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Research article

Characterization and identification of lactic acid bacteria isolated from Napier grass and sugarcane top silages and their application for silage preparation

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Abstract: In this study, we aimed to isolate and characterize lactic acid bacteria (LAB) from mixed Napier grass (NG) and sugarcane top (ST) silages, and to evaluate their effects on silage quality as alternative LAB inoculants. Among the 370 LAB strains isolated from silage and screened for tolerance to pH 3.7, we identified 17 strains with distinct colony shapes and sources. Strains XH146 and XH352 exhibited the strongest ability to produce acid in Man Rogosa Sharpe broth and were identified as *Lactiplantibacillus paracasei* and *Lactiplantibacillus plantarum*, respectively. Strains XH146 (LAB1), XH352 (LAB2), and *L. plantarum* Chikuso-1 (FG, a commercial inoculant) were used as additives for NG and ST silage preparation. All inoculated silages were better preserved than the control. LAB addition significantly reduced the pH of NG silage, with the lowest pH and the highest lactic acid content found in the combined LAB1 + LAB2 treatment. NH3-N contents decreased in the following order: Control > LAB1 > LAB2 > FG > LAB1 + LAB2. In conclusion, *L. paracasei* XH146 and *L. plantarum* XH352 demonstrated the highest ability to enhance silage quality, making them promising candidates for silage inoculants.

Keywords: identification; lactic acid bacteria; Napier grass; sugarcane top; silage inoculant

1. Introduction

Silage, a fermented feed produced by preserving fresh forage and grasses under anaerobic

conditions, has the ability to preserve nutrients, enhance palatability, balance seasonal feed supplies, reduce waste, and reduce costs, which underscores its value as a cornerstone of sustainable ruminant livestock nutrition [1]. As the global demand for animal products increases, silage production will play an increasingly vital role in optimizing feed efficiency, minimizing environmental impacts, and ensuring food security [2]. The fermentation process also enhances nutrient bioavailability. During ensiling, epiphytic bacteria, especially lactic acid bacteria (LAB), convert sugars into organic acids (e.g., lactic acid) and thus lowers the pH to 3.5–4.2. This suppresses the growth of harmful bacteria, and minimizes the risk of secondary fermentation, which stabilizes the feed and improves nutrients digestibility [3,4]. However, the types and quantities of microorganisms substantially differ across regions, and their colony structure and abundance exhibit dynamic changes during silage fermentation [5]. Moreover, the production of high-quality silage using conventional methods remains difficult because of limited populations of naturally occurring LAB in forage.

Future advancements in additive technologies (e.g., inoculants and enzymes) and precision ensiling methods will further elevate silage quality, solidifying its position as an indispensable feed resource. Inoculants enhance silage quality and animal performance by inhibiting the growth of harmful microorganisms, reducing fermentation losses, and improving forage palatability [6]. Silage additives are categorized by their effects on preservation into fermentation stimulants, fermentation inhibitors, aerobic deterioration inhibitors, and nutrients and absorbents [7]. LAB naturally emerge during fermentation and are usually added to enhance the ensiling process and improve the final fermentation quality [8]. LAB inocula play a key role in the preservation and fermentation of forage crops within inoculated silages [9]. However, not all commercial LAB inoculants ensure good fermentation, owing to differences in strains and varying adaptation to ensiling and environmental conditions [10,11].

Napier grass (*Pennisetum purpureum* Schumach; NG) is a herbaceous plant belonging to the Poaceae family and is used for livestock farming. It is characterized by a high biological yield, softness and juiciness, good palatability, and high nutritional value [12,13]. As an excellent forage source and important high-quality green feed for animals, NG is widely cultivated in tropical and subtropical regions [14]. However, NG production is highly seasonal. During the vigorous growth period in summer and autumn, biomass is often produced in excess, whereas biomass shortages occur during the 4-month grass-cutting period in winter and spring, leading to imbalances in NG availability throughout the year [15]. While NG is primarily used as fresh feed, it also serves as a valuable silage material and helps offsetting seasonal feed deficits. However, its relatively high water and low water-soluble carbohydrate (WSC) contents often result in poor fermentation quality when ensiled alone [16,17].

Sugarcane (*Saccharum officinarum* L.) is an important energy and sugar crop. It is an essential agricultural crop that is mostly cultivated in tropical and subtropical countries, accounting for approximately 80% of global sugar production [18]. In 2023, the sugarcane planting area in China reached 1.265 million hectares, producing 104.556 million tons of sugarcane [19]. Sugarcane top (ST) is the main byproduct of sugarcane harvesting and accounts for approximately 15 to 20% of the sugarcane plant [20]. ST has high yields, an elevated WSC content, and is highly nutritious, with an acceptable intake preference for livestock [21,22]. However, sugarcane harvesting is seasonal, resulting in resource wastage and suboptimal utilization of ST [23]. Following pretreatment methods such as steaming, steam explosion, or ensiling to retain the nutrients in ST, it is mostly used as forage for milk production and the fattening of ruminants worldwide [24]. The most rational use of ST is to cut it into silage during winter and spring, when roughage resources are scarce. Indeed, ST is an

important animal roughage resource in southern China, particularly in Guangxi [25,26].

Ensiling can preserve high-quality forage, but the high moisture and low WSC contents of NG make ensiling challenging. Moreover, high-quality ST silage is relatively difficult to prepare because of the physical structure and climatic disadvantages of untreated ST. The addition of exogenous LAB inoculants has been extensively studied for their beneficial effects on silage fermentation and quality [27]. However, only some researchers have reported the isolation and evaluation of dedicated LAB strains that are naturally adapted to tropical and subtropical forage, such as NG and ST, and their efficacy as silage inoculants. Therefore, we aimed to isolate and characterize LAB with probiotic potential in silages prepared with NG, ST, or their mixtures and evaluate their effects on silage characteristics to develop highly effective LAB for NG or ST silage.

2. Materials and methods

2.1. Silage samples and isolation of LAB

NG was obtained from the Herbage Base at the Buffalo Research Institute, Guangxi, Nanning, China. ST was manually collected after cane harvest in the Chongzuo City industrial sugar production area, China. NG and ST were ensiled separately or mixed at varying ratios (from 10:90 to 90:10) based on fresh matter (FM) contents. Approximately 1 kg of raw material from each of the six established replicates was packed into 160 × 250 mm plastic bags and sealed with a vacuum sealer. The silages were kept at 22–28 °C in the laboratory and sampled on days 1, 3, 5, 7, 15, 30, and 60. Twenty grams of silage samples were homogenized with 180 mL of sterilized water and subsequently subjected to serial dilution from 10−1 to 10−5 in sterilized water. The number of LAB was measured via plate counting on Man Rogosa Sharpe (MRS) agar (HuanKai Biology Co., Ltd, Guangzhou, China) incubated at 30 °C for 72 h. Each LAB colony was purified twice on MRS agar and then preserved at −80 °C in a 9:1 mixture of MRS broth and dimethyl sulfoxide for later analysis.

2.2. Morphological, physiological, and biochemical tests

After incubation on MRS agar for 24 h, LAB gram staining and morphology were examined. Catalase activity and glucose gas production were assessed using the method described by Cai et al. [28]. Growth was tested in MRS broth at 5 and 10 °C for 10 d and at 45 and 50 °C for 7 d. The ability to grow at pH levels of 2.5, 3.0, 3.5, 4.0, and 7.0 was assessed in MRS broth after incubation at 30 °C for 7 d. Salt tolerance was tested in MRS broth containing 3.0 and 6.5% NaCl over a period of 7 d. The ability to grow at pH levels of 2.5, 3.0, 3.5, 4.0, and 7.0 and salt tolerance in 3.0 and 6.5% NaCl was evaluated via MRS broth incubation at 30 °C for 7 d.

2.3. 16S rRNA gene sequence analysis

LAB was cultured in MRS broth at 37 °C for 48 h for DNA extraction and purification using a TSINGKE Plant DNA Extraction Kit (Product Code: TSP101, Tsingke Biotechnology Co., Ltd, Beijing China). The isolated DNA underwent polymerase chain reaction (PCR) with primers 27F (5′-AGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACT T-3′). Electrophoresis, gel cutting, recovery, and purification of PCR products were performed by Tsingke

Biotechnology Co., Ltd., and the Sanger method (Contig Express software) was used for two-way detection and splicing. The 16S rRNA gene sequence similarity was determined using the GenBank and BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cg). The sequences are available in GenBank with accession numbers. Nucleotide substitution rates were calculated, and phylogenetic trees were created using the maximum likelihood method.

2.4. Laboratory silage preparation and chemical analysis

NG was obtained in the vegetative stage approximately 10 cm above ground level from the Herbage Base at the Buffalo Research Institute, Guangxi, Nanning, China. ST was manually collected after cane harvest in the Chongzuo City industrial sugar production area, China, in November 2023. Isolates of LAB1 (*L. paracasei*; XH146), LAB2 (*L. plantarum*; XH352), and FG (*L. plantarum* Chikuso-1, isolated from a commercial inoculant, Snow Brand Seed Co., Ltd, Sapporo, Japan) were used to establish the following treatments: Silage without additives (Control) or silage with LAB1, LAB2, LAB1 + LAB2, or FG. The inoculated LAB level was 1.0×10^5 CFU/g FM. In the LAB1 + LAB2 treatment, LAB1 and LAB2 were added in equal amounts to add up to 1.0×10^5 conlony-forming units (CFU)/g FM. Approximately 1 kg of silage material was chopped into pieces with a length of approximately 20 mm, treated evenly with LAB, packed into 160 × 250-mm plastic bags, and sealed with a vacuum sealer. Five bags per treatment were stored at room temperature and in the dark for 60 d before chemical and fermentation analyses were performed. Dry matter (DM), crude protein (CP), and organic matter (OM) contents were analyzed using Association of Official Analytical Chemists methods 934.01, 976.05, and 942.05, respectively [29]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were analyzed following Van Soest et al. [30]. The WSC content was measured using the anthrone colorimetric method described by Udén [31]. Yeasts and molds were cultivated on potato dextrose agar and counted. Yeasts were distinguished from molds and bacteria based on their colony morphology and cell structure. Microbial populations were expressed as log10 (lg) CFU/g FM. The pH was measured using a pH meter. The content of lactic, acetic, propionic, and butyric acids was measured using high-performance liquid chromatography (1260 Infinity II; Agilent Technologies, Inc., Waldbronn, Germany). The NH_3-N content was measured using the method described by Byrne and McCormack [32].

2.5. Statistical analyses

Data were analyzed using one-way analysis of variance in the SAS version 9.2 software (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was used to identify differences ($p < 0.05$) between means.

3. Results

3.1. Characteristics of representative strains isolated from silage

Using MRS solid medium, a total of 370 LAB strains were preliminarily isolated from NG, ST, or mixed silage. Among these, 17 LAB strains (named XH1, XH15, XH49, XH122, XH124, XH146, XH184, XH209, XH335, XH341, XH343, XH345, XH348, XH352, XH356, XH360, and XH368)

with distinct colony shapes and sources were obtained through preliminary screening of acid production and growth characteristics (Table 1). The 17 strains were rod-shaped, gram-positive, catalase-negative, and homofermentative. Among the 17 strains, XH352 and XH146 showed the lowest pH values (3.46 and 3.50, respectively) when cultured in MRS broth medium for 24 h. Most strains thrived between pH 3.5 and 9, and between 5 and 50 ℃. Except for XH341, all strains grew normally at salt concentrations of 3 and 6.5% NaCl.

Table 1. Characteristics of representative strains isolated from Napier grass, sugarcane top, and mixed silages.

Strain	Shape	Gram	Fermentation	Catalase	24 h pH	Growth at temperature (°C)				Growth in NaCl		Growth at pH					
		stain	type	activity	in MRS broth	5	10	45	50	3.00%	6.50%	2.5	3	3.5	4	7	9
XH343	Rod	+	Homo	-	3.54	+	+	+	+	+	+	-	-	-	+	+	+
XH49	Rod	+	Homo	-	3.53	+	+	+	+	+	+	-	-	+	+	+	+
XH345	Rod	+	Homo	-	3.55	+	+	+	+	+	+	-	-	-	+	+	+
XH1	Rod	+	Homo	-	3.59	+	+	+	+	+	+	-	-	-	+	+	+
XH15	Rod	+	Homo	-	3.57	+	+	+	+	+	+	-	-	-	+	+	+
XH124	Rod	+	Homo	-	3.64	+	+	+	+	+	+	-	-	+	+	+	+
XH352	Rod	+	Homo	-	3.46	+	+	+	+	+	+	-	-	+	+	+	+
XH341	Rod	+	Homo	-	3.61	+	+	+	+	-	-	-	-	+	+	+	+
XH356	Rod	+	Homo	-	3.57	−	+	+	+	+	+	-	-	+	+	+	+
XH209	Rod	+	Homo	-	3.65	−	+	w	-	+	+	-	-	-	+	+	+
XH122	Rod	+	Homo	-	3.58		+	+	+	+	+	-	-	+	+	+	+
XH146	Rod	+	Homo	-	3.50	+	+	+	+	+	+	-	-	+	+	+	+
XH184	Rod	+	Homo	-	3.64	+	+	+	+	+	+	-	-	+	+	+	+
XH335	Rod	+	Homo	-	3.65	+	+	+	+	+	+	-	-	+	+	+	+
XH348	Rod	+	Homo	-	3.67	+	+	+	+	+	+	-	-	+	+	+	+
XH360	Rod	+	Homo	-	3.59	-	+	+	+	+	+	-	-	-	+	+	+
XH368	Rod	+	Homo	-	3.61	-	+	+	+	+	+	-	-	-	+	+	+

Note: +, positive; w, weakly positive; −, negative.

3.2. BLAST results of 16S rDNA sequences of LAB

BLAST alignment of 16S rDNA sequences revealed a 99 to 100% similarity between the 17 LAB strains and their closely related species (Table 2). According to the phylogenetic tree (Figure 1), the LAB strains formed two branches, with XH122, XH146, and XH184 clustering with a self-expansion support rate of 99%. Based on the BLAST similarity of their gene sequences to closely related species, they were identified as *L. paracasei*. The remaining 14 strains of LAB clustered with a self-expansion support rate of 99%, indicating a close genetic relationship. Based on their BLAST similarity, they were identified as *L. plantarum*.

Table 2. BLAST results of 16S rDNA sequences of lactic acid bacteria strains.

Gene ID	Accession	Species	Identity
X122	MT613613.1	*L. paracasei*	1424/1425(99.93)
X146, X184	MT457703.1	*L. paracasei*	1424/1424(100.00)
X368, XH1, XH124, X335, XH356	MT645503.1	*L. plantarum*	1372/1372(100.00)
XH15, X348	MT613627.1	*L. plantarum*	1398/1399(99.93)
X360	MN448306.1	*L. plantarum*	1397/1397(100.00)
XH209	MT613638.1	*L. plantarum*	1427/1428(99.93)
XH341	CP037429.1	*L. plantarum*	1384/1384(100.00)
XH343	MT613627.1	*L. plantarum*	1398/1399(99.93)
XH345, XH352	MT597745.1	*L. plantarum*	1403/1404(99.93)
XH49	MT645511.1	*L. plantarum*	1395/1396(99.93)

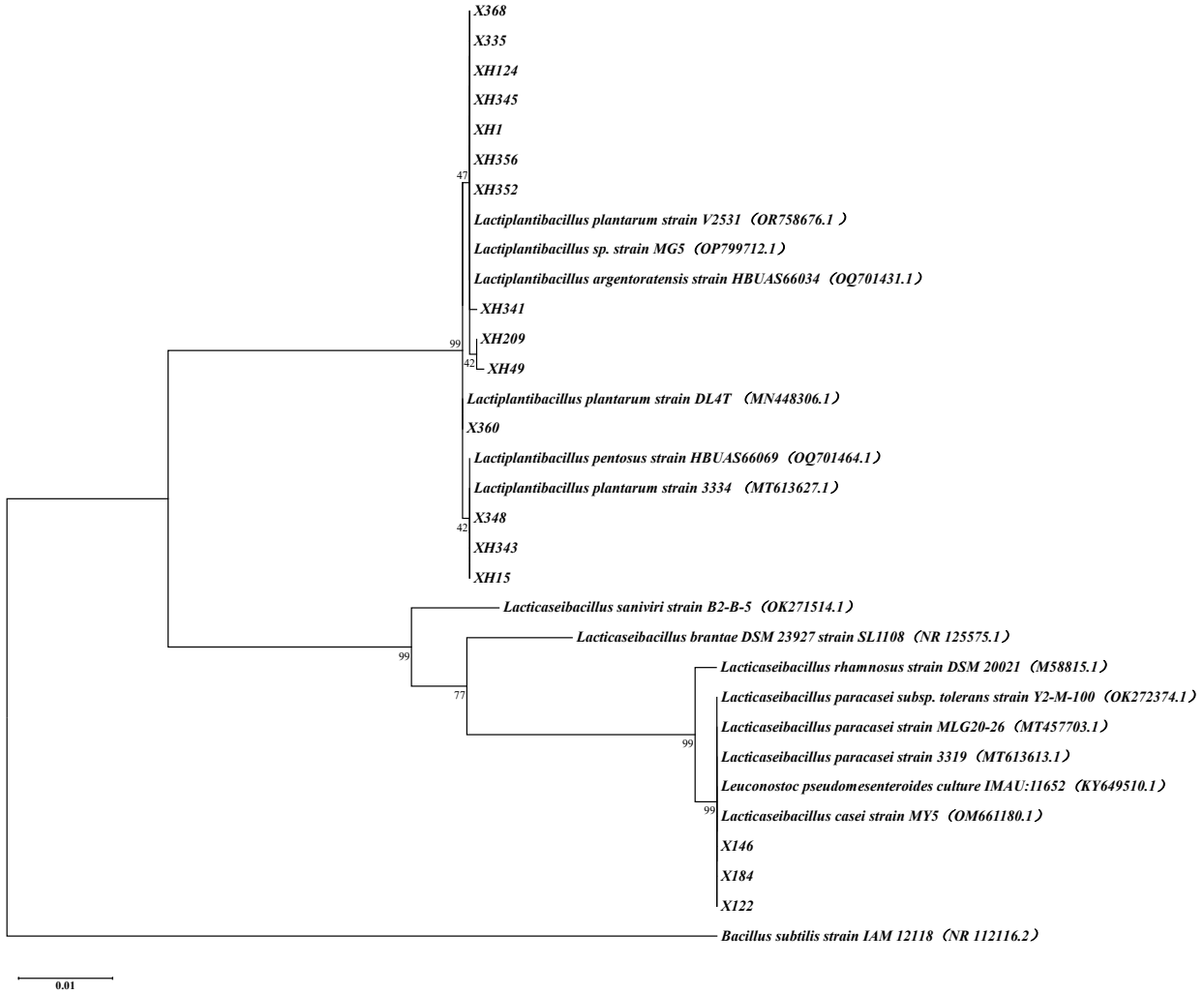

Figure 1. Phylogenetic tree showing the relative position of *L. plantarum* species as inferred by the maximum likelihood method used with 16S rRNA gene sequences. Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. *B. subtilis* was used as an outgroup. The bar indicates 1% sequence divergence.

3.3. Chemical composition of raw material and silage

Before ensiling, ST had higher contents of DM, OM, NDF, ADF, and lactic and acetic acids, along with higher numbers of LAB and yeasts, but lower pH and lower contents of CP and ash than NG (Table 3). The LAB1 + LAB2 treatment resulted in the highest DM but lowest WSC contents in NG and ST silages, significantly surpassing those of the control ($p < 0.05$; Table 4). The LAB2-, LAB1 + LAB2-, and FG-treated ST silage had CP contents comparable with that of the control ($p < 0.05$); however, the CP content in NG silage did not show significant differences across treatments ($p > 0.05$). NG silage without LAB (control) or NG silage treated with LAB1 had higher ash contents but lower OM contents than NG silage treated with LAB2, LAB1 + LAB2, and FG ($p < 0.05$), whereas ST silage treated with LAB2 and LAB1 + LAB2 had higher ash contents but lower OM contents than control silage ($p < 0.05$). Compared with the control, NG silage treated with LAB1 + LAB2 had significantly lower NDF and ADF contents ($p < 0.05$). In ST silage, the NDF content did not differ significantly among treatments ($p > 0.05$), but the ADF content in LAB2- and LAB1 + LAB2-treated ST silage was notably lower than that in the control ($p < 0.05$).

Table 3. Chemical composition, microbial population, organic acids, and pH of Napier grass and sugarcane top before ensiling.

Item	NG	ST
Chemical composition		
DM（%）	18.96	26.84
CP（% DM)	12.38	7.53
Ash（% DM)	10.76	5.43
OM（% DM)	89.24	94.57
NDF（% DM)	67.97	75.03
ADF（% DM)	41.79	45.56
WSC（% DM)	2.97	15.26
Microbial population		
LAB (lg CFU/g FM)	3.94	4.12
Yeasts (lg CFU/g FM)	3.23	3.67
Molds (lg CFU/g FM)	ND	ND
Organic acids		
Lactic acid (g/kg DM)	0.26	0.75
Acetic acid (g/kg DM)	0.39	0.31
Propionic acid (g/kg DM)	ND	ND
Butyric acid (g/kg DM)	ND	ND
pH	6.83	6.17

Note: ADF, acid detergent fiber; CP, crude protein; DM, dry matter; EE, ether extract; FM, fresh matter; ND, not detected; NDF, neutral detergent fiber; NG, Napier grass; LAB, lactic acid bacteria; and ST, sugarcane top.

Table 4. Chemical composition of untreated and LAB-treated Napier grass and sugarcane top silages.

Item		DM	CP	Ash	OM	NDF	ADF	WSC
		(%)	(% DM)					
Napier grass								
	Control	15.31^{b}	10.33	11.69^{a}	88.31^{b}	67.64^{a}	40.96^{a}	1.43^{a}
	LAB1	15.82^{ab}	10.76	11.45^{a}	88.55^{b}	66.58^{ab}	39.79^{ab}	1.14^{ab}
	LAB2	15.99^{ab}	10.83	11.04^{b}	88.96^{a}	66.53^{ab}	39.72^{ab}	1.07^{ab}
	LAB1 + LAB2	16.22^{a}	10.98	11.03^{b}	88.97^{a}	66.08^{b}	38.94^{b}	0.71^{b}
	FG	15.92^{ab}	10.74	11.01^{b}	88.99^{a}	66.90^{ab}	39.98^{ab}	1.12^{ab}
	SEM	0.2491	0.2142	0.1227	0.1227	0.4027	0.4333	0.1803
	P-value	0.0337	0.3305	0.0031	0.0031	0.0372	0.0355	0.0149
Sugarcane top								
	Control	22.31^{b}	6.44^{b}	5.64^{c}	94.36^{a}	74.87	42.86^{a}	11.32^{a}
	LAB1	23.05^{ab}	6.65^{ab}	5.84^{abc}	94.16^{abc}	74.45	41.68^{ab}	10.96^{ab}
	LAB2	23.32^{a}	6.73^{a}	5.99^{a}	94.01^{c}	73.60	40.42^{b}	10.17^{b}
	LAB1 + LAB2	23.93^{a}	6.80^{a}	5.89^{ab}	94.11^{bc}	72.42	40.40^{b}	9.94^{b}
	FG	23.16^{ab}	6.69^{a}	5.76^{bc}	94.24^{ab}	74.53	41.36^{ab}	10.03^{b}
	SEM	0.2956	0.0723	0.0586	0.0586	0.9000	0.5133	0.1873
	P-value	0.0172	0.0335	0.0164	0.0164	0.3415	0.0154	0.0261

Note: Means with lowercase superscripts in the same column differ significantly ($p < 0.05$) between the treatments. Abbreviations: ADF, acid detergent fiber; CP, crude protein; DM, dry matter; FG, *Lactobacillus plantarum* Chikuso-1, Snow Brand Seed Co., Ltd., Sapporo, Japan; LAB1, *Lactobacillus paracasei* XH146; LAB2, *Lactobacillus plantarum* XH352; NDF, neutral detergent fiber; OM, organic matter; SEM, standard error of the mean; and WSC, water-soluble carbohydrate.

3.4. Fermentation parameter of silage

The LAB1 + LAB2-treated NG silage had numerically lower pH values than the other LAB-treated groups; however, the difference was not significant ($p > 0.05$; Table 5). LAB1 + LAB2-treated silages also showed the highest lactic acid contents in NG and ST silages. However, the control silage had a higher acetic acid content than ST silage treated with LAB1 + LAB2 ($p < 0.05$). Propionic acid and molds were absent from NG and ST silage, while butyric acid was not detected in LAB-treated NG and ST silages. The NH_3-N content decreased significantly in the following order: Control > LAB1 > LAB2 > FG > LAB1 + LAB2 ($p < 0.05$) in both NG and ST silages. The highest numbers of LAB and the lowest counts of yeasts were found in NG and ST silages treated with LAB1 + LAB2; these numbers significantly differed from those in the control ($p < 0.05$).

Table 5. Fermentation parameters and microbial population for untreated and LAB-treated Napier grass and sugarcane top silages.

Item		pH	LA	AA	PA	BA	NH_3-N	LAB	yeasts	molds
			（g/kg DM)					(lg CFU/g FM)		
Napier grass										
	Control	4.85^{a}	27.74^{b}	14.70	ND	3.06^{a}	3.35^{a}	4.63^{b}	2.87^{a}	ND
	LAB1	4.31^{b}	29.69^{ab}	12.70	ND	ND	3.12^{ab}	5.19^{a}	2.33^{ab}	ND
	LAB2	4.27^{b}	29.48^{ab}	13.72	ND	ND	2.98^{b}	5.26^{a}	2.47^{ab}	ND
	LAB1 + LAB2	4.21^{b}	32.00^{a}	13.58	ND	ND	2.95^{b}	5.48^{a}	2.20^{b}	ND
	FG	4.26^{b}	30.16^{ab}	13.64	ND	ND	2.96^{b}	5.29^{a}	2.39^{ab}	ND
	SEM	0.0331	3.6047	1.8632	-	0.8502	0.0881	2.3749	0.0926	-
	P-value	<0.0001	0.0166	0.9697	-	0.0082	0.0188	0.0104	0.0249	-
Sugarcane top										
	Control	4.19^{a}	33.78^{b}	7.08^{a}	ND	ND	2.20^{a}	7.23^{b}	1.99^{a}	ND
	LAB1	4.07^{abc}	35.31^{b}	4.20^{ab}	ND	ND	2.14^{a}	7.38^{ab}	1.47^{ab}	ND
	LAB2	3.95^{bc}	39.47^{a}	3.96^{ab}	ND	ND	2.06^{a}	7.59^{a}	1.43^{ab}	ND
	LAB1 + LAB2	3.91^{c}	40.06^{a}	1.41^{b}	ND	ND	1.52^{b}	7.81^{a}	1.31^{b}	ND
	FG	4.11^{ab}	36.41^{ab}	3.09^{ab}	ND	ND	1.62^{b}	7.33^{ab}	1.46^{ab}	ND
	SEM	0.0547	1.2793	1.2678	-	-	0.0474	0.7846	0.2961	-
	P-value	0.0107	0.0100	0.0267	-	-	<0.0001	0.0079	0.0163	-

Note: Means with lowercase superscripts in the same column differ significantly ($p < 0.05$) between the treatments. Abbreviations: AA, acetic acid; BA, butyric acid; DM, dry matter; FG, *Lactobacillus plantarum* Chikuso-1, Snow Brand Seed Co., Ltd., Sapporo, Japan; LA, lactic acid; LAB, lactic acid bacteria; LAB1, *Lactobacillus paracasei* XH146; LAB2, *Lactobacillus plantarum* XH352; ND, not detected; PA, propionic acid; and SEM, standard error of the mean.

4. Discussion

4.1. Characteristics of representative strains in silage

During ensiling, different raw materials and growth environments lead to diverse microbial communities in silage [33]. Without microbial inoculants, silage fermentation quality is mostly related to the amount, type, and substrate content of LAB present on the raw materials' surface. A relatively low number of LAB (generally below 1×10^5 CFU/g FM) are attached to the surface of grass under natural conditions [34,35]. Silage can be well preserved when the number of LAB reaches at least 10^5 CFU/g FM [36]. Therefore, LAB fermentation agents have to be added during silage production to enhance the lactic acid level and improve silage fermentation quality. Thus, it is crucial to identify and screen LAB species that grow quickly, rapidly produce lactic acid, and possess strong stress resistance on the surface of forage [37,38]. The classification and identification of LAB are an important research field, with accurate biochemical and genetic identification being the foundation for LAB research, development, and utilization [39–42]. In this study, 17 LAB strains with different colony shapes and sources were obtained through preliminary screening via acid production tests. These strains were characterized as rod-shaped, gram-positive, catalase-negative, and homofermentative; most strains grew between pH 3.5 and 9, at temperatures between 5 and 50 °C, and at salt concentrations between 3 and 6.5% NaCl. The 16S rDNA sequence BLAST results and phylogenetic tree showed that three

LAB strains were *L. paracasei* and 14 were *L. plantarum*.

The growth and acid production rates of LAB are important indicators for the screening of high-quality LAB. A swift increase in LAB and lactic acid production can suppress harmful bacteria and minimize nutrient loss in forage. Researchers [43–47] established the following criteria for high-quality silage LAB: (1) Fast growth rate, which inhibits the growth of harmful microorganisms; (2) homofermentative, enabling the quick production of lactic acid; and (3) strong acid resistance and growth in an environment with a pH of less than 4. In this study, among the 17 strains, XH352 (*L. plantarum*) and XH146 (*L. paracasei*) exhibited the strongest ability to produce acid in MRS broth, with pH values of 3.46 and 3.50, respectively. These results suggest that both strains possess desirable characteristics of high-quality LAB and hold strong potential as silage starters to enhance fermentation quality. To date, *Lactobacillus* strains have been successfully isolated from crop silages and have improved lactic acid fermentation and silage quality as additives [48]. These results are confirmed by our findings that *L. paracasei* and *L. plantarum* effectively contribute to fermentation during ensiling.

4.2. Chemical composition of raw material and silage

The chemical and nutritional composition of silage is an important proxy for evaluating silage quality in production applications; this quality is essentially determined by the composition of raw materials [49]. The production of good silage requires an appropriate moisture content in the raw material and the application of bacterial inoculates [50,51]. In this study, the highest DM content was found in LAB1 + LAB2-treated silages. This suggests that the selected local LAB strains contribute positively to nutrient preservation and exhibit strong potential for silage use. The CP content in the LAB-treated ST silage, except for that treated with LAB1, was higher than that in the control silage. This may be because silage fermentation requires a suitable moisture content, and the DM of ST is higher than that of NG. LAB inoculants generally exert minimal influence on fiber degradation and nutrient digestibility in silage owing to their limited enzymatic capability [52,53]. Liu et al. [54] reported the distinct roles of LAB in the degradation of hemicellulose and cellulose in biomass residues during silage acidogenic fermentation. In this study, compared with the control, LAB1 + LAB2-treated NG silage had significantly lower NDF and ADF contents. The ADF content in LAB2- and LAB1 + LAB2-treated ST silage was significantly lower than that in the control. This may be because the two selected strains of LAB from this experiment may indirectly affect fiber decomposition via microbial succession or fermentation acidification, thereby enhancing silage nutritional value. WSC contents are essential for achieving satisfactory fermentation quality in the production of high-quality silage [55]. In this study, the addition of LAB lowered the silage WSC content, in which LAB1 + LAB2-treated NG and ST silages had a significantly lower WSC content than the respective controls. These results indicate that the conversion of WSC by LAB increases silage quality.

4.3. Fermentation characteristics of silage

LAB have antibacterial performance and the ability to ferment and generate significant amounts of lactic acid against harmful microorganisms that cause silage spoilage. This is of great significance in maintaining silage quality [56]. The rapid reproduction of LAB and decrease in silage pH are key to ensuring successful silage production. Bai et al. [57] reported that adding isolated *Lactiplantibacillus plantarum* enhances the quality of *Caragana korshinskii* silage by regulating

microbial composition and metabolic pathways. Costa et al. [58] reported that treating late-maturity corn silage with *Lactobacillus farraginis* CCMA1362 enhances silage conservation. You et al. [59] reported *L. plantarum* as the dominant forage-associated species capable of growing in low pH environments and promoting lactic acid fermentation, which makes it a suitable starter culture for improving sorghum-Sudan grass hybrid silage. Thus, to produce high-quality silage, the selection of superior LAB strains is essential. Good-quality silage generally has low pH, low NH_3-N contents, and high lactic acid contents. In this study, the addition of LAB reduced the pH value in NG silage, i.e., the pH in LAB1 + LAB2-treated ST silage was significantly lower than that in the FG-treated and control silages. Additionally, LAB1 + LAB2-treated NG and ST silages also had significantly higher lactic acid contents than the controls. These results were likely attributable to increases in the LAB count, which constributes to the high fermentation rate. LAB efficiently converted WSC into lactic acid during NG and ST silage fermentation, resulting in a quicker lactic acid buildup and reduced pH. However, the pH of the LAB1 + LAB2 treatment was the lowest in the NG group (though not significantly), while it was significantly lower in the ST group. The higher WSC content in ST (15.26% DM) than NG (2.97% DM) provides suitable substrate conditions for LAB fermentation, stimulating the acidification process in silage, and thus improving fermentation quality. However, the fermentation effect of a single bacterial inoculant was not as significant as that of the composite bacterial inoculant. Moreover, the proteolytic activity of plants and microbes alters nitrogen compounds in silage, resulting in an increase in soluble N and NH_3-N during the fermentation process [3]. In this study, the NH_3-N content decreased in the following order: Control > LAB1 > LAB2 > FG > LAB1 + LAB2 for NG and ST silages. This may be because a lower pH value has a strong inhibitory effect on plant proteases and harmful microbes that are crucial in proteolysis. These effects cause direct acidification and suppression of undesired spoilage bacteria, thereby enhancing silage preservation [60].

5. Conclusions

In this study, a highly efficient LAB candidate with strong acid production and growth performance was biochemically and genetically identified, demonstrating significant potential for application in NG and ST silage fermentation. *L. paracasei* XH146 and *L. plantarum* XH352 improved the quality of NG and ST silages and were confirmed to be highly effective LAB inoculants. Furthermore, combining the two LAB types for silage fermentation yielded better results.

Use of AI tools declaration

The authors declare that they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

Conceptualization, C.Y. and H.X.; methodology, H.X. and F.Z.; software, X.L., and J. L.; validation, C.Y., H.X. and L.L.; formal analysis, L.P., and F.Z.; investigation, H.X.; resources, L.Z.; data curation, X.L., and L.L.; writing—original draft preparation, H.X.; writing—review and editing, H.X. and C.Y.; supervision, C.Y.; project administration, H.X.; funding acquisition, H.X., and C.Y.

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