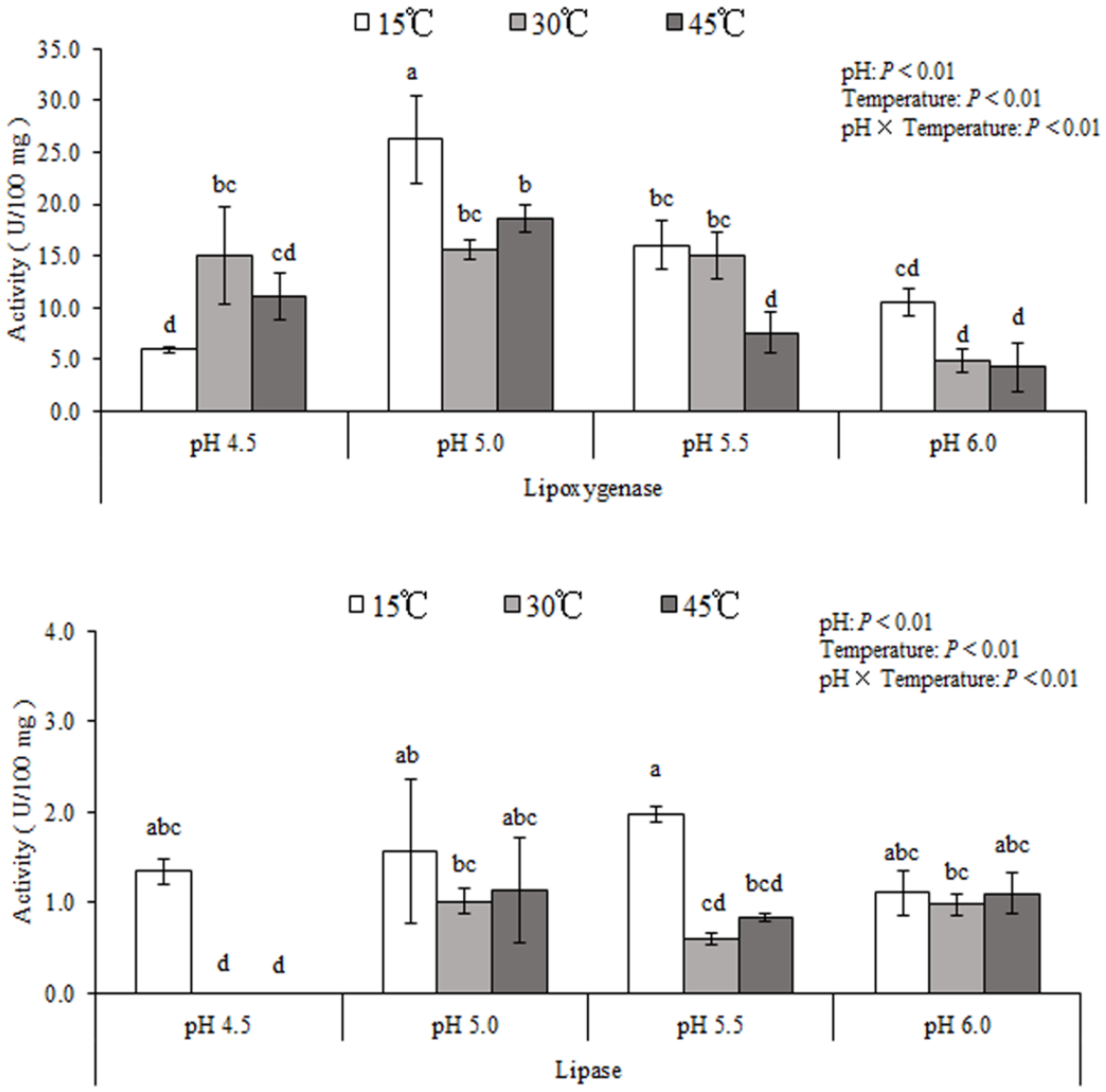

Figure 2 - The activity of lipoxygenase and lipase at different pH values and temperatures. Means with different lowercase letters above the column (a-d) indicated a significant difference according to Tukey's test at $P < 0.05$.

did not support the hypothesis of VAN RANST et al. (2009), who reported that total FA content remained stable irrespective of type and extent of fermentation. Regrettably, its reason was not yet elaborated. Fluctuation of contents of total FA, C18:2n-6 and C18:3n-3 in silage ensiled at 30°C and 45°C during ensiling from 3 to 65 days possibly was attributed to the intervention of different microbes and plant enzymes but which need further study. To our best knowledge, there were few reports for the decrease in contents of total FA, C18:2n-6 and C18:3n-3 in silage ensiled at 15°C. Based on our results, this could be attributed to the activity of microbes, showed by higher amount of aerobic bacteria and yeasts on most of ensiling days at 15°C than at 30°C and 45°C ($P < 0.05$ or $0.05 < P < 0.1$), and the higher activity of lipoxygenase at 15°C than at 30°C and 45°C ($P < 0.05$) (Figure 2). A significant interaction of temperature and ensiling time was on C16:0 ($P < 0.05$), shown by higher C16:0 content at 15°C and 30°C than 45°C during ensiling for 65 days ($P < 0.05$). This might be attributed to that C16:0 responded to different micro-ecologies

during ensiling, supported by lower aerobic bacteria and yeasts number at 45°C than at 15°C and 30°C ($P < 0.05$). This was similar to the result of ALVES et al. (2011), who reported that the use of formic acid as fermentation-inhibitor decreased microbial FA synthesis, and thus which made a decrease in the C16:0 as compared with the control.

In conclusion, after ensiling for 65 days, alfalfa silage ensiling at low temperature (15°C) had better fermentation quality than at high temperatures (30°C and 45°C). Temperatures could induce various interventions of plant enzymes and microbes in FA lipolysis, which resulted in different dynamics of change in FA profile in alfalfa silage during ensiling.

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