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Isolation and Characterization of Lactic Acid Bacteria: A Strategy to Produce Inoculum for Forage Ensiling

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ABSTRACT

Purpose: *Sri Lankan dairy sector faces challenges due to seasonal variability and suboptimal quality of forage for dairy cattle feeding. While silage offers year-round availability of forage, concerns arise regarding potential quality degradation during forage ensiling. Microbial inoculants are utilized to expedite the ensiling and minimize quality deterioration. This study involves the isolation and characterization of lactic acid bacteria (LAB) from silage, with the aim of identifying potential candidates to be used as silage inoculants.*

Research Method: *Lactobacillus were isolated from 10 silage samples of each forage type: sorghum, maize, and guinea grass. They were characterized using the 16S rRNA gene sequencing procedure. The anaerobic sugar fermentation ability of the isolated LAB was evaluated by inoculating them into sterilized sugar solutions, and measuring the titratable acidity (TA) and pH value over time.*

Findings: *The isolates were identified to be non-motile, non-spore-forming and Gram-positive rods. Further, they tested negative for catalase, triple sugar iron, citrate, urease, indole, gelatine hydrolysis, motility, and oxidase tests. The LAB isolated from guinea grass, sorghum, and maize corresponded to Lactobacillus oris, Lactobacillus rhamnosus, and Lactobacillus plantarum, respectively. The TA in all inoculated solutions was higher compared to the control (0.55-0.66% vs. 0.48%), with L. plantarum showing the highest TA (0.66%) at 30 hours incubation. Additionally, all sugar solutions treated with LAB isolates exhibited a lower pH value at 30 hours compared to the control (4.70-5.07 vs. 5.75). Notably, L. plantarum demonstrated the most significant pH value reduction at 18 hours (5.85 vs. 6.29-6.64).*

Value: *Lactobacillus oris, L. rhamnosus, and L. plantarum, isolated from guinea grass, sorghum, and maize silage, respectively, have the potential to be developed as inoculants for ensiling forage.*

Keywords: *DNA sequencing, Lactobacillus oris, L. plantarum, L. rhamnosus, pH value, Titratable acidity.*

INTRODUCTION

Dairy farms in Sri Lanka currently produces approximately 513.3 million milk liters annually, satisfying merely 38% of the domestic milk requirements (Central Bank of Sri Lanka, 2021). Inadequate year-round forage availability and poor nutritional value of existing forage contribute to low milk production in the country's dairy herds. Conserving forage through ensiling presents a viable solution to maintain a consistent forage supply year-round.

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Feeding high-quality maize silage with a pH value of 4 and 32.8% dry matter has been shown to enhance dry matter intake (22.2 kg/d) and promote high milk yield (32.5 kg/d) in dairy cows (Khan *et al.*, 2014). Forage ensiling involves the anaerobic fermentation of bacteria and yeast, resulting in the production of number of organic acids for example acetic acid, butyric acid, succinic acid, lactic acid, formic acid, and ethanol. The quality of forage silage depends on the speed and products of the ensiling process. The rapid drop in pH value caused by lactic acid facilitates the preservation of forage within a short period, minimizing nutrient and dry matter loss (Ellis *et al.*, 2016). Lactic acid bacteria (LAB) are primarily responsible for lactic acid production during ensiling. Hence, the rapid proliferation of LAB populations is crucial for ensuring high-quality forage silage.

However, the naturally occurring epiphytic LAB on forage is insufficient to achieve rapid lactic acid production during forage ensiling (Soundharrajan *et al.*, 2020). Inoculating LAB strains as additives has been proven effective in ensuring silage quality. Zielinska *et al.* (2015) reported that forage inoculated with LAB at levels above 10^5 CFU/g significantly reduced dry matter loss and prevented unfavourable microbial fermentation, including that caused by *Clostridium*. Moreover, the inoculation of specific *Lactobacillus* species during forage ensiling can enhance lactic acid production, inhibit fungal growth, and promote the aerobic stability of silage (Schmidt *et al.*, 2009).

However, the importation of silage inoculants in Sri Lanka is subject to additional scrutiny to ensure both the safety of the animals and the quality of the feed by the Animal Feed (Amendment) Act No. 15 (Parliament of the Democratic Socialist Republic of Sri Lanka, 2016). Therefore, it is crucial to produce a silage inoculum, using locally available *Lactobacillus* species to ensure a sustainable supply of nutritious forage for feeding dairy cattle in Sri Lanka. Ennahar *et al.* (2003) successfully isolated number of homofermentative and heterofermentative LAB from paddy rice (*Oryza sativa*) silage and characterized by 16S Ribosomal DNA Analysis. Peng *et al.* (2021) demonstrated the feasibility of isolating LAB from high-quality silage produced from rye grass (*Lolium perenne*), sorghum (*Sorghum dochna*), maize (*Zea mays*) and alfalfa (*Medicago sativa*) and using them as inoculants to enhance the quality of alfalfa silage. Based on this premise, the present study aimed to isolate and characterize *Lactobacillus* species from maize, sorghum, and guinea grass silage and evaluate their potential for anaerobic fermentation.

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria (LAB)

Ten silage samples from each forage type (maize, sorghum, guinea grass) were collected in airtight polythene bags and refrigerated at 4°C until LAB isolation was performed. Each silage sample (10 g) was blended with 100 mL of sterile distilled water and 1 mL of blended suspension was inoculated onto a broth of Man Rogosa and Sharpe (MRS). They were incubated for 48 hours at 37°C. Six bacteria pure cultures from each forage type were subsequently isolated by sub culturing on MRS agar plates, followed by incubation for 48 hours at 37°C.

Morphological and Molecular Characterization of the Isolated Lactic Acid Bacteria (LAB)

Morphology of the LAB colonies of pure cultures were observed and Gram staining was performed. Furthermore, the isolated LAB strains underwent biochemical tests, including catalase, triple sugar iron (TSI), citrate, urease, indole, gelatine hydrolysis, motility, and oxidase tests, following the methods described by Dick *et al.* (1993). The sugar fermentation ability of the isolated LAB was qualitatively investigated by inoculating them separately into 1% (w/v) sugar solutions (arabinose, galactose, glucose, mannitol), following the method described by Adikari *et al.* (2021).

Genomic DNA extraction was performed following the protocol provided by the manufacturer (*BactoSpin D* extraction kit, CEYGEN Biotech (Pvt.) Ltd., Sri Lanka). The PCR amplification of the 16S rRNA gene was carried out using the forward primer 63f; 5-CAG GCC TAA CAC ATG CAA GTC-3(Julian *et al.*, 1998) and the reverse primer 1387r;5-GGG CGG WGT GTA CAA GGC-3(Janda and Abbott, 2007). The PCR conditions were 95°C for 3 minutes for heating followed by 30 cycles of 1 minutes, at 95°C for denaturing, 1 minutes at 55°C for annealing 1.5 minutes at 72°C for extension (Janda and Abbot, 2007). Further, the PCR products were purified and sequenced using the ABI 3500 Genetic Analyzer (Applied Biosystems). The isolates were identified at the species level using the NCBI database and BLAST search. A maximum likelihood tree, constructed with complete 16S rRNA gene sequences, was employed to determine the relative positions of the isolated LAB with *Bacillus subtilis* used as an outgroup.

Evaluation of the Anaerobic Sugar Fermentation Ability of the Isolated Lactic Acid Bacteria (LAB)

The anaerobic fermentation potential of each isolated LAB species was assessed. The LAB species were individually inoculated into sterilized sugar solutions, while a sterilized sugar solution without LAB inoculant served as the control. The experiment was conducted as a completely randomized design (CRD) with three replicates. The inoculated and control sugar solutions were incubated anaerobically at 37°C for 30 hours. The titratable acidity (TA) percentage and pH value of the sugar solutions incubated for 0, 18, and 30 hours were measured throughout the incubation period. Subsequently, the effect of the isolated LAB on anaerobic sugar fermentation was statistically assessed using analysis of variance (ANOVA), followed by mean separation utilizing SAS software Version 9.2 (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Isolation and Identification of Lactic Acid Bacteria (LAB)

Epiphytic LAB species exhibit considerable diversity and abundance, which can vary significantly due to different environmental conditions (Pahlow *et al.*, 2003). The isolated LAB colonies exhibited round shapes, white color, and entire margins. The LAB strains isolated from guinea grass, sorghum, and maize appeared as curved long pleomorphic rods, short pleomorphic rods, and curved short pleomorphic rods, respectively. The colony and cell morphologies of the *Lactobacillus* species isolated from different substrates showed consistent results with previous studies (Adikari *et al.*, 2021; Yang *et al.*, 2016). All isolates were characterized as non-spore-forming, non-motile, and Gram-positive rods. They exhibited negative results for catalase, TSI, citrate, urease, indole, gelatine hydrolysis, and oxidase tests. Additionally, all isolates displayed the capacity for fermenting arabinose, galactose, glucose, and mannitol.

The topology of the maximum likelihood tree, constructed from complete 16S rRNA gene sequences (Fig. 1), validated the relative positions of the typical silage microbial isolates. The DNA sequencing confirmed that the *Lactobacillus* species isolated from guinea grass, sorghum, and maize silage were *Lactobacillus* oris, *Lactobacillus* rhamnosus, and *Lactobacillus* plantarum, respectively. The query cover for all *Lactobacillus* species was 98%. The percentage identity for *L. oris, L. rhamnosus,* and *L. plantarum* were 89.83%, 92.52%, and 91.69%, respectively.

Sugar Fermentation Ability of Isolated Lactic Acid Bacteria

As shown in the Table 1, the effect of *Lactobacillus* species and incubation period had a significant ($P<0.05$) effect on the TA percentage as well as the pH value of the sugar solution. *Lactobacillus plantarum* isolate significantly ($P<0.05$) increased the TA percentage of the fermented sugar solution as early as 18 hours. As expected, the TA percentage of all fermented sugar solutions was higher ($P<0.05$) at 30 hours compared to the initial inoculation (0.48-0.66% vs. 0.17%). Additionally, both *L. plantarum* and *L. rhamnosus* demonstrated significantly ($P<0.05$) higher TA percentages (0.66% and 0.64%, respectively) compared to the control (0.48%) after 30 hours of fermentation.

During the incubation period, all LAB-inoculated sugar solutions demonstrated a significant ($P<0.05$) reduction in pH value. The reduction in pH value was more rapid ($P<0.05$) in the sugar solution inoculated with *L. plantarum* compared to other *Lactobacillus* species (6.29 - 6.64 vs. 5.85 at 18 hours). Fermentative *Lactobacillus* species used in inoculants have been shown to affect silage quality parameters positively including low pH values and high lactic acid contents (Arriola *et al.*, 2011; Burns *et al.*, 2018). These microbial inoculants accelerate ensiling processes by enhancing acidification and lowering the pH values (Weinberg and Muck, 1996).

Lactobacillus plantarum, a facultative heterolactic bacterium exhibits a characteristic lactic acid production trait during substrate fermentation, contributing to its significance in silage preservation (Zielinska *et al.*, 2015). This bacterium significantly facilitates lactic acid production, leading to a reduction in pH value during forage ensiling (Puntillo *et al.*, 2020; Zhang *et al.*, 2021). The bacterium significantly lowered the pH level and ammonia nitrogen content, curbed butyric acid formation, boosted lactic acid levels, and greatly enhanced the fermentation quality of king grass, cassava foliage, and *Broussonetia papyrifera* (Liu *et al.*, 2022). Lactic acid, known for its potency, surpasses other organic acids in its effectiveness during forage ensiling. Although the lactic acid content was not measured in the present study, the rapid increase in TA percentage and the pH value drop observed in the sugar solution treated with *L. plantarum* can be attributed to the production of lactic acid by the bacterium. Yao *et al.* (2009) also

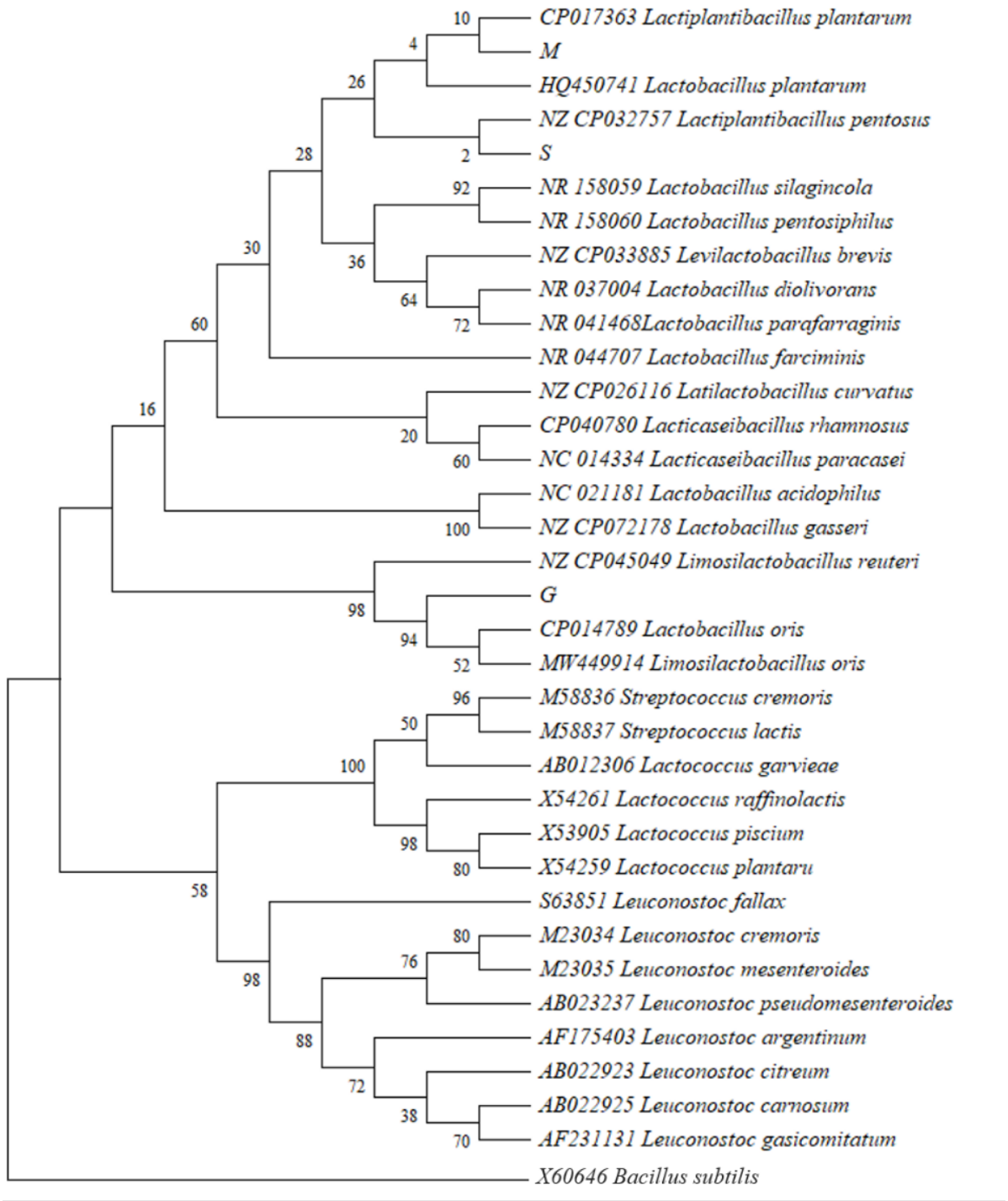

Figure 1: The topology of the maximum likelihood tree showing the relative positions of the common silage microbial isolates based on complete 16S rRNA gene sequences. (M; Maize, S; Sorghum, G; Guinea grass isolates)

Table 1: Effect of *Lactobacillus* species isolated from silage on titratable acidity and pH value of sugar solution during incubation period.

Bacteria species	Percentage titratable acidity (TA)[1]			pH value[1]		
	0 h	**18 h**	**30 h**	**0 h**	**18 h**	**30 h**
Lactobacillus plantarum	0.17 ± 0.01^{aC}	0.43 ± 0.04^{aB}	0.66 ± 0.01^{aA}	6.76 ± 0.01^{aA}	5.85 ± 0.19^{bB}	4.70 ± 0.09^{bC}
Lactobacillus rhamnosus	0.17 ± 0.01^{aB}	0.24 ± 0.03^{bB}	0.64 ± 0.02^{aA}	6.76 ± 0.01^{aA}	6.29 ± 0.07^{aB}	4.79 ± 0.19^{bC}
Lactobacillus oris	0.17 ± 0.01^{aB}	0.23 ± 0.02^{bB}	0.55 ± 0.06^{abA}	6.76 ± 0.01^{aA}	6.34 ± 0.01^{aB}	5.07 ± 0.13^{bC}
Control	0.17 ± 0.01^{aB}	0.20 ± 0.01^{bB}	0.48 ± 0.01^{bA}	6.76 ± 0.01^{aA}	6.64 ± 0.03^{aB}	5.75 ± 0.01^{aC}

[1] Mean ± SE. Within a column, means followed by different lower-case superscripts are significantly different ($P<0.05$). For each parameter, means within a row followed by different upper-case superscripts are significantly different ($P<0.05$).

reported that *L. plantarum* rapidly increases TA and decreases pH value during MRS broth incubation. After 16 hours of incubation, the changes in pH and TA were 1.2 and 1.7 times greater, respectively, compared to those observed with *Lactobacillus pentosus* inoculation.

Inoculation of homofermentative LAB results in a rapid drop in pH value due to the increased production of lactic acid during ensiling forage maize (Kung *et al.*, 2003). *Lactobacillus rhamnosus* is homofermentative bacteria, which has been primarily isolated from silage produced by Italian ryegrass at 45ºC has been recognized for its heat tolerance (Chen *et al.*, 2013). This species has demonstrated a greater capacity to produce antifungal agents, inhibiting fungal growth and mycotoxin production in silage more effectively than other bacteria. Therefore, *L. rhamnosus* is considered a favourable inoculant for forage ensiling, particularly in tropical and humid areas (Guan *et al.*, 2020).

Lactobacillus plantarum isolated from maize and sorghum silage has demonstrated its potential as an inoculant for forage ensiling (Puntillo *et al.*, 2020). Similarly, *L. rhamnosus*, isolated from corn and Napier grass also has demonstrated its potential as a silage inoculant (Guan *et al.*, 2020). Interestingly, *L. oris*, which was isolated from guinea grass silage in the present study, has not been previously reported in silage. *Lactobacillus plantarum* is commonly found in silage inoculants (Guan *et al.*, 2020). Obligatory heterolactic bacterium species yield more acetic acid in addition to lactic acid, improving the aerobic stability of silage (Zielinska *et al.*, 2015; Santos *et al.*, 2019). Thus, the combination of *L. plantarum* with obligatory heterolactic bacterium may result in high-quality forage silage.

CONCLUSION

Lactobacillus oris, L. rhamnosus, and L. plantarum, obtained from guinea grass, sorghum, and maize silage, respectively, exhibit the capacity to impact sugar fermentation, suggesting their suitability for use as inoculants in forage ensiling. Future investigations are advised to assess the potential benefits of combining *L. oris, L. rhamnosus,* and *L. plantarum* as silage inoculants.

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