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Effect of additives on fatty acid profile of high moisture alfalfa silage during ensiling and after exposure to air

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ABSTRACT

The objective of present study was to investigate the changes of total fatty acid (FA) content and fatty acid composition in high moisture alfalfa (DM = 229 g/kg fresh weight) silage treated without (Control) or with *Lactobacillus buchneri* (LB), *L. plantarum* (LP), propionic acid additive (PA) and tea polyphenols (TP) during ensiling and after exposure to air. A 750 g of alfalfa material was filled into the experimental silo (polyvinyl chloride bottle, 1000 mL capacity). After ensiling for 65 days at room temperature, a part of silages (200 g) was sampled and the others (450 g) were exposed to air for 6 days. Butyric acid (> 30 g/kg DM) was more than lactic acid (< 1 g/kg DM) in the control and TP silages due to the low contents of DM and water-soluble carbohydrates, and high buffer capacity, indicating poor fermentation quality. LB, LP, and PA promoted lactic fermentation, restrained butyric fermentation and thus improved fermentation quality of silages. Unsaturated fatty acids (UFA) lipolysis and saturated fatty acids (SFA) synthesis occurred in the control silage during ensiling, mainly the lipolysis of C18:2n-6 and C18:3n-3 and synthesis of C6:0. Compared with the control, LB, LP, and PA restrained butyric fermentation and synthesis of C6:0, meanwhile LB and LP did not relieve the lipolysis of UFA while PA relieved it; TP relieved synthesis of C6:0 and had a slight lipolysis of UFA. After exposure to air for 6 days, the control, LB, PA, and TP silages had better aerobic stability than LP silage, supported by higher temperature in LP silage than in the control, LB, PA, and TP silages. Like volatile FA, most of the FA composition had a positive correlation with aerobic stability. PA silage had the highest total FA content (33.8 g/kg DM), the proportions of UFA, C18:2n6 and C18:3n3 due to its lower pH (< 4.60) and amounts of aerobic bacteria (< 5.0 lg cfu/g FM) and yeasts (< 2.6 lg cfu/g FM) than the control, LB and LP silages, and its lower pH and aerobic bacteria number than TP silage. Therefore, PA kept silage aerobic stability and was a good additive to conserve FA of silage after exposure to air for 6 days.

1. Introduction

Fatty acids (FA) in fresh forage is dominated by a high proportion of unsaturated fatty acids (UFA) of linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3) (Clapham et al., 2005). High intake ration contained sufficient fresh forage has been confirmed to increase the concentration of UFA in ruminant products (Wood and Enser, 1997), and consequently be beneficial to human health

Abbreviations: aNDF, neutral detergent fiber assayed with a heat stable amylase and expressed inclusive of residual ash; ADF, acid detergent fiber expressed inclusive of residual ash; cfu, colony forming units; DM, dry matter; FA, fatty acids; FC, fermentation coefficient; FM, fresh matter; FW, fresh weight; LAB, lactic acid bacteria; LB, *Lactobacillus buchneri*; LP, *Lactobacillus plantarum*; mEq, milliequivalent; N, nitrogen; PA, propionic acid additive; SEM, standard error of the means; SFA, saturated fatty acids; TP, tea polyphenols; UFA, unsaturated fatty acids; VFA, volatile fatty acids; WSC, water soluble carbohydrates

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(Simopoulos, 2001). Therefore, FA composition in silages, mainly the changes of C18:2n-6 and C18:3n-3, has gained high attention during ensiling (Alves et al., 2011). Previous studies have reported changes of FA in silage as compared with fresh forage (Dewhurst and King, 1998; Boufaïed et al., 2003; Van Ranst et al., 2009; Alves et al., 2011). This was associated with the dry matter (DM) loss in a silo (Van Ranst et al., 2009; Ding et al., 2013). In order to decrease the DM loss, formic acid, *Lactobacillus plantarum* (LP) alone or combined with other lactic acid bacteria (LAB) were used to decrease DM loss during ensiling (Lattemae et al., 1996; Driehuis and van Wikselaar, 2000), but the desired results of reducing FA lipolysis was not completely obtained (Boufaïed et al., 2003; Jalč et al., 2009a; Jalč et al., 2009b; Van Ranst et al., 2009; Ding et al., 2013). The real reason is not yet completely clear. One proposed reason for this was that lipolytic enzymes are the contributor to the lipolysis in silage inoculated with LAB during ensiling (Lee et al., 2008). The activity of lipolytic enzymes and lipolysis could be restrained by polyphenols (Lourenço et al., 2008; Kalač and Samková, 2010). Tea polyphenols (TP) is a food antioxidant and has been used in silage for preventing FA oxidation in corn silage during ensiling (Han and Zhou, 2013). However, there is little information about the effect of TP on preventing FA oxidation of alfalfa silage during ensiling.

When a silo is opened, anaerobic conditions are no longer maintained, and aerobic microbes have an opportunity to grow. Once aerobic deterioration of silage caused by aerobic microbes, large DM loss in silage is inevitable (McDonald et al., 1991). In order to control aerobic deterioration of silage, *L. buchneri* (LB) and propionic acid (PA) were used and the desired effect was obtained (Kung et al., 2000; Nishino et al., 2004; Comino et al., 2014). Despite LB and PA had the ability to keep silage aerobic stability, the oxygen, microbes, light and plant lipoxygenases are still present and may induce oxidation (Khan et al., 2009). So far, limited information is available on the change of FA in LB- and PA-treated alfalfa silage after exposing to air. Silage with poor fermentation quality often keeps aerobic stability after exposure to air since it commonly contained a high content of volatile fatty acids (VFA, acetic acid, propionic acid, and butyric acid) and a positive correlation was found between VFA and aerobic stability (McDonald et al., 1991). In silage, non-volatile short-chain FA, medium- and long- chain FA, were present (McDonald et al., 1991). Some of them are of anti-fungal characteristic (Desbois and Smith, 2010; Huang et al., 2011; Pohl et al., 2011). It can be assumed that there might be a correlation between those FA and aerobic stability of silage.

The purpose of this study was to investigate the fatty acid profile in high moisture alfalfa silage treated without (Control) or with LB, LP, PA and TP during ensiling and after exposure to air for 6 days, and clarifying the correlation between FA and the aerobic stability of silage.

2. Materials and methods

2.1. Silage material and silage making

Alfalfa was planted on September 25, 2014, in an experimental field (Humid subtropical climate, Latitude 32°01′59.81″ N, Longitude 118°50′13.63″ E, Altitude above sea level 17 m) of Nanjing Agricultural University (Nanjing, China) which contained 7 plots. The area of each plot was 200 m^2. After the alfalfa was at the flowering stage on April 15, 2015, five plots were randomly selected and harvested for making silage, immediately. Fresh alfalfa was chopped into 1–2 cm-long pieces by a forage chopper (Sh-2000, Shanghai Donxe Industrial Co., Ltd., Shanghai, China). The *L. buchneri* strain, LB, was bought from China General Microbiological Culture Collection Center (Beijing, China). Strain LP was isolated from corn silage and was identified as *L. plantarum* by analyzing its 16S rDNA sequence (GeneBank accession Number: JN043516). LP was stored at −80 °C and was used after culturing in de Man Rogosa and Sharp broth (Guangdong Huankai Microbial Science and Technology Co., Ltd., Guangzhou, China) at 30 °C for 30 h. PA was propionic acid additive (Lupro-Mix® NC Liquid, containing a mixture of 38% propionic acid, 34% formic acid, 8% ammonia and 20% water), which was purchased from BASF (Shanghai, China). TP was antioxidant, which was purchased from a company (Rueyang biotechnology Co., Ltd., Wuxi, China).

Fresh alfalfa obtained from the 5 plots (1000 m^2) was mixed well. An experiment on high moisture alfalfa silage ensiled with 5 additive treatments (without additive as the Control, and with LB, LP, PA, and TP) × 4 replicates was designed. Each additive treatment was applied to a 750 g of alfalfa per silo in quadruplication. After treating and mixing well, alfalfa was filled to silo (polyvinyl chloride bottle, 1000 mL). According to McFarland turbidity standards, LB and LP were separately added to make the inoculation at 1×10^6 colony forming units (cfu)/g fresh matter (FM). PA was added to make the dose at 4 g/kg on FM basis. TP was added to make the dose at 4 g/kg on FM basis. The control was treated with 5 mL distilled water only. The total 20 silos were sealed with a screw top and plastic tape. The screw top of the silo was fitted with a plastic fermentation trap filled with water, which permitted the escape of gasses but prevented the entry of air. The silos were opened after ensiling at ambient temperature (18–30 °C) for 65 days.

2.2. Microbial and chemical analyses

Ten grams of the fresh alfalfa was shaken well with 90 mL of sterilized saline solution (8.50 g/L NaCl), and serial dilutions (10^{-1} through 10^{-6}) were made in sterile saline solution. LAB were 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 3 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 3 days.

Fifty grams of fresh alfalfa was taken before ensiling, mixed with 200 mL of distilled water, and stored at 4 °C for 18 h. The mixture was then filtered, and the filtrate was used for determination of pH value. The pH was measured by using a glass electrode pH meter (HI221, Hanna Ltd., Rome, Italy). The buffering capacity of fresh alfalfa was determined according to the procedures described by Playne and McDonald (1966). DM content of fresh alfalfa was determined by an oven drying at 70 °C for 48 h. Water-soluble carbohydrates (WSC) content of fresh alfalfa was determined by using the anthrone method (Murphy, 1958). The fermentation coefficient (FC) of alfalfa silage was predicted according to Addah et al. (2011), as follows: FC = DM% + 8 × WSC g/kg DM ÷ BC mEq/kg DM, where BC is the buffering capacity of the fresh alfalfa. FC expresses whether the fresh forage will ensile easily or will be difficult to ensile (FC > 45 = easy, FC < 35 = difficult to ensile). Crude protein of fresh alfalfa was analyzed according to AOAC-984.13 methods (1990). The aNDF content of fresh alfalfa was measured according to the AOAC Official First Action method (Mertens, 2002) with the inclusion of heat-tolerant alpha-amylase and sodium sulfite. Acid detergent fiber (ADF) was measured according to the AOAC (2005; method 973.18). Both procedures were modified for use of the ANKOM filter bag technique. The procedures were conducted sequentially and the results are expressed on an ash-inclusive basis. After ensiling for 65 days, silos were opened and the silages were mixed thoroughly. The silages were sampled and the DM, crude protein, aNDF, and ADF of silage was measured by the same method with the fresh alfalfa. DM loss of the silage was estimated by measuring the differences in DM weights in the same silo after sealing on day 1 and ensiling for 65 days. DM loss was calculated with using corrected DM content which was corrected by the formula of Porter et al. (1995). The filtrate was processed in the same way with the fresh alfalfa and was used for measuring pH value, ammonia-N, and organic acid content. Ammonia-N was determined by the phenol-hypochlorite procedure (Novozamsky et al., 1974). The organic acid content of the silage was analyzed using an Agilent HPLC 1260 (Agilent Technologies Inc., München, Germany; column: Carbomix® H-NP5, Sepax Technologies Inc., Newark, USA; Detector: refractive index detector, Agilent Technologies Inc., München, Germany; eluent: 0.5 mL/min, 2.5 mmol/L H_2SO_4; temperature: 55 °C).

2.3. Aerobic stability analyses

After silo opened, the 450 g silage was used for aerobic stability test. Temperature change was monitored in the untreated and treated silages which were placed in insulated polystyrene foam boxes at room temperature (24–30 °C). The boxes were covered with gauze to allow air to enter. Temperature change in silage and surrounding during aerobic exposure for 6 days was monitored by thermal recorders (MDL-1048A, Shanghai Tianhe Automation Instrumentation Co., Ltd., Shanghai, China). The temperature was recorded at 20 min intervals. The silage was recognized as undergoing aerobic stability before the temperature of silage exceeded the surrounding environment by 2 °C (Moran et al., 1996). Therefore, only the time points of 4 replicates of per additive treatment were included in the statistical analysis when the temperature of silage firstly exceeded the surrounding environment by 2 °C. At the end of aerobic exposure for 6 days, silages were thoroughly mixed. The pH values, lactic acid content, the amounts of aerobic bacteria and yeasts in silage were analyzed.

2.4. 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 5 mL preheated isopropanol in a glass tube, heated at 75 °C for 15 min, and then cooled to room temperature. The glass tube was added with 3 mL chloroform and 1 mL water and was incubated with shaking for 60 min. The liquid extract was transferred to a fresh tube, extract tissue was added with 4.5 mL chloroform: methanol (2:1 v/v) two times until tissues are grayish white. All extracts were combined and added 2 mL 1 mol/L KCl. After mixing, the extracts were centrifuged at 1820*g* at 16 °C, and 16 mL lower phase was obtained. After concentrated by Termovap sample concentrator (MD200-2, Allsheng Instruments Co., Ltd., Hangzhou, China) at 45 °C, the 2 mL sample was added 2 mg of nonadecanoic acid (C19:0; Sigma, Shanghai, China) as internal standard and 5 mL of 2.5% H_2SO_4 (v/v) in methanol, and then heated at 80 °C for 60 min to methyl esterification. A 1.5 mL of heptane was added and followed by 1 mL 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 (100 m × 0.25 mm i.d. × 0.2 μm, Agilent Technologies Inc., Shanghai, China). The temperature program was used: 150 °C for 2 min, 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 containing 37 FAME obtained from Sigma (Supelco 37 component, Supelco Inc. Bellefonte, PA, USA) was used as a standard to quantify individual FA (C6:0, C14:0, C16:0, C16:1, C18:0, C18:1 *trans*-9, C18:1 *cis*-9, C18:2n6, C18:2 *trans*-12 *trans*-15, C18:3n3, C18:3n6 and C20:2).

2.5. Data analyses

The statistical analyses were performed using the IBM Statistical Packages for the Social Sciences (IBM SPSS 20.0 for Windows). The data were analyzed by one-way analysis of variance (ANOVA, General Linear Models) (5 treatments × 4 replicates) to evaluate effects of additives on the fermentation characteristics and microbial composition of alfalfa silages. The data were analyzed by one-way ANOVA (fresh alfalfa material and 5 treatments × 4 replicates) to evaluate effects of ensiling and additives on FA profile of alfalfa silages. Only the time points of 4 replicates of per additive treatment were reported and analyzed using one-way ANOVA (5 treatments × 4 replicates) to evaluate effects of additives on aerobic stability when the temperatures of silages firstly exceeded the surrounding environment by 2 °C. After aerobic exposure for 6 days, the data were analyzed by one-way ANOVA (5 treatments × 4 replicates) to evaluate effects of additives on FA profile of alfalfa silages. The means were then compared for significance using

Table 1
Effects of additives on the chemical compositions of silages.

Items [a]	DM (g/kg FW)	DM loss (% DM)	Crude protein (g/kg DM)	aNDF	ADF	Hemicellulose	WSC
Control	197 d	15.6 a	159 d	473 a	397 a	76.0	15.6 bc
LB	211 bc	8.51 b	183 c	413 c	340 c	73.3	15.8 bc
LP	219 ab	6.41 bc	205 b	369 d	300 d	68.3	17.7 b
PA	227 a	4.36 c	223 a	388 d	310 d	78.0	20.9 a
TP	201 cd	13.6 a	165 d	442 b	367 b	75.0	13.6 c
SEM	2.4	0.821	4.2	6.7	6.5	2.62	0.69
P-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.178	< 0.001

Means with different letters in the same column (a–d) indicated a significant difference according to Tukey's test at $P < 0.05$.

[a] aNDF, neutral detergent fiber assayed with a heat stable amylase and expressed inclusive of residual ash; ADF, acid detergent fiber expressed inclusive of residual ash; Control, silage treated without additives; DM, dry matter; FW, fresh weight; LB, *Lactobacillus buchneri*; LP, *L. plantarum*; FW, fresh weight; PA, propionic acids additive; SEM, standard error of the means; TP, tea polyphenols; WSC, water-soluble carbohydrates.

Tukey's test at $P < 0.05$. Correlation analysis between aerobic stability and all measured or analyzed variables was performed using SPSS Correlation Procedure with Pearson correlation coefficient.

3. Result

3.1. Alfalfa characteristics before ensiling

Alfalfa had low DM (229 g/kg FW), WSC content (53.3 g/kg DM) and fermentation efficiency (24.8), and had high crude protein (173 g/kg DM), aNDF (404 g/kg DM) and ADF (317 g/kg DM) content and buffering capacity (226 mEq/kg DM). The pH value of fresh alfalfa was 6.30. Epiphytic LAB (5.89 lg cfu/g FM) on the alfalfa was less than aerobic bacteria (6.63 lg cfu/g FM) and yeasts (6.77 lg cfu/g FM).

3.2. Chemical compositions of silages

After ensiling for 65 days, DM content of the control silage lowered to 197 g/kg FW, which resulted in high DM loss (15.6% DM), contents of aNDF (473 g/kg DM) and ADF (397 g/kg DM), and low crude protein content (159 g/kg DM) (Table 1). Obviously, there were significant effects of additives on all chemical compositions ($P < 0.05$) except hemicellulose ($P > 0.05$). PA maximally increased contents of DM, crude protein and WSC ($P < 0.05$), and decreased DM loss, contents of aNDF and ADF as compared with the control ($P < 0.05$), which was followed by LB, LP, and TP, successively.

3.3. Fermentative quality of silages

As shown in Table 2, the control silage had high pH (> 5.4), contents of butyric acid (32.7 g/kg DM) and ammonia-N (144 g/kg N), low lactic acid content (< 1 g/kg DM) and ratio of lactic acid to acetic acid (< 0.1), indicated poor fermentation quality. Obviously, there were significant effects of additives on all fermentation characteristics ($P < 0.05$) except alcohol content and yeasts number ($P > 0.05$). LP maximally increased the lactic acid content, and thus decreased pH below 4.3 and contents of butyric acid (< 2 g/kg DM) and ammonia-N (< 0 g/kg N) as compared with the control ($P < 0.05$), indicated vigorous lactic fermentation and considerable improvement in fermentation quality, which was followed by PA and LB because lactic acid content in LB and PA silages

Table 2
Effects of additives on the fermentative characteristics of silages.

Items [a]	pH	Lactic acid (g/kg DM)	Acetic acid	Propionic acid	Butyric acid	Alcohol	VFA	NH_3-N (g/kg N)	Ratio	Lactic acid bacteria (lg cfu/g FM)	Aerobic bacteria	Yeasts
Control	5.43 a	0.59 c	32.5 a	11.0 b	32.7 a	3.30	76.2 a	155 a	0.010 c	7.54 ab	4.46 ab	2.90
LB	4.67 b	11.8 b	25.5 ab	3.27 d	12.9 b	3.20	41.7 b	70.6 c	0.553 b	7.08 b	2.33 c	< 2.00
LP	4.23 c	24.3 a	20.27 ab	1.83 e	1.43 c	4.17	23.5 c	49.8 d	1.190 a	6.97 b	3.09 bc	< 2.00
PA	4.41 c	10.9 b	15.7 b	21.97 a	0.73 c	3.30	38.4 b	47.3 d	1.280 a	5.04 c	3.40 bc	< 2.00
TP	5.30 a	0.36 c	32.6 a	10.3 c	31.5 a	2.10	74.4 a	129 b	0.013 c	8.36 a	4.88 a	< 2.00
SEM	0.075	2.589	3.805	0.213	1.399	0.698	4.09	3.33	0.096	0.184	0.296	0.300
P-value	< 0.001	< 0.001	0.038	< 0.001	< 0.001	0.404	< 0.001	< 0.001	< 0.001	< 0.001	0.001	0.208

Means with different letters in the same column (a–d) indicated a significant difference according to Tukey's test at $P < 0.05$.

[a] cfu, colony-forming units; Control, silage treated without additives; DM, dry matter; FM, fresh matter; LB, *Lactobacillus buchneri*; LP, *L. plantarum*; FM, fresh matter; NH_3-N, ammonia-N; N, nitrogen; PA, propionic acid additive; SEM, standard error of the means; TP, tea polyphenols, VFA, volatile fatty acids.

Table 3
Total fatty acid content (g/kg of DM) and fatty acid composition (g/100 g of total fatty acids) of fresh alfalfa and alfalfa silages.

Items [a]	Fresh alfalfa	Silages					SEM	P-value
		Control	LB	LP	PA	TP		
Total fatty acids	36.9 a	33.3 ab	25.4 c	16.2 d	25.8 c	30.2 bc	1.50	< 0.001
Fatty acid composition								
Saturated fatty acids	24.4 cd	43.0 a	31.5 bc	20.8 d	20.4 d	34.2 b	1.64	< 0.001
Unsaturated fatty acids	75.6 a	46.6 b	35.5 c	23.1 d	48.0 b	41.0 bc	2.06	< 0.001
C6:0	0.00 d	16.3 a	4.37 c	1.00 d	0.77 d	11.0 b	0.39	< 0.001
C14:0	0.00 b	0.43 ab	1.20 a	0.00 b	0.00 b	0.30 ab	0.22	0.013
C16:0	19.9 ab	21.8 a	21.2 a	16.1 b	16.1 b	20.2 ab	1.23	0.001
C16:1	1.90	1.77	1.80	1.93	1.77	2.10	0.194	0.810
C18:0	4.50 ab	4.57 ab	4.63 a	3.63 ab	3.53 b	3.97 ab	0.231	0.015
C18:1 *trans*-9	5.87	2.53	3.40	4.87	3.57	2.93	0.816	0.100
C18:1 *cis*-9	0.00 d	0.60 c	1.97 a	0.00 d	1.53 b	1.37 b	0.062	< 0.001
C18:2n6	16.2 a	12.1 bc	7.83 de	5.77 e	14.4 ab	9.93 cd	0.627	< 0.001
C18:2 *trans*-12 *trans*-15	1.40 a	0.97 b	0.53 c	0.60 bc	0.57 bc	0.60 bc	0.088	< 0.001
C18:3n3	43.1 a	28.8 b	21.9 c	9.93 d	27.7 bc	22.4 bc	1.452	< 0.001
C18:3n6	6.60 a	0.00 c	0.00 c	0.00 c	0.00 c	3.07 b	0.153	< 0.001
C20:2	0.00 b	0.50 a	0.00 b	0.00 b	0.00 b	0.00 b	0.001	< 0.001

Means with different letters in the same row (a–e) indicated a significant difference according to Tukey's test at $P < 0.05$.

[a] Control, silage treated without additives; LB, *Lactobacillus buchneri*; LP, *L. plantarum*; PA, propionic acid additives; TP, tea polyphenol; SEM, standard error of the means.

was lower than in LP silage ($P < 0.05$). PA and LB silages had better fermentation quality, which was supported by lower pH, contents of butyric acid and ammonia-N ($P < 0.05$) and higher lactic acid content and ratio of lactic acid to acetic acid as compared with the control ($P < 0.05$). In addition, PA silage had the highest propionic acid content when compared with the control and other treated silages ($P < 0.05$), and only LB decreased aerobic bacteria number as compared with the control ($P < 0.05$). In contrast, the fermentation quality of TP silage approximated the control silage because there were no differences in pH, contents of lactic acid, acetic acid and butyric acid and ratio of lactic acid to acetic acid ($P > 0.05$) except ammonia-N ($P < 0.05$).

3.4. FA in alfalfa before and after ensiling

As shown in Table 3, C16:0, C18:2n6 and C18:3n3 were the major FA composition in fresh alfalfa. The proportion of UFA was 3 times higher than the SFA. After alfalfa ensiling for 65 days, considerable decreases were observed in the proportions of UFA, C18:2n-6 and C18:3n-3 as compared with fresh alfalfa ($P < 0.05$); conversely, increases in proportions of SFA and C6:0 occurred ($P < 0.05$). Obviously, there were significant effects of additives on total FA content and the major FA composition (SFA, UFA, C6:0, C16:0, C18:2n6 and C18:3n3) ($P < 0.05$). Big decreases were found in the proportion of C6:0 and C16:0 in PA silage ($P < 0.05$), and thus PA silage had a lower proportion of SFA than the control silage ($P < 0.05$), which was followed by LP, LB, and TP silages, successively. On the other hand, small changes were observed in proportions of C18:2n6 and C18:3n3 in PA and TP silages as compared with the control silage ($P > 0.05$), and thus PA, TP and the control silages had a similar proportion of UFA ($P > 0.05$). In contrast, since there were considerable decreases in proportions of C18:2n6 and C18:3n3 in LB and LP silages as compared with the control silage ($P < 0.05$), the control silage had a higher proportion of UFA than LB and LP silages ($P < 0.05$).

3.5. Aerobic stability of silages

LP silage had the lowest aerobic stability when compared with the control and other treated silages ($P < 0.05$) because the temperature of LP silage firstly exceeded the surrounding environment 2 °C after aerobic exposure for 134 h (Fig. 1). Conversely, the temperatures of LB, PA, TP and the control silages did not exceed the surrounding environment 2 °C after aerobic exposure for 144 h.

After exposure to air for 6 days, LP silage had higher pH than PA silage ($P < 0.05$) and other silages ($P > 0.05$) (Table 4), but still had higher lactic acid content than the control, LB and TP silages ($P < 0.05$); PA silage had lower amounts of aerobic bacteria and yeasts than the control, LB, and LP silages ($P < 0.05$), and had lower amount of aerobic bacteria than TP silage ($P < 0.05$). In addition, as known from Tables 2 and 4, increases in pH, aerobic bacteria, and yeasts number were lower than 0.12, 4.90 lg cfu/g FM and 2.40 lg cfu/g FM in PA silage, orderly. In contrast, increases in amounts of aerobic bacteria and yeasts in control silage were more than 5 lg cfu/g FM, and an increase of pH in LB silage was 0.5 units; the pH and amounts of aerobic bacteria and yeasts in LP silage rose separately more than 0.5 units and 5 lg cfu/g FM, and the aerobic bacteria number in TP silage rose more than 5 lg cfu/g FM.

3.6. Changes of FA in alfalfa silages after exposure to air

After exposure to air for 6 days, there were significant differences in total FA content and major FA compositions (SFA, UFA, C6:0, C16:0, C18:2n6 and C18:3n3) among the control and additives treated silages ($P < 0.05$) (Table 5). PA silage had the highest total

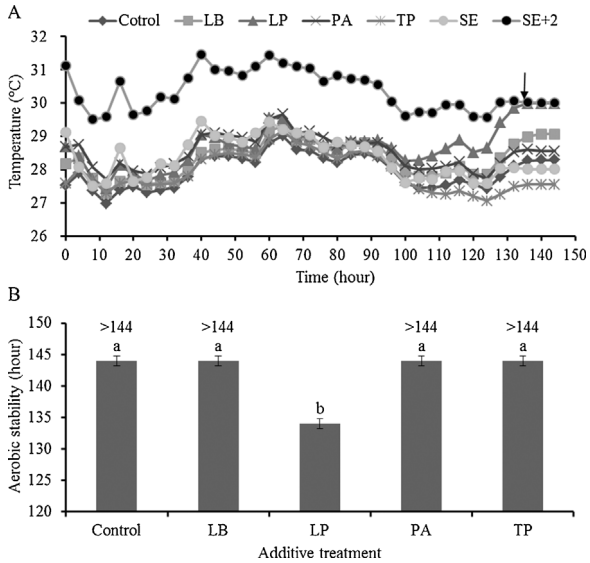

Fig. 1. Changes of temperature (A) and aerobic stability (B) of alfalfa silages after exposure to air (averaged by 4 replicates for each additive treatment). The arrows showed the temperature of silage exceeded the surrounding temperature 2 °C. Means with different letters in the same column (a–b) indicated a significant difference according to Tukey's test at $P < 0.05$. Control, silage treated without additives; LB, *Lactobacillus buchneri*; LP, *L. plantarum*; PA, propionic acid additives; SE, surrounding temperature; SE + 2, surrounding temperature plus 2 °C; TP, tea polyphenol.

Table 4
Effects of additives on the pH, lactic acid content, amounts of aerobic bacteria and yeasts of silages after exposure to air for 6 days.

Items [a]	pH	Lactic acid (g/kg DM)	Aerobic bacteria (lg cfu/g FM)	Yeasts
Control	5.41 ab	0.26 b	6.41 ab	5.58 a
LB	5.17 ab	3.50 b	5.17 bc	4.30 b
LP	5.87 a	13.45 a	6.69 a	4.60 ab
PA	4.52 b	12.19 a	4.90 c	2.50 c
TP	5.31 ab	0.56 b	5.60 abc	< 2.00 c
SEM	0.243	1.773	0.279	0.227
P-value	0.033	0.001	0.004	< 0.001

Means with different letters in the same column (a–c) indicated a significant difference according to Tukey's test at $P < 0.05$.

[a] cfu, colony-forming units; Control, silage treated without additives; DM, dry matter; FM, fresh matter; LB, *Lactobacillus buchneri*; LP, *L. plantarum*; FM, fresh matter; PA, propionic acid additive; SEM, standard error of the means; TP, tea polyphenols.

FA content and proportions of UFA, C18:2n6, C18:3n3 when compared with the control and other additives treated silages ($P < 0.05$), which was followed by the control and TP silages. In contrast, LB and LP silages had a lower proportion of UFA, C18:2n6 and C18:3n3 than the control and other additives treated silages ($P < 0.05$ or $P > 0.05$). C6:0, one major ingredient of SFA, was less in LP and PA silages than in the control, LB and TP silages ($P < 0.05$), and thus the control, LB and TP silages had higher proportion of SFA than LP ($P < 0.05$) and PA silages ($P < 0.05$ or $P < 0.05$).

3.7. Correlation analysis between aerobic stability and other variables

As shown in Table 6, aerobic stability had positive correlation with propionic acid, VFA, aNDF, ADF, total FA, SFA, UFA, C18:1 *cis*-9, C18:2n6 and C18:3n3 ($P < 0.05$), positive correlation with butyric acid, ammonia-N, C6:0 and C16:0 ($0.05 < P < 0.1$), and had negative correlation with lactic acid content and C18:1 *trans*-9 ($P < 0.05$).

4. Discussion

The present study showed that the control silage had high pH (> 5.4), contents of butyric acid (32.7 g/kg DM) and ammonia-N (155 g/kg N), low lactic acid content (< 1 g/kg DM), indicating poor fermentation quality. This could be attributed to the low fermentation coefficient (24.8), indicated by low contents of DM and WSC, and high buffer capacity being the deciding factors. Reports concluded that fresh alfalfa was not easy to be fermented by epiphytic LAB since it had low contents of DM and WSC, and high buffer capacity (Owens et al., 1999; Ke et al., 2015). It was suggested that measures might be needed to improve silage quality.

Many researches have focused on improving fermentation quality by inoculating LAB and acidic additive (Kung et al., 2004; Nkosi

Table 5
Total fatty acid content (g/kg of DM) and fatty acid composition (g/100 g of total fatty acids) of alfalfa silages after exposure to air for 6 days.

Items [a]	Control	LB	LP	PA	TP	SEM	P-value
FA	27.97 b	23.17 b	15.77 c	33.80 a	27.13 b	1.145	< 0.001
Fatty acid composition							
Saturated fatty acids	35.57 a	34.03 ab	19.63 c	25.90 bc	32.33 ab	1.778	< 0.001
Unsaturated fatty acids	38.77 b	26.93 c	21.50 c	65.10 a	39.50 b	1.598	< 0.001
C6:0	11.53 a	6.50 b	0.80 c	0.63 c	10.53 a	0.320	< 0.001
C14:0	0.63	1.03	0.33	1.13	1.00	0.213	0.114
C16:0	19.33 ab	18.37 ab	14.97 b	20.00 a	19.35 ab	1.062	0.047
C16:1	2.03	2.00	2.30	1.97	1.50	0.274	0.403
C18:0	4.07	4.10	3.50	4.17	4.33	0.261	0.284
C18:1 *trans*-9	5.90	4.30	3.03	3.67	5.70	0.766	0.094
C18:1 *cis*-9	1.40 b	1.87 a	1.87 a	0.60 c	1.67 ab	0.082	< 0.001
C18:2n6	9.80 b	6.33 cd	5.43 d	16.80 a	8.27 bc	0.465	< 0.001
C18:2 *trans*-12 *trans*-15	0.43	0.60	0.87	1.23	0.50	0.203	0.100
C18:3n3	20.60 b	13.10 c	9.93 c	39.67 a	18.20 b	1.076	< 0.001
C18:3n6	0.00 b	0.00 b	0.00 b	0.00 b	4.77 a	0.098	< 0.001
C20:2	0.00 b	0.50 b	0.00 b	1.73 a	0.53 b	0.255	0.004

Means with different letters in the same row (a–d) indicated a significant difference according to Tukey's test at $P < 0.05$.

[a] Control, silage treated without additives; LB, *Lactobacillus buchneri*; LP, *L. plantarum*; PA, propionic acid additives; TP, tea polyphenol; SEM, standard error of the means.

Table 6
Correlation analysis (Pearson coefficient) between aerobic stability (h) and other measured or analyzed variables of alfalfa silages.

Items [a]	Pearson coefficient	P-value
Fermentative characteristics		
pH	0.580	0.023
Lactic acid (g/kg DM)	−0.756	0.001
Acetic acid (g/kg DM)	0.281	0.311
Propionic acid (g/kg DM)	0.538	0.039
Butyric acid (g/kg DM)	0.504	0.055
Volatile fatty acids (g/kg DM)	0.61	0.014
Alcohol (g/kg DM)	−0.388	0.153
NH_3-N (g/kg N)	0.450	0.092
Microbial composition (lg cfu/g FM)		
Lactic acid bacteria	−0.007	0.982
Aerobic bacteria	0.301	0.275
Yeasts	0.159	0.572
Chemical composition		
Neutral detergent fiber (g/kg DM)	0.625	0.013
Acid detergent fiber (g/kg DM)	0.573	0.025
Total fatty acids (g/kg DM)	0.802	< 0.001
Fatty acid composition (g/100 g of fatty acids)		
Saturated fatty acids	0.514	0.050
Unsaturated fatty acids	0.811	< 0.001
C6:0	0.461	0.084
C14:0	0.343	0.210
C16:0	0.495	0.061
C18:0	0.369	0.176
C18:1 *trans*-9	−0.669	0.006
C18:1 *cis*-9	0.758	0.001
C18:2n6	0.674	0.006
C18:3n3	0.866	< 0.001

[a] DM, dry matter; FM, fresh matter; FW, fresh weight; N, nitrogen.

et al., 2009; Liu et al., 2016), which was confirmed in the present study. LB, LP, and PA decreased pH and remarkably restrained butyric fermentation of silage. Therefore, the nutrients in alfalfa were well preserved, as indicated by lower DM loss, contents of aNDF and ADF, and higher crude protein as compared with the control silage. TP did not improve fermentation quality of silage. This was consistent with the result of Kondo et al. (2004), who found that high dose (4 g/kg FM) of TP suppressed butyric fermentation, and inhibiting degree of TP on butyric fermentation varied due to the characteristics of sudangrass silage. In the present study, alfalfa had high moisture and low WSC content. Therefore, the dose of TP (4 g/kg FM) is insufficient and the effect of a higher dose of TP on the suppression of the butyric fermentation needs to be further studied. Although TP silage showed butyric fermentation, TP silage had lower ammonia-N content and higher crude protein content than the control silage. This is an indication that TP restrained

protein from being degraded to ammonia-N, which was consistent with the result of Kondo et al. (2004), who reported that TP prevented protein degradation during ensiling. Albrecht and Muck (1991) found a strong negative relationship between tannins, one integrant of TP, and soluble non-protein nitrogen in silages. In the present study, we considered that polyphenol combined with protein could reduce protein degradation from microorganisms.

There were inconsistent effects of ensiling on total FA content (Boufaïed et al., 2003; Whiting et al., 2004; Van Ranst et al., 2009; Alves et al., 2011). The real reason for the contrary results is not yet completely clear. The proposed explanation is that DM loss was responsible for higher total FA content in silage as compared with forage before ensiling, *e.g.,* fermentation or effluent loss (Van Ranst et al., 2009; Alves et al., 2011). In the present study, vigorous butyric fermentation occurred in control silage due to low DM content, which showed the greatest DM loss when compared with other silages. However, control silage had a loss (9.76%) of total FA content as compared with alfalfa before ensiling. This was attributed to the fact that synthesis of some SFA relieved loss of total FA content despite showing lipolysis of UFA in control silage. Indeed, there was UFA lipolysis similar to the results of previous studies (Ding et al., 2013; Ke et al., 2015), especially on C18:2n-6 degraded from 5.98 to 4.02 g/kg of DM and C18:3n-3 degraded from 15.90 to 9.59 g/kg of DM. Alves et al. (2011) and Ding et al. (2013) found that plant enzyme catalyzed C18:2n-6 and C18:3n-3 lipolysis as hydroperoxy polyunsaturated fatty acids that are further catabolized to yield a range of volatile compounds. Grossman (1972, 1974) found that leaves of alfalfa were rich in lipoxygenase. SFA increased after ensiling, mainly on C6:0 increasing from 0 to 5.43 g/kg of DM. This was inconsistent with the previous studies (Ding et al., 2013; Ke et al., 2015) because high DM alfalfa silage was an absence of vigorous butyric fermentation. McDonald et al. (1991) reported that C6:0 was a major FA produced by clostridia in poor fermentation quality of silage because the clostridial activity is stimulated by low contents of DM and WSC, and high buffer capacity of alfalfa.

Some reports showed that LAB inoculation had a small effect on increasing or decreasing total FA content, proportions of C18:2n-6 and C18:3n-3 in gramineae forage silage as compared with untreated silage (Boufaïed et al., 2003; Arvidsson et al., 2009; Alves et al., 2011). However, the contrary results were observed in the present study. This difference in different reports was attributed to the degree of butyric fermentation and species variation of ensiled material. Ding et al. (2013) found that LAB inoculated alfalfa silage with butyric fermentation had a big effect on FA, showed a big increase in total FA content proportions and concentrations of C18:2n-6 and C18:3n-3 as compared with the control silage. In the present study, LB and LP inhibited butyric fermentation and DM loss like previous studies (Gandra et al., 2016; Liu et al., 2016), and thus decreased the synthesis of C6:0. On the other hand, LAB inoculants did not eliminate the activity of lipolytic enzymes which were the contributor to the lipolysis in silage during ensiling (Lee et al., 2008). Actually, some LAB have the ability to enhance biohydrogenation of UFA, *e.g.*, C18:2n-6 and C18:3n-3 (Ogawa et al., 2005; Kishino et al., 2009). Therefore, LB and LP did not relieve but promote the lipolysis of UFA. Interestingly, LP silage had lower total FA content, proportions of C6:0, C16:0, C18:2n-6 and C18:3n-3 than LB silage. Based on our results, this might be attributed to weaker butyric fermentation and lower DM loss in LP silage as compared with LB silage. Otherwise, LP might have a bigger ability than LB in biohydrogenation of C18:2n-6 and C18:3n-3, which can be supported by the slightly higher proportion of C18:2 *trans*-12 *trans*-15 in LP silage. Kishino et al. (2011) found that linoleic acid isomerase in LP silage could make the biohydrogenation of C18:2n-6 and C18:3n-3. PA silage had a big effect on decreasing SFA, especially on decreasing proportions and concentrations of C6:0 and C16:0 as compared with the control silage, which was consistent with the results in C16:0 (Boufaïed et al., 2003; Arvidsson et al., 2009; Alves et al., 2011). PA inhibited butyric fermentation and microbes of silage in the present study, thus the synthesis of C6:0 and C16:0 was restrained. Compared with the control, PA had a slight effect on increasing or decreasing some UFA of silage, *e.g.* proportions of C18:2n-6 and C18:3n-3. This was similar to the findings of Van Ranst et al. (2009) and Alves et al. (2011). The proposed explanation was that propionic acid could inhibit lipolysis (Heimann et al., 2015). Compared with the control, TP decreased proportions of SFA, C6:0 and C16:0. The reason was that TP partially suppressed the activity of clostridia due to the antimicrobial activity of polyphenols (Kondo et al., 2004), showed slightly low butyric acid content and DM loss in TP silage as compared with the control silage, and polyphenols in TP could inhibit the activity of bacterial type II fatty acid synthase (Zhang and Rock, 2004). Meanwhile, TP slightly decreased proportions of UFA, C18:2n-6 and C18:3n-3 as compared with the control. This was inconsistent with the findings of Han and Zhou (2013). This difference could be attributed to the characteristic of forage since high moisture alfalfa had a big activity of plant lipases (Grossman, 1972, 1974; Ding et al., 2013). The dose of TP (4 g/kg FM) was insufficient to completely restrain the activity of plant lipases in low DM alfalfa. Han and Zhou (2013) found that high dose of TP enhanced the effect on restraining lipolysis of C18:2n-6 and C18:3n-3.

There were 3 definitions of aerobic stability. Moran et al. (1996) reported that the silage was recognized as undergoing aerobic stability before the temperature of silage exceeded the surrounding environment by 2 °C. Weinberg et al. (2008) reported that silages producing $CO_2 < 10$ g/kg DM or showing a change of < 0.5 units in pH over a 5-day period are deemed to be stable. Kung (2010) proposed that aerobic stability was a term that nutritionists had used to define the length of time that silage remained cool and did not spoil after exposed to air. Therefore, in the present study, aerobic stability was measured by monitoring the temperature change, final pH, aerobic bacteria, and yeasts count after exposure to air for 6 days. Similar to previous studies (Danner et al., 2003; Weiss et al., 2016), LP silage exhibited aerobic deterioration after exposing to air for 134 h due to its temperature exceeding surrounding environment by 2 °C. This was attributed to lower VFA content and higher lactic acid content in LP silage. McDonald et al. (1991) reported that VFA had a significantly positive correlation with aerobic stability while lactic acid had a significantly negative correlation with aerobic stability. In addition, the pH, amounts of aerobic bacteria and yeasts in PA silage rose lower than the control, LB, and LP silages, and the pH and aerobic bacteria number in PA silage rose lower than TP silage after aerobic exposure for 144 h. This indicated that PA silage was unspoiled while the control, LB, LP and TP silages spoiled after aerobic exposure for 144 h. Interestingly, total FA content and proportions of most of the FA composition, especially on C6:0, C16:0, C18:2n6 and C18:3n3 had a positive correlation with aerobic stability, respectively. This was consistent with the results of silages keeping aerobic stability for

6 days and containing high total FA content, proportions and concentrations of C6:0, C16:0, C18:2n6 and C18:3n3 at the opening. Indeed, C6:0, C16:0, C18:2n6 and C18:3n3 are of antifungal characteristics (Pohl et al., 2011; Huang et al., 2011). However, with the exception of C6:0, there is litter information about C16:0, C18:2n6 and C18:3n3 and how they can individually improve aerobic stability of silage. Further study is necessary to investigate the exact roles of C16:0, C18:2n6 and C18:3n3 in improving aerobic stability of silage.

The environment of the silo dramatically changes during opening and feeding of the silages. When the FA of silage is exposed to air, the presence of oxygen, light, microbes and plant lipoxygenases all can induce oxidation (Khan et al., 2009). In the present study, PA best conserved FA of silage after aerobic exposure for 6 days, mainly in UFA, C18:2n6 and C18:3n3. This could be attributed to that PA restrained multiplication of aerobic bacteria and yeasts of silage, supported by lower pH (< 4.60) and amounts of aerobic bacteria (< 5.0 lg cfu/g FM) and yeasts (< 2.6 lg cfu/g FM) than the control, LB and LP silages, and lower pH and aerobic bacteria number than TP silage after exposure to air for 6 days. Khan et al. (2009) proposed that oxidation of FA was of microbial origin and was further induced by light or by plant lipoxygenases. Furthermore, the total FA content, proportions and concentrations C16:0, C18:2n6 and C18:3n3 in PA silage were slightly lower than the fresh alfalfa before ensiling. Based on present results, this phenomenon was difficult to explain. FA increase in PA silage after exposure to air might be caused by moisture loss as an effluent loss which resulted in FA enrichment of silages during ensiling (Alves et al., 2011). However, the exact explanation can be clarified by a further experiment.

5. Conclusion

Untreated high moisture alfalfa was poor nutritional value and fermentation quality, supported by low contents of crude protein and high butyric acid. Compared with control, LB, LP, and PA improved the nutritional value and fermentation quality of silage because of restraining butyric fermentation but TP did not. UFA lipolysis and SFA synthesis occurred in high moisture alfalfa silage during ensiling, mainly the lipolysis of C18:2n-6 and C18:3n-3 and synthesis of C6:0. Compared with the control, LB, LP, and PA restrained the synthesis of C6:0, and LB and LP did not relieve the lipolysis of UFA but PA relieved it; TP relieved synthesis of C6:0 and had a slight lipolysis of UFA. Similar to VFA, most of the FA composition had a positive correlation with aerobic stability. After exposure to air for 6 days, PA kept silage aerobic stability and best conserved FA of silage, mainly in UFA, C18:2n6 and C18:3n3.

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