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# Isolation of tropical antifungal lactic acid bacteria and their role in fermentation, aerobic stability, and ruminal digestibility of sorghum silage

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**Abstract:** In this study, native lactic acid bacteria (LAB) with antifungal activity were isolated, and their effects on the sorghum (*Sorghum bicolor* L. Moench) silage's chemical composition, fermentation quality, aerobic stability, as well as *in vitro* ruminal digestibility were assessed. In the first experiment, LAB were separated from sorghum silage and chosen according to their capacity to inhibit *Fusarium graminearum*. Based on 16S rRNA gene sequencing, four isolates (AF-2, AF-24, AF-25, and AF-34) with the greatest antifungal activity were chosen and identified as *Lactiplantibacillus plantarum* (≥99.6% similarity). In experiment 2, sorghum forage (28.7% dry matter) was ensiled for 60 days without inoculant (CON) or with each isolate (INO1–INO4; $1 \times 10^5$ colony-forming units (CFU)/g fresh weight). A total of five silos per treatment served as experimental replicates. The results showed that inoculation of silages did not affect chemical composition. Whereas CON silage had more acetic acid (AA) ($P < 0.05$), INO2 silage had more lactic acid (LA). Mould counts were reduced in all inoculated silages compared to CON ($P < 0.05$), while yeast populations declined in INO1 and INO4. *In vitro* ruminal digestibility was not impacted by inoculation, however INO1 and INO2 silages had stronger aerobic stability than CON ($P < 0.05$). *Lactiplantibacillus plantarum* AF-2 and AF-24 improved silage quality by inhibiting mould, and enhanced aerobic stability without any negative effects on ruminal digestibility. These findings indicated that antifungal compounds from indigenous inoculants could improve silage preservation and aerobic stability, offering a practical method to enhance ruminant feed quality.

**Keywords:** biopreservation; epiphytic microbiota; feed-out phase; inoculant screening; shelf life; strain selection

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Silage production under tropical conditions is characterised by several challenges, including high ambient temperatures, elevated crop moisture, and water-soluble carbohydrates (WSC) low concentrations in some forages (Borreani et al. 2018). Because high temperatures as well as humidity encourage the bacteria's growth either before or after ensiling, contamination of spoilage microbes in silage production is a major problem in tropical countries (Bernardes et al. 2018). Yeasts and moulds also cause aerobic degradation during the feed-out phase, which results in significant nutrition and energy losses (Carvalho et al. 2016).

Sorghum (*Sorghum bicolor* L. Moench) silage, which has substantial nutritional content and can be successfully stored to sustain ruminant production, is a viable forage source for ruminants in tropical settings (McCary et al. 2020; Guo et al. 2024; Florit et al. 2025). Sorghum forage exhibits strong adaptability to marginal environments and drought tolerance through physiological and molecular mechanisms associated with abiotic stress resistance (Zheng et al. 2023). Despite the advantages, sorghum crops are highly susceptible to fungal infection, particularly by *Fusarium* spp., which cause plant diseases and mycotoxin contamination (Ackerman et al. 2021). These difficulties are exacerbated in tropical areas, where high humidity along with temperatures encourage the fungal growth and the mycotoxins' formation both before as well as after harvest (Carvalho et al. 2016; Bernardes et al. 2018).

The most practical solution to these problems is the lactic acid bacteria (LAB) utilisation as a silage additive in place of chemical additives to prevent fungal infestation (Muck et al. 2018; Ardiansyah et al. 2025), particularly in sorghum silage (Junior et al. 2025). In consideration of tropical conditions, LAB with specific antifungal activity have shown potential for controlling fungal spoilage in silage. Additionally, they can inhibit the mould's and yeast's growth throughout the feed-out and ensiling stages (Paradhipta et al. 2020; Paradhipta et al. 2021). Nevertheless, LAB isolated from tropical sorghum silage are still little understood, and antifungal activity is quite strain-specific. Locally adapted strains may perform better under high temperature and humidity conditions typical of tropical environments (Muck et al. 2018), providing a natural and sustainable alternative to chemical additives. The demand for sorghum silage in Indonesia is increasing to supply the requirements of dairy cattle, where maize is primarily prioritised for poultry feed. Accordingly, the purpose of this work is to isolate and distinguish LAB with antifungal capabilities from sorghum silage and assess their impact on aerobic stability, *in vitro* ruminal digestibility, as well as fermentation characteristics. We hypothesised that indigenous antifungal LAB isolated from tropical sorghum silage would improve fermentation quality and aerobic stability without adversely affecting ruminal digestibility.

## MATERIAL AND METHODS

### Experiment 1 – Isolation and selection of LAB with antifungal activity

Sorghum (*Sorghum bicolor* L. Moench cv. Samurai-2, BRIN) silage ensiled for 100 days without additives was used as the source of LAB. A 20 g of silage was diluted with 180 ml sterile deionised water, filtered through cheesecloth, as well as spread-plated (0.1 ml) on de Man, Rogosa, and Sharpe (MRS; Merck, Germany) agar. Subsequently, plates were propagated at 30 °C for 48 h under $CO_2$-enriched circumstances (BINDER GmbH, Germany) and colonies with typical LAB morphology were purified twice in MRS broth.

A total of 100 isolates were screened based on optical density ($OD_{600}$) using a microplate spectrophotometer (Multiskan SkyHigh Microplate Spectrophotometer; Thermo Scientific, USA) and acidification according to the pH value quantified with a pH meter (Ohaus Starter 2100, USA). The 25 best-performing isolates were selected based on the highest $OD_{600}$ values and the lowest pH values, indicating superior bacterial growth and acidification ability. All measurements were performed in two replications. The 25 selected isolates were continued for measurement of antifungal activity against *Fusarium graminearum* strain YP0124 with two replicates following the procedures by Paradhipta et al. (2020). Antifungal activity was evaluated on potato dextrose agar (PDA; Merck, Germany) by streaking LAB isolates 2 cm from a centrally placed *Fusarium graminearum* colony and incubating at 30 °C for 7 days. Inhibition zones were measured as the distance between bacterial and fungal growth margins, with four replicates for each isolate.

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The 16S rRNA gene sequencing was utilised to identify the four most prevalent isolates. The 16S rRNA gene was amplified using primers 27F/1492R and then commercially sequenced (Genetika Science, Indonesia). BLAST (NCBI) was utilised to estimate sequence similarity, and MEGA 7.0's neighbour-joining approach was used for phylogenetic analysis (Suyabatmaz et al. 2022).

### Experiment 2 – Effects of selected LAB isolates on sorghum silage

***Silage production.*** *Sorghum bicolor* L. Moench cv. Samurai-2 was planted in Yogyakarta, Indonesia, and harvested at 87 days with a DM content of approximately 28.7% and chopped into 3–5 cm. Forage was kept as silage into a mini-silo (5 kg) using a plastic bag combined with a bucket to maintain the density following the recommendation of Paradhipta et al. (2024). Treatments consisted of control (CON) and four LAB isolates (INO1–INO4). The four LAB isolates showing the highest antifungal activity against *Fusarium graminearum* among the 25 pre-selected isolates were used in the silage experiment. These isolates were designated as INO1 (AF-2), INO2 (AF-24), INO3 (AF-25), and INO4 (AF-34). An isolate of LAB was applied at 4 ml to achieve $1 \times 10^5$ CFU/g fresh forage, with five silos per treatment serving as replicates. For 60 days, silos were kept at room temperature after being anaerobically sealed.

***Chemical compositions.*** Silage samples were dried at 55 °C for 3–5 days and ground through a 1-mm screen. Dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), neutral detergent fibre (NDF), and acid detergent fibre (ADF) were analysed according to AOAC International (2016). DM was determined by oven drying (934.01), OM by ashing at 550 °C (942.05), CP by the Kjeldahl method (954.01), EE by Soxhlet extraction (920.39), NDF was determined according to AOAC Official Method 2002.04 using heat-stable α-amylase, and ADF according to AOAC Official Method 973.18, using an ANKOM A200 Fibre Analyser (ANKOM Technology, USA).

***Fermentation characteristics.*** Silage pH was measured using a pH meter (OHAUS AB-23PH-F, USA) and $NH_3$-N was determined by the steam distillation–colorimetric method (Chaney and Marbach 1962; AOAC International 2016). Organic acids were analysed by HPLC equipped with a diode-array detector and a Shim-pack GIST C18 column (Shimadzu LC-2030C; Japan) following Castillo Vargas et al. (2020). Microbial populations of LAB, yeasts, and moulds were determined by plate counting according to Paradhipta et al. (2021), using MRS agar for LAB and PDA for yeasts and moulds, and expressed as $\log_{10}$ CFU/g fresh matter.

***Aerobic stability.*** Aerobic stability was evaluated by exposing 3 kg of silage to air under ambient tropical conditions (23–35 °C). Silage temperature was recorded hourly using a digital thermometer (Traceable® 4052; USA). Aerobic stability was defined as the time required for silage temperature to increase by 2 °C above ambient temperature (Fitriani et al. 2024; Ardiansyah et al. 2025).

***Physical appearances.*** Physical appearances were performed to assess colour, aroma, and texture. A total of 30 untrained panellists with general familiarity with silage completed a questionnaire covering multiple evaluation parameters following Fitriani et al. (2024) and Ardiansyah et al. (2025). Sensory characteristics were evaluated descriptively by 30 panellists based on colour, aroma, texture, and visible mould. The sensory observations were summarised as percentages for each category and were not subjected to inferential statistical analysis.

***In vitro ruminal digestibility.*** Rumen fluid was collected before morning feeding from two adult female Bali cattle fitted with rumen cannulas, following ethical approval (LPPT UGM No. 00007/III/UN1/LPPT/EC/2024). The animals were maintained on a standard diet consisting of *Pennisetum purpureum* forage and commercial concentrate at a ratio of 60 : 40, formulated to be iso-nitrogen and iso-energy, generating approximately 11–13% CP and 58–62% total digestible nutrients (TDN). *In vitro* incubations were performed following the Adesogan et al. (2005) procedure. Samples (0.5 g DM) were propagated in 40 ml rumen fluid–buffer mixture (1 : 2, v/v) at 39 °C for 48 h under $CO_2$ conditions. For every silo, two blanks and four replications were made. According to Fitriani et al. (2024), residues were filtered, dried, and examined for DM and OM digestibility following incubation. Rumen pH was measured during the experiment using the supernatant.

### Statistical analysis

Data from Experiment 1 ($OD_{600}$, pH, and inhibition zone) were summarised descriptively. Data from

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Experiment 2 were analysed by one-way analysis of variance using a completely randomised design, with individual silos considered as experimental units ($n$ = 5 per treatment). The statistical model included treatment as a fixed effect. The normality of model residuals was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated using Levene's test. When treatment effects were significant ($P < 0.05$), means were compared using Duncan's multiple range test. Organoleptic characteristics evaluated by 30 panellists were summarised descriptively and were not subjected to inferential statistical analysis. Analyses were performed using SAS® Studio Release v3.82 (Enterprise Edition; SAS Institute Inc., Cary, NC, USA).

Relationships between aerobic stability and fermentation (acetate and lactate to acetate ratio) were further evaluated using individual silo observations ($n$ = 25). Pearson correlation analysis was performed between aerobic stability and acetate concentration, and lactate-to-acetate ratio. Exploratory linear regression analyses were subsequently performed to assess the contribution of these variables to variation in aerobic stability. Statistical significance was declared at $P < 0.05$.

## RESULTS AND DISCUSSION

### Experiment 1 – LAB isolation and selection with antifungal activity

A total of 100 LAB isolates were obtained from sorghum silage fermented for 100 days without additives. Preliminary screening based on growth ($OD_{600}$) and acidification (pH) identified 25 isolates for further antifungal evaluation (Figures 1 and 2).

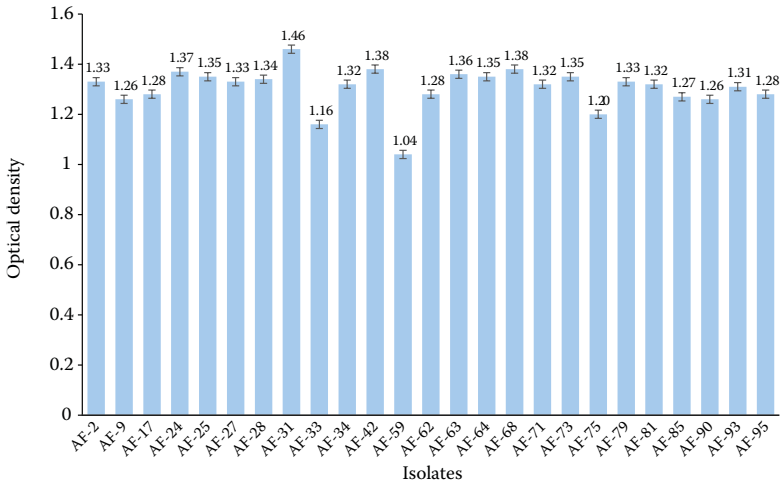


Figure 1. The result of optical density from 25 selected isolates

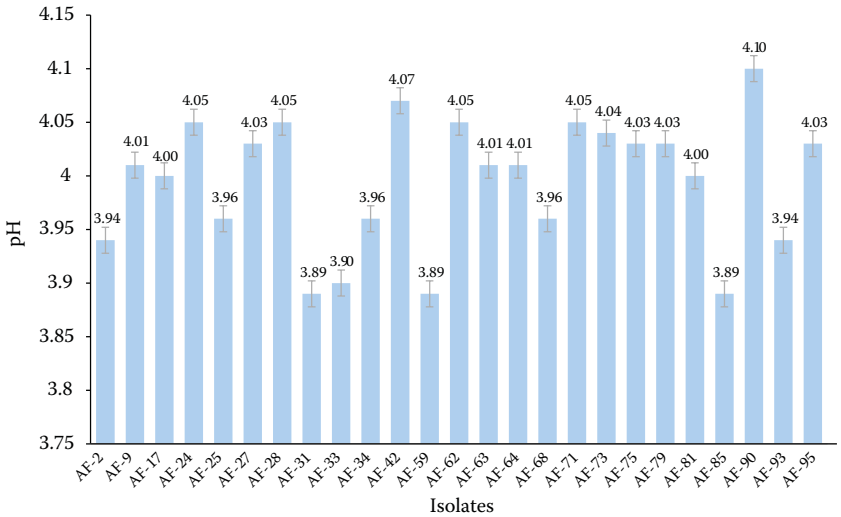


Figure 2. The result of acidification (pH value) from 25 selected isolates

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After 48 h of incubation, $OD_{600}$ and pH ranged from 1.04 to 1.46 and 3.89 to 4.10, respectively. Antifungal activity against *Fusarium graminearum* YP0124 varied among isolates, with inhibition zones ranging from 0.22 to 1.05 cm (Figures 3 and 4). AF-2, AF-24, AF-25, and AF-34 showed the largest inhibition zones (1.05 ± 0.07, 1.03 ± 0.04, 0.94 ± 0.01, and 0.83 ± 0.05 cm, respectively) and were identified as *Lactiplantibacillus plantarum* with ≥99.6% sequence identity (Table 1). Sequence lengths ranged from 1 395 to 1 405 bp, and phylogenetic analysis confirmed clustering within the *Lactiplantibacillus plantarum* clade (Figure 5).

The $OD_{600}$ and pH values indicated that the selected indigenous LAB were able to grow and acidify MRS medium. The observed $OD_{600}$ values were comparable to those previously reported for *Lactiplantibacillus plantarum* under similar culture conditions, where higher $OD_{600}$ values reflected greater bacterial growth and biomass accumulation (Marwah et al. 2023). Differences in $OD_{600}$ and pH among isolates may reflect strain-

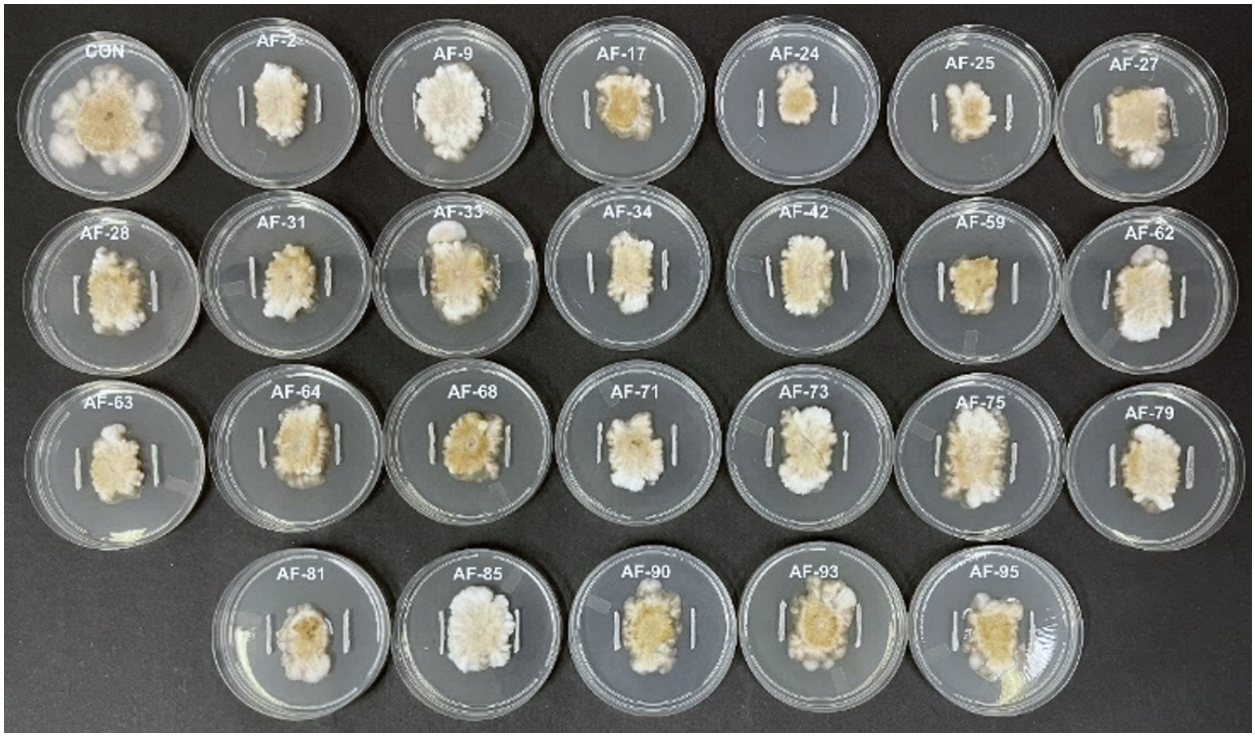


Figure 3. Antifungal activity test of 25 selected isolates against *Fusarium graminearum* using the plate assay method

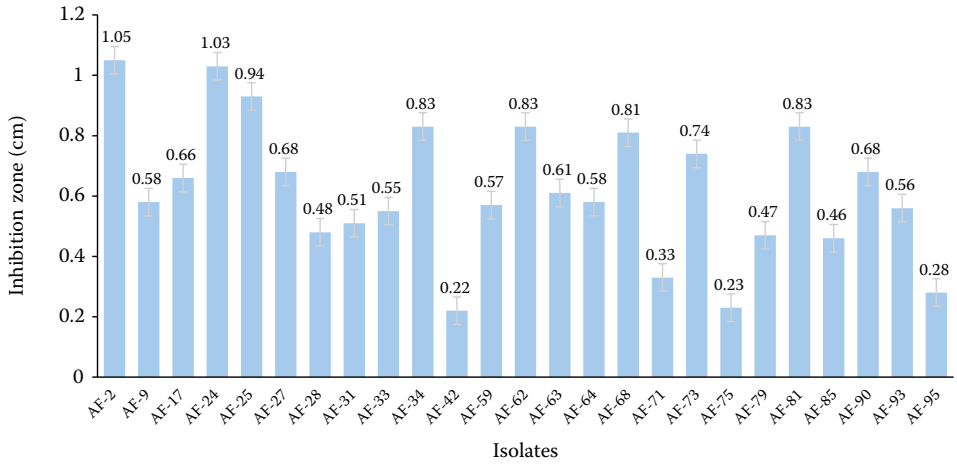


Figure 4. The result of antifungal activity from 25 selected isolates in making inhibition area against *Fusarium graminearum*

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Table 1. Identification of lactic acid bacteria (LAB) isolates using 16S rRNA gene sequencing

| Isolate | Nucleotide (bp) | Species | Identity (%) |
|---|---|---|---|
| AF-2 | 1 404 | *Lactiplantibacillus plantarum* | 99.6 |
| AF-24 | 1 395 | *Lactiplantibacillus plantarum* | 99.9 |
| AF-25 | 1 405 | *Lactiplantibacillus plantarum* | 99.9 |
| AF-34 | 1 405 | *Lactiplantibacillus plantarum* | 99.7 |

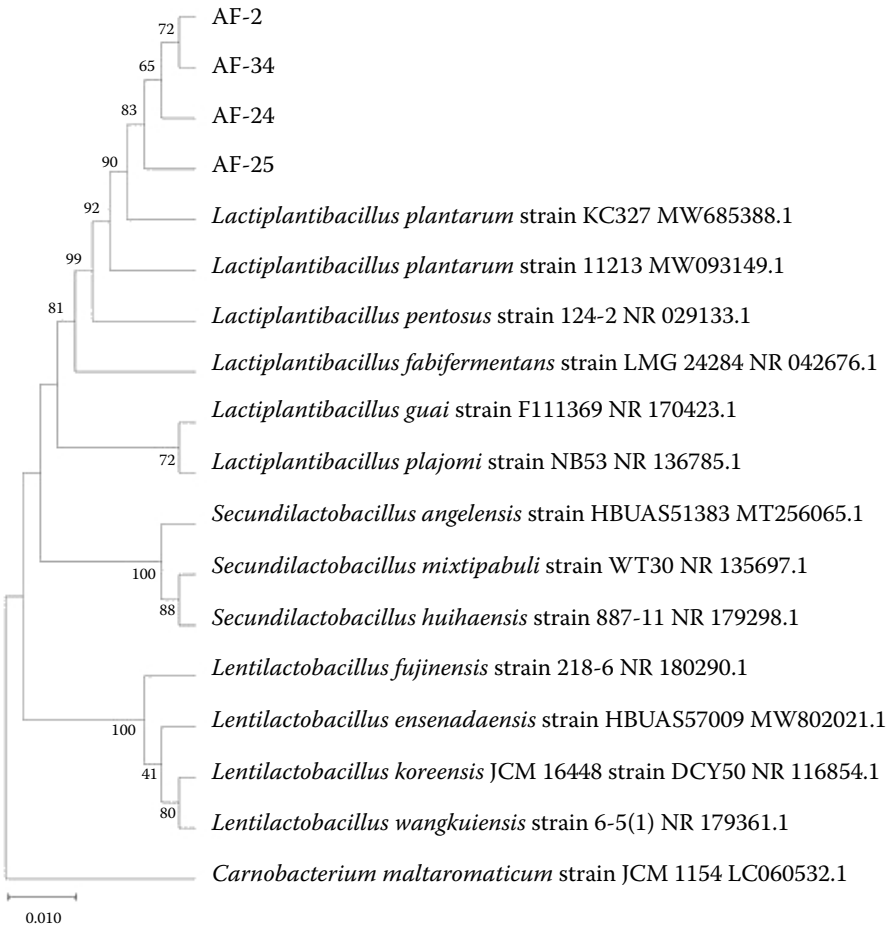


Figure 5. Phylogenetic tree showing the genetic relationship of lactic acid bacteria (LAB) isolates AF-2, AF-24, AF-25, and AF-34

dependent variation in growth kinetics and metabolic activity, which is commonly observed within LAB populations (Stasiak-Rozanska et al. 2021). Despite their ability to grow and acidify the medium, the isolates exhibited different levels of antifungal activity, suggesting that fungal inhibition was not solely associated with bacterial growth or acidification. Similar strain-specific antifungal responses have been reported among LAB and may be related to differences in the production of organic acids, hydrogen peroxide, bacteriocins, and other antifungal metabolites (Cizeikiene et al. 2013; Valan Arasu et al. 2013; Bao et al. 2024). Molecular identification further confirmed that the four isolates with the greatest antifungal activity belonged to *L. plantarum*, a species commonly associated with silage fermentation and previously reported to possess antifungal properties (Paradhipta et al. 2021).

**Experiment 2: Effects of selected LAB isolates on sorghum silage**

***Chemical composition.*** The chemical composition of fresh sorghum forage before ensiling

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Table 2. Chemical composition of sorghum forage before ensiling

| Item | Value of content |
|---|---|
| Dry matter (g/kg fresh matter) | 287 |
| Organic matter (g/kg DM) | 896 |
| Crude protein (g/kg DM) | 63.4 |
| Ether extract (g/kg DM) | 22.9 |
| Neutral detergent fibre (g/kg DM) | 666 |
| Acid detergent fibre (g/kg DM) | 396 |

DM = dry matter

is presented in Table 2. LAB inoculation did not affect dry matter (DM) loss ($P$ = 0.071; Figure 6) or the chemical composition of the silages, including organic matter (OM), crude protein (CP), ether extract (EE), neutral detergent fibre (NDF), and acid detergent fibre (ADF) after 60 days of ensiling (Table 3; $P$ > 0.05). Dry matter loss was low (16.9–21.4%), indicating limited dry matter loss during fermentation.

The absence of significant differences in chemical composition indicates that the selected LAB isolates preserved nutrients without adversely affecting the major chemical fractions. The concentrations of DM, OM, CP, EE, NDF, and ADF were within ranges previously reported for sorghum silage (McCary et al. 2020; Fitriani et al. 2024; Florit et al. 2025; Junior et al. 2025). The stable CP concentration, together with low $NH_3$-N production, suggests limited proteolysis during ensiling

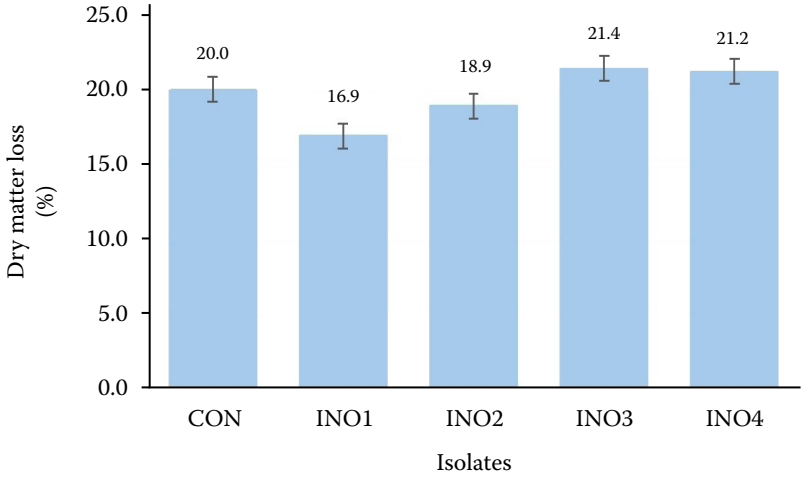


Figure 6. Percentage of dry matter loss of sorghum silage after 60 days of fermentation
CON = sorghum silage without inoculant; INO1 = sorghum silage with lactic acid bacteria (LAB) AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant; LAB = lactic acid bacteria

Table 3. Effects of selected isolates on chemical composition of sorghum silage

| Item | Treatment | | | | | SEM | $P$-value |
|---|---|---|---|---|---|---|---|
| | CON | INO1 | INO2 | INO3 | INO4 | | |
| DM (g/kg fresh matter) | 263 | 259 | 259 | 257 | 262 | 1.71 | 0.854 |
| OM (g/kg DM) | 902 | 899 | 904 | 905 | 902 | 0.89 | 0.196 |
| CP (g/kg DM) | 56.3 | 57.8 | 52.0 | 56.3 | 57.7 | 0.84 | 0.351 |
| EE (g/kg DM) | 23.9 | 24.3 | 26.7 | 22.5 | 26.1 | 1.78 | 0.092 |
| NDF (g/kg DM) | 513 | 514 | 520 | 520 | 507 | 16.3 | 0.715 |
| ADF (g/kg DM) | 387 | 384 | 389 | 420 | 387 | 25.7 | 0.413 |

ADF = acid detergent fibre; CON = sorghum silage without inoculant; CP = crude protein; DM = dry matter; EE = ether extract; INO1 = sorghum silage with LAB AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant; LAB = lactic acid bacteria; NDF = neutral detergent fibre; OM = organic matter; SEM = standard error of the mean

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(Borreani et al. 2018; Kung et al. 2018). The use of five silos per treatment may have limited the ability to detect treatment effects for parameters with relatively high variability, as observed for ADF (SEM = 25.7). A larger number of replicates may therefore be beneficial in future studies.

Sun et al. (2024) reported that NDF and hemicellulose can be degraded during sweet sorghum ensiling, indicating that changes in cell-wall carbohydrate fractions may occur during fermentation. In the present study, ADF remained relatively stable between the fresh forage (396 g/kg DM) and ensiled silages (384–420 g/kg DM), whereas NDF decreased substantially from 666 g/kg to 507–520 g/kg DM. Because ADF mainly represents cellulose and lignin, the greater reduction in NDF may be associated with changes in the hemicellulose fraction during ensiling. However, hemicellulose was not determined separately in the present study, and the comparison between fresh forage and silage was descriptive because the fresh forage was not included as a replicated treatment. Therefore, this apparent change in the hemicellulose fraction should be interpreted cautiously.

***Fermentation characteristics.*** Silage pH and $NH_3$-N were not affected by LAB inoculation (Table 4; $P > 0.05$). INO2 had a higher lactate concentration than CON (26.4 vs 22.5 g/kg DM; $P = 0.001$), whereas acetate was lower in all inoculated silages than in CON (15.3–18.9 vs 29.7 g/kg DM; $P = 0.001$). The lactate-to-acetate ratio was higher in INO2 than in CON ($P < 0.05$). Propionate and butyrate were not detected.

These fermentation characteristics indicate successful acidification across treatments, while the differences in lactate and acetate demonstrate strain-dependent fermentation patterns. Homofermentative LAB such as *Lactiplantibacillus plantarum* primarily convert water-soluble carbohydrates into lactate, which contributes to rapid acidification and inhibition of undesirable microorganisms (Kung et al. 2018). The higher acetate concentration in CON may reflect greater activity of epiphytic heterofermentative LAB. Similar acetate production has been reported in sorghum silage associated with heterofermentative fermentation (Fitriani et al. 2024; Ardiansyah et al. 2025). The absence of detectable butyrate further suggests that clostridial fermentation was effectively suppressed (Kung et al. 2018).

***Microbial characteristics.*** Microbial populations differed among treatments (Table 5; $P = 0.001$). INO1 had the highest LAB count (6.38 $\log_{10}$ CFU/g fresh matter), whereas INO4 had the lowest (5.74 $\log_{10}$ CFU/g fresh matter). Yeast counts were lower in INO4 than in CON, while INO3 had the highest yeast population. Mould was not detected in INO1, INO2, and INO3, whereas INO4 did not differ from CON.

The absence of detectable mould in INO1–INO3 supports the antifungal activity observed during isolate screening and indicates that antifungal effectiveness was strain-specific. Certain *Lactiplantibacillus plantarum* strains can produce organic acids, bacteriocins, and other antifungal metabolites capable of suppressing fungi

Table 4. Effects of selected isolates on fermentation characteristics of sorghum silage

| Item | Treatment | | | | | SEM | *P*-value |
|---|---|---|---|---|---|---|---|
| | CON | INO1 | INO2 | INO3 | INO4 | | |
| pH | 3.57 | 3.54 | 3.62 | 3.71 | 3.68 | 0.123 | 0.193 |
| $NH_3$-N (g/kg DM) | 0.24 | 0.25 | 0.25 | 0.24 | 0.22 | 0.025 | 0.394 |
| Lactate (g/kg DM) | 22.5$^{b}$ | 24.6$^{ab}$ | 26.4$^{a}$ | 19.5$^{bc}$ | 19.1$^{c}$ | 2.84 | 0.001 |
| Acetate (g/kg DM) | 29.7$^{a}$ | 18.9$^{b}$ | 18.5$^{b}$ | 15.3$^{b}$ | 17.3$^{b}$ | 3.04 | 0.001 |
| Propionate (g/kg DM) | n.d. | n.d. | n.d. | n.d. | n.d. | n.a. | n.a. |
| Butyrate (g/kg DM) | n.d. | n.d. | n.d. | n.d. | n.d. | n.a. | n.a. |
| Lactate : acetate | 0.77$^{c}$ | 1.27$^{ab}$ | 1.41$^{a}$ | 1.29$^{a}$ | 1.12$^{b}$ | 0.128 | 0.001 |

$^{a–c}$Different superscripts within the same row indicate significant differences ($P < 0.05$)

CON = sorghum silage without inoculant; DM = dry matter; INO1 = sorghum silage with LAB AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant; LAB = lactic acid bacteria; n.a. = not applicable; n.d. = not detected in HPLC analysis; SEM = standard error of the mean

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Table 5. Effects of selected isolates on microbial characteristics of sorghum silage [$log_{10}$ colony-forming units (CFU)/g fresh matter]

| Item | Treatment | | | | | SEM | *P*-value |
|---|---|---|---|---|---|---|---|
| | CON | INO1 | INO2 | INO3 | INO4 | | |
| Lactic acid bacteria | 6.23$^{ab}$ | 6.38$^{a}$ | 6.07$^{b}$ | 5.98$^{bc}$ | 5.74$^{c}$ | 0.061 | 0.001 |
| Yeast | 5.50$^{b}$ | 5.23$^{bc}$ | 5.41$^{bc}$ | 5.92$^{a}$ | 5.08$^{d}$ | 0.063 | 0.001 |
| Mould | 5.03$^{a}$ | n.d.$^{b}$ | n.d.$^{b}$ | n.d.$^{b}$ | 4.61$^{a}$ | 0.334 | 0.001 |

$^{a–d}$Different superscripts within the same row indicate significant differences ($P < 0.05$) CON = sorghum silage without inoculant; INO1 = sorghum silage with LAB AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant; LAB = lactic acid bacteria; n.d. = not detected; SEM = standard error of the mean

such as *Fusarium* and *Aspergillus* (Valan Arasu et al. 2013; Bao et al. 2024). However, the specific antifungal compounds produced by AF-2, AF-24, and AF-25 were not identified in the present study and require further investigation.

***Aerobic stability.*** Aerobic stability differed among treatments ($P < 0.05$; Figures 7 and 8), with INO1 showing the longest stability at 44.4 hours. During aerobic exposure, temperature increased earlier in the less stable silages, indicating more rapid aerobic deterioration. Aerobic deterioration is primarily associated with the growth of lactate-assimilating yeasts and moulds after oxygen exposure, resulting in heat production and nutrient losses (Carvalho et al. 2016; Borreani et al. 2018). The longer aerobic stability of INO1 occurred despite its lower acetate concentration than CON, suggesting that acetate was not the sole factor contributing to stability. The response may also be associated with the strain-specific antifungal activity of AF-2 and its ability to suppress spoilage microorganisms. As AF-2 is a homofermentative LAB, its effect on aerobic stability may not be explained by acetate production alone but may also be associated with the production of antifungal substances. LAB can produce several antifungal metabolites that contribute to fungal inhibition independently of acetate concentration (Muhialdin et al. 2020). The specifics of antifungal substances should be identified in our future studies.

An important limitation of the present study is the absence of a validated commercial or reference inoculant as a positive control. Because antifungal activity and silage performance of *Lactiplantibacillus*

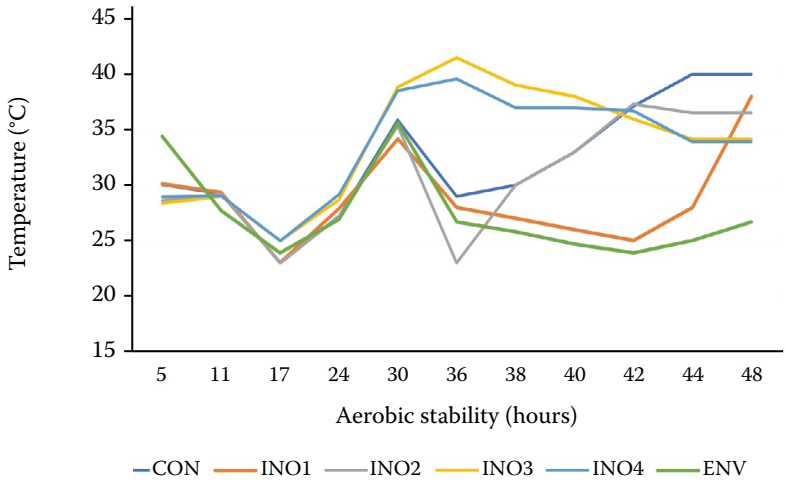


Figure 7. Aerobic stability graph of sorghum silage
CON = sorghum silage without inoculant; ENV = ambient environment; INO1 = sorghum silage with LAB AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant; LAB = lactic acid bacteria

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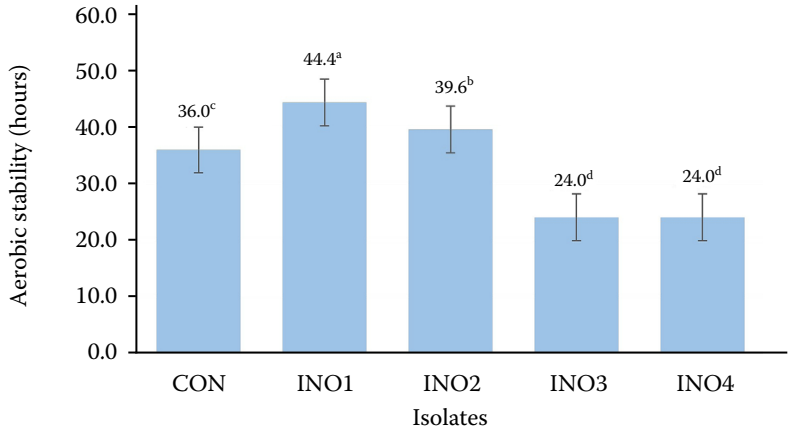


Figure 8. Aerobic stability graph of sorghum silage

[a–d]Different superscripts indicate significant differences ($P < 0.05$)

CON = sorghum silage without inoculant; INO1 = sorghum silage with LAB AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant; LAB = lactic acid bacteria

*plantarum* are highly strain-dependent, comparisons with results reported for other strains across different studies should be interpreted cautiously. Therefore, although AF-2 and AF-24 improved aerobic stability and suppressed mould relative to the uninoculated control, the present study cannot determine whether these isolates provide superior performance or added value compared with established commercial inoculants. Direct comparisons with validated reference or commercial strains under the same experimental conditions are required in future studies.

The correlation analysis did not reveal a significant linear association between aerobic stability and either acetate concentration ($r = 0.008$, $P = 0.968$) or the lactate : acetate ratio ($r = 0.110$, $P = 0.602$) across the 25 individual silos. Thus, the variation in aerobic stability could not be explained by these fermentation variables alone. The absence of a significant association is also supported by the treatment-level data, as INO1 exhibited the longest aerobic stability despite having lower acetate concentration than CON, whereas INO3 had the lowest acetate concentration but the shortest aerobic stability. These findings suggest that acetate concentration and the lactate : acetate ratio alone were insufficient to explain the differences in aerobic stability observed among treatments. The contribution of other strain-specific or microbial factors should therefore be considered, although the mechanism underlying the response of INO1 could not be conclusively determined in the present study.

***Organoleptic characteristics.*** Organoleptic characteristics were evaluated descriptively by 30 panellists (Figures 9–12). All silages were predominantly brownish-green in colour, had an acidic aroma, and showed mainly non-clumped and non-slimy textures. No visible mould was reported in inoculated silages, whereas a small proportion (6%) was observed in CON (Figure 12). The organoleptic characteristics generally supported the chemical and microbiological results and indicated acceptable fermentation quality. The acidic aroma was consistent with organic acid production, while the absence of slime and clumping suggested appropriate preservation conditions (Kung et al. 2018). The absence of visible mould should, however, be distinguished from microbiological plate counts, because viable mould was still detected in INO4 (Table 5).

***In vitro ruminal digestibility.*** LAB inoculation did not affect *in vitro* dry matter digestibility (IVDMD), *in vitro* organic matter digestibility (IVOMD), or rumen fluid pH (Table 6; $P > 0.05$). IVDMD and IVOMD ranged from 53.9% to 56.3% and 63.3% to 65.6%, respectively, while rumen fluid pH ranged from 6.47 to 6.55.

The lack of differences in IVDMD and IVOMD indicates that inoculation with the selected LAB isolates did not impair ruminal nutrient degradability. LAB inoculation does not consistently alter ruminal or *in vitro* digestibility, particularly when fibre fractions remain unchanged (Weinberg et al. 2007; Bernardes et al. 2018).

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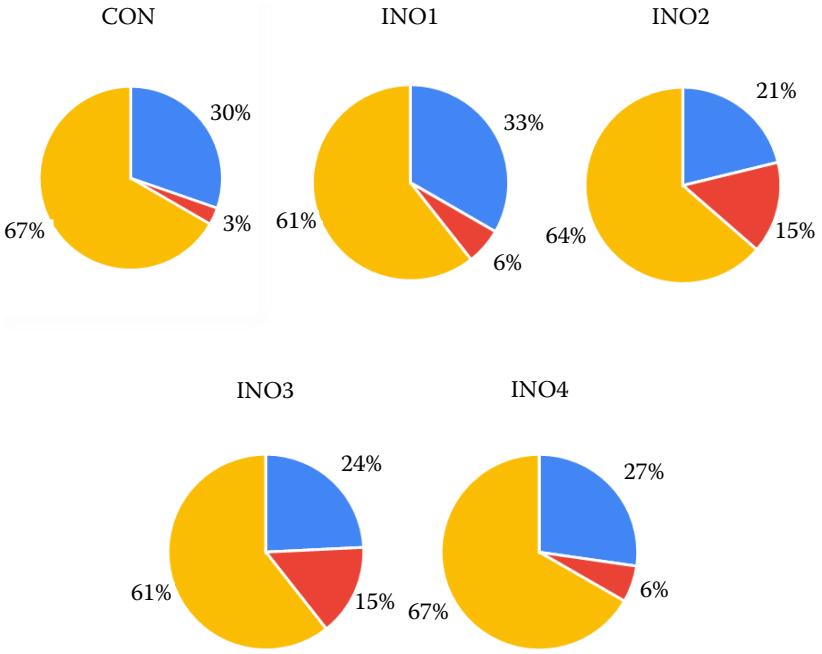


Figure 9. Colour of sorghum silage with selected lactic acid bacteria (LAB) isolates
(■) Greenish brown, (■) greenish yellow, (■) yellow
CON = sorghum silage without inoculant; INO1 = sorghum silage with LAB AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant; LAB = lactic acid bacteria

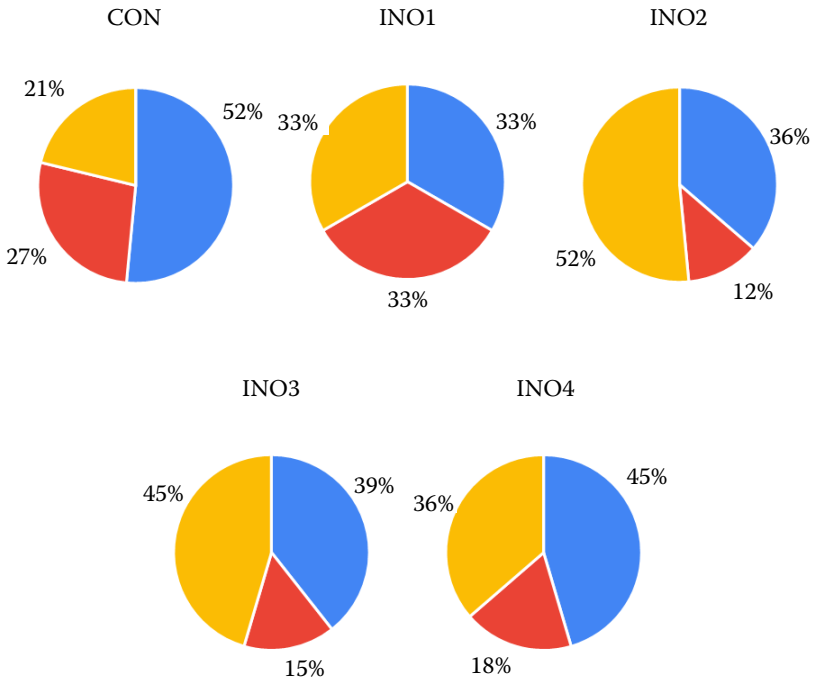


Figure 10. Aroma of sorghum silage with selected lactic acid bacteria (LAB) isolates
(■) Slightly sour, (■) sour, (■) very sour and fresh
CON = sorghum silage without inoculant; INO1 = sorghum silage with LAB AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant

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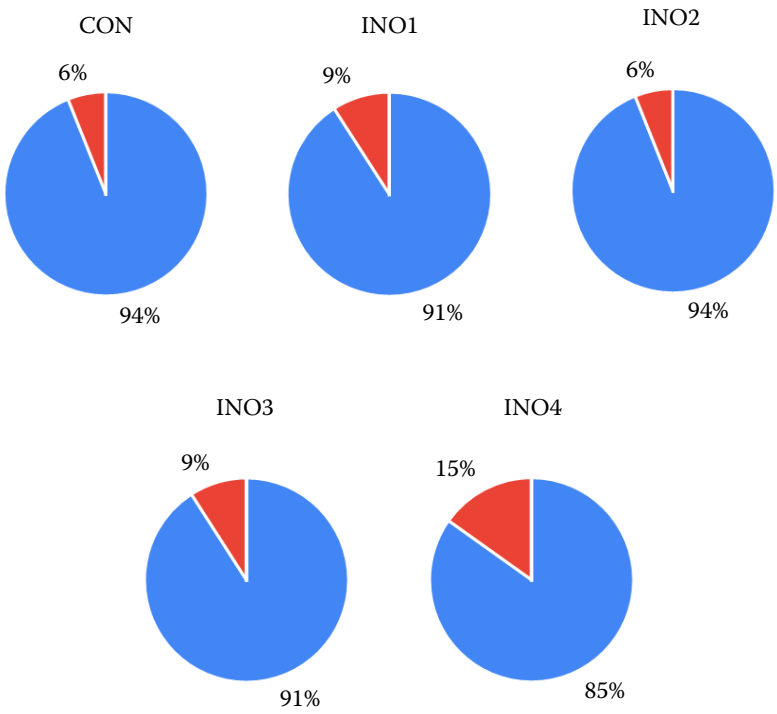


Figure 11. Texture of sorghum silage with selected lactic acid bacteria (LAB) isolates
(■) Non-clumped and non-slimy, (■) slightly clumped
CON = sorghum silage without inoculant; INO1 = sorghum silage with LAB AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant

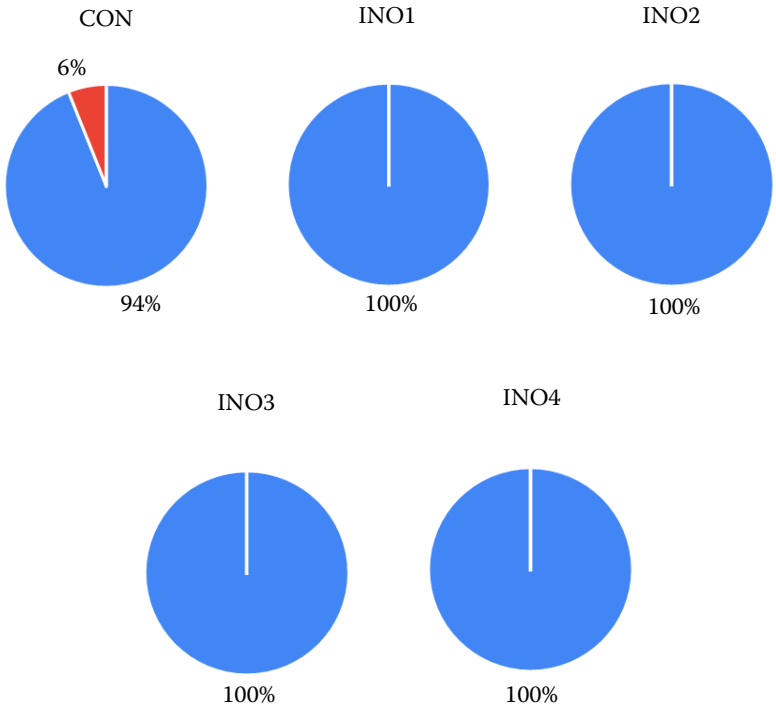


Figure 12. Mould growth in sorghum silage with selected lactic acid bacteria (LAB) isolates
(■) None, (■) slight
CON = sorghum silage without inoculant; INO1 = sorghum silage with LAB AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant

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Table 6. Rumen fermentation characteristics of sorghum silage with lactic acid bacteria (LAB) isolates

| Item | Treatment | | | | | SEM | *P*-value |
|---|---|---|---|---|---|---|---|
| | CON | INO1 | INO2 | INO3 | INO4 | | |
| Nutrient digestibility | | | | | | | |
| DMD (%) | 56.3 | 53.9 | 54.0 | 55.8 | 55.8 | 1.83 | 0.386 |
| OMD (%) | 65.3 | 64.1 | 63.3 | 65.6 | 65.4 | 1.52 | 0.353 |
| Rumen pH | 6.47 | 6.50 | 6.55 | 6.50 | 6.52 | 0.063 | 0.467 |

CON = sorghum silage without inoculant; DMD = dry matter digestibility; INO1 = sorghum silage with LAB AF-2 inoculant; INO2 = sorghum silage with LAB AF-24 inoculant; INO3 = sorghum silage with LAB AF-25 inoculant; INO4 = sorghum silage with LAB AF-34 inoculant; OMD = organic matter digestibility; SEM = standard error of the mean

Overall, AF-2 combined strong antifungal activity with the longest aerobic stability without impairing digestibility.

Although the selected LAB strains were isolated and evaluated under tropical conditions, their potential application may not necessarily be restricted to tropical regions. In some subtropical countries, the summer conditions may be similar to those in tropical areas. The suppression of mould growth and improvement in aerobic stability observed in this study are associated with strain-specific antifungal activity, which could also be relevant to silage preservation in other climatic regions. Nevertheless, temperature strongly affects silage fermentation dynamics, microbial succession, and aerobic deterioration, and silage preservation presents distinct challenges under hot and cold climatic conditions (Bernardes et al. 2018). Therefore, the effectiveness of AF-2 and AF-24 under temperate and other non-tropical conditions should be validated before their broader application can be recommended.

A limitation of the present study is the absence of a commercial or validated reference inoculant as a positive control. Because *Lactiplantibacillus plantarum* is widely used as a silage inoculant, the present results cannot be interpreted as evidence that the selected indigenous isolates are superior to established commercial strains.

Moreover, antifungal activity is strain- and method-dependent, which limits direct comparison among studies. Therefore, the present findings demonstrate the potential of the selected indigenous isolates under the conditions evaluated, while their performance relative to commercial inoculants remains to be established. Future studies should include a validated commercial inoculant as a reference treatment.

## CONCLUSION

In conclusion, this study successfully isolated antifungal LAB from sorghum silage and identified the most effective strains as *Lactiplantibacillus plantarum*. Among the isolates evaluated in this study, AF-2 showed the most promising combination of antifungal activity and aerobic stability without adversely affecting chemical composition or *in vitro* ruminal digestibility. These isolates show strong potential as functional inoculants for improving sorghum silage preservation under tropical conditions.

Their antifungal properties may also have relevance to silage systems in other climatic regions; however, their effectiveness under temperate and other non-tropical conditions requires further validation. However, direct comparison with validated commercial or reference inoculants is required to determine whether these indigenous strains provide additional benefits over established silage inoculants.

### Conflict of interest

The authors declare no conflict of interest.

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