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Production of Lactobacillus Inoculants and Their Influence on Ensiling Forage Maize

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ABSTRACT

Purpose: *Lactic acid bacteria inoculants used to enhance forage ensiling. They are neither imported nor produced in Sri Lanka. The present research produced freeze-dried Lactobacillus plantarum, Lactobacillus rhamnosus, and Lactobacillus oris inoculants, and assessed their influence on forage maize ensiling.*

Research Method: *Lactobacillus mother cultures were prepared from isolates from maize, sorghum, and Guinea grass silage using MRS broth (37ºC, 18 h). These were further incubated in 10% skim milk broth (37ºC, 72 h) to study growth kinetics. Skim milk broth (10%) was selected as the lyophilization medium. Pelleted cultures (10 mL) were resuspended in lyophilization medium (30 mL) and freeze-dried inoculants were produced from suspensions (0.5 mL). Forage maize was inoculated with these inoculants at 10^6 CFU/g fresh forage, and their effect on ensiling was analyzed.*

Findings: *In growth kinetics study, all Lactobacillus species exceeded 10^9 CFU/mL population by 24 hours and reduced the pH to a range of 3.92–4.11, by 72 h. The influence of inoculation of Lactobacillus species was significant ($P < 0.05$) on silage pH. Significantly ($P < 0.05$) low pH was recorded when forage maize was inoculated with L. oris and L. rhamnosus at 2 and 52 weeks, respectively. An increasing trend in organic matter digestibility and metabolizable energy was evident with inoculation of L. rhamnosus and L. oris.*

Originality/ Value: *Locally produced freeze-dried L. rhamnosus and L. oris can effectively enhance the ensiling of forage maize, offering a solution for the unavailability inoculants for silage production in Sri Lanka.*

Keywords: *Growth kinetics, Forage ensiling, Lactobacillus oris, Lactobacillus rhamnosus, Lactobacillus plantarum, Lyophilization medium.*

INTRODUCTION

The average daily milk productivity of 100,460 improved cows at milk (63%) in Sri Lanka is only 6.63 L. With the current feeding regime, both temperate and tropical crossbred dairy cows experience postpartum negative energy balance. The country's crude protein and total digestible nutrients deficiencies are 38.1% and 24.02%, respectively. Native forage such as Guinea grass (*Megathyrsus maximus*) is low quality, while availability of improved forages is seasonal. Forage silage offers a year-round supply of high-quality forage. *Zea mays* (maize) is globally preferred for silage due to its favorable properties for fermentation. During ensiling, lactic acid bacteria (LAB) ferment water-soluble carbohydrates to organic acids under anaerobic conditions.

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However, low densities of epiphytic LAB can delay acidification, leading to nutrient losses. Inoculation with selected LAB enhances homolactic fermentation and rapid acidification. Compatibility between forage and LAB strain is critical for successful silage fermentation. *Lactobacillus plantarum* functions as a homofermentative strain when glucose is abundant and as a facultative heterofermentative strain when limited. Previous studies confirm the benefits of *L. rhamnosus* under tropical conditions.

Despite the growing interest in silage production, microbial inoculant importation faces regulatory hurdles in Sri Lanka. Therefore, developing inoculants from local strains is essential. This study developed freeze-dried inoculants from local isolates of *L. plantarum*, *L. rhamnosus*, and *L. oris*, and evaluated their efficacy on maize ensiling.

MATERIALS AND METHODS

Growth kinetics of Lactobacillus species

Lactobacillus plantarum, *L. rhamnosus* and *L. oris* isolated from maize, sorghum, and guinea grass silage, respectively were studied. The isolates had been stored in glycerol in clearly labelled screw-cap tubes at -20°C. The *Lactobacillus* isolates were cultured on de Man, Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, UK) in anaerobic jars at 37°C for 18 h (MRS mother culture). Skim milk broth (10% w/v) was prepared by dissolving non-fat milk (5 g) in warm distilled water (50 mL, 60°C) and autoclaved at 121°C for 15 minutes. The sterilized skim milk broth was inoculated with 1 mL of actively growing *Lactobacillus* mother culture separately and incubated under ideal conditions (i.e. in anaerobic jars, at 37°C) for 72 h. This process ensured consistency across the *Lactobacillus* cultures. Sterilized milk without inoculum was also incubated at 37°C for 72 h, as the control. The study was on complete randomized design (CRD) with 4 replicates. During the incubation period, a number of *Lactobacillus* colony forming units (CFU/1 mL), and pH value of the cultures were recorded until 48 h and 72 h, respectively.

Production of freeze-dried *Lactobacillus* inoculants

Each *Lactobacillus* species was cultured separately on 250 mL MRS broth at 37 °C for 18 h in anaerobic jars (mother culture). The study was on complete randomized design (CRD) with 4 replicates. Bacteria pellets were prepared by centrifuging (15,000 rpm), 10 mL of MRS broth at 4 °C for 10 minutes. Each pellet was suspended in 30 mL of 10% (w/v) non-fat milk. The suspensions (0.5 mL) were dispensed into freeze drying vials and frozen at -40 ° C for 3–4 h. The frozen *Lactobacillus* in vials were freeze-dried using a laboratory freeze drier (SUPERMODULYO-230, Thermo Electron Corporation) and inoculants were prepared. The freeze-dried *Lactobacillus* inoculants were cultured on MRS broth for 24 h in anaerobic jars at 37 ° C, and Colony Forming Units(CFU) were recorded to determine the viability of *Lactobacillus* cells in inoculants.

Assessment of the influence of inoculation of freeze-dried Lactobacillus on maize ensiling

Forage maize at semi-hard kernel stage was harvested and chopped (15 - 20 mm). The experiment was devised as a complete randomized design (CRD) with 4 replicates. Each freeze-dried LAB inoculant was dissolved in 15 mL deionized water and sprayed on 1 kg fresh, chopped forage at the inoculation rate of 10^6 CFU/g of fresh forage. The control was sprayed with 15 mL of deionized water without *Lactobacillus* inoculant. The treated forage and control were pressed into airtight glass jars (Laboratory silo, 1 L) and ensiled at room temperature ranged 25 - 30 ° C. The influence of *Lactobacillus* inoculation on forage maize ensiling was assessed at 2 weeks and 52 weeks ensiling. The pH (AFIA 2011), DM content and lactic acid content (Barnett 1951) were determined. The organic matter digestibility (OMD) and metabolizable energy content (ME) were determined following *in-vitro* gas fermentation assay (Makkar, 2009). Prior to commencing the experiment, the approval (ECC/2022/E/049) to conduct the experiment was sought from the Ethical Review Committee (ERC) of the Faculty of Agriculture, University of Peradeniya. The rumen fluid donor sheep used for *in-vitro* gas fermentation assay were managed following standards set by the ERC in order to minimize pain or discomfort.

Statistical Analysis

The data gathered on growth kinetics of *Lactobacillus* isolates, cell viability of freeze-dried *Lactobacillus* inoculants and quality of silage at 2 weeks and 52 weeks ensiling were subjected to analysis of variance (ANOVA) procedures followed by mean comparison considering 95 % probability level for significance in the Version 9.1 of the SAS statistical package (SAS Inc., 2009).

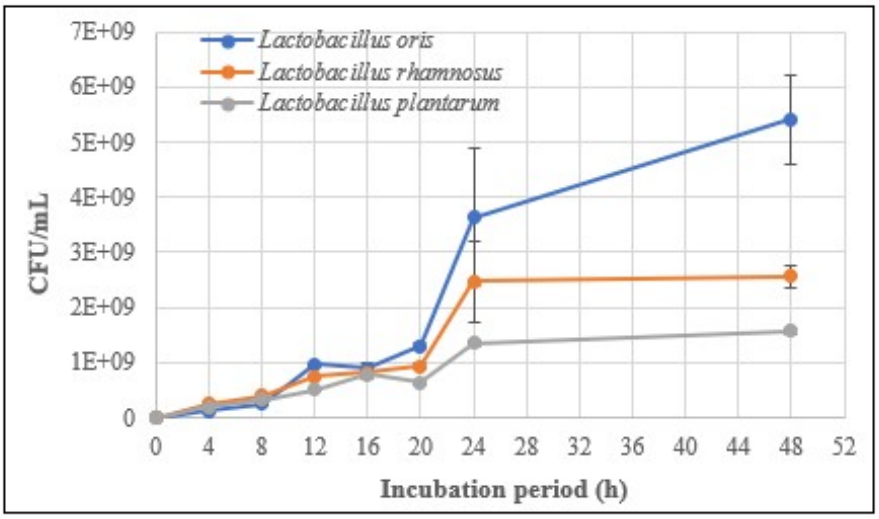

Figure 1: Growth of *Lactobacillus* species in skim milk lyophilization medium during the incubation period

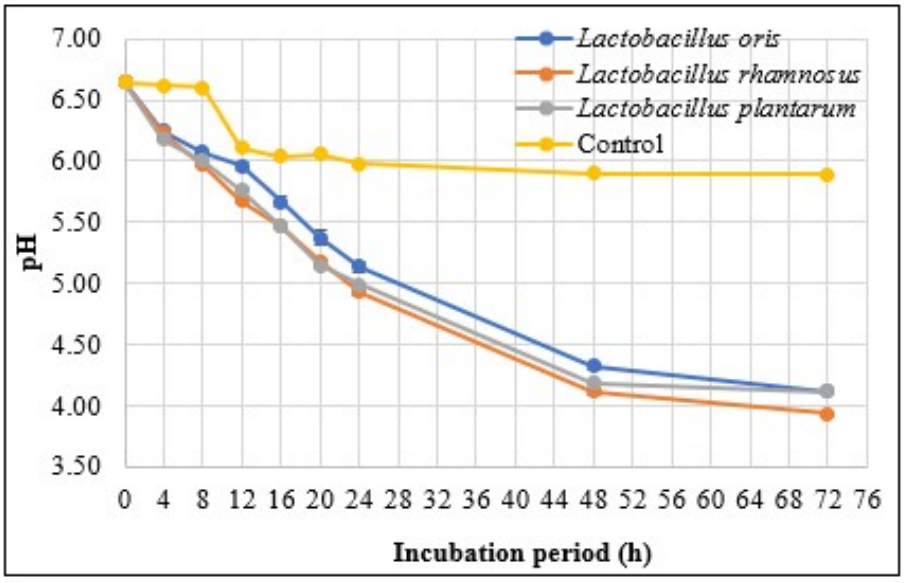

Figure 2: Variation of pH in skim milk lyophilization medium during the incubation period

RESULTS AND DISCUSSION

Growth kinetics of Lactobacillus and variation in pH of Lactobacillus cultures

The critical estimation of growth kinetics is crucial for enhancing the analysis of microbial behavior, leading to significant improvements in microbial applications. *Lactobacillus plantarum*, *L. rhamnosus* and *L. oris* were satisfactorily cultured in 10 % skim-milk broth. The variation in the CFU of *Lactobacillus* species in the inoculated skim milk broth during the 48-hour incubation period is depicted in Figure 1. The CFU were highest ($P<0.05$) in *L. oris*, followed by *L. rhamnosus* and *L. plantarum* throughout the incubation period. Although, *L. oris* maintained marked increments of CFU until 48 h, the increments were marginal in *L. rhamnosus* and *L. plantarum* cultures after 24 h. More importantly, all *Lactobacillus* species exceeded a concentration of 10^9 CFU/mL by 24 h. Puntillo *et al.* (2020) cultured *L. plantarum* strains isolated from maize silage on Maize Based Media. The results reported a significant difference in cell counts among (between) strains after 24 h and 48 h of incubation. The cell counts of majority isolates ranged from 1 to 1.5 log orders, while some isolates achieved notably greater cell counts at 2 log orders. As shown in Figure 2, skim milk broths inoculated with *Lactobacillus* showed a continuous reduction in pH value, irrespective of the species. By the end of the 72 h incubation period, while the un-inoculated skim milk with *Lactobacillus* species reduced the pH value to 5.89, the broths inoculated with *Lactobacillus* species reduced the pH values between 3.92 to 4.11. The present results are in agreement with Puntillo *et al.*, (2020), where *L. plantarum* strains cultured on maize-based medium recorded pH values

from 6.9 to 3.91–4.25 after 24 h, and to 3.78–3.90 after 48 h. Variation in the reduction of pH recorded in different *Lactobacillus* inoculated skim milk cultures suggests the variation in the ability of *Lactobacillus* species in ensiling forages when used as an inoculant.

Production of Lactobacillus inoculants and their stability

Freeze-drying is a multistep process used to preserve bacteria including *Lactobacillus* species. The process involves culturing of microbes, suspending them in a lyophilization medium (buffer), performing freeze-drying, and storing the freeze-dried product appropriately (Peiren *et al.* 2015). Lyoprotectants ensure the survival of bacteria during the freeze-drying process. They stabilize the bacterial cells when water is removed during freeze-drying and serve as a matrix that ensures the entire content retains its shape during and after the process. Thus, the survival rate of freeze-dried bacteria is significantly influenced by the lyoprotective media (Zhang *et al.*, 2020). In 10% skim milk broth, all isolated *Lactobacillus* species exceeded 10^9 CFU/mL concentration by 24 h and continuously reduced the pH during the incubation period (Figure 1). Skim milk, sucrose, specific buffers, etc., have been successfully used as lyophilization media (Oluwatosin *et al.*, 2022). . Therefore, 10% skim milk broth was determined to be a successful lyophilization medium to produce freeze-dried *Lactobacillus* inoculants. Viability of *L. plantarum*, *L. rhamnosus*, and *L. oris* in the freeze-dried inoculants were 3×10^9 CFU/mL, 1.6×10^9 CFU/mL, and 3.4×10^9 CFU/mL, respectively. Therefore, the protocol adopted for the production of freeze-dried *Lactobacillus* inoculants using the *Lactobacillus* species isolated from silage in the present study can be confirmed.

Effect of freeze-dried Lactobacillus inoculants on ensiling maize

The species diversity and abundance of epiphytic LAB exhibit significant variability, influenced by a range of environmental conditions (Pahlow *et al.*, 2003). . Lactic acid fermentation may spontaneously occur when anaerobiosis, water activity, and thermal conditions are favorable on the plant materials, making LAB dominant during the ensiling process (Di Cagno *et al.*, 2013). Once harvested forage is compressed, fermentation is typically initiated by LAB, namely *Enterococcus faecalis* and *Leuconostoc mesenteroides*, which are subsequently replaced by acid-tolerant *L. brevis*, *L. plantarum*, and *L. buchneri* (Holzer *et al.*, 2003). . The success of forage preservation hinges on the establishment of precise biological and chemical conditions that facilitate a substantial decline in pH value in silage. Moreover, these conditions are crucial for stabilizing the resulting low pH value, which is a key factor in successful silage(Ferraretto *et al.*, 2018). Promotion of the growth of LAB has been a widely used strategy in inducing lactic acid formation during forage ensiling (Puntillo *et al.*, 2020). . The inoculants used for commercial silage production contain one or more fermentative *Lactobacillus* species (Arriola *et al.*, 2011; Burns *et al.*, 2018). The present study investigated the influence of *Lactobacillus* inoculants containing a single species of *Lactobacillus* on forage maize ensiling. Microbial inoculants influence the quality parameters of silages, including lactic acid content and pH value. They often accelerate ensiling by enhancing acidification, thereby lowering the pH value and reducing protein degradation in silage (Weinberg and Muck, 1996). The effect of *Lactobacillus* inoculation (10^6 CFU/kg fresh matter) of fodder maize on lactic acid content and pH value of silage at 2 and 52 weeks has been presented in Table 1 and Table 2, respectively. A tendency for increased lactic acid concentration appeared due to inoculation of *Lactobacillus* as early as 2 weeks. Further, at 2 weeks ensiling, the lactic acid percentage is the highest with *L. plantarum* followed by *L. rhamnosus* and *L. oris*. Accordingly, a significant ($P<0.05$) reduction in pH value was observed with *L. rhamnosus* and *L. oris* inoculated silage, providing evidence for noticeable organic acid formation by the *Lactobacillus* species during ensiling. Surprisingly, the lactic acid content of maize silage inoculated with *L. oris* was significantly ($P<0.05$) low at 52 weeks ensiling. The existence of *L. oris* in silage has never been reported in the literature. The species was isolated from excellent quality Guinea grass silage in our lab in previous research (Disanayaka *et al.*, 2025) and produced freeze-dried inoculant in the present experiment.

At 52 weeks ensiling, the pH was significantly ($P<0.05$) low in *Lactobacillus* inoculated maize silage compared to the control irrespective of the *Lactobacillus* species. The lowest ($P<0.05$) pH value was recorded in silage inoculated with *L. rhamnosus*. The resulted low level of lactic acid ($P<0.05$) along with low pH ($P<0.05$) value in maize silage inoculated with *L. oris* provide evidence for formation of organic acids other than lactic acid when forage maize was inoculated with *L. oris*, presumably acetic acid or butyric acid. Unfortunately, these acids were not analyzed in the present experiment. At 52 weeks ensiling stage, the DM and pH value recorded are measures of the acidity of completely ensiled silage. Khan (2015) reported 3.7 pH value for very-wet-silage with DM less than 22.9%. The DM and pH value of maize

Table 1: Effects of *Lactobacillus* inoculation (10^6 CFU/g Fresh Forage) on quality of forage maize silage at 2 weeks ripening

Inoculated *Lactobacillus* species	Lactic acid content (%)	pH value
Without inoculum (Control)	14.872 ± 0.055[a]	3.475 ± 0.005[a]
Lactobacillus plantarum	15.577 ± 0.263[a]	3.465 ± 0.015[ab]
Lactobacillus rhamnosus	15.142 ± 0.152[a]	3.430 ± 0.010[bc]
Lactobacillus oris	15.061 ± 0.325[a]	3.410 ± 0.010[c]

[1] Mean ± SE.
Within a column, means followed by different letters are significantly different (P<0.05).
Dry matter percentage of silage was 20.16%.

Table 2: Effects of *Lactobacillus* inoculation (10^6 CFU/g Fresh Forage) on quality of forage maize silage at 52 weeks ripening

Inoculated *Lactobacillus* species	Lactic acid content (%)	pH value	OMD (%)	ME (MJ/kg DM)
Without inoculum (Control)	18.545 ± 0.205[a]	3.570 ± 0.001[a]	49.430 ± 0.115[a]	7.303 ± 0.017[a]
Lactobacillus plantarum	17.725 ± 0.305[ab]	3.440 ± 0.001[b]	47.356 ± 3.152[a]	6.986 ± 0.482[a]
Lactobacillus rhamnosus	17.390 ± 0.380[b]	3.390 ± 0.001[c]	52.666 ± 2.814[a]	7.796 ± 0.430[a]
Lactobacillus oris	15.785 ± 0.125[c]	3.440 ± 0.001[b]	51.656 ± 2.075[a]	7.636 ± 0.318[a]

[1] Mean ± SE.
Means within a column followed by different lower-case letters are significantly different (P<0.05).
Dry matter and crude protein percentage of silage ranged between 17.73% to 19.67% and 8.62% to 9.14%, respectively.

silage inoculated with *L. plantarum* (18.36%, 3.44), *L. rhamnosus* (18.88%, 3.39) and *L. oris* (19.67%, 3.44) at 52 weeks ensiling were in agreement with very-wet-silage. The present pH values reported are also comparable with the pH value (3.86) reported for forage maize inoculated with LAB (Puntillo *et al.*, 2020). During forage ensiling, bacteria ferment water-soluble carbohydrates (WSC) in forage. Thus, both species of LAB and the concentration of WSC are crucial for the successful silage fermentation. Inoculation of forage with LAB reduces the silage pH and concentration of WSC (Ali *et al.*, 2021). In agreement with them, forage maize inoculated with *L. rhamnosus* also recorded significantly (P<0.05) low pH value and WSC content (3.39 and 2.570%, respectively) compared to forage maize that did not inoculate (3.57 and 2.835%, respectively) at 52 weeks ensiling.

Inoculation of homofermentative LAB results in a rapid drop in pH value due to the increased production of lactic acid during ensiling forage maize (Marbun *et al.*, 2020). Both *L. plantarum* (Zhang *et al.*, 2020) and *L. rhamnosus* (Coudeyras *et al.*, 2008) are homofermentative bacteria. Variation in pH levels among treatments could arise from factors such as the species and rate of inoculation of LAB, the forage species inoculated, concentration of soluble carbohydrates, and the duration of ensiling (Kung *et al.*, 2018). The pH value reflects the ultimate acidity of the ensiled product and thus serves as a key indicator of both ensiling efficiency and preserving ability of silage. Silage with low pH is often associated with poor feed intake and rumen digestion stability. Contrary, silage with higher pH frequently associated with elevated ammonia nitrogen content, resulting in low feed intake and compromised preservation ability (Zhang *et al.*, 2021). Fast acidification is a favorable characteristic of microbial inoculants because they preserve silage by inactivating plant proteases, reducing the growth of pathogenic and spoilage microorganisms (Dunière *et al.*, 2013).

A trend in enhancing the *in-vitro* OMD and in-vitro ME due to forage maize inoculated with *L. rhamnosus* and *L. oris* is evident at 52 weeks ensiling stage. Quick lowering of pH value by lactic acid reduces proteolytic enzyme activity and thereby prevents break down of forage proteins during ensiling. This protected protein enhances the OMD of maize silage (Sucu, 2009). Nevertheless, *in-vitro* responses of microbial inoculants on OMD greatly depends on the species or strain of microorganism, and the substrate (Aydin and Denek, 2023). The key determinant of successful microbial inoculants is the compatibility of plant and microorganism (Muck, 2008).

Lactobacillus rhamnosus and *L. oris* inoculants produced in the present study had been isolated from sorghum and guinea grass silage (Disanayaka *et al.*, 2025). Microbial inoculants produced from strains selected from environments different from the environment of the ensiling forage, as well as from different forage cultures, can perform well in ensiling forages (Puntillo *et al.*, 2020). Therefore, *L. rhamnosus* and *L. oris* inoculants, which produced silage with significantly ($P < 0.05$) low pH and showed a tendency to increase *in-vitro* OMD and ME, would be better for ensiling forage maize. Further, inoculation of forage maize with *L. rhamnosus* and *L. oris* inoculants at rates exceeding 10^6 CFU/g fresh forage may result in significantly accelerated organic acid production, reduction of pH, and enhancement of OMD and ME, thereby improving the nutritive value of maize silage.

CONCLUSION

The present study confirmed the potential for the production of freeze-dried inoculants from *L. plantarum*, *L. rhamnosus*, and *L. oris*, isolated from maize, sorghum, and Guinea grass silage, respectively. Skim milk was found to be a satisfactory medium for lyophilization of *Lactobacillus* species. Ensiling forage maize with freeze-dried *L. rhamnosus* inoculants at 10^6 CFU/kg fresh matter ensures a rapid drop in pH value in maize silage. Inoculation with *L. rhamnosus* and *L. oris* inoculants at concentrations exceeding 10^6 CFU/g fresh forage may result in further acceleration of the ensiling process; thereby significantly increasing organic acid content, OMD, and ME in maize silage. Further studies are warranted to investigate the potential of inoculants produced from a mixture of *L. rhamnosus* and *L. oris* species and at concentrations exceeding 10^6 CFU/g fresh forage for ensiling forage maize.

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