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Research Article

Characterization and Identification of Lactic Acid Bacteria by 16S rRNA Gene Sequence and Their Effect on the Fermentation Quality of Elephant Grass *(Pennisetum purpureum)* Silage

Azizza SIFEELDEIN [1] Xianjun YUAN [1] Zhihao DONG [1] Junfeng LI [1]
Hager YOUNS [1] Tao SHAO [1]

[1] Institute of Ensiling and Processing of Grass, Nanjing Agricultural University, Weigang 1, Nanjing 210095, CHINA

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Abstract

The purpose of this study is to isolate and identify lactic acid bacteria from elephant grass *(Pennisetum purpureum)* silage and examine their effect on the silage fermentation quality. Three of lactic acid bacteria strains were isolated from elephant silage and their characterization, identification, and influence on silage quality after 90 d of ensiling was studied. All three strains were Gram-positive, catalase-negative, and were grown in 6.5% NaCl and pH 4.00. Strains AZZ1, was identified as for genus *pediococcus*, whereas, AZZ4 and AZZ7 strains were classified as genus *Lactobacillus* according to the phenotype, 16S rRNA, and RecA gene analysis. Three strains were used as additives at 1.0×10^6 CFU/g of fresh material of elephant grass. Strain AZZ4 is found to be the most effective in improving the fermentation quality of the elephant grass silage, as indicated by a lower ($P<0.0001$) dry matter losses, pH value, water-soluble carbohydrates, acetic acid content, butyric acid, propionic acid and ammonia-N. However, the lactic acid content was higher ($P<0.0001$) compared to the control and other treatments. In conclusion, these results suggest that, for well-preserved silage, the isolates may be useful as inoculants for silage making, and could play a major role in developing silage production.

Keywords: *Isolation, Identification, Lactic Acid Bacteria, Sequence, Silage Quality*

16S rRNA Gen Sekansı Kullanılarak Laktik Asit Bakterilerinin Fil Çimeninde *(Pennisetum Purpureum)* Karakterizasyonu, Tanımlanması ve Fermantasyon Kalitesine Etkileri

Özet

Bu çalışmanın amacı Fil çimeninden *(Pennisetum purpureum)* laktik asit bakterilerin izolasyonu, tanımlanması ve silaj fermantasyon kalitesine etkilerini araştırmaktır. Üç laktik asit bakteri suşu fil silajından izole edildi ve bunların karakterizasyonu, tanımlanması ve 90 gün sonrasında silaj kalitesi üzerine etkileri çalışıldı. Üç suşun hepsi de Gram-pozitif ve katalaz-negatif olup %6.5 NaCl ve pH 4.00'de büyüme gösterdi. 16S rRNA ve RecA gen analiz sonuçlarına göre AZZ1 suşları pediococcus genusunda tanımlanırken AZZ4 ve AZZ7 suşları *Lactobacillus* genusunda sınıflandırıldı. Üç suş taze fil çimenine 1.0×10^6 CFU/g oranında katılarak kullanıldı. AZZ4 suşu daha düşük kuru madde kaybı ($P<0.0001$), pH değeri, suda çözünür karbohidratlar, asetik asit, burik asit, propiyonik asit ve amonyum-N miktarları baz alındığında fil çimen silajının fermantasyon kalitesini artırmada en etkili olan olarak belirlendi. Ancak laktik asit miktarı kontrol ve diğer uygulamalar ile karşılaştırıldığında daha yüksek olarak tespit edildi ($P<0.0001$). Sonuç olarak, iyi saklanmış bir silaj için elde edilen izolatların silaj yapımında faydalı olabileceği ve silaj üretimini geliştirmede önemli olabileceği düşünülmektedir.

Anahtar sözcükler: *İzolasyon, Tanımlama, Laktik asit bakterileri, Sekans, Silaj kalitesi*

İletişim (Correspondence)
+86 1380 9021517
taoshaolan@163.com

INTRODUCTION

Elephant grass *(Pennisetum purpureum)* is a monocot C4 perennial grass in the Poaceae family, and it is among the highest yielding tropical grasses. However, its high cell walls and low water-soluble carbohydrates can cause a significant inhibition of the ensiling process. The dry matter yields of elephant grass vary between 15 and 30 ton ha^{-1} a year [1]. Despite this, this grass concentrates too much moisture content at its best nutritional stage, which caused a reduction in the silage quality due to the high proteolysis; moreover, there are substantial nutrient losses due to such moisture levels [2]. Nevertheless, elephant grass is regarded as one of the most important tropical forages because of its high potential for biomass production, easy adaptation to diverse ecosystems and good acceptance by animals. Furthermore, it is extensively used to feed herds in the form of grazing [1]. However, its low crude protein content [3] and high structural carbohydrate contents [4] usually lead to the low nutritive value of silage. Recently, to avoid the aerobic deterioration of silage, heterofermentative LAB species, such as *L. buchneri* and, *L. brevis* have been developed as silage additives [5].

Lactic acid fermentation, an ancient conservation technique, currently preferred as a "natural" procedure to improve the products (dairy, vegetable, and meat) [6]. Epiphytic LAB colonies on plant material are responsible for the metabolism of water-soluble carbohydrates (WSC) into organic acids, mainly lactic acid, thus resulting in pH decline and subsequent forage preservation [7]. There is a few number of homofermentative LAB commonly used in silage inoculants, including *Lactobacillus acidophilus, Lactobacillus plantarum, Enterococcus faecium and Pediococcus acidilactici* [8]. Commercially available microbial inoculant contains one or more of these bacteria, is selected based on their ability to achieve efficient fermentation. The silage quality could be improved by the addition of inoculants, then lactic acid production occurs more rapidly, thereby, the loss of nutrients during ensilage can be reduced [9]. Therefore, homofermentative bacteria can be used to improve silage preservation by accelerating and promoting the initial stage of the conservation procedure through the fermentation of water-soluble carbohydrates (WSC) into lactic acid followed by a rapid reduction in pH [10].

The processes of isolation, screening and identification of lactic acid bacteria from natural sources are routinely employed as the best means of gaining useful and genetically-stable strains for industrially-important products [11]. LAB that has industrial potential should be homofermentative, *i.e.*, they mainly produce lactic acid [11]. Facile recovery of lactic acid and consequent purification is also a main requirement in a homofermentative process [11].

To our knowledge, no information is available on the microbial ecology isolated from elephant silage, especially about the indigenous LAB and their effects during the fermentation process.

This study set out to isolate, screen and identify lactic acid bacteria colonizing elephant silage during the fermentation process. Isolated strains were identified biochemically, and selected strains were identified depending on the phenotype, 16S rRNA, and RecA gene analysis of sequence amplification product. As well as, some of the assessed to excellent LAB strains were used to inoculate elephant silage to determine their influence on the fermentation quality.

MATERIAL and METHODS

Isolation of LAB from Elephant Silage

Ten grams of elephant grass fresh material were blended with 90 mL of sterilized saline solution (8.50 g L^{-1} NaCl) and serially diluted from 10^{-1} to 10^{-6} in sterilized water. The number of the LAB were measured by the plate count method described by Cai et al.[12]. Three strains were isolated from elephant silage with GYP agar, and Lactobacilli MRS broth (Difco Laboratories, Detroit, MI) containing 1.5% agar incubated at 37°C for 2 d under anaerobic conditions. The separate colonies with different morphology were picked using a tooth pick and grown in MRS broth. Three strains were selected randomly from the plates containing between 30 and 300 colonies and purified by MRS agar, and each LAB strain was isolated and purified twice by streaking on MRS agar plates [12]. Pure cultures were grown on MRS agar at 37°C for 48 h, before being shifted to nutrient broth (Difco) and kept as standard cultures at -80°C with 10% glycerol for further examination.

Morphological and Physiological Tests of LAB

Morphological, physiological, gram-staining, catalase activity and gas production from glucose were determined according to the method described by Kozaki et al.[13]. Growth at different temperatures was observed in MRS broth after incubation at 5°C and 10°C for 14 days and at 45°C and 50°C for 7 days. Whereas, the growth at different pH 3.0, 3.5, 4.0, 4.5,7 and 8.0 was examined in MRS broth (the pH was adjusted with HCl or NaOH), after incubation at 30°C for 48 h. The acidity resistance of the LAB was tested in MRS broth containing 3.0 and 6.5% NaCl at 30°C for 2 days [10]. A preliminary identification assay of the LAB isolates based on the phenotypic characteristics was achieved according to the criteria of Bergey's Manual of Determinative Bacteriology. The purified strains were cultured on MRS plates in anaerobic vessels, and the obtained colonies were grown in 5 mL MRS media at 30°C overnight. A cell suspension was applied to API 50 CH strip wells (bioM_erieux. L'Etoile, France), which were coated with liquid paraffin, and the strips were incubated at 30°C. The results were assessed after 24 h and verified after 48 h. The fermentation of carbohydrates in the medium

was indicated by a yellow color, except for esculine (dark brown), and color reactions were scored by a chart provided by the manufacturer.

Genomic DNA Extraction

The selected LAB isolates were cultured for 24 h in MRS agar at 30°C and used for DNA extraction and purification. Seven aliquots of 10 mL from the homogenates used for microbiological analysis were centrifuged at 13.000×g for 5 min at 4°C. Pellets were washed twice with phosphate saline buffer (PBS) and incubated at 37°C for 1 h in 400 µL of lysis buffer (0.2 mg/L sucrose, 1.5 g/L Tris-HCl, 3 g/L NaCl and 3 g/L EDTA) containing lysozyme (10 g/L). Then 20 µL of SDS solution (100 g/L) and 2 µL of proteinase K solution (20 g/L) were added to each tube, mixed gently and incubated at 37°C for 1.5 h. After incubation, DNA was extracted using the phenol: chloroform method and precipitated with ethanol and sodium acetate. The final DNA sample was resuspended in 100 µL of Tris-EDTA (TE) buffer containing RNase (0.1 g/L), and the quality of the extracts was checked on agarose gels (0.1 g/L) and quantified by spectrophotometry.

The 16S rRNA gene sequence coding region was amplified by PCR thermocycler (Takara Shuzo Co., Ltd., Ohtsu, Japan) as described by Suzuki et al.[14]. PCR primers used were 27F (5′-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5′GGT TACCTTGTTACGACTT-3). The PCR was performed in a final volume of 50 µL using reagents provided by Bioline (London, UK). Initial DNA denaturation was performed at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and elongation at 68°C for the 40s, and then a final elongation step at 68°C for 10 min. The expected size of the amplified fragments was verified on agarose gels (0.2 g/L).

The nucleotide sequences for the 16S rDNA described in this study were deposited in Gen Bank under accession no. of AZZ1, AZZ4, and AZZ7 were KY495875, KY584256, and KY584253, respectively.

Sequence Alignments and Phylogenetic Implications

The 16S rDNA sequences were aligned with 16S rRNA sequences from GenBank to identify organisms using nBLAST analysis. Then, the sequence information from representative organisms was introduced into the CLUSTALW program for assembly and alignment [15]. The 16S rRNA gene sequences of isolates were compared with sequences from LAB-type strains held in GenBank. Nucleotide substitution rates were calculated [16], and phylogenetic trees were constructed by the neighbor-joining method [17].

Silage Preparation and Treatment with LAB Strains

Elephant grass *(Pennisetum purpureum)* was harvested at dough stage from 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). After harvesting, the grass was chopped manually to an approximate length of 2-3 cm. 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. Strains AZZ1 *(Pediococcus acidilactici)*, AZZ4 *(Lactobacillus plantarum subsp. plantarum)*, AZZ7 *(Lactobacillus plantarum subsp. argentoratensis)* were selected as additives at 1.0×10^6 CFU/g of fresh material to elephant grass; then elephant grass were treated with (1) no additives (control), (2) AZZ1. (3) AZZ4 and AZZ7, 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 12 laboratory silos were made (4 treatments × 3 replicates) for each treatment and kept at 25°C in ambient temperature. All silos were opened after 90 d of ensiling.

Chemical Analysis

On the sampling day, the content of each silo was removed, well-mixed, and divided into two silage samples. The first silage sample was used to determine the content of ammonia nitrogen (AN) and pH value, following the methodology used by Viana et al.[18], the other sample was dried using a forced-draft oven at 65°C for 48 h. For the chemical analysis of the fresh forage and silages, samples were finely ground to approximately 1 mm particle size in a Willey type laboratory mill in order to determine the dry matter (DM) content. The total nitrogen (TN) content of the fresh forage and silage samples was examined according to the Kjeldahl method [19]. Crude protein (CP) was analyzed and calculated as the TN multiplied by 6.25. Water-soluble carbohydrates (WSC) contents were determined by a colorimetric method after reacting with an anthrone reagent [20]. The neutral detergent fiber and acid detergent fiber content were performed according to the techniques described by Van Soest et al.[21]. 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.[22]. Ammonia nitrogen (AN) was measured according to the method of phenol-hypochlorite reaction [20]. The pH of fresh forage and silage were measured using a pH meter (F-23; Horiba, Tokyo, Japan).

Statistical Analyses

Silage fermentation data were analyzed as a completely randomized design using the general linear model (GLM) procedure of Statistical Analysis System [23]. Least significant

difference was used to separate means when significant effects (P<0.05) were detected.

RESULTS

The Morphological and Physiological Properties of Characteristic Strains Isolated from Elephant Silage

All isolates were identified as gram-positive, catalase negative, rod-shaped bacteria, and their morphological and physiological characteristics are presented in *Table 1*. Strains AZZ1 and AZZ4, grew normally at 10°C, whereas, AZZ7 grew weakly at 10°C and strains AZZ1 grew weakly at 15°C, strain AZZ1 was unable to grew at 45°C while AZZ4 and AZZ7 were grow. Moreover, no growth was detected at 50°C. All strains were able to grew at pH 3.5. Apart from the weak growth of AZZ4 and AZZ7 at pH 3.5, all strains could grow in the rang of pH 3.5-8.0, however, the growth of all strains seems to be inhibited at pH 3.0. All strains showed homofermentative products, and all were able to fermented glucose and fructose and other sugars.

16S rRNA Gene Sequence Analysis

After blasting the 16S rRNA sequence, Strains AZZ4 and AZZ7 were clustered in the genus *Lactobacillus* with 99% similarity among their 16S rDNA gene sequences, on the other hand, strain AZZ1 was clustered in the genus *pediococcus* with 98% similarity in their 16S rDNA gene sequences, as shown in the phylogenetic tree *(Fig. 1*, bootstrap between 51%-100%).

Amplification Products Obtained from the recA Gene Multiplex Assay

Fig. 2 shows the amplification products obtained from the recA multiplex assay; lane M contained a 600 bp PLUS DNA ladder (Tiangen Biotech Co, Ltd., Beijing, China). Lanes 1, 2, and 3, are PCR amplification products from *Pediococcus acidilactici*, (AZZ1), *L. plantarum* subsp. *Plantarum* (AZZ4), *L. plantarum subsp. Argentoratensis* (AZZ7), respectively. Sequences of the three strains were deposited with GenBank under accession numbers of KY495875, KY584256 and KY584253 for AZZ1, AZZ4 and AZZ7, respectively.

Characteristics of Elephant Grass Before Ensiling

The chemical and microbiological compositions of fresh elephant grass before ensiling are shown in *Table 2*. The number of lactic acid bacteria was low on the material before ensiling which is common for tropical grasses to have a low number of lactic bacteria, less than 10^6 CFU/g fresh forage

Effects of LAB Isolate on Silage Quality After 90 d of Ensiling

The effects of LAB strains on the fermentation quality of elephant silage after 90 d of ensiling are shown in *Table 3*. At the ensiling day, the analysis of the chemical composition

Table 1. *The characteristics and sugar fermentation patterns of lactic acid bacteria isolates used for silage inoculation*

Characteristic	AZZ1	AZZ4	AZZ7
Shape	Rod	Rod	Rod
Gram stain	+	+	+
Gas from glucose	-	-	-
Fermentation type	Homo	Homo	Homo
Catalase activity	-	-	-
Growth at 5°C	-	-	-
Growth at 10°C	+	+	W
Growth at 15°C	W	+	-
Growth at 45°C	-	+	+
Growth at 50°C	-	-	-
Growth in 3.5%NaCl	+	+	+
Growth in 6.5%NaCl	+	+	+
Growth at pH 3.0	-	-	-
Growth at pH 3.5	+	+	w
Growth at pH 4.0	+	+	+
Growth at pH 4.5	+	+	+
Growth at pH 7.0	+	+	+
Growth at pH 8.0	+	+	+
Carbohydrate fermentation			
L-Arabinose	+	+	+
Ribose	+	+	+
D-Xylose	+	+	+
ß-Methyl-xyloside	-	+	+
Galactose	+	+	+
D-Glucose	+	+	+
D-Fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	+	+	+
Rhamnose	+	-	-
Inositol	+	+	+
Mannitol	+	+	+
Sorbitol	+	+	+
α-Methyl-D-mannoside	-	+	+
α-Methyl-D-glucoside	-	+	+
N-Acetyl glucosamine	+	+	+
Amygdalin	+	+	+
Esculin	+	+	+
Salicine	+	+	+
Cellobiose	+	+	+
Maltose	+	+	+
Lactose	+	+	+
Melibiose	+	+	+
Saccharose	+	+	+
Melezitose	+	+	+
D-Raffinose	-	+	-
ß-Gentiobiose	+	+	+
D-Tagatose	+	-	+
Gluconate	+	+	+

+, normal growth; -, no growth; ***w,*** *weak growth;* ***Homo,*** *homofermentative;* ***Hetero,*** *heterofermentative*

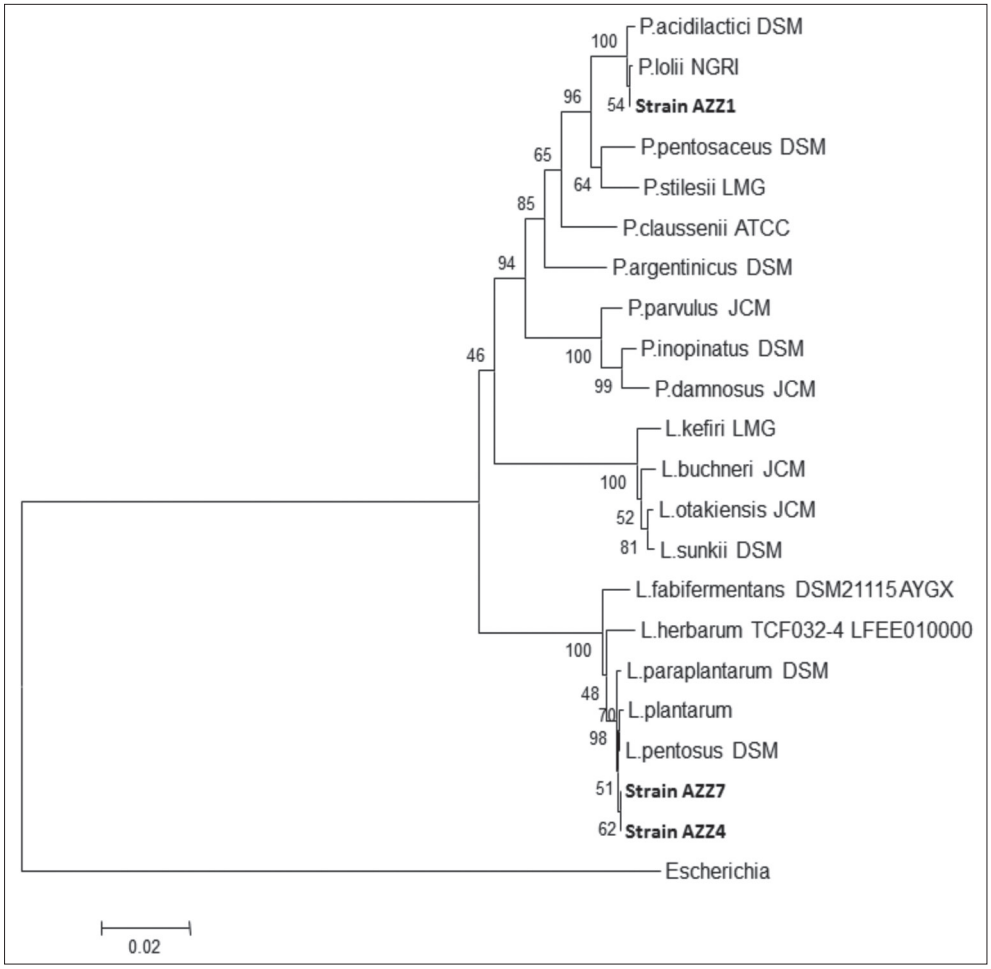

Fig 1. Polygenetic tree showing the relative positions of strains AZZ1, AZZ4, and AZZ7 isolate from elephant silage and related species, as inferred by the neighbor-joining method using complete 16S rRNA sequences. Bootstrap values for a total of 1000 replicates are shown at the nodes of the tree. *B. subtilis* is used as an outgroup. The GenBank accession no of the 16S rRNA gene of each strain is shown behind the strain

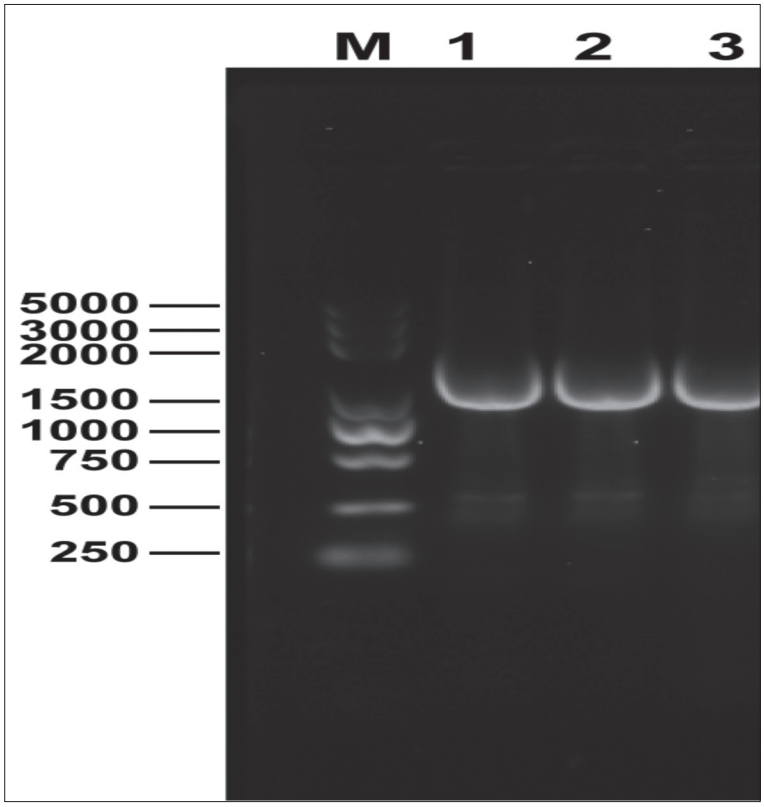

Fig 2. Amplification products obtained from the recA multiplex assay. Lane M contained a 600 bp PLUS DNA ladder (Tiangen Biotech Co, Ltd., Beijing, China). Lanes 1, 2 and 3, PCR amplification products from *Pediococcus acidilactici*, (AZZ1), L. *plantarum* subsp. *Plantarum* (AZZ4), *L. plantarum subsp. Argentoratensis (AZZ7)*, respectively

Table 2. *Chemical and microbial composition of elephant grass before ensiling*

Component	Content
Dry matter (g/kg FM)	286.5
pH	5.97
Crude protein (g-kg DM)	272.6
Neutral detergent fiber (g/kg DM)	697.4
Acid detergent fibre (g/kg DM)	390.3
Water-soluble carbohydrates (g/kg DM)	55.6
Buffering capacity (mEq/kg DM)	284
Lactic acid bacteria (log^{10} CFU/g FM)	4.74
Aerobic bacteria (log10 CFU/g FM)	6.53
Mould (log^{10} CFU/g FM)	4.36
Yeast (log^{10} CFU/g FM)	4. 85

FM, *fresh matter;* ***DM,*** *dry matter;* ***mEq,*** *milligram equivalent;* ***CFU,*** *colony-forming units*

phenotypic methods is inconsistently successful [24], while the reported use of 16S rRNA sequence analysis is considered a good approach to identify LAB strains at both the genus and species level. However, some LAB species, for example, *L. plantarum* and *L. pentosus* have very similar 16S rRNA gene sequences, differing only by 2 bp [25]. This finding is in agreement with Pang et al.[26] who found carbohydrate fermentation patterns showed ambiguity. Although the pattern of strains isolated from silage and two type strains *L. pentosus* and *L. plantarum* were quite similar, they could not be identified at the species level based on the 16S rRNA gene sequence and API 50 CHL analysis. Therefore, other phylogenetic analysis methods were required to distinguish these strains accurately. In the present study, the selected strains were gram-positive, catalase-negative rods that produced major metabolic products such as lactate from glucose.

Following phylogenetic analysis of 16S rRNA gene

Table 3. *Inoculating effects of LAB strains on the fermentation quality of elephant grass silage at 90 d of ensiling*

Item	Treatment				SEM	P-value
	Control	AZZ1	AZZ4	AZZ7		
DM (g/kgDM)	244.03^{a}	235.35^{c}	239.31^{b}	236.34^{bc}	0.290	0.0004
pH	5.42^{a}	3.19^{b}	3.12^{b}	3.46^{b}	1.080	<.0001
Lactic acid (g/kg DM)	30.26^{d}	47.74^{c}	61.70^{a}	57.82^{b}	3.680	<.0001
Acetic acid (g/kg DM)	19.43^{a}	15.26^{b}	8.31^{d}	11.51^{c}	1.286	<.0001
Propionic acid (g/kg DM	5.19^{a}	2.44^{b}	1.41^{c}	1.48^{c}	0.467	<.0001
Butyric acid (g/kg DM)	8.98^{a}	4.33^{b}	2.08^{b}	3.39^{b}	0.812	<.0001
Water soluble carbohydrates (g/kgDM)	5.63^{a}	3.97^{c}	4.93^{b}	4.75^{b}	0.183	<.0001
Ammonia nitrogen (g/kg total nitrogen)	55.36^{a}	37.66^{b}	26.90^{c}	35.46^{b}	3.217	<.0001
LAB (log cfu/g of FM)	4.78^{b}	5.82^{a}	5.90^{a}	5.70^{a}	0.145	0.0008
Aerobic bacteria (log cfu/g of FM)	6.65a	6.48a	5.58^{b}	6.21^{a}	0.134	0.0024
Yeasts (log cfu/g of FM)	5.50^{a}	5.23^{ab}	4.91^{b}	5.13^{ab}	0.074	0.0116

DM, *dry matter;* ***FM,*** *fresh matter;* ***log,*** *denary logarithm of the numbers;* ***cfu,*** *colony-forming units;* ***LAB,*** *lactic acid bacteria;* ^{a,b,c,d} *Significant differences in means when used multiple comparison* based on *Tukey's test (P<0.05) within the control silages over the 90 day fermentation; Control;* ***AZZ1,*** *Pediococcus acidilactici;* ***AZZ4,*** *Lactobacillus plantarum subsp. plantarum;* ***AZZ7*** *Lactobacillus plantarum subsp. argentoratensis;* ***SEM,*** *standard error of means*

revealed that the contents of DM in the AZZ4 treated silages presented a significant difference (P=0.0004) compared with other treatments. The comparison of the fermentation quality compared with the control showed that all LAB treated silages presented lower values of pH, Water soluble carbohydrates, butyric acid, propionic acid, and NH_3-N content (P<0.0001), while higher lactic acid content and LAB count (P<0.0001) were revealed. Additionally, the AZZ4 treated silages exhibited the lowest values of pH, propionic acid, butyric acids and NH_3-N content compared with all other treatments and control samples.

DISCUSSION

Differentiation between isolates of known species using sequences, selected strains AZZ4, and AZZ7 were identified as for genus *Lactobacillus* and AZZ1 was identified as the genus *Pediococcus*. However, they could not be identified to the species level by phenotypic characteristics. There have been several reports of *Lactobacilli* composing the dominant microbial population of forage crops and silage, where they may contribute to silage fermentation. Some silage-associated lactobacilli have been characterized by phenotypic features and 16S rRNA gene sequences and have been described as different species: for example, *L. paraplantarum, L. brevis, L. buchneri, L. acidophilus, L. plantarum, L. fermentum, L. casei and L. pentosus* [8,10,12,26,27].

In recent years, the phylogenetic relationships of the LAB have been studied extensively in 16S rDNA sequence

ribotyping, and DNA-DNA hybridization experiments and a new species *L. nasuensis* isolated from silage has been added [28]. In the present study, the strains AZZ1, AZZ4 and AZZ7 had a high similarity (>98%) of 16S rDNA sequences to their corresponding known strains within the *Lactobacillus* and *Pediococcus* families, and furthermore confirming that they are most closely related to *L. plantarum* and *P. acidilactici* genera.

The lactic acid bacteria species identified in this study were natural populations of a diversity of forage crops and silages. This finding was in agreement with the results of other studies, which demonstrated that the natural fermentation methods in grass silages and forage crop are dominated by *Enterococcus, Lactococcus, Leuconostoc, Pediococcus and Lactobacillus* species [6,29]. Additionally, a study of Ennahar et al.[8] reported that the LAB species of paddy rice silage in Japan also included *Pediococcus*, which is in consistence with the current study findings. The main reason might be that, the bacterial colonization of plants and fresh crops is controlled by many factors, most notably the climate variations [30]. Therefore, examining the microflora of elephant grass samples from various locations could provide more information about the LAB inhabitation and facilitate the optimization of inoculant characteristics to in order to achieve better silage fermentation quality.

The aim of adding LAB inoculants at ensiling is to guarantee rapid and vigorous fermentation, which leads to faster accumulation of lactic acid, lower pH values during the early stages of ensiling, and inhibition of some pathogenic bacterial growth [31]. On the first stage of the screening method (data not shown), three strains (AZZ1, AZZ4, and AZZ7) produced the highest amounts of lactic acid in the elephant broth and were subsequently selected as the inoculants for elephant silage. All selected isolates increased the lactic acid content and the number of lactic acid bacteria, while decreased, the pH, ammonia nitrogen, water-soluble carbohydrates, butyric acid, and propionic acid when compared to the control group. A small amount of butyric acid was detected except in the control and fresh treatments. A decreased of butyric acid could be related to the reduction in pH caused by the addition of the isolates, which may have inhibited the growth and proteolytic activity of microorganisms such as Clostridia [32,33]. It is accepted that proteolytic activity decreases in the ensiled forage at lower pH.

The low pH values could also explain the decrease in NH_3-N content in response to treatment with the isolated strains. Similar results were reported previously [34,35]. According to the outcomes of Cai et al.[31], *L. plantarum* FG10, a LAB strain isolated from Italian ryegrass, increased the lactic acid content and decreased the butyric acid, NH_3-N content and DM loss of Italian ryegrass silage [10]. This improvement of silage quality was observed when AZZ1, AZZ4, and AZZ7 strains where employed for silage fermentation. Lactic acid is the main reason for the pH decline in high silage quality.

In general, a typical selection criteria for the LAB in silage fermentation would be the ability to survive in various fermentation environments, the ability to produce lactic acid as the main product of carbohydrate fermentation, as well as the competitiveness between inoculants and natural population. Our study evaluated the effects of various lactic acid bacteria on silage quality and found that, the inoculant effects varies at the strain level within species [36]. Also, some studies have found a significant correlation between silage inoculated with LAB and animal performance. Cao et al.[37] found that, *L. plantarum* inoculated with vegetable residue silage had the lowest methane production and highest in vitro DM digestibility [37].

In conclusion; this study showed the isolation of naturally evolved LAB species in the elephant silage and the positive effects of inoculating the silage regarding quality and preservation, which leads to a good feed source for livestock diets. *Lactobacillus Plantarum* subsp. *plantarum* (AZZ4) presented the highest content of lactic acid and lowest NH4-N and pH value among the three inoculated LAB stains, thereby, it could be regarded as a suitable strain for improving elephant silage quality and, thus, is a potential inoculant for the production of silage.

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Conflict of Interest

All author declares that they are no conflict of interest.

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