Kafkas Univ Vet Fak Derg
24 (3): 371-378, 2018
DOI: 10.9775/kvfd.2017.18999

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://submit.vetdergikafkas.org

Research Article

Effect of Applying *Lactobacillus plantarum* and *Pediococcus acidilactici Isolated* on Fermentation Dynamics, Microbial Community and Aerobic Stability of Napier Grass *(Pennisetum purpureum)* Silage

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Article Code: KVFD-2017-18999 Received: 13.11.2017 Accepted: 01.03.2018 Published Online: 02.03.2018

How to Cite This Article

Sifeeldein A, Yuan X, Dong Z, Li J, Shao T: Effect of applying *Lactobacillus plantarum* and *Pediococcus acidilactici* Isolated on fermentation dynamics, microbial community and aerobic stability of napier grass *(Pennisetum purpureum)* silage. *Kafkas Univ Vet Fak Derg,* 24 (3): 371-378, 2018. DOI: 10.9775/kvfd.2017.18999

Abstract

The aim of this study was to determine the effect of applying three of lactic acid bacteria isolates AZZ1, AZZ4, AZZ7 and one commercial (CB) on the fermentation dynamics, microbial community and aerobic stability of Napier grass silage. The grass was ensiled with or without the addition of a lactic acid bacteria inoculant at 1.0×10^6 CFU/g of fresh material to Napier grass silage. Napier grass was ensiled in laboratory silos and treated as follows: no additives (control), *Pediococcus acidilactici* (AZZ1), *Lactobacillus plantarum* subsp. *plantarum* (AZZ4), *Lactobacillus plantarum* subsp. *argentoratensis* (AZZ7) and *Lactobacillus plantarum,* Ecosyl MTD/1 (CB). To follow the fermentation dynamics during ensiling samples were taken on days 7, 14, 30, 60 and 90 of ensiling for chemical and microbiological analysis. Aerobic stability was determined on day 90 of ensiling. The experimental design was a completely randomized design, with a $5 \times 5 \times 3$. The inoculant resulted in a more decreased ($P<0.0001$) in pH, water soluble carbohydrate, ammonia nitrogen, organic acid, aerobic bacteria and yeast, while a higher ($P<0.0001$) level of lactic acid content and lactic acid bacteria compared with control group. Treatment with AZZ4 had the better ($P<0.0001$) fermentation results. The aerobic stability of Napier silage was reduced with strains treatment compared with the control. In conclusion, the addition of bacteria inoculant resulted in better preservation and reduced the aerobic stability of silage.

Keywords: *Lactobacillus plantarum, Pediococcus acidilactici, Silage, Aerobic stability, Napier grass*

Lactobacillus plantarum ve *Pediococcus acidilactici* Kullanımının Fil Otu *(Pennisetum purpureum)* Silajının Fermantasyon Dinamikleri, Mikrobiyal İçerik ve Aerobik Stabilitesi Üzerine Etkisi

Öz

Bu çalışmanın amacı, üç adet izole edilmiş AZZ1, AZZ4 ve AZZ7 ile bir adet ticari (CB) laktik asit bakteri izolatları kullanımının Fil otu silajında fermentasyon dinamikleri ve aerobik stabilitesi üzerine etkisini belirlemektir. 1.0×10^6 CFU/g laktik asit bakteri inokulantı içeren ve içermeyen Fil otu silajı hazırlandı. Fil otu laboratuvar silosunda silajlandı ve şu gruplar oluşturuldu; katkı yok (kontrol), *Pediococcus acidilactici* (AZZ1), *Lactobacillus plantarum* subsp. *plantarum* (AZZ4), *Lactobacillus plantarum* subsp. *argentoratensis* (AZZ7) ve *Lactobacillus plantarum,* Ecosyl MTD/1 (CB). Silajlama süresince fermantasyon dinamiklerini takip etmek amacıyla 7, 14, 30, 60 ve 90. günlerde örnekler alınarak kimyasal ve mikrobiyolojik analizler gerçekleştirildi. Silajlamanın 90. gününde aerobik stabilite belirlendi. Deneysel çalışma, tamamıyla rastgele dizaynda ve $5 \times 5 \times 3$ olarak gerçekleştirildi. İnokulant kullanımı kontrol grubu ile karşılaştırıldığında pH, suda eriyebilen karbonhidrat, amonyum nitrojen, organik asit, aerobik bakteri ve mayada daha fazla azalmaya ($P<0.0001$) laktik asit içeriği ve laktik asit bakteri miktarında ise artmaya neden oldu ($P<0.0001$). AZZ4 ile muamele daha iyi ($P<0.0001$) fermantasyon sonuçlarına neden oldu. Fil otu silajının aerobik stabilitesi kontrol ile karşılaştırıldığında suşlar ile muamele edilenlerde azaldı. Sonuç olarak, bakteri inokulant ilavesi silajda daha iyi muhafaza ve aerobik stabilitede azalmaya neden oldu.

Anahtar sözcükler: *Lactobacillus plantarum, Pediococcus acidilactici, Silaj, Aerobik stabilite, Fil otu*

INTRODUCTION

Napier grass *(Pennisetum purpureum* Schum.), is an important tropical grass with high biomass yield, now widely planted in southern regions of China, where it is commonly used for silage making. However, it has high

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moisture content and insufficient water soluble carbohydrate (WSC) at vegetative stage, which sometimes resulted in clostridial fermentation [1]. Furthermore, its low crude protein content [2] and high structural carbohydrate contents [3,4] usually lead to low nutritive value of silage. Hay making is not practical in southwest China due to high humidity, and forage supply for livestock tends to be sufficient in summer, and scanty in spring, respectively. Therefore, ensiling is a common method of preserving the nutritive value of forage and supplying domestic animals with moderate feedstuff in spring.

When forage is ensiled, the epiphytic lactic acid bacteria (LAB) convert WSC mainly into lactic acid (LA) under anaerobic conditions, decreasing the pH to the point at which undesirable microbes are not able to survive [5]. Thus, the forage can be preserved as feedstuff. Air does harm to silage quality because undesirable microbes, such as yeasts and molds, will become active when exposed to air. During exposure to air in the storage and feeding phase, aerobic spoilage of silage is a major cause of low nutritive value, and it also brings about proliferation of potentially disgusting microbes [6]. Susceptibility to spoilage is a vital problem for ensiling forages in warm climates [7]. Hence, additives that protect the silage upon exposure to air may be helpful in subtropical areas. Biological additives are more suitable because they are safe and easy to use, do not pollute the environment, non-corrosive to machinery, and are natural products also they are preferred over chemical additives such as formic acid and formaldehyde [8]. It has been shown by McDonald [9] that one of the key factors for the successful application of microbial additives in silages is the harmony between the ensiled forage plant and the microorganisms used. A number of studies [10-12] reported positive effects on silage fermentation from using some LAB inoculants as silage additives, relatively few [11,12] have reported their effect on silage deterioration. In this experiment, selected lactobacilli and *Pediococcus* strains isolated from elephant grass were used as silage additives, and their effect on fermentation dynamics, microbial community and aerobic deterioration of silage was examined.

MATERIAL and METHODS

Napier grass *(Pennisetum purpureum)* was grown at the experimental field of Nanjing Agricultural University Jiangsu, China (Latitude 32°01_19"N, Longitude 118°51_08" E, at Altitude 17 m above sea level). The Napier grass at dough stage was harvested for the first cutting on 13 October 2016, and chopped manually to an approximate length of 2-3 cm. The chopped forage were inoculated with three isolated strains of LAB *Pediococcus acidilactici* (AZZ1), *Lactobacillus plantarum subsp. plantarum* (AZZ4), *Lactobacillus plantarum subsp. Argentoratensis* (AZZ7) and commercial LAB inoculant *Lactobacillus Plantarum*, Ecosyl MTD/1 (CB) Ecosyl Product Inc. USA. All strains were isolated from previously fermented juice of elephant grass via our laboratory, identified by phenotype, 16S rRNA, and RecA gene analysis., then suspended in 20% glycerol and stored at -20°C. The grass was subsequently mixed homogeneously, packed, and compressed manually into approximately 1.32 L (9.5 cm diameter × 18.7 cm height), then ensiled in a laboratory silo and sealed airtight with a screw top. Five treatments were prepared, including: (1) no addtives (control), (2) AZZ1 inoculant, (3) AZZ4 inoculant, (4) AZZ7 inoculant and (5) CB inoculant were applied as additives at 1.0×10^6 CFU/g of fresh material to Napier grass silage, control treatment was sprayed with equal distilled water. Additives were applied using a hand sprayer by spraying uniformly onto the mixture that was constantly hand mixed. After treating and thorough mixing, each treated batch was used to fill a silo, which was sealed with a screw top and plastic tape. A total of 75 laboratory silos were made (5 days × 5 treatments × 3 replicates) for each treatment and kept at 25°C in ambient temperature. Triplicate jars for each treatment were opened and sampled evenly under constant mixing on days 7, 14, 30, 60 and 90.

Chemical Analyses

The DM contents of pre-ensiling forages and silages were determined at 65°C in a forced-air oven for 48 h. Total nitrogen (TN) was analysed by the Kjeldahl method [13], CP was determined as 6.25 multiplied by TN. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed using the method of Van Soest et al.[14]. The WSC content were determined by colourimetric method after reaction with anthrone reagent [15]. The ammonia-N (NH_3-N) was determined according to the method of phenol-hypochlorite reaction [16]. The pH of fresh forage and silage were measured using a pH meter (F-23; Horiba, Tokyo, Japan). Organic acids contents of silage, including the lactic acid (LA), acetic acid (AA), propionic acid (PA) and butyric acid (BA) were analyzed by high-performance liquid chromatography (Agilent Technologies, CA, USA) according to the methods described by Liu et al.[17].

Microbial Population

A sub-sample (10 g) of wet silage of each sample were added to 90 mL of sterilized saline solution (8.50 g L^{-1} NaCl), completely immersed, and shaken well. Decimal dilutions of 10^{-1} to 10^{-6} were prepared from these extracts for microbiological counting. The enumeration of LAB, aerobic bacteria, and yeast was carried out by using de Man, Rogosa, and Sharpe agar, nutrient agar, and potato dextrose agar, respectively. The Petri dishes were incubated at 37ºC, and the bacteria enumeration was done manually after the certain time of incubation determined for the growth of the microorganism (48 to 72 h). Finally, the overall microbial data were transformed to log10 and presented on a wet weight basis.

Aerobic Stability

After 90 d of ensiling, a total of 45 silos (1.32 L capacity)

per treatment were opened for aerobic stability test. Napier grass silages from each silo were mixed thoroughly and placed into separate new sterile plastic laboratory silos (approximately 6.2 L capacity, 17.3 cm diameter × 26.5 cm height) without compression, kept uncovered and stored at ambient temperature (18-25°C). Three silos from each treatment were removed at 0, 3, 5 and 7 d after aerobic exposure, and samples were taken for subsequent chemical and microbial analyses, including, the pH value and the content of LA, AA, PA, BA, NH_3-N and WSC as well as the counts of the LAB, aerobic bacteria and yeast.

Statistical Analyses

All microbial data were transformed to log units and presented on a wet weight basis. Results collected from different time points were analyzed using the general linear model (GLM) procedure of Statistical Analysis System [18]. The model used for the analysis was: Y = I + treatment + time + treatment time + E where Y = observation, I = general mean, treatments = effect of control or AZZ1 or AZZ4 or AZZ7or CB, time = days, treatment ×time = interaction between treatment and time, E = residual error. Data from the aerobic stability test were analyzed using the general linear model (GLM) procedure of Statistical Analysis System [18]. The model used for the analysis was: Y = I + treatment + time + treatment time + E where Y = observation, I = general mean, treatments = effect of control or AZZ1 or AZZ4 or AZZ7or CB, time = days, treatment ×time = interaction between treatment and time, E = residual error. Duncan multiple comparisons have been used to contrast means among different treatment groups. The significant difference was declared at $P<0.05$.

RESULT

Chemical Composition of Materials

The chemical and microbiological compositions of fresh Napier grass before ensiling are shown in *Table 1*. The number of lactic acid bacteria was low on the material before ensiling. However, tropical grasses had a low number of lactic bacteria, lower than 10^6 CFU/g fresh forage.

Fermentation Characteristics of Napier Grass Silages

The dynamics of pH, DM, WSC, and ammonia- N of Napier grass silages are shown in *Table 2*. The treatment, day of fermentation and their interaction significantly influenced the pH, DM, WSC, and ammonia- N of Napier grass silages *(P*<.0001). The pH values of all silages decreased gradually after 7 d of fermentation; the lowest value was observed at d 90. The pH values of all of the LAB-treated silages were lower *(P*<.0001) than those of control silages. The pH value decrease with increasing fermentation period *(P*<.0001), silage treated with AZZ4 had the lowest pH value (4.11), whereas that of the control was still 4.60 after 90 d of ensiling. The DM content decreased significantly *(P*<.0001) as the time of fermentation, and decreased slightly from the 7th d of ensiling, reaching the lowest *(P*<.0001) at the end of ensiling (d 90). The lowest dry matter was observed in silage treated with CB compare with silage treated with AZZ1, AZZ4 and AZZ7.

Table 1. *The chemical and microbial composition of Napier grass before ensiling*

Items	Elephant Grass
DM (g/kg FW)	285
CP (g/kg DM)	128
NDF (g/kg DM)	287
ADF (g/kg DM)	351
WSC (g/kg DM)	55.6
Buffer capacity (mEq/kg DM)	284
pH	5.97
LAB (log cfu/g FW)	4.74
Aerobic bacteria (log cfu/g FW)	6.53
Mold(log cfu/g FW)	4.36
Yeast(log cfu/g FW)	4.85

ADF: Acid detergent fiber; NDF: Neutral detergent fiber; CP: Crude protein; DM: Dry matter; FW; Fresh weight; LAB: lactic acid bacteria; mEq: milligram equivalent; WSC: water soluble carbohydrates

The residual WSC decreased *(P*<.0001) over the first 14 d of ensiling from the original values of fresh forage, and then slowly declined until the end of the ensiling period *(Table 2)*. At the 7th d of ensiling the control silage showed significantly *(P*<.0001) higher WSC content than treated silages, however, this difference was reversed by the end of the ensiling period. The total of NH_3-N slightly increased *(P*<.0001) during the first 14 d of ensiling and then significantly increased *(P*<.0001) at 90 d of ensiling *(Table 2)*. The content of NH_3-N in the silage treated with CB was significantly higher *(P*<.0001) compare with silage treated with AZZ1, AZZ4 and AZZ7.

The content of LA, AA, BA and PA content of experimental silage are presented in *Table 2*. Treatment, day of fermentation and their interaction significantly affects LA, AA, BA, PA and LA/AA content of Nnapier silage *(P*<.0001). Lactic acid was produced rapidly during the first 7 d in treated silages, with the LA content was more than three times that of the control silage, and this difference was maintained up to 90 d of ensiling. The highest LA content was observed in the silages treated with AZZ4 compare with control silages. Acetic acid (AA) concentration increased gradually in all silages during the storage period *Table 2*. Treated silages showed significantly *(P*<.0001) lower AA content than the control, and the silage treated with AZZ1 and CB had the highest AA concentration during the full fermentation course.

Changes in the PA and BA concentrations showed a similar trend to the AA concentrations. Propionic acid and BA concentrations tended to increase with prolonged storage, and were significantly *(P*<.0001) lower in treated silages as

***Table 2**. Changes on fermentation characteristics of the Napier grass silages during ensiling periods*

Days of Fermentation	Treatments	pH	DM (g/kg DM)	WSC (g/kg DM)	NH_3-N (g/kg DM)	LA (g/kg DM)	AA (g/kg DM)	PA (g/kg DM)	BA (g/kg DM)	LA/ AA (g/kg DM)
7	Control	5.27^{a}	282.76^{a}	11.58^{a}	28.69^{a}	15.65^{b}	16.79^{a}	1.5^{8a}	5.50^{C}	0.93^{c}
	CB	4.81^{b}	279.96^{a}	7.48^{c}	25.91^{c}	41.51^{a}	8.42^{a}	1.52^{c}	3.59^{c}	4.92^{b}
	AZZ1	4.77^{b}	280.43^{a}	7.91^{c}	25.73^{b}	41.84^{b}	7.95^{b}	1.34^{ab}	3.14^{b}	5.22^{b}
	AZZ4	4.43^{b}	281.66^{a}	9.56^{b}	23.85^{c}	45.38^{a}	6.45^{d}	0.79^{c}	2.36^{c}	7.03^{a}
	AZZ7	4.68^{b}	281.22^{a}	8.69^{a}	24.77^{bc}	42.71^{a}	7.30^{a}	0.97^{bc}	2.91^{cb}	5.85^{b}
	SE	0.09	0.50	0.42	0.56	0.03	1.25	0.10	0.36	0.70
14	Control	5.10^{a}	281.99^{a}	10.20^{a}	30.49^{a}	17.22^{c}	17.28^{a}	1.88^{a}	5.75^{c}	0.99^{d}
	CB	4.65^{a}	279.71^{a}	6.21^{c}	26.87^{d}	44,93^{b}	12.97^{b}	1.95^{b}	3.68^{b}	3.46^{b}
	AZZ1	4.53^{b}	280.23^{a}	6.48^{d}	26.60^{b}	45.45^{c}	12.67^{b}	1.80^{a}	3.31b	3.58^{c}
	AZZ4	4.32^{b}	281.46^{a}	8.41^{b}	24.62^{d}	59.60^{a}	6.86^{d}	1.07^{c}	2.59^{bc}	8.68^{a}
	AZZ7	4.50^{a}	281.13^{a}	7.90^{c}	25.58^{c}	46.83^{b}	7.79^{c}	1.39^{b}	2.98^{bc}	6.01^{b}
	SE	0.09	0.54	0.40	0.67	0.03	1.25	0.10	0.37	0.92
30	Control	4.57^{a}	280.89^{a}	7.44^{aD}	49.23^{a}	22.45^{d}	17.80^{a}	4.33^{a}	6.63^{aC}	1.26^{dA}
	CB	4.33^{a}	279.55^{a}	4.59^{a}	29.79^{c}	45.28^{b}	13.88^{b}	2.64^{d}	3.84^{b}	3.26^{bC}
	AZZ1	4.21^{b}	279.98^{a}	4.95^{c}	29.48^{b}	45.92^{c}	13.69^{a}	2.23^{b}	3.51^{bA}	3.35^{cB}
	AZZ4	3.99^{b}	280.16^{a}	6.58^{ab}	25.26^{d}	59.86^{a}	7.41^{c}	1.44^{d}	2.95^{c}	8.07^{aA}
	AZZ7	4.09^{b}	280.11^{a}	5.69^{cb}	27.55^{c}	55.67^{b}	9.37^{b}	1.83^{c}	3.19b^{c}	5.94^{bC}
	SE	0.07	0.54	0.30	2.88	0.14	0.83	0.33	0.45	0.71
60	Control	4.39^{a}	279.77^{a}	6.19^{a}	53.54^{a}	28.84^{d}	18.68^{a}	4.93^{a}	7.77^{a}	1.54^{c}
	CB	4.25^{b}	277.69^{a}	4.31^{b}	37.03^{b}	46.89^{a}	14.17^{c}	3.86^{c}	3.97^{b}	3.30^{a}
	AZZ1	4.15^{b}	278.38^{a}	4.39^{c}	36.54^{b}	47.74^{c}	13.96^{b}	3.47^{b}	3.93^{b}	3.41^{b}
	AZZ4	3.94^{c}	278.93^{a}	5.52^{b}	25.89^{c}	60.35^{a}	10.72^{c}	1.93^{d}	2.99^{c}	5.62^{a}
	AZZ7	4.10^{b}	278.74^{a}	4.12^{c}	35.88^{b}	57.82^{b}	13.86^{b}	2.54^{c}	3.86^{b}	4.17^{a}
	SE	0.04	0.82	0.25	2.99	0.03	0.89	0.34	0.58	0.42
90	Control	4.60^{a}	275.43^{a}	6.06^{a}	77.68^{a}	29.60^{d}	24.01^{a}	4.94^{a}	13.19^{a}	1.23^{d}
	CB	4.16^{c}	274.47^{b}	3.73^{b}	41.62^{b}	47.14^{a}	17.89^{c}	3.96^{c}	5.97^{b}	2.63^{a}
	AZZ1	4.25^{b}	274.87^{d}	4.05^{c}	38.49^{b}	48.16^{b}	16.79^{b}	3.59^{cc}	4.33^{b}	2.87^{b}
	AZZ4	4.11^{c}	275.11^{b}	4.47^{b}	30.42^{c}	62.10^{a}	13.33^{b}	2.69^{c}	3.75^{b}	4.65^{c}
	AZZ7	4.17^{c}	274.96^{c}	5.98^{b}	35.91^{b}	61.04^{a}	16.49^{c}	2.86^{b}	3.97^{b}	3..70^{a}
	SE	0.05	0.36	3.21	0.18	0.13	0.85	0.46	0.81	0.4
P-value	T	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	D	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	T*D	0.0054	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

Means of triplicate. Means for the same inoculation treatment with different letters (a, b, c, d) are significantly different (P<0.0.05) DM, dry matter; WSC, water soluble carbohydrates, NH_3-N ammonia nitrogen LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid; LA/AA; Lactic acid/acetic acid. CB, Commercial Bacteria; AZZ1, Pediococcus acidilactici; AZZ4, Lactobacillus plantarum subsp. plantarum; AZZ7 Lactobacillus plantarum subsp. Argentoratensis; T, effect of treatment; D, effect of day of fermentation; T × D, interaction between treatment and day of fermentation

compared to the control silages *Table 3*. The ratio of LA/ AA in treated silages reached a peak on day 14 of ensiling, and then gradually decreased until the end of the ensiling period (d 90). In contrast, the ratio of LA/AA in the control silage increased during the ensiling period and by the end of the ensiling period (d 90) was significantly *(P*<.0001) lower as compared to the treated silages.

Treatment, day of fermentation and interaction between them significantly *(P*<0.05) affect the amounts of lactobacilli, yeast, and aerobic bacteria, the highest number of aerobic bacteria was observed on day 90. The higher amounts of lactobacilli were observed in the silage inoculated with AZZ4 compared with control, AZZ1, AZZ7 and CB *Table 3*.

Aerobic Stability of Napier Silages

Chemical and microbial effects on the aerobic stability of Napier grass silages are presented in *Table 4* and *Table 5*. The effect of AZZ1, AZZ4 and AZZ7 on fermentative quality of the Napier grass silages during aerobic exposure was

Table 3. *Changes in LAB, aerobic bacteria, and yeast during ensiling periods*

Days of Fermentation	Treatments	log10 cfu/g FW		
		LAB	Aerobic Bacteria	Yeasts
7	Control	5.13^{d}	2.16^{b}	1.26^{a}
	CB	5.19^{a}	1.97^{b}	1.24^{b}
	AZZ1	5.37^{c}	1.90^{a}	1.18^{a}
	AZZ4	5.77^{a}	1.13^{c}	0.92^{b}
	AZZ7	5.58^{b}	1.51^{b}	1.03^{b}
	SE	0.07	0.08	0.04
14	Control	5.23^{d}	2.30^{a}	2.19^{a}
	CB	5.34^{a}	2.31^{c}	1.79^{c}
	AZZ1	5.41^{c}	2.13^{ab}	1.77^{b}
	AZZ4	6.18^{a}	1.77^{c}	1.09^{d}
	AZZ7	5.89^{b}	1.95^{bc}	1.52^{c}
	SE	0.11	0.06	0.12
30	Control	5.40^{c}	3.34^{a}	3.81^{a}
	CB	5.52^{a}	2.69^{a}	2.99^{b}
	AZZ1	5.93^{b}	2.77^{b}	2.96^{a}
	AZZ4	6.39^{a}	2.31^{c}	2.40^{b}
	AZZ7	6.25^{a}	2.57^{cb}	2.73^{ab}
	SE	0.11	0.12	0.07
60	Control	5.75^{d}	4.75^{a}	3.97^{a}
	CB	5.97^{b}	4.46^{a}	3.94^{a}
	AZZ1	6.15^{c}	4.17^{a}	3.91^{a}
	AZZ4	6.68^{a}	3.33^{d}	3.40^{a}
	AZZ7	6.51^{b}	3.61^{c}	3.61^{a}
	SE	0.10	0.16	0.09
90	Control	5.78^{b}	5.50^{a}	4.48^{a}
	CB	6.25^{a}	5.56^{b}	4.72^{a}
	AZZ1	6.70^{a}	5.23^{ab}	4.65^{a}
	AZZ4	6.90^{a}	4.91^{b}	4.21^{a}
	AZZ7	6.82^{a}	5.13^{ab}	4.58^{b}
	SE	0.14	0.07	0.13
P-value	T	<.0001	<.0001	<.0001
	D	<.0001	<.0001	<.0001
	T*D	0.0054	<.0001	<.0001

Means of triplicate. Means for the same inoculation treatment with different letters; (a, b, c, d) are significantly different (P<0.0.05); Cfu, colony-forming units; FW, fresh weight; LAB, lactic acid bacteria; CB, Commercial Bacteria; AZZ1, Pediococcus acidilactici; AZZ4, Lactobacillus plantarum subsp. plantarum; AZZ7 Lactobacillus plantarum subsp. Argentoratensis; T, effect of treatment; D, effect of day of fermentation; T × D, interaction between treatment and day of fermentation

significant (*P*<0.0001). Days of aerobic exposure also had a significant effect (*P*<0.0001) on the aerobic stability, in all treatments. The interaction between aerobic exposure period and treatments had a significant effected on PA, BA, NH_3-N, Yeasts, and Aerobic bacteria (*P*<0.0001), except in LA content (*P*=0.1059) and LAB count (*P*=0.3060). The LA content showed a significant decrease (*P*<.0001) during 3 d of aerobic exposure, and then the trend was maintained in silages treated with AZZ4, while LA content in both control and AZZ1 samples were quickly decreased (*P*<0.0001) till the end of the aerobic exposure period. We observed a small variation in LA contents in all Napier grass silages during the 7 d of aerobic exposure test.

The pH gradually increased along the aerobic exposure and pH in all treatment silages significantly rose (*P*<0.0001) and exceeded initial pH value, and reached pH 6 after 7 d of aerobic exposure. Silages treated with AZZ4 showed lower pH during the prolonged days of aerobic exposure. The content of AA, BA and PA in all silages significantly decreased (*P*<0.0001) over the course of aerobic exposure. Whereas, the amount of NH_3-N increased along with aerobic exposure in all treated silages, and reached the highest value after 7 d. Silages treated with AZZ4 and AZZ7 were significantly lower (*P*<0.0001) pH than that of the AZZ1 and control throughout the aerobic exposure. However, the WSC content significantly decreased (*P*<0.0001) throughout the aerobic exposure period, and this decreased was observed in the initial 3 d of aerobic exposure where the WSC content in silages were significantly lower (*P*<0.0001) than the opening d (d 0 of the aerobic exposure test). Details of the microorganism counts from Napier silages during the aerobic exposure are shown in *(Table 5)*. Effects of treatment, aerobic exposure d and interaction between them on the population of yeast and aerobic bacteria were noticeable (*P*<0.0001), except for the effect of the interaction on the lactic acid bacteria counts (*P*=0.3060). The population of LAB gradually decreased after exposure to air, and the LAB counts of AZZ4 and AZZ7 silages remained higher (*P*<0.0001) than that of other silages after 7 d of the aerobic exposure period. Yeast and aerobic bacteria counts in all silages significantly increased (*P*<0.0001) after 7 d of aerobic exposure.

DISCUSSION

The good silage should be achieved by a stable fermentation [19]. The addition of LAB inoculant caused higher level of LA which resulted in more decreased in pH compare to the control silage. Meesk et al.[3] found similar results when adding a LAB inoculant to *E. curvula* (subtropical grass). The WSC content of tropical grasses is generally lower than that of temperate species [9]. The low WSC content of Napier grass and lower level of lactic acid bacteria may have contributed to the very slow rate in pH decreased of the control silage. Meesk et al.[3] found that the WSC content of maize at ensiling was 107 gkg^{-1} DM and the pH dropped to four after two days of ensiling. After seven days of ensiling the average pH of the inoculated Napier silage was still 4.67. The low amount of available WSC may have restricted the growth of lactic acid bacteria, preventing a faster decreased in pH in the inoculated silage. It has been shown that grasses of tropical and subtropical area accumulated starches composed of amylose and amylopectin instead of fructans in their vegetative tissues [20]. The concentration

Table 4. *Changes on fermentation characteristics of the Napier grass silages during aerobic exposure*

Days of Fermentation	Treatments	pH	WSC (g/kg DM)	NH_3-N (g/kg DM)	LA (g/kg DM)	AA (g/kg DM)	PA (g/kg DM)	BA (g/kg DM)
0	Control	4.60^{a}	6.06^{a}	77.68^{a}	29.60^{c}	24.01^{a}	4.94^{a}	13.19^{a}
	CB	4.16^{a}	3.73^{b}	41.62^{c}	47.14^{a}	17.89^{a}	3.96^{ab}	5.97^{b}
	AZZ1	4.25^{a}	6.05^{a}	38.49^{b}	48.16^{b}	16.79^{a}	3.59^{a}	4.33^{c}
	AZZ4	4.11^{b}	4.47^{b}	30.42^{c}	62.10^{a}	13.33^{b}	2.69^{b}	3.75^{b}
	AZZ7	4.17^{ab}	5.98^{a}	35.91^{c}	61.04^{ab}	16.49^{a}	2.86^{ab}	3.97^{b}
	SE	0.10	0.22	5.76	1.45	1.25	0.32	1.47
3	Control	5.67^{a}	4.60^{a}	79.48^{a}	13.18^{c}	29.51^{a}	13.02^{a}	16.62^{a}
	CB	5.58^{b}	3.31^{a}	65.23^{d}	13.87^{a}	25.54^{b}	11.72^{a}	13.68^{a}
	AZZ1	5.53^{a}	3.69^{b}	57.28^{b}	14.56b^{c}	23.54^{ab}	10.76^{b}	11.74^{b}
	AZZ4	4.80^{c}	3.27^{ab}	41.86^{c}	22.5^{a}	22.97^{b}	7.06^{d}	7.78^{c}
	AZZ7	5.21^{b}	4. 26^{ab}	53.41^{b}	18.78^{a}	23.18^{b}	8.69^{c}	9.40^{c}
	SE	0.10	0.11	4.16	1.19	1.07	0.68	1.01
5	Control	6.25^{a}	3.30^{a}	79.99^{a}	7.66^{b}	33.31^{a}	19.03^{a}	19.83^{a}
	CB	6.13^{d}	3.12^{a}	69.51^{a}	8.22^{b}	33.02^{a}	18.62^{b}	17.29^{d}
	AZZ1	5.95^{b}	3.17^{a}	65.59^{b}	9.32^{ab}	32.33^{a}	17.60^{a}	15.38^{b}
	AZZ4	5.34^{d}	2.50^{a}	49.81^{d}	21.64^{a}	26.28^{a}	12.58^{c}	10.58^{d}
	AZZ7	5.71^{c}	2.59^{a}	61.83^{c}	13.09^{ab}	30.78^{a}	14.63^{b}	12.85^{c}
	SE	0.10	0.14	2.13	2.02	0.96	0.77	1.04
7	Control	6.52^{a}	2.48^{a}	80.16^{a}	2.84^{b}	37.75^{a}	21.67^{a}	23.75^{a}
	CB	6.38^{b}	1.35^{c}	77. 23^{b}	4.29^{c}	36.65^{b}	20.48^{c}	20.52^{c}
	AZZ1	6.29 ^{b}	1.94^{b}	73.69^{a}	5.34^{a}	35.97^{a}	19.66^{ab}	18.64^{b}
	AZZ4	6.08^{c}	1.23^{c}	67.11^{c}	7.94^{a}	31.03^{b}	15.11^{c}	15.02^{d}
	AZZ7	6.22^{bc}	1.50^{c}	71.00^{b}	6.37^{a}	34.78^{ab}	18.81^{b}	16.83^{c}
	SE	0.05	0.14	0.94	0.61	0.83	0.74	0.99
P-value	T	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	D	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
	T*D	0.0011	0.0024	<.0001	0.1059	0.0068	<.0001	<.0001

Means of triplicate. Means for the same inoculation treatment with different letters (a, b, c, d) are significantly different (P<0.005). WSC, water soluble carbohydrates, NH_3-N ammonia nitrogen LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid. CB, Commercial Bacteria; AZZ1, Pediococcus acidilactici; AZZ4, Lactobacillus plantarum subsp. plantarum; AZZ7 Lactobacillus plantarum subsp. Argentoratensis; T, effect of treatment; D, effect of day of fermentation; T × D, interaction between treatment and day of fermentation

of AA increased with the ensiling period, and the ratio of LA/AA tended to decline in the treated silages after 7 d of ensiling. This might be due to the changes of fermentation pattern from homofermentation to heterofermentation, and is consistent with other studies as reported by Shao et al.[21], who found that there was a significant shift from homofermentative to heterofermentative activity of LAB after 5 d of ensiling. McDonald et al.[9] also reported that in well preserved silages, acidification was initiated by homofermentative strains, but after only 4 d 85% of the strains were heterofermentative, whose tolerance to AA was greater than homofermentative strains. The treated silages showed significantly lower AA, PA and BA concentrations as compared with the control silage. The BA concentration in the control was always higher than 5 g/kg DM, which is recognized as the critical value of BA content for well silages [22], whereas only small amounts of PA and BA were detected in treated silages. This may be explained as the fast LA production and pH reduction in treated silages could inhibit the aerobes microbes during the early stage of ensiling.

Treatment, day of fermentation, and their interaction increased lactobacilli and also decreased yeast and aerobic bacteria numbers. These results are in approval with those reported by Júnior et al.[8]. Filya et al.[23] found decreased fungal populations in silages treated with LAB; these results also agree with [24] who mentioned that a successful silage is one in which LAB replaces the initial microbial composition of the plant.

When the fermentation is completed, and the silage is exposed to air during storage, heating in the silo is usually initiated by the production of yeast metabolites [25]. As the climate is inherently unstable and there are large differences between day and night temperatures in Nanjing

Table 5. *Changes in LAB, aerobic bacteria, and yeast during aerobic exposure of silage for 0, 3, 5, and 7 d*

Days of Fermentation	Treatments	log10 cfu/g FW		
		LAB	Aerobic Bacteria	Yeasts
0	Control	5.78[a]	5.50[a]	4.48[a]
	CB	6.25[b]	5.56[b]	4.72[b]
	AZZ1	6.70[a]	5.23[ab]	4.65[d]
	AZZ4	6.90[a]	4.91[b]	4.21[c]
	AZZ7	6.82[a]	5.13[ab]	4.58[b]
	SE	0.20	0.11	0.09
3	Control	2.18[b]	6.90[a]	6.82[a]
	CB	3.84[a]	6.88[a]	6.78[a]
	AZZ1	4.14[a]	6.85[a]	6.74[a]
	AZZ4	4.21[a]	6.27[b]	6.07[b]
	AZZ7	4.14[a]	6.75[a]	6.67[a]
	SE	0.26	0.07	0.09
5	Control	1.70[b]	7.42[a]	7.53[a]
	CB	2.98[ab]	7.40[b]	7.89[a]
	AZZ1	3.44[a]	7.36[a]	6.81[b]
	AZZ4	3.75[a]	6.93[a]	6.18[c]
	AZZ7	3.49[a]	7.09[b]	6.69[d]
	SE	0.25	0.06	0.22
7	Control	1.51[b]	8.53[a]	7.71[a]
	CB	2.15[aC]	8.17[b]	7.86[b]
	AZZ1	2.70[aC]	7.87[b]	7.77[a]
	AZZ4	2.86[aC]	7.08[a]	6.88[c]
	AZZ7	2.82[aC]	7.62[b]	7.37[b]
	SE	0.17	0.15	0.11
P-value	T	<.0001	<.0001	<.0001
	D	<.0001	<.0001	<.0001
	T*D	0.3060	<.0001	<.0001

Means of triplicate. Means for the same inoculation treatment with different letters; (a, b, c, d) are significantly different (P<0.0.05); Cfu, colony-forming units; FW, fresh weight; LAB, lactic acid bacteria; CB, Commercial Bacteria; AZZ1, Pediococcus acidilactici; AZZ4, Lactobacillus plantarum subsp. plantarum; AZZ7 Lactobacillus plantarum subsp. Argentoratensis; T, effect of treatment; D, effect of day of fermentation; T × D, interaction between treatment and day of fermentation

area, it is hard to control the temperature change of silage in the open air conditions. Measuring the temperature change of silages in the open air could not reflect their aerobic stability adequately. Moreover, there is no information on evaluating the aerobic stability of Napier grass silage by measuring the temperature changes. Therefore, our assessment of the aerobic stability of Napier grass silage was based on changes in chemical composition and microbial populations under laboratory conditions with the time of aerobic exposure in the open air.

The pH of the control and treated silages increased gradually throughout the time of aerobic exposure which might be attributed to the decrease in the LA content. The pH was an indicator for the aerobic deterioration of the silage because yeasts can consume LA during aerobic exposure, making the silage more suitable for the growth of other undesirable microorganisms such as mold and aerobic bacteria [26].

Examining the LA contents during aerobic exposure showed significant reductions of about 64% in the 7th d aerobic exposure. It has been suggested that silage with high LA and WSC were more aerobically unstable than silage with low values because lactic acid and WSC were a potential source of readily available substrate for the growth of undesirable bacterial when the silages were exposed to air. Cai et al.[27] concluded that although silage inoculation with homofermentative LAB improved the fermentation quality, it did not inhibit the growth of yeasts and silages deterioration upon exposure to air. Similarly, Weinberg et al.[28] applied cellulase and hemicellulase to pea and wheat silages and found that, higher concentrations of residual WSC and LA enhanced lactate assimilating yeast and mold growth upon exposure.

NH_3-N concentration in silage reflected the degree of protein degradation; extensive proteolysis adversely affected the utilization of nitrogen by ruminants. In the present experiment, NH_3-N in all silages increased gradually, accompanied with the increase of pH over the course of aerobic exposure, which indicates the protein breakdown by undesirable bacteria during the exposure to air. It is well known that yeasts are primarily responsible for the onset of aerobic silage spoilage, silages with a high yeast population (over 10^5 CFU/g FW) are prone to be deteriorated in the presence of air [29,30]. A study of Tabacco et al.[31] also found that, the aerobic stability of maize silage decreased exponentially with the increase of yeast count regardless of the additive treatment. In the present experiment, the yeast count in all treated silages increased significantly during the time of air exposure. Courtin et al.[32] reported that aerobic bacteria can also induce aerobic deterioration. Our results suggest that the population of aerobic bacteria have a similar increasing trend like that of yeast, and the stability of Napier grass silage appears to be negatively affected by this trend.

The aerobic stability of treated silages significantly reduced compared to the control silage which might be attributed to the lower content of AA, BA and PA in the treated silage during the aerobic exposure period. This is in consistent with the outcome of Moon [33], which reported that, AA, BA and PA could protect silage against aerobic yeasts and molds. On the other hand, the application of AZZ1, AZZ4 and AZZ7 led to the production of more stable silages after air exposure; this might be referred to as the positive effect of this treatment in improving the fermentation properties, and therefore controlling the growth of yeasts.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgements

This work was supported by the Natural Science Foundation of China (31672488), the Key Technologies R&D Program of China during the 13th Five-Year Plan period (2016YFC0502005), and the special project focusing on the grass in the Tibetan Autonomous Region for the "13th Five-Year" Plan, China (2017ZDKJZC).

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