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ISOLATION AND APPLICATION OF A LACTIC ACID BACTERIUM FROM *PENNISETUM GIGANTEUM* TO IMPROVE THE SILAGE QUALITY OF KING GRASS

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Abstract

In this study, a dominant strain of *Lactiplantibacillus plantarum* was isolated from *Pennisetum giganteum* and used alone or in combination with cellulase, xylanase, and glucose oxidase in a silage experiment with King grass. The results showed that all additives improved the aroma, color, and texture of the silage, as well as enhanced its nutritional content and fermentation quality. Comprehensive analysis indicated that the combination of *L. plantarum* and xylanase was the most effective, significantly increasing lactic acid content, reducing pH and ammonia nitrogen levels, and improving overall silage quality. This combination shows strong potential for application and provides theoretical and technical support for the utilization of King grass as feed.

Introduction

King grass is a perennial, upright, and clump-forming species from the *Pennisetum genus* within the Poaceae (Sun *et al.* 2024). It is extensively grown in China's tropical and subtropical areas because of its strong adaptability, good palatability, high yield, and rich nutritional value (Luo *et al.* 2023). King grass grows well under conditions of sufficient sunlight and moist climate but lags in growth during winter or dry seasons, making it difficult to achieve a balanced supply throughout the year (Zi *et al.* 2021).

Silage is a technique for preserving moist forage by cutting it into small pieces and allowing it to ferment in an oxygen-free environment, preventing the proliferation of harmful bacteria (Xie *et al*. 2022). Silage helps in prolong storage period while largely preserving the nutrient content of the forage, addressing the issue of uneven supply of green feed. Additionally, silage softens the plant fibers in the forage, improving its palatability and promoting digestion by livestock (Zhang *et al*. 2017). Therefore, using King grass for silage can fully exploit its resources, alleviate feed shortages, and effectively solve the problem of uneven annual supply of forage resources.

King grass has a high crude fiber and low water-soluble carbohydrate content, making it prone to rot and poor palatability when directly ensiled, resulting in low-quality silage (Kou *et al.* 2021). Therefore, appropriate additives are needed to ensure proper fermentation. *Lactiplantibacillus plantarum* is capable of fermenting water-soluble carbohydrates into lactic acid, which lowers the pH, suppresses harmful bacterial growth, and minimizes the loss of dry matter and protein in silage, thereby effectively preserving the feed's nutritional value during the fermentation process (Shah *et al*. 2018). Cellulase and xylanase are frequently used as additives in silage to facilitate the breakdown of plant cell walls, producing soluble compounds that serve as fermentation substrates for lactic acid bacteria, thereby enhancing the overall fermentation process

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of the forage (Nkosi *et al*. 2012). Glucose oxidase can reduce oxygen levels during the ensiling process, improving anaerobic conditions, supporting the growth of lactic acid bacteria, and speeding up fermentation. This results in the production of significant amounts of lactic acid, lowering the pH and ensuring proper fermentation (Zhao and Zhang 2007). However, there is a lack of extensive research on how dominant strains selected from forage surfaces and enzyme preparations together impact the silage quality of King grass. Therefore, this study explored the impact of *L. plantarum*, isolated from *P. giganteum*, along with the individual or combined use of cellulase, xylanase, and glucose oxidase on the silage quality of King grass. The findings offer a theoretical foundation for enhancing silage quality and promoting the broader use of King grass.

Materials and Methods

The King grass used in this study was supplied by Yunnan Bergler Animal Husbandry Co., Ltd. It was harvested on July 25, 2024, from the Chuxiong Forage Experimental Base in Yunnan, with a cutting length of about 1.5 meters and a fresh grass moisture content of about 70%. The grass was shredded into 2-3 cm pieces utilizing a shredder and then ensiled in bales. Cellulase (activity of 20,000 U/g, dosage of 0.5 g/kg), xylanase (activity of 100,000 U/g, dosage of 0.25 g/kg), and glucose oxidase (activity of 1,000 U/g, dosage of 0.04 g/kg) were supplied by Kunming Aikeite Biotechnology Co. Ltd.

The dominant strain *L. plantarum* screened from *P. giganteum* and the four enzymes were used as additives and mixed with King grass for ensiling. The experiment included one control group and seven experimental groups: control group (CK) with sterile water; group A with cellulase; group B with xylanase; group C with glucose oxidase; group D with *L. plantarum*; group E with a mixture of cellulase and *L. plantarum*; group F with a mixture of xylanase and *L. plantarum*; and group G with a mixture of glucose oxidase and *L. plantarum*. After mixing various silage additives and sterile water with the crushed king grass, the mixture is packed into silage bags, compressed tightly, vacuum-sealed, and sealed. Each bag contains 1 kg and each treatment group is designed with three parallel silage bags. After being stored at room temperature for 60 days, sensory quality evaluation, as well as the determination of nutritional components and fermentation quality, is conducted.

A 1 gram sample of *P. giganteum* silage was added to 100 mL of sterile water, thoroughly mixed using a vortex oscillator at 30°C and 180 rpm, and left undisturbed for 5 min. The resulting supernatant was collected, and serial dilutions of 10^{-3}, 10^{-4}, and 10^{-5} were prepared. A 100 µl aliquot from each dilution was plated on MRS agar and cultured at 36 ± 1°C for 48 hrs. Selected colonies were isolated and streaked for purification. Gram staining was performed to determine morphological characteristics, and physiological and biochemical identification was conducted using the HBI Lactobacillus Biochemical Identification Kit (HaiBo Biotechnology Co. Ltd.). The screened strains were sent to Tsingke Biotechnology Co. Ltd. for DNA sequencing. The obtained sequences were assembled using SeqMan software, and homology comparisons were performed using the BLAST program in the NCBI database. Evolutionary trees were generated in MEGA11 using ITS sequences and homologous sequences retrieved from the GenBank database to identify the species.

The sensory quality of King grass was evaluated based on color, odor, and structure following the scoring standards set by the German Agricultural Society (DLG), the detailed evaluation criteria are based on the method of Li *et al.* (2016). The nutritional constituents of King grass, such as dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and water-soluble carbohydrates (WSC), were assessed. DM was quantified using the drying method (Liao *et al*. 2024), CP was assessed following the method of Zhang (2007), while

NDF and ADF contents were determined based on the Van Soest *et al.* (1991) method. WSC content was evaluated using the anthrone-sulfuric acid colorimetric technique (Owens *et al.* 1999). Fermentation quality was assessed by measuring pH, ammonia nitrogen (NH_3-N), lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA). pH was recorded using a pHS-3D pH meter, NH_3-N concentration was determined via the phenol-sodium hypochlorite colorimetric method (Kung *et al.* 2018), while LA, AA, PA, and BA concentrations were analyzed using liquid chromatography with high resolution (HPLC) (Liu *et al.* 2015).

The membership function approach was employed to conduct a comprehensive assessment of the nutritional value and fermentation quality of King grass silage (Ling *et al.* 2023). Positive indicators included DM, LA, AA, PA, CP, and WSC, while negative indicators included pH, NH_3-N, NDF, ADF, and BA. The membership degrees were summed, and the total membership degree was ranked to determine the best treatment. The calculation formulas are based on the Ling *et al.* (2023) method.

The raw data were organized using Excel 2013, and the nutritional content and silage sample data from various treatments were evaluated through one-way ANOVA in SPSS 26.0 software. For multiple comparisons, Duncan's method was applied, and results are presented as mean ± standard deviation, with $P < 0.05$ considered statistically substantial.

Results and Discussion

The isolated bacterial strains appeared as milky white colonies on MRS solid medium. Under the microscope, the strains were short rods and stained purple with Gram stain. In terms of physiological and biochemical characteristics, the strain was capable of utilizing a variety of carbon sources within 24-48 hrs, including maltose, cellobiose, lactose, sucrose, raffinose, mannitol, sorbitol, salicin, and inulin. It was also tested positive for esculin hydrolysis. These results indicate a broad carbohydrate fermentation profile and high metabolic activity, which are consistent with the typical sugar metabolism pattern of lactic acid bacteria and further support the preliminary identification of the strain as belonging to the genus *Lactobacillus*. Based on the morphological features, the strains were preliminarily identified as *Lactobacillus*. Then the ITS regions of 16S rDNA of the isolated strains were sequenced and analyzed for molecular identification. The sequences were then subjected to homology comparison in NCBI, and a phylogenetic tree was generated with the help of MEGA11. The results of molecular identification are shown in Fig. 1. Combining morphological, physiological, biochemical, and molecular identification, the strain corroborated 100% homology with *Lactobacillus plantarum.*

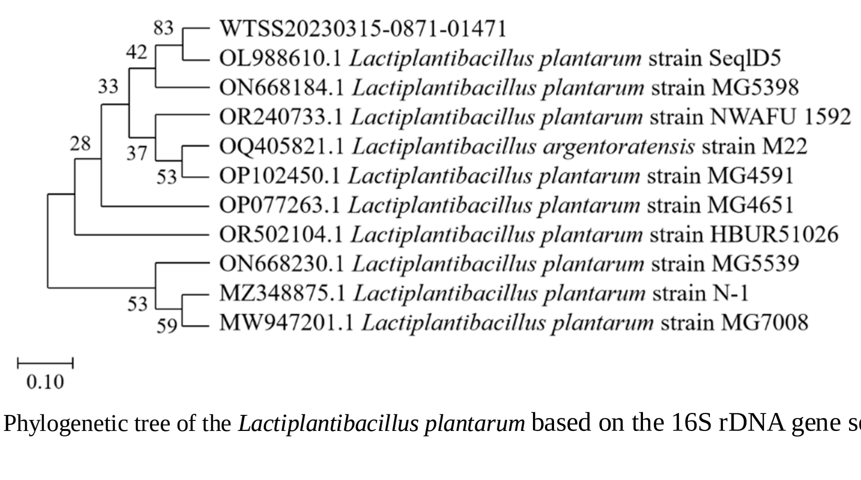

Fig. 1. Phylogenetic tree of the *Lactiplantibacillus plantarum* based on the 16S rDNA gene sequence.

Sixty days into the ensiling process, the sensory characteristics of King grass were assessed in terms of odor, texture, and color (Table 1). Regarding odor, the control group (CK) along with groups A and C exhibited a sour scent, which could affect livestock feed intake. Groups B, D, E, F, and G had a wine-like aroma, scoring the highest at 14 points, meeting the standard for high-quality silage. In terms of structure, all treatment groups except CK maintained good structure, being loose and non-sticky, with no signs of decay or contamination. In terms of color, groups B, E, and F had colors similar to the raw material, while CK, A, C, D, and G had a slight yellowish tint, indicating slightly poorer color. Overall, CK scored the lowest at 13 points, while groups A and C scored 15 points, rated as "good," indicating less ideal ensiling results. Groups B, D, E, F, and G performed the best, rated as "excellent," with scores of 20 points.

Sensory evaluation is a common method for assessing the quality of silage. In practice, evaluators often judge the quality of silage based on visual, olfactory, and tactile indicators such as odor, structure, and color, which can provide a quick and intuitive assessment of the forage quality (Jiang *et al.* 2024). In this study, the sensory ratings for all groups treated with additives were higher compared to the control group, aligning with the results of Wang *et al.* (2024) regarding the sensory quality of alfalfa treated with composite bacteria and enzymes. This suggests that it is difficult to achieve the anaerobic environment required for lactic acid bacteria fermentation under conventional ensiling conditions. Additive treatments can effectively improve the sensory quality of King grass, this is likely because the addition of enzymes and bacteria fosters the creation of an anaerobic environment, leading to the production of substantial amounts of lactic acid, which suppresses the growth of harmful bacteria. As a result, the structure of King grass silage is preserved, and its odor and color are enhanced.

Table 1. Sensory evaluation of King grass silage.

Group	Odor and Score	Structure and Score	Color and Score	Total Score	Grade
CK	Strong sour smell (10 points)	Poor structure (2 points)	Light yellow (1 point)	13 points	Good
A	Weak butyric smell (10 points)	Good structure (4 points)	Light yellow (1 point)	15 points	Good
B	Wine-like aroma (14 points)	Good structure (4 points)	Similar to raw material (2 points)	20 points	Excellent
C	Weak butyric smell (10 points)	Good structure (4 points)	Light yellow (1 point)	15 points	Good
D	Wine-like aroma (14 points)	Good structure (4 points)	Light yellow (1 point)	16 points	Excellent
E	Wine-like aroma (14 points)	Good structure (4 points)	Similar to raw material (2 points)	20 points	Excellent
F	Wine-like aroma (14 points)	Good structure (4 points)	Similar to raw material (2 points)	20 points	Excellent
G	Wine-like aroma (14 points)	Good structure (4 points)	Similar to raw material (2 points)	20 points	Excellent

The contents of DM, NDF, ADF, CP, and WSC in King grass silage are presented in Table 2. Except for group G, the DM content in all experimental groups was substantially greater than that of the control group, with groups E and F showing the most significant effects. For NDF content, except for group C, all experimental groups exhibited a markedly lower value than the control group ($P < 0.05$). The ADF content in all experimental groups was notably reduced compared to the control group ($P < 0.05$), with group E showing the lowest ADF level at 42.99%. Except for group D, the CP content in all experimental groups was markedly higher than in the control group. Similarly, the WSC content of all experimental groups exceeded that of the control group, with groups E and F displaying significantly greater WSC levels compared to the others.

The nutritional composition of feed is the most intuitive indicator for assessing feed quality. By determining the composition of feed, a preliminary evaluation of its quality can be directly made (Liu *et al*. 2019). In silage, the loss of DM often involves valuable nutrients. In this study, the treatment involving both xylanase and *L. plantarum* led to the smallest DM loss, indicating that the addition of xylanase and *L. plantarum* can utilize WSC to produce organic acids, primarily LA, this, in turn, reduces the pH of the fermentation system, effectively preventing the growth of spoilage microorganisms and minimizing DM loss (Zheng *et al*. 2024). Higher CP content indicates better nutritional value of forage (Li *et al*. 2020), but CP degradation during ensiling is inevitable. In this study, the CP content in all experimental groups exceeded that of the control group, indicating that the inclusion of *L. plantarum* and the three enzymes helped preventing the degradation of CP by spoilage microorganisms, effectively reducing nutrient loss. WSC acts as an essential nutrient for the growth and proliferation of lactic acid bacteria, which is vital for effective fermentation (Nkosi *et al*. 2012). A high WSC content in silage fermentation materials is a key factor for ensuring proper fermentation. NDF and ADF are important indicators affecting feed palatability and digestibility. NDF content is negatively correlated with animal feed intake, while ADF is difficult for livestock to digest and absorb. Lower ADF content indicates higher digestible dry matter (Ma *et al*. 2025).

Table 2. Nutrient composition of King grass silage.

Group	DM（%）	NDF（%）	ADF（%）	CP（%）	WSC（%）
CK	23.24 ± 0.29e	70.74 ± 0.44a	49.60 ± 0.28ab	4.27 ± 0.01e	33.45 ± 0.65f
A	25.46 ± 0.24c	67.07 ± 0.84bc	45.30 ± 0.46cde	5.21 ± 0.01bc	34.53 ± 0.26cde
B	25.51 ± 0.54bc	65.65 ± 0.54d	45.15 ± 0.81cde	5.46 ± 0.020a	38.42 ± 0.11bc
C	24.40 ± 0.36d	70.41 ± 0.48a	47.07 ± 0.49c	5.23 ± 0.015bc	34.88 ± 0.65e
D	25.78 ± 0.59bc	67.34 ± 0.52bc	46.98 ± 3.06c	4.59 ± 0.0057de	34.48 ± 0.40de
E	26.12 ± 0.18ab	64.01 ± 0.28e	42.99 ± 0.29e	5.28 ± 0.026b	39.27 ± 0.13ab
F	26.67 ± 0.16ab	66.18 ± 0.71bc	45.05 ± 1.56cde	5.43 ± 0.028a	39.93 ± 0.28ab
G	24.27 ± 0.14de	67.13 ± 0.39bc	46.62 ± 0.75cd	5.21 ± 0.026bc	35.35 ± 0.21cd

Mean values within the column followed by the same letters are not significantly different at $P < 0.05$, different letters within the same column represent significant differences at $P<0.05$. according to DMRT.

Table 3 presents the fermentation quality of King grass. When compared to CK, the LA content in all experimental groups was considerably higher, with group F having the highest LA content (2.55%). The AA content in groups A, B, and D was notably higher than that in CK, whereas no significant difference was observed in the other experimental groups compared to CK. In terms of PA content, except for groups A and C, all other experimental groups exhibited significant differences when compared to CK. Except for group A, the BA content in all experimental groups was notably lower than that of CK, with group D having the lowest BA content at 0.78%. The pH levels in all experimental groups were notably lower than that of CK, with group F having the lowest pH at 3.24. Regarding NH_3-N, all experimental groups, except for groups A and E, had significantly reduced levels compared to CK. Overall, group F showed the most significant increase in LA content and the lowest pH, indicating the best performance.

pH is an essential parameter for assessing silage quality, with high-quality silage typically having a pH of 4.2 or less (Wang *et al.* 2017). In this study, the pH levels in all experimental groups were below 4.2, with the combined treatment of enzymes and bacteria showing more pronounced effects and better silage quality. Organic acids are metabolic products of microorganisms during ensiling and are key indicators for evaluating silage quality. High LA content and low BA content are characteristics of high-quality silage. LA is the primary metabolic product of lactic acid bacteria and is found in high levels in silage, where it effectively suppresses the growth of harmful microorganisms and enhances silage quality (Xie *et al.* 2022). In this study, the LA levels in all experimental groups were considerably higher as compared to the control group, with the highest concentration observed in the treatment combining xylanase and *L. plantarum*. This suggests that the inclusion of *L. plantarum* encourages the rapid growth of lactic acid bacteria, leading to increased LA production, and that xylanase and *L. plantarum* exhibit a synergistic effect. BA is primarily produced by the fermentation of harmful anaerobic clostridia. Under conventional treatment, silage tends to accumulate high levels of BA. The addition of combined enzymes and bacteria significantly reduced BA content, this suggests that the combined treatment effectively suppressed the proliferation of spoilage microorganisms, ensuring high-quality silage (Wang *et al.* 2014). Higher NH_3-N content indicates greater degradation of amino acids and proteins in silage, leading to greater nutrient loss. Therefore, the NH_3-N content serves as a key indicator of protein degradation during ensiling and plays a crucial role in assessing silage quality (Di *et al.* 2024). In this study, all treatment groups exhibited lower NH_3-N levels compared to the control group, indicating that the additives inhibited protein degradation during fermentation and improved silage quality.

Table 3. Fermentation quality of King grass silage.

Group	LA（%）	AA（%）	PA（%）	BA（%）	pH	NH3-N（%）
CK	0.47 ± 0.11d	0.39 ± 0.03de	0.16 ± 0.01d	2.06 ± 0.23a	5.5 ± 0.02a	4.21 ± 0.20a
A	1.25 ± 0.24c	0.82 ± 0.02ab	0.16 ± 0.0057d	1.94 ± 0.043a	4.06 ± 0.02c	3.54 ± 0.12ab
B	1.58 ± 0.16c	0.7 ± 0.005bc	0.23 ± 0.020bc	0.75 ± 0.020f	4.006 ± 0.01d	3.37 ± 0.24bc
C	1.44 ± 0.11c	0.41 ± 0.026de	0.15 ± 0.015d	0.93 ± 0.075def	4.19 ± 0.005b	3.07 ± 0.16c
D	1.53 ± 0.057c	0.48 ± 0.005bc	0.24 ± 0.011abc	0.78 ± 0.03ef	3.8 ± 0.01e	3.49 ± 0.12b
E	2.11 ± 0.10b	0.32 ± 0.030ef	0.23 ± 0.011abc	1.51 ± 0.16b	3.31 ± 0.01f	3.58 ± 0.46ab
F	2.55 ± 0.38ab	0.41 ± 0.032de	0.26 ± 0.01ab	1.16 ± 0.10de	3.24 ± 0.01f	3.31 ± 0.12bc
G	1.41 ± 0.13c	0.40 ± 0.011de	0.21 ± 0.0057c	1 ± 0.052def	3.36 ± 0.01f	3.05 ± 0.12c

Mean values within the column followed by the same letters are not significantly different at $P < 0.05$, different letters within the same column represent significant differences at $P < 0.05$ according to DMRT.

In this study, the membership function method was applied to evaluate the silage quality of King grass based on 10 distinct indicators.: DM, NDF, ADF, CP, WSC, LA, AA, PA, BA, pH, and NH_3-N. The higher average membership degree indicates better silage quality. As shown in Table 4, group F performed the best, this suggests that the mixture of *L. plantarum* and xylanase was the most efficient. The comprehensive quality ranking of the treatment groups was F > E > G > D > B > A > C > CK.

Due to the interactions between *L. plantarum* and the various enzymes, the trends of different indicators varied across treatment groups. Relying solely on a single or a few indicators to evaluate the quality of forage silage is insufficient. Therefore, to scientifically and reasonably assess its nutritional value, this study employed the membership function method to assess silage quality holistically using multiple indicators. Through this analysis, the treatment combining xylanase and *L.plantarum* had the highest membership function value, indicating that it is the most suitable for the ensiling of King grass.

Table 4. Comprehensive evaluation and ranking of King grass silage quality.

Measurement item	Group							
	CK	A	B	C	D	E	F	G
DM	0.587	0.400	0.403	0.407	0.537	0.561	0.569	0.450
NDF	0.448	0.502	0.413	0.515	0.533	0.803	0.482	0.478
ADF	0.461	0.518	0.459	0.609	0.384	0.589	0.391	0.609
CP	0.500	0.500	0.583	0.444	0.333	0.600	0.667	0.400
WSC	0.339	0.551	0.500	0.391	0.531	0.347	0.424	0.367
LA	0.349	0.503	0.474	0.478	0.333	0.428	0.687	0.469
AA	0.619	0.600	0.333	0.400	0.667	0.556	0.611	0.333
PA	0.500	0.333	0.583	0.555	0.667	0.333	0.500	0.667
BA	0.393	0.375	0.583	0.619	0.500	0.458	0.444	0.600
pH	0.500	0.583	0.667	0.333	0.500	0.556	0.667	0.667
NH3-N	0.466	0.555	0.503	0.583	0.556	0.365	0.556	0.555
Average Membership Degree	0.470	0.493	0.500	0.485	0.504	0.509	0.5454	0.508
Ranking	8	6	5	7	4	2	1	3

The findings of this study indicate that *L. plantarum* is the predominant strain isolated from *P. giganteum*. The addition of *L. plantarum* with cellulase, xylanase, and glucose oxidase, either individually or in combination, improved the sensory quality (odor, color, texture) of King grass silage to some extent, enhancing fermentation quality and nutritional value. Among the treatments, the combination of xylanase and *L. plantarum* yielded the best results and is recommended for the mixed ensiling of King grass.

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