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Effects of fibrolytic enzymes on grass silages, inoculation and storage conditions on whole-plant corn silage, and a meta-analysis of storage temperature in silages

Gabriel Ferreira de Lima Cruz
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GABRIEL FERREIRA DE LIMA CRUZ

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Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Karina Guimaraes Ribeiro

Co-adviser: Odilon Gomes Pereira

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To my grandfather José Alves de Lima (In memoriam),
for being an example of perseverance, integrity, and love. With these qualities, he inspired everyone, including me, to become upstanding citizens.

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“Wherever you are, you must always show up with excellence [… my Galician singer]”

(José Alves de Lima)

ABSTRACT

CRUZ, Gabriel Ferreira de Lima, D.Sc., Universidade Federal de Viçosa, November, 2024. **Effects of fibrolytic enzymes on grass silages, inoculation and storage conditions on whole-plant corn silage, and a meta-analysis of storage temperature in silages**. Adviser: Karina Guimaraes Ribeiro. Co-adviser: Odilon Gomes Pereira.

This study was mainly divided into three chapters: Chapter 1 – Two experiments were carried out to evaluate whether the addition of cellulase and/or xylanase enzymes interacts with regrowth age (RA) in mombasa grass and elephant grass cv. BRS Capiaçu to alter nutritive, fermentation profile and ruminal degradation kinetics. Mombasa and elephant grass plots were mowed and harvested at two different RA (7- and 11-wk for mombasa grass; 12- and 16-wk for elephant grass) and submitted to the following additive treatments: no additive (control), xylanase enzyme, cellulase enzyme, and cellulase + xylanase enzymes, in a factorial arrangement of 4 (additives) × 2 (regrowth ages), with three replicates. In both trials, lower degradability was observed for late harvest. For mombasa grass silage, lower DM losses and more extensive fermentation were observed for early harvest, while the opposite was observed for elephant grass silage. Cellulase improves the fermentation profile of Mombasa grass silage, while xylanase enhances the dry matter and neutral detergent fiber degradability of elephant grass silage. The combined application of these enzymes does not appear to warrant recommendation. Chapter 2 – This study evaluated the effects of a heterofermentative inoculant, storage temperature and storage length on fermentation profile, aerobic stability, and nutrient composition of whole-plant corn silage. The experiment was a completely randomized design with a 2 (microbial inoculation) × 2 (storage temperature) × 4 (storage length) factorial arrangement of treatments. Corn forage was either inoculated with distilled water (CON) or with 300,000 CFU/g of fresh forage of Pediococcus acidilactici DSM 16243, Lentilactobacillus buchneri DSM 12856, and L. diolivorans DSM 32074 (LBLD). Silos were stored for 7, 15, 30 or 90 d at either 22 (NT) or 40? (HT). Soluble crude protein and ammonia-N concentrations, and in vitro starch digestibility increased with SL and were greater for silage stored at HT. Inoculation increased pH and LAB counts. Three-way interactions were observed for acetic acid and 1-propanol concentrations, and aerobic stability, which LBLD-22 was greater than other treatments at 90 d. Greater temperature negatively impacted fermentation profile and increased spoilage after aerobic exposure, whereas the heterofermentative inoculant enhanced aerobic stability. However, improvements in fermentation profile and aerobic stability with

microbial inoculation were alleviated when silage was stored at greater temperatures. Chapter 3 – A meta-analysis of 42 peer-reviewed articles was conducted to examine the effects of temperature ranges on nutritive and fermentation profile of silage. A complementary meta-analysis of 15 articles examined the effects on whole-plant corn silage (WPCS) specifically. The effects were evaluated by ranges of temperature and the treatments were classified into the following categories: 1) = 10°C; 2) > 10 and = 20°C; 3) > 20 and = 30 °C; 4) > 30 and = 40°C; 5) > 40°C. Overall, The environmental temperature during fermentation affected the activity of LAB, and both high and low temperatures reduced acid production. Therefore, the temperature range of 21-30°C may be more suitable for silage storage and fermentation due to greater total acid production and more rapid decline in pH, which enhances nutrient preservation.

Keywords: degradability; fermentation profile; nutritive value

RESUMO

CRUZ, Gabriel Ferreira de Lima, D.Sc., Universidade Federal de Viçosa, novembro de 2024. **Efeitos de enzimas fibrolíticas em silagens de gramíneas, condições de inoculação e armazenamento em silagem de milho de planta inteira e uma meta-análise da temperatura de armazenamento em silagens**. Orientadora: Karina Guimaraes Ribeiro. Coorientador: Odilon Gomes Pereira.

Esse estudo foi dividido principalmente em três Capítulos: Capítulo 1 – Dois experimentos foram realizados para avaliar se a adição de enzimas celulase e/ou xilanase interage com a idade de rebrota para alterar o perfil nutritivo, fermentativo e a cinética de degradação ruminal de capim-mombaça e capim-elefante cv. BRS Capiaçu. Parcelas de capim-mombaça e capim-elefante foram cortadas e colhidas em duas idades diferentes (7 e 11 semanas para capim-mombaça; 12 e 16 semanas para capim-elefante) e submetidas aos seguintes tratamentos aditivos: sem aditivo (controle), enzima xilanase, enzima celulase e enzimas celulase + xilanase, em um arranjo fatorial de 4 (aditivos) × 2 (idades de rebrota), com três repetições. Em ambos os ensaios, foi observada menor degradabilidade para a colheita tardia. Para silagens de capim-mombaça, foram observadas menores perdas de MS e fermentação mais extensa para a colheita precoce, enquanto o oposto foi observado para silagens de capim-elefante. A celulase melhora o perfil de fermentação da silagem de capim-mombaça, enquanto a xilanase melhora a degradabilidade da matéria seca e da fibra em detergente neutro da silagem de capim-elefante. A aplicação combinada dessas enzimas não parece justificar recomendação. Capítulo 2 – Este estudo avaliou os efeitos de um inoculante heterofermentativo, temperatura de armazenamento e tempo de armazenamento no perfil de fermentação, estabilidade aeróbia e composição de nutrientes da silagem de planta inteira de milho. O experimento foi um delineamento inteiramente casualizado com um arranjo fatorial de tratamentos 2 (inoculação microbiana) × 2 (temperatura de armazenamento) × 4 (duração de armazenamento). A forragem de milho foi inoculada com água destilada (CON) ou com 300.000 UFC/g de forragem fresca de Pediococcus acidilactici DSM 16243, Lentilactobacillus buchneri DSM 12856 e L. diolivorans DSM 32074 (LBLD). Os silos foram armazenados por 7, 15, 30 ou 90 dias a 22 (NT) ou 40? (HT). As concentrações de proteína bruta solúvel e amônia-N e a digestibilidade in vitro do amido aumentaram com o tempo de armazenamento e foram maiores para a silagem armazenada em HT. A inoculação aumentou as contagens de pH e LAB. Interações triplas foram observadas para concentrações de ácido acético e 1-propanol e

estabilidade aeróbia, que LBLD-22 foi maior do que outros tratamentos em 90 dias. A alta temperatura impactou negativamente o perfil de fermentação e aumentou a deterioração após exposição aeróbia, enquanto o inoculante heterofermentativo aumentou a estabilidade aeróbia. No entanto, as melhorias no perfil de fermentação e na estabilidade aeróbia com inoculação microbiana foram aliviadas quando a silagem foi armazenada em altas temperaturas. Capítulo 3 – Uma meta-análise de 42 artigos revisados por pares foi conduzida para examinar os efeitos das faixas de temperatura no perfil nutritivo e de fermentação das silagens. Uma meta-análise complementar de 15 artigos examinou os efeitos na silagem de milho de planta inteira (WPCS) especificamente. Os efeitos foram avaliados por faixas de temperatura e os tratamentos foram classificados nas seguintes categorias: 1) = 10 °C; 2) > 10 e = 20 °C; 3) > 20 e = 30 °C; 4) > 30 e = 40 °C; 5) > 40 °C. No geral, a temperatura ambiente durante a fermentação afetou a atividade do LAB, e tanto as temperaturas altas quanto as baixas reduziram a produção de ácido. Portanto, a faixa de temperatura de 21-30 °C pode ser mais adequada para armazenamento e fermentação de silagem devido à maior produção total de ácido e declínio mais rápido no pH, o que melhora a preservação de nutrientes.

Palavras-chave: degradabilidade; perfil de fermentação; valor nutritivo.

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GENERAL INTRODUCTION

Under normal aerobic conditions, harvested crops are prone to spoilage, reducing the safety and nutritional value of grains and forage. Silage making is a preservation process where anaerobic bacteria ferment plant sugars into organic acids, and acidification is crucial for nutrient retention and preventing spoilage microorganisms (Pahlow et al., 2003). Initially developed for regions with seasonal forage production, silage is now an integral part of ruminant diets worldwide.

Given its global spread, various forage species are studied and used for silage production. For high-quality silage, forage should ideally contain appropriate levels of dry matter (DM), water-soluble carbohydrates (WSC), and buffering capacity (BC) (Moraes et al., 2023). Forage crops should also provide high yields per area and maintain strong nutritive value, especially at the optimal harvest stage for ensiling. This is often achieved with corn but can be more challenging with tropical grasses. Recognizing the differences between whole-plant corn silage and tropical grasses is essential for understanding fermentation dynamics and identifying strategies to enhance fermentation quality.

Corn is the primary silage crop in Brazilian dairy cattle farms (Daniel et al., 2019) and worldwide, containing substantial concentration of WSC and adequate DM range, combined with low buffering, which enables rapid pH reduction during initial fermentation phase (Ferrero et al., 2018). However, WPCS has low production of antifungal acids, increasing its susceptibility to aerobic degradation during the feedout phase (Borreani et al., 2018).

Tropical grass silages are also widely used, including Mombasa [*Megathyrsus maximus* (Jacq.) B.K. Simon & S.W.L. Jacobs (syn. *Panicum maximum* Jacq.)] and Elephant grass [*Cenchrus purpureus* (Schumach.) Morrone (syn. *Pennisetum purpureum* Schumach.)]. Although these tropical grasses are highly productive, they typically have high moisture and fiber contents with low sugar levels (Daniel et al., 2019; Gomes et al., 2021), making it challenging to achieve efficient fermentation (Moraes et al., 2023).

Moisture content is a primary factor in silage fermentation. High-moisture forages require elevated WSC concentration to reach optimal fermentation due to acid dilution by water content (Carvalho et al., 2024). Hence, producing tropical grass silage with high nutritive value requires strategies that promote desirable fermentation and inhibit the proliferation of undesirable bacteria (e.g., enterobacteria, clostridia, and others) (McDonald et al., 1991).

Dry matter content is generally driven by plant maturity. Therefore, harvesting at the optimal maturity stage is essential for effective fermentation. Unlike WPCS, achieving both

adequate DM content and optimal nutritive value is challenging in tropical grasses (Daniel et al., 2019), requiring strategies to adjust DM concentration or enhance the fermentability of the ensiled material.

The use of additives has traditionally aimed to reduce energy and DM losses by inhibiting undesirable fermentations, improving aerobic stability, and enhancing nutrient digestibility (Muck et al., 2018). Among these additives, exogenous fibrolytic enzymes have become a significant research focus. These enzymes target cell walls, breaking down hemicellulose and cellulose into simple sugars like glucose and certain pentoses (Khota et al., 2018). This increased substrate availability for lactic acid fermentation can promote a more rapid pH drop (Khota et al., 2016). Enhanced fiber solubility may also improve silage DM content and fiber degradability (Li et al., 2014; Khota et al., 2018). Nevertheless, results in the literature remain inconsistent (Del Valle et al., 2018).

Xylanase mainly targets hemicellulose, releasing xylooligosaccharides that are specifically utilized by obligate heterofermentative lactic acid bacteria (LAB) (Del Valle et al., 2018; Elghandour et al., 2016). These LAB ferment pentoses through the phosphoketolase pathway, producing both acetic and lactic acids as end products (Heinl & Grabher, 2017). Lactic acid tends to lower pH due to its strength—approximately 10 times stronger (pKa 3.8) than short-chain fatty acids like acetic acid (pKa 4.76; Kung et al., 2018). Nonetheless, acetic acid plays a vital role in silage prone to aerobic deterioration, such as high-soluble carbohydrate silage like BRS Capiaçu silage and WPCS, as it inhibits yeast growth, the primary microorganism involved in aerobic spoilage (Kleinschmit & Kung, 2006; Pahlow et al., 2003).

Cellulase, on the other hand, acts on the cellulosic fraction, releasing cello-oligosaccharides (e.g., cellobiose and cellotriose) and glucose (Zhang et al., 2019). In this case, in heterofermentative LAB, the 6-phosphogluconate/phosphoketolase pathway is inhibited by the glycolytic pathway, preventing the WSC released during enzymatic hydrolysis from being fermented through the heterolactic pathway (Rooke & Hatfield, 2003). As a result, there is an increase in lactic acid production, leading to a rapid pH drop and suppression of undesirable microorganisms. Based on these findings, the use of cellulases could be particularly beneficial in forages with low WSC content and slow pH reduction, such as Mombasa grass.

As a concluding remark, few studies on enzyme-treated silage have focused on tropical grasses. Understanding how fibrolytic enzymes can influence the fermentation of tropical grass silage at different maturity stages is a key area of research to improve silage production, particularly in Brazil and other tropical countries. This is especially important given the

considerable variation in responses and the limited number of studies examining the interaction of these factors.

Microbial inoculants offer numerous benefits in fermentation, making them the most common additives used in silage production (Muck, 2010). Many commercial inoculants used today would be classified as combination inoculants, in that they contain both homo- and heterofermentative LAB to achieve the benefits of both types of inoculants in a single product (Muck et al., 2018). In a combination inoculant, the homofermentative LAB would control the initial stages of fermentation through rapid acidification, while the heterofermentative LAB would later convert some of the lactic to acetic acid. *Lentilactobacillus buchneri*, the most used heterofermentative LAB in silage inoculants, enhances aerobic stability by partially converting lactic into acetic acid and 1,2-propanediol (Oude Elferink et al., 2001). In addition to acetic acid, propionic acid is recognized for its strong antifungal properties (Moon, 1983). *Lentilactobacillus diolivorans*, another obligate heterofermentative LAB, utilizes 1,2-propanediol as a carbon source for growth, producing propionic acid and 1-propanol as the primary fermentation products (Krooneman et al., 2002). *L. diolivorans* has also been reported to produce acetic acid (Schein et al., 2018). However, research on the ability of the combination of those species to inhibit yeast and mold growth and enhance the aerobic stability of WPCS is currently limited.

Generally, heterofermentative species require a longer storage period to effectively modulate silage fermentation. While recent studies have shown that acetic acid production, yeast suppression, and improvements in aerobic stability can begin as early as 7–15 d of storage (Fernandes et al., 2020; Saylor et al., 2020; Arriola et al., 2021), these effects are typically more pronounced after extended fermentation of 30 to 60 d or longer (Muck et al., 2018; Ferrero et al., 2021; Arriola et al., 2021).

Storage temperatures can also influence fermentation patterns of silage and the efficacy of microbial inoculants (Bernardes et al., 2018; Bai et al., 2022). Low temperatures reduce microbial activity in the silo, leading to slower fermentation (Ferrero et al., 2021). Nevertheless, high temperatures increase the risk of aerobic deterioration and can also reduce LAB activity, leading to greater DM losses (Borreani et al., 2018), even though many strains used in silage inoculants are selected for their performance at temperatures typical of warmer climates (Weinberg and Muck, 1996).

While controlling environmental temperatures during silage fermentation is not feasible, understanding the potential negative effects of high temperatures is essential. Additionally, evaluating how commercial inoculants perform at different storage temperatures

and storage lengths is crucial for both the scientific community and producers. Furthermore, there is a lack of meta-analyses in the literature summarizing the main effects of temperature on the fermentation and chemical composition of silage, not only for WPCS but also for other crops used worldwide.

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CHAPTER I

Effect of fibrolytic enzymes and regrowth ages on fermentation profile, nutrient composition, and ruminal degradation kinetics of Mombasa grass and elephant grass silages[1]

Fibrolytic Enzymes Affect Grass Silage Harvested at Different Regrowth Ages

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Abbreviations: AA, acetic acid; ADF, acid detergent fiber; ADIN, acid detergent insoluble nitrogen; AS, aerobic stability; BA, butyric acid; BC, buffering capacity; CEL, cellulase application; CON, control; CP, crude protein; DM, dry matter; DMD, dry matter digestibility; DML, dry matter losses; EFE, exogenous fibrolytic enzyme; ENZ,

exogenous fibrolytic enzymes application; Fraction a, ruminal rapidly degradable fraction; Fraction b, dry matter ruminal slowly degradable fraction; Fraction B, neutral detergent fiber ruminal slowly degradable fraction; Fraction I, undegradable fraction; FC, fermentability coefficient; kd, degradation rate constant of fraction "b"; LA, lactic acid; LA:AA, lactic-to-acetic acid ratio; LAB, lactic acid bacteria; MIX, cellulase plus xylanase application; NDF, neutral detergent fiber; NDFap, neutral detergent fiber corrected for ash and protein; NDFD, neutral detergent fiber digestibility; NH_3-N, ammonia nitrogen; PA, propionic acid; RA, regrowth age; WSC, water soluble-carbohydrates; XYL, xylanase application; λ, joint fractional rate of latency and degradation.

ABSTRACT

Two experiments were carried out to evaluate whether the addition of cellulase and/or xylanase enzymes interacts with regrowth age (RA) in mombasa grass (*Megathyrsus maximus*) and elephant grass cv. BRS Capiaçu (*Cenchrus purpureus*) to alter nutritive, fermentation profile and ruminal degradation kinetics. Mombasa and elephant grass plots were mowed and harvested at two different RA (7- and 11-wk for mombasa grass; 12- and 16-wk for elephant grass). After forages were chopped, they received one of the following additive treatments: no additive (control), xylanase enzyme (300 mg/kg DM; XYL), cellulase enzyme (1 g/kg fresh matter; CEL), and cellulase + xylanase enzymes (same dosages as before; MIX), in a factorial arrangement of 4 (additives) × 2 (regrowth ages), with three replicates. Greater lactic acid and lower acetic acid, butyric acid and NH_3-N concentrations were observed for CEL in mombasa grass silage. Nevertheless, CEL decreased aerobic stability in elephant grass silage and increased effluent production in both species. Xylanase increased DM and NDF degradability, especially in elephant grass silage. In both trials, lower degradability was observed for late harvest. For mombasa grass silage, lower DM losses and more extensive fermentation were observed for early harvest, while the opposite was observed for elephant grass silage. Cellulase improves the fermentation profile of Mombasa grass silage, while xylanase enhances the dry matter and neutral detergent fiber degradability of elephant grass silage. The combined application of these enzymes does not appear to warrant recommendation.

Keywords: aerobic stability; *Cenchrus purpureus*; effluent; *Megathyrsus maximus*; microbial population.

INTRODUCTION

Many species of tropical grasses utilized for silage have high moisture and fiber concentrations with a low sugar concentration (Daniel et al., 2019; Gomes et al., 2021). These factors promote undesirable fermentations, characterized by greater production of ammonia nitrogen (NH_3-N), butyric acid, and effluent, which increase dry matter losses (Kung et al., 2018). Hence, producing tropical grass silage with high nutritive value requires strategies that promote desirable fermentation and inhibit the proliferation of undesirable bacteria (e.g., enterobacteria, clostridia, and others) (McDonald et al., 1991).

Exogenous fibrolytic enzymes (EFE) have been used as silage additives because they promote the solubilization of the most digestible parts of cell wall carbohydrates. With greater substrate for lactic acid fermentation, the decline in pH can be more rapid (Khota et al., 2016). This has proven to be an efficient alternative for improving the fermentation profile of silage with low sugar concentrations and/or high buffering capacity (Khota et al., 2016, 2018). Fiber solubility might also be beneficial because it can enhance silage dry matter (DM) content and fiber degradability (Li et al., 2014; Khota et al., 2018). However, inconsistent results are found in literature (Del Valle et al., 2018).

Mombasa grass and elephant grass cv. BRS Capiaçu are very productive forages commonly used for ensilage in tropical regions. Usually, the timing of harvest for tropical grasses for silage is based on days of regrowth. For elephant grass cv. BRS Capiaçu, 90 to 120 days was recommended as harvest interval (Monção et al., 2019). However, depending on the interval between harvests, high variation in nutrient composition and the fermentability

coefficient (FC) of the forage would be expected. Tomaz et al. (2018) found that mombasa grass had a greater FC when harvested at a height of 130 cm, although protein concentration, organic matter degradability and neutral detergent fiber (NDF) were compromised.

Although many studies have evaluated the fermentation profile and degradability of enzyme-treated silage, few have focused on tropical grasses. Thus, we hypothesized that cellulase and xylanase application would improve the chemical composition, fermentation profile and degradability of mombasa grass and elephant grass cv. BRS Capiaçu silage, and that they may also exhibit a synergistic effect when applied together. In addition, the magnitude of the EFE effect would depend on regrowth age.

In this study, our objective was to evaluate the effect of EFE on nutrient composition, fermentation profile, aerobic stability and in vitro degradation kinetics of mombasa grass and elephant grass cv. BRS Capiaçu ensiled at two regrowth ages (RA).

MATERIALS AND METHODS

Silage Preparation and Treatments

Mombasa grass [*Megathyrsus maximus* (Jacq.) B.K. Simon & S.W.L. Jacobs (syn. *Panicum maximum* Jacq.)] and Elephant grass cv. BRS Capiaçu [*Cenchrus purpureus* (Schumach.) Morrone (syn. *Pennisetum purpureum* Schumach.)] forage were obtained from different plots (third year of production) at the Teaching, Research and Extension Unit in Forages of the Department of Animal Science at the Universidade Federal de Viçosa (Viçosa, MG, Brazil). The plots were situated at an altitude of approximately 649 m with a south latitude of 20°45'014" and west longitude of 42°52'54". The climate in the area is classified as Cwa (subtropical climate; Köppen, 1918), with an average annual precipitation and temperature of approximately 1200 mm and 21 °C, respectively (Universidade Federal de Viçosa, 2021). The monthly

averages for precipitation, temperature, humidity, and calculated temperature-humidity index during regrowth are in Table 1.

Prior to harvest for this experiment, plots were mowed at 10 cm above ground level, followed by top-dressing fertilization with 40 kg/ha of nitrogen and 40 kg/ha of potassium, using urea and potassium chloride as sources, respectively. Mombasa grass with 7- and 11-wk regrowth (129.8 ± 19.72 and 151.7 ± 5.9 cm in height, respectively) and Elephant grass cv. BRS Capiaçu with 12- and 16-wk regrowth (3.56 ± 0.27 and 4.25 ± 0.16 m in height, respectively) were cut at 10 cm stubble height and immediately collected without wilting. Each species was evaluated separately, in a different experiment with each forage species. Elephant grass was harvested over two different years [spring-summer/2021-2022 (First) and summer-fall/2023 (Second)] while mombasa grass was harvested only in the first season.

For each period, forage of both species was manually harvested in the same day and chopped to a particle size of 1.5 cm using a stationary chopper (model PN Plus 2000, Nogueira S.A., São João da Boa Vista, SP, Brazil), and the forage was homogenized and subsamples were collected for nutrient composition before ensilage. Subsamples of approximately 10 kg and 4 kg each (for elephant and mombasa grass, respectively) from each period were randomly assigned to 1 of 8 treatments (for each trial), which were a combination of four exogenous fibrolytic enzymes applications (ENZ) and two regrowth ages (RA). Fibrolytic enzymes were applied by hand and consisted of: (1) 10 mL distilled water (CON), (2) 400 mg/kg DM (XYL; Smizyme xylanase, 10.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd, Beijing, China), (3) 1000 mg/kg of fresh forage of cellulase (CEL; Smizyme cellulase, 3.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd, Beijing, China), or (4) both enzymes applied at same dosages previously mentioned (MIX). The enzymatic products used are commercially available for use in total mixed ration diets; however, the use of Smizyme xylanase as a silage additive has been

previously investigated and the dosage followed their results (Del Valle et al., 2018). The dosage of cellulase followed previous studies with other cellulase products (Khota et al., 2016).

After enzyme application, mombasa grass was packed into 3.6 L plastic buckets at a density of approximately 99 ± 9.7 and 122 ± 5.4 kg of DM/m^3 (490 ± 48 and 560 ± 25 kg of FM/m^3) for 7- and 11-wk of RA, respectively. For elephant grass, 10 L plastic buckets were used, with a density of approximately 107 ± 8.0 and 123 ± 9.5 kg of DM/m^3 (579 ± 43 and 602 ± 46 kg of FM/m^3) for 12- and 16-wk in the first season; and 132 ± 8.0 and 178 ± 8.6 kg of DM/m^3 (685 ± 39 and 657 ± 32 kg of FM/m^3) for 12- and 16-wk in the second season, respectively. Prior to packing, sandbags were prepared by drying sand at 105 °C for 48 h and weighing approximately 400 g (for mombasa grass silos) and 2 kg (for elephant grass silos) into a cotton bag. Sandbags were then placed in the bottom of silos to create a depth of approximately 10 cm for accumulation and quantification of effluent production. To account for the weight of the sand and buckets, empty silo weights were taken before forage was added to silos. After being filled, silos were sealed with plastic lids and tape to limit aerobic exposure and weighed at opening. Mini silos were stored at room temperature (approximately 23.6 ± 4 °C) for 60 d. Thus, each experiment consisted of 8 treatments (2 regrowth ages × 4 enzyme applications) in triplicate. A total of 24 mini-silos for mombasa grass and 48 mini-silos for elephant grass (including two seasons which were used as a random effect) were used.

Sample Preparation and Analysis

After 60 d of storage, silos were weighed and opened. The material from the top 5 cm was discarded, and the remaining material from each mini-silo was removed and mixed. Representative subsamples of silage were collected for analysis conducted in the Forage and Silage Microbiology Laboratory of the Department of Animal Science at the Universidade Federal de Viçosa (Viçosa, MG, Brazil). Full buckets and empty buckets (with sandbags, but without silage) were weighed.

Approximately 100 g of fresh samples were dried in duplicate at 55 °C for 72 h in a forced air oven, and 250 g was frozen for later analysis. Dried samples were ground using a model R-TE-650-1 knife mill (Tecnal®, Piracicaba, SP, Brazil) and passed through a 1 mm sieve to evaluate nutrient composition and in vitro degradation kinetics. The ground samples were analyzed for: DM (method 934.01, AOAC, 1990), crude protein (CP; N × 6.25; method 984.13, AOAC 1990), ash (method 942.05; AOAC 1990), acid detergent fiber and lignin (method 973.18, AOAC 1990). Neutral detergent fiber corrected for ash and protein (NDFap) was analyzed using heat-stable α-amylase and without sodium sulfite (Mertens, 2002) and acid detergent insoluble nitrogen (ADIN) was analyzed according to Licitra et al. (1996).

The pH and microbial counts of fresh, untreated forage were evaluated soon after the forage was collected. A 25 g undried, unground sub-sample was diluted 10-fold (mass basis) in 225 mL sterile Ringer's solution (Oxoid™, Hampshire, England) using an industrial blender (Model-LB10S, Waring Laboratory®, Toronto, Canada) in an orbital mixer for 1 min, filtered through a two layers of sterile cotton gauze to extract silage juice, and divided into three aliquots. The first aliquot (50 mL) was used to measure pH in duplicate using a digital potentiometer (model W38, Tecnal®, Sao Paulo, SP, Brazil); the second aliquot (20 mL) was treated with 1:1 H_2SO_4 (stored at – 20 °C) for colorimetric analysis of WSC (Nelson, 1944) and NH_3-N through the phenol-hypochlorite method (Okuda et al., 1965). The third aliquot (10 mL) was used to quantify the lactic acid bacteria (LAB), enterobacteria, yeast and mold counts. Serial dilutions were made in sterile Ringer's solution and plated using the pour-plate technique in different culture media (Kung et al., 2003). Lactic acid bacteria counts were evaluated on De Man, Rogosa & Sharpe (MRS *Lactobacilli* Agar, Merck®, Darmstadt, Germany) and incubated at 37 °C for 48 h. Enterobacteria counts were evaluated on Violet Red Bile (VRB agar, Oxoid™, Hampshire, England) and incubated at 37 °C for 24 h. Dicloran Rose Bengal Chloramphenicol agar (DRBC agar, Difco™, Franklin Lakes, NJ, USA) media was used at 25 °C to evaluate

counts of yeasts after 72h and filamentous fungi (mold) after 96 h of incubation. Colony forming units (CFU) were counted on plates containing 25 to 250 colonies (Salfinger & Tortorello, 2015).

Fermented samples were analyzed for organic acids (lactic acid, acetic acid, butyric acid, and propionic acid) and ethanol at the Laboratory of Microorganism Physiology (BIOAGRO) of the Universidade Federal de Viçosa, by high-performance liquid chromatography using a model SPD-10AVP device (Shimadzu®, Tokyo, Japan) under the following conditions: BIO-RAD Aminex HPX 87H (300 × 7.8 mm) and pre-column of the same phase (Bio-Rad Laboratórios Brasil, Rio de Janeiro, Brazil); refractive index detector (Shimadzu RID-20A, Kyoto, Japan); column oven temperature: 45 °C; sample volume: 20 µL; and mobile phase, 5 mmol/L H_2SO_4 with flow rate at 0.7 mL/min (Siegfried et al., 1984). Equipment operation and data processing were done using LabSolutions LC software (Shimadzu®, Tokyo, Japan).

After subsamples were collected from the silos, clean buckets were used to evaluate aerobic stability with approximately 2 kg of the remaining elephant grass silage. Aerobic stability was only evaluated in elephant grass silage, by placing temperature loggers (Cryopark iMINI™, Cryopark Company Inc, VA, USA) in the geometric center of the bucket. Loggers recorded temperature every 15 min, and three additional sensors were used to monitor environmental temperature. Environmental temperature averaged 25.3 ± 1.5 °C in the first season; and 21.1 ± 0.9 °C in the second season of the trial. Buckets were covered with aluminum foil and two layers of cheesecloth to prevent drying (Borreani et al., 2007), and silos were left exposed to aerobic conditions to 168 h. Aerobic stability was defined as the number of hours silo temperature increased 2 °C above the environmental temperature.

Effluent production was calculated by subtracting the weight of the empty silo and sandbag prior to ensiling from the weight of the empty silo and sandbag at the day of opening

and dividing this value by the weight of forage (Fresh basis) at ensiling. Dry matter losses (DML) during fermentation were calculated by the difference in weight (WD) between the beginning and end of fermentation by the following equation proposed by Weissbach (2005):

$$DML[\%] = 100\frac{WD[g]}{DMensiled[g]} + 2.5 \quad (1)$$

Dry matter losses during aerobic exposure (DML 7d) were also calculated by dividing the weight of material at the 7th day of exposure to air by the weight of material at the day of opening, after correcting both weights with the DM concentration of deteriorated and ensiled material, respectively, and subtracting the result from 100.

The buffering capacity (BC) of the fresh forages was measured according to Playne and McDonald (1966), wherein macerated forage was first titrated to pH 3 with 0.1 M HCl to release bicarbonate as CO_2, and then titrated with 0.1 M NaOH until pH 6, and expressed as MEq/kg DM. To calculate the fermentability coefficient of the fresh forages, BC was expressed as g lactic acid/kg DM through the following equation [$R^2 = 0.95$; Clavin et al., 2017; (O'Kiely and Pahlow, 2003)]:

$$BC\ (g\ lactic\ acid\ kg^{-1}\ DM) = 0.0154 \times BC\ mEq\ kg^{-1} DM - 0.2115 \quad (2)$$

Fermentability coefficient was calculated using the equation proposed by Weissbach et al. (1974):

$$FC = DM\ (g\ 100^{-1}) + \frac{8 \times WSC\ (g\ kg^{-1} DM)}{BC\ (g\ lactic\ acid\ kg^{-1}\ DM)} \quad (3)$$

In vitro ruminal kinetics and degradation

Ground and dried samples of replicates were mixed to obtain a composite sample for each treatment, and approximately 0.5 g of samples were weighed into F-57 filter bags (Ankom® Technology Corporation, Fairport, NY, USA) in quadruplicate for each time point.

Eight timepoints were used (0, 3, 6, 12, 24, 48, 72 and 96-h) to determine DM and NDF degradation kinetics.

Ruminal fluid for *in vitro* incubations was obtained from three ruminally cannulated heifers fed a mixed diet composed of elephant grass forage and concentrate in a ratio of 80:20 on a DM basis. The concentrate was composed of corn bran, soybean meal, mineral supplement, urea, and ammonium sulfate. The animals were acclimated to this diet for 14 days before collecting ruminal fluid (Machado et al., 2016). Rumen fluid was strained through four layers of cheesecloth into warm thermoses, and immediately transported to a warm room. Rumen fluid from each heifer was mixed in a 1:4 ratio with buffer solution (McDougall, 1948) that was pre-warmed (39 °C) and flushed with CO_2 (to reach pH around 6.8), without the addition of urea. Approximately 2 L of the inoculum was used for each of four glass bottles placed in Daisy^{II} incubators (Ankom® Technology Corporation, Fairport, NY, USA), with one enzyme treatment per bottle. Three consecutive runs were conducted and used as replicates.

The incubation was carried out in decreasing order of time so that all bags were removed simultaneously from bottles in the end. After removal, bags were washed manually with cold tap water until the water ran clear (Zanetti et al., 2017). The zero-h bags were not incubated but were washed with other bags. All bags were dried in an oven at 55 °C for 72 h, weighed, and were analyzed for DM and NDF by methods described before.

In vitro degradability of DM was estimated by the equation proposed by (Ørskov & McDonald, 1979). The degradability of NDF was estimated by the logistic model described by (Van Milgen et al., 1991):

$$Y(t) = a + b \times (1 - e^{(kd \times t)}) \quad (4)$$

$$RNDF(t) = b \times [1 + (\lambda \times t)] \times e^{(-\lambda \times t)} + I \quad (5)$$

Where: Yt = degraded fraction of DM (g/kg); a = soluble fraction (g/kg); b = potentially degradable fraction in the rumen (g/kg); I = undegradable fraction; kd = rate constant for degradation of b (g/kg per h); t = time independent variable (h); RNDFt = non-degraded NDF residue at time "t" (g/kg); λ = joint fractional rate of latency and degradation (per h).

The parameters were estimated using PROC NLIN procedure (version 9.4, SAS Institute Inc., Cary, NC, USA), using the Marquardt algorithm for convergence.

The NDF degradation rate was calculated based on λ, using the properties of the Γ (2) distribution (Ellis et al., 1994):

$$kd = (0.59635 \times \lambda) \times 1000 \quad (6)$$

Where: kd = rate constant for degradation of b (g/kg per h); λ = joint fractional rate of latency and degradation (per h).

Statistical analyses

For trial 1 (mombasa grass), data were analyzed as a completely randomized design with a 2 (regrowth ages) × 4 (enzyme applications) factorial arrangement of treatments using PROC GLIMMIX of SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). Fixed effects included regrowth ages, enzyme application, and their two-way interaction. For trial 2 (elephant grass), data were also analyzed as a 2 (regrowth ages) × 4 (enzyme applications) factorial arrangement, with the same fixed effects as trial 1. However, in this trial, the season of harvest was used as a random effect.

Means were determined using the least-square means statement and were compared using Tukey's test adjustment after an overall significant *F*-test. Significance was declared when $P \leq 0.05$. Main effects were presented and discussed only if the interaction was not significant ($P > 0.05$).

RESULTS

Table 1 shows the average precipitation, temperature, humidity, and temperature-humidity index (THI) during both study periods. Briefly, average temperatures were mild during both periods, and relative humidity was high, characteristic of the region's humid climate. Precipitation was the main difference between both periods of study. Precipitation was elevated during the regrowth of mombasa grass and the first year of the elephant grass study, as the standardization cut was performed at the beginning of the rainy season. However, the second regrowth ages of elephant grass study had lower precipitation averages, as the standardization cut was performed during the transition from the rainy to the dry season.

Nutrient composition, pH, microbial counts, and FC of the fresh, untreated mombasa grass and elephant grass prior to ensiling, are shown in Table 2. Briefly, in both trials, increasing RA decreased CP and increased dry matter, NDFap, lignin, and FC. The concentration of WSC was greater at 11- than 7-wk regrowth for mombasa grass, and lower for elephant grass at 16- than 12-wk regrowth. The DM, NDFap, and WSC varied considerably between the two seasons in which the elephant grass study was grown. This was likely due to different weather conditions mentioned above.

Mombasa grass silage (Experiment 1)

An interaction was observed for pH ($P = 0.005$; Table 3) where at 11-wk regrowth, CON and XYL had greater pH than CEL and MIX, and when compared to CON and XYL at 7-wk. Harvesting mombasa grass at 7-wk regrowth increased lactic acid concentration (LA; $P = 0.001$) compared to 11-wk regrowth. The LA concentrations were greater ($P = 0.001$) for CEL and MIX and the lactic-to-acetic acid ratio (LA:AA) ($P = 0.002$) decreased, especially in CEL. Acetic acid (AA) was lower ($P = 0.001$) in CEL compared to CON and XYL, while MIX did not differ from any other treatment. Butyric acid (BA) was lower ($P = 0.02$) in CEL and MIX compared to CON and XYL, and propionic acid (PA) was lower ($P = 0.01$) compared to XYL.

Ethanol concentrations were not affected by ENZ or RA ($P > 0.05$). Enterobacteria counts were lower ($P = 0.002$) for silage harvested at 7-wk than from 10-wk regrowth and were also lower ($P = 0.003$) for MIX than other treatments. Lactic acid bacteria counts were greater ($P = 0.04$) for MIX and CEL than XYL. Yeast and mold counts were affected by the interaction of ENZ × RA ($P = 0.02$ and $P = 0.03$, respectively). At 7-wk regrowth, yeast counts were lower for XYL, CEL and MIX than CON. Mold counts were also lower for CEL and MIX than CON at 7-wk regrowth. The concentrations of NH_3-N were lower ($P = 0.02$) for CEL and MIX than CON, while effluent losses were greater ($P = 0.001$; Table 4).

Dry matter losses were 4.0 g/kg DM lower ($P = 0.01$; Table 4), and DM concentrations were 10.9 g/kg as fed lower ($P = 0.001$) for 7-wk than 11-wk regrowth. However, greater ($P = 0.001$) ash, CP and WSC concentrations were observed for 7-wk regrowth compared to 11-wk regrowth. Lower ADF concentrations ($P = 0.02$) and greater CP concentrations ($P = 0.001$) were observed for CEL and MIX than CON. There was an interaction between ENZ × RA on NDFap ($P = 0.007$) where at 11-wk regrowth, NDFap concentrations were greater for CON and XYL than at 7-wk regrowth, and when compared to CEL and MIX at 11-wk regrowth. An interaction was also observed on ADIN ($P = 0.01$), where CON, XYL and CEL at 11-wk had greater ADIN than at 7-wk. Lower ADIN was also observed for CEL than MIX at 7-wk.

There was an interaction between ENZ × RA ($P = 0.02$; Table 5) for the soluble fraction (a) of DM, where at 11-wk regrowth, greater fraction ‘a’ average for MIX than XYL and CON was observed. The Dry matter degradability (DMD) after 72h was greater ($P = 0.03$) for silage at 7-wk regrowth. The fraction ‘B’ was greater ($P = 0.01$) and fraction ‘I’ was lower ($P = 0.01$) for 7-wk than 11-wk regrowth. The fraction ‘I’ was also lower ($P = 0.03$) for XYL than CEL. The NDF degradability (NDFD) after 72h was greater ($P = 0.002$) for 7-wk than 11-wk regrowth, and greater ($P = 0.01$) for XYL than CEL.

Elephant grass silage (Experiment 2)

There were no differences in pH, LAB, yeast counts, or mold counts among treatments ($P > 0.05$; Table 6). The LA and AA concentrations were greater ($P = 0.002$ and $P = 0.005$, respectively) for late harvest (16-wk) than early harvest (12-wk). Butyric acid concentrations were affected by ENZ ($P = 0.004$), where CON had the greatest concentrations and CEL and MIX silages had the lowest, while XYL did not differ from any other treatments. No differences among treatments were observed ($P > 0.05$) in PA, ethanol and LA:AA.

There was an interaction between ENZ × RA on NH_3-N ($P = 0.03$; Table 6) and effluent production ($P = 0.02$; Table 7). The concentrations of NH_3-N were lower for CEL and MIX than CON at 16-wk regrowth. Effluent production was lower for XYL and CON than CEL and MIX at 16-wk regrowth.

Dry matter losses were 16.21 g/kg DM greater ($P = 0.03$) for 12-wk than 16-wk regrowth. There was an interaction between ENZ × RA on AS ($P = 0.001$), where AS was greater for MIX at 12-wk than and mix at 16-wk regrowth. In addition, CEL had lower AS than CON, XYL and MIX at 12-wk regrowth, while CEL and MIX had lower AS than CON and XYL at 16-wk regrowth. Interactions were also observed for pH ($P = 0.02$) and mold counts ($P = 0.006$) after 7d of aerobic exposure. At 16-wk regrowth, lower pH 7d were observed for CON and XYL than CEL and MIX. Greater mold counts after 7d of aerobic exposure were observed for MIX at 16-wk than MIX at 12-wk regrowth. In addition, at 16-wk regrowth, mold counts were greater for CEL and MIX than CON. Greater maximum temperature were observed ($P = 0.02$) for CEL and MIX than CON. No differences were observed for DML after 7d of AS ($P > 0.05$).

Dry matter, lignin and ADIN concentrations were 9.55, 56.9, and 14.9 g/kg lower ($P = 0.001$; Table 8), respectively, for silage at 12-wk than 16-wk regrowth. Conversely, greater ash and CP ($P = 0.001$) concentrations were observed for 12-wk regrowth. The concentration of

ADIN was greater (*P* = 0.001) for CON, and CON had greater NDFap (*P* = 0.001) than other treatments. The NDFap concentrations were also affected by RA (*P* = 0.001), where 16-wk was 45 g/kg DM greater compared to 12-wk regrowth. The concentration of ADF was lower (*P* = 0.04) for CEL and MIX compared with CON. There was an interaction between ENZ × RA on WSC (*P* = 0.009) where at 12-wk, WSC concentrations were greater for CEL and MIX than CON and XYL. In addition, greater WSC concentrations were observed for CEL and MIX at 12-wk than at 16-wk.

An interaction was observed (*P* = 0.001; Table 9) for soluble fraction ‘a’. At 12-wk regrowth, CEL had the greatest average value, while XYL and MIX were intermediate, and CON the lowest. At 16-wk regrowth, CEL and MIX had the greatest value, XYL was intermediate, and CON the lowest. In addition, a greater fraction ‘a’ average for XYL at 12-wk than at 16-wk regrowth were observed. An interaction was also observed (*P* = 0.03) for fraction ‘b’. At 12-wk regrowth, XYL was more digestible than CEL and MIX. Control silage at 12-wk also had greater average than MIX, while CEL did not differ from CON and MIX. Greater degradability was also observed for XYL when harvested at 12-wk than at 16-wk regrowth.

A greater rate of degradation (kd) for DM (*P* = 0.009; Table 9), DMD 24h (*P* = 0.001), and 48h (*P* = 0.001) were observed in silage harvested at 12- than 16-wk regrowth. In addition, DMD 48h (*P* = 0.001) was greater for XYL than CON and MIX. The main effect of ENZ also affected DMD 24h (*P* = 0.001), but no differences among treatments were observed after Tukey corrections. An interaction was observed for DMD 72h (*P* = 0.003). At 12-wk regrowth, XYL was more digestible than CON and MIX. Greater degradability was also observed for CON, XYL and CEL when harvested at 12-wk than at 16-wk regrowth.

For NDF ruminal kinetics, kd, and λ rates were greater (*P* = 0.004; Table 9) when elephant grass was harvested at 12-wk. The NDFD 24h was greater (*P* = 0.006) for 12-wk than 16-wk regrowth. An interaction was observed (*P* = 0.001) for ruminal slowly degradable

fraction 'B'. At 12-wk regrowth, XYL had the greatest fraction 'B', while CON was intermediate, MIX the lowest, and CEL did not differ from CON and XYL but was greater than MIX. In addition, CON, XYL and CEL harvested at 12-wk were more digestible than at 16-wk. An interaction (ENZ × RA) was observed for NDF undegradable fraction (I; $P = 0.001$) where XYL and CON harvested at 12-wk regrowth had lower values than at 16-wk. At 12-wk regrowth, XYL had the lowest fraction 'I' values, while CON was intermediate, MIX the lowest, and CEL did not differ from CON and MIX but was greater than XYL. An interaction (ENZ × RA) was also observed for NDFD 48h ($P = 0.04$) and 72 h ($P = 0.01$). For both time points, XYL at 12-wk regrowth was more digestible than at 16-wk. At 12-wk regrowth, greater degradability was observed for XYL than MIX, while for NDFD 48h XYL at 12-wk was also greater than CEL.

DISCUSSION

Nutrient composition and microbial counts of the fresh forages in this study were typical of mombasa grass and elephant grass harvested at similar RA (Khota et al., 2016; Monção et al., 2019). Tropical grass silage usually has less than 25% DM (unwilted), which indicates risk of effluent loss and activity of undesirable microorganisms such as *Clostridium* spp. (McDonald et al., 1991). To improve fermentation profile under such low DM conditions, a rapid pH decline is necessary and the ratio of WSC to BC must be greater than forages with a higher DM to stabilize pH values (Carvalho et al., 2024). Mombasa grass had a lower FC than the recommended minimum (FC ≥ 35%) to ensure desirable fermentation (Weissbach, 1974). However, the cultivar BRS Capiaçu can accumulate high WSC concentration (Table 2), ensuring an FC above the minimum reference.

Strategies can be adopted to improve FC and consequently the fermentation profile of tropical grass silage, such as applying EFE during harvest. However, only small improvements

in nutritive value and fermentation profile in XYL silage have been observed previously (Del Valle et al., 2018). Therefore, the lack of effect on pH and lactic acid may be related to the lower capacity of LAB's in utilizing pentoses (in this case, xylan) to produce lactic acid. Degradation of xylan produces mainly acetic acid as an end-product during fermentation (Dehghani et al., 2012; J. Li et al., 2018). Rasmussen et al. (2014) reported that cleavage of the acetyl linkages in xylan lead to an acetic acid formation, which is nearly ten times less effective at reducing pH compared to lactic acid (Kung et al., 2018). The greater propionic acid concentration in mombasa grass treated with XYL is probably related to the reduction in LAB counts and clostridia activity, as it is doubtful that other propionic acid producing bacteria like *Propionibacterium* sp. can flourish in most silage (Kung et al., 2018).

Applying cellulase alone or in combination with xylanase (MIX) improved the fermentation profile of mombasa grass silage. This silage had a greater lactic-to-acetic acid ratio, reflecting greater capacity for lactic acid production and a more rapid decline in pH. In addition, secondary fermentations and proteolysis were reduced. Similar results were also previously reported in grass silage fermentation studies that evaluated EFE(Li et al., 2014; Li et al., 2018; Zhao et al., 2021). The more rapid decrease in pH provided less proteolysis in CEL and MIX silages, so CP concentration was greater compared to XYL and CON silages, and protein quality was preserved (Bureenok et al., 2019).

Khota et al. (2016) evaluated different combinations and dosages of microbial inoculants and cellulase in unwilted and wilted mombasa and Napier grass silage. They observed that when applied at 1000 mg/kg FM (the same dosage used in the present study), the cellulase improved fermentation profile, by decreasing pH and increasing lactic acid compared to control and/or LAB treatments. In our study, the improvement in fermentation in CEL elephant grass silage was less pronounced than in mombasa grass. However, lower NH_3-N and butyric acid concentrations for CEL compared to CON were observed. When applied to

sorghum and Napier grass with high WSC concentrations, CEL and XYL did not improve silage fermentation (Bureenok et al., 2019; Khota et al., 2017). Elephant grass had high concentrations of WSC (> 70 g/kg DM) before ensiling in this study, which were likely sufficient to promote desirable fermentation without EFE applications, unlike the lower WSC concentrations in Napier grass (24.4 g/kg; Khota et al., 2016).

The improvement in fermentation profile for MIX can be attributed to cellulase activity, as XYL were not affected. However, a combination of EFE could exert a synergistic effect on mombasa grass silage, suppressing enterobacteria and enhancing silage quality. Apparently, in this study the synergistic activity of EFE may have caused a rapid recline in pH in the initial period of fermentation. This can limit the proliferation of enterobacteria, known for reducing NO_3, NH_3-N, and biogenic amines (Queiroz et al., 2018). Furthermore, enterobacteria does not thrive when pH is below 4.5 (Heron et al., 1993).

Due to the low DM of silage in this study, DML above 100 g/kg DM were observed in both trials, regardless of the treatment. For mombasa grass at 11-wk regrowth (late harvest) greater DML were observed, which is likely related to the greater enterobacteria and yeast counts. The competition of these microbes for substrate with LAB reduces the rate of decline in pH (lactic acid production), increasing losses (McDonald et al., 1991). However, for elephant grass the greatest DML were observed at 12-wk regrowth (early harvest). The small increase in DM (which also increased FC) at later harvest improved fermentation profile, by increasing lactic and acetic acid concentrations and decreasing DML in elephant grass silage (Ali et al., 2022). The conversion of lactic acid into acetic and 1,2-propanediol could be the reason for similar pH among RA.

Interactions between ENZ × RA were observed, highlighting a clear regrowth-response relationship. Depending on the variable, the response was slightly more favorable at late harvest, such as NDFap solubilization and pH decline rate for mombasa grass CEL and MIX

silages, and lower NH_3-N concentrations for elephant grass CEL and MIX silages. However, the opposite occurred for early harvest, such as lower yeast and mold counts for enzyme treated mombasa grass silage, while greater effluent production increased only at late harvest for elephant grass silage.

The decrease in yeast and mold counts for CEL and MIX mombasa grass silages at 7-wk regrowth were likely caused by greater LAB activity and total organic acid production after enzymatic activity. In elephant grass silage, counts were similar among treatments, likely because pH, lactic acid concentration, and ethanol concentration did not differ. Molds are less acid-tolerant than yeast, and yeast is a well-known ethanol producer during fermentation in the silo (Kung et al., 2018; Queiroz et al., 2018).

Yeast growth is the principal concern after silos are opened, as they assimilate lactic acid and increase pH (Kung et al., 2018). The increase in pH allows other undesirable microbes, such as molds and aerobic bacteria, to reproliferate in silage and increase DML and temperature (Queiroz et al., 2018). In this context, high concentrations of residual WSC are undesirable, since they provide substrate for microbial metabolism unless undesirable microbes are inhibited by antifungal acids, like acetic or propionic acid (Kung et al., 2018).

Cellulase and MIX provided a lower aerobic stability than XYL and CON, in agreement with previous reports (Zhang & Kumai, 2000). Those silages had lower acetic acid and greater WSC concentrations at opening (> 19 g/kg DM). With more substrate and less antifungal acids, WSC were rapidly oxidized by aerobic bacteria, yeast, and molds, which explains the reduction in WSC concentrations (< 5.5 g/kg DM) after seven days of air exposure (McDonald et al., 1991). Additionally, greater pH, and maximum temperature were observed after seven days of exposure for these silages.

Greater effluent losses were observed for silage treated with CEL and MIX in both grass silages, which can increase the loss of organic acids and highly digestible components. Effluent

also has a high biochemical oxygen demand and a low pH, so it is classified as a farm pollutant (Savoie & Jofriet, 2003). Solubilization of the cell wall, such as by EFE, releases water contained within cells, along with sugar, into the extracellular environment (Campana et al., 2023; Zhang & Kumai, 2000). Those results, in addition to the remained underwent butyric acid fermentation (> 3 g/kg DM; Kaiser et al., 2002) in mombasa grass and lower aerobic stability in elephant grass (regardless of plant RA), may limit the adoption and use of cellulase alone in grass silage. For example, chemical additives containing sodium nitrite and hexamine may be preferred over cellulase and have demonstrated greater efficacy to inhibit clostridial activity in grass silage (Gomes et al., 2021; Moraes et al., 2023). Alternatively, a combined application of these additives with cellulase may improve fermentation profile more than cellulase alone, and can be an alternative to tropical grass silage production, depending on the costs.

The effect of RA on nutritional value has been well documented (Ali et al., 2022; Moraes et al., 2023), and the changes in nutrient composition and degradability observed in this trial were expected. The RA with the greatest nutritive value in tropical forages is generally not the best for silage fermentation, due to low DM, WSC concentrations, and high BC (Tomaz et al., 2018). Therefore, harvest must be timed to best balance nutritive value, degradability, productivity, and fermentation profile.

The older the plant, the smaller the proportion of leaves and cell contents (such as total and soluble nitrogen), decreasing the nutritional value (Moraes et al., 2023). Furthermore, stem elongation promotes thickening of cell wall (Ali et al., 2022; Santos et al., 2014). This occurs particularly in long-stem forages, such as elephant grass BRS Capiaçu, which can reach heights above 5 m that requiring sturdy stems to prevent the plant lodging (Jung et al., 2012). Stiffer stems are difficult for microorganisms to degrade, as the covalent bonds between lignin and other polysaccharides act as a barrier that hinders microorganisms from degrading fiber in the rumen (Hatfield et al., 2017). The degradability of protein is also affected, considering the

increase in ADIN due to the association of nitrogen with lignin (Licitra et al., 1996). Enzyme-treated elephant grass silage also had lower concentrations of ADIN, probably due to less proteolysis that decreases the easily degradable portion of protein and increases ADIN concentrations, as observed in control silage.

Elephant grass silage harvested at 16-wk regrowth had lower degradable fractions and greater undegradable fractions. This decreased kd for DM and NDF, which can increase ruminal retention time and decrease passage rate, along with dry matter intake and performance (Oba & Allen, 1999). Even without differences in lignin concentration for mombasa grass silage, lower DMD and NDFD 72h, and fractions B and I of NDF were observed for 11-wk compared to 7-wk regrowth. This can be attributed to changes in leaf/steam ratio, as stem elongation increased canopy height (21 cm higher) for 11-wk regrowth, along with NDF and indigestible contents, since leaves are more digestible than the stem. These results agree with Moraes et al. (2023), who observed lower degradability in mombasa grass silage at late harvest and suggested that if grass silage is intended to supply nutrients in the diet (i.e., not only supply physically effective fiber) the practice of late harvest should be discouraged.

In the current study, lower NDFap and protein loss during ensiling were observed for CEL, conserving more digestible substrates (fraction a). However, the lower NDF concentration was accompanied by a greater undegradable NDF fraction. Enzymes act on the most digestible portion of NDF, meaning silage treated with EFE usually has lower NDF concentration and a similar concentration of undegradable NDF, increasing the proportion of undegradable NDF in the fiber fraction (Del Valle et al., 2018).

Greater NDFD 72h for XYL compared with CEL was observed for mombasa grass silage, as well as NDFD 24h, when compared with MIX, for elephant grass silage. Both silages treated with XYL had a lower undegradable fraction, which contributed to these effects. Xylanase also increased DMD 48 and 72h. Furthermore, greater b fraction, DMD 72h, and

NDFD 48 and 72h were observed for XYL at early harvest (12-wk). Apparently, xylanase can act in less digestible fractions of the cell wall and appears to enhance attachment and colonization by rumen microorganisms (Nsereko et al., 2002). Acting in synergy with other ruminal enzymes, xylanase can promote a balance of rapidly and slowly degradable fractions which may improve microbial fermentation since more energy is available for microbial growth (Elghandour et al., 2016; Sakita et al., 2020). In addition, xylanase-treated silage had lower effluent production than cellulase-treated silage, and probably less nutrient losses as a result.

CONCLUSIONS

Our results indicate that cellulase is the better option to reduce clostridia activity and proteolysis than xylanase or mix on mombasa grass silage, due to greater total acid production and more rapid decline in pH.

Both enzymes reduced cell wall constituents, but only xylanase improved DM and NDF degradability of elephant grass silage, mainly at 12-wk regrowth age, and without any benefits in fermentation. In addition, nutritive value and degradability of mombasa and elephant grass silages were greater for early RA. No improvement in the combined application of enzymes appears to justify its recommendation.

However, further research is warranted to evaluate the synergetic effects of cellulase with chemical, absorbent and microbial additives, to focus on avoiding effluent losses and improving aerobic stability, where were negatively affected by cellulase application.

AUTHOR CONTRIBUTIONS

Conceptualization, G.F.L.C., W.A.S., K.G.R.; methodology, G.F.L.C., K.G.R.; software, G.F.L.C, E.C.D.; data curation, G.F.L.C., K.G.R.; investigation, G.F.L.C. R.L.F.; validation, G.F.L.C., K.G.R.; formal analysis, G.F.L.C. R.L.F.; supervision, K.G.R.; funding acquisition,

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1. Average precipitation, temperature, humidity, and temperature-humidity index (THI) during the first and second period of study.

	Season Interval[1]							
	First (2021-2022)[2]				Second (2023)[3]			
Month	Precipitation,[4] mm	Temperature,[4] °C	Relative humidity,[4] %	THI[5]	Precipitation,[4] mm	Temperature,[4] °C	Relative humidity,[4] %	THI[5]
October	7.0	20.4	84.0	67.7	-	-	-	-
November	388	20.8	82.3	68.3	-	-	-	-
December	224	214	83.5	69.4	-	-	-	-
January	346	22.7	82.4	71.4	11.4	23.2	77.9	71.9
February	323	21.7	88.3	70.2	356	22.1	85.7	70.8
March	-	-	-	-	36.2	22.8	78.1	71.3
April	-	-	-	-	33.6	20.9	82.2	68.4
May	-	-	-	-	19.2	19.2	82.7	65.8

[1] Elephant grass study (Experiment 2) was evaluated in two different growing seasons, while mombasa grass study (Experiment 1) was only evaluated in the first season.

[2] Standardization cut for both forages was made on October 29th. After 28 days additional cuts were made on mombasa and elephant grass plots designed to early harvest. On January 15th mombasa grass was harvested after 7- and 11-wk regrowth, and on February 17th elephant grass was harvested after 12- and 16-wk regrowth.

[3] Standardization cut was made on January 19th. After 28 days an additional cut was made on plots designed to early harvest (12-wk). On May 15th elephant grass was harvested after 12- and 16-wk regrowth.

[4] Data were obtained from the Meteorological Bulletin of Universidade Federal de Viçosa (2023).

[5] Temperature-humidity index was calculated by THI = (1.8 x temperature + 32) – (0.55 – 0.55 x relative humidity/100) x [(1.8 x temperature + 32) – 58] according to Segnalini et al. (2011).

Table 2. Nutrient composition, pH, microbial counts, and fermentability coefficient of fresh, untreated mombasa grass silage (*n* = 6) and elephant grass (*n* = 12) at two different regrowth ages.

Item	Mombasa grass		Elephant grass	
	7 wk	11 wk	12 wk	16 wk
pH	5.79 ± 0.02	5.66 ± 0.03	6.02 ± 0.26	6.10 ± 0.09
Dry Matter, g/kg as fed	201 ± 5.5	219 ± 3.0	177 ± 9.0	223 ± 20.9
Organic Matter, g/kg DM	885 ± 3.7	888 ± 1.9	943 ± 19.8	949 ± 3.7
Crude Protein, g/kg DM	80.3 ± 0.6	64.4 ± 0.8	67.3 ± 4.3	60.8 ± 7.9
NDFap, g/kg DM	654 ± 0.4	700 ± 6.2	677 ± 23.6	711 ± 16.1
WSC, g/kg DM	11.3 ± 0.6	21.0 ± 0.5	90.4 ± 8.6	70.7 ± 1.6
Ash, g/kg DM	115 ± 4.7	112 ± 2.3	77.1 ± 4.9	74.3 ± 23.9
Lignin, g/kg DM	33.3 ± 2.6	43.5 ± 1.8	59.4 ± 10.6	88.6 ± 7.9
ADIN, g/kg TN	42.3 ± 1.1	36.9 ± 1.9	70.5 ± 2.5	64.5 ± 1.7
BC, mEq kg/DM	533 ± 16.2	420 ± 18.7	236 ± 32.7	197 ± 27.6
FC (%)	21.4 ± 0.32	24.5 ± 0.41	38.9 ± 0.45	42.3 ± 3.68
Yeast, log cfu/g of fresh forage	5.05 ± 0.15	5.14 ± 0.37	5.50 ± 0.11	5.04 ± 0.18
Mold, log cfu/g of fresh forage	4.81 ± 0.21	4.49 ± 0.70	4.75 ± 0.75	4.90 ± 0.18
LAB, log cfu/g of fresh forage	5.57 ± 0.03	5.83 ± 0.03	5.71 ± 0.03	5.89 ± 0.82
Enterobacteria, cfu/g of fresh forage	5.62 ± 0.02	5.38 ± 0.43	ND	ND

NDFap: neutral detergent fiber corrected for ash and protein; WSC: water soluble-carbohydrates; ADIN: acid detergent insoluble fiber; FC: Fermentability coefficient; LAB: lactic acid bacteria; TN: total nitrogen; ND: not determined (detection limit was set at 2.0 cfu/g of fresh forage)

Table 3. Fermentation profile and microbial counts of mombasa grass silage (n = 24) treated with fibrolytic enzymes and harvested at different regrowth ages after 60 days of storage.

		Enzyme Application				RA General		P-value		
Item[1]	Regrowth Age	Control	Xylanase	Cellulase	Mix	mean	SEM	ENZ	RA	ENZ × RA
pH	7 wk	4.54^{Ba}	4.54^{Ba}	4.53^{Aa}	4.45^{Aa}	4.52	0.087	0.001	0.005	0.005
	11 wk	5.00^{Aa}	5.02^{Aa}	4.49^{Ab}	4.35^{Ab}	4.72				
	ENZ General mean	4.77	4.78	4.51	4.40					
Lactic acid, g/kg DM	7 wk	11.3	10.8	14.6	14.9	12.9^{A}	0.565	0.001	0.001	0.68
	11 wk	9.2	9.7	12.9	12.4	11.1^{B}				
	ENZ General mean	10.3^{b}	10.2^{b}	13.8^{a}	13.7^{a}					
Acetic acid, g/kg DM	7 wk	27.2	34.3	17.6	24.7	26.0	2.618	0.002	0.18	0.38
	11 wk	25.6	26.5	19.1	22.4	23.4				
	ENZ General mean	26.4^{a}	30.4^{a}	18.3^{b}	23.6^{ab}					
Butyric acid, g/kg DM	7 wk	16.3	12.8	7.36	7.37	10.9^{A}	1.070	0.001	0.006	0.06
	11 wk	10.8	8.97	7.19	7.15	8.54^{B}				
	ENZ General mean	13.6^{a}	10.9^{b}	7.28^{c}	7.26^{c}					
Propionic acid, g/kg DM	7 wk	1.39	2.50	1.56	1.40	1.71	0.202	0.01	0.45	0.09
	11 wk	1.74	1.77	1.40	1.50	1.60				
	ENZ General mean	1.57^{ab}	2.14^{a}	1.48^{b}	1.45^{b}					
Ethanol, g/kg DM	7 wk	4.88	8.59	9.17	8.78	7.85	3.371	0.99	0.33	0.52
	11 wk	13.5	9.44	8.96	9.04	10.2				
	ENZ General mean	9.06	9.20	8.91	9.01					
LA:AA	7 wk	0.438	0.326	0.827	0.606	0.549	0.0616	0.001	0.28	0.63
	11 wk	0.360	0.369	0.714	0.558	0.500				
	ENZ General mean	0.399^{c}	0.348^{c}	0.770^{a}	0.582^{b}					
NH_3-N, g/kg total nitrogen	7 wk	108	68.0	76.0	73.9	81.5	13.80	0.02	0.29	0.13
	11 wk	117	119	65.6	65.8	92.2				
	ENZ General mean	112^{a}	93.8^{ab}	70.8^{b}	69.8^{b}					
	7 wk	3.02	3.03	3.08	1.96	2.77^{B}	0.204	0.003	0.002	0.33

Enterobacteria, cfu/g fresh silage	11 wk	3.43	3.46	3.36	2.96	3.30^{A}				
	ENZ General mean	3.22^{a}	3.25^{a}	3.22^{a}	2.46^{b}					
Lactic acid bacteria, cfu/g fresh silage	7 wk	7.51	7.05	7.36	7.64	7.39				
	11 wk	7.12	7.11	8.17	7.83	7.56	0.247	0.04	0.36	0.15
	ENZ General mean	7.32^{ab}	7.08^{b}	7.77^{a}	7.73^{a}					
Yeast, cfu/g fresh silage	7 wk	4.99^{Aa}	3.16^{Ab}	3.48^{Ab}	2.69^{Ab}	3.58				
	11 wk	4.51^{Aa}	3.96^{Aa}	4.34^{Aa}	3.72^{Aa}	4.13	0.229	0.01	0.004	0.02
	ENZ General mean	4.75	3.56	3.91	3.21					
Mold, cfu/g fresh silage	7 wk	4.46^{Aa}	3.14^{Aab}	2.52^{Ab}	2.41^{Ab}	3.13				
	11 wk	3.82^{Aa}	3.61^{Aa}	4.11^{Aa}	2.97^{Aa}	3.60	0.336	0.004	0.06	0.03
	ENZ General mean	4.14	3.38	3.32	2.64					

^{A,B,a,b} Different letters within a row with lowercase and within a column with uppercase are significantly different (Tukey test, α = 0.05).

[1] ENZ: control (distilled water), xylanase (400 mg/kg of dry matter weight, Smizyme xylanase with 10.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd), cellulase (1.000 mg/kg of fresh weight, Smizyme cellulase with 3.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd) or Mix (both enzymes at same dosage). RA: 7- and 11-wk of regrowth.

DM: dry matter; LA:AA: lactic-to-acetic acid ratio.

SEM: greatest standard error of the mean.

Table 4. Nutrient composition and losses of dry matter and effluent of mombasa grass silage (n = 24) treated with fibrolytic enzymes and harvested at different regrowth ages after 60 days of storage.

		Enzyme Application				RA General	P-value			
Item[1]	Regrowth Age	Control	Xylanase	Cellulase	Mix	mean	SEM	ENZ	RA	ENZ × RA
Dry matter losses, g/kg DM	7 wk	10.4	9.96	11.5	12.2	11.0^{B}				
	11 wk	15.8	14.6	15.9	13.8	15.0^{A}	1.97	0.92	0.01	0.78
	ENZ General mean	13.1	12.3	13.6	12.3					
Effluent, kg/t fresh silage	7 wk	63.7	59.6	85.1	91.8	75.1				
	11 wk	40.6	37.9	85.6	86.6	62.7	9.45	0.001	0.08	0.52
	ENZ General mean	52.2^{b}	48.8^{b}	85.4^{a}	89.2^{a}					
Dry matter, g/kg as fed	7 wk	197	197	199	198	198^{B}				
	11 wk	203	205	212	216	209^{A}	3.20	0.12	0.001	0.28
	ENZ General mean	200	201	205	207					
Ash, g/kg DM	7 wk	144	145	142	140	143^{A}				
	11 wk	120	127	122	117	121^{B}	2.88	0.14	0.001	0.72
	ENZ General mean	132	136	132	129					
Crude protein, g/kg DM	7 wk	63.0	64.3	68.0	67.3	65.7^{A}				
	11 wk	50.0	49.4	61.0	62.0	55.5^{B}	1.96	0.001	0.001	0.08
	ENZ General mean	56.5^{b}	56.8^{b}	64.5^{a}	64.6^{a}					
NDFap, g/kg DM	7 wk	628^{Ba}	603^{Ba}	606^{Aa}	614^{Aa}	613				
	11 wk	669^{Aa}	646^{Aab}	624^{Ab}	607^{Ab}	636	6.88	0.001	0.001	0.007
	ENZ General mean	649	624	615	611					
Acid detergent fiber, g/kg DM	7 wk	301	288	284	286	290				
	11 wk	303	289	282	277	288	6.29	0.02	0.72	0.82
	ENZ General mean	302^{a}	288^{ab}	283^{b}	283^{b}					
Lignin, g/kg DM	7 wk	46.9	46.3	54.4	39.3	47.0				
	11 wk	47.1	54.4	52.3	46.6	50.1	5.33	0.28	0.39	0.72
	ENZ General mean	47.0	50.4	53.3	43.0					
	7 wk	4.54	4.98	5.00	3.73	4.92^{A}	0.210	0.85	0.001	0.13

Water-soluble carbohydrates, g/kg DM	11 wk	4.14	3.76	4.01	3.73	3.91^{B}				
	ENZ General mean	4.34	4.37	4.51	4.45					
Acid detergent insoluble nitrogen, g/kg total nitrogen	7 wk	46.4^{Bab}	49.6^{Bab}	40.5^{Bb}	69.2^{Aa}	51.4				
	11 wk	93.0^{Aa}	88.5^{Aa}	70.7^{Aa}	76.4^{Aa}	82.2	5.24	0.02	0.001	0.01
	ENZ General mean	69.7	69.1	55.6	72.8					

[A,B,a,b] Different letters within a row with lowercase and within a column with uppercase are significantly different (Tukey test, α = 0.05).

[1] ENZ: control (distilled water), xylanase (400 mg/kg of dry matter weight, Smizyme xylanase with 10.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd), cellulase (1.000 mg/kg of fresh weight, Smizyme cellulase with 3.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd) or Mix (both enzymes at same dosage). RA: 7- and 11-wk of regrowth.

NDFap: neutral detergent fiber corrected for ash and protein.

SEM: greatest standard error of the mean.

Table 5. In vitro degradation parameters and digestibility at 24, 48 and 72h of dry matter and neutral detergent fiber of mombasa grass silage (*n* = 24) treated with fibrolytic enzymes and harvested at different regrowth ages after 60 days of storage.

		Enzyme Application				RA General	P-value			
Item[1]	Regrowth Age	Control	Xylanase	Cellulase	Mix	mean	SEM	ENZ	RA	ENZ × RA
	7 wk	176^{Aa}	186^{Aa}	198^{Aa}	187^{Aa}	187				
a, g/kg DM	11 wk	167^{Ab}	167^{Ab}	188^{Aab}	210^{Aa}	183	7.59	0.001	0.39	0.02
	ENZ General mean	172	176	193	198					
	7 wk	610	617	576	573	594				
b, g/kg DM	11 wk	582	626	580	589	594	23.3	0.19	0.99	0.74
	ENZ General mean	596	622	578	581					
	7 wk	25.4	26.6	25.0	31.0	27.0				
kd, g/kg per h	11 wk	25.7	28.1	25.7	27.4	26.7	3.98	0.56	0.90	0.83
	ENZ General mean	25.6	27.4	25.4	29.2					
	7 wk	479	496	477	519	493				
DMD 24h, g/kg	11 wk	465	505	478	509	489	20.3	0.09	0.79	0.90
	ENZ General mean	472	500	477	514					
	7 wk	611	636	618	648	628				
DMD 48h, g/kg	11 wk	606	622	616	619	616	11.6	0.15	0.14	0.64
	ENZ General mean	608	629	617	633					
	7 wk	674	674	676	670	674^{A}				
DMD 72h, g/kg	11 wk	629	668	668	672	659^{B}	11.5	0.08	0.03	0.06
	ENZ General mean	652	671	672	671					
	7 wk	645	656	630	639	643^{A}				
B, g/kg NDF	11 wk	607	633	607	632	620^{B}	11.0	0.14	0.01	0.60
	ENZ General mean	626	645	618	636					
	7 wk	349	352	383	374	365^{B}				
I, g/kg NDF	11 wk	392	369	403	383	387^{A}	14.0	0.03	0.01	0.40
	ENZ General mean	371^{ab}	361^{b}	393^{a}	379^{ab}					
λ, per h	7 wk	0.0668	0.0694	0.0650	0.0773	0.0696	0.0065	0.52	0.41	0.19

	11 wk	0.0777	0.0781	0.0680	0.0667	0.0727				
	ENZ General mean	0.0723	0.0737	0.0665	0.0720					
	7 wk	39.8	41.4	38.7	46.1	41.5				
kd, g/kg per h	11 wk	46.3	46.6	40.6	39.8	43.3	3.85	0.52	0.41	0.19
	ENZ General mean	43.1	44.0	39.7	43.0					
	7 wk	337	344	305	371	339				
NDFD 24h, g/kg	11 wk	370	381	316	310	344	26.0	0.24	0.78	0.24
	ENZ General mean	353	362	310	340					
	7 wk	521	531	491	549	523				
NDFD 48h, g/kg	11 wk	521	530	487	474	503	14.7	0.07	0.08	0.06
	ENZ General mean	521	531	489	512					
	7 wk	597	587	569	576	582^{A}				
NDFD 72h, g/kg	11 wk	565	583	544	547	560^{B}	8.57	0.01	0.002	0.35
	ENZ General mean	581^{ab}	585^{a}	557^{b}	562^{ab}					

[A,B,a,b] Different letters within a row with lowercase and within a column with uppercase are significantly different (Tukey test, α = 0.05).

[1] ENZ: control (distilled water), xylanase (400 mg/kg of dry matter weight, Smizyme xylanase with 10.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd), cellulase (1.000 mg/kg of fresh weight, Smizyme cellulase with 3.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd) or Mix (both enzymes at same dosage). RA: 7- and 11-wk of regrowth.

DM: dry matter; NDF: neutral detergent fiber; DMD: dry matter digestibility; NDFD: neutral detergent fiber digestibility; a: ruminal rapidly degradable fraction; b: ruminal slowly degradable fraction; kd: degradation rate constant of fraction “b”; I: undegradable fraction; λ: joint fractional rate of latency and degradation.

SEM: greatest standard error of the mean.

Table 6. Fermentation profile and microbial counts of elephant grass silage (n = 48) treated with fibrolytic enzymes and harvested at different regrowth ages after 60 days of storage.

		Enzyme Application				RA General	P-value			
Item[1]	Regrowth Age	Control	Xylanase	Cellulase	Mix	mean	SEM	ENZ	RA	ENZ × RA
	12 wk	3.44	3.50	3.39	3.42	3.44				
pH	16 wk	3.77	3.53	3.39	3.42	3.53	0.14	0.08	0.15	0.17
	ENZ General mean	3.60	3.39	3.51	3.42					
	12 wk	44.4	42.6	44.5	41.4	43.3^{B}				
Lactic acid, g/kg DM	16 wk	41.7	58.1	62.8	64.0	56.7^{A}	5.69	0.25	0.002	0.14
	ENZ General mean	43.1	50.4	53.7	52.7					
	12 wk	12.7	12.2	12.7	11.3	12.3^{B}				
Acetic acid, g/kg DM	16 wk	14.5	20.6	18.6	17.2	17.7^{A}	2.641	0.71	0.005	0.66
	ENZ General mean	13.6	16.4	15.6	14.3					
	12 wk	4.34	2.31	1.57	2.03	2.56				
Butyric acid, g/kg DM	16 wk	5.14	3.10	1.57	2.93	3.18	1.593	0.004	0.29	0.94
	ENZ General mean	4.74^{a}	2.71^{ab}	1.57^{b}	2.48^{b}					
	12 wk	2.76	3.40	2.72	2.75	2.91				
Propionic acid, g/kg DM	16 wk	3.93	3.83	3.17	2.95	3.47	0.48	0.36	0.10	0.77
	ENZ General mean	3.35	3.62	2.94	2.85					
	12 wk	12.5	14.1	12.4	13.7	13.2				
Ethanol, g/kg DM	16 wk	13.8	9.6	10.5	9.2	10.8	3.464	0.87	0.15	0.57
	ENZ General mean	13.1	11.9	11.4	11.5					
	12 wk	4.21	3.74	3.70	3.91	3.87				
LA:AA	16 wk	2.95	2.96	4.07	3.91	3.47	0.542	0.71	0.29	0.39
	ENZ General mean	3.58	3.35	3.88	3.86					
	12 wk	118^{Aa}	131^{Aa}	110^{Aa}	121^{Aa}	120				
NH_3-N, g/kg total nitrogen	16 wk	173^{Aa}	139^{Aab}	80.1^{Ab}	90.6^{Ab}	121	46.3	0.009	0.94	0.03
	ENZ General mean	145	135	95.1	106					
	12 wk	7.08	7.18	6.53	6.95	6.93	0.36	0.25	0.75	0.91

Lactic acid bacteria, cfu/g fresh silage	16 wk	7.47	7.11	6.61	6.88	7.02				
	ENZ General mean	7.28	7.14	6.57	6.91					
Mold, cfu/g fresh silage	12 wk	3.60	3.28	3.44	2.91	3.31				
	16 wk	3.39	3.11	2.54	3.01	3.01	0.24	0.11	0.09	0.21
	ENZ General mean	3.50	3.19	2.99	2.96					
Yeast, cfu/g fresh silage0	12 wk	3.28	3.66	3.60	3.59	3.53				
	16 wk	3.67	2.97	4.17	4.10	3.73	0.51	0.29	0.43	0.25
	ENZ General mean	3.47	3.32	3.89	3.85					

[A,B,a,b] Different letters within a row with lowercase and within a column with uppercase are significantly different (Tukey test, $\alpha = 0.05$).

[1] ENZ: control (distilled water), xylanase (400 mg/kg of dry matter weight, Smizyme xylanase with 10.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd), cellulase (1.000 mg/kg of fresh weight, Smizyme cellulase with 3.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd) or Mix (both enzymes at same dosage). RA: 12- and 16-wk of regrowth.

DM: dry matter; LA:AA: lactic-to-acetic acid ratio.

SEM: greatest standard error of the mean.

Table 7. Dry matter and effluent losses, aerobic stability, and fermentation parameters after 7d of air exposure of elephant grass silage (n = 48) treated with fibrolytic enzymes and harvested at different regrowth ages after 60 days of storage.

Item[1]	Regrowth Age	Enzyme Application				RA General mean	P-value			
		Control	Xylanase	Cellulase	Mix		SEM	ENZ	RA	ENZ × RA
Effluent, kg/t fresh silage	12 wk	61.4^{Aa}	66.5^{Aa}	67.3^{Aa}	68.3^{Aa}	65.9				
	16 wk	50.4^{Ab}	50.8^{Ab}	72.3^{Aa}	75.2^{Aa}	62.1	18.7	0.001	0.22	0.02
	ENZ General mean	55.9	58.6	69.8	71.7					
Dry matter losses, g/kg DM	12 wk	108	124	106	126	116^{A}				
	16 wk	90.9	101	108	99.4	100^{B}	30.7	0.57	0.03	0.52
	ENZ General mean	99.6	112	107	113					
Aerobic stability, h	12 wk	111^{Aa}	108^{Aa}	41.5^{Ab}	97.5^{Aa}	89.7				
	16 wk	131^{Aa}	149^{Aa}	44.4^{Ab}	40.5^{Bb}	91.1	27.7	0.001	0.84	0.001
	ENZ General mean	121	129	43.0	69.0					
pH 7d	12 wk	5.82^{Aa}	5.87^{Aa}	6.65^{Aa}	5.85^{Aa}	6.05				
	16 wk	5.15^{Ab}	4.47^{Ab}	6.79^{Aa}	7.25^{Aa}	5.91	1.27	0.001	0.67	0.02
	ENZ General mean	5.49	5.17	6.72	6.65					
Yeast 7d, cfu/g fresh silage	12 wk	6.98	7.17	8.96	7.85	8.09				
	16 wk	6.99	7.35	8.96	9.06	7.74	0.28	0.001	0.09	0.12
	ENZ General mean	6.99^{b}	7.26^{b}	8.96^{a}	8.46^{a}					
Mold 7d, cfu/g fresh silage	12 wk	7.18^{Aa}	7.26^{Aa}	7.29^{Aa}	6.83^{Ba}	7.14				
	16 wk	6.60^{Ab}	7.20^{Aab}	8.28^{Aa}	8.55^{Aa}	7.66	0.33	0.04	0.03	0.006
	ENZ General mean	6.89	7.23	7.78	7.69					
Water-soluble carbohydrates, g/kg DM	12 wk	4.04	4.02	5.67	5.05	4.69				
	16 wk	3.70	3.13	5.42	4.93	4.29	0.69	0.001	0.11	0.70
	ENZ General mean	3.87^{b}	3.57^{b}	5.55^{a}	4.99^{a}					
Dry matter losses 7d, g/kg DM	12 wk	46.7	37.6	61.8	41.4	46.9				
	16 wk	42.1	57.1	41.4	42.0	48.9	9.55	0.90	0.76	0.16
	ENZ General mean	44.4	47.3	51.6	48.3					
	12 wk	25.5	26.4	29.9	26.6	27.1	3.36	0.02	0.57	0.25

Maximum temperature, °C	16 wk	24.3	24.1	27.9	29.7	26.5
	ENZ General mean	24.9[b]	25.2[ab]	28.9[a]	28.2[a]	

[A,B,a,b] Different letters within a row with lowercase and within a column with uppercase are significantly different (Tukey test, α = 0.05).

[1] ENZ: control (distilled water), xylanase (400 mg/kg of dry matter weight, Smizyme xylanase with 10.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd), cellulase (1.000 mg/kg of fresh weight, Smizyme cellulase with 3.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd) or Mix (both enzymes at same dosage). RA: 12- and 16-wk of regrowth.

DM: dry matter.

SEM: greatest standard error of the mean.

Table 8. Nutrient composition elephant grass silage (n = 48) treated with fibrolytic enzymes and harvested at different regrowth ages after 60 days of storage.

Item[1]	Regrowth Ages	Enzyme Application				RA General mean	P-value			
		Control	Xylanase	Cellulase	Mix		SEM	ENZ	RA	ENZ × RA
Dry matter, g/kg as fed	12 wk	178	172	169	166	171^{B}				
	16 wk	225	227	229	231	228^{A}	11.6	0.97	0.001	0.61
	ENZ General mean	202	200	199	198					
Ash, g/kg DM	12 wk	74.7	78.1	79.9	80.8	78.4^{A}				
	16 wk	66.1	66.9	73.9	70.4	69.4^{B}	2.93	0.12	0.001	0.82
	ENZ General mean	70.4	72.5	76.9	75.6					
Crude protein, g/kg DM	12 wk	60.9	62.9	66.7	63.0	63.4^{A}				
	16 wk	53.5	54.2	58.2	57.7	55.9^{B}	2.37	0.15	0.001	0.89
	ENZ General mean	57.2	58.5	62.5	60.4					
NDFap, g/kg DM	12 wk	709	658	628	654	662^{B}				
	16 wk	736	721	667	703	707^{A}	10.5	0.001	0.001	0.41
	ENZ General mean	723^{a}	689^{b}	648^{c}	650^{c}					
Acid detergent fiber, g/kg DM	12 wk	323	320	311	313	317				
	16 wk	326	316	309	312	316	6.04	0.04	0.82	0.94
	ENZ General mean	325^{a}	318^{ab}	310^{b}	313^{b}					
Lignin, g/kg DM	12 wk	64.2	56.1	57.6	54.5	58.1^{B}				
	16 wk	69.1	65.4	67.6	68.7	67.7^{A}	2.94	0.15	0.001	0.40
	ENZ General mean	66.6	60.8	62.6	61.6					
Water-soluble carbohydrates, g/kg DM	12 wk	5.69^{Ab}	5.23^{Ab}	23.9^{Aa}	27.1^{Aa}	16.5				
	16 wk	3.82^{Aa}	4.61^{Aa}	14.6^{Ba}	11.1^{Ba}	7.49	5.42	0.001	0.001	0.009
	ENZ General mean	4.75	4.92	19.2	19.1					
Acid detergent insoluble nitrogen, g/kg total nitrogen	12 wk	67.9	43.1	40.3	40.5	48.0^{B}				
	16 wk	71.1	64.9	55.3	59.9	62.9^{A}	13.2	0.001	0.001	0.29
	ENZ General mean	69.5^{a}	54.0^{b}	47.8^{b}	50.3^{b}					

[A,B,a,b] Different letters within a row with lowercase and within a column with uppercase are significantly different (Tukey test, $\alpha = 0.05$).

[1] ENZ: control (distilled water), xylanase (400 mg/kg of dry matter weight, Smizyme xylanase with 10.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd), cellulase (1.000 mg/kg of fresh weight, Smizyme cellulase with 3.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd) or Mix (both enzymes at same dosage). RA: 12- and 16-wk of regrowth.

NDFap: neutral detergent fiber corrected for ash and protein; DM: dry matter.

SEM: greatest standard error of the mean.

Table 9. In vitro degradation parameters and digestibility at 24, 48 and 72h of dry matter and neutral detergent fiber of elephant grass silage (n = 24) treated with fibrolytic enzymes and harvested at different regrowth ages after 60 days of storage.

		Enzyme Application				RA General	P-value			
Item[1]	Regrowth Age	Control	Xylanase	Cellulase	Mix	mean	SEM	ENZ	RA	ENZ × RA
	12 wk	126^{Ac}	171^{Ab}	200^{Aa}	180^{Ab}	170				
a, g/kg DM	16 wk	133^{Ac}	152^{Bb}	193^{Aa}	179^{Aa}	164	5.00	0.001	0.03	0.03
	ENZ General mean	131	161	197	180					
	12 wk	528^{Aab}	549^{Aa}	481^{Abc}	469^{Ac}	507				
b, g/kg DM	16 wk	483^{Aa}	498^{Ba}	458^{Aa}	479^{Aa}	479	12.44	0.001	0.002	0.03
	ENZ General mean	506	523	470	474					
	12 wk	36.5	35.3	36.4	36.1	36.1^{A}				
kd, g/kg per h	16 wk	31.8	31.3	31.4	27.5	30.5^{B}	3.53	0.80	0.009	0.82
	ENZ General mean	34.1	33.3	33.9	31.2					
	12 wk	480	530	519	500	507^{A}				
DM 24h, g/kg	16 wk	412	439	451	426	432^{B}	17.89	0.04	0.001	0.82
	ENZ General mean	446	484	485	463					
	12 wk	586	629	613	565	598^{A}				
DM 48h, g/kg	16 wk	525	546	554	547	543^{B}	10.66	0.01	0.001	0.055
	ENZ General mean	556^{b}	587^{a}	584^{ab}	556^{b}					
	12 wk	610^{Ab}	676^{Aa}	637^{Aab}	601^{Ab}	631				
DM 72h, g/kg	16 wk	565^{Ba}	585^{Ba}	590^{Ba}	586^{Aa}	581	10.26	0.001	0.001	0.003
	ENZ General mean	588	630	613	594					
	12 wk	565^{Ab}	619^{Aa}	571^{Aab}	513^{Ac}	567				
B, g/kg NDF	16 wk	502^{Ba}	509^{Ba}	495^{Ba}	509^{Aa}	504	13.37	0.001	0.001	0.001
	ENZ General mean	534	565	533	564					
	12 wk	454^{Bb}	400^{Bc}	476^{Aab}	512^{Aa}	460				
I, g/kg NDF	16 wk	507^{Aa}	488^{Aa}	510^{Aa}	484^{Aa}	497	13.97	0.001	0.001	0.001
	ENZ General mean	480	444	493	498					
λ, per h	12 wk	0.0875	0.0909	0.0898	0.0903	0.0896^{A}	0.00901	0.51	0.004	0.48

	16 wk	0.0800	0.0845	0.0767	0.0698	0.0778^{B}				
	ENZ General mean	0.0838	0.0877	0.0833	0.0801					
	12 wk	52.2	54.2	53.5	53.9	53.4^{A}				
kd, g/kg per h	16 wk	47.7	50.4	45.8	41.6	46.4^{B}	5.39	0.51	0.004	0.48
	ENZ General mean	50.0	52.3	49.6	47.7					
	12 wk	358	395	345	300	349^{A}				
NDF 24h, g/kg	16 wk	289	321	283	275	292^{B}	27.32	0.006	0.001	0.47
	ENZ General mean	323^{ab}	358^{a}	314^{ab}	287^{b}					
	12 wk	495^{Aab}	526^{Aa}	475^{Aab}	430^{Ab}	482				
NDF 48h, g/kg	16 wk	425^{Aa}	447^{Ba}	418^{Aa}	443^{Aa}	433	15.90	0.03	0.001	0.04
	ENZ General mean	460	486	447	437					
	12 wk	525^{Aab}	585^{Aa}	510^{Ab}	470^{Ab}	522				
NDF 72h, g/kg	16 wk	485^{Aa}	487^{Ba}	473^{Aa}	476^{Aa}	480	16.66	0.003	0.001	0.01
	ENZ General mean	505	536	491	473					

^{A,B,a,b,c} Different letters within a row with lowercase and within a column with uppercase are significantly different (Tukey test, α = 0.05).

[1] ENZ: control (distilled water), xylanase (400 mg/kg of dry matter weight, Smizyme xylanase with 10.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd), cellulase (1.000 mg/kg of fresh weight, Smizyme cellulase with 3.000 U/g, Beijing Smile Feed Sci. & Tech. Co., Ltd) or Mix (both enzymes at same dosage). RA: 12- and 16-wk of regrowth.

DM: dry matter; NDF: neutral detergent fiber; DMD: dry matter digestibility; NDFD: neutral detergent fiber digestibility; a: ruminal rapidly degradable fraction; b: ruminal slowly degradable fraction; kd: degradation rate constant of fraction “b”; I: undegradable fraction; λ: joint fractional rate of latency and degradation.

SEM: greatest standard error of the mean.

CHAPTER II

Effect of microbial inoculation, storage temperature and storage length on nutrient composition, fermentation profile and aerobic stability of whole-plant corn silage

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ABSTRACT

This study evaluated the effects of a heterofermentative inoculant, storage temperature and storage length on fermentation profile, aerobic stability, and nutrient composition of whole-

plant corn silage. The experiment was a completely randomized design with a 2 (microbial inoculation) × 2 (storage temperature) × 4 (storage length) factorial arrangement of treatments. Corn forage was either inoculated with distilled water (CON) or with 300,000 CFU/g of fresh forage of *Pediococcus acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074 (LBLD). Silos were stored for 7, 15, 30 or 90 d at either 22 (NT) or 40°C (HT) for the entire storage length (SL) in 20 L laboratory silos. Interactions between MI × ST as well as ST × SL were observed for pH ($P = 0.02$ and $P = 0.001$, respectively) and LAB ($P = 0.03$ and $P = 0.001$, respectively), and CON had a lower pH compared to LBLD. Additionally, pH had a greater decrease at 30 d for NT than HT. An interaction between ST × SL ($P = 0.001$) was observed for lactic acid concentration, which was greater for NT at 30 d and 90 d of storage. Three-way interactions were observed ($P = 0.001$) for acetic acid and 1-propanol concentrations, and aerobic stability, which LBLD-22 was greater than other treatments at 90 d. Soluble crude protein and ammonia-N concentrations, and in vitro starch digestibility increased ($P < 0.05$) with SL and were greater ($P < 0.05$) for silage stored at HT. After aerobic exposure, mold and yeast counts increased ($P = 0.001$) for silage stored at HT, indicating greater spoilage and deterioration. Greater temperature negatively impacted fermentation profile and increased spoilage after aerobic exposure, whereas the heterofermentative inoculant enhanced aerobic stability. However, improvements in fermentation profile and aerobic stability with microbial inoculation were alleviated when silage was stored at high temperatures.

Keywords: aerobic stability, storage temperature, *L. buchneri*, *L. diolivorans*, 1,2-propanediol.

INTRODUCTION

Inoculants composed of obligate heterofermentative lactic acid bacteria (LAB) are widely recommended to prevent spoilage and reduce nutrient losses of whole-plant corn silage (WPCS) once silos have been opened and exposed to oxygen (Arriola et al., 2021). These bacteria can produce both lactic acid and antifungal acids (i.e. acetic and propionic acid), which inhibit the growth of yeast and mold; hence, improving aerobic stability (Oude Elferink et al., 2001; Danner et al., 2003). *Lentilactobacillus buchneri*, the most commonly used heterofermentative LAB in silage inoculants, enhances aerobic stability by partially converting lactic into acetic acid and 1,2-propanediol (Oude Elferink et al., 2001). Recently, the use of another heterofermentative bacteria, *Lentilactobacillus diolivorans*, has been proposed for silage inoculants. This bacterium can convert 1,2-propanediol into propionic acid, which is also an antifungal compound (Moon, 1983), or 1-propanol (Krooneman et al., 2002). Together, these two bacteria could complement each other by modulating fermentation and enhancing the aerobic stability of WPCS more than either alone (Diepersloot et al., 2022). Generally, heterofermentative species require a longer storage period to effectively modulate silage fermentation. While recent studies have shown that acetic acid production, yeast suppression, and improvements in aerobic stability can begin as early as 7–15 d of storage (Fernandes et al., 2020; Saylor et al., 2020a; Arriola et al., 2021), these effects are typically more pronounced after extended fermentation of 30 to 60 d or longer (Muck et al., 2018; Ferrero et al., 2021; Arriola et al., 2021).

Exposure to high temperatures (HT) during ensiling and fermentation can also influence corn silage fermentation patterns (Windle and Kung, 2016; Bai et al., 2022). While it is not feasible to control environmental temperatures during the fermentation of silage, understanding the potential negative effects caused by high temperatures is crucial. According to Wilkinson and Muck (2019), temperatures beyond 40°C are expected to become more common in the

future, highlighting the need for studies that investigate fermentation patterns and aerobic spoilage under these conditions. However, research on the use of heterofermentative microbial inoculants in WPCS at high temperatures is limited and further studies are warranted.

Therefore, this study aimed to evaluate the effects of microbial inoculation, storage temperature, and storage length on the fermentation profile, microbial counts, nutrient composition, and aerobic stability of WPCS. Our hypothesis was that greater storage temperatures would be associated with impaired fermentation, reduced aerobic stability, and greater spoilage after aerobic exposure. Additionally, our hypothesis was that microbial inoculation with a combination of *L. buchneri*, *L. diolivorans* and *P. acidilactici* would increase acetic and propionic acid concentrations, as well as aerobic stability, with these effects becoming more pronounced over longer storage periods.

MATERIAL AND METHODS

Whole-plant corn forage (9658-32, Stine Seed, Adel, IA) was obtained from the University of Wisconsin-Madison Arlington Agricultural Research Station (Arlington, WI) on September 21, 2023 at approximately 40% DM. The forage was harvested using a self-propelled forage harvester (Claas of America, Omaha, NE) set to a theoretical cut length of 26.5 mm, with a kernel processor roll gap of 2 mm. Corn silage was cut at a height of 25 cm from four randomly locations (block) selected within a field. After harvest, forage from each location (block) was homogenized, and subsamples (n = 4) were collected for nutrient characterization and microbial counts.

Subsamples of approximately 15 kg each from each location were collected and assigned to 1 of 16 treatments which were a combination of two microbial inoculants, two storage temperatures and four storage lengths. Microbial inoculation treatments were a heterofermentative microbial inoculant containing a combination of *Pediococcus acidilactici*

DSM 16243, *L. buchneri* DSM 12856, and *L. diolivorans* DSM 32074 (LBLD; 300,000 CFU/g of whole-plant corn forage) or the same amount of distilled water (CON), applied manually with spray bottles and hand-mixed immediately prior to ensiling. Each laboratory silo was treated individually. Inoculation rates were based on manufacturer recommendations. Whole-plant corn silage was packed into 20 L plastic buckets (Uline, Kenosha, WI, United States) to a density of approximately 295 kg of DM/m^3 and sealed with lids and tape. The silos were weighed and randomly assigned to be stored at either normal (air-conditioned room) temperature (NT; 22 ± 1.52°C) or a high (warm room) temperature (HT; 40 ± 2.12°C), until reaching their assigned storage length (7, 15, 30 or 90 d). This experiment consisted of 16 treatments combinations (2 storage temperatures, 2 microbial inoculants, 4 storage lengths) in quadruplicate for a total of 64 mini silos.

Approximately 50 g of representative fresh samples were dried in duplicate at 60°C for 48 h in a forced air oven to determine DM concentration, while approximately 100 g of representative fresh samples in triplicate were frozen for later analysis. An additional 250 g of fresh samples was frozen to save as a backup. For microbial counts, 20 g of undried and unground sample was homogenized and diluted 10-fold (mass basis) in 0.1% peptone water (Oxoid CM0090, Blasingstoke, UK). The mixture was blended for 60 s in a high-speed blender, and then filtered through two layers of cheesecloth to collect the silage extract. The extract was divided into 2 subsamples. The first subsample was used to determine pH in duplicate using a pH electrode (Accumet AP85 Portable Waterproof; Thermo Fisher Scientific Inc., Waltham, MA). The second subsample was placed in a 50 mL plastic tube for microbial enumeration. Lactic acid bacteria, yeast, and mold were counted using a pour plating method with a 10-fold serial dilution on malt agar (Difco 211220, Thermo Fisher Scientific Inc., Waltham, MA) acidified with 85% lactic acid for yeast and molds counts, and on Man, Rogosa, Sharpe agar

(Oxoid, Blasingstoke, UK) for LAB counts. Agar plates were incubated at 35°C for 48 h for LAB, while yeast and mold were evaluated on the same plate after 72 h and 120 h, respectively.

Frozen fresh samples were submitted to Dairyland Laboratories Inc. (Arcadia, WI). Samples were dried as previously described and ground to 1-mm using a Wiley mill (Thomas Scientific, Swedesboro, NJ). Subsequently, samples were analyzed by near-infrared reflectance spectroscopy (NIRS) using a Foss 5000 (Foss North America Inc, Eden Prairie, MN). Calibration equations, based on wet chemistry procedures, were used to determine ash (method 942.05; AOAC, 2005), crude protein (CP, method 990.03; AOAC, 2012), neutral detergent fiber treated with α-amylase and sodium sulfite, corrected for residual ash (aNDFom; method 2002.04, AOAC, 2005), ether extract (EE, method 920.39; AOAC, 2005), water-soluble carbohydrates (WSC; Deriaz, 1961), 240 h in vitro ruminal undigested NDF (uNDF; Huhtanen et al., 1994) and starch concentrations (Vidal et al., 2009). In vitro starch disappearance after 7h (StarchD; Richards et al., 1995) and the in vitro digestibility of NDF after 30 h (NDFD) were determined using methods adapted from Goering and Van Soest (1970), with residue analyzed for aNDFom concentration by NIRS as described. Additionally, borate-phosphate soluble crude protein (SCP) was determined by wet chemistry (Krishnamoorthy et al., 1982).

Silos were weighed at the time of closure and opening, for quantification of fermentative DM losses. Dry matter losses during fermentation were calculated by the difference in weight (WD) between the beginning and end of fermentation by the following equation proposed by Weissbach (2005):

$$DML[\%] = 100\frac{WD[g]}{DMensiled[g]} + 2.5 \quad (1)$$

When laboratory silos reached their assigned storage lengths, they were opened, and the top 5 cm of silage discarded. The remaining material in each silo was mixed, and representative subsamples were collected and frozen for later analysis, as described previously. Following subsample collection, the remaining silage from each laboratory silo was assessed for aerobic

stability. Two layers of cheesecloth was placed over each bucket to prevent drying, and aerobic stability was measured by placing two wireless temperature sensors (HOBO® temperature data logger 64k; Onset Computer Corporation, Cape Cod, MA) in the geometric center of each bucket that contained approximately 3 kg of silage. The sensors recorded temperatures every 30 min for 240 h. The environmental temperature during the aerobic stability assay matched the ST (22 or 40°C) depending on the ST treatment. Aerobic stability was defined as the time (h) before silage temperature increased by 2°C above the baseline temperature. For silage stored for 15, 30 and 90 d, additional 3.78 L buckets with approximately 2.5 kg of silage were placed and sampled after 0, 2, 4, 6, 8, and 10 d of exposure to air to monitor changes in microbial counts and pH during aerobic exposure. Silage samples collected during aerobic stability were frozen for later analysis of pH, yeast counts, and mold counts which were evaluated as described previously.

Frozen silage samples were sent to Dairyland Laboratories Inc. (Arcadia, WI) to evaluate lactic acid and ammonia-N concentrations. Lactic acid was measured by extracting silage fluid: 15 g of silage was blended with 200 mL of distilled water for 2 min, the filtrate was then centrifuged at 4°C and 21,500 g for 20 min, and the supernatant was analyzed using the method of Canale et al. (1984) with a high-performance liquid chromatograph (L7485, Hitachi, Tokyo, Japan) equipped with an UV detector (Spectroflow 757, ABI Analytical Kratos Division, Ramsey, NJ). Ammonia nitrogen (ammonia-N) was evaluated using colorimetry using a Technicon Automatic Analyzer (RFA-300, Alpkem Corporation, Wilsonville, OR), following a method adapted from Noel and Hambleton (1976).

Additional frozen samples were sent to the Laboratory of Food Analysis and Animal Nutrition (State University of Maringá, Maringá, PR, Brazil) for the analysis of volatile fatty acids, alcohols and esters, following the method described by Moraes et al. (2023). Briefly, an undiluted portion of the aqueous extract was centrifuged at 10,000g for 15 min, and the

supernatant was analyzed using a gas chromatograph (Nexis GC-2030, Shimadzu, Kyoto, Japan) with an autoinjector (AOC-20i Plus, Shimadzu, Kyoto, Japan) and a Stabilwax capillary column (Restek, Bellefonte, PA; 60 m, 0.25 mm ø, 0.25 μm polyethylene glycol crossbond carbowax). Compounds were identified by their retention times and quantified using external standards.

Samples of WPCS were analyzed to evaluate the effects of microbial inoculation, storage temperature and storage length. Laboratory silos were experimental units. Microbial data were $\log_{10}$-transformed before statistical analysis and presented as $\log_{10}$ values. Data were analyzed using a randomized complete block design with a 2 (microbial inoculant) × 2 (storage temperature) × 4 (storage length) factorial arrangement of treatments. Location (replications were four different locations within an experimental field) was considered a blocking factor. Analysis was performed using PROC GLIMMIX in SAS (version 9.4; SAS Institute Inc., Cary, NC). Fixed effects included microbial inoculation, storage temperature and storage length, and their two- and three-way interactions, while block (location) was the random effect. A split-plot design in time was used to analyze the samples collected during aerobic exposure (AE; yeast counts, mold counts, and pH). Data from 15, 30 and 90 d of storage length were analyzed separately. Buckets from each storage temperature and microbial inoculation combination were considered the main plot (which included main effects and their interaction), while the day that samples were collected during aerobic stability test was treated as the subplot. The storage temperature, microbial inoculation, day of aerobic exposure, and all their interactions were considered fixed effects, and location (replicate) was treated as a random effect. Means were determined using the LSMEANS statement and compared with the sequentially rejective Bonferroni *t*-test adjustment following an overall significant *F*-test. If a three-way interaction was detected ($P \leq 0.05$), effects were partitioned by storage length using the SLICE option. Statistical significance was declared at $P \leq 0.05$. Two-way interactions are reported and

discussed only if three-way interactions were not significant (P > 0.05). Main effects are presented and discussed if no interaction effects were significant (P > 0.05).

Regression analyses were performed using the REG procedure of SAS, to explore relationships between volatile organic compounds in silage fermentation. The sum of ethyl lactate and ethyl acetate concentrations was regressed on ethanol concentration; and concentration of 2,3-butanediol was regressed on 1-propanol concentration.

RESULTS

Nutrient composition, pH, and microbial counts for both fresh, uninoculated WPCS are presented in Table 1.

Fermentation profile

Probability significance values (P-values) for storage temperature, microbial inoculation, storage length, and their two- and three-way interactions for fermentation profile are presented in Table 2. Some organic volatile compounds were detected only in specific treatments and only at 90 d of storage: acetone in LBLD-40 (28.0 mg/kg DM of average) and 2-butanol and n-propyl acetate in LBLD-22 (21.7 and 30.5 mg/kg DM on average, respectively). Propionic and iso-butyric acids were analyzed but not detected in any treatment. Valeric acid concentration was below the detection limit of 30.0 mg/kg DM for all treatments. Thus, these variables will not be presented or discussed.

An interaction between MI × ST as well as an interaction between ST × SL were observed for pH (Figure 1A; P = 0.02 and P = 0.001, respectively). For the interaction between MI × ST, the pH was greatest for LBLD-40 (3.94), intermediate for LBLD-22 (3.89), and lowest for CON-22 and CON-40 (3.83 and 3.84, respectively). For the interaction between ST × SL, pH was greater for HT than NT at 30 d (3.97 vs. 3.85) and 90 d of storage (3.79 vs. 3.65), but not at 7 d (3.94, on average) or 15 d (3.92, on average). Likewise, an interaction between MI ×

ST or between ST × SL were observed for concentration of total acids (Figure 1B; *P* = 0.002 and *P* = 0.001, respectively). For the interaction between MI × ST, total acids concentration was greatest for LBLD-22 (7.20% DM), intermediate for CON-22 (6.81% DM), and lowest for LBLD-40 and CON-40 (5.93% DM, on average). For the interaction between ST × SL, total acids concentration was greater for NT than HT at 15 d (6.56 vs. 5.87% DM), 30 d (7.08 vs. 5.81% DM) and 90 d of storage (8.65 vs. 6.45% DM), but not at 7 d (4.79% DM, on average).

An interaction between ST × SL was observed for lactic acid concentration (Figure 1C; *P* = 0.001), which was greater for NT than HT at 30 d (4.74 vs. 3.79% DM) and 90 d (5.52 vs. 4.25% DM), but not at 7 d (3.91% DM, on average) or 15 d (4.19% DM, on average). A three-way interaction was observed for acetic acid concentration (Figure 1D; *P* = 0.001); at 90 d of storage, LBLD-22 was greater (4.39% DM), than CON-22, CON-40 and LBLD-40 (2.34% DM, on average), but no differences among treatments were observed at 7, 15 and 30 d. Likewise, a three-way interaction was detected for LA:AA ratio (Figure 1E; *P* = 0.003), which a lower ratio for LBLD-22 (1.10) than LBLD-40, CON-22, CON-40 (2.03, on average), was observed at 90 d of storage, but not at 7, 15 or 30 d. Similarly, a three-way interaction was detected for aerobic stability (Figure 1F; *P* = 0.001), which at 90 d was greatest for LBLD-22 (240 h) than LBLD-40, CON-22, and CON-40 (71 h, on average), but not at 7 d (45 h, on average), 15 d (85 h, on average), or 30 d of storage (88 h, on average).

Ethanol concentration was 0.3 percentage-units lower (Figure 2A; *P* = 0.001) for HT compared to NT. Ethanol concentration was also 0.1 percentage-units lower (*P* = 0.02) for 7 d compared to 90 d of storage, but not than 15 d and 30 d of storage (0.9% DM, on average). An interaction between MI × ST as well as an interaction between ST × SL were observed for methanol concentration (Figure 2B; *P* = 0.02 and *P* = 0.03, respectively). For the interaction between MI × ST, methanol concentration was greatest for LBLD-22 and LBLD-40 (145 and 142 mg/kg DM, respectively), intermediate for CON-40 (139 mg/kg DM) and lowest for CON-

22 (128 mg/kg DM). Despite observing an interaction between ST × SL, no differences among treatments were observed after Bonferroni corrections.

A three-way interaction was detected for 1-propanol concentration (Figure 2C; P = 0.001), which was greater for LBLD-22 (0.536 vs. 0.004% DM, on average) than other treatments at 90 d, but not at 7, 15 or 30 d. A two-way interaction of MI × ST was detected (Figure 2D; P = 0.001) for 1,2-propanediol concentration, which was greatest for LBLD-40 (0.102% DM), intermediate for LBLD-22 (0.043% DM), and lowest for CON-40 (0.009% DM), while CON-22 (0.032% DM) did not differ from either LBLD-22 and CON-40. The concentration of 1,2-propanediol was 0.046 percentage-units lower (P = 0.005) for 7 d compared to 90 d, but not 15 d and 30 d of storage (0.045% DM, on average).

An interaction between ST × SL was observed for ethyl acetate concentration (Figure 3A; P = 0.004), which was greater for NT than HT at 7 d (62.9 vs. 26.5 mg/kg DM), 30 d (78.9 vs. 31.1 mg/kg DM) and 90 d of storage (81.9 vs. 24.2 mg/kg DM), but not at 15 d of storage (48.5 mg/kg DM, on average). A two-way interaction of ST × SL was also detected for ethyl lactate concentration (Figure 3B; P = 0.001), which was greater for NT than HT at 15 d (104 vs. 67mg/kg DM), 30 d (139 vs. 81 mg/kg DM), and 90 d of storage (225 vs. 120 mg/kg DM), but not at 7 d (18 mg/kg DM, on average). Three-way interactions were observed for concentrations of 2,3-butanediol (Figure 3C; P = 0.001) and n-butyric acid (Figure 3D; P = 0.001). The 2,3-butanediol concentration was greater for LBLD-22 than other treatments (2963 vs. 941 mg/kg DM, on average) at 90 d, but did not differ at 7, 15 and 30 d. For n-butyric acid, at 7 d of storage, LBLD-40 had the greatest concentration (33.0 mg/kg DM), CON-40 and LBLD-22 intermediate (20.5 and 17.7 mg/kg DM, respectively), and CON-22 the lowest (8.7 mg/kg DM). At 15 d, LBLD-40 also had the greatest concentration (32.2 mg/kg DM), CON-40 the intermediate (25.2 mg/kg DM), and CON-22 the lowest (14.5 mg/kg DM), while the LBLD-22 (22.0 mg/kg DM) did not differ from CON-40 and LBLD-40. At 30 d, LBLD-40 (36.7 mg/kg

DM) and CON-40 (35.0 mg/kg DM) concentrations were greater than LBLD-22 (21.2 mg/kg DM) and CON-22 (18.2 mg/kg DM). Again, LBLD-40 had the greatest concentration (58.7 mg/kg DM) at 90 d, CON-40 the intermediate (37.5 mg/kg DM), and CON-22 and LBLD-22 the lowest (22.2 and 17.0 mg/kg DM, respectively). Positive relationships between concentrations of ethyl lactate + ethyl acetate and ethanol concentration (R^2 = 0.58, P = 0.001; Figure 4A) as well as 2,3-butanediol and 1-propanol concentrations (R^2 = 0.88; P = 0.001; Figure 4B) were observed.

Two-way interactions between MI × ST and ST × SL were observed for LAB counts (Figure 5A; P = 0.001 and P = 0.03, respectively). For the interaction between MI × ST, LAB counts were greatest for CON-22 (8.11 CFU/g) and LBLD-22 (8.33 CFU/g), intermediate for LBLD-40 (5.55 CFU/g), and lowest for CON-40 (5.02 CFU/g). For the interaction of ST × SL, LAB counts were lower for HT than NT at all storage lengths, but the magnitude of the difference increased at 30 d and 90 d of storage. Mold counts were greater (Figure 5C; P = 0.001) for HT (1.96 CFU/g) compared to NT (1.48 CFU/g). In addition, mold counts were lower (P = 0.001) at 90 d (1.79 CFU/g) than 15 d and 30 d of storage (2.55 CFU/g, on average). Mold counts were not detected at 7 d.

A three-way interaction was observed for yeast counts (Figure 5B; P = 0.001). At 15 d of storage, CON-40 (5.05 CFU/g) and LBLD-40 (3.88 CFU/g) had greater yeast counts than CON-22 (2.25 CFU/g) and LBLD-22 (1.96 CFU/g). At 30 d of storage, the yeast counts were greater for CON-22 (6.44 CFU/g) than CON-40, LBLD-22 and LBLD-40 (3.37 CFU/g, on average). No differences were observed at 7 d (7.44 CFU/g, on average) and 90 d of storage (2.27 CFU/g, on average). Dry matter losses were lower (Figure 5D; P = 0.001) at 7 d (1.86% DM) than 15, 30 and 90 d (4.20% DM, on average).

Nutrient composition

Probability significance values (*P*-values) for storage temperature, microbial inoculation, storage length, and their two- and three-way interactions for nutrient composition is in Table 3.

The DM concentration was 0.5 percentage-units greater (Figure 6A; *P* = 0.03) for LBLD than CON. In addition, the concentration of DM was greater (*P* = 0.001) at 7 d (42.1%) than at 15 d, 30 d and 90 d of storage (40.7%, on average). A two-way interaction between ST × SL was observed for WSC (Figure 6B; *P* = 0.001). Water-soluble carbohydrates concentration was lower for NT than HT at 15 d (2.58 vs. 3.19% DM), 30 d (2.60 vs. 3.46% DM) and 90 d of storage (2.22 vs. 3.60% DM), but not at 7 d of storage (3.10% DM, on average). A three-way interaction was observed (Figure 6C; *P* = 0.05) for CP, but no differences among treatments were observed at any SL after Bonferroni corrections. The concentration of SCP was greater (Figure 6D; *P* = 0.001) for HT than NT (50.9 vs. 58.2% CP). Soluble CP concentration also increased by 17.9 percentage-units as SL increased from 7 to 90 d. A two-way interaction between MI × ST was observed for ammonia-N concentration (Figure 6E; *P* = 0.001); ammonia-N concentration was greatest for LBLD-40 (5.82% N), intermediate for CON-40 (5.18% N), and lowest for LBLD-22 and CON-22 (3.59% N, on average). In addition, a two-way interaction of ST × SL was observed for ammonia-N concentration, which was greater for HT than NT at 7 d (3.55 vs. 2.42% N), 15 d (4.61 vs. 3.06% N), 30 d (5.93 vs. 3.54% N) and at 90 d of storage (7.93 vs. 5.36% N), but the magnitude of this effect was greater as storage length progressed. An interaction between MI × ST was observed for aNDFom concentration (Figure 6F; *P* = 0.001). The concentration of aNDFom was 2.3 percentage-units lower for LBLD-40 compared to CON-40 and LBLD-22, while CON-22 did not differ from any other treatment combination. In addition, an interaction of ST × SL (*P* = 0.01) was detected for aNDFom

concentration, but no differences among ST were detected at any SL after the Bonferroni corrections.

In vitro ruminal NDF digestibility was greater (Figure 7A; *P* = 0.02) at 90 d (59.8% NDFom) than 7 d (58.3% NDFom), while 15 and 30 d did not differ from other treatments (59.4% NDFom, on average). A two-way interaction of MI × ST was observed for uNDF concentration (Figure 7B; *P* = 0.001), which was lowest for LBLD-40 (7.91% vs. 8.69% uNDF, on average). Although an effect was observed for SL (*P* = 0.04), no differences among treatments were observed after Bonferroni corrections. Interactions between MI × ST and ST × SL were observed for starch concentration (Figures 7C; *P* = 0.001 and *P* = 0.05, respectively). For the interaction between MI × ST, starch concentration was greater for LBLD-40 and CON-22 (43.4 and 42.5% DM, respectively) than CON-40 (40.5% DM), while LBLD-22 (41.4% DM) did not differ from any other treatment. Despite observing an interaction between ST × SL, no differences among ST were detected at any SL after Bonferroni corrections. Greater StarchD was observed (Figure 7D; *P* = 0.001) for HT than NT (71.8 vs. 69.3% starch). Starch digestibility also increased (*P* = 0.001) with SL from 67.9% at 7 d to 73.9% starch at 90 d of storage. A two-way interaction of MI × ST was detected (Figure 7E; *P* = 0.01) for ash concentration, which was lowest for LBLD-40 (3.35% vs. 3.56% DM, on average). Ash concentration increased (*P* = 0.001) by 0.32 percentage-units as SL progressed. Two-way interactions of ST × SL and MI × ST (Figure 7F; *P* = 0.008 and *P* = 0.007, respectively) were observed for EE concentration, but no differences among treatments were observed for any treatments after Bonferroni corrections.

Aerobic exposure measurements

Probability significance values (*P*-values) for storage temperature, microbial inoculation, aerobic exposure, and their two- and three-way interactions for nutrient composition is in Table 4.

At 15 d of storage, a two-way interaction between ST × AE was observed for pH (Figure 8A; $P = 0.001$). The pH was greater at 4 d of aerobic exposure for HT (5.01) than NT (3.94), while no differences were observed at 0 d (3.85, on average), 2 d (3.90, on average), 6 d (5.33, on average), 8 d (5.87, on average), and 10 d of aerobic exposure (6.02, on average). A two-way interaction between ST × AE was also observed for yeast counts (Figure 8B; $P = 0.001$) with greater counts observed for HT than NT at 2 d (6.53 vs. 2.45 CFU/g), 4 d (8.77 vs. 3.11 CFU/g), 6 d (8.95 vs. 4.72 CFU/g), and at 8 d (8.58 vs. 4.34 CFU/g), but not at 0 d (2.89 CFU/g, on average) or 10 d of aerobic exposure (6.22 CFU/g, on average). Likewise, a two-way interaction (ST × AE) was observed for mold counts (Figure 8C; $P = 0.001$) with greater mold counts observed for HT than NT at 2 d (5.78 vs. 2.43 CFU/g), 4 d (7.39 vs. 2.41 CFU/g), 6 d (7.39 vs. 2.90 CFU/g), 8 d (8.27 vs. 3.02 CFU/g), and 10 d of AE (8.74 vs. 3.69 CFU/g), but not 0 d (2.89 CFU/g, on average).

At 30 d of storage, a three-way interaction was observed for pH (Figure 9A; $P = 0.003$), but no differences among treatments were observed after Bonferroni corrections. A three-way interaction was also observed for mold counts (Figure 9B; $P = 0.003$). At 8 d of aerobic exposure, greater mold counts were observed for CON-40 than other treatments (8.05 vs. 3.40 CFU/g, on average), but no differences were observed for other days of AE.

At 90 d of storage, a three-way interaction was detected for pH (Figure 10A; $P = 0.001$), with similar counts at 0 d (3.77, on average), 2 d (3.76, on average), 4 d (3.89, on average), and 6 d (4.63, on average), but at 8 d greater pH was observed for LBLD-40 (6.43) than LBLD-22 (3.75), while CON-22 and CON-40 (5.34, on average) did not differ from either treatment combination. Also, at 10 d, LBLD-40 (6.99), CON-40 (6.23) and CON-22 (5.95) were greater than LBLD-22 (3.81). A two-way interaction between ST × AE was observed for mold counts (Figure 10B; $P = 0.001$) with similar counts at 0 d (2.39 CFU/g, on average) and 2 d (2.54

CFU/g, on average), but greater counts observed for HT than NT at 4 d (3.95 vs. 1.45 CFU/g), 6 d (6.10 vs. 1.85 CFU/g), 8 d (7.35 vs. 1.49 CFU/g), and 10 d (8.19 vs. 2.42 CFU/g).

DISCUSSION

Previous studies have shown that temperature affects silage fermentation, with microbial and enzymatic activities both being impacted by environmental conditions such as humidity and temperature (Weinberg et al., 2001; Muck et al., 2003). Greater storage temperatures (≥ 40°C) impact the rate of fermentation and the dominant microbial species, affecting the ratio and concentration of organic acids in silage (Borreani et al., 2018; Bai et al., 2022). This is consistent with our findings of impaired fermentation in silage stored at HT. High temperatures in this study slightly reduced agar-culturable LAB, which is consistent with previous studies (Weinberg et al., 2001; Kim and Adesogan, 2006; Guan et al., 2020). Although most LAB can adapt to high temperatures (38-45°C) during the initial phase of ensiling, their activity typically declines over extended periods of high temperatures (Guan et al., 2020; Bai et al., 2022; Wali et al., 2022).

The LBLD-40 had greater LAB counts than CON-40, indicating that microbial inoculation increased agar-culturable LAB despite the high temperatures. Lower lactic acid concentration and greater silage pH was observed for silage stored at HT compared with NT at 30 and 90 d of storage. Silage pH directly affects silage nutritive value, as a lower pH would be expected to inhibit the growth of undesirable microorganisms. Usually, lactic acid causes a decline in pH because is about 10 times stronger (pKa of 3.8), than short-chain fatty acids (pKa of 4.8; Kung et al., 2018). Silage treated with *L. buchneri* typically have a pH 0.1-0.2 units greater than untreated silage (Oude Elferink et al., 2001; Kleinschmit and Kung, 2006a), as observed in our study, due the ability of *L. buchneri* to convert lactic acid into acetic acid or 1,2-propanediol (Oude Elferink et al. 2001). Despite these differences, the average pH of all

silage remained adequate, below 4.2 (McDonald et al., 1991). Silage pH decreases more rapidly in WPCS during the first few days of fermentation, even at high storage temperatures (Weinberg et al., 2001; Bai et al., 2022).

Oude Elferink et al. (2001) reported that the rate of lactic acid degradation to acetic acid is influenced by temperature, with an optimum range for conversion between 20 and 30°C. Previous research from our lab observed greater acetic acid conversion in earlier stages of fermentation, after 14 d of storage, when *L. buchneri* was inoculated with *L. diolivorans* (Diepersloot et al., 2022; Saylor et al., 2022). However, in this current study, inoculation with LBLD increased acetic acid concentration only in silage stored at NT at 90 d of storage. No reductions in lactic acid concentration were observed due to microbial inoculation, which could also be an influence of the homofermentative bacteria used in the microbial inoculant. This fermentation pattern explains why a reduction in LA:AA ratio was only observed for LBLD-22 at 90 d of fermentation. Silage inoculated with *L. buchneri* typically has a lower LA:AA ratio than untreated silage due to the ability of converting lactic into acetic acid and 1,2-propanediol during silage fermentation. Additionally, in a petri dish study, *L. diolivorans* also proved to produce acetic acid through this pathway (Schein et al., 2018), contributing to the reduction in the LA:AA ratio.

Kung et al. (2018) noted that silage with higher LA:AA ratio may be more aerobically unstable if the acetic acid concentration is insufficient to inhibit lactate-assimilating yeasts. Accordingly, aerobic stability was greater for LBLD-22 at 90 d of storage, maintaining stability until the end of the 240-h evaluation period. These results highlight that the activity of microbial inoculants at high temperatures may depend on their ability to tolerate warmer conditions (Weinberg et al., 2001). Greater aerobic stability was most likely due to the greater acetic acid concentration, which is known to have antifungal properties that inhibit yeast growth,

enhancing aerobic stability (Oude Elferink et al., 2001; Danner et al., 2003; Kim and Adesogan, 2006).

Ethanol concentration was lower for silage stored at HT, likely due to reduced heterofermentative microbial activity at high temperatures. Depending on microbial redox potential and environment conditions, traces of ethanol can be produced through the phosphoketolase enzyme and through lactic acid consumption (Heinl and Grabherr, 2017). In well-fermented WPCS, ethanol concentration typically ranges from 0.5 to 1.5% DM (Kung et al., 2018). Ethanol plays a prominent role in ester formation through the catalytic action of yeast and LAB enzymes (Hafner et al., 2013). This may explain greater ethyl acetate and ethyl lactate concentrations at NT, which had a concomitant greater ethanol concentration compared to HT. The correlation between the sum of these esters and ethanol in this study ($R^2 = 0.58$) was lower than previously reported ($R^2 = 0.69$) by Weiss et al. (2016), who also observed increased ester formation in WPCS stored at cooler (20°C) compared to warmer (35°C) temperatures.

Methanol is not an alcohol commonly associated with known bacterial or fungal species found in silage. Instead, its presence in silage is usually attributed to plant enzyme activity before ensiling, particularly the hydrolysis of pectin by methylesterase (Hafner et al., 2013). On the other hand, 1,2-propanediol is a common alcohol observed in silage, especially those inoculated with heterofermentative inoculants. Thus, the production of 1,2-propanediol can serve as an indicator of *L. buchneri* activity. Likewise, *L. diolivorans* is capable of degrading 1,2-propanediol under anaerobic conditions, producing propionic acid and 1-propanol (Krooneman et al., 2002). Despite the inoculation of *L. diolivorans*, propionic acid was not detected in any treatment, likely due to its metabolization during fermentation or the inoculant's lack of production capacity. This agrees with previous studies from our laboratory using similar microbial inoculants in high-moisture corn, and sorghum and corn silage (Diepersloot et al., 2021, 2022; Saylor et al., 2022). Petri dish studies indicate that *L. diolivorans* has low potential

for propionic acid production in the presence of WSC (Zhang et al., 2010; Zielińska et al., 2017; Schein et al., 2018).

Inoculating silage with *L. buchneri* has the potential to reduce concentration of 2,3-butanediol in silage fermentation (Nishino et al., 2007). However, the high correlation between 1-propanol and 2,3-butanediol ($R^2 = 0.88$) in this trial suggests that this fermentation pattern may stimulate other microbes who possesses the butanediol fermentation pathway (i.e., enterobacteria, LAB, and bacilli; McDonald et al., 1991), although this association remains unexplained in the literature. Few studies have investigated 2,3-butanediol, and its involvement with lactic acid bacteria is not well understood (Nishino and Shinde, 2007). The increase in n-butyric acid concentration in silage stored at HT is noteworthy; however, the very low concentration observed, along with the absence of propionic acid, indicates this is not biologically meaningful.

Dry matter concentration decreased with SL and DM losses increased at 15, 30, and 90 d compared to 7 d. It is widely recognized that prolonged in-silo fermentation can increase DM and nutrient losses (Borreani et al., 2018). As storage length increases, secondary fermentations carried out by yeasts and heterofermentative bacteria can produce carbon dioxide, ethanol, and organic acids such as acetic and butyric (Borreani et al., 2018; Kung et al., 2018). In this current study, no differences in DM losses were observed due to high storage temperatures, which contrasts with previous literature (Guan et al., 2020). It is well-documented that ensiling at high temperatures or under wet conditions can increase DM losses (Weinberg et al., 2001; Ashbell et al., 2002).

An effect of microbial inoculation was observed on DM concentration, with a reduction of 0.5% for the CON silage. However, this reduction is unlikely to be biologically meaningful. As expected, starchD increased as fermentation progressed, corroborating that proteolysis occurred with increasing storage length. Hoffman et al. (2011) suggested that hydrophobic zein

proteins within the starch-protein matrix could be degraded during ensiling through solubilization or proteolytic activity. The degradation of zein proteins due to proteolytic activity is often associated with greater ammonia-N and SCP concentrations with increasing storage length (Hoffman et al., 2011; Der Bedrosian et al., 2012; Fernandes et al., 2020). Ammonia-N and soluble CP concentrations were positively correlated with starchD [$P < 0.001$, $R^2 = 0.87$ and $R^2 = 0.90$, respectively (data not shown)], as previously reported by Ferraretto et al. (2014). Furthermore, SCP and ammonia-N concentrations and starchD were also affected by temperature and were greater in silage stored at HT. High-temperature conditions can increase proteolysis, leading to the hydrolysis of proteins into amino acids and further degradation to ammonia-N by microorganisms (Kung et al., 2018), which appears to have increased starchD due to the degradation of zein proteins in this trial. Moreover, it has been suggested that heterofermentative inoculants might promote the growth of proteolytic bacteria (Junges et al., 2017; Saylor et al., 2020b), which is supported by the increase in ammonia-N concentration observed in LBLD-40 silage.

The LBLD-40 treatment had the lowest concentrations of uNDF and aNDFom. This reduction may be related to the ability of inoculants like *P. acidilactici* and *L. buchneri* to alter the fibrous portion of the plant during fermentation (Reich and Kung, 2010), although the mechanism is not well understood. However, this effect was not observed by Diepersloot et al. (2022) when evaluating the same microbial inoculant under room temperature conditions. High temperatures may also contribute to the hydrolysis of hemicellulose into xylose and arabinose, as the optimal temperature for hemicellulases is typically between 30 to 40°C (Dewar et al., 1963). Additionally, cellulose hydrolysis is also more pronounced at 35°C compared to 25°C due to increased cellulase activity at a higher temperature (Pitt, 1990). Thus, an interaction between high temperature and the microbial inoculant evaluated in this trial may have affected the fibrous portion. The reduction in aNDFom concentration may partially explain for the

observed differences in starch and ash concentrations due to a dilution effect. Greater ash concentration was observed with increasing storage length, which can be attributed to reductions in other nutrients (i.e. WSC) during fermentation.

However, NDFD increased with storage length. Continued hydrolytic activity, either from forage enzymatic activity or due to acidic conditions within the silo, has been associated with changes in structural carbohydrates after long-term ensiling (Rooke and Hatfield, 2003). However, the exact cause of these differences remains unclear, as NDF concentration and digestibility are not typically affected or reduced by fermentation due to the utilization of soluble fiber fractions (Kung et al., 2018).

High temperatures often promote the growth of yeast and molds, as the levels of naturally occurring antifungal acids are typically insufficient to inhibit their proliferation (Bernardes et al., 2018; Wali et al., 2022). At 90 d of storage, the pH of LBLD-22 remained below 4.0 throughout the period of aerobic exposure, effectively preventing the growth of lactate-utilizing yeast and lactic acid oxidation (Borreani et al., 2018). However, at early SL, greater mold (and yeast in silage stored for 15 d) proliferation was observed after AE when silage was stored at HT, despite little or no change in pH during the exposure to oxygen. This result suggests that when high concentration of WSC is available, pH may not increase during aerobic deterioration. Conversely, when lactic acid is the primary energy source for microorganisms, pH increases (Weinberg et al., 2001).

CONCLUSIONS

This study confirmed the negative impact of storage temperature on the fermentation, aerobic stability, and nutritive value of WPCS. Greater temperature impaired fermentation and allowed greater yeast and mold proliferation after aerobic exposure. However, inoculating WPCS with a heterofermentative inoculant enhanced acetic acid concentration and improved

aerobic stability for silage stored in lower temperatures. But this modulation in fermentation and improvement of aerobic stability was not as pronounced under high temperatures, underscoring the influence of temperature on the effectiveness of microbial inoculants.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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Table 1. The nutrient composition of uninoculated whole-plant corn forage.

Item[1]	Average	SD[2]
pH	6.17	0.12
Dry Matter, % as fed	41.3	1.99
WSC, % DM	7.77	0.42
Crude Protein, % DM	7.57	0.35
SCP, % CP	30.2	2.88
aNDFom, % DM	30.8	3.68
NDFD 30 h, % NDFom	53.4	1.54
uNDF 240h, % DM	10.1	1.15
Starch, % DM	42.7	4.67
StarchD 7h, % starch	65.4	0.93
Ash, % DM	2.57	0.43
Ether Extract, % DM	2.57	0.33
LAB, log CFU/g of fresh weight	5.49	0.53
Yeast, log CFU/g of fresh weight	7.19	0.07
Mold, log CFU/g of fresh weight	4.04	0.17

[1]DM: dry matter; WSC: water-soluble carbohydrates; CP: crude protein; SCP: soluble crude protein; aNDFom: neutral detergent fiber treated with α-amylase and sodium sulfite, corrected for residual ash; NDFD: 30 h in vitro ruminal NDFom digestibility; uNDF: 240h in vitro ruminal undigested NDF; StarchD: in vitro starch disappearance after 7h.

[2]SD: standard deviation.

Table 2. The statistical analysis (*P*-values) of the effect of storage temperature (ST), microbial inoculation (MI), storage length (SL), and their interactions on the fermentation profile of whole-plant corn silage[1]

Item[2]	MI	ST	SL	MI × ST	MI × SL	ST × SL	MI × ST × SL
pH	0.002	0.001	0.001	**0.02**	0.16	**0.001**	0.27
Total Acids, % DM	0.47	0.001	0.001	**0.002**	0.49	**0.001**	0.07
Lactic acid, % DM	0.17	0.001	0.001	0.24	0.98	**0.008**	0.92
Acetic acid, % DM	0.001	0.001	0.001	0.001	0.001	0.001	**0.001**
LA:AA ratio	0.001	0.66	0.001	0.24	0.30	0.01	**0.004**
Aerobic Stability, h	0.001	0.001	0.001	0.02	0.007	0.001	**0.001**
Ethanol, % DM	0.70	**0.001**	**0.02**	0.34	0.69	0.28	0.66
Methanol, % DM	0.001	0.18	0.001	**0.02**	0.16	**0.03**	0.33
1-Propanol, % DM	0.001	0.001	0.001	0.001	0.001	0.001	**0.001**
1,2-Propanediol, % DM	0.001	0.04	**0.005**	**0.001**	0.25	0.32	0.06
Ethyl acetate, mg/kg DM	0.46	0.001	0.46	0.29	0.40	**0.004**	0.27
Ethyl lactate, mg/kg DM	0.83	0.001	0.001	0.11	0.19	**0.001**	0.56
n-butyric acid, mg/kg DM	0.001	0.001	0.001	0.001	0.001	0.001	**0.001**
2.3 butanediol, mg/kg DM	0.001	0.001	0.001	0.001	0.001	0.001	**0.001**
LAB, log CFU/g	0.001	0.001	0.001	**0.03**	0.29	**0.001**	0.45
Yeast, log CFU/g	0.001	0.04	0.001	0.001	0.001	0.001	**0.001**
Mold, log CFU/g	0.37	**0.001**	**0.001**	0.20	0.38	0.40	0.45
DM Losses, % DM	0.75	0.38	**0.001**	0.84	0.72	0.78	0.91

[1]MI: LBLD (*P. acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage; Provita Supplements Inc., Mendota Heights, MN) or CON (distilled water); ST: 22°C or 40°C; SL: 5, 15, 30 or 90 d of storage. Propionic acid and iso-butyric acid were measured, but not detected in any treatments. [2]LAB: lactic acid bacteria.

Table 3. The statistical analysis (*P*-values) of the effect of storage temperature (ST), microbial inoculation (MI), storage length (SL), and their interactions on nutrient composition of whole-plant corn silage[1]

Item[2]	MI	ST	SL	MI × ST	MI × SL	ST × SL	MI × ST × SL
DM, % as fed	**0.03**	0.48	**0.001**	0.99	0.92	0.66	0.90
WSC, % DM	0.60	0.001	0.17	0.18	0.05	**0.001**	0.18
CP, % DM	0.70	0.13	0.001	0.05	0.58	0.66	**0.05**
SCP, % CP	0.16	**0.001**	**0.001**	0.99	0.08	0.47	0.21
Ammonia-N, % N	0.001	0.001	0.001	**0.002**	0.96	**0.001**	0.47
aNDFom, % DM	0.15	0.17	0.02	**0.001**	0.41	**0.02**	0.50
NDFD, % NDFom	0.29	0.07	**0.02**	0.43	0.64	0.17	0.24
uNDF, % DM	0.10	0.001	**0.04**	**0.001**	0.86	0.07	0.35
Starch, % DM	0.08	0.93	0.001	**0.001**	0.73	**0.05**	0.42
StarchD, % starch	0.07	**0.001**	**0.001**	0.45	0.67	0.25	0.72
Ash, % DM	0.13	0.02	**0.001**	**0.01**	0.75	0.81	0.07
EE, % DM	0.50	0.74	0.001	**0.007**	0.96	**0.008**	0.68

[1]MI: LBLD (*P. acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage; Provita Supplements Inc., Mendota Heights, MN) or CON (distilled water); ST: 22°C or 40°C; SL: 5, 15, 30 or 90 d of storage. [2]DM: dry matter; WSC: water soluble carbohydrates; CP: crude protein; SCP: soluble crude protein; aNDFom: neutral detergent fiber treated with α-amylase and sodium sulfite, corrected for residual ash; NDFD: 30 h in vitro ruminal NDFom digestibility; uNDF: 240 h in vitro ruminal undigested NDF; StarchD: in vitro starch disappearance after 7h; EE: ether extract.

Table 4. The statistical analysis (*P*-values) of the effect of microbial inoculation (MI), storage temperature (ST), aerobic exposure (AE), and their interactions on pH and microbial counts of whole-plant corn silage.[1]

Item	MI	ST	AE	MI × ST	MI × AE	ST × AE	MI × ST × AE
15 d of storage							
pH	0.88	0.02	0.001	0.30	0.52	**0.001**	0.37
Yeast, log CFU/g	0.58	0.001	0.001	0.34	0.78	**0.001**	0.84
Mold, log CFU/g	0.61	0.001	0.001	0.23	0.62	**0.001**	0.24
30 d of storage							
pH	0.99	0.29	0.001	0.62	0.40	0.38	**0.003**
Mold, log CFU/g	0.32	0.001	0.001	0.89	0.001	0.001	**0.001**
90 d of storage							
pH	0.40	0.01	0.001	0.04	0.35	0.001	**0.001**
Mold, log CFU/g	0.21	0.001	0.001	0.10	0.64	**0.001**	0.39

[1]Microbial inoculation treatments: LBLD (*Pediococcus acidilactici* DSM 16243, *Lactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage; Provita Supplements Inc., Mendota Heights, MN), or CON (distilled water); Storage temperature treatments: 22℃ or 40℃; Storage length treatments: 15, 30 or 90 d of storage; Aerobic exposure treatments: 0, 2, 4, 6, 8 and 10 d of air exposure.

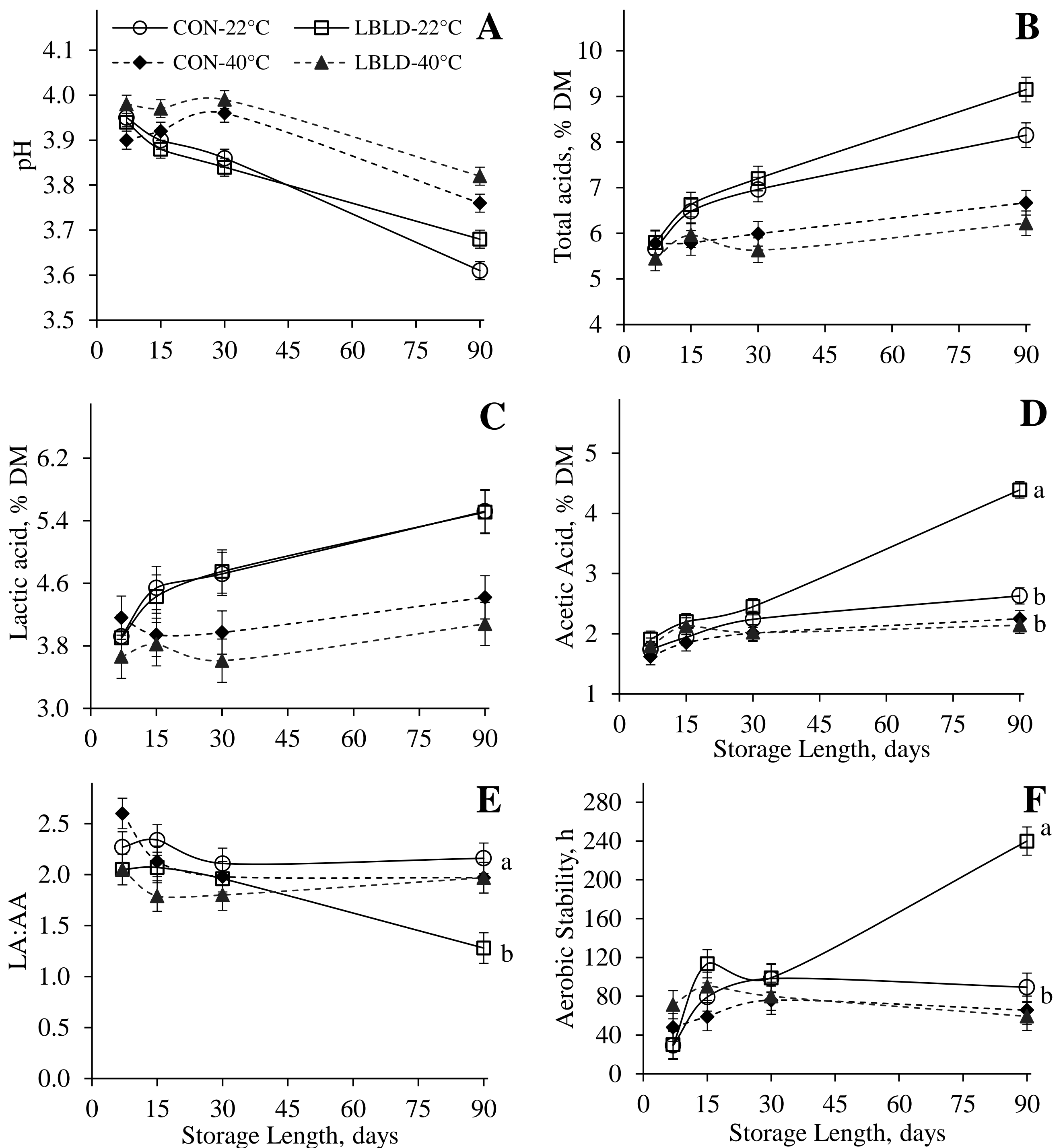

Figure 1. The effect of microbial inoculation (MI), storage temperature (ST), storage length (SL) on pH, and concentrations of total acids, lactic acid, acetic acid, LA:AA ratio, and aerobic stability of whole-plant corn silage (n = 64). Means within the same day with different superscripts differ (P < 0.05). Whole-plant corn silage was treated with distilled water (CON) or LBLD (*P. acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage) and stored at different ST (22 or 40°C). Silage

was stored for 7, 15, 30 or 90 d. Each treatment combination of MI, ST and SL has 4 observations. Error bars represent standard error of the mean (SEM). (A) pH; P = 0.27; SEM = 0.02; (B) Total acids, % DM; P = 0.07; SEM = 0.24; (C) Lactic acid, % DM; P = 0.92; SEM = 0.28; (D) Acetic acid, % DM; P = 0.001; SEM = 0.13; (E) LA:AA ratio; P = 0.004; SEM = 0.15; (F) Aerobic stability, h; P = 0.001; SEM = 14.55.

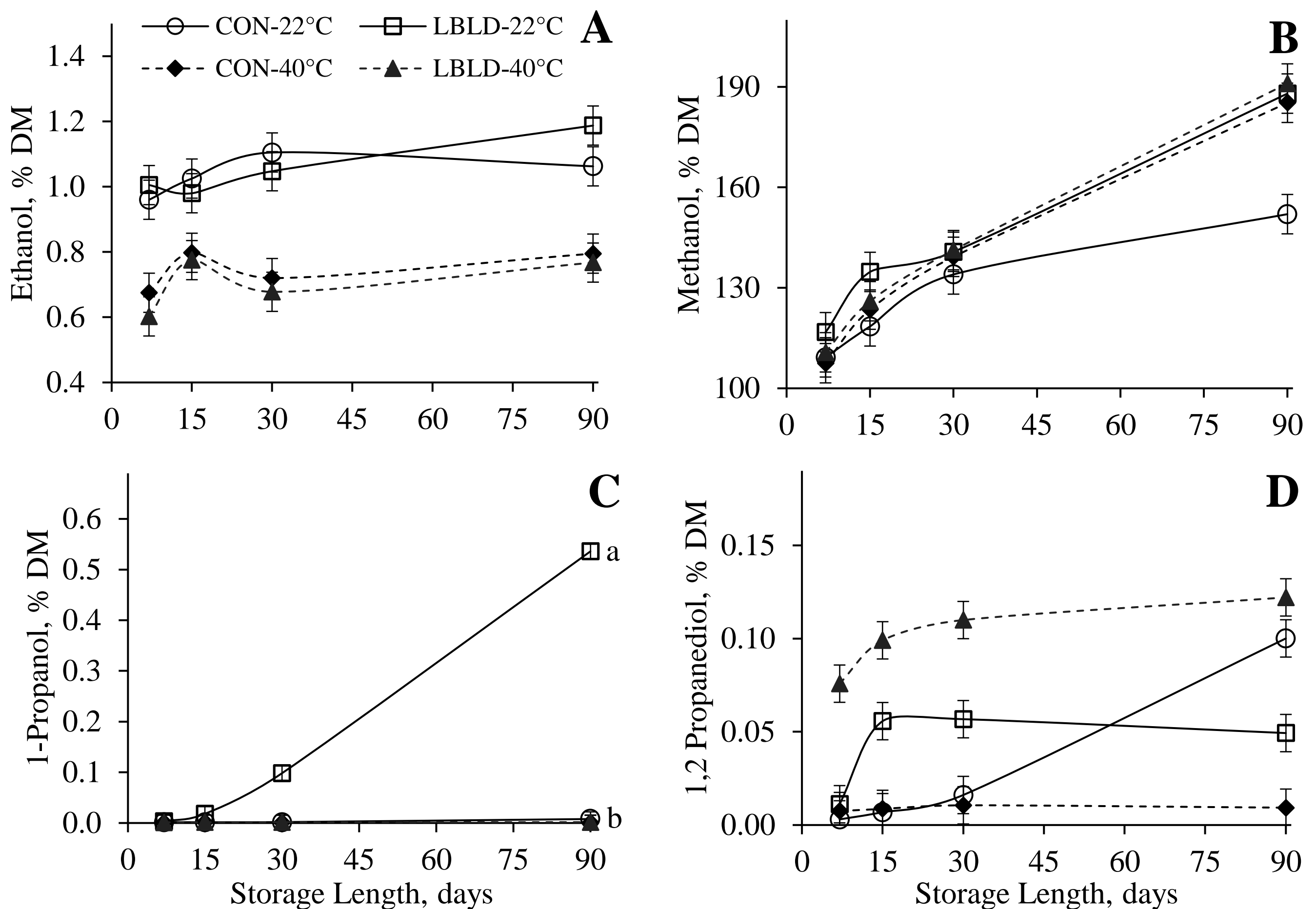

Figure 2. The effect of microbial inoculation (MI), storage temperature (ST), storage length (SL) on concentrations of ethanol, methanol, 1-propanol and 1,2-propanediol of whole-plant corn silage (n = 64). Means within the same day with different superscripts differ (P < 0.05). Whole-plant corn silage was treated with distilled water (CON) or LBLD (*P. acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage) and stored at different ST (22 or 40°C). Silage was stored for 7, 15, 30 or 90 d. Each treatment combination of MI, ST and SL has 4 observations. Error bars represent standard error of the mean (SEM). (A) Ethanol, % DM; P = 0.66; SEM = 0.06; (B) Methanol, mg/kg; P = 0.33; SEM = 5.87; (C) 1-propanol, % DM; P = 0.001; SEM = 0.01; (D) 1,2-propanediol, % DM; P = 0.06; SEM = 0.01.

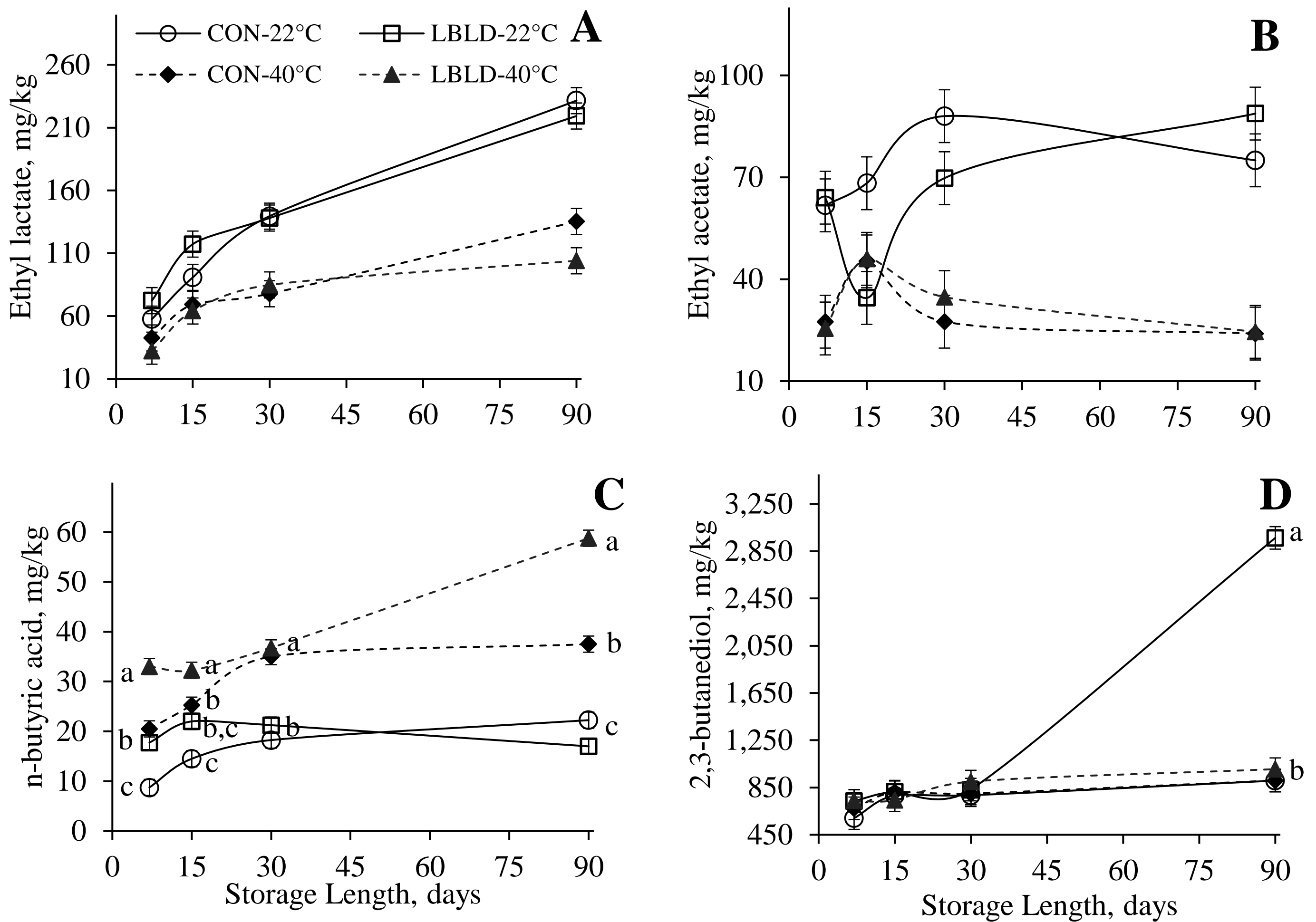

Figure 3. The effect of microbial inoculation (MI), storage temperature (ST), storage length (SL) on concentrations of ethyl lactate, ethyl acetate, n-butyric acid and 2,3-butanediol of whole-plant corn silage (n = 64). Means within the same day with different superscripts differ (P < 0.05). Whole-plant corn silage was treated with distilled water (CON) or LBLD (*P. acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage) and stored at different ST (22 or 40°C). Silage was stored for 7, 15, 30 or 90 d. Each treatment combination of MI, ST and SL has 4 observations. Error bars represent standard error of the mean (SEM). (A) Ethyl lactate, mg/kg; P = 0.56; SEM = 10.38; (B) Ethyl acetate, mg/kg; P = 0.28; SEM = 7.77; (C) n-butyric acid, mg/kg; P = 0.001; SEM = 1.62; (D) 2,3-butanediol, mg/kg; P = 0.001; SEM = 95.19.

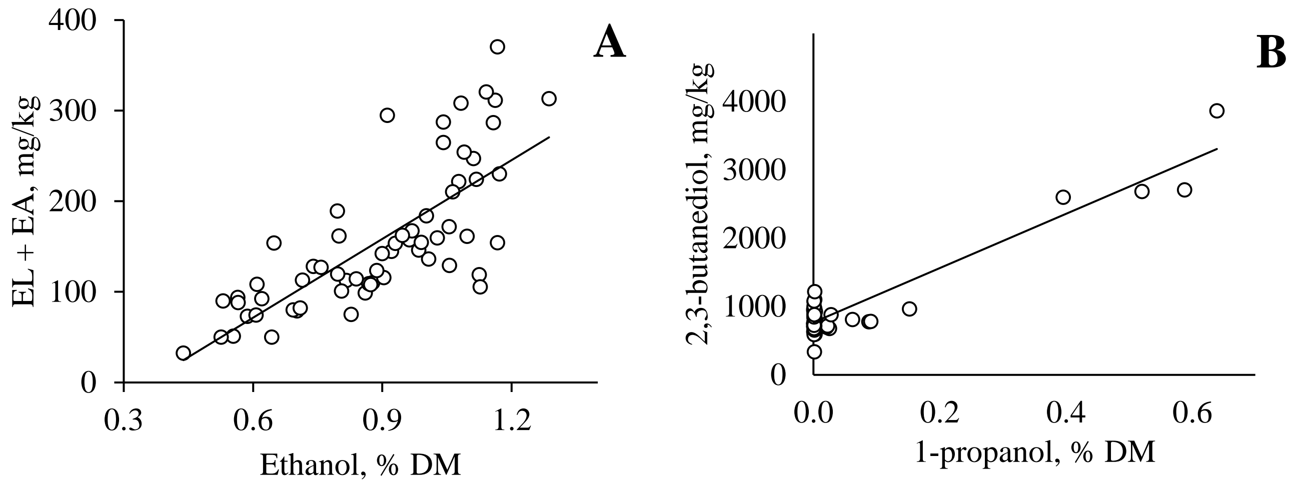

Figure 4. Relationship between ethyl lactate + ethyl acetate (EL + EA) and ethanol; 2,3-butanediol and 1-propanol of whole-plant corn silage (n = 64). (A) Ethanol prediction equation: y = 289.84x – 102.42, root mean square error (RMSE) = 51.46, R^2 = 0.58; (B) 1-propanol prediction equation: y = 3971.4x + 768.3, RMSE = 192.93, R^2 = 0.88.

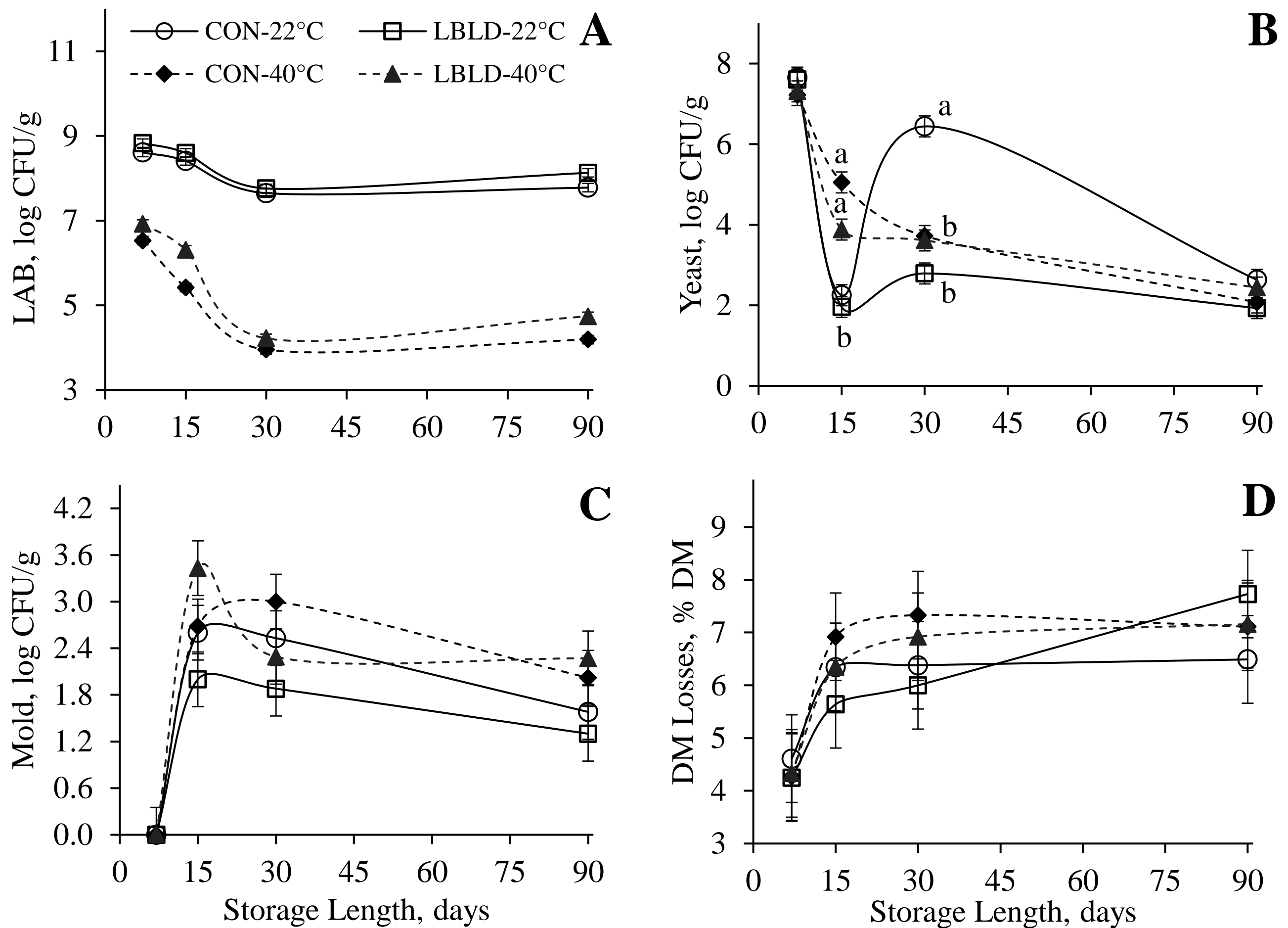

Figure 5. The effect of microbial inoculation (MI), storage temperature (ST), storage length (SL) on dynamic counts of lactic acid bacteria (LAB), yeast and mold, and dry matter losses of whole-plant corn silage (n = 64). Means within the same day with different superscripts differ (P < 0.05). Whole-plant corn silage was treated with distilled water (CON) or LBLD (*P. acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage) and stored at different ST (22 or 40°C). Silage was stored for 7, 15, 30 or 90 d. Each treatment combination of MI, ST and SL has 4 observations. Error bars represent standard error of the mean (SEM). (A) LAB, log CFU/g; P = 0.45; SEM = 0.10; (B) Yeast, log CFU/g; P = 0.001; SEM = 0.26; (C) Mold, log CFU/g; P = 0.45; SEM = 0.35; (D) DM losses, % DM; P = 0.91; SEM = 0.83.

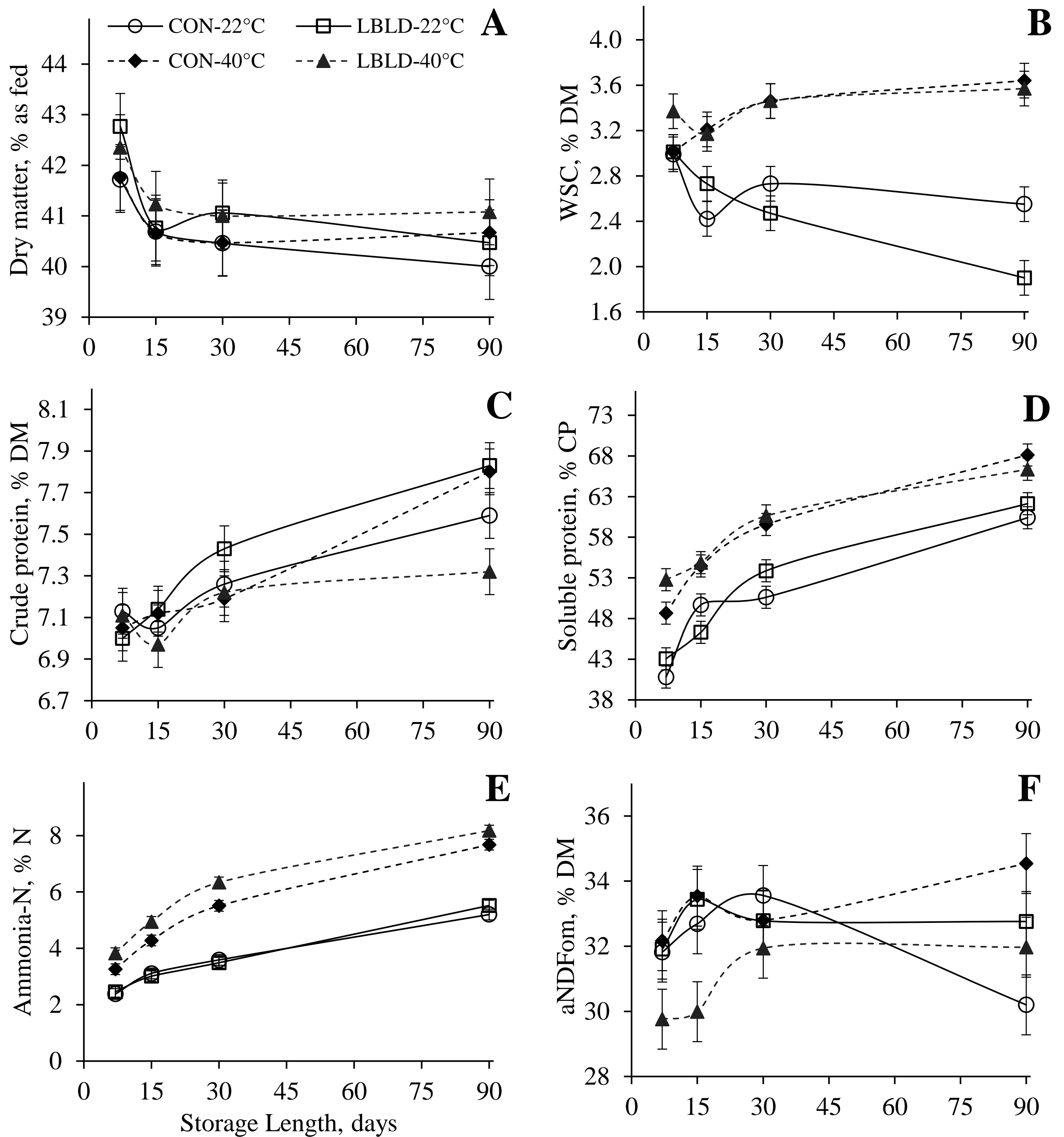

Figure 6. The effect of microbial inoculation (MI), storage temperature (ST), storage length (SL) and their interactions on concentrations of dry matter (DM), water-soluble carbohydrates (WSC), crude protein, soluble protein (SCP), ammonia-N, and neutral detergent fiber treated with α-amylase and sodium sulfite corrected for residual ash (aNDFom) of whole-plant corn silage (n = 64). Means within the same day with different superscripts differ ($P < 0.05$). Whole-plant corn silage was treated with distilled water (CON) or LBLD (*P. acidilactici* DSM 16243,

Lentilactobacillus buchneri DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage) and stored at different ST (22 or 40°C). Silage was stored for 7, 15, 30 or 90 d. Each treatment combination of MI, ST and SL has 4 observations. Error bars represent standard error of the mean (SEM). (A) Dry matter, % as fed; P = 0.90; SEM = 0.65; (B) WSC, % DM; P = 0.18; SEM = 0.15; (C) Crude protein, % DM; P = 0.04; SEM = 0.11; (D) Soluble Protein, % CP; P = 0.21; SEM = 1.36; (E) Ammonia-N, % N; P = 0.47; SEM = 0.19; (F) aNDFom, % DM; P = 0.50; SEM = 0.92.

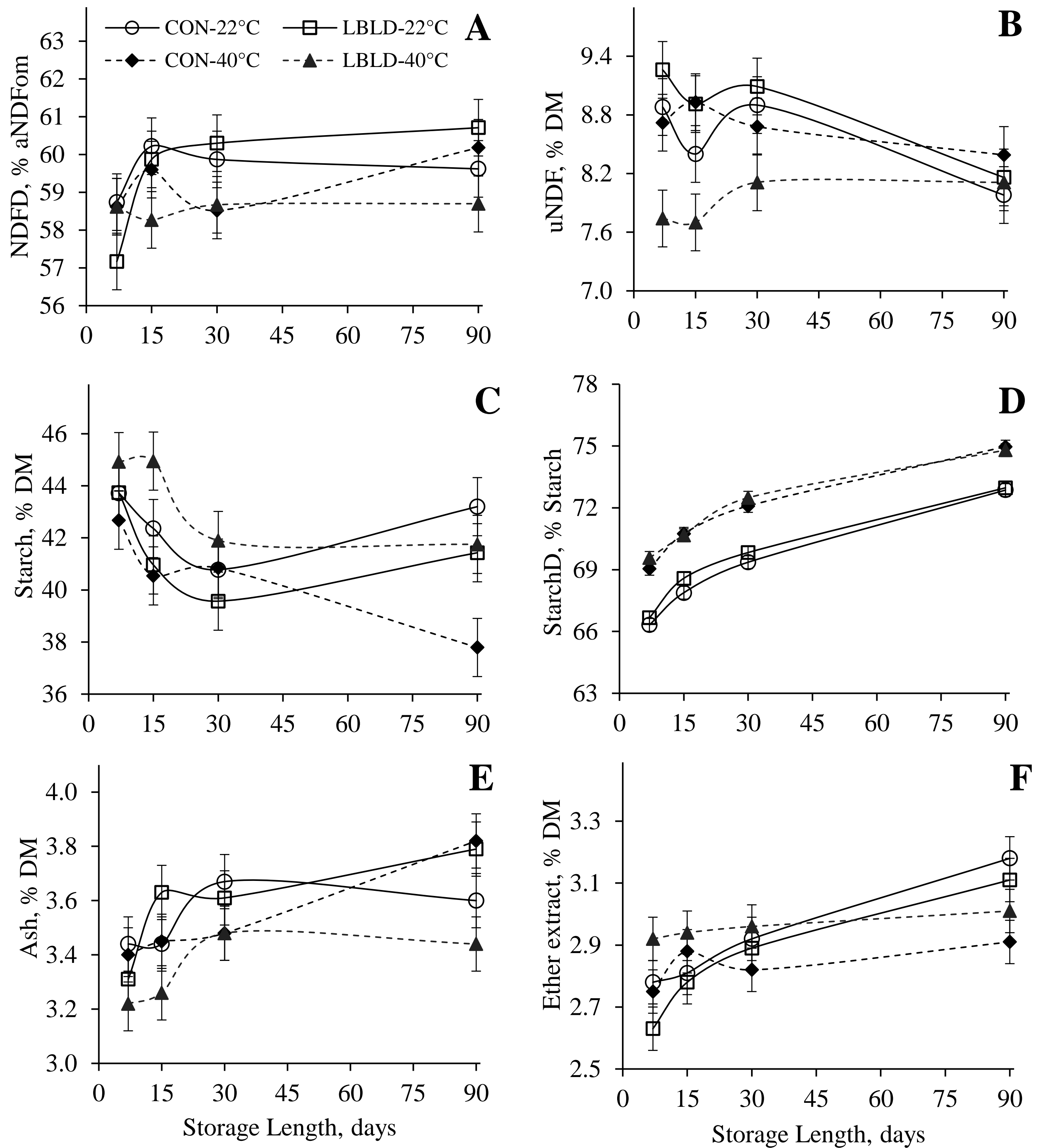

Figure 7. The effect of microbial inoculation (MI), storage temperature (ST), storage length (SL) and their interactions on 30 h in vitro ruminal NDF digestibility (NDFD), 240 h in vitro undigested NDF (uNDF), starch concentration, in vitro starch disappearance after 7h (starch-D), ash, and ether extract concentrations of whole-plant corn silage (n = 64). Means within the same day with different superscripts differ ($P < 0.05$). Whole-plant corn silage was treated with distilled water (CON) or LBLD (*P. acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM

12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage) and stored at different ST (22 or 40°C). Silage was stored for 7, 15, 30 or 90 d. Each treatment combination of MI, ST and SL has 4 observations. Error bars represent standard error of the mean (SEM). (A) NDFD, % aNDFom; $P = 0.24$; SEM = 0.75; (B) uNDF, % DM; $P = 0.35$; SEM = 0.29; (C) Starch, % DM; $P = 0.42$; SEM = 1.12; (D) StarchD, % starch; $P = 0.72$; SEM = 0.31; (E) Ash, % DM; $P = 0.07$; SEM = 0.10; (F) Ether extract, % DM; $P = 0.68$; SEM = 0.07.

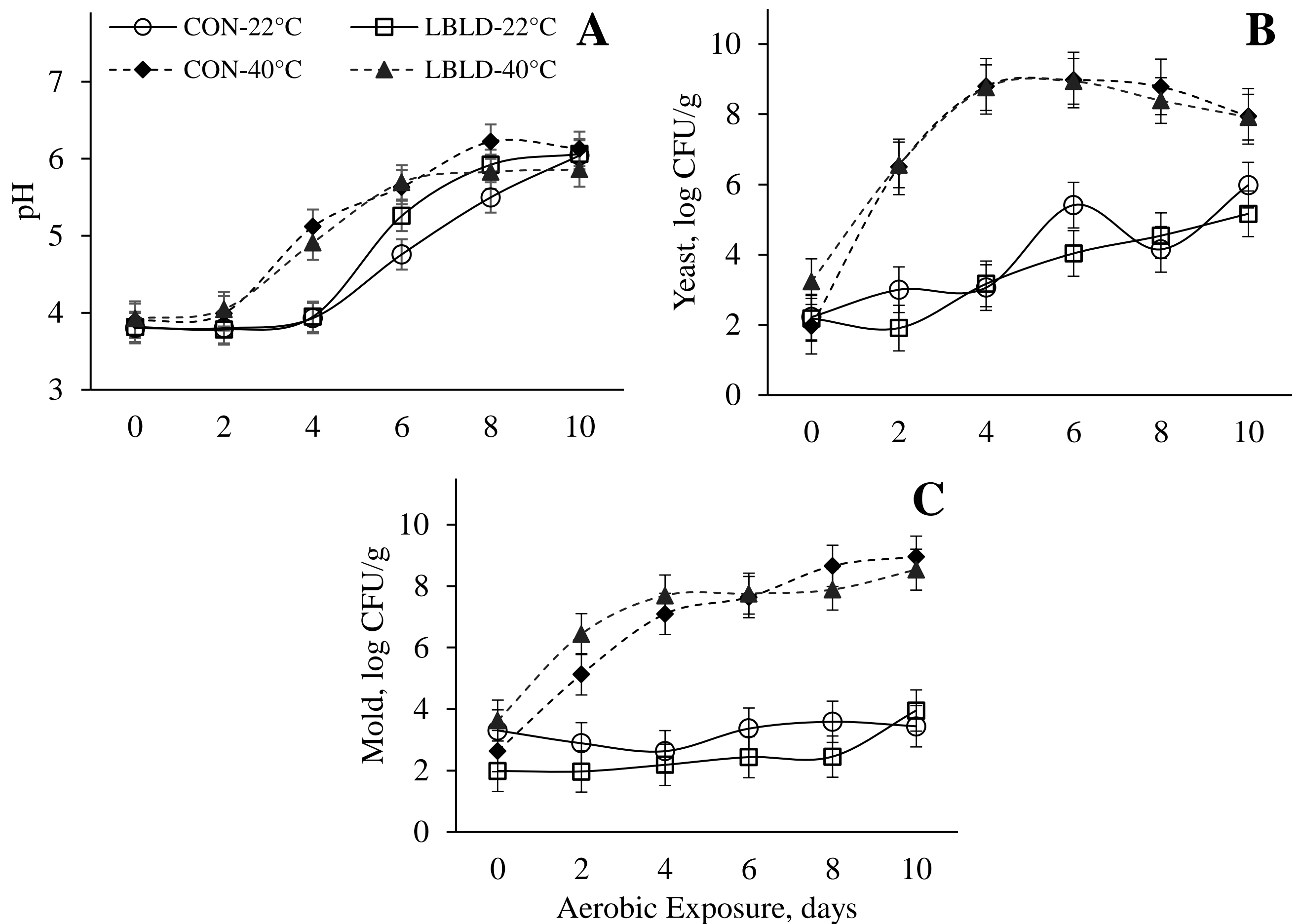

Figure 8. The effect of microbial inoculation (MI), storage temperature (ST), and aerobic exposure (AE) on pH, dynamic counts of yeast and mold of whole-plant corn silage stored for 15 d (n = 96). Me5ans within the same day with different superscripts differ (P < 0.05). Whole-plant corn silage was treated with distilled water (CON) or LBLD (*P. acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage) and stored at different ST (22 or 40°C) for 15 d. Silage was exposed to the air for 0, 2, 4, 6, 8 and 10 d. Each treatment combination of MI, ST and AE has 4 observations. Error bars represent standard error of the mean (SEM). (A) pH; P = 0.37; SEM = 0.22; (B) Yeast, log CFU/g; P = 0.84; SEM = 0.65; (C) Mold, log CFU/g; P = 0.67; SEM = 0.24.

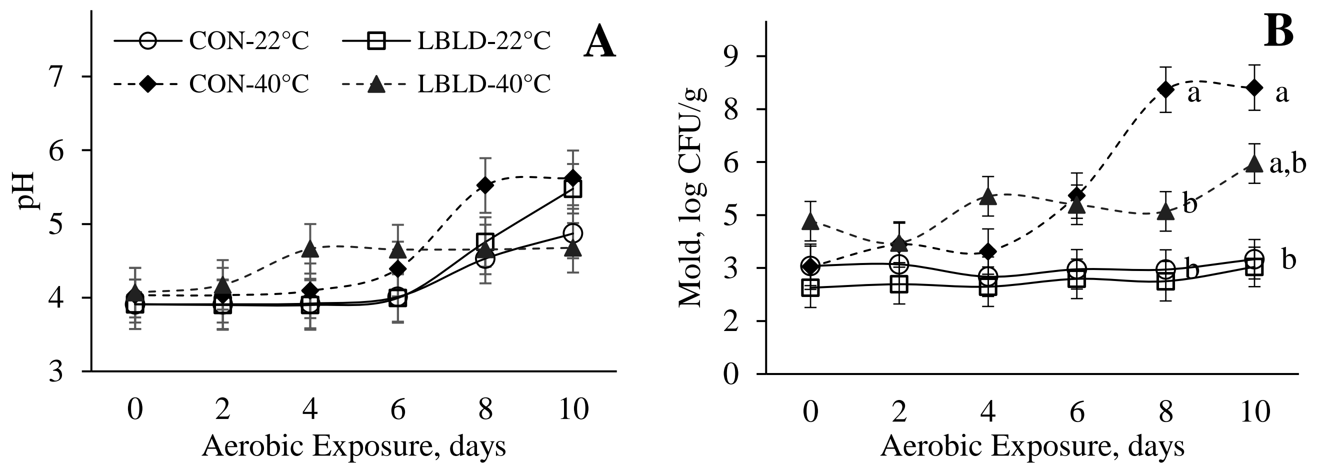

Figure 9. The effect of microbial inoculation (MI), storage temperature (ST), and aerobic exposure (AE) on pH and mold counts of whole-plant corn silage stored for 30 d (n = 96). Means within the same day with different superscripts differ ($P < 0.05$). Whole-plant corn silage was treated with distilled water (CON) or LBLD (*P. acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage) and stored at different ST (22 or 40°C) for 30 d. Silage was exposed to the air for 0, 2, 4, 6, 8 and 10 d. Each treatment combination of MI, ST and AE has 4 observations. Error bars represent standard error of the mean (SEM). (A) pH; P = 0.003; SEM = 0.32; (B) Mold, log CFU/g; P = 0.001; SEM = 0.56.

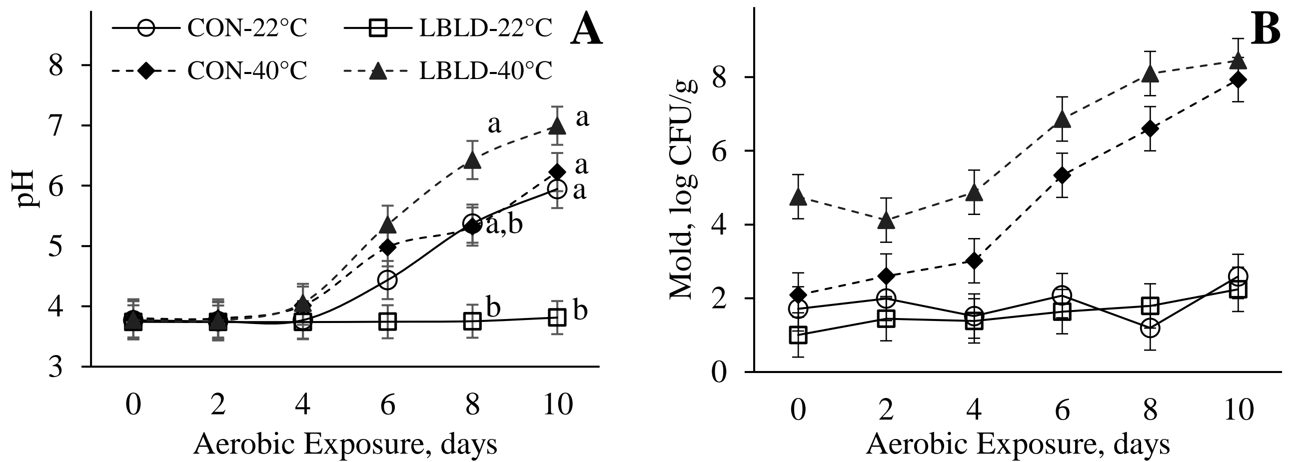

Figure 10. The effect of microbial inoculation (MI), storage temperature (ST), and aerobic exposure (AE) on pH and mold counts of whole-plant corn silage stored for 90 d (n = 96). Means within the same day with different superscripts differ ($P < 0.05$). Whole-plant corn silage was treated with distilled water (CON) or LBLD (*P. acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074; 300,000 CFU/g forage) and stored at different ST (22 or 40°C) for 90d. Silage was exposed to the air for 0, 2, 4, 6, 8 and 10 d. Each treatment combination of MI, ST and AE has 4 observations. Error bars represent standard error of the mean (SEM). (A) pH; P = 0.001; SEM = 0.32; (B) Mold, log CFU/g; P = 0.39; SEM = 0.60.

CHAPTER III

A meta-analysis of the effects of storage temperature on the nutrient composition, fermentation profile and aerobic stability of silage[1]

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ABSTRACT

Temperature has been previously reported as one of the most critical factors influencing microbial fermentation and silage production. However, effects of environmental temperature during storage are still unclear. A meta-analysis of 42 peer-reviewed articles was conducted to examine the effects of temperature ranges on nutritive and fermentation profile of silage. A complementary meta-analysis of 15 articles examined the effects on whole-plant corn silage

(WPCS) specifically. The effects were evaluated by ranges of temperature and the treatments were classified into the following categories: 1) ≤ 10°C; 2) > 10 and ≤ 20°C; 3) > 20 and ≤ 30 °C; 4) > 30 and ≤ 40°C; 5) > 40°C. Overall, fermentation temperature decreased linearly pH and yeast counts, and increased linearly dry matter, soluble crude protein and lignin for both meta-analysis. Butyric acid concentrations had a quadratic effect when different silage groups were evaluated together, and dry matter losses, NH_3-N and aerobic stability increased for WPCS meta-analysis. Concentrations of neutral detergent fiber, acid detergent fiber, starch, and ash were not affected by storage temperature. The storage temperature affected the activity of lactic acid bacteria, and both extreme temperature ranges reduced acid production. The environmental temperature during fermentation affected the activity of LAB, and both high and low temperatures reduced acid production. Therefore, the temperature range of 21-30°C may be more suitable for silage storage and fermentation due to greater total acid production and more rapid decline in pH, which enhances nutrient preservation.

Keywords: aerobic stability; corn silage; ensiling; fermentation temperature.

INTRODUCTION

The environmental temperature can affect DM yield and nutritive value of crops harvested for silage production, for example with further effects on the ensiling process and fermentation profile (Bernardes et al., 2018). Although environmental temperature cannot be controlled during harvesting and ensiling, understanding the extent of damage that temperature may cause to the silage can be important. Such knowledge can provide insights into management strategies to reduce nutritive losses under challenging conditions (Ashbell et al., 2002).

Previous studies have reported that storage temperature (ST) can affect silage bacterial community in a greater magnitude compared with microbial inoculants and silage density (Bai et al., 2022a; Bai et al., 2022b). Lactic acid bacteria (LAB) are responsible for the rapid decrease in silage pH and despite their ability to grow under a wide temperature range (5–45°C; Oude Elferink et al., 2001), optimal temperatures vary between 27 and 38°C (Yamamoto et al., 2011). This suggests that a mild temperature (20 to 30°C) may be desirable for silage fermentation (Weinberg et al., 2001).

Furthermore, silage temperature can increase above 40°C at the start of ensiling due to continuous plant respiration and the activity of aerobic microorganisms when oxygen is still present in plant gaps (Bernardes et al., 2018). This occurs more frequently in tropical regions (Adesogan, 2009) and when crops are ensiled under hot conditions in temperate climates (Kung, 2011). Once fermentation stabilizes, the temperature gradually decreases, depending on environmental conditions and silo size. However, in warm climates, high silage temperatures may persist for several months (Weinberg et al., 2001). In contrast, in cooler environments, the lower average daily temperature can also negatively impact the fermentation process, delaying the pH decline during the initial days after silo sealing (Zhou et al., 2016).

Although silage reviews are available in the literature (Bernardes et al., 2018; Borreani et al., 2018) evaluating the effects of environmental temperature on silage production, fermentation profile, and nutritive losses, little is known about its impact. A meta-analytic approach is valuable for synthesizing findings from multiple studies in the literature, enhancing the power to detect treatment effects and increasing the ability to explore sources of variation in responses (Glass, 1976; Higgins, 2008). Therefore, our objective was to conduct a meta-analysis to evaluate the magnitude of the effects of environmental temperature on nutritive value and fermentation profile of silages. In addition, our objective was to assess the effects of environmental temperature on nutritive value and fermentation profile of whole-plant corn silage only. Our hypothesis was that both low and high temperature ranges would reduce nutritive value, fermentation profile and aerobic stability of silage.

MATERIALS AND METHODS

A comprehensive search of the literature published in the English language from 1984 to 2024 was searched to identify experiments with silage evaluated under different fermentation temperatures. A literature search was conducted using US National Library of Medicine and National Institutes of Health through PubMed (http://www.ncbi.nlm.nih.gov/pubmed), ISI Web of Science (http://apps.webofknowledge.com), and Google Scholar (https://scholar.google.com) online databases with the keywords "silage temperature", "silage fermentation temperature", and "silage storage temperature". Furthermore, references reported in the collected manuscripts were also checked.

Inclusion Criteria

For inclusion in the database, the studies were required to include (1) evaluation of temperature within fixed or small range temperatures; (2) temperature remained constant during the storage period; (3) tested simultaneously at least two fermentation temperatures as

treatment means; (4) evaluated at least 12h of ensiling period to ensure changes on silage fermentation.

Data Extraction

Figure 1 shows a PRISMA diagram (Moher et al., 2009) depicting the data collection process for the meta-analysis. After initial screening, 105 full-text articles were assessed to determine their eligibility to be included in the meta-analysis and 62 publications were excluded for the following reasons: (1) did not control temperature treatments (large variation of temperature daily [8 experiments]); (2) had variable temperatures when the targeted storage period was reached (4 experiments); (3) ST were not considered treatment means (34 experiments); (4) publications not available in peer-reviewed journals (16 experiments). Data from 42 peer-reviewed papers met the selection criteria, with a total of 253 treatment means to assess the effects of ST on silage nutritive value (Table 1). The experiment with whole-plant corn silage (WPCS) in Weinberg et al. (2001) had a treatment with large variation throughout the storage length; hence it was not included in the current dataset. Similarly, treatment with oscillating temperature during storage from Zhou et al. (2019) was not included. Total *n* was corrected accordingly for both peer-reviewed papers. Additional classifications included silage type [whole-plant corn, grass, cereal, legume, total mixed ration silage, and others (crop residues, by-products, specific plant materials)]; storage length; temperature range (in groups of 10°C, achieving the following ranges: 1) ≤ 10°C; 2) > 10 and ≤ 20°C; 3) > 20 and ≤ 30°C; 4) > 30 and ≤ 40°C; 5) > 40°C. An additional meta-analysis was performed in the studies with WPCS as silage source, which were included 15 peer-reviewed papers with a total of 96 treatment means to evaluate effects of temperature on silage nutritive value, maintaining similar classifications as described previously.

The number of replicates, treatment means, and sources of errors were collected from tables of the published manuscripts for the following response variables: pH, and concentrations

of dry matter (DM), neutral detergent fiber (NDF), acid detergent fiber (ADF), crude protein (CP), water-soluble carbohydrates (WSC), ash, lignin, DM losses, lactic acid, acetic acid, lactic-acetic acid ratio, propionic acid, butyric acid, ethanol, counts of LAB, yeast, and mold (log cfu/g fresh forage), and aerobic stability (h). Responses from different publications were standardized to the same unit using the metric system. A few articles reported NH_3-N concentration on a DM basis, and values were converted to % N. Similarly, total N concentrations was converted to % CP (CP = N x 6.25); DM recovery in % was converted to DM Losses in % (DM Losses = 100 – DMR); and organic matter to ash concentration % DM (ash = 100 – organic matter). Data were transferred to Excel spreadsheets (Microsoft Corp., Redmond, WA) and reviewed by two people to ensure information collected was accurately transcribed from the manuscripts into the spreadsheets before statistical analysis.

Statistical analysis

In the 4 articles (one was with WPCS) that reported SD, standard error of the mean was calculated as described in the equation below:

$$SEM = \frac{SD}{\sqrt{n}}$$

Where n is the number of replications per treatment.

Data were analyzed using a mixed model procedure in SAS (version 9.4; SAS Institute Inc., Cary, NC) using the first-order autoregressive, compound symmetry, or spatial power covariance structure, whichever provided the best fit according to Sawa's Bayesian information criterion. Storage length and silage type were used as continuous and categorical covariates, respectively. The model included storage temperature range as fixed effect. For WPCS only, silage type was removed in the model. A random intercept was specified for each study to account for between-study variability, allowing each study to have its own baseline level of the response variable, thereby adjusting for the hierarchical structure of the data. The covariance structure for the random effects was set to unstructured (‘type=un’). Study was

considered as a random effect. The weight of each study was calculated as the inverse of the square of the variance as proposed by Roman-Garcia et al. (2016). Degrees of freedom were calculated using the Kenward-Roger option. Means were determined using the LSMEANS statement and compared using the Tukey's test option after a significant overall treatment *F* test. Orthogonal contrasts were used to evaluate linear and quadratic effects of storage temperature. Statistical significance was considered at $P \leq 0.05$.

RESULTS

Data from fourth-two peer-reviewed studies were collected to investigate the effects of temperature on silage fermentation, aerobic stability, and nutritive value of whole-plant corn (33.3% of the studies; 27.6% of the experiments), whole-plant sorghum (2.4% of the studies; 1.7% of the experiments), other cereals (14.3% of the studies; 8.6% of the experiments), alfalfa (11.9% of the studies; 17.2% of the experiments), grasses (16.6% of the studies; 22.4% of the experiments), other legumes (11.9% of the studies; 8.6% of the experiments), TMR (7.15% of the studies; 5.2% of the experiments), and by-products/other silage (11.4% of the studies; 8.6% of the experiments). A total of 28.6% of the studies evaluated the temperature effect at different SL, and 66.6% had an SL ≥ 60d. The most common fermentation temperature ranges were between 21 to 30°C and 11 to 20°C, which were present in 78.6 and 71.4% of the selected studies, respectively.

Our dataset revealed a considerable variation in fermentation end products (e.g. lactic and volatile acids, NH_3-N), microbial counts (LAB, yeast and mold counts), and nutrient composition (e.g. DM, CP, NDF concentrations), indicating the broad representation of the dataset (Table 2). When the meta-analysis covered only the subset of WPCS studies the variation was reduced, but as DM concentration at harvest covered 21 percentage-units, a large variation in fermentation end products was observed (Table 3). The variation in fermentation end products

revealed that silage fermentation was either restrained or stimulated across treatments and studies.

Temperature Effects on Silage Fermentation and Nutritive Value

Microbial counts and fermentation profile of silage stored at different temperature ranges are presented in Table 4. Overall, pH decreased linearly ($P = 0.001$), whereas lactic acid concentration quadratically increased ($P = 0.004$) as the ST range increased. Acetic acid concentration was affected ($P = 0.03$) by ST, but no differences were observed after Tukey-Kramer test corrections. Storage temperature quadratically increased ($P = 0.001$) LA:AA ratio and butyric acid concentration ($P = 0.01$). Due to the higher variation, only a Tukey test significance was observed for propionic acid concentration ($P = 0.003$). The ST ranges of > 20 to ≤ 30°C and > 30 to ≤ 40°C differed from the ≤ 10°C range (0.196, 0.205, and -0.0429 % DM, respectively), while the other ST ranges did not differ from any treatment. No differences among ST range treatments were observed for ethanol concentration ($P > 0.05$). A Tukey test significance was also observed for NH_3-N concentration ($P = 0.001$). The concentration of NH_3-N was greater for the ranges of > 20 to ≤ 30°C (7.26 % N) and > 30 to ≤ 40°C (7.32 % N) than > 10 to ≤ 20°C (6.12 % N), while the ranges of ≤ 10°C (6.53 % N) and > 40°C (6.17 % N) did not differ from any other treatment. A linear reduction was observed for LAB ($P = 0.001$), yeast ($P = 0.001$), and mold counts ($P = 0.02$). No differences among ST range treatments were observed for DM losses ($P > 0.05$).

Chemical composition of silage stored at different temperature ranges is presented in Table 5. Dry matter concentration linearly increased with ST ($P = 0.003$), whereas ST quadratically increased WSC and CP concentrations ($P = 0.001$ and $P = 0.001$, respectively). Storage temperature linearly increased SCP ($P = 0.001$). No differences among ST range treatments were observed for NDF, ADF, and starch concentrations ($P > 0.05$). Lignin concentration increased linearly with ST ($P = 0.01$), while ash concentration was not affected by ST ($P > 0.05$).

Temperature Effects on Corn Silage Fermentation, Aerobic Stability and Nutritive Value

All data associated with microbial and fermentative profile of WPCS stored at different temperature ranges are presented in Table 4. A linear increase was observed for pH (Table 6; $P = 0.001$), while lactic acid concentration was affected ($P = 0.001$) by ST, which was greater for > 20 to ≤ 30°C (5.63 % DM) than ≤ 10°C, > 10 to ≤ 20°C, > 30 to ≤ 40°C, and > 40°C ST ranges (3.58 % DM, on average). No differences among ST range treatments were observed for acetic acid concentration ($P > 0.05$). Lactic to acetic acid ratio quadratically increased ($P = 0.01$) as the ST range increased. Again, due to the higher variation, only a Tukey test significance was observed for propionic acid concentration ($P = 0.01$). The ST range of > 20 and ≤ 30 °C (0.243% DM) had greater concentration of propionic acid than ≤ 10°C (-0.048% DM) and > 30 to ≤ 40°C (0.079% DM), while > 10 to ≤ 20°C (0.207% DM) did not differ from other treatments. Ethanol and NH_3-N concentrations were linearly increased ($P = 0.003$ and $P = 0.001$, respectively) and 1,2-propanediol concentration quadratically increased ($P = 0.004$). Lactic acid bacteria ($P = 0.001$) and yeast counts ($P = 0.02$) were linearly decreased with ST, while mold counts were not affected ($P > 0.05$) by ST ranges. Dry matter losses and aerobic stability were linearly increased ($P = 0.001$) as the ST increased.

Chemical composition of WPCS stored at different temperature ranges is presented in Table 5. As observed for the meta-analysis with all types of silage, a linear increased effect was observed for DM (Table 7; $P = 0.003$). Water-soluble carbohydrates were quadratically decreased ($P = 0.001$) with ST, while CP was linearly reduced ($P = 0.004$) and SCP was linearly increased ($P = 0.001$) as the ST increased. No differences among ST ranges were observed for NDF, ADF, starch and ash ($P > 0.05$). Lignin was linearly increased ($P = 0.001$) with ST.

DISCUSSION

Storage temperature directly affects microbial activity and fermentation during ensiling (McDonald et al., 1966), as environmental conditions like humidity and temperature regulate microbial growth and enzymatic activities (Weinberg et al., 2001; Muck et al., 2003). Research shows that temperature alters bacterial communities shortly after silo sealing, driving distinct bacterial successions and fermentation patterns across different temperature ranges (Bai et al., 2022a). For that reason, ST affects the rate of pH decline, determining the time needed to reach a stable low pH (Ali et al., 2015).

The metabolic theory of ecology suggests that microbial metabolism is generally more active in warmer environments (Gillooly et al., 2001; Price et al., 2012). This trend is observed in silage fermentation, where lactic acid production had a quadratic response with the full dataset (different silage types), and with a decrease in lactic acid in WPCS stored > 30°C compared to > 20 and ≤ 30 °C. Despite this, pH decreased linearly with rising temperature. A lower pH improves silage quality by inhibiting undesirable microorganisms, and lactic acid is nearly 10 times more effective than acetic acid at lowering pH (Kung et al., 2018). However, LAB populations in the final silage may be inhibited by low pH (McDonald et al., 1991), leading to reduced LAB counts at higher temperatures. Regardless the pH effect, although most LAB initially adapt to high temperatures (38-45°C), their activity declines over time potentially resulting in lower LAB counts in higher temperature ranges (Bai et al., 2022a; Guan et al., 2020; Wali et al., 2022). This likely explains the linear reduction in LAB counts across silage types, despite a quadratic effect in the WPCS dataset.

Zhou et al. (2016) found that the pH drop in WPCS was influenced by incubation temperatures, particularly at ≤ 10°C. Our results align, due to greater pH and lower lactic acid concentrations, along with increased yeast and mold counts. Despite greater LAB counts, silage stored at low temperatures has reduced microbial activity, including LAB (Jungbluth et al.,

2017). In silage, elevated pH typically indicates poor fermentation, characterized by low lactic acid, reduced LAB, and increased yeast and mold activity (Weinberg et al., 2001), which were observed in the meta-analysis. Greater yeast counts and slower fermentation rates in WPCS were linked to reduced aerobic stability (< 20 h at ≤ 10°C). These findings are consistent with Ferrero et al. (2021), who reported that low temperatures can create fermentative profiles that diminish aerobic stability, as yeast survives longer in such conditions.

Weinberg and Muck (1996) noted that microbial inoculants are less effective at low temperatures. The meta-analysis of Bernardi et al. (2019) evaluating inoculants in WPCS included temperature as subgroup, and also agree with that statement. Reduced activity of heterofermentative LAB under these conditions limits the production of acetic and propionic acids, which possess antifungal properties that inhibit yeast growth and enhance aerobic stability (Ferrero et al., 2021). Although acetic acid levels did not differ, a quadratic increase for the lactic-to-acetic acid ratio in both datasets (with silage types and WPCS only) was observed, driven by the quadratic increase in lactic acid concentration. This also led to a quadratic effect on residual WSC concentration, indicating that fermentation rates were lower at extreme temperatures.

1,2-Propanediol is a common alcohol in silage, particularly when heterofermentative inoculants like *L. buchneri* are used. This bacterium anaerobically converts 1 mole of lactate into half a mole each of acetate, 1,2-propanediol, and carbon dioxide, without an electron acceptor (Oude Elferink et al., 2001). Lower 1,2-propanediol concentration in WPCS stored at ≤ 10°C indicates reduced lactic acid conversion to acetic acid by heterofermentative species. Additionally, propionic acid concentrations in WPCS also decreased at ≤ 10°C. *L. diolivorans* (Krooneman et al., 2002), *L. reuteri* (Sriramulu et al., 2008), and a novel strain of *L. buchneri* A KKP 2047p (Zielińska et al., 2017) can convert 1,2-propanediol into propionate in the presence of cobalamin. In currently meta-analysis, studies with WPCS typically evaluated 1,2-

propanediol and propionic acid concentrations in factorial designs, including temperature, inoculant, and sometimes storage length as factors.

Krooneman (2002) assessed the growth of *L. buchneri* (strain LMG 6892^T) and *L. diolivorans* (LMG 19667^T and LMG 19668) at 12, 20, 30, and 42°C, finding that none of the strains grew at 12 or 42°C. *L. diolivorans* strain LMG 19667T exhibited optimal anaerobic growth at 30-32°C when utilizing 1,2-propanediol as a substrate. Oude Elferink et al. (2001) reported that the degradation rate of lactic acid to acetic acid by *L. buchneri* is temperature-dependent, with optimal activity between 20 and 30°C. PCR–denaturing gradient gel electrophoresis analysis of silage indicated that 15°C might represent the lower growth limit for *L. buchneri*, while other heterofermentative species like *L. sakei* and *L. curvatus* dominated the LAB population after 60 days of fermentation at temperatures below 15°C (Zhou et al., 2016). These findings suggest that the activity of heterofermentative inoculants is adversely affected under extreme temperature conditions. Recently, new strains claimed to be resistant to high or low temperatures have been proposed and tested for their activity and effectiveness under such conditions (Chen et al., 2020; Xia et al., 2024). However, further research is needed to evaluate their effects across various situations and silage types.

These results indicate that low temperatures can adversely affect silage production in both small silos (e.g., bale silage) and the peripheral areas of large horizontal silos. This issue is particularly concerning in northern regions (e.g., North America, Canada, and Northern Europe), where ensiling often occurs in late fall as temperatures decrease during fermentation and conservation (Zhou et al., 2016; Bernardes et al., 2018). Silage produced under these conditions typically exhibit elevated pH values, slow rates of pH decline (Ali et al., 2015), low acid production, increased residual WSC, and higher yeast counts (Kung, 2010; Zhou et al., 2016).

Conversely, high temperatures and warm weather are known to promote the growth of spoilage organisms under aerobic conditions, with naturally produced antifungal fermentation products often insufficient to inhibit their proliferation (Bernardes et al., 2018; Kung et al., 2018; Wali et al., 2022). Our recent trial with corn silage stored at 22 and 40°C confirmed increased spoilage by yeast and molds following air exposure (Cruz et al., unpublished). In that study, after silos opening, the silage was exposed to the same environmental temperatures as during fermentation, with samples collected every two days. Silage stored at 40°C exhibited higher yeast and mold counts, along with increased pH. Additionally, the heterofermentative LAB inoculant did not effectively increase acetic acid concentration or enhanced aerobic stability at high temperatures.

High temperatures also negatively impact silage production. For example, corn silage yield decreases by 0.5 tons per hectare for each degree Celsius increase in temperature above 30°C (Bassu et al., 2014). Additionally, higher fiber and lignin deposition occur compared to corn grown in cooler or temperate climates (Adesogan, 2010). Damage also occurs during fermentation temperatures, which is the focus of our study. Higher ST ($\geq$ 40°C) affect both the fermentation rate and the dominant microbial species, ultimately influencing the ratio and concentration of organic acids produced (Borreani et al., 2018). The study findings support this, as lower LAB counts and lactic acid concentration were observed, along with higher residual WSC.

Increased environmental temperatures often promote protein degradation in silage. High-temperature conditions elevate the activity of plant proteases, leading to extensive protein hydrolysis. The hydrolysis of proteins into amino acids can subsequently degrade into NH_3-N by microorganisms (Kung et al., 2018). Increased proteolysis correlates with microbial activity; undesirable microorganisms, such as *Clostridia* and *Enterobacteria*, are particularly affected by temperature, especially in the initial days of fermentation. *Clostridium* spp. thrive at high

temperatures (>30°C), with optimal growth occurring at 37°C (McDonald et al., 1991). Additionally, Li et al. (2021) observed a greater abundance of the *Enterobacter* genus in oat silage stored at 25°C compared to 20, 15, 10, and 5°C.

Butyric acid concentrations exhibited a quadratic effect, with higher concentration observed in the ST range of > 20 and ≤ 30 °C. This finding highlights that temperatures considered more typical also promote clostridial activity in silage, particularly in tropical grass and legume silage, which often have low sugar concentrations, as well as higher moisture, buffering capacity, and consequently pH levels (Kung et al., 2018). However, our results indicate that lactic fermentation and mass preservation were more favorable within this temperature range. Despite the reported greater concentration of butyric acid, no differences in dry matter losses were found.

In our study, DM losses increased linearly in WPCS. This may result from greater microbial activity with rising temperatures, leading to increased pH acidification and subsequent enhanced heterofermentation. Meta-analysis had already reported an increase of DM losses due the activity of *L. buchneri* in WPCS (+ 1.0, 2.1 and 0.8% DM losses, respectively; Kleinschmit and Kung, 2006; Bernardi et al., 2019; Arriola et al., 2021) due to carbon dioxide formation during the conversion of lactic acid to acetic acid and 1,2-propanediol. Rees (1982) reported DM losses of 1.7% for every 10°C increased temperature in laboratory scale silos. The DM of silage, specifically WPCS, increased with ST, likely due to a slight drying effect and evaporation of intracellular water. This phenomenon can also occur in the more superficial areas of poorly compacted and sealed commercial silos (McDonald et al., 1991).

Bernardes et al. (2018) reviewed silage from both hot and cold regions, reporting that the digestibility of corn silage generally decreases at high temperatures due to reduced starch concentration and increased lignin and fiber deposition in the plant. This effect may also extend

to other types of forages. However, our meta-analysis focused specifically on the effects of temperature during fermentation, with all silage harvested under similar conditions. While no differences were observed in fiber contents, an increase in lignin content appears to occur with rising temperatures, likely due to structural changes in the plant material after sealing. High temperatures can accelerate certain chemical reactions in the forage, which are related to enzymatic activity. Heat production is a normal occurrence during the ensiling process, and a temperature increase of up to 12°C above the harvesting temperature is common, even in well-managed silos (Adesogan and Newman, 2014). Furthermore, the temperature inside the silo can be greater than the environmental temperature surrounding the silage.

The Maillard reaction produces various compounds, including melanoidins, which are brown-colored polymers formed from the interaction of sugars and amino acids (Shakoor et al., 2022). These products can bind to lignin, rendering it less accessible to the microbes involved in fermentation and leading to an increase in lignin content in the final silage. The browning reaction, characterized by a cured tobacco odor and a dark brown color, typically occurs in silage exposed to prolonged temperatures of ≥ 40°C. This reaction results from protein damage (denaturation) (Muck and Pitt, 1993), which may reduce the efficiency of nitrogen utilization from forage by ruminants (Bernardes et al., 2018; Broderick, 2018). Future investigations should assess how silage ST affect animal performance and safety after fed these silages. Studies focusing the dynamic changes of silage microbiome can also provide new insights in the isolation and inoculation of silage with heat- and cold-resistant heterofermentative strains, prioritizing the enhance of aerobic stability under these conditions.

CONCLUSIONS

The environmental temperature during fermentation affected the activity of LAB, and both high and low temperatures reduced acid production. Therefore, the temperature range of

21-30°C may be more suitable for silage storage and fermentation due to greater total acid production and more rapid decline in pH, which enhances nutrient preservation.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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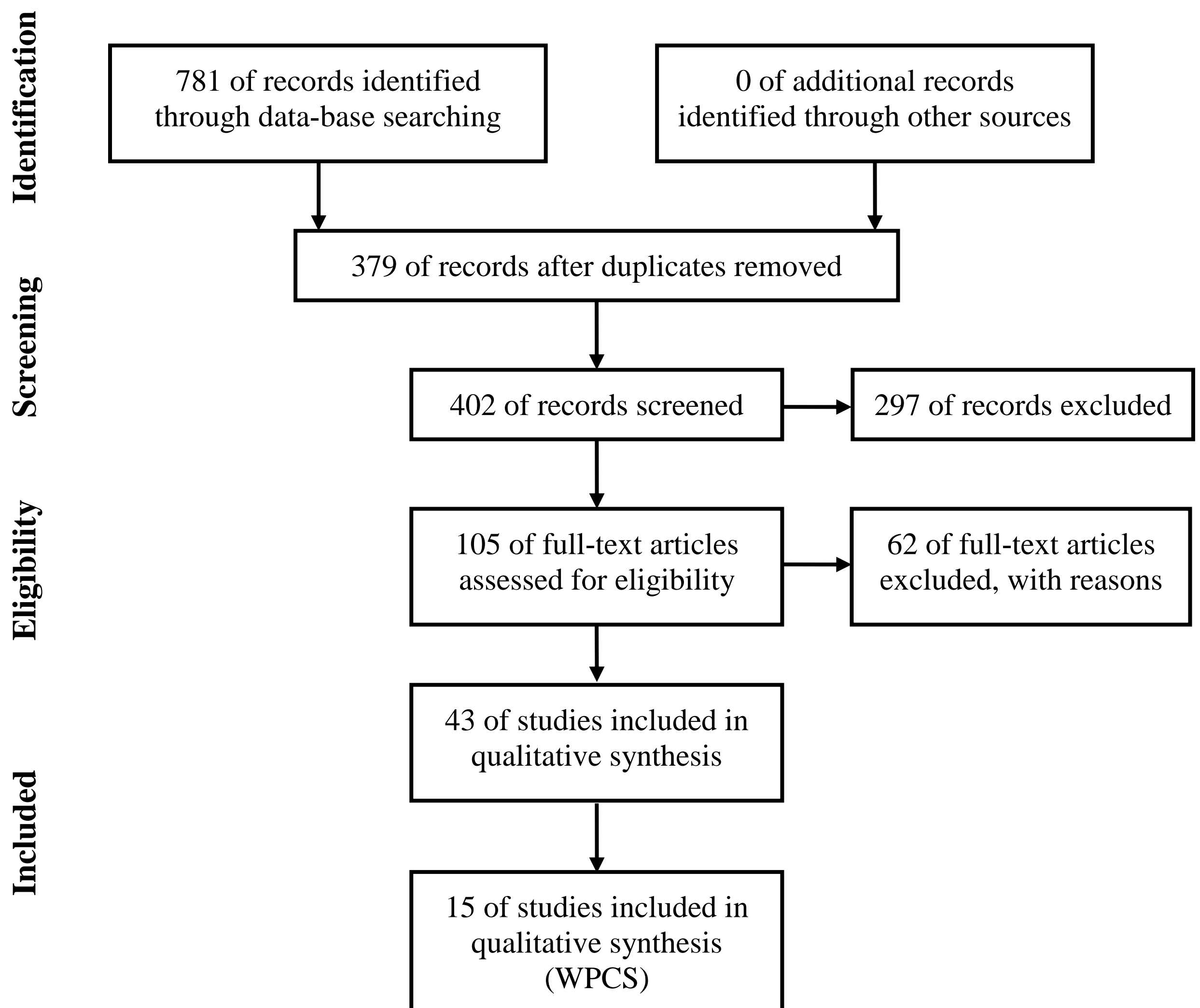

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram adapted from Moher et al. (2009). The flow describes the systematic review from initial search and screening to final selection of publications to be included in the meta-analysis. The 43 articles selected for inclusion in the meta-analysis contained on or multiple experiments.

Table 1. Complete dataset used for meta-analysis and their classifications[1]

Reference	Study	Exp	Substrate[2]	Ensiling period (d)	Temperature (°C)
Ali et al. (2015)	1	1 2	WPC	28, 56, 112	5, 12, 18
Bai et al. (2022a)	2	3	WPC	60	20, 30
Bai et al. (2022b)	3	4	WPC	60	10, 25
Chai et al. (2022)	4	5	Oat	60	25, 35, 45
Cruz et al., unpublished (2025)	5	6	WPC	7, 15, 30, 90	22, 40
Colombatto et al. (2004)	6	7	WPC	120	17.5, 40
Ferrero et al. (2021)	7	8	WPC	15, 30, 100	20, 14.6, 12.2, 4.6
Guan et al. (2020)	8	9	WPC	3, 7, 14, 70	30, 45
Gulfam et al. (2016)	9	10	Napier grass	50	30, 40, 50
Kim and Adesogan (2006)	10	11	WPC	82	20, 40
Koc et al. (2009)	11 12	12 13	WPC Vetch-grain	45 45	20, 30, 37
Hou and Nishino (2022)	13	14 15	Guinea grass	3, 30, 60	25, 40
Bolsen et al. (1988)	14	16 17 18	Alfalfa	90	15.5, 32.2
Coskuntuna et al. (2010)	15	19	Wet brewer's grain	45	20, 30, 37
Kondo et al. (2016)	16	20	TMR	30, 90	15, 30
Liu et al. (2012)	17	21	Stylo	45	20, 30, 40
Liu et al. (2016)	18	22	Napier grass	70	15, 30, 45
Liu et al. (2018)	19	23	Alfalfa	1, 3, 7, 21, 39, 65	15, 30, 45
Liu et al. (2011)	20	24	Stylo	45	10, 20, 30, 40
Li et al. (2019a)	21	25	Corn stalk	7, 14, 30, 60	25, 40
Li et al. (2019b)	22	26	King grass, Paspalum, White Popinac and Stylo	30, 60	28, 40
Li et al. (2021)	23	27	Oat	60	5, 10, 15, 25
Muck et al. (1988)	24	28 29 30 31	Alfalfa	40	15, 25, 35
Ren et al. (2020a)	25	32	Maize straw + Cabbage waste	30, 60, 90, 120	-3, 18, 34
Ren et al. (2020b)	26	33	Cauliflower leaf	30	20, 25, 30, 35, 40, 45
Wang and Nishino (2013)	27	34	TMR	10, 30, 90	5, 15, 25, 35
Wang et al. (2018a)	28	35	Italian ryegrass	30	10, 15, 25
Wang et al. (2018b)	29	36	WPC	55	20, 28, 37

Wang et al. (2019)	30	37	Moringa	60	15, 30
Weinberg et al. (1988)	31	38	Wheat	60	25, 41
Weinberg et al. (2001)	32	39	WPC	63	28, 37
		40	Wheat	60	24, 41
Weiss et al. (2016)	33	41	WPC	141	20, 35
Tamada et al. (1999)	34	42 43 44 45 46	Napier grass	45	40, 30
Tian et al. (2023)	34	47	TMR	56	15, 30, 40
Zhou et al. (2016)	35	48	WPC	60	5, 10, 15, 20, 25
	36	49		28	
Zhou et al. (2019)	37	50	WPC	60	10, 20
Zhang et al. (2010)	38	51 52 53 54	Barley straw Guinea grass	50	20, 30
Zhang et al. (2018)	39	55	Alfalfa	60	20, 30, 40
Zhu et al. (2022)	40	56	Oat	60	5, 15
Hu et al. (2024)	41	57	Sorghum	2, 5, 15, 40	10, 20, 30, 40
Liu et al. (2024)	42	58	Alfalfa	60	20, 35

[1]Silage was stored at different environmental temperatures.

[2]WPC: whole-plant corn; TMR: total mixed ration.

Table 2. Descriptive statistics of the dataset used in the meta-analysis[1]

Item[2]	n[3]	Mean	SD[4]	Minimum	Maximum
DM, %	412	32.1	11.3	12.5	61.2
CP, %	214	9.95	5.17	2.64	27.5
SCP, %	38	48.3	12.2	22.5	68.1
NDF, % DM	256	46.2	15.2	3.87	75.1
ADF, % DM	258	29.1	11.0	2.73	53.6
Lignin, % DM	90	4.22	2.26	1.34	14.4
Starch, % DM	74	35.3	8.26	8.21	44.9
WSC, % DM	225	3.03	3.13	0.090	14.0
Ash, % DM	98	4.10	1.52	2.30	8.70
DM Losses, % DM	162	5.19	4.42	0.430	23.3
pH	548	4.44	0.700	3.41	7.07
Lactic Acid, %DM	568	4.43	2.99	0.00	18.5
Acetic Acid, %DM	568	1.50	1.48	0.00	10.8
LA:AA	238	3.27	1.93	0.010	10.63
Propionic acid, % DM	214	0.18	0.28	0.00	2.43
Butyric acid, %DM	182	0.63	0.92	0.00	3.89
Ethanol, % DM	162	0.87	0.71	0.014	5.54
1,2-propanediol, % DM	46	0.04	0.07	0.00	0.33
NH_3-N, % N	448	7.50	6.42	0.160	45.6
LAB, log CFU/g	274	6.77	1.85	0.00	10.9
Yeast, log CFU/g	224	3.13	2.37	0.00	7.68
Mold, log CFU/g	133	1.82	1.73	0.00	5.80
Aerobic Stability, h	66	81.0	52.6	10.7	240

[1]Silage was stored at different environmental temperatures.

[2]DM: dry matter; CP: crude protein; SCP: soluble crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; WSC: water-soluble carbohydrates; LA:AA: lactic to acetic acid ratio; LAB: lactic acid bacteria.

[3]Number of means.

[4]Standard deviation.

Table 3. Descriptive statistics of the nutritive value and fermentation profile of whole-plant corn silage meta-analysis[1]

Item[2]	n[3]	Mean	SD[4]	Minimum	Maximum
DM, %	125	35.2	5.67	21.8	43.3
CP, %	96	6.88	0.82	9.10	5.60
SCP, %	22	47.0	14.3	22.5	68.1
NDF, % DM	94	40.7	7.78	30.8	59.0
ADF, % DM	94	21.3	4.49	16.4	37.8
Lignin, % DM	22	5.06	0.48	4.31	6.15
Starch, % DM	68	36.0	8.23	8.21	44.9
WSC, % DM	80	3.66	3.62	0.40	14.05
Ash, % DM	90	3.83	1.21	2.30	7.52
DM Losses, % DM	70	3.95	2.84	1.20	13.3
pH	177	4.28	0.75	3.41	6.64
Lactic Acid, %DM	175	4.90	2.10	0.83	10.2
Acetic Acid, %DM	175	1.79	1.58	0.00	8.00
LA:AA	143	3.85	2.02	0.910	10.6
Propionic acid, % DM	63	0.164	0.23	0.00	0.830
Ethanol, % DM	68	1.04	0.380	0.199	1.97
1,2-propanediol, % DM	46	0.04	0.07	0.00	0.330
NH_3-N, % N	129	5.07	3.72	0.160	19.7
LAB, log CFU/g	81	7.36	1.26	3.95	9.39
Yeast, log CFU/g	95	4.24	2.06	0.00	7.68
Mold, log CFU/g	73	1.81	1.53	0.00	4.99
Aerobic Stability, h	66	81.0	52.6	10.7	240

[1]Whole-plant corn silage was stored at different environmental temperatures.

[2]DM: dry matter; CP: crude protein; SCP: soluble crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; WSC: water-soluble carbohydrates; LA:AA: lactic to acetic acid ratio; LAB: lactic acid bacteria.

[3]Number of means.

[4]Standard deviation.

Table 4. The effects of storage temperature on fermentation profile, microbial counts, and aerobic stability of silage[1]

Item[2]	Temperature range, °C					SEM[3]	*P*-value	Contrast[4]	
	≤10	11 - 20	21 - 30	31 - 40	> 40			L	Q
pH	5.21a	4.52b	4.11c	4.19c	4.22bc	0.088	**0.001**	**0.001**	0.37
Lactic Acid, %DM	3.20b	3.88c	5.00a	4.04b	4.83ab	0.286	**0.001**	**0.02**	**0.004**
Acetic Acid, %DM	1.40	1.16	1.09	0.983	1.28	0.099	**0.03**	0.08	0.06
LA:AA ratio	3.11ab	2.96b	3.95a	2.60b	4.52a	1.68	**0.001**	0.54	**0.001**
Butyric acid, %DM	0.266b	0.257b	0.669a	0.277b	0.309ab	0.143	**0.01**	0.71	**0.01**
Propionic acid, % DM	-0.043b	0.150ab	0.196a	0.205a	0.117ab	0.236	**0.003**	0.06	0.08
Ethanol, % DM	0.721	0.886	0.836	0.834	0.829	0.472	0.69	0.47	0.52
NH_3-N, % N	6.53ab	6.12b	7.26a	7.32a	6.17ab	0.773	**0.001**	0.09	0.76
LAB, log CFU/g	7.71a	7.68a	6.85b	5.54c	5.81c	0.983	**0.001**	**0.001**	0.21
Yeast, log CFU/g	4.09a	4.24a	2.87b	2.78b	0.752c	1.46	**0.001**	**0.001**	**0.001**
Mold, log CFU/g	2.41a	2.16a	1.47b	1.72ab	1.44ab	0.497	**0.02**	**0.02**	0.28
DM Losses, % DM	2.92	3.63	4.74	4.44	3.72	0.565	0.37	0.23	0.99

[1]Silage was stored at different environmental temperatures and were grouped and analyzed within five ranges of temperature.

[2]DM: dry matter; LA:AA: lactic to acetic acid ratio; LAB: lactic acid bacteria.

[3]SEM: standard error of the mean.

[4]L: linear; Q: quadratic.

Table 5. The effects of storage temperature on the chemical (DM basis) composition of silage[1]

Item[2]	Temperature range, °C					SEM[3]	*P*-value	Contrast[4]	
	≤ 10	11 - 20	21 - 30	31 - 40	> 40			L	Q
DM, %	30.5b	30.1b	31.0b	30.9ab	32.5a	0.732	**0.001**	**0.003**	0.34
WSC, % DM	4.85b	2.19c	2.54c	2.45c	7.76a	0.409	**0.001**	**0.01**	**0.001**
CP, %	9.59ab	9.79a	9.49b	9.55ab	8.72c	0.416	**0.001**	**0.003**	**0.001**
SCP, %	-	43.3c	48.9b	53.4a	-	2.18	**0.001**	**0.001**	0.44
NDF, % DM	48.0	46.2	46.8	46.7	46.1	1.36	0.23	0.79	0.48
ADF, % DM	29.2	29.9	30.1	29.7	30.3	2.68	0.16	0.65	0.86
Starch, % DM	32.6	33.6	37.9	36.3	36.8	2.16	0.16	0.14	0.58
Lignin, % DM	3.06b	4.99a	5.25a	5.46a	5.68a	1.13	**0.002**	**0.01**	0.20
Ash, % DM	4.42	4.41	4.34	3.82	-	0.203	0.33	0.06	0.13

[1]Silage was stored at different environmental temperatures and were grouped and analyzed within five ranges of temperature.

[2]DM: dry matter; CP: crude protein; SCP: soluble crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; WSC: water-soluble carbohydrates.

[3]SEM: standard error of the mean.

[4]L: linear; Q: quadratic.

Table 6. The effects of storage temperature on fermentation profile, microbial counts, and aerobic stability of whole-plant corn silage[1]

Item[2]	Temperature range, °C					SEM[3]	*P*-value	Contrast[4]	
	≤ 10	11 - 20	21 - 30	31 - 40	> 40			L	Q
pH	5.64a	4.56b	3.95c	4.07bc	3.92bc	0.1849	**0.001**	**0.001**	0.75
Lactic Acid, %DM	3.04b	4.26b	5.63a	3.68b	3.34b	0.4811	**0.001**	0.56	0.22
Acetic Acid, %DM	1.84	1.60	1.56	1.59	1.27	0.3020	0.89	0.52	0.88
LA:AA	3.67ab	3.56b	4.69a	2.18b	3.31ab	0.5145	**0.001**	0.10	**0.01**
Propionic acid, % DM	-0.048b	0.207ab	0.243a	0.079b	-	0.0518	**0.01**	0.64	0.48
Ethanol, % DM	1.04ab	1.16a	0.958ab	0.831b	-	0.0757	**0.01**	**0.003**	0.94
1,2-propanediol, % DM	-0.091b	0.0570a	0.0690a	0.0680a	-	0.0221	**0.001**	**0.03**	**0.004**
NH_3-N, % N	2.67c	4.13b	5.02a	6.25a	5.15a	0.4943	**0.001**	**0.001**	0.11
LAB, log CFU/g	7.53a	7.91a	7.96a	5.57b	-	0.1786	**0.001**	**0.001**	**0.001**
Yeast, log CFU/g	5.37a	4.54ab	3.60b	3.94b	-	0.3594	**0.02**	**0.02**	0.49
Mold, log CFU/g	1.32	1.74	2.13	2.38	-	0.3915	0.35	0.11	0.39
DM Losses, % DM	1.71c	1.97c	4.22b	4.59b	7.58a	0.3719	**0.001**	**0.001**	**0.002**
Aerobic Stability, h	16b	52ab	108a	77a	-	27.51	**0.01**	**0.001**	0.86

[1]Whole-plant corn silage was stored at different environmental temperatures and were grouped and analyzed within five ranges of temperature.

[2]DM: dry matter; LA:AA: lactic to acetic acid ratio; LAB: lactic acid bacteria.

[3]SEM: standard error of the mean.

[4]L: linear; Q: quadratic.

Table 7. The effects of storage temperature on the chemical (DM basis) composition of whole-plant corn silage[1]

Item[2]	Temperature range, °C					SEM[3]	*P*-value	Contrast[4]	
	≤ 10	11 - 20	21 - 30	31 - 40	> 40			L	Q
DM, %	32.9b	33.0b	34.0a	33.7a	-	1.378	**0.001**	**0.001**	0.63
WSC, % DM	6.60b	2.53c	3.18c	2.84c	12.4a	0.8798	0.001	**0.02**	**0.001**
CP, %	7.25b	7.47a	7.13b	7.04b	6.55b	0.3402	**0.001**	**0.004**	0.06
SCP, %	-	39.6c	45.1b	58.9a	-	3.065	**0.001**	**0.001**	0.08
NDF, % DM	41.7	41.1	41.9	39.4	39.9	1.476	0.07	0.28	0.34
ADF, % DM	21.5	20.9	21.6	20.4	21.0	0.8185	0.28	0.58	0.22
Starch, % DM	33.4	34.4	38.7	37.2	-	1.941	0.09	0.12	0.87
Lignin, % DM	-	4.61b	5.00a	5.20a	-	0.0957	**0.001**	**0.001**	0.56
Ash, % DM	4.08	4.06	4.01	3.58	-	0.2445	0.46	0.16	0.33

[1]Whole-plant corn silage was stored at different environmental temperatures and were grouped and analyzed within five ranges of temperature.

[2]DM: dry matter; CP: crude protein; SCP: soluble crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; WSC: water-soluble carbohydrates.

[3]SEM: standard error of the mean.

[4]L: linear; Q: quadratic.

CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

Substantially research in silage production aims to improve the fermentation profile and quality of corn and tropical grass silage; however, the focus and strategies often diverge due to their distinct fermentation patterns. The experiments described here were conducted to evaluate how strategies such as the use of additives, including enzymes and microbial inoculants, and storage length affect silage quality. Additionally, decisions like determining the optimal regrowth age for tropical grasses and examining the influence of storage temperature on fermentation, aerobic exposure, and interactions with microbial inoculants were addressed. Results obtained from the storage temperature trial with whole-plant corn silage (WPCS) motivated us to conduct a meta-analysis to further elucidate the effects of storage temperature on the quality of WPCS, as well as other silage types. We hope that our results contribute to a better understanding of how the factors studied influence silage dynamics and open pathways for further research to advance silage fermentation science and microbiology globally.

The first experiment evaluated how exogenous fibrolytic enzymes, specifically cellulase and hemicellulase, applied individually or in combination, affect the nutrient composition, fermentation profile, aerobic stability, and in vitro degradation kinetics of Mombasa grass and elephant grass (cv. BRS Capiaçu) ensiled at two regrowth ages. Overall, cellulase improved the fermentation profile of Mombasa grass silage by increasing LAB and lactic acid concentration, while reducing clostridial activity, which led to lower butyric acid and ammonia-N contents. In contrast, xylanase did not improve the fermentation profile but did enhance the dry matter and neutral detergent fiber degradability, particularly for 12-wk regrowth BRS Capiaçu silage. Additionally, silage from both grasses had higher nutritive value and degradability at earlier regrowth ages, indicating that delaying harvest to increase tropical grass dry matter content may not be beneficial to the farm nutritional program and profit, as

delaying harvest may reduce silage digestibility, potentially offsetting productivity gains. Using both enzymes together would increase production costs without providing sufficient returns. It is also important to note that cellulase increased effluent losses for both forage species and reduced aerobic stability for BRS Capiaçu silage, which may be a concern for more conservative producers in terms of silage management. Further research should explore the synergetic effects of cellulase with chemical, absorbent, and microbial additives to mitigate these limitations.

The second experiment assessed the effects of microbial inoculation, storage temperature, and storage length on the fermentation profile, microbial counts, nutrient composition, and aerobic stability of WPCS. Results indicated that the strains *Pediococcus acidilactici* DSM 16243, *Lentilactobacillus buchneri* DSM 12856, and *L. diolivorans* DSM 32074 increased acetic acid concentrations, which subsequently enhanced aerobic stability. However, these benefits only occurred when the silage was stored at moderate temperatures around 22 °C. High storage temperatures (40 °C) not only limited inoculant efficacy but also reduced fermentation intensity and led to increased yeast and mold proliferation after exposure to air. Moreover, high temperatures promoted greater proteolysis, increasing ammonia-N and soluble crude protein concentration, as well as enhancing in vitro starch digestibility. Results did not support the hypothesis that *L. diolivorans* inoculation would increase propionic acid concentrations, likely due to substrate availability, environmental conditions, and redox potential, resulting instead in increased 1-propanol as the only product from 1,2-propanediol metabolism.

The third experiment was a meta-analysis conducted to assess the effects of storage temperature on the fermentation profile and nutritive value of silage across various types, regions, and cultivation conditions. Given the global significance of WPCS in silage production, we also conducted a sub-meta-analysis focused exclusively on studies involving

WPCS. Findings indicated that extreme temperature ranges, high and low, affect LAB activity, reducing acid production and compromising silage fermentation. This suggests that a temperature range of 21-30 °C may be more conducive for silage storage and fermentation due to greater total acid production and a faster pH decline. Additionally, lower temperatures were associated with increased counts of undesirable microorganisms such as yeasts and molds, which decreased aerobic stability. Future research should explore how silage storage temperature affects animal performance and safety when feeding these silages. Studies examining the dynamic changes in silage microbiomes could also provide new insights, particularly into isolating and inoculating silage with heat- and cold-resistant heterofermentative strains to improve aerobic stability under variable temperature conditions.

Given the critical importance of silage production to farm profitability, the interest and investment in research focusing on enhance precision in silage production will persist. In my view, scientific trends in the coming years will likely focus on the silage microbial community and the development of additives, including new enzymatic sources and novel bacterial strains for inoculants. The interactions of these additives with both the substrate and the bacterial community are not yet well understood, and this knowledge gap could be a focal point for future studies. Producing silage under various abiotic conditions, such as storage temperature, will challenge the use of these additives, which may become essential for enhancing the dairy industry's resilience to increasingly adverse conditions expected in the future.