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Research article

# Lactic acid bacteria and propionic acid affect the fermentation quality and deterioration rate of maize silage after aerobic exposure

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## Abstract

The experiment aimed to assess and compare the effect of bacterial inoculants, propionic acid and their combinations on the fermentation process, along with variations in pH, yeast and mould count after aerobic exposure. Maize fodder was harvested at one-third to half milk line stage (average dry matter 30.62 %). Lactic acid bacterial inoculants and their combination with chemical additives were used on a fresh matter basis for silage preparation. The treatments were control (no additive), *Lactiplantibacillus plantarum* (LP), *Limosilactobacillus fermentum* (LF), propionic acid (PA), and a combination of LP+PA, LF+PA, LP+LF and LP+LF+PA. Silage fermentation parameters and aerobic stability were evaluated after 30 days of ensiling. Additive treatment significantly reduced silage pH, whereas lactic acid, acetic acid content and dry matter recovery were increased. Lactic acid concentration was noticeably greater in the LP+LF+PA (7.91) treated silage compared to the other treatments. In comparison to the hetero-fermentative additive (LF) treated group, dry matter recovery was greater in the LP, PA and their respective combination treated groups. On various days of aerobic exposure, silage treated with the treatments of LF+PA and LP+LF+PA showed the lowest pH, yeast and mould counts. Overall, these findings indicate that the additive combination LP+LF+PA was successful in raising quality indicators, but the combination of LF+PA was more beneficial in reducing silage deterioration after aerobic exposure. The additives used in the experiment were effective in improving the silage fermentation characteristics. A combination of bacterial inoculants and chemical additives significantly decreased aerobic spoilage in maize silage. The pH values and the yeast-mould count remained more static as the exposure period increased.

**Keywords:** Aerobic deterioration rate, Bacterial inoculants, Fermentation quality, Maize silage

## Introduction

Silage is gaining popularity as it provides a constant supply throughout the year. The basis of ensiling phenomena is the naturally occurring anaerobic fermentation of fodder in the presence of lactic acid-producing bacteria, which convert readily fermentable carbohydrates into organic acids, primarily lactic acid (Koc *et al*., 2008). Ensiling is inherently an uncontrolled process that depends on the epiphytic microflora. Silage additives can control the course of the ensiling process and improve fermentation quality and aerobic stability (Muck *et al*., 2018; Chauhan *et al*., 2022). Various additive combinations are used to enhance the nutritive value of silage, but bacterial inoculants are generally used separately from chemical preservatives. Based on an analysis of various silage experiments, homofermentative lactic acid bacteria increased DM recovery during the fermentation and increased the efficiency of the ensiling process (Oliveira *et al*., 2017; Muck *et al*., 2018).

It has been noted that silage high in carbohydrates with high lactic acid concentrations and low quantities of volatile fatty acids (acetic acid) are particularly susceptible to aerobic degradation (McDonald *et al*., 1991). The main cause of the aerobic deterioration of silage is the growth of yeasts and moulds, which remain latent in anaerobic conditions and quickly multiply when exposed to air again. The obligately heterofermentative lactic acid bacteria have been proven to improve silage aerobic stability by producing acetic acid (Kleinschmit and Kung, 2006). The fermentation of undesirable bacteria is frequently inhibited by chemical additives (propionic acid), which lowers losses and stops the growth of yeasts that initiate aerobic deterioration in silages (Muck *et al*., 2018).

Homofermentative bacterial inoculants are effective in improving the fermentation profile. In contrast, heterofermentative bacterial inoculants and chemical additives effectively increase silage's aerobic stability by inhibiting the bacteria that cause unwanted fermentation and spoilage. Consequently, indenting additives related to silage fermentation that prevent fodder deterioration have been the main focus of studies on maize silage in order to minimise losses. Additionally, not much has been researched about the effects of propionic acid, *Lactiplantibacillus plantarum*, and *Limosilactobacillus fermentum* individually or in combination on the fermentation profile and changes in pH, yeast, and mould count after aerobic exposure of maize silage.

Thus, the purpose of this study was to assess how different bacterial inoculants, chemical additives, and their combinations affected the fermentation process of maize silage, as well as how pH, yeast and mould counts changed during aerobic exposure.

## Materials and Methods

***Silage preparation and additive treatment*:** The study was carried out at the National Dairy Research Institute, Karnal, Haryana, located at a height of 250 meters above sea level, with a latitude and longitude of 29° 42″ N and 79° 54″ E, respectively. Maize fodder was collected from the NDRI field at one-third to half the milk line stage (30.62 %DM). Fodder (leaves and stem combination) was chopped using an electrical chaff cutter to an average chop length of 1.5-2.0 cm and ensiled in a plastic container equipped with a lid that enables gas release and vacuum creation. For silage preparation, the following additives were used on a fresh basis. The bacterial inoculants include homo-fermentative bacteria (*Lactiplantibacillus plantarum,* LP), NCDC No.-344 ($1*10^6$ CFU $g^{-1}$) and hetero-fermentative bacteria (*Limosilactobacillus fermentum,* LF), NCDC No.-214 ($2*10^6$CFU $g^{-1}$) and chemical additive propionic acid (PA) @ 0.1%. Treatments were used in the experiment that were control (no additive), LP, LF, PA (propionic acid), LP+PA, LF+PA, LP+LF and LP+LF+PA. Three plastic containers of size 2.5 to 3 kg were filled with maize fodder for each treatment after proper mixing with additives to evaluate the effect of additives individually or in various possible combinations. The containers were tightly closed, weighed and stored at room temperature. After 30 days of ensiling, the samples were analysed in triplicate for fermentation and quality parameters, microbial composition, and aerobic stability. Pre-ensiled samples were taken for chemical analysis.

***Chemical composition, fermentation parameters and silage quality assessment*:** The chemical composition (DM, CP, OM and EE) of fresh maize fodder was determined as per the method described by AOAC (2005). The pH of fresh fodder and silage was determined using the Eutech pH meter from the aqueous extract. The water-soluble carbohydrate (WSC) content of fresh fodder was determined by a spectrophotometer after a reaction with an anthrone reagent (Yemm and Willis, 1954). The fermentation coefficient (FC) of maize fodder was calculated using dry matter, water-soluble carbohydrates and buffering coefficient (Weissbach and Honig, 1996). The weight of the forage mass in the plastic container and its DM content at day 0 and the $30^{th}$ day were used to calculate the DM recovery (DMR) (da Silva *et al*., 2020). Lactic acid estimation was done as per the method described by Barnett (1951). Acetic acid was estimated with the help of Nucon's gas-liquid chromatography. Flieg point was calculated from the pH value and DM of silage at the end of the fermentation period with the following equation (Moselhy *et al*., 2015):

Flieg point = 220 +[(2*DM-15)] -40*pH

LAB count was done by pour-plating 10-fold serial dilutions on de Man, Rogosa, and Sharpe agar (De Man *et al*., 1960) from Himedia Laboratories Pvt Ltd, Mumbai, India. The Petri plates were incubated at 37°C for 48 hours to enumerate LAB in fresh maize fodder and silages. The total numbers of yeasts and moulds were determined by pour-plating 10-fold serial dilutions on potato dextrose agar that was acidified with 0.5% (vol/vol) of 85% lactic acid after autoclaving. These plates were incubated aerobically for 72 hr at 25 °C. Clostridia spore concentration in fresh silage samples was determined by the most probable number (MPN) procedure (Tabacco *et al*., 2009).

Fitness values (Davies *et al*., 2000) were modified as follows for the present experiment conditions: Modified fitness value =

$$\left(\frac{1}{1+\left[\text{pH wtg}*\left(\frac{\text{pH}}{\text{cntlpH}}\right)\right]+\left[\text{DML wtg}*\left(\frac{\text{DML}}{\text{cntlDML}}\right)\right]+\left[\text{Amm}-\text{Nwtg}*\left(\frac{\text{AmmN}}{\text{cntlAmm}-\text{N}}\right)\right]}\right)$$

Weightage for different parameters pH- 4, DM Loss- 3, Ammonia-N 3; where wtg is weightage, cntl is control silage, DML is dry matter loss and Amm-N is ammonia nitrogen content.

***Assessment of pH, yeast and moulds count after aerobic exposure*:** Aerobic stability was estimated by a change in pH level, yeast and mould count of silage after aerobic exposure of silage. Silage (approximately 2 kg) samples were placed in plastic buckets and kept at room temperature (25ºC). The silage was sampled during the aerobic exposure (0, 2, 4, 6 and 8 days) to evaluate the pH and the numbers of yeast and mould (Dolci *et al*., 2011). The counting of yeasts and moulds was done on a plate of potato dextrose agar acidified with lactic acid. The plates

were incubated at 37°C for counting of yeast and moulds at 48 and 96 h, respectively, based on morphology. The plates with colonies between 30 and 300 were counted to obtain the number of colony-forming units (CFU).

***Statistical design*:** Data of the fermentation parameters were analyzed by one-way analysis of variance (ANOVA) using SPSS (26.0) software. Using the general linear model, the aerobic exposure data were subjected to a two-way analysis of variance with the fixed effects of additives, ensilage period, and additives × ensilage period. For LAB, yeast and mould used $log_{10}$-transformed data. Pair-wise comparisons of the mean values were tested by Duncan's multiple range tests (Duncan, 1955) at the significance level (P<0.05).

## Results and Discussion

***Chemical composition of maize green fodder*:** The composition of the maize fodder before ensiling (Table 1) depicts that the dry matter (DM), CP, EE and TA content of maize before ensiling were 30.62, 9.46, 2.76 and 6.05%, respectively. The pH and water-soluble carbohydrate content of maize fodder were 6.05 and 12.73, respectively. The epiphytic LAB of maize fodder was 5.99 ($log_{10}$CFU/g) and the yeast & mould count was 3.20 ($log_{10}$ CFU/g).

The fresh maize fodder had the appropriate dry matter (30-35%) and water-soluble carbohydrate content (6-12 %) required for ensiling (Tyrolová *et al*., 2017). According to Johansson (2010), if DM is <30 % it increases the risk of bacterial and fungal spoilage. The chemical composition of maize fodder was similar to that in a previous study (Arriola *et al*., 2011). The pH and water-soluble carbohydrates were within the range reported for maize fodder (Tyrolova *et al*., 2017). The epiphytic LAB count (5.99 $log_{10}$CFU/gm) of maize fodder agreed with Contreras-Govea *et al*. (2013). In contrast to the present finding, Hafner *et al*. (2015) and Queiroz *et al*. (2013) reported a higher number of epiphytic microbes (lactic acid bacteria), *i.e*., 7.04 and yeast count 5.77$log_{10}$CFU/g. Addah *et al*. (2011) observed 8.57 $log_{10}$CFU/g *Lactobacillus* counts on corn silage. According to Lin *et al*. (1992), the number of epiphytic LABs on fresh plants is highly variable, ranging from less than 10 CFU/g to $10^4$ CFU/g and depends on crop species, climatic conditions, maturity stage and chopping process.

**Table 1.** Chemical composition (%DM), fibre fraction, microbial count and energy content of fresh maize fodder

| Items | Maize fodder before ensiling | †SE |
|---|---|---|
| Dry matter | 30.62 | 0.40 |
| Crude protein | 9.46 | 0.02 |
| Ether extract | 2.76 | 0.08 |
| Organic matter | 93.95 | 0.50 |
| Total Ash | 6.05 | 0.04 |
| pH | 6.05 | 0.01 |
| Water-soluble carbohydrate | 12.70 | 0.03 |
| Fermentation coefficient | 40.58 | 0.50 |
| Lactic acid bacteria (log10CFU/g) | 5.99 | 0.05 |
| Yeast and mould (log10CFU/g) | 3.20 | 0.04 |

†SE: Standard error: CFU: Colony forming unit

***Effect of additives on pH, lactic acid, acetic acid and their ratio in maize silage*:** The fermentation parameters of maize silage treated with the different additive combinations were evaluated after 30 days of ensiling (Table 2). The pH values significantly ($p$ <0.05) decreased and lactic acid concentration was increased in all inoculated silages compared to the control. The additives improved the fermentation characteristics, including pH and lactic acid content. The lowest lactic acid content was in control (6.23%), while the highest (7.91%) was detected in LP+LF+PA inoculated maize silage, suggesting that PA did not interfere with the action of LP and LF. The acetic acid concentration was uniformly ($p$ <0.05) higher in additive-treated silages than in untreated silage.

Acetic acid content was significantly higher in LP+LF (2.15%) and LP+LF+PA (2.15%), respectively, than control treatment. Silages treated with heterofermentative bacterial inoculant and with combinations had higher acetic acid concentration (LF, LP+LF, LP+LF+PA) and lower lactic acid to acetic acid ratio (LF, 3.16; LP+LF, 3.37; LF+PA, 3.40) compared to homofermentative and control groups. The inoculation of additives improved the fermentation characteristics, including pH and lactic acid content of maize silage. Brar *et al*. (2019) recorded pH values of different maize silage samples between 3.6 to 4.3, which were within the ideal limits. The findings of lower pH and increased lactic acid concentration in inoculated silages were in agreement with Nkosi *et al*. (2012), who studied the application of bacterial inoculants and cellulase enzyme on the fermentation quality of silage made from sorghum forage in laboratory jars. The findings from the above authors concluded that inoculation reduced pH and increased lactic acid content in inoculated silage when compared with the control. Similarly, Sucu and Filya (2006) reported higher lactic acid concentration and lower pH value in additive-treated corn silage, likewise Acosta *et al*. (2012) reported that inoculation of whole maize fodder at ensiling with commercial additive (a blend of homo and hetero-fermentative lactic acid bacteria) increased the fermentation quality with a significantly lower pH and increased concentration of lactic acid compared to untreated silage. According to

**Table 2.** Fermentation parameters of maize silage treated with the different additive combinations

| Treatments | pH | Lactic acid (%DM) | Acetic acid (%DM) | L: A |
|---|---|---|---|---|
| Control | $4.16^{c} \pm 0.02$ | $6.23^{a} \pm 0.27$ | $1.81^{a} \pm 0.05$ | $3.44^{ab} \pm 0.21$ |
| LP | $4.04^{ab} \pm 0.02$ | $7.13^{bc} \pm 0.29$ | $2.03^{bc} \pm 0.03$ | $3.52^{ab} \pm 0.47$ |
| LF | $4.09^{b} \pm 0.01$ | $6.66^{ab} \pm 0.19$ | $2.11^{cd} \pm 0.03$ | $3.16^{a} \pm 0.05$ |
| PA | $4.06^{ab} \pm 0.01$ | $6.88^{bc} \pm 0.19$ | $1.94^{b} \pm 0.01$ | $3.54^{b} \pm 0.11$ |
| LP+PA | $4.06^{ab} \pm 0.01$ | $7.18^{c} \pm 0.10$ | $1.99^{bc} \pm 0.05$ | $3.61^{b} \pm 0.07$ |
| LF+PA | $4.03^{a} \pm 0.01$ | $7.13^{bc} \pm 0.09$ | $2.10^{cd} \pm 0.02$ | $3.40^{ab} \pm 0.07$ |
| LP+LF | $4.06^{ab} \pm 0.02$ | $7.24^{c} \pm 0.11$ | $2.15^{d} \pm 0.01$ | $3.37^{ab} \pm 0.07$ |
| LP+LF+PA | $4.03^{a} \pm 0.01$ | $7.91^{d} \pm 0.11$ | $2.15^{d} \pm 0.06$ | $3.69^{b} \pm 0.15$ |
| SEM | 0.009 | 0.105 | 0.025 | 0.457 |
| *p-value* | 0.01 | 0.01 | 0.01 | 0.04 |

Mean values with different superscripts within a column differ significantly (P<0.05); L: A- the lactic acid to acetic acid ratio; LP: *Lactiplantibacillus plantarum*; LF: *Limosilactobacillus fermentum*; PA: Propionic acid; Other treatments are combinations of these.

many researchers, the bacterial inoculants stimulate lactic acid production, increase the speed of pH decrease and improve preservation of sugarcane top silage (Chauhan *et al*., 2021; Singh *et al*., 2022).

Higher acetic acid concentration results in LF-treated silage, supporting the unique mode of action of heterofermentative bacteria. According to earlier observations, heterofermentative LAB species produced a blend of lactic and acetic acids (Kleinshmit and Kung, 2006; Schmidt *et al*., 2009). The heterofermentative bacteria produce acetic acid in addition to lactic acid by fermenting soluble carbohydrates. This mode of action results in lower lactic acid concentrations, higher acetic acid and higher silage pH in LF-inoculated silage. This finding agrees with Muck and Kung (1997), who reported that there is a slightly higher ratio of lactic acid to acetic acid in silages treated with homolactic acid bacteria inoculants, particularly legume silages, because homolactic lactic acid bacteria mainly produce lactic acid. The silage's higher lactate-to-acetate ratio indicates the fermentation was more towards homofermentative (Chauhan *et al*., 2021; Chauhan *et al*., 2023; Singh *et al*., 2022).

Silages treated with heterofermentative bacterial inoculants had higher acetic acid concentration and lower lactic acid ratio to acetic acid relative to untreated silage. The present finding on a higher lactic acid to an acetic acid ratio in silage inoculated with homofermentative bacterial inoculant is supported by the work of Hashemzadeh-Cigari *et al*. (2014) when alfalfa silage was treated with a combination of homofermentative and propionate-producing bacterial inoculants. Similarly, Kleinschmit and Kung (2006) reported *L. buchneri*-treated silage had higher acetic acid concentrations and a lower lactic acid to acetic acid ratio than untreated silage. This might be due to the degradation of certain lactic acid to acetic acid with the production of 1, 2-propanediol and traces of ethanol by heterofermentative bacteria (Oude Elferink *et al*., 2001). Ideally, the lactic acid to acetic acid ratio should not be less than 3:1; a higher ratio is ideal (Kung and Shaver, 2001).

***Effect of additives on microbial count*:** The variations of the lactobacilli and yeast-moulds count in the maize silage were recorded (Fig. 1). The lactic acid bacteria count was significantly ($p$ <0.05) higher in additive-treated maize silage as compared to the control. Among the treatments, the lactic acid bacteria ($log_{10}$CFU/g) count was higher in LP+LF+PA (9.43) and LP+LF (9.47) inoculated silage. The yeast and mould counts ranged from 2.44 to 4.97 ($log_{10}$CFU/g), the lowest count was observed in LP+LF+PA (2.44 $log_{10}$CFU/g) and LF+PA (2.76 $log_{10}$CFU/g) treated silage, respectively. The yeast and mould count was significantly ($p$ <0.05) reduced in additive-treated silage compared to control silage and among the treatments, it was relatively low in silage treated with a combination of chemical and heterofermentative bacterial inoculants. Clostridia spore was not detected in silage samples. There was no significant difference in lactic acid bacterial count in silage treated with PA and bacterial inoculants exclusively and combined with PA. This suggests that PA did not adversely affect the *lactobacillus* counts of silage.

The lactic acid bacteria count was significantly higher in additive-treated maize silage than the control. This finding agrees with the findings of Cai *et al*. (1999) and Kumari *et al*. (2023) who concluded that the inoculation of silage with homofermentative LAB had beneficial effects in promoting LAB growth. Among the treatments, the lactic acid bacteria ($log_{10}$ CFU/g) count was higher in LP+LF (9.47) and LP+LF+PA (9.43) inoculated silage. This might be due to the synergistic effect of bacterial inoculants. The PA at the level of 0.1% did not interfere with the growth of both lactic acid bacteria groups. The LF inoculants in the present study decreased yeast

**Table 3.** Quality parameters of maize silage treated with varying additive combinations

| Treatments | Modified fitness value | Flieg point | DM recovery (%) |
|---|---|---|---|
| Control | $0.0909^{a} \pm 0.0004$ | $89.63^{a} \pm 1.58$ | $87.05^{a} \pm 0.12$ |
| LP | $0.1039^{cd} \pm 0.0007$ | $96.47^{b} \pm 1.53$ | $89.95^{bc} \pm 0.21$ |
| LF | $0.0989^{b} \pm 0.0014$ | $93.86^{ab} \pm 1.31$ | $87.95^{ab} \pm 0.58$ |
| PA | $0.1038^{cd} \pm 0.0009$ | $95.26^{b} \pm 0.45$ | $90.04^{bc} \pm 0.38$ |
| LP+PA | $0.1050^{cd} \pm 0.0006$ | $94.29^{b} \pm 1.25$ | $90.45^{d} \pm 0.26$ |
| LF+PA | $0.1011^{bc} \pm 0.0009$ | $96.43^{b} \pm 0.83$ | $88.33^{abc} \pm 0.39$ |
| LP+LF | $0.1030^{bcd} \pm 0.0020$ | $95.76^{b} \pm 2.20$ | $88.46^{abc} \pm 1.31$ |
| LP+LF+PA | $0.1069^{d} \pm 0.0021$ | $97.16^{b} \pm 1.76$ | $89.60^{bc} \pm 1.01$ |
| SEM | 0.001 | 0.635 | 0.304 |
| *p-value* | 0.00 | 0.05 | 0.02 |

Values with different superscripts within a column differ significantly ($p$ <0.05)

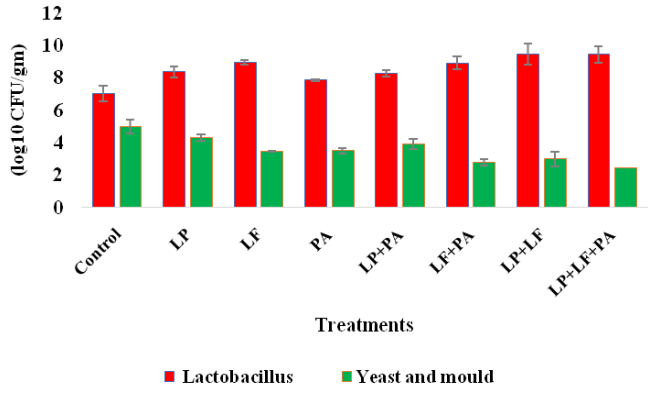


**Fig 1.** Microbial count of maize silage treated with various additive combinations

growth more effectively than LP due to the higher acetate production. Acetic acid inhibits yeasts that are responsible for aerobic spoilage might be due to the antifungal property of acetic acid. However, Danner *et al*. (2003) also identified that acetate has antimicrobial properties against undesirable microbes. The chemical additive reduces yeast population may be due to altering membrane functions (Stratford *et al*., 2013) or cytosolic acidification (Stratford *et al*., 2020).

***Quality parameters of maize silage treated with different additive combinations*:** The modified fitness value, flieg point and DM recovery of the maize silage were recorded (Table 3). In comparison to the control, modified fitness value, flieg point and DMR were significantly ($p$ <0.05) higher in all additive-treated silages compared to the control. Modified fitness value was highest in LP+LF+PA (0.1069), whereas lowest in the control (0.0909). Modified fitness value depends on the silage's pH, dry matter loss and ammonia nitrogen content. The pH point of all silages was significantly higher (P<0.05) than the control, but there was no significant variation among the treatments. The flieg point depends on pH and dry matter content of the silage. The percent dry matter recovery of maize silage ranges from 87.05 (Control) to 90.45 (LP+PA). The additives used in this study effectively increased the dry matter recovery. Dry matter recovery was significantly ($p$ <0.05) higher in silage treated with homofermentative bacterial (89.95%) inoculants as compared to heterofermentative bacterial inoculants (87.95%) treated silage. Among the treatments, homofermentative and chemical additive and their combinations (LP, PA, LP+LF+PA and LP+PA) treated silage had higher dry matter recovery than heterofermentative bacterial inoculated silage. PA did not alter the LP and LF effect on DMR.

The modified fitness value is based on pH, ammonia nitrogen concentration (% of total nitrogen) and dry matter loss. The modified fitness value is indicative of the efficacy of silage additives. Kilic (1986) developed the quick method (flieg points) for quality evaluation using dry matter and pH. According to this, silage was considered very high quality when it scored between 81 and 100. Based on the flieg point evaluation, all the silages were classified as very good quality silage. Treatment with inoculants containing LF resulted in lower DMR. The probable reason might be the more extensive heterolactic fermentation and $CO_2$ production. The conversion of lactic acid and carbohydrates to acetic and propionic acids by heterolactic bacteria causes DML and is characterized by $CO_2$ production (Filya, 2003). Similarly, Arriola *et al*. (2021) and Li *et al*. (2021) reported that treatment with inoculants containing *L. buchneri* resulted in lower DMR than untreated silage and Kim *et al*. (2015) concluded that dry matter loss was higher in silage treated with a heterofermentative bacterial inoculant. Muck *et al*. (2018) reported that the dry matter loss was similar or greater in silage treated with *L. buchneri* than in the control treatment. Typically, these losses fall between 2 and 4% (Zimmer, 1981). The predominant bacterial species and the fermentable substrates influence fermentation-related DM loss. The LAB that ferments glucose with a homofermentative fermentation pathway produces mostly lactate and no DM loss occurs, while the LAB that ferments glucose by a heterofermentative fermentation pathway produces 1 mole of carbon dioxide per mole of glucose, resulting in a loss of 2 to 4% (Zimmer, 1981). Better DMR from a chemical additive in high DM silage was observed previously (Silva *et al*., 2016), and this is probably because yeasts' fermentative activity was suppressed (McDonald *et al*., 1991).

***Changes in pH and microbial count after aerobic exposure of maize silage*:** The pH values on different aerobic exposure days (Day 0, 2nd, 4th, 6th and 8th) of additive-treated maize silage were recorded (Table 4). All the silages had low pH values at the zero-day of aerobic exposure and after that pH value increased as the aerobic exposure days increased ($p$ <0.05). The pH was significantly ($p$ <0.05) lower in silages treated with heterofermentative bacterial inoculants than homofermentative bacterial inoculants on 4th, 6th, 8th day of aerobic exposure. The lowest mean value of pH (4.59) at different aerobic exposure days was observed in silage treated with LF+PA followed by LP+LF+PA inoculated (4.72) and LF treated (4.90) silage.

The yeast count of additive-treated maize silage during aerobic exposure was recorded (Fig. 2). Propionic acid and LF-inoculated silage exhibited the lowest yeast counts while simultaneously exhibiting the highest aerobic stability. PA and LF improved aerobic stability over untreated and LP-inoculated silage ($p$ <0.05). The yeast growth was higher (P<0.05) in LP, followed by control, LF and PA inoculated silage.

The mould count ($log_{10}$CFU/g) of additive-treated maize silage on different aerobic exposure days was recorded (Fig. 3). The lowest mould count was observed in LF+PA (4.74 $log_{10}$CFU/g) treatment. Silage deterioration indicators were pH change and an increase in yeast and mould numbers.

A lower pH value after aerobic exposure to silage indicates that the more aerobic stability or growth of silage spoilage bacteria is less. Silage treated with a combination of heterofermentative bacterial inoculants, chemical additive (LF+PA) followed by chemical additive (PA) and heterofermentative bacterial inoculants silage had lower mean pH values on different aerobic exposure. As the aerobic exposure time increases, the pH of the silage increases; this might be because of many yeasts' species' growth, which degrades the lactic acid into $CO_2$ and $H_2O$ under aerobic conditions and the degradation of lactic acid causes a rise in silage pH,

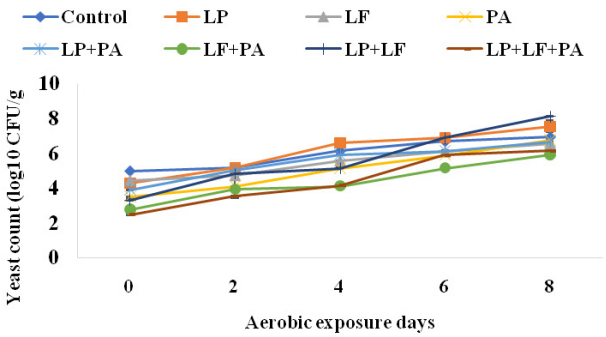


**Fig 2.** Effect of additives and aerobic exposure days on yeast count (log10 CFU/g) of maize silage

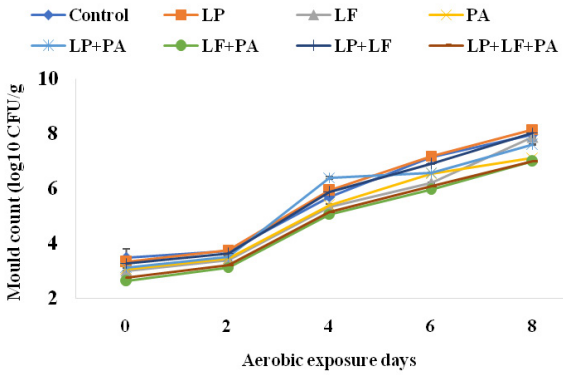


**Fig 3.** Effect of additives and aerobic exposure days on mould count (log10 CFU/g) of maize silage

**Table 4.** Effect of additives and combinations on pH of maize silage on various aerobic exposure days

| Treatment | Aerobic exposure days | | | | | Mean | SEM | Significance | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | | | P | T | P×T |
| Control | $4.16^{dA}$ | $4.49^{bcB}$ | $5.53^{fC}$ | $5.71^{dD}$ | $6.17^{dE}$ | $5.21^{F}$ | 0.208 | 0.01 | 0.01 | 0.01* |
| LP | $4.04^{cA}$ | $4.59^{cB}$ | $5.63^{fC}$ | $5.84^{efC}$ | $6.31^{eD}$ | $5.28^{G}$ | | | | |
| LF | $4.09^{cdA}$ | $4.39^{bcB}$ | $5.02^{cC}$ | $5.26^{bD}$ | $5.74^{bE}$ | $4.90^{C}$ | | | | |
| PA | $4.06^{cA}$ | $4.31^{bB}$ | $5.42^{eC}$ | $5.58^{cD}$ | $5.76^{bE}$ | $5.02^{D}$ | | | | |
| LP+PA | $4.06^{cA}$ | $4.35^{bB}$ | $5.55^{fC}$ | $5.75^{deD}$ | $5.97^{cE}$ | $5.14^{E}$ | | | | |
| LF+PA | $4.03^{cA}$ | $4.27^{bB}$ | $4.58^{bC}$ | $4.89^{aD}$ | $5.17^{aE}$ | $4.59^{A}$ | | | | |
| LP+LF | $4.06^{bA}$ | $4.43^{dB}$ | $5.15^{dC}$ | $5.43^{gD}$ | $6.00^{fE}$ | $5.01^{D}$ | | | | |
| LP+LF+PA | $4.02^{aA}$ | $4.31^{aB}$ | $4.69^{aC}$ | $5.03^{fD}$ | $5.58^{dE}$ | $4.72^{B}$ | | | | |
| Mean | $4.07^{a}$ | $4.39^{b}$ | $5.20^{c}$ | $5.44^{d}$ | $5.84^{e}$ | | | | | |

P: Aerobic exposure period; T: Treatment; P×T: Interaction of period and treatment; *$p$ <0.05 significant; SEM: Standard error of the means; $^{a-e}$ Values with distinct small letters indicate statistically significant variations between treatment in the same aerobic exposure days ($p$ <0.05); $^{A-G}$ Significant variations aerobic exposure days in the same treatment are shown by values with distinct capital letters ($p$ <0.05)

which in turn triggers the growth of many other spoilage organisms (McDonald *et al*., 1991). Silage treated with homofermentative bacterial inoculants was less aerobic stable than control and heterofermentative bacteria-treated silage. These findings agree with Filya and Succu (2007). This might be due to homofermentative bacteria inoculated silage containing more lactic acid and less acetic acid than heterofermentative bacterial inoculants treated silage. Yeast and mould used lactic acid as a substrate and converted it into $CO_2$ and other products, subsequently rapidly increasing the silage pH.

The yeast count was lower in LF+PA-treated silage because of the organic acids (acetic acid and propionic acid) that inhibit yeast growth. Chemical additives improved aerobic stability over that of untreated and LP-inoculated silage. Silage treated with LPhad has lower aerobic stability because of more lactic acid production or a lower lactic acid to acetic acid ratio. Lactic acid fermentation is usually associated with reduced formation of acetic acid; this finding agrees with Wilkinson and Davies (2013). Yeasts are commonly the initiators of aerobic deterioration (Pahlow *et al*., 2003); decreased aerobic stability of silages inoculated with LP may be explained by greater yeast numbers. Inoculation with LP may also result in lower levels of acetic acid production, which might hasten yeast development and diminish aerobic stability. According to Carvalho *et al*. (2015), lactate-assimilating yeasts *Candida spp., Hansenula spp, Pichia spp, Issatchenkia spp* and *Saccharomyces spp* are mainly responsible for the aerobic deterioration of silage. The stability of silage after aerobic exposure can be zero if the yeast count exceeds more than>6 log10 CFU/g of silage (Chauhan *et al*., 2023). The administration of heterofermentative LAB during ensiling enhanced the acetate concentration and in turn, the aerobic stability of silage increased (Danner *et al*., 2003; Filya & Sucu, 2007). Like a prior study, a high acetate content in LF silages led to a lower yeast count than LP silages (Ranjit and Kung, 2000).

Heterofermentative bacterial inoculants were more effective in reducing mould growth than homofermentative bacterial inoculants due to acetic acid production. *Lactobacillus buchneri* was effective in improving aerobic stability in various crops, including corn silage, high-moisture corn and alfalfa (Kung *et al*., 2003). Similarly, treatment with chemical additives improved legumes, grasses, corn silage and high-moisture corn's aerobic stability (Knicky and Spörndly, 2015; Kung *et al*., 2018).

Improved aerobic stability in LF inoculated silage due to more acetic acid production by *Limosilactobacillus fermentum*, whereas improved stability from PA was from the propionic acid availability in silage. Short-chain organic acids such as acetic acid and PA can be fungistatic and or fungicidal because they lower the pH of yeast cells (cytosolic acidification), inhibiting glycolysis and decreasing the concentration of ATP (Krebs *et al*., 1983). Chemical additive (sorbates and benzoates) directly alters membrane functions (Stratford *et al*., 2013) in some yeasts, in addition to cytosolic acidification (Stratford *et al*., 2020) The increased rate of deterioration when silages were treated with the only homolactic acid type of LAB is because this type of fermentation reduces the accumulation of compounds (e.g., acetic acid) with antifungal properties (Muck *et al*., 2018). Undissociated acetic acid, along with other short-chain fatty acids, is also known to suppress the growth of yeasts and moulds, but lactic acid is mainly ineffective against these initiators of the aerobic degradation process (Danner *et al*., 2003; Wilkinson and Davies, 2013). Propionic acid is effective in reducing yeast and moulds, which are responsible for the aerobic deterioration in silages. Antimycotic action of PA increases as pH drops, making it a prime choice for boosting corn silage's aerobic stability in low-pH environments (Beck, 1975). According to Chauhan *et al*. (2023), if mould count reaches up to 7 log10CFU/g of silage, then the nutritional quality of the silage is reducedand it can be considered as aerobically deteriorated.

***Cluster analysis of maize silage treated with various additives*:** To facilitate the categorization and understanding of maize silage with different treatments, a hierarchical clustering analysis was conducted. This analysis aimed to identify similarities and differences among various parameters and group them accordingly. A heat map was generated using a Euclidean distance matrix of quantitative values (Fig. 4). Each cell represents a specific parameter value for a particular silage sample in the heat map. The intensity of colour in each cell indicates the concentration level of that parameter in the sample. Higher colour intensity suggests a greater concentration of a specific parameter. The pattern observed in the heat maps aligned with the findings previously discussed in this study, reinforcing the consistency of the results. The heat map clearly predicts a strong positive correlation of LP+LF+PA along with Fleig point and positive correlation with LA:AA, Acetic acid and LAB, while it is negatively correlated with pH of silage.

Principal Component Analysis (PCA) was applied to analyse the dynamic variance of the microbial population and fermentation quality under different conditions. The results (Fig 5) indicate that the first two components account for 68.55 and 22.29% of the total variance, respectively. In the PCA plot, the x-axis represents the results of the first component, while the y-axis represents the second component. The overview plot displays the median intensities of each cluster, with darker lines indicating higher intensities. The control treatment of silage forms a distinct group that is separate from the other treatments. This indicates that the control samples have significantly different values compared to the other

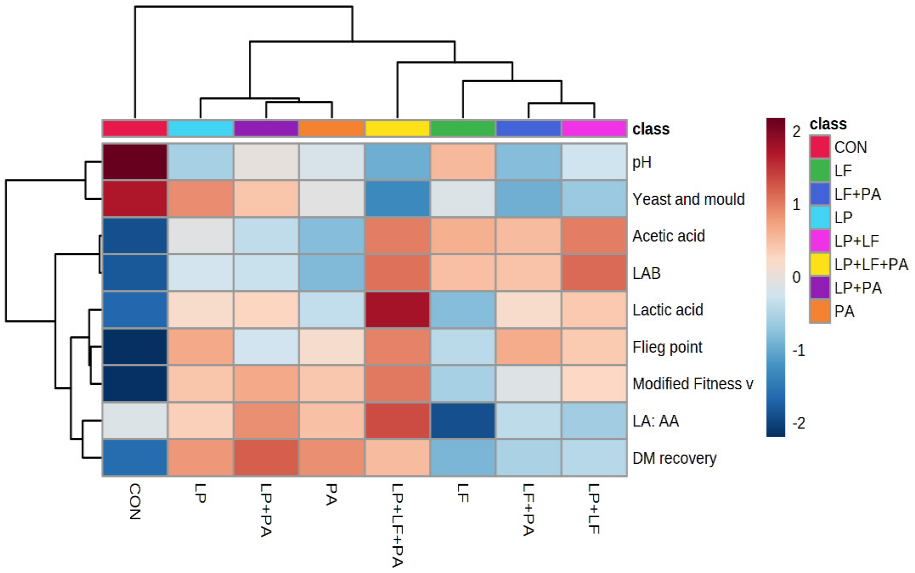


**Fig 4.** Heat map of hierarchal clustering analysis of quality parameters of maize silage for different treatments [Coloured cells correspond to concentration value (the cell's increased colour intensity indicates a larger concentration of particular parameters value in the silage sample); Samples in column and quality parameters in row]

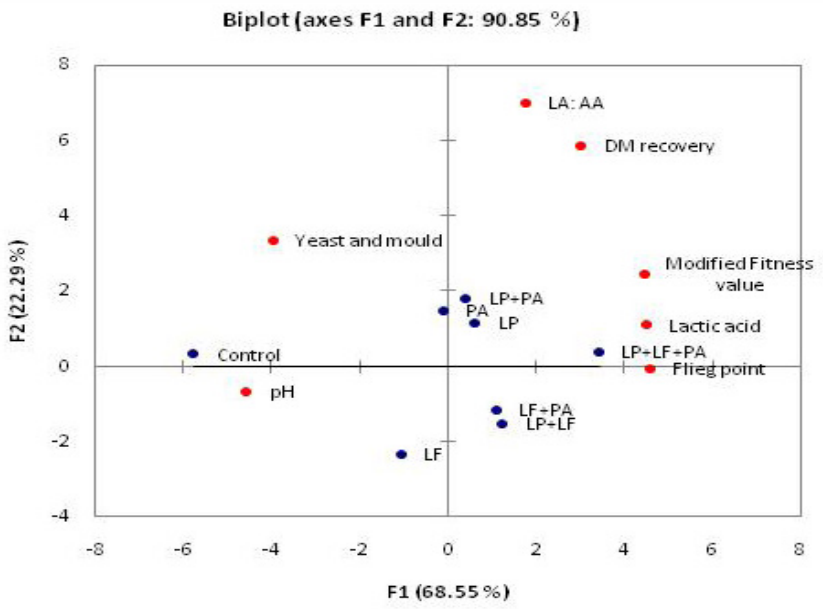


**Fig 5.** Principal component analysis (PCA) plot of different variables of additive-treated maize silage

seven treatments. These findings align well with the results of the present study, which include chemical and quality profiling of the silage samples.

The observations further highlight that the other treatments are relatively distant from each other, indicating greater variability in the considered characteristics between the control and the remaining treatments. LP+LF+PA stands out with higher scores along with F1. This suggests that the LP+LF+PA combination shows promising results in improving the fermentation quality of maize silage. Based on our study, LP+LF+PA emerges as the most favourable additive combination for enhancing the fermentation quality of maize silage.

## Conclusion

The additives effectively improved the maize silage quality parameters, quality attributes after aerobic exposure of silage. Lactic acid bacterial inoculants stayed effective in the presence of propionic acid. Among the treatments, the combination of LP+LF+PA was effective

in improving the fermentation quality of silage, while the combination of LF+PA has shown more potential to reduce the yeast and moulds after aerobic exposure.

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