Effects of addition of malic or citric acids on fermentation quality and chemical characteristics of alfalfa silage

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ABSTRACT

We studied the effects on alfalfa preservation and chemical composition of the addition of different levels of malic acid and citric acid at ensiling as well as the utilization efficiency of these 2 organic acids after fermentation. Alfalfa was harvested at early bloom stage. After wilting to a dry matter content of approximately 40%, the alfalfa was chopped into 1- to 2-cm pieces for ensiling. Four levels (0, 0.1, 0.5, and 1% of fresh weight) of malic acid or citric acid were applied to chopped alfalfa at ensiling with 4 replicates for each treatment, and the treated alfalfa forages were ensiled for 60 d in vacuum-sealed polyethylene bags (dimensions: 200 mm × 300 mm) packed with 200 to 230 g of fresh alfalfa per mini silo and an initial density of 0.534 g/cm³. The application of malic or citric acids at ensiling for 60 d led to lower silage pH than was observed in the control silage (0% of malic or citric acids). Application of the 2 organic acids led to higher lactic acid concentration in alfalfa silage than in the control silage except with the application rate of 1% of fresh weight. Silages treated with both organic acids had lower nonprotein nitrogen concentrations than the control silages, and the nonprotein nitrogen concentrations in ensiled forages decreased with the increase in malic or citric acid application rates. The application of the 2 organic acid additives led to lower saturated fatty acid proportions and higher polyunsaturated fatty acid proportions in ensiled alfalfa than in the control silage. The amount of malic and citric acids degraded during ensiling of alfalfa was 1.45 and 0.63 g, respectively. At the application rate of 0.5% of fresh weight, residues of malic acid and citric acid in alfalfa silage were 11.1 and 13.6 g/kg of dry matter. These results indicate that including malic or citric acids at the ensiling of alfalfa effectively improved silage fermentation quality, limited proteolysis,

improved fatty acid composition of the ensiled forage, and could provide animals with additional feed additives proven to promote animal performance. However, when the application rate of both organic acids reached 1%, the concentration of lactic acid in silages decreased notably. Additionally, 0.5 and 1% application rates also increased the yeast count in ensiled alfalfa.

Key words: malic acid, citric acid, alfalfa silage, organic acid

INTRODUCTION

Extensive research over recent years has explored the potential of using organic acids (e.g., fumaric, malic, citric, and succinic acids) as alternatives to feed antibiotics to improve performance and feed efficiency. As a key intermediate in the citric acid cycle of biological tissues and in the succinate-propionate pathway of ruminal bacteria (Castillo et al., 2004), malic acid has been introduced as a feed additive for ruminants because of its numerous beneficial effects. Many *in vitro* studies have shown that malic acid could stimulate rumen fermentation, increase rumen pH (Martin and Streeter, 1995; Carro and Ranilla, 2003), improve microbial N production and microbial efficiency (Sniffen et al., 2006), decrease methane production (Carro and Ranilla, 2003), and increase feed digestibility (Carro et al., 1999). Feeding trials also confirmed that malic acid had a positive effect on increasing milk production in dairy cows (Sniffen et al., 2006; Devant et al., 2007; Wang et al., 2009b) and improving beef cattle performance (Martin et al., 1999; Castillo et al., 2007). Citric acid is another essential intermediate in the citric acid cycle, which has a similar function in stimulating rumen fermentation and improving animal performance (Packett and Butcher, 1963; Sun et al., 2008; Wang et al., 2009a).

To date, little information is available on the application of malic acid or citric acid in ensiled forage, and whether these organic acids have positive effects on promoting the fermentation quality of ensiled alfalfa

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is not clear. Even so, the existing literature shows that malic acid or citric acid could accelerate the growth of lactic acid bacteria (**LAB**; Branen and Keenan, 1970; Salou et al., 1991; Passos et al., 2003). Therefore, it is plausible that the application of malic acid or citric acid could possibly improve the fermentation of ensiled forage and consequently decrease proteolysis by promoting pH decline. Based on the positive effects of the abovementioned organic acids in stimulating microbial fermentation and their use as feed additives for improving animal performance, we propose that the application of these organic acids at ensiling may not only improve fermentation quality and nutritive value of ensiled forage but could also indirectly provide animals with feed additives after ingestion of organic acid-treated silages. However, whether these 2 acids have different effects on fermentation of ensiled forage is not clear. It is also necessary to clarify the degradation of the added organic acids after fermentation to see whether the residues in silages are enough to meet the amount of organic acids for improving animal performance as suggested by previous studies. In addition, the fatty acid (**FA**) composition in ensiled forages has been of interest. It has been reported that a high intake of PUFA could increase the concentration of PUFA in ruminant products (Wood and Enser, 1997; Kalač and Samková, 2010) and consequently be beneficial to human health (Simopoulos, 2001). It was reported that ensiling led to lower PUFA but higher SFA compared with fresh forage due to lipolysis and biohydrogenation (Alves et al., 2011). Because malic and citric acids are well-known antioxidants, they might inhibit the lipolysis caused by lipoxygenases during ensiling. Thus, the objectives of this study were to investigate the effects of different levels of malic or citric acids on fermentation quality, proteolysis, and lipolysis of alfalfa silage and to evaluate the utilization efficiency of these 2 organic acids in ensiled alfalfa after fermentation.

MATERIALS AND METHODS

Experimental Treatments and Mini Silo Preparation

Alfalfa (*Medicago sativa* L.) was mowed in the late bud to early bloom stage and wilted to a DM content of approximately 400 g/kg of fresh weight (**FW**). A total of 8 kg of the wilted forage was then chopped into 1- to 2-cm pieces using a paper cutter within 40 min. The chopped forage was then treated with malic acid or citric acid at application rates of 0, 0.1, 0.5, and 1% of FW. To apply the organic acids to the chopped alfalfa forage, malic acid or citric acid was dissolved in 12 mL of distilled water and mixed thoroughly with

the forages after uniform spraying onto the piles prepared for each treatment. For the untreated forage, the same amount of distilled water alone was applied. The pH values in alfalfa treated with malic acid at 0.1, 0.5, and 1% at ensiling were 6.02 ± 0.01 , 5.21 ± 0.02 , and 4.95 ± 0.06 , respectively, and the pH values in alfalfa treated with citric acid at 0.1, 0.5, and 1% at ensiling were 6.0 ± 0.02 , 5.3 ± 0.01 , and 4.79 ± 0.02 , respectively. To accurately trace the degradation of the organic acids before and after fermentation, laboratory vacuum packed mini silos were used (Johnson et al., 2005; Hoedtke and Zeyner, 2011). All the treated forages were packed into polyethylene plastic bags with initial density of 0.534 g/cm^3 (dimensions: 200 mm $\times$ 300 mm; Embossed Food saver bag; Taizhou Wenbwu Soft-Packing Color-Printing Co. Ltd., Zhejiang, China) and vacuum sealed tightly. Each polyethylene bag was packed with 200 to 230 g of wilted forage, and there were 4 replicates for each treatment. The silos were then stored at an ambient temperature of $25 \pm 2^\circ\text{C}$ for 60 d.

Chemical and FA Analyses

After 60 d of ensiling, the bags were opened and a portion of silage immediately was frozen (-20°C) for further analysis. Initial fresh forage samples were taken before samples were ensiled. A 20-g FW sample from each bag was placed in a juice extractor (BA-828, Mannengda Plastics Co. Ltd., Guangzhou, China), diluted with 180 mL of distilled water, squeezed for 30 s at a high speed, and filtered through 4 layers of medical gauze. The filtrate was divided into 2 parts. The pH was measured immediately; then, one part of the filtrate was acidulated with $7.14 \text{ M H}_2\text{SO}_4$ and filtered with a $0.45\text{-}\mu\text{m}$ dialyzer. Lactic acid, acetic acid, propionic acid, and butyric acid were analyzed by HPLC (Shodex KC-811 column; Shimadzu, Kyoto, Japan; oven temperature: 50°C ; flow rate: 1 mL/min ; SPD-10Avp, Shimadzu: 210 nm) as described previously (Ding et al., 2013). A total of 1 mL of 250 g/L (wt/vol) trichloroacetic acid was added to 4 mL of the second part of the filtrate from each bag. This solution was held overnight at 4°C to precipitate the protein and was centrifuged at $18,000 \times g$ for 15 min at 4°C . Next, the supernatant fluid was analyzed for ammonia nitrogen (**NH₃-N**) and AA nitrogen (**AA-N**) according to Broderick and Kang (1980) and for water-soluble carbohydrates (**WSC**) using the method of Thomas (1977). The NPN content of forage samples was analyzed after precipitating protein by using trichloroacetic acid as described by Licitra et al. (1996). Enumeration of LAB, yeasts, and molds in fresh alfalfa

and ensiled forage was performed according to methods described by Reich and Kung (2010). Briefly, samples (10 g) were homogenized in 100 mL of sterile Ringer's solution (Oxoid BR52, Basingstoke, UK) for 1 min and serially diluted (10-fold). The number of LAB was enumerated on spread plates using Rogosa agar (Oxoid CM627, Oxoid) and incubated at 30°C for 48 to 72 h. Yeasts and molds were determined by pour plating serial 10-fold dilutions of water extracts on malt extract agar (Oxoid CM0059) that had been acidified with lactic acid (concentration of 850 g/kg added at 50 g/kg, vol/vol). Plates were incubated at 32°C for 48 to 72 h. Colonies were counted from plates where appropriate dilutions yielded 30 to 300 colonies.

The DM content of the fresh forage and the remaining ensiled forage from each silo were measured by drying the samples in a forced-air oven at 65°C for 72 h. Dried samples were ground with a mill (1-mm screen). Ground samples were analyzed for Kjeldahl N (AOAC, 1990). Crude protein was calculated as Kjeldahl N $\times$ 6.25. The content of NDF assayed with heat-stable amylase (**aNDF**) and ADF was determined according to the methods of Van Soest et al. (1991) using an Ankom 200 fiber analyzer (Ankom Technology, Fairport, NY). During the analysis procedure, heat-stable α -amylase and sodium sulfite were added. The aNDF and ADF were expressed in terms of residual ash.

To analyze FA in the forages, samples were lyophilized using a freeze dryer (Labconco, Kansas City, MO) and then milled through a 1-mm screen. Fatty acids of samples (0.6 g each) were extracted using methylbenzene and methyl esterified according to the method of Sukhija and Palmquist (1988) except that 1 mL of an internal standard [1 mg/L of methyl 10-heptadecenoate (C17:0)] dissolved in chloroform was added. Extracted lipids were then dissolved in 5 mL of hexane, and 0.5 mL of Na-methylate (0.5 M) was added. The mixture was vortexed and allowed to react for 5 min. Following this, 1 g of NaHSO₄ was added and, after vortexing for a further 2 min, the samples were centrifuged at 1,400 $\times g$ for 5 min at 4°C. The upper liquid layer was used for the determination of FAME by GC with attached MS (model 6890N-5975C, Agilent Technologies, Santa Clara, CA) fitted with a fused-silica capillary column (30 m $\times$ 0.25 mm $\times$ 0.50 μ m; DB-FFAP, Agilent) as described previously (Ding et al., 2013).

Malic and Citric Acid Analyses

Malic acid and citric acid in fresh and ensiled forages were extracted from the ground samples by the method of Callaway et al. (1997). Briefly, ground samples (0.8 g) were mixed with 20 mL of distilled water in a 100-

mL screw bottle and then boiled in a 100°C water bath for 30 min. After it was stirred thoroughly with a glass stirring rod, the slurry was filtered through a Buchner funnel fixed with a filter paper. An additional 20 mL of distilled water was used to rinse excess organic acid and residue from the screw bottle. The filtrate was collected and then centrifuged for 10 min at 10,000 $\times g$ at room temperature. After filtering through a 0.22- μ m membrane, the filtrate was used for the measurement of malic acid and citric acid by using HPLC (KC-811 column, Shodex; oven temperature: 50°C; SPD: 210 nm). Samples were eluted from the column with 6 mM H₄ClO₄ at a flow rate of 0.5 mL/min.

Statistical Analysis

Data were analyzed using the GLM procedure of SAS (SAS Institute Inc., Cary, NC) according to the model for a 2 $\times$ 4 factorial treatment design:

$$Y_{ijk} = \mu + \alpha_i + b_j + (\alpha \times b)_{ij} + e_{ijk},$$

where Y_{ijk} represents the chemical composition and fermentative characteristics of the ensiled forages, μ is the overall mean, α_i is the effect of organic acid treatments i ($i = 1, 2$), b_j is the effect of application rates j ($j = 1, 2, 3$, or 4), $(\alpha \times b)_{ij}$ is the effect of interaction between organic acid treatments and application rates, and e_{ijk} is the error term. Polynomial contrast was used to examine effects of the unequally spaced application rate (by first generating the coefficients using PROC IML of SAS) as well as the organic acid type $\times$ application rate interactions. Comparisons between organic acid types within each application rate of organic acid were made using t -tests when at least one of the contrasts of organic acid type $\times$ application rate was significant. Differences were considered significant at $P < 0.05$ and a trend at $P < 0.10$.

RESULTS

Chemical Composition and Epiphytic Microflora of Forage Before Ensiling

The chemical composition and epiphytic microflora of the freshly chopped alfalfa are shown in Table 1. Before ensiling, fresh alfalfa was wilted to a DM content of 432 g/kg, and the pH value was 6.21. The counts of epiphytic microbes in fresh alfalfa such as LAB, yeasts, and molds were 6.37, 6.65, and 6.51 log₁₀ cfu/g, respectively. Only small amounts of malic acid (17.11 g/kg of DM) and citric acid (9.16 g/kg of DM) were detected in the untreated alfalfa at ensiling.

Table 1. Chemical composition and epiphytic microflora of fresh alfalfa before ensiling (mean $\pm$ SD)

Item	Value
DM, g/kg	432 $\pm$ 8.12
pH	6.21 $\pm$ 0.06
CP, g/kg	200 $\pm$ 2.34
aNDF, ¹ g/kg of DM	384 $\pm$ 8.0
ADF, g/kg of DM	287 $\pm$ 7.4
WSC, ² g/kg of DM	60.7 $\pm$ 0.86
Lactic acid bacteria, log ₁₀ cfu/g of FM ³	6.37 $\pm$ 0.02
Yeasts, log ₁₀ cfu/g of FM	6.65 $\pm$ 0.04
Molds, log ₁₀ cfu/g of FM	6.51 $\pm$ 0.02
Malic acid, g/kg of DM	17.1 $\pm$ 0.13
Citric acid, g/kg of DM	9.2 $\pm$ 0.03

¹aNDF = NDF assayed with a heat-stable amylase and expressed inclusive of residual ash.

²WSC = water-soluble carbohydrate.

³FM = fresh matter.

Fermentation Characteristics of Alfalfa Silages Ensiled for 60 d

The application of malic or citric acids at ensiling of alfalfa led to lower silage pH ($P < 0.05$; Table 2) compared with the control silage. Lower silage pH on average was observed in citric acid-treated silages than in malic acid-treated silages, but the difference was extremely small and not of practical significance (4.81 vs. 4.85; $P < 0.01$). The pH decreased and the lactic acid concentration increased linearly and quadratically with increases in the application rate of either acid ($P < 0.01$). Lactic acid concentration was greater in malic acid-treated silages when the application rates were 0.1 or 0.5% but was greater in citric acid-treated silages when the application rate reached 1% (linear interaction; $P < 0.05$). Application of these 2 organic

acids at the low level of 0.1% led to higher lactic acid concentration and lower yeast amounts compared with the control silage ($P < 0.05$), but when the application rates reached 0.5 or 1%, higher yeast amounts in alfalfa silage were observed. The yeasts increased linearly ($P < 0.001$) with the increases in application rate (from 0.1 to 1%). Treatments with 0.1% malic acid had a higher yeast population than did those with 0.1% citric acid, but lower yeast populations were observed in 0.5 or 1% malic acid-treated silages compared with 0.5 or 1% citric acid-treated silages (linear interaction; $P < 0.001$). Both malic and citric acids at all application rates decreased mold populations in ensiled forage ($P < 0.05$).

Chemical Composition of Alfalfa Silages Ensiled for 60 d

The chemical composition of alfalfa silages ensiled for 60 d is shown in Table 3. The increased application rates of organic acid linearly increased silage DM content ($P < 0.001$). The DM contents were greater in citric acid-treated silages when the application rate was 0.1 or 0.5% but were greater in malic acid-treated silages when the application rate was 1% (linear and quadratic interactions; $P < 0.05$). On average, the concentration of WSC was higher in silages treated with malic acid than in those treated with citric acid (15.45 vs. 10.64 g/kg of DM; $P < 0.001$), and with the increase of organic acid application rates (from 0.1 to 1%), the WSC concentration in ensiled forages increased accordingly. The NPN concentration decreased linearly ($P < 0.01$) with the increase of citric acid application rates, and the effect of malic acid was curvilinear with

Table 2. Fermentation characteristics of alfalfa silages ensiled for 60 d

Item	Acid type ¹	Application rate (%)					P-value ²				
		0	0.1	0.5	1	SEM	A	AR-L	AR-Q	AR-L $\times$ A	AR-Q $\times$ A
pH	Malic acid	5.19	4.85	4.58	4.79	0.006	0.01	<0.001	<0.001	0.015	0.005
	Citric acid	5.19	4.79	4.61	4.67						
Lactic acid, g/kg of DM	Malic acid	67.0	92.2	81.1	37.1	1.75	0.309	<0.001	<0.001	0.026	0.156
	Citric acid	67.0	88.3	78.9	58.1						
Acetic acid, g/kg of DM	Malic acid	13.2	10.4	10.8	12.2	0.39	0.244	0.048	0.006	0.046	0.244
	Citric acid	13.2	10.1	10.7	16.5						
Lactic acid bacteria, log ₁₀ cfu/g of FM ³	Malic acid	7.44	7.10	7.14	6.51	0.024	0.102	<0.001	0.228	0.292	0.001
	Citric acid	7.44	7.39	6.84	6.85						
Yeasts, log ₁₀ cfu/g of FM	Malic acid	4.18	3.60	4.43	4.42	0.022	<0.001	<0.001	<0.001	<0.001	0.001
	Citric acid	4.18	3.16	5.19	5.10						
Molds, log ₁₀ cfu/g of FM	Malic acid	4.40	3.16	3.16	3.16	0.049	0.094	<0.001	<0.001	0.439	0.126
	Citric acid	4.40	3.09	2.69	3.00						

¹Organic acid-treated alfalfa silages.

²A = acid type; AR-L = linear effect of application rate; AR-Q = quadratic effect of application rate; AR-L $\times$ A = interaction of linear application rate effect with acid type; AR-Q $\times$ A = interaction of quadratic application rate effect with acid type.

³FM = fresh matter.

Table 3. Chemical composition of alfalfa silages ensiled for 60 d

Item	Acid type ¹	Application rate (%)				SEM	P-value ²				
		0	0.1	0.5	1		A	AR-L	AR-Q	AR-L × A	AR-Q × A
DM, g/kg	Malic acid	379	373	383	429	0.67	<0.001	<0.001	0.864	<0.001	<0.001
	Citric acid	379	384	415	410						
WSC, ³ g/kg of DM	Malic acid	8.60	7.30	11.0	28.1	0.11	<0.001	<0.001	<0.001	<0.001	<0.001
	Citric acid	8.60	6.00	10.1	15.8						
CP, g/kg of DM	Malic acid	215	218	219	222	1.09	0.167	0.003	0.523	0.187	0.608
	Citric acid	215	218	225	229						
NPN, g/kg of total N	Malic acid	696	609	651	550	5.86	0.756	<0.001	0.713	0.243	0.165
	Citric acid	696	632	607	549						
AA-N, ⁴ g/kg of total N	Malic acid	187	235	218	83	2.12	<0.001	<0.001	0.004	<0.001	<0.001
	Citric acid	187	164	117	146						
NH ₃ -N, g/kg of total N	Malic acid	132	100	38.1	11.3	0.62	<0.001	<0.001	<0.001	<0.001	<0.001
	Citric acid	132	68.2	24.9	14.8						
aNDF, ⁵ g/kg of DM	Malic acid	429	416	404	389	3.39	0.675	0.0152	0.031	0.142	0.096
	Citric acid	429	414	389	418						
ADF, g/kg of DM	Malic acid	329	308	299	289	3.42	0.566	0.031	0.020	0.158	0.194
	Citric acid	329	306	290	316						

¹Organic acid-treated alfalfa silages.²A = acid type; AR-L = linear effect of application rate; AR-Q = quadratic effect of application rate; AR-L × A = interaction of linear application rate effect with acid type; AR-Q × A = interaction of quadratic application rate effect with acid type.³WSC = water-soluble carbohydrate.⁴AA-N = free AA nitrogen.⁵aNDF = NDF assayed with a heat-stable amylase and expressed inclusive of residual ash.

an increase in NPN in the 0.5% malic acid treatment. Additionally, silages treated with either of the organic acids had lower NPN concentrations than did the control silages ($P < 0.05$). An interaction between organic acid type and application rate was observed in AA-N and NH₃-N. The AA-N and NH₃-N were higher in malic acid-treated silages under the application rates of 0.1 or 0.5% but higher in citric acid-treated silages under the highest application rate of 1% (linear and quadratic interactions; $P < 0.05$). There were effects of organic acid type and their application rates on the concentration of NH₃-N in ensiled forages, and lower NH₃-N concentrations on average were observed in citric acid-treated silages than in the malic acid-treated silages (35.9 vs. 49.9 g/kg of total N; $P < 0.05$). Moreover, application of malic or citric acids at ensiling led to lower NH₃-N concentrations than those observed in the control silages ($P < 0.05$).

FA Composition of Fresh Alfalfa and Alfalfa Silages Ensiled for 60 d

On average, total FA in citric acid-treated silages was greater than that in malic acid-treated silages (22.05 vs. 19.18 g/kg of DM; $P < 0.05$; Table 4). On average, the application of the 2 organic acid additives led to lower SFA proportions and higher PUFA proportions in ensiled alfalfa ($P < 0.05$) than in the control

silages. In addition, citric acid-treated silage had a lower proportion of SFA and a greater proportion of PUFA in total FA at the respective application rates than malic acid-treated silage ($P < 0.05$). There were organic acid type × application rate linear interactions on SFA and PUFA ($P < 0.001$). The SFA decreased and PUFA increased linearly and quadratically ($P < 0.01$) in silage with the increased application rates of the organic acids. As expected, a higher proportion of C16:0 in total FA was observed after alfalfa was ensiled for 60 d than in fresh alfalfa. Application of these acids led to a lower proportion of C16:0 than in the control silage, and C16:0 was higher in malic acid-treated silages than in citric acid-treated silages under an application rate of 0.1% ($P < 0.05$). Although there was no difference between the 2 additives in the proportion of C16:0 in total FA, applying malic acid or citric acid at ensiling led to a markedly higher proportion of *cis*-9,*cis*-12,*cis*-15 C18:3 in total FA of ensiled forage than in the control silage ($P < 0.05$), and the FA proportion of *cis*-9,*cis*-12,*cis*-15 C18:3 in the silage treated with citric acid was greater than that in the silage treated with malic acid ($P < 0.05$). There was also an organic acid type × application rate interaction for the proportion of *cis*-9,*cis*-12,*cis*-15 C18:3. The increased application rates of citric acid resulted in increased proportion of *cis*-9,*cis*-12,*cis*-15 C18:3 in silage, but no differences in the proportion were observed among the silages treated with the 3 application rates of malic acid.

Table 4. Total fatty acid (FA) content (mg/g of DM) and the major FA composition (g/100 g of total FA) of fresh alfalfa and alfalfa silages ensiled for 60 d

Item	Fresh alfalfa ¹	Acid type ²	Application rate (%)				SEM	<i>P</i> -value ³				
			0	0.1	0.5	1		A	AR-L	AR-Q	AR-L × A	AR-Q × A
Total FA	20.3	Malic acid	17.3	19.0	18.5	20.1	0.163	<0.001	<0.001	0.046	0.002	0.022
		Citric acid	17.3	20.6	22.2	23.4						
C16:0	18.8	Malic acid	27.7	25.7	23.3	22.8	0.106	0.939	<0.001	<0.001	0.057	0.592
		Citric acid	27.7	24.6	23.9	23.3						
C18:2 <i>cis</i> -9, <i>cis</i> -12	16.7	Malic acid	16.2	15.4	17.4	16.8	0.059	0.578	0.003	0.001	<0.001	0.101
		Citric acid	16.2	16.6	16.6	16.2						
C18:3 <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15	43.7	Malic acid	41.1	43.5	43.4	43.2	0.051	<0.001	<0.001	<0.001	<0.001	0.226
		Citric acid	41.1	44.4	45.5	46.7						
SFA	35.1	Malic acid	39.5	37.7	35.9	36.6	0.070	<0.001	<0.001	<0.001	<0.001	0.527
		Citric acid	39.5	35.9	34.9	34.1						
MUFA	4.58	Malic acid	3.21	3.38	3.24	3.37	0.034	0.009	0.610	0.434	0.175	0.849
		Citric acid	3.21	3.12	3.04	3.02						
PUFA	60.4	Malic acid	57.3	58.9	60.9	60.0	0.076	<0.001	<0.001	<0.001	<0.001	0.618
		Citric acid	57.3	61.0	62.1	62.9						

¹Alfalfa before ensiling.²Organic acid-treated alfalfa silages.³A = acid type; AR-L = linear effect of application rate; AR-Q = quadratic effect of application rate; AR-L × A = interaction of linear application rate effect with acid type; AR-Q × A = interaction of quadratic application rate effect with acid type.

Degradation and Residue of Malic Acid and Citric Acid in Alfalfa Silages Ensiled for 60 d

The residue and utilization efficiency of malic and citric acids in alfalfa silages are shown in Table 5. After ensiling, concentrations of malic acid and citric acid decreased from 17.9 and 9.2 g/kg of DM to 2.7 and 4.23 g/kg of DM, respectively. As expected, on average higher residues of malic acid were found in silages treated with malic acid than in those treated with citric acid (12.3 vs. 8.4 g/kg of DM; $P < 0.05$). The degraded amounts of malic acid in ensiled forage did not increase with the increase of malic acid application rates from 0.1 to 1% of FW, but the increased application rates of citric acid decreased the amount of degraded malic acid in ensiled forage ($P < 0.05$). The highest degradation rate of malic acid was observed in silages treated with 0.1% of malic acid. The average degradation rates of malic acid in silages treated with malic and citric acids were 62 and 55 g/100 g, respectively. Small amounts of citric acid were degraded in silages treated with 0.1 and 0.5% of malic acid, and an even higher amount of citric acid (11.6 g/kg of DM) was detected in silage treated with 1% of malic acid compared with fresh forage (9.2 g/kg of DM). The amount of degraded citric acid decreased with the increased application rates of malic acid ($P < 0.05$), whereas the increased application rates of citric acid did not affect the amount of citric acid degraded in silage. The average degradation rates of citric acid in silages treated with malic and citric acids were 10.9 and 37.7 g/100 g, respectively.

DISCUSSION

It is well known that it is difficult to generate good silage using alfalfa because of its high buffer capacity and low WSC concentration (Owens et al., 1999). Therefore, inoculants and organic acids such as formic acid, propionic acid, and benzoic acid are commonly used to promote a rapid decline of silage pH and to improve the fermentation quality of ensiled forage (Mills and Kung, 2002; Tyrolova and Vyborna, 2008; Li et al., 2016). However, the corrosive effect of these organic acids on machinery during application is a problem in practice despite buffered organic acids currently being used. Malic and citric acids are widely used in the food industry because of their safety, in addition to many other uses. In the present study, the lower pH, greater lactic acid, and lower acetic acid concentrations in silages treated with malic and citric acids (at application rates of 0.1 or 0.5% of FW) indicate that including malic or citric acids at ensiling could improve or alter the fermentation process of the ensiled alfalfa. These acids were also reported to stimulate *in vitro* mixed ruminal microorganism fermentation (Martin and Streeter, 1995; Carro and Ranilla, 2003; Wang et al., 2009a). Nevertheless, when the application level of these 2 acids reached 1% of FW, remarkable declines in the lactic acid concentration and the number of LAB were observed, which suggests that the growth and fermentation of LAB were inhibited by the high concentration of malic or citric acids in ensiled forage. These results are probably attributable to the low pH

Table 5. Residue and degradation of malic acid and citric acid in alfalfa silages ensiled for 60 d

Item	Acid type ¹	Application rate ² (%)				SEM	P-value ³				
		0	0.1	0.5	1		A	AR-L	AR-Q	AR-L × A	AR-Q × A
Residue of malic acid, g/kg of DM	Malic acid	2.70	2.60	11.1	23.3	0.185	<0.001	<0.001	0.174	<0.001	<0.001
	Citric acid	2.70	3.47	7.88	13.8						
Amount of malic acid degraded, ⁴ g	Malic acid	1.33	1.42	1.60	1.32	0.011	<0.001	<0.001	<0.001	<0.001	<0.001
	Citric acid	1.33	1.23	0.91	0.38						
Degradation rate of malic acid, g/100 g	Malic acid	86.7	88.8	66.9	39.8	0.429	<0.001	<0.001	0.174	<0.001	0.032
	Citric acid	86.7	82.7	56.8	25.6						
Residue of citric acid, g/kg of DM	Malic acid	4.23	5.68	8.11	11.6	0.395	<0.001	<0.001	<0.001	<0.001	0.073
	Citric acid	4.23	6.37	13.6	26.0						
Amount of citric acid degraded, ⁵ g	Malic acid	0.50	0.32	0.19	-0.26	0.009	<0.001	<0.001	0.367	<0.001	0.073
	Citric acid	0.50	0.52	0.75	0.62						
Degradation rate of citric acid, g/100 g	Malic acid	61.0	42.6	24.5	-34.4	1.177	<0.001	<0.001	<0.001	<0.001	0.953
	Citric acid	61.0	52.5	38.6	21.9						

¹Organic acid-treated alfalfa silages.

²Total amounts of each acid added at ensiling under application rates of 0.1, 0.5, and 1% were 0.19, 0.97, and 1.97 g, respectively.

³A = acid type; AR-L = linear effect of application rate; AR-Q = quadratic effect of application rate; AR-L × A = interaction of linear application rate effect with acid type; AR-Q × A = interaction of quadratic application rate effect with acid type.

⁴The amount of malic acid degraded was calculated as total amount of malic acid in alfalfa silos at ensiling minus the total residue amount of malic acid in alfalfa silos after ensiling.

⁵The calculation was the same as that of malic acid.

value of acid-treated alfalfa at ensiling. When adding malic acid and citric acid at the level of 1% of FW, the pH values of alfalfa at ensiling were 4.95 and 4.79, respectively; however, at the application levels of 0.1 or 0.5% of FW, the pH values of alfalfa at ensiling ranged from 5.21 to 6.03.

Compared with the untreated silages, application of malic or citric acids at a low level of 0.1% of FW decreased the number of yeasts in ensiled alfalfa. Interestingly, yeast growth was promoted after the 2 acids were applied at higher levels of 0.5 and 1% of FW. Moreover, our results showed that citric acid was more effective than malic acid in promoting the growth of yeast in ensiled alfalfa when their application rates were over 0.5% of FW. The results of previous studies suggest that malic acid or citric acid can be used by some yeast strains as a carbon and energy source (Zhao et al., 2004; Seo et al., 2007). Therefore, the growth of yeasts might be more competitive than LAB when higher levels of malic or citric acids are applied to ensiled alfalfa. Adding malic or citric acids at ensiling decreased the number of molds in alfalfa silage after 60 d of fermentation, but application levels of malic or citric acid did not affect the number of molds in ensiled alfalfa.

Because including malic or citric acids provided additional substrates to the silage fermentation, the DM and WSC content in ensiled alfalfa increased with the increased application of malic or citric acids. Reduction in proteolysis during ensilage is a conventional but prevalent concern of ensiled legume forage because extensive proteolysis in silage, mainly caused by plant proteases (Ding et al., 2013), normally results in a low

efficiency of N utilization by ruminants (Givens and Rulquin, 2004). Lowering pH value in ensiled forage can effectively inhibit proteolysis because plant enzymes are quickly inactivated with a decrease in pH (Kung and Bedrosian, 2010). A greater reduction of NPN and NH₃-N concentrations in silages treated with higher levels of malic or citric acids was mainly attributed to the lower pH values caused by acid treatment of fresh alfalfa at ensiling and by the fermentation of alfalfa after ensiling. When applying the 2 organic acids at rates of 0.1 or 0.5%, citric acid was more effective than malic acid in reducing AA-N and NH₃-N in silage, but the opposite was true as the application rate reached 1%.

Additionally, the FA composition in ensiled forages has been of interest. Generally, linoleic acid (C18:2n-6) and α -linolenic acid (C18:3n-3) make up a major proportion of the FA composition of fresh and ensiled forage (Clapham et al., 2005; Ding et al., 2013). It was reported that a high intake of PUFA could increase the concentration of PUFA in ruminant products (Wood and Enser, 1997; Kalač and Samková, 2010) and consequently be beneficial to human health (Simopoulos, 2001). Therefore, most of the studies on the FA composition of silages are concerned with losses of C18:2n-6 and C18:3n-3 during ensiling (Alves et al., 2011). In agreement with previous reports (Vanhatalo et al., 2007; Ding et al., 2013), ensiling led to lower total FA compared with fresh forage. In addition, including malic or citric acids at ensiling caused a better preservation of total FA in ensiled alfalfa, especially in citric acid-treated silages. Application of malic or citric acids had a small effect on C18:2n-6 but improved the pro-

portion of C18:3n-3 in total FA. The increased proportions of C18:3n-3 and PUFA as well as the decreased SFA in silages treated with malic or citric acids might be attributable to inhibition of lipoxygenases by these 2 acids because malic acid and citric acid are well-known antioxidants (Brittain, 2001; Aktac et al., 2003). Moreover, citric acid was more effective in increasing the proportions of C18:3n-3 and PUFA than malic acid. With the increased application rates of citric acid, the proportion of C18:3n-3 in silage increased accordingly, but there was no effect of the increased application rates of malic acid.

Compared with the fresh forage, extensive degradation of malic acid and citric acid were observed after ensiling. Concentrations of malic acid and citric acid decreased from 17.9 and 9.2 g/kg of DM in fresh forage to 2.7 and 4.23 g/kg of DM in the control silage, respectively. Based on the results of degradation of malic and citric acids during ensiling of alfalfa (1.45 vs. 0.63 g), it can be concluded that the microbes in ensiled alfalfa preferred to use malic acid as a substrate rather than citric acid. However, the fermentation quality and chemical composition of ensiled alfalfa treated by either malic acid or citric acid were comparable. The observed negative value of citric acid degradation in 1% of FW of malic acid-treated silage was probably attributable to conversion of malic acid into citric acid during the fermentation of ensiled alfalfa by microbes, as indicated in the tricarboxylic acid cycle.

Previous studies indicate that a supplement of 50 g (Sniffen et al., 2006) or 84 g (Devant et al., 2007) of malic acid/cow per day could increase milk production by approximately 2 kg/d when fed to dairy cows with a TMR in which silage composed around 50% of dietary DM. Martin et al. (1999) also reported that providing steers with 80 g of malic acid/d effectively led to higher ADG of steers compared with the control group (2.11 vs. 1.86 kg). Moreover, supplementation of citric acid at 100 g/steer per day was proven to increase fiber digestibility by steers (Wang et al., 2009a). Li (2005) reported that supplying dairy cows with 20 g of citric acid/d could effectively improve daily milk production. Our results showed that applying 0.5% of FW of malic or citric acids at ensiling was optimal to improve fermentation quality and FA composition and to effectively inhibit proteolysis in ensiled alfalfa. At this application rate, the residues of malic acid and citric acid in alfalfa silage were 11.1 and 13.6 g/kg of DM, respectively. In this case, if a dairy cow consumes 20 kg of malic acid- or citric acid-treated alfalfa silage/d, the malic or citric acid provided by alfalfa silage would be approximately 80 to 100 g, which meets the reported amounts of these 2 organic acids required to improve animal performance.

CONCLUSIONS

Including malic or citric acids at low application rates of 0.1 or 0.5% at the ensiling of alfalfa could effectively improve silage fermentation quality, limit proteolysis, and improve FA composition of the ensiled forage. When the application of both organic acids reached 1% of FW, fermentation in ensiled forage tended to decrease. The effects of malic acid and citric acid on fermentation quality and reduction of NPN in ensiled forage were comparable, but citric acid was more effective than malic acid in improving FA composition of the ensiled forage. Supplementation with 0.5% of malic or citric acids at ensiling of forage improves silage fermentation and can provide additional feed additives that have proven to promote animal performance. However, the negative effect of increased amounts of yeast after application of a high dose of either acid should be considered, and considering the costs of these 2 organic acids, citric acid is more acceptable in practice.

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