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Evaluation of preharvest and postharvest factors on forage crop quality, physiology, and ensiling characteristics

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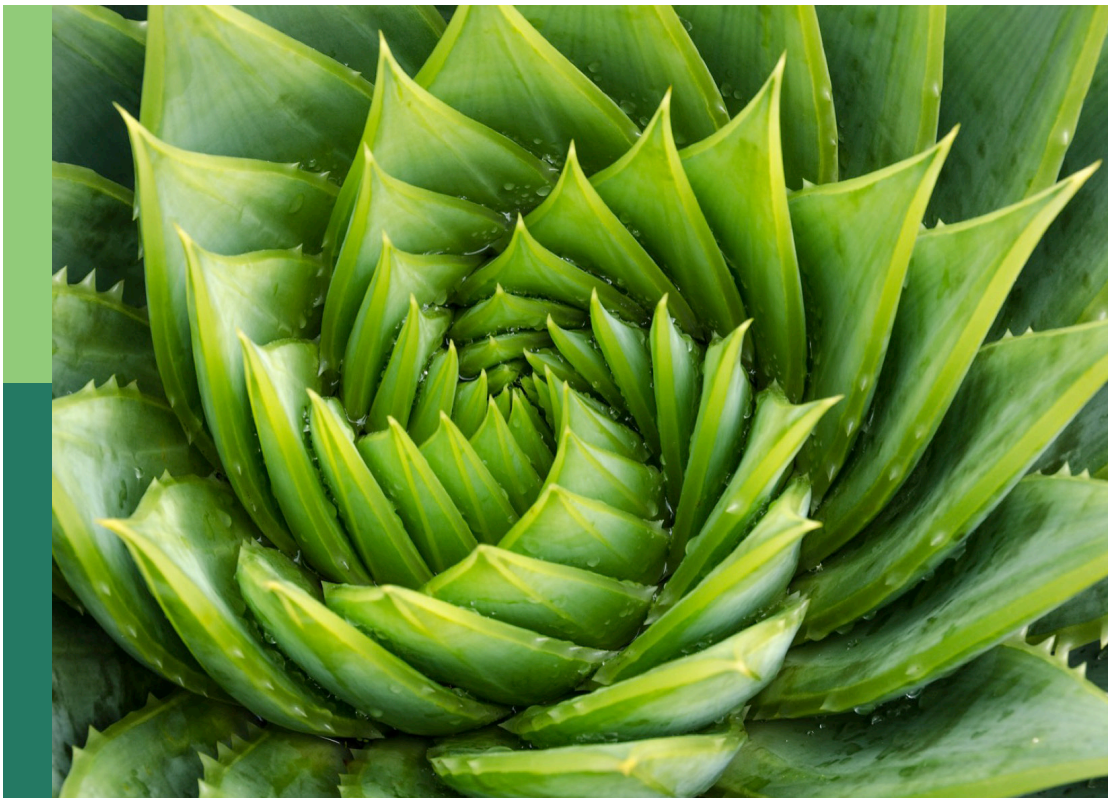

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Evaluation of preharvest and postharvest factors on forage crop quality, physiology, and ensiling characteristics

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Editorial: Evaluation of preharvest and postharvest factors on forage crop quality, physiology, and ensiling characteristics

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forage, microbial community, nutritive value, ensiling, silage, crop management

Editorial on the Research Topic

Evaluation of preharvest and postharvest factors on forage crop quality, physiology, and ensiling characteristics

The purpose of making silage is to preserve the nutritive value of the harvested crop. The preharvest factors of forages that affect silage nutritional value are plant physical structure and chemical composition, which are mainly affected by maturity at harvest, plant genotypes (species, cultivars within species, hybrids), crop management (sowing time, planting method, irrigation, fertilization, weeds and pest control, plant microbiology), epiphytic and endophytic microbiota (particularly lactic acid producing bacteria (LAB)), and the environment in which the crop is planted and harvested (temperature, rainfall, weather, soil, day light, and some nature disasters). The postharvest factors include harvest management (machinery, cutting height, wilting, bruising and chopping), ensiling management (type of silo, compaction/density, sealing, type and dose of silage additives) and feeding management (exposure time to air). Silo management is also a key factor that affects quality of ensiled forages, including the speed and degree of filling silo, the storage method, and the rate of consuming silage. Recently, most researchers focused on the improvement of forage yield and fermentation quality through breeding, optimizing crop harvest maturity, and using various biological and chemical additives for improving silage fermentation quality and post-ensiling stability. However, the aforementioned factors are

often not integrated with field management and methods of harvesting and ensiling in improving silage fermentation and nutritive value. Hence, this Research Topic aimed to explore the preharvest and postharvest factors affecting forage physiology, nutritional quality, fermentation characteristics and post-ensiling quality.

Our Research Topic comprises 8 original research articles and 2 reviews contributed by 67 authors. In this topic, Wang et al. (2024) evaluated the effects of adding *Bacillus velezensis* to whole-plant corn silage (WPCS), to explore the factors contributing to the fermentation characteristics of WPCS. They found that *B. velezensis* enhanced the silage quality and aerobic stability of WPCS during the milk-ripe stage by promoting the proliferation of LAB. In comparison to *Lactiplantibacillus plantarum*, *B. velezensis* rapidly lowered the pH of WPCS, caused greater degradation of fiber compositions, and improved water-soluble carbohydrate (WSC) level, and aerobic stability of the silage.

Crop–livestock integrated systems can optimize the efficiency of inputs and nutrients (re)-utilization, and promote the production and sustainability in agriculture. Nevertheless, limited studies investigated the triple intercropping of forages for ensiling. Prado et al. (2023) evaluated the dry biomass yield, ensiling characteristics, and nutritional components of sorghum silage intercropped with *Stylosanthes* cv. Bela and *Tamani guinea* grass as an integrated cropping system for silage production. The results indicated intercropping of sorghum with tropical forages could be used for producing mixed silage with several advantages, such as greater biomass production per unit area, superior nutritional quality, and pasture availability after harvesting crop for grazing and producing silage, finally improving the land-use efficiency with a sustainable way. Mixed silage from sorghum with *Stylosanthes* cv. Bela and *Tamani guinea* grass presented better fermentation quality, higher total digestible nutrient and ether extract contents than monocropped-forage silage. Mixed cropping with tropical forages increased the crude protein (CP) content as compared to monocropped-sorghum silage, which is expected to probably decrease costs with acquisition of protein supplements. Finally, they suggested the triple intercropping of Bela, sorghum, and *Tamani guinea* grass for producing mixed silage, due to higher yield, better fermentation and nutritional value of silage, and fresh forage availability for a longer period.

The WPCS is an important feedstuff of dairy rations globally whereby its great biomass yield and metabolizable energy. Whereas, the nutritive value of WPCS changed in different seasons during the developing stage. The interactions among management (M) × environment (E) × genotype (G) influenced the partitioning to grain (Harvest Index, HI). Hence, modelling tool could be used to accurately predict the variations of HI during crop growth. Ojeda et al. (2023) conducted a study to (i) explore the main factors affecting HI variability and grain yield, (ii) check the APSIM system (Agricultural Production Systems Simulator) to evaluate the growth of crop and crop partitioning with sufficient experimental data, and (iii) study the main resources of HI difference with a large scale of combined M × E × G. Sowing date, nitrogen rates, plant density, harvest date, genotype data, and irrigation rates were utilized from experimental fields to evaluate the main factors of HI variability and to check the growth of corn with APSIM. They concluded that the model supplies some good insights into the improvement of nutritional value of corn silage, and choosing the genotype and harvest timing when making decision.

Some plants could tolerate the poor soil and drought and high-temperature conditions, thus they could be utilized as feed sources for ruminants under different harsh environments. Amaranth (*Amaranthus hypochondriacus*) is one of such plants. Furthermore, a lower content of lignin (4% DM) and lower levels of nitrate and oxalic acid, and a higher content of CP (25% DM) were found in amaranth compared to corn. Ma et al. (2023) selected LAB, glucose and cellulase as silage additives, and investigated the impacts of various additives on the ensiling characteristics, ruminal digestion, and aerobic stability of mixed silages containing corn straw and amaranth. They found that the combined addition of cellulose, glucose, and LAB enhanced CP, lactic acid, DM contents and LAB populations, and reduced neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents. The combined inoculant also decreased mold and aerobic bacteria population and enhanced ruminal digestion and aerobic stability of mixed silages containing corn straw and amaranth.

The saline–alkali soils are widely distributed in the world. Alfalfa (*Medicago sativa* L.) is a good legume forage with high palatability and nutritive value. The rood system of alfalfa is very deep, contributing to fixing the nitrogen, thus planting alfalfa can improve the soil fertility. In saline–alkali soils, alfalfa could supply a large amount of quality protein, with ensiling being one of good utilization and preservation ways, especially in some fields with climatic limitations. Shi et al. (2023) evaluated the effects of endogenous potassium (K^+) and sodium (Na^+) in crops on the silage quality and bacterial community compositions during the ensiling of alfalfa. The results showed that the level of Na^+ in silage enhanced as the salt stress increasing. Enhanced salt stress resulted in changes in bacterial compositions, with increased abundances of *Pantoea* and *Lactococcus*, particularly under high salt stress. Moreover, the accumulation of endogenous Na^+ in alfalfa limited bacterial growth under salt stress, thus suppressing protein degradation during ensiling. Higher levels of Na^+ can be found in alfalfa silage under higher salt stress. The good silage quality of alfalfa under salt stress could be ascribed to the dominant genera *Lactobacillus* and *Lactococcus* in alfalfa silage.

Corn often has lower counts of epiphytic LAB, and contents of lactic acid and WSC contents, and poor aerobic stability and silage quality in high-temperature environment. Khan et al. (2023) systematically assessed the impacts of molasses, homofermentative LAB (homLAB), heterofermentative LAB (hetLAB) and their mixture (MIX) on fermentative profile, chemical compositions, carbohydrate fractions, *in vitro* digestibility of DM (IVDMD), microbial populations, and aerobic stability of corn in high-temperature environment (30~45°C). They concluded that applying additives could enhance DM recovery, nutritive value, silage quality, and aerobic stability of corn under high-temperature condition. The

MIX inoculant also performed well in improving the silage quality and aerobic stability, whereas more studies are required, especially in the effect of dose rate.

The level of STC (kernel starch) can indicate the nutritive value of corn, and is directly related to the grains' aroma and taste. The magnesium (Mg) and calcium (Ca) are important nutrients for the development and growth of corn and the STC content. To assess the physiological mechanisms of Mg and Ca impacts on the production of STC in corn kernel and impacts of endogenous enzymes and hormones synthesis in corn leaves on STC, He et al. (2024) added foliar Mg and Ca fertilizers at different doses to corn before pollination. They reported that the production of STC was evidently increased by Mg and Ca addition with modulating the levels of endogenous hormone and activity of key enzymes. Their results explore a new way to enhance the nutritive value of corn.

Recently, volatile metabolomics and microbiomics have become important tools of modern biotechnology, showing potential for more applications in food nutrition and science field. Microbiomics gives some insights into the abundance, species, and functionality of various microbes with studying the microbial community, which can also present the correlations with the flavor and quality of fermentative products. Volatile metabolomics could be used to identify the relationship between microbial activity and fermentation products via studying the variation and compositions of volatile compounds in foods. Deng et al. (2023) analyzed the silage quality, volatile metabolites, and bacterial communities of oat silages harvested at two growth stages, and examined the relationship between volatile metabolites and microbes. They concluded that the growth stages had great impacts on nutritional compositions, fermentative products, and flavor characteristics of oat, with the fermentation profile predominated by *Lactiplantibacillus* resulting in favorable flavor, while the fermentation profile predominated by *Enterococcus* resulted in unpalatable flavor.

In poultry and livestock production, some plant extracts can be used as additives to replace the antibiotics. Plant extracts are extracted from plants, and contain some bioactive components and pharmacological property, thus they need to be further studied. Furthermore, they are an ecofriendly and sustainable additive because they possess natural biodegradable characteristics and can decrease the application of chemicals. Plant essential oils can replace the antibiotic as feed additives due to their inhibition on bacteria and fungi. With the widely application of plant essential oils, their effects on improving the silage quality have been studied. Chen et al. (2023) reviewed the effects of essential oils and their activity on inhibiting bacteria and fungi, and explored the contribution of plant essential oils to silage quality, and provided some knowledge to the application and development of plant essential oils as feed additives in silage production. They found that plant essential oils can inhibit the growth and activity of various harmful microorganisms in silages whereby their ability to affect the permeability of cell membrane, ATPase activity and cell division, and limit biofilm formation. The application of suitable doses of plant essential oils in producing silage could affect microbial compositions via limiting the growth of some undesirable microbes such as yeast, Fusarium and Clostridium in silages, and indirectly accelerate LAB strains to become predominant microbes, decrease nutritional loss in silages, enhance aerobic stability and fermentation quality of silages.

With the rapid enhancement of global population and economy, the demand for animal products like milk, egg and meat are increasing. The shortage of feeds in animal industry is a global issue limiting the development of livestock. Natural woody plants can be found in many districts with a big biomass yield. The fresh branches and leaves of many woody plants have high levels of nutritional compositions like amino acids, crude protein, minerals and vitamins, and could be utilized to produce silages for animals. Hence, it is important to develop and use the woody plants for producing the clean and sustainable feeds in livestock. Du et al. (2023) reviewed the research progress, current status and development prospects of natural woody plant feed resources. The nutritive value and application of natural woody plants, the main factors influencing the silage quality of woody plant and the interaction mechanisms between secondary metabolites and microbial co-occurrence network were studied. Different preparing technologies for clean fermentation of woody plant silages were summarized, which presented a sustainable way for enhancing the production efficiency of livestock. Hence, woody plants are very important as a potential source of natural feeds in reducing feed shortage and accelerating sustainable development of livestock products.

In summary, this Research Topic explored the preharvest and postharvest factors influencing forage quality, physiology, and fermentation characteristics using various approaches. This Research Topic provided interesting results, and provided solution to some unresolved issues that need more exploration in producing silage. However, there is still a deep study gap identifying the impact of preharvest and postharvest factors on animal response. Therefore, further studies should focus on improving the field management and harvesting methods in silage production and in relation to animal performance.

Author contributions

SW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. QZ: Data curation, Methodology, Supervision, Writing – review & editing. LS: Formal analysis, Project administration, Validation, Writing – review & editing. HP: Data curation, Formal analysis, Methodology, Supervision, Writing – review & editing. PL: Formal analysis, Project administration, Validation, Writing – review & editing. NK: Data curation, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Field and in-silico analysis of harvest index variability in maize silage

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Maize silage is a key component of feed rations in dairy systems due to its high forage and grain yield, water use efficiency, and energy content. However, maize silage nutritive value can be compromised by in-season changes during crop development due to changes in plant partitioning between grain and other biomass fractions. The partitioning to grain (harvest index, HI) is affected by the interactions between genotype (G) × environment (E) × management (M). Thus, modelling tools could assist in accurately predicting changes during the in-season crop partitioning and composition and, from these, the HI of maize silage. Our objectives were to (i) identify the main drivers of grain yield and HI variability, (ii) calibrate the Agricultural Production Systems Simulator (APSIM) to estimate crop growth, development, and plant partitioning using detailed experimental field data, and (iii) explore the main sources of HI variance in a wide range of G × E × M combinations. Nitrogen (N) rates, sowing date, harvest date, plant density, irrigation rates, and genotype data were used from four field experiments to assess the main drivers of HI variability and to calibrate the maize crop module in APSIM. Then, the model was run for a complete range of G × E × M combinations across 50 years. Experimental data demonstrated that the main drivers of observed HI variability were genotype and water status. The model accurately simulated phenology [leaf number and canopy green cover; Concordance Correlation Coefficient (CCC)=0.79-0.97, and Root Mean Square Percentage Error (RMSPE)=13%] and crop growth (total aboveground biomass, grain + cob, leaf, and stover weight; CCC=0.86-0.94 and RMSPE=23-39%). In addition, for HI, CCC was high (0.78) with an RMSPE of 12%. The long-term scenario analysis exercise showed that genotype and N rate contributed to 44% and 36% of the HI variance. Our study demonstrated that APSIM is a suitable tool to estimate maize

HI as one potential proxy of silage quality. The calibrated APSIM model can now be used to compare the inter-annual variability of HI for maize forage crops based on G × E × M interactions. Therefore, the model provides new knowledge to (potentially) improve maize silage nutritive value and aid genotype selection and harvest timing decision-making.

KEYWORDS

silage quality, APSIM, crop modelling, calibration, forage, *Zea mays* L.

1 Introduction

Maize (*Zea mays* L.) silage is a key component of the dairy cow feed ration in intensive dairy production systems and is also used in temperate pasture-based systems to supplement cows' diets when pasture availability is low (Wales and Kolver, 2017). The inclusion of maize in dairy cows' diets has increased over the past 30 years due to its high total yield (Ferraretto et al., 2018), digestible energy content (Sucu et al., 2016), and the opportunity for relatively long-term storage as silage with limited nutritive value loss when ensiled properly (Borreani et al., 2018).

Crop growth, phenology, and yield are also affected by genetics (Barlow et al., 2012; Borreani et al., 2018; Gruber et al., 2018), environmental (Bernardes et al., 2018), and management factors (Nilahyane et al., 2020; Petković et al., 2022). For the latter, the inputs of nitrogen (N) fertiliser and water have the greatest economic and environmental impact with regards to maize production (Petković et al., 2022). Optimal irrigation of maize crops can increase biomass partitioning to grain (Li et al., 2020) and the management of irrigation and N (defined by rate and timing) can affect dry matter allocation to different parts of the plant, and, therefore, maize silage nutritive value (Nilahyane et al., 2020; Zhou et al., 2021). Maize silage of high quality can result in a feed ingredient with a high recovery of dry matter, energy, and highly digestible nutrients compared with the fresh crop (Kung et al., 2018). More specifically, starch content and, therefore, grain yield can markedly improve silage forage quality (Nazli et al., 2019) and, consequently, milk production of dairy systems (Boerman et al., 2015).

However, maize yield (grain and total plant) and climatic conditions can vary greatly both spatially and temporally (Khalili et al., 2013; Nilahyane et al., 2020). Thus, predictive tools that provide a quantification of the variability in the grain proportion relative to biomass, i.e., the ratio between grain yield and aboveground biomass (HI) of maize crops grown for silage, are urgently required. Such tools would allow farmers and advisors to predict the inter-annual variability of maize HI based on genotype (G) × environment (E) × management (M) interactions and, therefore, provide a decision support system to aid genotype selection (Hütsch and Schubert, 2017; Gruber et al., 2018) and harvest timing (Borreani et al., 2018; Ferraretto et al., 2018) decision making for optimising ration formulations. Mechanistic crop models (hereafter 'models') are effective tools for understanding the complexity of system interactions to achieve productivity and environmental goals (Archontoulis et al., 2014). Models enable the analysis of climate variability on crop growth and thus inform the development of adaptation strategies (Challinor et al., 2013; Jahangirlou et al., 2023). Despite the increasing use of models, there are implicit model uncertainties (i.e., deviations derived from a probability distribution of simulations generated using different parameters) and prediction uncertainties (i.e., deviations between simulations and observations commonly named residuals) (Chapagain et al., 2022). Therefore, there is a need for a standardised approach to quantify both model and prediction uncertainty (Chapagain et al., 2022) to increase model accuracy.

The Agricultural Production Systems Simulator (APSIM) (Holzworth et al., 2014) is a mechanistic biophysical crop model that estimates crop growth and development in response to G × E × M interactions. This model has been widely used for maize under several productive scenarios (Archontoulis et al., 2014; Ojeda et al., 2018b; Rotili et al., 2020; Kamali et al., 2022; Jahangirlou et al., 2023). These studies demonstrated that APSIM had a reasonable to very good accuracy for biomass and maize grain yield estimations. Apart from some exceptions (Pembleton et al., 2013; Teixeira et al., 2017; Ojeda et al., 2018a; Ojeda et al., 2018b; Morel et al., 2020), previous studies assessing the performance of APSIM to estimate crop yield have been largely focused on maize crops grown for grain, using specific grain yield harvest-destined genotypes and crop management to achieve high grain yields. While these studies mainly assessed the effect of crop management and environment on maize biomass and grain yield, the effect of genotype on HI has not been assessed under a wide range of crop management conditions. There is only one study where maize quality was estimated by combining APSIM yield simulations with logistic regression models with a focus on grain composition although it used grain genotypes and generated predictions based on simulated grain dry weight (Jahangirlou et al., 2023). The model's ability to estimate crop growth and phenological responses for maize silage genotypes and management under contrasting environments and focus on the prediction of HI across a wide range of G × E × M is still required.

The objectives of this study were to (i) identify the main drivers of grain yield and HI variability, (ii) calibrate APSIM to estimate crop growth, development, and plant partitioning using detailed experimental field data, and (iii) explore the primary sources of HI variability of silage maize in a wide range of G×E×M combinations.

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2 Material and methods

In this study, we applied two levels of analysis which are described in detail in 2.1 and 2.5. First, we identified the main drivers of HI (ratio between grain yield and biomass) variability at plot level using a non-complete factorial combination of fields which was analysed by experiment depending on the factors present in each experiment. Second, we used APSIM to generate results from a complete factorial combination (and therefore interactions) where we used a widely known Analysis of Variance approach to decompose the effect of each factor and their interactions on HI variability. For the analysis of simulated data, we applied a G × E × M model to disentangle the effect of G, E, and M on HI variability at one site. The E component was implicit in the inter-annual variability of climate across years.

2.1 Experimental datasets

Data from four field experiments conducted by the University of Sydney in different locations (P-farm, Westwood, and Mayfarm) near Camden, NSW, Australia (33° 59' 53.52" S; 150° 36' 45.72" E) were used to determine the key drivers of grain yield and HI variability and to calibrate the maize crop module in APSIM Classic (v7.10; https://www.apsim.info/) to predict biomass partitioning (Table 1). The Mayfarm site included two experimental years (MayfarmY1 and MayfarmY2) and several in-season crop measurements. A detailed description of these two experiments is provided by Islam et al. (2011); Islam et al. (2012) and Islam and Garcia (Islam and Garcia, 2012; Islam and Garcia, 2014). P-farm and Westwood sites included one experiment each and data at the final harvest for silage. For P-farm, hybrid forage maize (Pioneer 2307) was sown on 4 November 2012 in 20 × 20 m blocks within a 30-ha paddock. Maize was planted at two plant densities (6.6 plants m^{-2} and 7.4 plants m^{-2}) with row-to-row distances of 79 cm and harvested at three different stages of maturity [31%, 38%, and 45% whole plant dry matter (DM)] on 14 March, 27 March, and 8 April in 2013, respectively, for both sowing densities. Each treatment (density × maturity stage at harvest) was replicated in three blocks. For Westwood, two hybrid forage maize (PacificX and PioneerX) were grown in 60 × 15 m blocks within a 20-ha paddock. Both maize forages were sown on 20 November 2012 at two plant densities (5.5 plants m^{-2} and 8.2 plants m^{-2}) with row-to-row distances of 68 cm and harvested at three different stages of maturity (31%, 42%, and 45% DM) on 20 March, 8 April, and 15 April in 2013, respectively, for both sowing densities. In both P-farm and Westwood, each treatment was

TABLE 1 Description of maize field experiments, factors of analysis, treatments, and observations (in-season and at final harvest) used to calibrate the Agricultural Production Systems Simulator.

Experiments	P-farm		Westwood		MayfarmY1		MayfarmY2	
	Factor	*Value*	*Factor*	*Value*	*Factor*	*Value*	*Factor*	*Value*
Factorial combinations	Harvest date	120 DAS*	Harvest date	120 DAS	Sowing date	20-Oct	Irrigation rates	0 mm (0%)
		133 DAS		139 DAS		3-Nov		153 mm (33%)
		145 DAS		141 DAS	N fertilisation rate pre-sowing	0 kgN ha^{-1}		305 mm (66%)
	Sowing density	6.3-6.9 plants m^{-2}	Genotype	PioneerX		135 kgN ha^{-1}		480 mm (100%)
		7.2-7.7 plants m^{-2}		PacificX	N fertilisation rate post-sowing	0 kgN ha^{-1}	N fertilisation rate pre-sowing	0 kgN ha^{-1}
			Sowing density	5.2-6 plants m^{-2}		79 kgN ha^{-1}		135 kgN ha^{-1}
				6.4-9.3 plants m^{-2}		158 kgN ha^{-1}	N fertilisation rate post-sowing	0 kgN ha^{-1}
								79 kgN ha^{-1}
								158 kgN ha^{-1}
Treatments (replications)	6 (3)		12 (4)		12 (4)		24 (4)	
Obs. variables in-season by treatment					12 (2)		24 (2)	
Obs. Variables at harvest by treatment	6 (3)		12 (4)		12 (4)		24 (4)	

*DAS, days after sowing.

assigned to a plot of 5 × 3 m and 5 × 2 m, respectively, with a 1 m buffer on each side of the plot, and plots were randomised within each block. All experiments included combinations of different factors (crop management and genotypes) as shown in Table 1. For all experiments, total aboveground biomass and its partitioning between plant organs (leaf weight, stem weight, and grain + cob weight) were measured. Total biomass was measured by harvesting the whole plot, while leaf, stem, and grain + cob weight were estimated based on the proportions determined from one sampled plant per plot (Islam et al., 2012; Islam and Garcia, 2014). For the Mayfarm site, leaf number and normalised difference vegetation index (NDVI) were also measured sequentially over the whole growing period. The number of observations varied between experiments and variables assessed (Table 2). The NDVI observations were converted to the proportion of intercepted photosynthetically active solar radiation, commonly known as fPAR or cover green in APSIM, using the equation proposed by Pellegrini et al. (2020) as follows:

$$Green\ \ Cover = (1.5\ \times NDVI) - 0.29 \qquad \text{Eq. (1)}$$

2.2 Model description

We used the maize crop module within APSIM Classic 7.10 (Holzworth et al., 2014). The model has been described at https://www.apsim.info/documentation/model-documentation/crop-module-documentation/maize/ and calibrated with satisfactory results across an extensive range of environments (Ojeda et al., 2018b; Wu et al., 2021). A complete description of the model structure and parameters was provided by Brown et al. (2014a) and Brown et al. (2019). The model contains algorithms that simulate crop phenology, growth, and soil-plant C and N dynamics. In brief, crop phenology is simulated using thermal time thresholds for each phenological stage. The model accumulates thermal time until a target is achieved, which defines the change of the stage. The growing period for the whole crop cycle of maize in APSIM is mainly influenced by the cumulative thermal time from emergence to the end of the juvenile phase and flowering (R1; Ritchie et al., 1996) to physiological maturity (R6), as well as by the phyllochron (leaf appearance rate). When water and N conditions are optimum, crop daily growth rate is only limited by photosynthetically active solar radiation (PAR) and is calculated as the product of intercepted PAR and radiation use efficiency. When the crop is under water stress, biomass accumulation is calculated as the product of potential crop transpiration (limited by available soil moisture within field capacity and permanent wilting point, root extent, and water uptake capacity) and transpiration efficiency, adjusted for atmospheric vapour pressure deficit (Brown et al., 2014b). Table S1 shows details on key embedded processes in the APSIM-Maize model.

Potential biomass partitioning among plant parts in APSIM depends on the crop development stage and uses partitioning ratios (Brown et al., 2019). Between emergence and flag leaf (the last leaf to appear and expand), the fraction of biomass that is provisionally allocated to the growing leaves decreases as the number of fully expanded leaves increases. Between tassel initiation and flag leaf, the biomass remaining after allocation to the leaves is partitioned between the stem and the developing cobs with a fixed ratio. After flag leaf, biomass is partitioned between the stem and the cobs only, until partitioning to the grain starts at the onset of grain filling (Soufizadeh et al., 2018). Grain yield is calculated as the product of grain number and grain size. Grain number is estimated from the average daily growth rate per plant during a thermal time window defined as the critical period, generally starting at 227°Cd before flowering (Otegui and Bonhomme, 1998) and finishing at the start of grain filling and the potential grain number per cob (Liu et al., 2022). Total grain assimilate demand is the product of grain number and a potential grain growth rate, which is based on potential grain size and grain filling duration. A detailed description of the association between potential grain number and grain size has been described by Gambín et al. (2006).

2.3 Simulation configuration

2.3.1 Climate and soil

Experiment geolocations were used to retrieve climate and soil data to calibrate the model. Historical daily climate data (daily

TABLE 2 The number of observations per variable (in-season and at final harvest) by experiment.

	P-farm	Westwood	MayfarmY1	MayfarmY2	Total
Leaf number			54	144	198
Canopy green cover			54		54
Aboveground biomass weight	6	12	64	96	178
Grain/cob weight	6	12	64	96	178
Stover weight	6	12	64	96	178
Leaf weight	6	12	64	168	250
Stem weight	6	12	64	144	226
Grain number	6	12	12		30
Grain size	6	12	12		30

rainfall, maximum and minimum air temperature, and global solar radiation) from SILO (https://www.longpaddock.qld.gov.au/silo/point-data/) were used as inputs to the model. This daily interpolated climate dataset has been widely tested with weather station data across Australia (Jeffrey et al., 2001; Beesley et al., 2009) and also used for crop modelling purposes (Ojeda et al., 2020). Soil data were extracted from the Soil and Landscape Grid of Australia Database, which generates a synthetic APSIM soil derived from pedotransfer functions (https://www.asris.csiro.au/ASRISApi#!/APSIM32Services/ApsoilFromGrid_getApSoilTypeMap). Due to the high spatial proximity of the experiments and after consultation with the experimentalists about the soil types of the experiments, we decided to use a single soil profile for the model setup in all experiments. A complete description of the soil parameters used to configure the soil profile in APSIM can be found in Table S2.

2.3.2 Crop management

Actual crop management practices implemented in the field were used to parametrise the crop management settings in the model for in-silico experiments. Sowing density varied among experiments from 5.5 to 11.4 plants m^{-2}, and the distance between rows was the same for all experiments (680 mm) except for P-farm (790 mm). Sowing and harvest date varied among experiments although all crops were harvested for silage purposes (i.e., at a whole plant DM content of 21-47%; Table S3). In all simulations, a harvesting rule was set to remove the biomass at a height of 150 mm as per standard practice in commercial farms. We used two model scripts to configure the actual N fertilisation rates of the field experiments: one at the sowing date and the other at V6. Nitrogen fertilisation was applied as urea in all experiments and the rates varied from 0 to 293 kg N ha^{-1} (Table S3). The irrigation module was parametrised to mimic the actual irrigation amounts applied in the field. A detailed description of the crop management practices used to set up the model can be found in Table S3.

2.3.3 Genotypic parametrisation

There are more than 90 maize genotypes available in APSIM. Therefore, the computing cost to test the accuracy of the model using all these APSIM default genotypes against our experimental data would be too high and impossible to implement. In addition, all hybrids used in the experiments employed in this study were silage genotypic types unavailable in the APSIM cultivar library. Thus, the parameterisation of new genotypes was required. We classified the genotypic parameters into parameters for parametrisation for optimisation or default model parameters (Table S4). This classification helped us to identify the calibration method to be implemented for each group of parameters. A *parameter for parametrisation* can be calibrated using field observations, while a *parameter for optimisation* is uncertain and, therefore, requires to be optimised. We defined a *default parameter* when there was a lack of data available to include them in the optimisation procedure and therefore the original genotypic parameter in APSIM was used for that parameter. The genotypic parameter values after calibrating the model can be found in Table 3.

2.3.4 Optimisation procedure

We carried out a multi-step optimisation procedure to select the best combination of values for the most sensitive genotypic parameters (Figure 1). We followed a series of steps: *Step 1)* We generated a logical range of possible values of parameters for optimisation based on the literature. We used the wide range of maize parameters reported by Rotili et al. (2020) based on in-farm and on-research station trials conducted across New South Wales and Queensland, Australia, over three seasons. *Step 2)* We created artificial genotypes in the Maize.xml plant module in APSIM using all combinations of parameters previously selected. *Step 3)* We differentiated the experimental treatments by genotype used. *Step 4)* We ran APSIM using a range of values for five parameters that affect biomass partitioning to grain: *frac_stem_flower* (0.05, 0.4, and 0.75), *stem_trans_frac* (0.05, 0.4, and 0.75), *GNk* (0.27, 0.33, and 0.39), *GNmaxCoef* (215, 282.5, and 350 grains $plant^{-1}$), and *potKernelWt* (299, 319, and 339 mg $grain^{-1}$) (Table S4). *Step 5)* We selected the set of parameters that optimised the grain yield simulations, i.e., the highest reduction in the prediction uncertainty (simulated grain yield – observed grain yield) for this model output at the experiment level (one genotype by experiment).

2.4 Data analysis, calculations, and model evaluation

First, we curated and formatted the data available from the four above-mentioned experiments to unify units and names before implementing the APSIM modelling calibration. Data curation and analysis were done using Python 3.8.12 (https://www.python.org) through the Jupyter Lab interface (https://jupyter.org/). The code developed during this procedure is hosted in a private GitHub repository (https://github.com/Jjguri/DairyUp) (access to the repository can be provided by request to the corresponding author). We used boxplots and scatter plots to investigate relationships and general trends of grain yield, HI, and biomass partitioning. In this paper, we considered HI as the ratio between grain yield and aboveground biomass independent of the maize growth stage when the measurements were collected (considering mainly differences in the percentage of DM for silage purposes). The correlation between observed HI and plant components was assessed for all experiments (Figure S1). This analysis allowed us to identify the main causes of variance for observed data.

Second, we calculated statistical coefficients, including the Root Mean Square Error (RMSE) and the Root Mean Square Percentage Error (RMSPE), the Nash–Sutcliffe model efficiency coefficient (NSE), and Lin's Concordance Correlation Coefficient (CCC). The RMSE and RMSPE provide a quantification of the differences between simulated and observed values in the unit of the variable and percentage, respectively. On the other hand, the NSE is a normalised statistic that determines the relative magnitude of the residual variance compared to the observed data variance. The CCC

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TABLE 3 Description of calibrated genotypical parameters used in the Agricultural Production Systems Simulator by experiment.

	P-farm	Westwood		MayfarmY1	MayfarmY2	
Genotype	Pioneer 2307	PacificX	PioneerX	PioneerY	Pioneer 31H50	
Parameter	value					unit
aX0	0.57	0.61	0.67	0.67	0.67	proportion
largestLeafParams	1666 -1.17 0.047	1000-1.17 0.047	1200 -1.17 0.047	3400 -1.17 0.047	3050 -1.17 0.047	–
leaf_app_r1	65	65	65	85	90	°Cd
leaf_app_r2	36	36	36	45	45	°Cd
leaf_app_r3	36	36	36	45	45	°Cd
leaf_no_rate_ch1	8	8	8	8	9	leaves
leaf_no_rate_ch2	3	3	3	3	6	leaves
ph1	12.5	12.5	12.5	12.5	12.5	h
ph2	20	20	20	20	20	h
ph_s	0	0	0	0	0	h
tt_emerg_to_endjuv	310	350	330	230	270	°Cd
tt_flag_to_flower	50	50	50	50	50	
tt_flower_to_start_grain	170	170	170	170	170	°Cd
tt_flower_to_maturity	800	600	1000	700	600	°Cd
tt_maturity_to_ripe	1	1	1	1	1	°Cd
frac_stem2flower	0.26	0.46	0.12	0.47	0.55	0-1
stem_trans_frac	0.61	0.4	0.4	0.26	0.275	0-1
potKernelWt	315	332	311	319	315	mg grain^{-1}
GNk	0.33	0.38	0.366	0.354	0.321	–
GNmaxCoef	323	339	309.5	350	297	grains plant^{-1}

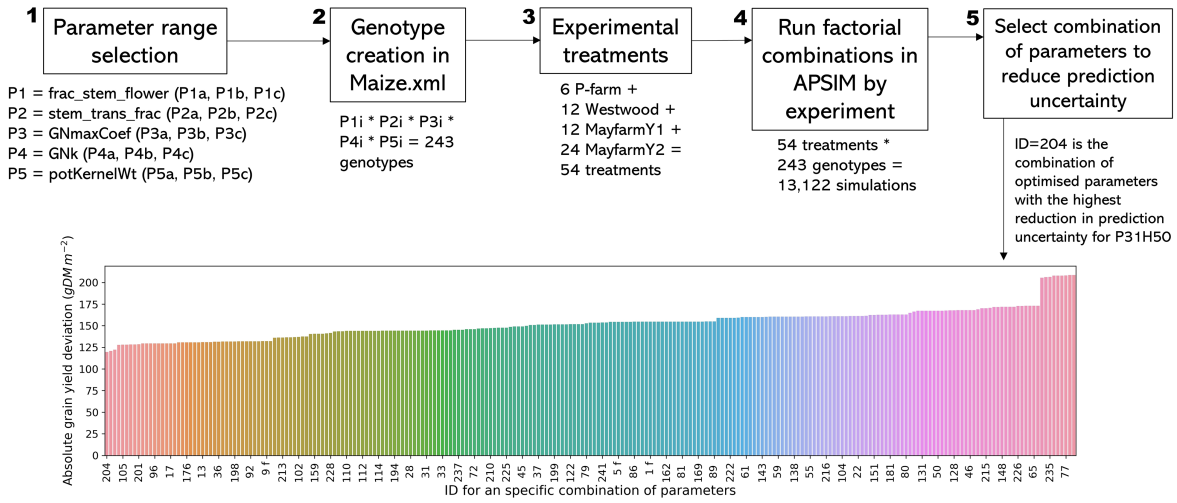

FIGURE 1
Optimisation process description for genotype parameters. Steps 1-4 apply for all genotypes, and step 5 and the bar plot show a specific example for MayfarmY2 for genotype Pioneer 31H50. Subscript letters in steps 1 and 2 indicate each level of a given parameter (i.e., for P1a, a = 0.4). Subscript 'i' in step 2 represents 'a', 'b', or 'c' from step 1. Labels in the x-axis represent one ID combination of every four possible combinations for display purposes.

integrates precision through Pearson's correlation coefficient, which represents the proportion of the total variance in the observed data that can be explained by the model and accuracy by a bias which indicates how far the regression line deviates from the concordance (y=x) line. All statistical indicators are suitable to evaluate model performance; however, CCC integrates model precision and accuracy, and the evaluation can be interpreted by assessing a single number from -1 to 1. Following the approach proposed by Ojeda et al. (2017), we created a categorical variable to evaluate the model performance based on CCC. The model performance (defined as the sum of accuracy and precision) to simulate all variables was evaluated as: "very good" when CCC ≥ 0.90, "satisfactory" when 0.8 ≤ CCC< 0.9, "acceptable" when 0.4 ≤ CCC< 0.8, and "poor" for other values (Tedeschi, 2006). We used the *NumPy* and *pandas* packages in Python to assess the model prediction uncertainty (simulated values – observed values). The statistical coefficients were used to create a heatmap of global model performance for the variables described in Table 2. Additionally, we visually compared observed vs. simulated state variables by experiment for each observation date (in-season and final harvest).

2.5 Scenario analysis of harvest index variance

To analyse the variance of HI across a wide range of scenarios, we ran APSIM with the calibrated and validated genotypes across a 50-year period (1972-2021) for several combinations of crop management factors (described below). We used climate and soil information from the Mayfarm site to run the long-term simulations. We combined the effect of irrigation (rainfed, deficit, and well-irrigated), sowing date (20 Oct and 3 Nov), sowing density (5.2, 7.2, and 9.3 pl m^{-2}), N application rate (0, 125, and 250 kg N ha^{-1}), genotype [early (PacificX; 1170°Cd from emergence to physiological maturity) and late (PioneerX; 1550°Cd from emergence to physiological maturity)], and harvest date (125, 138, and 150 days after sowing). The levels of all factors were defined based on the data range of the field experimental data used for the calibration. Three irrigation levels were defined based on the ratio between actual soil water and soil field capacity (0, rainfed; 0.4, deficit irrigation; 0.8, well irrigated). The model quantified this ratio every day and applied irrigation if the ratio exceeded the indicated thresholds for each treatment. In total, we generated 324 scenarios (three irrigations × two sowing dates × three sowing densities × three N rates × three harvest dates × two genotypes) across 50 years (16,200 scenario x year combinations). The main drivers of HI variability were assessed through boxplots and analysis of variance.

To determine the contribution of each factor to the total HI variability, variance-based sensitivity indices were computed (Monod et al., 2006; Kamali et al., 2022). According to this method, the variance of the model output is decomposed into fractions that can be attributed to various factors. These methods measure the sensitivity of each factor independently and also quantify the effect of interactions. Two indices, namely, Main Effect (*ME*) (Eq. 2) and Total Effect (*TE*) (Eq. 3), were used to disentangle the variance caused by one source from the variance caused by the interaction.

$$ME_i = \frac{Variance(E[HI\ X_i])}{variance\ (Y)} \quad (2)$$

And

$$TE_i = 1 - \frac{Variance\ (E[HI\ X_{-i}])}{Variance\ (Y)} \quad (3)$$

where $E[HI\ X_i]$ denotes the expected value of HI across all sources X_i (irrigation, sowing date, sowing density, N rate, harvest date, and genotype), while $E[HI\ X_{-i}]$ is the expected value of HI across all sources except for X_i. In other words, *ME* explains the share of the components to HI variability without interactions, i.e., if $ME = 1$, the assessed factors explain the entire proportion of HI variability, but if $M < 1$, residuals exist, which means additional factors are required to explain this variability. *TE* represents the interaction of a given factor with other factors, i.e., high *TE* values for a given factor denote high interactions of that factor with other factors; therefore, *TE* does not include residuals.

3 Results

3.1 Drivers of observed harvest index variability

Observed biomass and grain yield varied across experiments depending on the genotype and crop management. On average, the observed biomass ranged from 1,558 g DM m^{-2} (MayfarmY2) to 2,922 g DM m^{-2} (MayfarmY1). Grain yield ranged from 772 g DM m^{-2} (MayfarmY2) to 1,096 g DM m^{-2} (MayfarmY1) and stover weight ranged from 786 g DM m^{-2} (MayfarmY2) to 1,826 g DM m^{-2} (MayfarmY1). While the average observed HI was relatively similar (0.472 to 0.487) between P-farm, Westwood, and MayfarmY2, it was considerably lower on MayfarmY1 (0.374). Consequently, we found the highest stover proportion at MayfarmY1 (0.626) (Figure 2).

In the experiments with the highest plant densities (MayfarmY1 and MayfarmY2), observed HI was significantly correlated with observed grain yield. However, it was only associated with aboveground biomass and stover weight at MayfarmY2 (Figure S1; Table 4). The associations were mainly affected by irrigation rate (i.e., water crop status) and the genotype used in the experiments. While HI considerably increased when the irrigation rate increased up to 66% at MayfarmY2, it remained relatively constant when irrigation rates surpassed this threshold (Figure S1). A complete description of the relationship between observed HI and aboveground biomass, stover, and grain weight is provided in Figure S1.

3.2 Crop phenology model calibration

Overall, APSIM showed a very good performance to estimate crop leaf number (CCC = 0.97) and satisfactorily estimated canopy green cover (CCC = 0.79) (Figure 3 and Figure S2). Therefore, the model was able to capture leaf senescence and dry matter

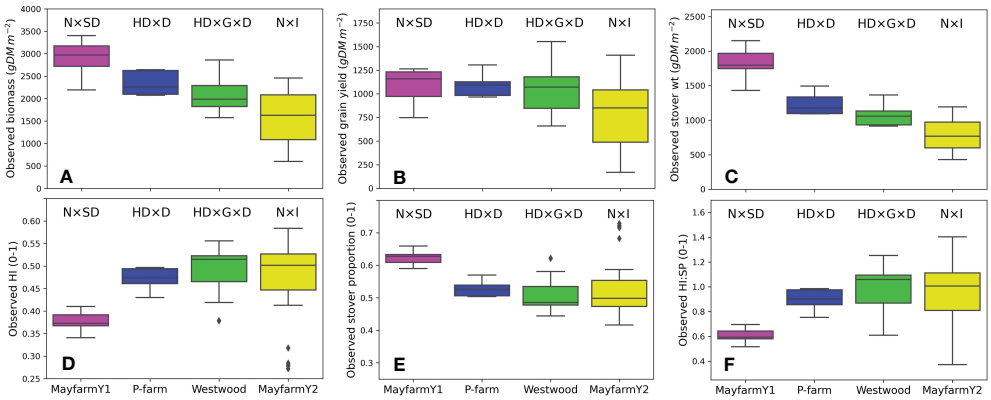

FIGURE 2
Observed **(A)** aboveground biomass, **(B)** grain yield, **(C)** stover weight, **(D)** harvest index (HI), **(E)** stover proportion (SP; stover weight/aboveground biomass), and **(F)** harvest index:stover proportion (HI : SP) by experiment. The letters above each boxplot indicate the combinations of factors assessed in each experiment. N, N fertilisation rate; SD, sowing date; HD, harvest date; D, plant density; G, genotype; I, irrigation rate. Grain yield data in **(B)** include only final harvest measurements (i.e., it does not include cob weight measurements).

TABLE 4 The statistical description of correlations between grain yield and harvest index (HI) vs. aboveground biomass, grain yield, and stover weight in each experiment.

Experiment	Dependent Variable (y)	Independent Variable (x)	R^2	Regression equation	P-value
MayfarmY1	grain yield	aboveground biomass	0.95	y = 0.4251x – 152.23	<0.001
	HI	aboveground biomass	0.18	y = 0.0607ln(x) – 0.1099	0.024
		grain yield	0.44	y = 0.0772ln(x) – 0.1652	<0.001
		stover weight	0.05	y = 0.0335ln(x) + 0.123	0.292
MayfarmY2	grain yield	aboveground biomass	0.98	y = 0.6311x – 211.05	<0.001
	HI	aboveground biomass	0.84	y = 0.2003ln(x) – 0.9904	<0.001
		grain yield	0.93	y = 0.1388ln(x) – 0.4339	<0.001
		stover weight	0.63	y = 0.2596ln(x) – 1.2558	<0.001
P-farm	grain yield	aboveground biomass	0.76	y = 0.4172x + 125.76	<0.001
Westwood	grain yield	aboveground biomass	0.94	y = 0.6917x – 410.54	<0.001

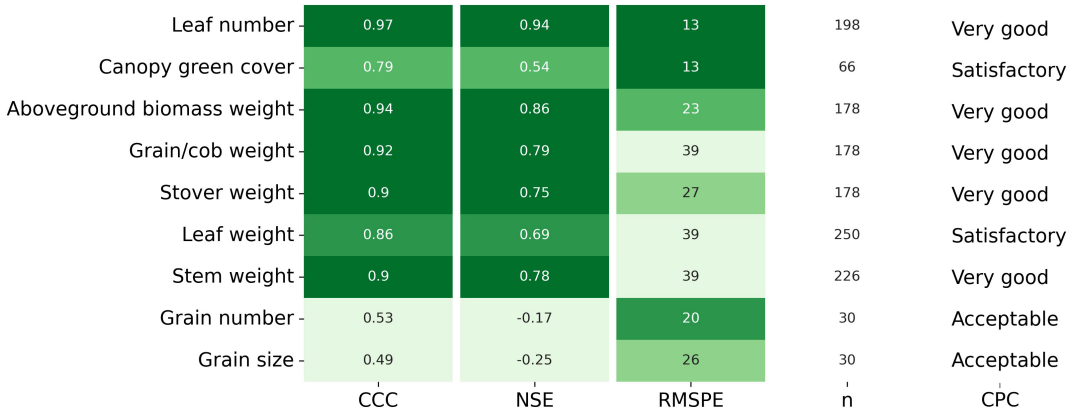

FIGURE 3
Lin's Concordance Correlation Coefficient (CCC), Nash Sutcliffe model Efficiency coefficient (NSE), Root Mean Square Percentage Error (RMSPE), number of observations (n), and Categorical Performance Classification (CPC; Acceptable, Satisfactory, Very good) for each variable measured in-season and at final harvest. The darker green colour indicates better model performance.

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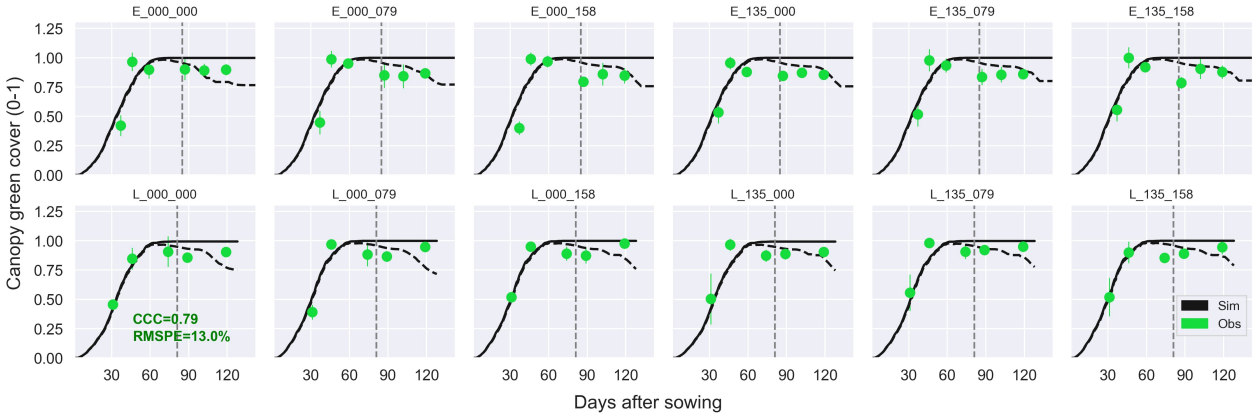

FIGURE 4
Observed (circles) and simulated (dashed black line) canopy green cover and total cover (live and dead canopy; solid black line) during the crop growing season at the MayfarmY1 experiment. Each subplot represents a treatment combination of sowing date × N fertilisation rate pre-sowing × N fertilisation rate post-sowing. For example, E_135_158 indicates an early sowing date, 135 kg N ha^{-1} is applied pre-sowing, and 158 kg N ha^{-1} is applied post-sowing. Vertical dashed lines indicate the flowering date for each treatment. The CCC and RMSPE indicate Lin's Concordance Correlation Coefficient and the Relative Root Mean Square Percentage Error.

remobilisation demonstrated by the capability to estimate green cover, which only includes active photosynthetic crop canopy (Figure 4). Although the ability of APSIM to estimate leaf number was very good, there were slight under-estimations of this variable under high N rates and irrigation amounts (Figure 5). Canopy cover variables presented the lowest RMSPE of the model calibration (13%).

3.3 Model calibration of crop growth and partitioning

The general model assessment (i.e., using in-season and final harvest data) demonstrated that APSIM had a very good performance to simulate total aboveground biomass (CCC = 0.94), grain + cob weight (CCC = 0.92), stover weight (CCC =

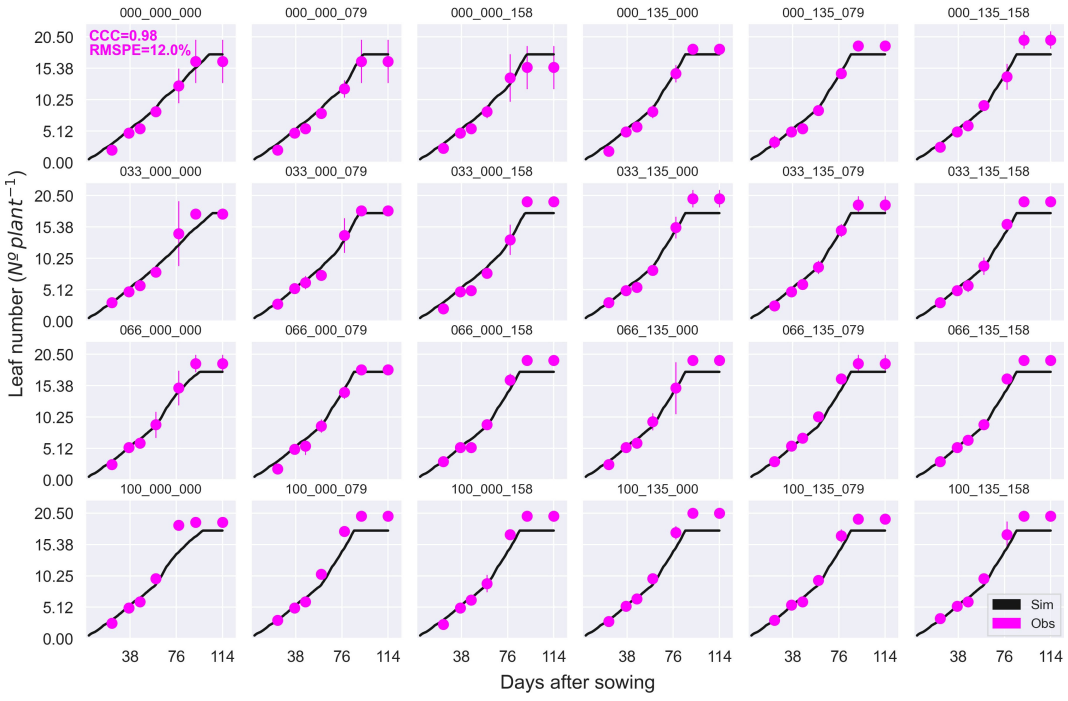

FIGURE 5
Observed (circles) and simulated (solid black line) leaf number during the crop growing season at the MayfarmY2 experiment. Each subplot represents a treatment combination of the percentage of irrigation × N fertilisation rate pre-sowing × N fertilisation rate post-sowing. For example, 100_135_158 indicates 100% irrigation, 135 kg N ha^{-1} is applied pre-sowing, and 158 kg N ha^{-1} is applied post-sowing. CCC and RMSPE indicate Lin's Concordance Correlation Coefficient and the Relative Root Mean Square Percentage Error.

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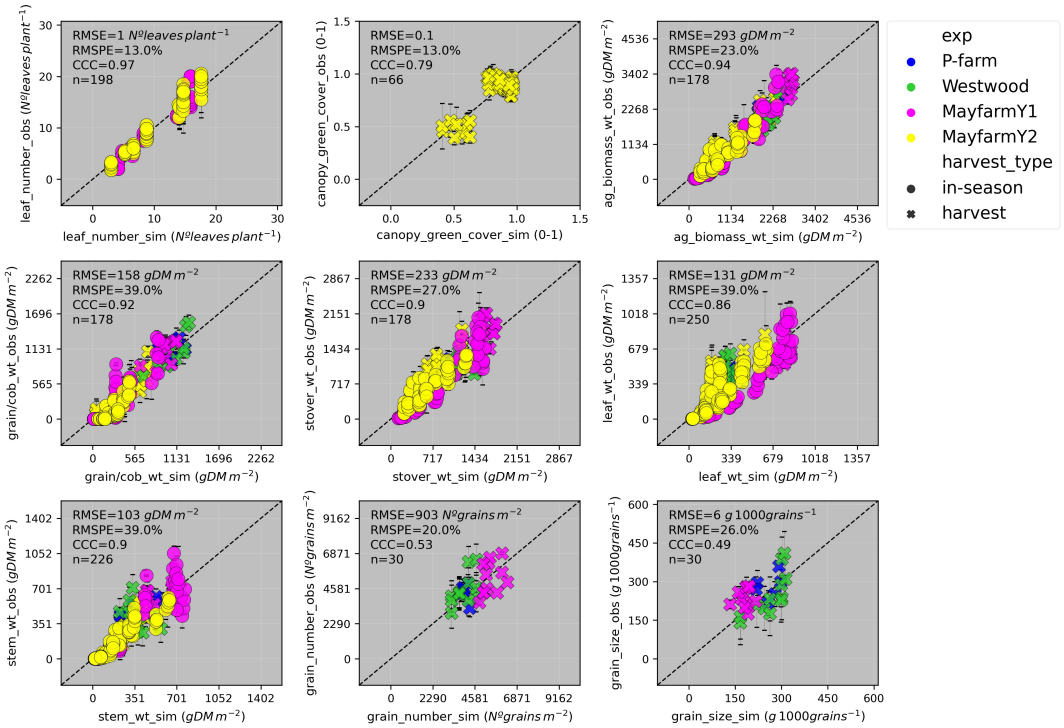

FIGURE 6
Observed vs. simulated leaf number, canopy green cover, aboveground biomass, grain + cob weight, stover weight, leaf weight, stem weight, grain number, grain size by experiment (P-farm, MayfarmY1, MayfarmY2, and Westwood), and type of harvest. Data include in-season and final harvest measurements. The solid grey line represents the line 1:1, that is, y = x and the solid black line represents the regression line adjusted to the complete dataset. RMSE, Root Mean Square Error; RMSPE, Relative Root Mean Square Percentage Error; CCC, Lin's Concordance Correlation Coefficient; n, number of observations.

0.9), and stem weight (CCC = 0.9) (Figures 3 and 6). Leaf weight was simulated with satisfactory model performance (CCC = 0.86). On the contrary, grain yield components (grain number and size) had an acceptable modelling performance (CCC = 0.49). The highest RMSPE was found for leaf, stem, and grain + cob weight (39%). In general, as N fertilisation (from left to right in Figure 7 and Figure S3) and irrigation rates increased (from top to bottom in Figure 7 and Figure S3), the model performance for different aboveground crop components increased too. Seasonal patterns of observed and simulated data demonstrated that the model was able to simulate specific crop partitioning change events. This includes the flag leaf stage, when the crop stops partitioning assimilates to the leaf component and starts translocating assimilates to the grain component (Figure 7). Partitioning to grain and cob started earlier in the model than in the observed data, which generated grain and cob weight over-estimations. However, the final values (close to 90 and 120 days after sowing) were satisfactorily simulated (Figure 7). Most importantly, the model satisfactorily estimated HI (CCC = 0.78) with a relatively low RMSPE (12.1%) (Figure 8). A complete statistical description of model deviations for all assessed crop variables is provided in Table S5.

3.4 Sources of model prediction uncertainty

The ability of the model to estimate the maize HI was mainly affected by the accuracy to estimate grain yield (Figure 9). Although the model prediction uncertainty (simulated values – observed values) was higher than the observed standard deviations for most crop variables, it was equal and lower than the observed standard deviations for grain number and grain size, respectively (Figure S4). This demonstrated that the model was able to capture the variability of biomass partitioning across treatments and environments. The model prediction uncertainty was also affected by the sampling method applied for specific crop variables (leaf weight, stem weight, grain yield + cob weight, and aboveground biomass), particularly for grain yield + cob weight (Figure S5).

3.5 Assessing the harvest index variability through scenario analysis

The scenario analysis demonstrated that genotype and N application rates were the most influential crop management factors affecting the variance of HI (Figures 10 and 11). Nitrogen rate

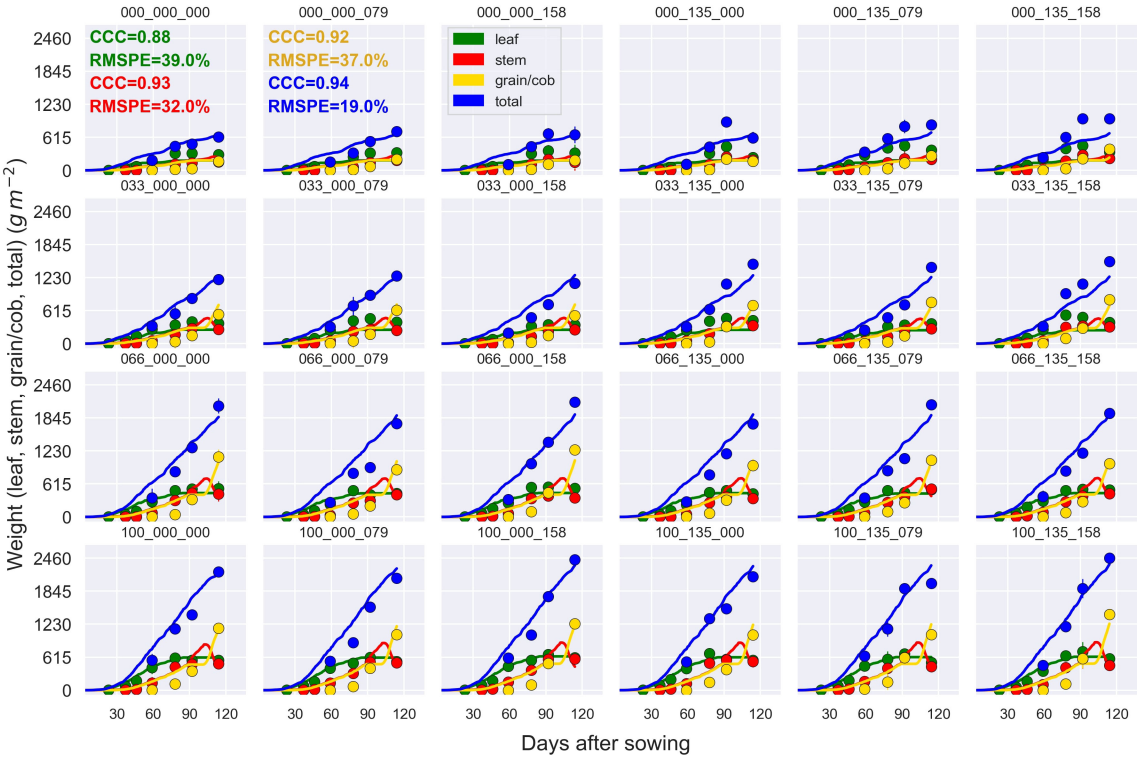

FIGURE 7
Observed (circles) and simulated (lines) leaf weight, stem weight, cob + grain weight, and aboveground biomass (total) during the crop growing season (das, days after sowing) at the MayfarmY2 experiment. Each subplot represents a treatment. For example, 100_135_158 indicates 100% irrigation, 135 kg N ha^{-1} at sowing, and 158 kg N ha^{-1} post-sowing. CCC and RMSPE indicate Lin's Concordance Correlation Coefficient and the Relative Root Mean Square Percentage Error across treatments.

outweighed the effect of irrigation which strongly affected grain yield, and therefore HI, independent of the water status (Figures S6 and S7). The harvest index varied from 0 (for scenarios without N rate applications) to 0.71 (for late sowing × high density × early genotype scenarios) (Table S6). The median HI across years and factors was 0.51. Genotype and N rate contributed to 44% and 36% of the HI variance (ME = 0.44 and ME = 0.36, respectively) (Figure 11A). These factors were also the most interactive across all crop management practices (Figure 11B). Harvest date, sowing density, sowing date, and irrigation had a lower contribution to HI variance (ME< 0.04).

4 Discussion

4.1 Drivers of observed grain yield and harvest index variability

We showed how genotype impacted the partitioning of maize assimilate to cob and HI and the ratio between HI and stover proportion (HI : SP) across experiments (Figure 2) , but the main driver of observed HI variability for a given genotype was crop water status (Figure S1). These results align with previous studies which assessed the effect of maize genotypes under a wide range of water conditions (Gambin et al., 2016). Harvest index has been shown to be affected by various biotic and abiotic stresses, including water, temperature, N, diseases, and pests (Bender et al., 2013; Khalili et al., 2013; Hütsch and Schubert, 2017; Liu et al., 2022). Our results highlight the importance of genotype selection (based on its HI : SP) and the maintenance of optimum water conditions during the maize growing season in order to achieve high HI and, therefore, greater silage quality (Hernandez et al., 2020).

Interestingly, the crop growing period driven by the thermal time between sowing and flowering and between flowering and maturity was similar between the genotypes used in this work (Table 3). In fact, although the HI in MayfarmY1 was significantly lower than in the other experiments, the genotype used in this experiment had a similar vegetative:reproductive thermal time ratio to other genotypes, such as PioneerX in Westwood (Table 3). For a given genotype, increasing grain number per plant or decreasing vegetative shoot biomass are the two main manipulations for increasing maize HI (Hütsch and Schubert, 2017). As such, the ideal maize plant for silage would have both high vegetative shoot biomass and a high grain number per plant (Johnson et al., 1999). Although the duration of the vegetative and reproductive phases may change HI (Capristo et al., 2007), the growing conditions explored during the critical period for grain set and the grain filling period and the intrinsic grain set efficiency of a given genotype are the main factors governing this variable in maize (Tollenaar et al., 2006). Accordingly, modern maize hybrids have similar vegetative biomass at flowering but a lower shoot-dry matter threshold for yield when compared with older maize hybrids, mainly due to greater biomass partitioning to cobs, higher kernel set efficiency, and more grain number per plant (Ciancio et al., 2016). At the same

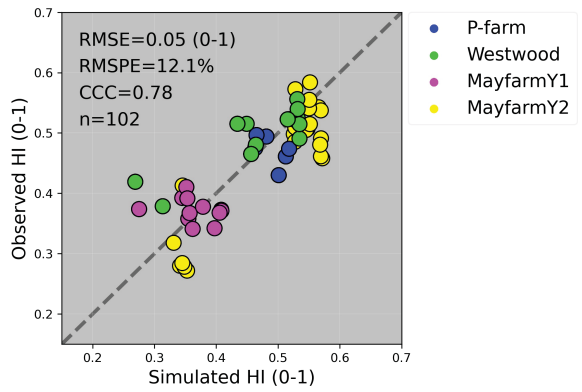

FIGURE 8
Observed vs. simulated harvest index (HI) by experiment. The solid grey line represents the line 1:1, that is, y = x and the solid black line represents the regression line adjusted to the complete dataset. RMSE, Root Mean Square Error; RMSPE, Relative Root Mean Square Percentage Error; CCC, Lin's Concordance Correlation Coefficient; n, number of observations.

time, a lower susceptibility of the crop to low soil water availability could be the cause of greater grain yield stability (Messina et al., 2022). In this study, we found maximum grain yields (1,159 g m^{-2}; Figure 2B) at MayfarmY1 under well-irrigated treatments (accumulated irrigation during growing season = 534 mm), but the HI in this experiment was on average the lowest (0.37; Figure 2D). One reason for this may be the genotype used in this experiment which produced a greater stover proportion with a reduced partitioning to cobs (Figure S1C). Variability in HI has been observed between silage maize genotypes (Tsakmakis et al., 2021), supporting the genotypic patterns observed in the present work. Our results highlight the need to incorporate a wide range of genotype maturity types and the HI : SP ratio in further analyses when HI of silage genotypes are compared across environments.

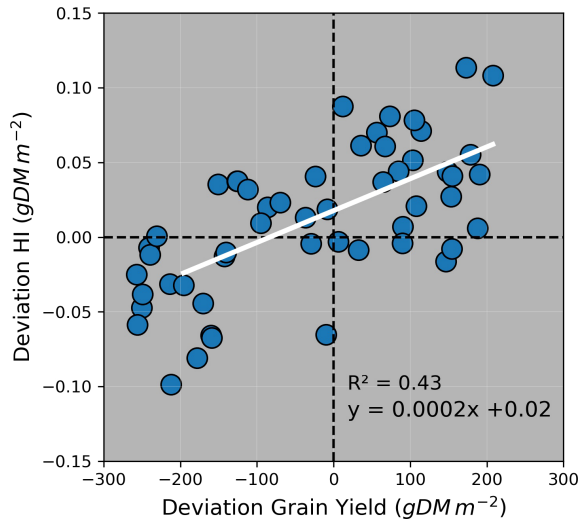

FIGURE 9
Deviation (i.e., simulated value – observed value) of harvest index (HI) vs. deviation of grain yield in all experiments. Colours and symbols indicate irrigation and N rates applied during the growing season. Dashed horizontal and vertical lines represent no deviation between simulated and observed values.

4.2 Drivers of simulated grain yield and harvest index variability

We calibrated APSIM to estimate crop growth, development, and plant biomass partitioning (and therefore HI) and explored the main sources of prediction uncertainty. Four detailed crop experiments on maize grown for silage were used to successfully calibrate APSIM, enhancing the value and applicability of historical field experimental data not collected for crop modelling purposes. Although we calibrated APSIM for biomass partitioning and grain yield, we carried it out on a more detailed level than common calibration practices usually performed in published papers, including variables such as grain size and grain number which usually are not included. This paper calibrated APSIM for forage maize using 1,322 field observations across a wide range of factors (sowing date, sowing density, genotype, N rate, and irrigation), highlighting the novelty and uniqueness of this calibration. Also, we calibrated the model in parallel for nine model outputs, i.e., we considered the trade-offs between state variables in the model using this approach.

Previous studies parametrised APSIM using datasets from Australian environments and grain maize genotypes (Hammer et al., 2010; Soufizadeh et al., 2018; Rotili et al., 2020). However, in this study, we calibrated this model for maize growth in Australia across nine crop variables, five silage genotypes, and six factors of analysis (Table 1). Although APSIM has been used to simulate the biomass production of silage maize under temperate environments (Pembleton et al., 2013; Teixeira et al., 2017; Ojeda et al., 2018a; Ojeda et al., 2018b) and extremely high latitudes (Morel et al., 2020), the model has been not previously tested for its capacity to predict the biomass partitioning for silage genotypes. Our work is the first study that provides reasonable APSIM estimations of grain yield and aboveground biomass (Figures 3 and 6) and satisfactory estimations of HI (Figure 8). The estimations of grain number and grain size presented in this study are comparable with other results reported in Gatton, Australia, (Rotili et al., 2020) although the prediction uncertainty for grain size in our work was considerably lower (RMSE = 6 mg $grain^{-1}$ vs. 38 mg $grain^{-1}$). These results provide confidence to use the model for nutritive quality predictions for maize silage as this crop's HI is correlated with neutral detergent fibre content and *in vitro* true digestibility (due to different grain:stover ratio through a HI gradient) (Tsakmakis et al., 2021). Overall, the model overpredicted HI when the crop was growing underwater limitations (Figure 9); however, these overestimations were relatively low on average (HI = 0.04). Therefore, this paper demonstrates that APSIM can model a set of crop traits and processes that directly affect the silage quality, expecting deviations lower than 10% in HI predictions (considering the average observed HI was 0.465).

In this study, we also demonstrated that a satisfactory model calibration could be achieved by parametrising the crop model for both phenology and crop growth. It is particularly important to properly simulate the occurrence of key crop stages related to

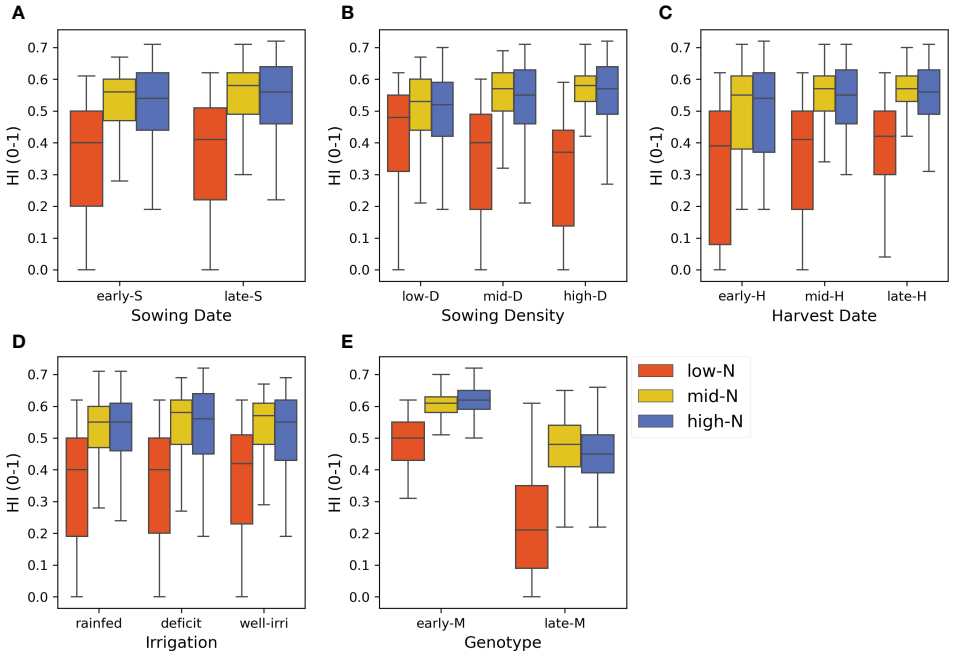

FIGURE 10
Harvest index (HI) variance for each level of crop management factor (subplots). **(A)** Sowing dates (20 Oct and 3 Nov), **(B)** sowing density (5.2, 7.2, and 9.3 pl m^{-2}), **(C)** harvest date (125, 138, and 150 days after sowing), **(D)** irrigation (rainfed, deficit, and well-irrigated), **(E)** genotype (early and late maturity) across N application rates in colours (0, 125 and 250 kg N ha^{-1}), and years (50 seasons).

carbon allocation to different plant components (Brown et al., 2019) and, therefore, define the HI and stover proportion derived from leaf and stem partitioning. Previous modelling studies have applied several methods to create new genotypes in crop models, including the minimisation of statistical metrics (e.g., RMSE) between observed and simulated values (Messina et al., 2006), gene-to-phenotype multi-trait link function integration (Cooper et al., 2021), and sensitivity analysis of cultivar trait parameters (Sexton et al., 2017). However, in this research, we conducted an exhaustive and robust procedure to generate new silage genotypes in the model, integrating model parametrisation (using observations to reduce the RMSE) and model optimisation based on sensitivity analysis (Monod et al., 2006) (Figure 1).

Previous studies showed the strong effect of genotype and N application rates on maize grain yield (Rossini et al., 2016; Ruiz et al., 2022). These highlighted the importance of the maize growing period length (particularly the reproductive phase) and crop N availability to determine HI. However, some specific features of our

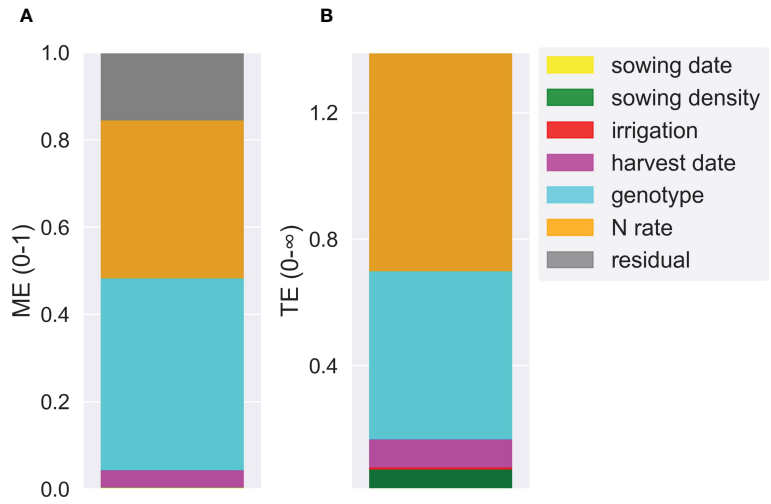

FIGURE 11
Main effect (ME) **(A)** and total effect (TE) **(B)** of different crop management factors (sowing date, sowing density, harvest date, irrigation, genotype, and N rate) explaining simulated harvest index variance.

study are novel when compared to the previous knowledge. Genotype and N application rate were the main drivers of simulated HI variability across years (i.e., under contrasting rainfall patterns) (Figure 10E), which corresponded with our findings from field experimental data (Section 4.1; Figure 2). However, the differences in HI were higher between genotypes under N-limited crops (N rate = 0 kg N ha^{-1}) than N fertilised crops (125 and 250 kg N ha^{-1}) because late maturity genotypes had higher biomass in relation to grain yield than early maturity genotypes, which considerably reduced HI (Figure 10E). This highlights the importance of N fertilisation to achieve high HI, independent of the genotype used.

The in-silico simulations allowed us to capture the interactions between N rate application and other factors of variance (mainly irrigation) across years, something that was not possible to identify in the field despite using a similar range of N application rates (0-158 kg N ha^{-1}) due to lack of inter-annual rainfall variability. Maize biomass and grain yield maximisation are usually conditioned by the existence of water and N co-limitation, in which limitations due to one resource depend on limitations due to the other resource (Cossani and Sadras, 2018). The response to the addition of one resource depends on the level at which the other is limiting (obeying the law of optimum rather than the law of minimum). The ability of the crop to uptake N depends on the water stress (Hammad et al., 2017); water-stressed crops have high N concentrate, and synergistic effects have been found between N and water stress in maize (Rossini et al., 2016). In our simulated scenarios, the responses of HI to irrigation were only expressed under non-N limited conditions (>125 kg N ha^{-1}), indicating that the effect of N rate on grain yield and, therefore HI, outweighed the effect of irrigation. It must be noted that the synergistic effects of water and N stress depend on the magnitude and timing of both stresses (Rossini et al., 2016), and the constant magnitude of the water limitations simulated across the whole crop cycle could have conditioned the co-limitation level. Also, the long-term analysis showed that maize crops limited by N (N rate = 0 kg N ha^{-1}) had much lower vegetative biomass than under high-N rates and, therefore, under these conditions, the crop generally did not experience water stress due to the amount of soil water available, which was enough to provide for the low water requirements under those conditions (Figure S6). The main differences of HI were found between the treatment with 0 *vs.* 125 kg N ha^{-1} of applied N, while HI was maximised above 125 kg N ha^{-1} and therefore, the results of this scenario analysis suggest that, under high-rainfall years, the treatment with 125 kg N ha^{-1} maximises HI and, therefore, can potentially improve maize silage quality.

4.3 Implications of this study and future work

This work provides a solid basis for additional parameterisation of APSIM for silage maize with the overarching goal of accurately predicting changes in plant's partitioning and composition and, from these, potentially predicting the nutritive value of whole plant maize silage. As previously highlighted by Archontoulis et al. (2014), accurate calibration of crop phenology is the primary priority as the partitioning of carbohydrates strongly depends on the phenology stage. Our results showed the need to integrate different approaches to achieve accurate genotype parameters to reproduce the G×M×E interactions in the model. This was demonstrated by the high accuracy to estimate grain yield independent of the genotype, environment, and crop management conditions (Figure 6). Therefore, future studies should focus on a detailed phenological parametrisation of the genotypes used to calibrate models using field or remote sensing-based phenological data as demonstrated in this study and by Yang et al. (2022) and Della Nave et al. (2022).

In addition, the overall satisfactory performance of APSIM to accurately predict phenology and HI found in this study has several implications for future work. A number of silage quality components are directly correlated with HI (i.e., starch content, neutral detergent fibre content, and forage digestibility) (Tsakmakis et al., 2021; Jahangirlou et al., 2023). Therefore, further work should be conducted to calibrate the model to estimate these quality components and quantify the prediction uncertainty. Further work is also required to determine the impact of N plant content on silage crude protein, starch, fibre, and energy and their interactions with HI to allow the modelling of these fine-tuning relationships from a functional perspective. Moreover, these results may be used to estimate the grain proportion of silage in advance (i.e., from the early stages of the crop), guiding diet balance and supplement purchases at the farm level. Future work should be done by integrating this kind of analysis across regions and at the farm level which will allow to cover a broader range of soil types and spatial soil variability.

We found modelling performance to differ when the same variables were collected using different harvest methodologies, i.e., harvesting the whole plot vs. proportions determined from one sampled plant per plot (Figure S5). This is particularly important because it generates uncertainty in the observations, which generally is not accounted for in crop modelling studies as a source of uncertainty (Chapagain et al., 2022). The quantification of the observational uncertainty is a key step in any model calibration procedure as it allows model users to target different model accuracy thresholds to finish the calibration. It highlights the need to use common protocols for crop sampling if experiments are targeted for crop modelling purposes, particularly for biomass partitioning. Future modelling studies should carefully consider the ground sampling method applied to calibrate models for crop biomass partitioning.

5 Conclusions

In this study, we present the first crop modelling study that calibrates APSIM for HI and plant partitioning using a detailed field dataset of forage maize and considering nine crop variables simultaneously. While field experimentation during one growing season demonstrated that the main drivers of HI variability were genotype and water status, the long-term scenario analysis highlighted the importance of N rate application as a second driver. We also applied an integrated and robust approach for

phenology and crop growth parameter calibration. In this study, we demonstrated that the calibrated APSIM model:

- has a satisfactory performance using five silage genotypes for maize grown in Australia across a wide range of G×E×M combinations. This is one of the first attempts to use a crop model to predict silage maize HI under contrasting crop growth scenarios.
- is a suitable tool to estimate maize HI as a potential proxy of silage quality for forage purposes.
- could now be used to compare inter-annual variability of maize HI based on G×E×M interactions and, therefore, assist in real-world farming conditions towards better synchronisation of crop management interactions focusing on high maize silage quality.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JO, RI, and SG contributed to the conception and design of the study. JO and RI organised the database. JO performed the statistical analysis and modelling. JO wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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APSIM is provided free for research and development use (see www.apsim.info for details).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1206535/full#supplementary-material

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Silage additives improve fermentation quality, aerobic stability and rumen degradation in mixed silage composed of amaranth and corn straw

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The objective of this research was to investigate effects of different additives on the fermentation quality, aerobic stability and rumen degradation of mixed silage composed of amaranth and corn straw. The mixture ratio of amaranth to corn straw was 78%: 22%. Three additives were selected in this study and five groups were as follows: control group (CON, without additive), lactic acid bacteria group (LAB, 5 mg/kg, *Lactobacillus plantarum* ≥ 1.6×10^{10} CFU/g and *L. buchneri* ≥ 4.0×10^{9} CFU/g), glucose group (GLU, 30 g/kg), cellulase group (CEL, 2 mg/kg) and lactic acid bacteria, glucose and cellulase group (LGC, added at the same levels as in individual group). The period of ensiling was 60 days. Fermentation quality, chemical composition and aerobic stability of mixed silage were analyzed. Four cows with permanent ruminal fistula were selected as experimental animals. Nylon bag technique was used to study rumen degradation characteristic of dry matter (DM), crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF) of mixed silage. Compared with CON group, the addition of different silage additives could improve mixed silage quality of amaranth and corn straw to some extent. Combining three additives significantly increased ($P < 0.05$) the DM, CP and lactic acid contents, whereas decreased ($P < 0.05$) the ADF and NDF contents as well as pH and ammonia nitrogen/total nitrogen. Moreover, the aerobic stability and rumen degradation of DM, CP and NDF were significantly improved ($P < 0.05$) in LGC group when compared to other groups. In conclusion, the combined addition of lactic acid bacteria, glucose and cellulase increased DM, CP and lactic acid contents as well as lactic acid bacteria count, decreased NDF and ADF contents and aerobic bacteria and mold counts, improved aerobic stability and rumen degradation of amaranth and corn straw mixed silage.

KEYWORDS

amaranth, corn straw, silage additive, fermentation quality, rumen degradability

1 Introduction

In dairy cows' production, the roughages (e.g. alfalfa hay, Chinese wildrye and corn silage) can provide essential nutrients for animals, which usually account for 30~70% in the ration (NRC, 2001). In order to preserve the quality of roughage, silage is commonly used in dairy farming. Corn is one of primary crops for ensiling; however, compared with other silage such as alfalfa, the contents of crude protein (CP) (<10% dry matter [DM] basis) and rumen degradable nutrients in corn silage are lower (Fernandes et al., 2022). In addition, due to the limited land resources, water scarcity and poor soils, the yield of some crops (e.g. corn and sorghum) is limited in some parts of the world (Jacobsen, 2014). Thus, it is of great importance in dairy farming by making full use of various roughage resources. In recent years, the utilization of non-conventional feed resource with high CP content, digestibility and yield has attracted increasing attention.

Some crops that can adapt to water shortage, high temperature and poor soils can be used as feedstuff resources for ruminants' industry under certain harsh conditions (Li et al., 2018b; Taghipour et al., 2021). Amaranth (*Amaranthus hypochondriacus*) is one of such crops. As a C_4 dicotyledonous crop, amaranth can grow in the areas with poor soils, water shortage and high temperature (Sarmadi et al., 2016). According to the survey, the yield of amaranth can reach up to 85 t/ha (fresh weight) and 16 t/ha (DM) (Abbasi et al., 2012). Moreover, compared with corn, the amaranth has a higher CP concentration (approximately 25%, DM basis), a lower lignin content (approximately 4%, DM basis) (Aderibigbe et al., 2022) and lower concentrations of oxalic acid and nitrate (Rahjerdi et al., 2015). In ruminants' production, the partial substitution of amaranth silage for maize silage in the ration of dairy cows (Rezaei et al., 2015) and fattening lambs (Rezaei et al., 2014) does not affect animal health and performance.

Our previous research found that the optimal growth stage of amaranth was from peak flowering stage to heading stage for ensiling (Ma et al., 2019). We also found that the fresh amaranth moisture content is high and water soluble carbohydrate (WSC) is low. The contents of moisture and WSC are key factors to determine the silage quality (Zi et al., 2022b). In China, the corn straw is rich in resources and the DM content of corn straw is high. In this study, we used amaranth and corn straw as raw materials for ensiling. On the other hand, adding silage additives can improve the fermentation characteristics and nutritional value of silage, especially for grass silage (Li et al., 2022). The lactic acid bacteria can promote the fermentation process by consuming WSC to produce lactic acid and inhibit the growth of harmful bacteria, then improve the alfalfa silage quality (Li et al., 2018a). In hybrid *Pennisetum* silage, the cellulase inoculation can improve fermentation quality by degrading structural carbohydrates to provide fermentation substrate (Xiong et al., 2022). In the current study, we selected lactic acid bacteria, glucose and cellulase as silage additives. For ruminants, an important parameter of nutritional value evaluation in roughage is the ruminal degradation rate of nutrients (Stirling et al., 2022). Generally, the nylon bag technology is utilized to evaluate the ruminal degradation rate of feedstuffs (Diao et al., 2020). Therefore, this study was conducted to evaluate the effects of different additives on the fermentation quality, chemical composition, aerobic stability and ruminal degradation characteristics of mixed silage composed of amaranth and corn straw.

2 Materials and methods

2.1 Experimental field and preparation of silage

The amaranth was planted in the experimental field (covers an area of 800 m^2, 41°25′ E longitude and 88°40′ N latitude) at the Heshuo County, Bayingolin Mongol Autonomous Prefecture. The altitude of this area is approximately 2217 m, with annual average temperature and rainfall of 11.4°C and 58.6 mm respectively. The soil at the experimental field is sandy clay and the pH is approximately 7.8. During the growth stage of amaranth in 2021 (May to October), the rain capacity was 30.3 mm. Before sowing, the nitrogenous (400 kg/ha) and potassic (60 kg/ha) fertilizers were provided in the amaranth field as base fertilizers. The nitrogen fertilizer was urea (N content was 46%) and the potassium fertilizer was potassium chloride (K content was 62%). The seeding rate was approximately 0.8 kg/ha.

The amaranth seeds were sown manually on the 10th of May (2021) and harvested at heading stage. The whole-plants was cut to a 5-cm stubble height with reaping hook. Then, the harvested fresh amaranth and corn straw were chopped into fragments of 1.5 to 2 cm in length using a forage chopper (Zhoushi Shengzhuoxin Machinery Processing Factory, Suzhou, Jiangsu, China) before making silage. In the current study, according to the principle that the water content of mixed silage materials was about 65%, the mixture ratio of amaranth and corn straw was 78%: 22%. The chemical compositions of amaranth and corn straw are presented in Supplementary Table S1.

2.2 Experimental design

In this study, lactic acid bacteria (*Lactobacillus plantarum* ≥ 1.6×10^{10} CFU/g and *L. buchneri* ≥ 4.0×10^{9} CFU/g, Silage Legend Technology Co., LTD., Hohhot, Inner Mongolia, China), glucose (99% purity, Shengxing Chemical Co., LTD., Jining, Shandong, China) and cellulase (5000 U/g, Xiasheng Biotechnology Co., LTD., Beijing, China) were used as silage additives. The five treatments were as follows: (1) control group without any additive (CON); (2) ensiled amaranth and corn straw inoculated with lactic acid bacteria (LAB, added level was 5 mg/kg); (3) mixed silage supplementation with glucose (GLU, added level was 30 g/kg); (4) mixed silage supplementation with cellulase (CEL, added level was 2 mg/kg) (5) lactic acid bacteria, glucose and cellulase group (LGC, the dose was same as that added separately). All the additives were mixed into water and then evenly sprayed onto the silage materials. The CON group was sprayed with equivalent water. Subsequently, the mixed amaranth and corn straw were tightly compacted and sealed in a fermentation container (2 L capacity) to make silage. Each

treatment had four replicates. The fermentation containers were stored at the laboratory. After 60 days of fermentation, the containers were opened, and samples were collected for analysis of chemical composition, fermentation quality, aerobic stability and ruminal degradability.

2.3 Chemical component, fermentation quality, microbial composition and aerobic stability analysis

The matured silage samples were weighed and dried at 65°C in a forced-air oven for 48 h to a constant weight. Next, the air-dried samples were ground to pass through a 1-mm sieve (Taifeng Machinery Equipment Co., LTD., Yantai,Shandong, China). Subsequently, the samples were used to determine the contents of DM (105 °C), CP (No. 988.05) and OM (No. 942.02) reference to the AOAC procedure (AOAC, 2006). The NDF and ADF concentrations of silage samples were analyzed according to the methods described by Van Soest et al. (1991).

Fresh mixed silage of 20 g from each container was blended with 180 mL distilled water and stored at 4 °C. Samples were leached for 24 h and filtered through four layers of gauze. Then, the pH value was determined by pH meter (Ruizhen Electronic Technology Co., Ltd., Shanghai, China). The WSC concentration was measured using anthrone colorimetry (Hansen and Møller, 1975). Total nitrogen (TN) was analyzed via a nitrogen analyzer (Youpu General Technology Co., LTD., Beijing, China) and phenol- hypochlorite method was used to measure ammonia nitrogen (NH_3-N) (Broderick and Kang, 1980). In addition, the contents of organic acids, including lactic acid, acetic acid, propionic acid and butyric acid, were analyzed using high-performance liquid chromatography (APS8026, DE Aupos Scientific) as described by Chen et al. (2022).

Plate count method was used to count the lactic acid bacteria, yeast, aerobic bacteria, mold and coliform bacteria in the mixed silage. The 10 g fresh silage samples of each container were mixed with 90 mL sterilized water, and serially diluted to enumerate the microbial composition in a sterile solution. The number of lactic acid bacteria, yeast, aerobic bacteria, mold and coliform bacteria was determined according to the procedure described by Sun et al. (2021).

After 60 days of fermentation, the mixed silage was conducted to 5 days aerobic stability experiment reference to procedure described by He et al. (2020). In the current experiment, the numbers of lactic acid bacteria, yeast, aerobic bacteria, mold and coliform bacteria were used as spoilage parameters. Furthermore, the aerobic stability time was defined that the silage temperature exceeded the environmental temperature above 2 °C. The temperature was collected using a multipoint real-time temperature recorder (Mike Sensor Technology Co., LTD., Hangzhou, Zhejiang, China).

2.4 Ruminal nutrient degradability

In this study, four Holstein cows (560.2 ± 13.8 kg of body weight; dry period) with a permanent ruminal fistula were selected to determine the ruminal degradability of DM, CP, NDF and ADF using the nylon bag technology. All animals were fed the total mixed ration which was formulated according to the NRC (NRC, 2001). The dietary ratio of concentrate and roughage was 40:60. The feed ingredients and nutrient contents of diet are shown in Supplementary Table S2. Cows were regularly provided diet twice daily at 08:00 and 17:00 and had free access to water during the experiment. All cows had 20 days to adapt the diet.

The nylon bag was sewed to 8 × 12 cm with a pore size of 50 μm and the air-dried silage samples were smashed through a 4-mm sieve. Five grams of samples were accurately weighed and sealed into nylon bags. Then, the nylon bags were fixed in the soft rubber hose and placed into the nylon net. Nylon net was put into the rumen through the permanent ruminal fistula before morning feeding and the other end of net was fixed to the fistula. In the rumen of cows, the silage samples were incubated for 4, 8, 16, 24, 36, 48 and 72 h. At each time point, the samples had 4 replicates.

After serially taking out the bags at the corresponding time point, the nylon bag was rinsed using cold tap-water until the outlet water was clear. Next, the bags were dried in a forced-air oven (65° C) to a constant weight. Residues were weighted and smashed via a micromill to pass a 1-mm screen sieve, and used to determine the nutrient contents (DM, CP, NDF and ADF) according to the methods mentioned earlier. The ruminal degradability (P) of nutrients at each time (t) was estimated using an exponential curve as $P = a + b\ (1 - e^{-ct})$ and the effective degradability (ED) of nutrients was calculated by $ED\ (\%) = a + (b \times c)/(c + k)$ according to previous reference (Øskov and McDonald, 1979). In the above-mentioned equations, "a" is the rapidly degradable fraction of samples to be tested; "b" is the insoluble but potentially degradable fraction that degrades at a constant fractional rate (c); "e" is the base of natural logarithm; "k" is the rumen outflow rate. A fixed outflow rate of "k" was 0.031/h according to our previous study (Ma et al., 2019). The values of a, b and c were calculated by the non-linear regression program of SAS software (version 9.2).

2.5 Statistical analysis

Data were analyzed by one-way ANOVA procedure of the SPSS statistical software (version 20.0). Duncan test was used to determine the differences among groups. Data were presented as means and standard error of mean (SEM). The significance level was indicated at $P < 0.05$, and $0.05 \leq P < 0.10$ represented a tendency.

3 Results

3.1 Chemical composition of mixed silage

The DM content of LAB and LGC groups was higher ($P < 0.05$) than that of CON, GLU and CEL groups (Table 1). LGC group showed highest CP content, which was increased by 20.34% as compared to CON group ($P < 0.05$). No significant difference ($P > 0.05$) of OM concentration was found among all groups. Compared

with CON and GLU groups, the NDF and ADF contents of CEL and LGC groups were significantly decreased ($P < 0.05$). In addition, the WSC concentration of GLU group was higher ($P < 0.05$) than CON and LAB groups.

3.2 Fermentation quality of mixed silage

As shown in Table 2, the pH of CEL and LGC groups was lower ($P < 0.05$) than that of CON group. The NH_3-N/TN of LGC group was minimum and lower ($P < 0.05$) than CON, LAB and GLU groups. On the contrary, LGC group displayed highest lactic acid content, which was increased by 58.36%, 21.26%, 13.06% and 18.40% respectively as compared with CON, LAB, GLU and CEL groups ($P < 0.05$). The contents of acetic and propionic acids in CON group were higher ($P < 0.05$) than those in GLU, CEL and LGC groups. No butyric acid was detected in LAB, GLU and LGC groups. Additionally, compared with CON, LAB and CEL groups, the lactic acid/acetic acid of LGC group was significantly increased ($P < 0.05$).

3.3 Microbial population of mixed silage

Notably, the number of lactic acid bacteria in LAB and LGC groups was higher ($P < 0.05$) than other groups (Figure 1A)). There was no significant difference ($P > 0.05$) of yeast (Figure 1B) and coliform bacteria (Figure 1E) counts among all treatments. However, compared with LAB, GLU and LGC groups, the aerobic bacteria count in CON and CEL groups was significantly increased ($P < 0.05$) (Figure 1C). All the inoculated treatments exhibited significantly decreased ($P < 0.05$) mold count (Figure 1D).

3.4 Aerobic stability of mixed silage

After aerobic exposure for 5 days, except for CEL group, other inoculated treatments significantly extended ($P < 0.05$) the aerobic stability time of mixed silage compared with CON group (Figure 2A). The LAB, GLU and LGC groups showed significantly higher ($P < 0.05$) lactic acid bacteria count than CON and CEL groups (Figure 2B). Compared with CON group, the yeast count was reduced by 26.22% and 22.97% in LAB and LGC groups respectively ($P < 0.05$) (Figure 2C). The numbers of aerobic bacteria, mold and coliform bacteria in inoculated treatments were lower ($P < 0.05$) than those in CON group (Figures 2D-F). In addition, the aerobic bacteria and mold counts of LGC group was significantly decreased ($P < 0.05$) as compared with GLU and CEL groups (Figures 2D, E).

3.5 Ruminal dry matter degradation of mixed silage

The ruminal DM degradation of LGC group at 72 h was higher ($P < 0.05$) than that of CON and GLU groups (Table 3). At 4 h and 8 h, the LGC and LAB groups exhibited higher ($P < 0.05$) DM degradation as compared with CON group. The DM degradation velocity of all mixed silage was faster before 24 h and then tended to be stable. The rapidly degradable fraction of LAB and LGC groups was higher ($P < 0.05$) than other groups (Table 4). Besides, the LGC group had maximum slowly degradable fraction and higher than ($P < 0.05$) CON, GLU and CEL groups. Compared with CON and GLU groups, the DM effective degradability of LGC group was significantly increased ($P < 0.05$).

3.6 Ruminal crude protein degradation of mixed silage

The ruminal CP degradation of LGC group at 4 h and 24 h was higher ($P < 0.05$) than that of CON, LAB and GLU groups (Table 5). At 72 h, the CP degradation rate of all groups ranged from 74.52% to 80.40%. Similar to DM degradability, the CP degradation occurred mainly before 24 h. No significant difference ($P > 0.05$) of slowly degradable fraction was observed among all groups (Table 6). However, the rapidly degradable fraction of CEL and LGC groups was higher ($P < 0.05$) than that of other groups. Similarly, the total degradable fraction of CEL and LGC groups was significantly enhanced ($P < 0.05$) as compared to CON and GLU

TABLE 1 Effects of different treatments on the chemical composition of mixed silage (DM basis, %).

Items	Treatments					SEM	*P*-value
	CON	LAB	GLU	CEL	LGC		
DM	24.93^{b}	29.56^{a}	25.12^{b}	25.42^{b}	30.69^{a}	0.679	0.001
CP	5.85^{b}	6.76^{a}	6.44^{ab}	6.27^{ab}	7.04^{a}	0.139	0.048
OM	86.19	87.06	85.84	85.46	87.43	0.582	0.842
NDF	60.04^{a}	56.20^{ab}	58.87^{a}	54.10^{b}	53.13^{b}	0.829	0.015
ADF	39.96^{a}	38.43^{ab}	41.08^{a}	34.75^{b}	35.43^{b}	0.737	0.006
WSC	2.19^{b}	1.82^{b}	2.90^{a}	2.26^{ab}	2.24^{ab}	0.117	0.038

CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg.
DM, dry matter; CP, crude protein; OM, organic matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water soluble carbohydrate.
In the same row, values with different letter mean significant differences ($P < 0.05$).

TABLE 2 Effects of different treatments on the fermentation characteristics of mixed silage.

Items	Treatments					SEM	*P*-value
	CON	LAB	GLU	CEL	LGC		
pH	4.16^{a}	4.04^{ab}	3.94^{abc}	3.89^{bc}	3.78^{c}	0.042	0.026
NH_3-N/TN	4.76^{a}	3.80^{b}	3.60^{b}	3.04^{c}	2.87^{c}	0.159	<0.001
Lactic acid (%, DM)	3.17^{c}	4.14^{b}	4.44^{b}	4.24^{b}	5.02^{a}	0.153	<0.001
Acetic acid (%, DM)	2.03^{a}	1.73^{ab}	1.33^{bc}	1.36^{bc}	1.11^{c}	0.094	0.003
Propionic acid (%, DM)	0.032^{a}	0.011^{bc}	0.019^{b}	0.016^{bc}	0.007^{c}	0.002	0.001
Butyric acid (%, DM)	0.003	ND	ND	0.002	ND	0.001	0.141
Lactic acid/Acetic acid	1.59^{c}	2.40^{bc}	3.52^{ab}	3.31^{b}	4.77^{a}	0.306	0.002

CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg.
DM, dry matter; NH_3-N, ammonia nitrogen; TN, total nitrogen; ND, not detected.
In the same row, values with different letter mean significant differences ($P < 0.05$).

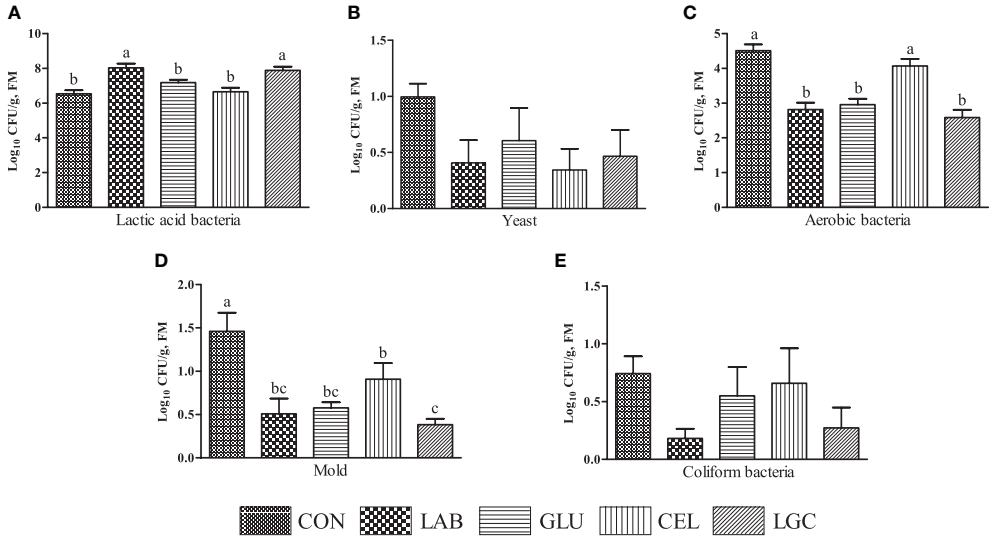

FIGURE 1
Effects of different treatments on the microbial population of mixed silage. **(A)** lactic acid bacteria; **(B)** yeast; **(C)** aerobic bacteria; **(D)** mold; **(E)** coliform bacteria. CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg. FM, fresh matter. Columns with different superscript letters mean significant differences ($P < 0.05$).

groups. Furthermore, the effective degradability of inoculated treatments was more than 60% and the effective degradability of LGC group was higher ($P < 0.05$) than CON, LAB and GLU groups.

3.7 Ruminal neutral detergent fiber degradation of mixed silage

At 24 h, the NDF degradability of LAB, CEL and LGC groups was higher ($P < 0.05$) than that of other groups (Table 7). The ruminal NDF degradability of mixed silage in LGC group at 72 h was significantly increased ($P < 0.05$) when compared to CON, LAB and GLU groups. Unlike DM and CP degradability, the NDF degradation of mixed silage occurred mainly after 24 h. The rapidly degradable fraction of all groups was lower and it had no significant difference ($P > 0.05$) (Table 8). However, the slowly degradable fraction of LGC group was significantly increased ($P < 0.05$) as compared with CON, LAB and GLU groups. The NDF effective degradability of all treatments ranged from 29.07% to 34.50%. Moreover, the effective degradability of CEL and LGC groups was higher ($P < 0.05$) than other groups.

3.8 Ruminal acid detergent fiber degradation of mixed silage

The ADF degradability did not show significant difference ($P > 0.05$) among all groups from 4 h to 16 h (Table 9). At 24 h, the ADF

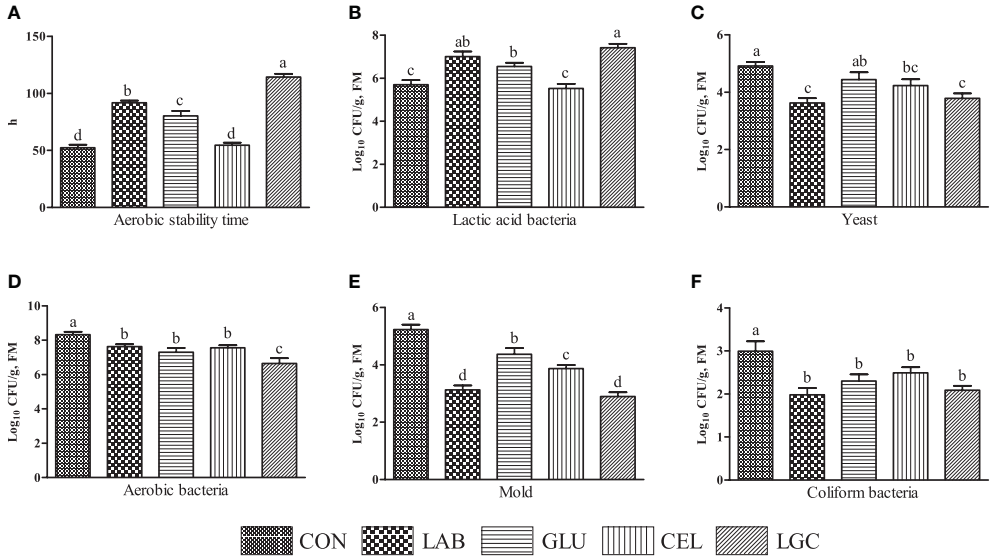

FIGURE 2
Effects of different treatments on the aerobic stability of mixed silage. **(A)** aerobic stability time; **(B)** lactic acid bacteria; **(C)** yeast; **(D)** aerobic bacteria; **(E)** mold; **(F)** coliform bacteria. CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg. FM, fresh matter. Columns with different superscript letters mean significant differences ($P < 0.05$).

TABLE 3 Rumen degradability of dry matter of mixed silage at different time points (%).

Time point (h)	Treatments					SEM	*P*-value
	CON	LAB	GLU	CEL	LGC		
4	26.61^{b}	31.98^{a}	28.60^{ab}	29.57^{ab}	33.33^{a}	0.818	0.049
8	32.65^{b}	38.69^{a}	36.25^{ab}	35.91^{ab}	41.69^{a}	1.002	0.035
16	42.31	47.57	47.99	44.98	51.05	1.049	0.072
24	53.10	55.89	56.14	53.11	59.34	1.052	0.320
36	58.35	61.16	59.28	59.93	63.91	0.811	0.232
48	62.51	65.40	64.52	65.76	68.11	0.798	0.279
72	64.34^{c}	70.14^{ab}	66.56^{bc}	67.33^{abc}	71.94^{a}	0.874	0.027

CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg.
In the same row, values with different letter mean significant differences ($P < 0.05$).

degradability of GLU group was lower ($P < 0.05$) than other groups. In addition, compared with other groups, the ADF degradability at 72 h of CEL and LGC groups was significantly increased ($P < 0.05$). In accordance with NDF, the ADF of all mixed silage degraded slowly before 16 h; then from 16 h to 48 h, the degradation speed of all groups was faster. The rapidly degradable fraction of mixed silage was lower and the peak value was only 4.03% (Table 10). No significant difference ($P > 0.05$) of rapidly and slowly degradable fractions was found among all groups. The effective degradability of LGC group was maximum and higher ($P < 0.05$) than that of LAB and GLU groups.

4 Discussion

The forage grass characteristics directly affect the quality of silage. Our previous study found that the moisture content of amaranth was high, whereas the WSC content was low (Liu et al., 2017; Ma et al., 2019). Thus, the quality of amaranth silage alone was relatively low. The corn straw has high DM content. In the current study, we used corn straw as mixed material to improve the quality of amaranth silage by adding different additives. The chemical compositions affect feeding value of silage. After 60 days of ensiling, mixed silage inoculation with lactic acid bacteria

TABLE 4 Rumen degradation parameters of dry matter of mixed silage.

Items	Treatments					SEM	*P*-value
	CON	LAB	GLU	CEL	LGC		
a (%)	22.56^{b}	26.55^{a}	23.18^{b}	24.20^{b}	26.67^{a}	0.460	<0.001
b (%)	39.75^{c}	45.39^{ab}	42.62^{bc}	41.74^{c}	46.64^{a}	0.701	0.002
c (%/h)	0.035^{d}	0.037^{cd}	0.046^{b}	0.053^{a}	0.040^{c}	0.002	<0.001
a + b (%)	62.31^{b}	71.94^{a}	65.80^{b}	65.94^{b}	73.31^{a}	1.059	<0.001
ED (%)	43.47^{c}	51.26^{ab}	48.49^{b}	50.56^{ab}	52.87^{a}	0.828	<0.001

CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg.
a: rapidly degradable fraction; b: slowly degradable fraction; a + b: total degradable fraction; c: degradation rate of slowly degradable fraction; ED: effective degradability.
In the same row, values with different letter mean significant differences ($P < 0.05$).

TABLE 5 Rumen degradability of crude protein of mixed silage at different time points (%).

Time point (h)	Treatments					SEM	*P*-value
	CON	LAB	GLU	CEL	LGC		
4	35.89^{b}	37.02^{b}	34.99^{b}	41.75^{a}	40.55^{a}	0.701	<0.001
8	48.68^{ab}	48.34^{ab}	45.31^{b}	51.77^{a}	54.32^{a}	1.043	0.043
16	56.97^{b}	59.17^{b}	61.33^{ab}	59.15^{b}	64.98^{a}	0.898	0.035
24	62.78^{b}	65.61^{b}	64.38^{b}	68.57^{ab}	71.04^{a}	0.903	0.010
36	67.12^{c}	68.71^{bc}	69.22^{bc}	71.79^{ab}	73.32^{a}	0.724	0.027
48	69.75	72.98	71.15	74.74	74.05	1.121	0.653
72	75.36^{b}	77.54^{ab}	74.52^{b}	78.11^{ab}	80.40^{a}	0.688	0.033

CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg.
In the same row, values with different letter mean significant differences ($P < 0.05$).

TABLE 6 Rumen degradation parameters of crude protein of mixed silage.

Items	Treatments					SEM	*P*-value
	CON	LAB	GLU	CEL	LGC		
a (%)	34.20^{b}	36.40^{b}	36.91^{b}	40.19^{a}	39.76^{a}	0.623	0.001
b (%)	38.25	42.67	39.79	41.26	42.70	0.762	0.292
c (%/h)	0.051^{c}	0.059^{ab}	0.053^{bc}	0.056^{abc}	0.060^{a}	0.001	0.024
a + b (%)	72.45^{c}	79.06^{ab}	76.69^{b}	81.45^{a}	82.46^{a}	0.991	0.001
ED (%)	57.89^{d}	64.20^{bc}	61.92^{c}	66.76^{ab}	67.92^{a}	0.913	<0.001

CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg.
a: rapidly degradable fraction; b: slowly degradable fraction; a + b: total degradable fraction; c: degradation rate of slowly degradable fraction; ED: effective degradability.
In the same row, values with different letter mean significant differences ($P < 0.05$).

significantly increased DM and CP contents. In the fermentation process of silage, a series of biochemical changes occur because of the action of a variety of microorganisms, resulting in nutrients loss (Zhang et al., 2022). A previous study found that the DM content of silage inoculated with *L. plantarum* was increased (Ren et al., 2020), which was consistent with our result. The reason may be related to less production of silage liquid by lactic acid bacteria action, which contains about 8% DM (Yang et al., 2020). However, the underlying mechanism of action still needs further study. Additionally, the inoculation with *L. plantarum* can promote homo-fermentation and prevent organic degradation caused by insufficient lactic acid yield (Ren et al., 2020), which are beneficial for reducing the loss of nutrients contents. Overall, the DM content of mixed silage of amaranth and corn straw was elevated as compared to individual amaranth silage (Liu et al., 2017). Corn straw is an effective mixed material to improve the quality of amaranth silage.

TABLE 7 Rumen degradability of neutral detergent fiber of mixed silage at different time points (%).

Time point (h)	Treatments					SEM	*P*-value
	CON	LAB	GLU	CEL	LGC		
4	7.17^{c}	9.04^{abc}	8.41^{bc}	10.44^{ab}	11.06^{a}	0.431	0.012
8	13.73	14.30	13.49	17.02	15.65	0.469	0.070
16	18.14	20.37	17.36	19.69	21.09	0.514	0.103
24	27.05^{b}	33.76^{a}	27.68^{b}	32.35^{a}	33.50^{a}	0.862	0.006
36	38.31^{a}	40.51^{a}	33.91^{b}	39.71^{a}	38.44^{a}	0.719	0.017
48	45.34^{bc}	47.83^{abc}	44.80^{c}	48.71^{ab}	50.71^{a}	0.683	0.016
72	47.52^{c}	50.38^{bc}	48.79^{c}	52.96^{ab}	55.11^{a}	0.785	0.002

CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg.
In the same row, values with different letter mean significant differences ($P < 0.05$).

TABLE 8 Rumen degradation parameters of neutral detergent fiber of mixed silage.

Items	Treatments					SEM	*P*-value
	CON	LAB	GLU	CEL	LGC		
a (%)	4.15	3.92	4.05	4.50	5.03	0.329	0.863
b (%)	44.52^{c}	45.69^{bc}	45.40^{bc}	48.77^{ab}	50.40^{a}	0.708	0.018
c (%/h)	0.040	0.041	0.044	0.045	0.044	0.001	0.237
a + b (%)	48.67^{b}	49.61^{b}	49.44^{b}	53.27^{a}	55.43^{a}	0.707	<0.001
ED (%)	29.07^{b}	29.99^{b}	30.60^{b}	33.19^{a}	34.50^{a}	0.561	0.001

CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg.
a: rapidly degradable fraction; b: slowly degradable fraction; a + b: total degradable fraction; c: degradation rate of slowly degradable fraction; ED: effective degradability.
In the same row, values with different letter mean significant differences ($P < 0.05$).

TABLE 9 Rumen degradability of acid detergent fiber of mixed silage at different time points (%).

Time point (h)	Treatments					SEM	*P*-value
	CON	LAB	GLU	CEL	LGC		
4	8.14	6.01	5.16	6.54	8.05	0.467	0.180
8	13.03	9.12	9.59	11.11	11.07	0.478	0.060
16	15.04	13.22	12.04	13.70	13.98	0.437	0.295
24	22.86^{a}	25.77^{a}	18.54^{b}	24.30^{a}	22.55^{a}	0.721	0.007
36	32.70	32.67	30.60	35.65	34.88	0.704	0.155
48	41.64^{b}	40.15^{b}	39.84^{b}	46.60^{a}	46.35^{a}	0.902	0.009
72	43.14^{b}	42.85^{b}	42.65^{b}	48.63^{a}	50.28^{a}	0.976	0.007

CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg.
In the same row, values with different letter mean significant differences ($P < 0.05$).

Research has reported that sufficient WSC concentration (≥ 5%) was necessary to ensure the silage quality (Zi et al., 2022a). The WSC was low in mixed materials; thus, we added glucose as additive to improve the silage quality. With the progress of silage fermentation, a large amount of WSC was consumed by lactic acid bacteria, leading to WSC content to below 3% after fermentation of 60 days. The cellulase can promote fermentation of lactic acid bacteria by degrading structural carbohydrates to provide

TABLE 10 Rumen degradation parameters of acid detergent fiber of mixed silage.

Items	Treatments					SEM	*P*-value
	CON	LAB	GLU	CEL	LGC		
a (%)	4.03	3.55	2.93	3.06	3.62	0.246	0.663
b (%)	54.12	47.02	45.90	51.71	51.45	1.087	0.066
c (%/h)	0.022^{c}	0.025^{bc}	0.024^{c}	0.031^{ab}	0.034^{a}	0.001	0.004
a + b (%)	58.15^{a}	50.57^{bc}	48.83^{c}	54.76^{ab}	55.07^{ab}	1.069	0.021
ED (%)	26.51^{abc}	24.73^{bc}	22.62^{c}	28.66^{ab}	30.18^{a}	0.809	0.007

CON, control group without additive; LAB, mixed silage inoculated with lactic acid bacteria 5 mg/kg; GLU, mixed silage supplementation with glucose 30 g/kg; CEL, mixed silage supplementation with cellulase 2 mg/kg; LGC, mixed silage supplementation with lactic acid bacteria 5 mg/kg, glucose 30 g/kg and cellulase 2 mg/kg.
a: rapidly degradable fraction; b: slowly degradable fraction; a + b: total degradable fraction; c: degradation rate of slowly degradable fraction; ED: effective degradability.
In the same row, values with different letter mean significant differences ($P < 0.05$).

substrate (Agustinho et al., 2021). Our results showed that mixed silage treated with cellulase (CEL and LGC groups) significantly decreased NDF and ADF contents when compared to CON and GLU groups. Similar result was found in Xiong et al. (2022) study, who reported that hybrid *Pennisetum* silage treated with cellulase reduced NDF and ADF contents. Three additives combinations showed highest DM and CP contents and lowest NDF content. This may be associated with synergistic action among different additives. The glucose and cellulase provided enough fermentation substrate for lactic acid bacteria to produce lactic acid, which were helpful for decreasing nutrients loss. In terms of chemical composition, the mixed silage of amaranth and corn straw inoculated with lactic acid bacteria, glucose and cellulase can improve nutritional value.

After 60 days of ensiling, pH in all groups reduced below 4.2, an important index that suggested silage was well preserved (McDonald et al., 1991). Compared with CON and LAB groups, the pH of LGC group was significantly reduced. The lactic acid bacteria can decrease the silage pH by utilizing WSC to produce lactic acid. Although the silage in LAB group was inoculated with lactic acid bacteria, the fermentation substrate was inadequate. Thus, the silage could not produce enough lactic acid to reduce pH, which was matched to the lactic acid result. The addition of glucose and cellulase provided substance for bacteria in the silage to promote fermentation process and was conducive to decreasing pH. A previous study in native grass silage found that the addition of lactic acid bacteria and molasses reduced pH and increased lactic acid content (Li et al., 2022), which was in accordance with our study.

High content of lactic acid content can inhibit the growth of harmful microorganisms, thus reducing the production of butyric acid (McDonald et al., 1991). In ruminants production, feeding silage with high butyric acid will increase the probability of metabolic disease such as ketosis in animals (Vicente et al., 2014). Butyric acid was detected in CON and CEL groups, which suggested that the mixed silage was contaminated by mold as seen by the microbial population results, and silage quality decreased. Propionic acid and butyric acid can consume some of the metabolic energy during production. The conversion of lactic acid to butyric acid leads to the loss of more than half of DM content (Zhao et al., 2022), which has negative effect on feed intake of animals. In the present study, mixed silage inoculated with additives significantly reduced propionic acid content. Besides, the acetic acid of CON group was higher than GLU, CEL and LGC groups. The lower WSC content in CON and LAB silage may accelerate the conversion from homo-fermentation to hetero-fermentation, contributing to higher acetic acid concentration in mixed silage of CON and LAB groups after ensiling was finished (Muck, 2013).

In general, NH_3-N production is associated with CP breakdown caused by enzymes and microorganisms in silage and NH_3-N/TN can be used to reveal the extent of proteolysis (Wang et al., 2019). Compared with CON group, inoculation with lactic acid bacteria, glucose and cellulase alone or in combination markedly reduced NH_3-N/TN in mixed silage. Among them, the LGC group showed lowest NH_3-N/TN, indicating that undesirable proteolytic bacteria were inhibited effectively in additives-treated silages. After 60 days of fermentation, the decline of NH_3-N/TN in LGC group might be that the synergistic effect of different additives could cause nitrification, which transferred NH_3-N to nitrate nitrogen (Fang et al., 2022). The future researches should be paid more attention to the effects of different additives on nitrogen transformation in fermentation process of amaranth silage. In the fermentation of silage, protein hydrolysis can be inhibited by acid environment, which prevents proteolytic enzyme activity and decreases NH_3-N concentration (Li et al., 2018c). In our study, combined addition of lactic acid bacteria, glucose and cellulase could maintain lowest acid environment (pH=3.78) to maximum the NH_3-N reduction of mixed silage, which was line with CP result. The results mentioned earlier suggested that different additives in the fermentation process had the ability to improve fermentation quality, and the combined inoculation efficiently promoted the fermentation.

The production of NH_3-N and organic acids is closely related to microbial population. In the current experiment, the lactic acid bacteria count was significantly increased in LAB and LGC groups. Correspondingly, the harmful microorganisms counts, including aerobic bacteria and mold, were reduced. Moreover, inoculation of glucose also had significant reduction of aerobic bacteria and mold counts. The additional supplementation of lactic acid bacteria and an enough supply of carbohydrate source as substrates could explain the decrease in harmful microorganisms counts (So et al., 2020). Previously, a study has found that the combination of lactic acid bacteria and molasses can inhibit growth of yeast, mold and

coliform bacteria in soybean silage (Ni et al., 2017), which was basically consistent with our study. The positive effects were most likely because of the additive' ability to produce sufficient lactic acid to reduce pH and create acid experiment, thus preventing the growth of a variety of unwanted microorganisms in silage fermentation process (Henderson, 1993). In silage, yeast, mold and coliform bacteria play a critical role in butyric acid production by secreting amino acid decarboxylases to generate butyric acid (Jia et al., 2021). Therefore, higher these microorganisms counts in CON and CEL groups induced increased butyric acid, which was not beneficial for silage quality. Overall, these results verified that additives can reduce the number of undesirable microorganisms in mixed silage of amaranth and corn straw, with the LGC groups showing the greatest influence.

After exposure to air, the aerobic microorganisms activity of silage begin to enhance and release a mass of heat because of metabolizing and consuming nutrients, resulting in increased pH and nutrients loss (Yuan et al., 2015). Thus, the change of temperature is usually used as an important parameter to evaluate the silage aerobic stability. In our study, combination of all additive significantly lengthened the aerobic stability. Particularly, yeast is deemed to be the promoter of aerobic spoilage, the number of which is closely related to high temperature of silage (He et al., 2020). The improvement in aerobic stability of LGC group could be attributed to a reduction of yeast count. After 5 days of aerobic exposure, the number of lactic acid bacteria was increased and the aerobic bacteria and mold counts were decreased in additives treatments. The possible reason was mainly because although the lactic acid bacteria activity of additives group was not insufficient to maintain aerobic stability long time after mixed silage was opened, it could still prevent the growth of harmful microorganisms via maintaining the acid environment and antibacterial substances generation within a short period (da Silva et al., 2018). In ruminants' production, coliform bacteria can cause diseases such as diarrhea and mastitis, which lead to considerable economic damage (Ursula et al., 2022). After aerobic exposure, coliform bacteria of all group was increased as compared with ensiling stage, but the CON group had highest counts. In short, inoculation with silage can improve the aerobic stability of mixed silage of amaranth and corn straw.

In addition to fermentation quality and chemical composition, the utilization rate of nutrients by animals is a critical index to assess the silage quality. Therefore, we used dairy cows as experimental animals to investigate the effects of different additives on the ruminal degradation characteristics of mixed silage composed of amaranth and corn straw. In dairy cows' production, dry matter intake is important to maintain lactation and the ruminal DM degradability is positively related to dry matter intake (Hao et al., 2020). In our study, the LGC group had the highest DM degradation at 72 h and effective degradability. A previous study reported that the addition of lactic acid preparation can improve the DM digestibility (Rowghani and Zamiri, 2009), which was in accordance with our result. This result may be related to higher DM content in LGC group. Similarly, the ruminal CP degradation of LGC group showed greatest effects. The CP degradation rate is affected by true protein concentration and amino acid composition proportion of CP in the feedstuff (Cardozo et al., 2004). The combined inoculation of additives may have positive effects on the amino acid composition of mixed silage, but it needs future study. In addition, the CP total degradable fraction of LGC and CEL groups exceeded than that of CON and GLU groups. A plausible explanation for this result was that the mixed silage inoculated with cellulase contained high content of soluble true protein including the form of non-ammonia N (Wang et al., 2022a), as seen by the NH_3-N result mentioned earlier.

Increased crude fiber degradation rate is conducive to rumen fermentation and can result in elevated contents of volatile fatty acids that provide more energy for dairy cows to maintain production performance (Abbasi et al., 2018). Previous researchers have found that the addition of additive enhanced the ruminal NDF and ADF degradation of silage (Jiao et al., 2021; Wang et al., 2022b), which were consistent with our results. Furthermore, inoculation of cellulase showed significant improvement of ruminal degradability of NDF and ADF. The result was likely attributed to the breakdown of connection between polyester and cellulose by cellulase treatment, which could enable the degradation and utilization of structural carbohydrates by microbiota in the rumen (Li et al., 2018a). In the future, metagenomics and other multi-omics technologies can be used to explore effects of different additives on the microbial community and function in mixed silage of amaranth and corn straw.

5 Conclusions

The results obtained from current study provided evidence that the lactic acid bacteria inoculation increased DM, CP and lactic acid contents and decreased propionic acid content and aerobic bacteria and mold counts of mixed silage. Glucose addition had no significant effect on the chemical composition, where increased lactic acid content and decreased aerobic bacteria and mold counts of mixed silage. Cellulase treatment reduced NDF and ADF contents as well as NH_3-N/TN and mold count. Combining the three additives contributed in a number ways of mixed silage quality, including promotion in the fermentation, reduction in the harmful bacteria counts and improvement of aerobic stability and nutrients composition. In addition, the rumen degradation of nutrients was improved in inoculation of three additives.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Guangdong Ocean University (Zhanjiang, Guangdong, China).

Author contributions

JM, XF and SG conceived and designed the research. JM, XF, ZM, XH, and MT performed the experiment and sample analysis. JM, XF, and ZZ analyzed the data. JM and XF wrote the original manuscript. JM, XF, FY, and SG reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1189747/full#supplementary-material

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Silages of sorghum, Tamani guinea grass, and *Stylosanthes* in an integrated system: production and quality

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Crop–livestock integration systems are efficient technologies for diversifying production and promoting agricultural sustainability. However, less is known about the triple intercropping of crops for silage production. The objective of this study was to evaluate the dry mass production, fermentation profile, and nutritive value of sorghum silage intercropped with Tamani guinea grass and *Stylosanthes* cv. Bela in integrated systems. We used an experimental design with randomized blocks with four replicates. The treatments consisted of silage of (1) sorghum in monocropped; (2) Tamani guinea grass in monocropped (*Panicum maximum* cv. BRS Tamani); (3) *Stylosanthes* cv. Bela in monocropped (*Stylosanthes guianensis* cv. BRS Bela); (4) sorghum intercropped with Tamani guinea grass; (5) sorghum intercropped with *Stylosanthes* cv. Bela; (6) *Stylosanthes* cv. Bela intercropped with Tamani guinea grass; and (7) sorghum intercropped with Tamani guinea grass and *Stylosanthes* cv. Bela, totaling 28 experimental silos. Our results demonstrated that intercropping sorghum with tropical forages can be utilized in integrated silage production systems. This practice led to an increase in silage mass production per unit area while also providing pasture forage after the crop harvest for silage production, ultimately enhancing land-use efficiency in a sustainable manner. Silage produced from sorghum intercropped with Tamani guinea grass and *Stylosanthes* cv. Bela exhibited improved fermentative characteristics, as well as higher ether extract and total digestible nutrient contents compared with silage from monocropped forages. Tropical forages contributed to an increase in the crude protein content of monocropped sorghum silage, which could potentially reduce costs associated with acquiring protein salts for ruminant feed supplementation. Consequently, we recommend the triple intercropping of sorghum, Tamani guinea grass, and Bela for silage production, as it offers advantages for the cultivation of annual and tropical forage crops.

KEYWORDS

fiber fraction, *Panicum maximum* cv. BRS Tamani, crude protein, *Sorghum bicolor*, *Stylosanthes guianensis* cv. Bela, fermentation profile

Introduction

The sustainability of agribusiness has become a prerequisite for maintaining a position in the global market, encompassing both socioenvironmental appeal and the necessity for integrated negotiation (Borsellino et al., 2016). In this regard, conservative production systems that integrate crops and livestock have emerged as an established strategy for intensifying food production while addressing environmental concerns (Simões et al., 2023). Integrated crop–livestock systems are widely recognized as one of the most sustainable and competitive technologies for advancing agribusiness (Dias et al., 2020).

The ecosystem services provided by integrated systems have been documented in numerous studies, including enhanced land use efficiency and increased grain production (Muniz et al., 2021); reduced soil compaction, improved water infiltration rates, and lower erosion risks (Linhares et al., 2020); maintenance of soil fertility through enhanced nutrient cycling (Dias et al., 2020); greater carbon sequestration, increased soil organic matter, and improved microclimatic conditions (Vincent-Caboud et al., 2019); pasture regeneration (Santos et al., 2020); and improved silage production during the dry season (Oliveira et al., 2020). However, due to the intricate interactions between annual and tropical forage crops, integrated systems have become dynamic and complex, requiring more sophisticated technologies to consolidate environmental and productive sustainability (Soussana and Lemaire, 2014).

Among the annual crops employed in integrated systems, *Sorghum bicolor* L. (sorghum) stands as a significant forage crop in numerous global regions owing to its adaptability across diverse environments (Perazzo et al., 2017), capability to flourish in low soil fertility conditions, potential for regrowth post-grain harvest, and high tolerance against water deficits (Buffara et al., 2018). These characteristics enable a wider range of sowing seasons and greater resilience to adverse environmental factors compared with maize (Mateus et al., 2016). Consequently, sorghum has been recognized as crucial in recent years for the production of preserved forage (Oliveira et al., 2020), offering high dry mass productivity, preservation of nutritional value, and favorable fermentation patterns (Cruz et al., 2020).

However, sorghum silage typically exhibits a lower crude protein content compared with tropical forages (Ribeiro et al., 2017), necessitating the adoption of cultivation strategies to enhance the crude protein content of sorghum silage. Notably, Oliveira et al. (2020) demonstrated the advantageous effects of intercropping annual and tropical forage crops in integrated systems for silage production. Intercropping not only enhances the quality of sorghum silage but also intensifies production systems, resulting in higher yields and improved silage nutritional value. Moreover, this approach mitigates the challenges associated with silage fermentation in exclusive grass and legume systems.

Among tropical forages, Tamani guinea grass possesses significant potential as a conserved forage resource due to its high mass yield, slender stems and leaves, and high tillering capacity (Dias et al., 2020). The exceptional quality and adaptability of this grass make it suitable for silage production (Paludo et al., 2020). Additionally, tropical legume silage has gained attention due to its superior nutritional value (Silva et al., 2023). Recently introduced *Stylosanthes* cv. Bela (*Stylosanthes guianensis* cv. BRS Bela) has shown positive results in ruminant production, owing to its elevated crude protein content (Braga et al., 2020) and ability to fix biological nitrogen. Given the escalating costs of mineral fertilizers and their significant contribution to greenhouse gas emissions, the integration of legumes into integrated systems emerges as a promising technology for increasing crop productivity and ensuring enhanced sustainability (Epifanio et al., 2019a,b). This approach allows for the partial or complete replacement of mineral (nitrogen) fertilizers, ensuring adequate plant nutrition, soil conservation, fertility maintenance, and carbon sequestration (Bourscheidt et al., 2023; Silva et al., 2023).

In this context, sorghum silage, tropical grasses, and legumes present a favorable combination for achieving a balanced nutritional value, improved qualitative characteristics in dry matter (DM), and higher nutrient production per unit area (Perazzo et al., 2017). Moreover, their versatility in use establishes them as significant alternative food sources during the off-season period (Oliveira et al., 2020).

However, limited knowledge exists regarding triple intercropping (sorghum + tropical grass + legumes) for silage production. Understanding the optimal intercropping approach can enhance both silage production and quality within integrated systems while also facilitating crop diversification to meet the demand for high-quality feed during periods of limited forage availability. Therefore, we hypothesized that double and triple intercropping of sorghum with forage crops would have a positive impact on the bromatological characteristics and yield of the ensiled mass, without compromising the fermentation process required for silage preparation. The objective of this study was to assess the production of dry mass, fermentation profile, and nutritive value of silage derived from sorghum intercropped with Tamani guinea grass and *Stylosanthes* cv. Bela in integrated systems.

Materials and methods

Description of the area and crop establishments

This experiment was conducted under field conditions at Instituto Federal Goiano, Campus Rio Verde, located in the municipality of Rio Verde, State of Goiás, Brazil (17°48′ 22″S, 50°54′ 11″W; 832 m altitude). According to the Köppen–Geiger classification, the climate of the region is defined as tropical (Aw), with a dry season in winter.

Before the implementation of the experiment, soil samples were collected from the 0 to 20 cm layer for physicochemical characterization. The soil in the experimental area was characterized as Dystroferric Red Latosol (Santos et al., 2018). The soil characteristics were as follows: it has 364, 83, and 553 g kg^{-1} of clay, silt, and sand, respectively; pH in $CaCl_2$: 5.4; Ca: 2.69 $cmol_c$ dm^{-3}; Mg: 1.00 $cmol_c$ dm^{-3}; Al: 0.01 $cmol_c$ dm^{-3}; Al + H: 3.79 $cmol_c$ dm^{-3}; K: 0.69 $cmol_c$ dm^{-3}; cation exchange capacity: 8.6 $cmol_c$ dm^{-3}; current base saturation of the soil (V1): 56%; P (Mehlich): 3.8 mg dm^{-3}; S: 8.5 mg dm^{-3}; Cu: 3.7 mg dm^{-3}; Zn: 1.0 mg dm^{-3}; Fe: 17.3 mg dm^{-3}; organic matter (OM): 39.8 g

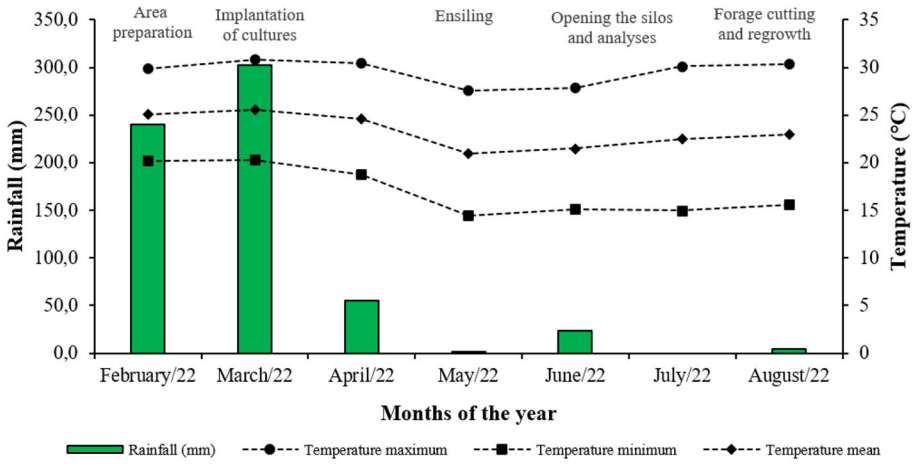

FIGURE 1
Monthly precipitation and minimum, average and maximum temperatures recorded from February to August 2022 in Rio Verde—GO, Brazil.

dm^{-3}. No irrigation system was installed during the experiment. Precipitation, maximum, average, and minimum temperatures were monitored throughout the duration of the study, as shown in Figure 1.

Experimental design and treatments

The experimental design consisted of randomized blocks with four replicates. The treatments consisted of the silage of (1) sorghum in monocropped; (2) Tamani guinea grass in monocropped (*Panicum maximum* cv. BRS Tamani); (3) *Stylosanthes* cv. Bela in monocropped (*Stylosanthes guianensis* cv. BRS Bela); (4) sorghum intercropped with Tamani guinea grass; (5) sorghum intercropped with *Stylosanthes* cv. Bela; (6) *Stylosanthes* cv. Bela intercropped with Tamani guinea grass; and (7) sorghum intercropped with Tamani guinea grass and Bela *Stylosanthes*, totaling 28 experimental silos. The sorghum used was grain sorghum (AG 1077) with an early cycle and high production potential.

For monocropped cultures, a spacing of 0.5 m between rows was used (Figures 2A–C). In the double intercropping of annual and forage crops, sorghum was sown at 0.5 m spacing and Tamani guinea grass and/or legume between rows was sown at a 0.25 m distance from the sorghum row (Figures 2D, E). In the double intercropping of forage plants, Tamani guinea grass was sown at 0.25 m of the *Stylosanthes* cv. Bela (Figure 2F). In the triple intercropping, sorghum was sown at 0.9 m spacing, with Tamani guinea grass and *Stylosanthes* cv. Bela sown between the rows at a 0.3 m distance from the sorghum row, as shown in Figure 2G. No herbicides were applied to suppress grass or legume growth during the intercropping.

The seeding of forage systems was carried out manually on 7 March 2022, and 120 kg ha^{-1} of P_2O_5 and 20 kg ha^{-1} of FTE BR 12 (9% Zn, 1.8% B, 0.8% Cu, 2% Mn, 3.5% Fe, and 0.1% Mo) were applied in the planting furrow using simple superphosphate and Frit sources, respectively. A total of 12 sorghum seeds were used per meter, and for grass and legumes, 3 kg of pure viable seeds were used per hectare.

When sorghum plants were at the stage of three and six fully developed leaves, two fertilizer applications of 80 and 60 kg ha^{-1} of N and K_2O from urea and potassium chloride, respectively, were applied to the following systems: sorghum in monocropped, Tamani guinea grass in monocropped, and sorghum intercropped with Tamani guinea grass. For the systems intercropped with legumes (sorghum intercropped with *Stylosanthes* cv. Bela, Tamani guinea grass intercropped with *Stylosanthes* cv. Bela, and sorghum intercropped with Tamani guinea grass and Bela), 60 kg ha^{-1} of K_2O and only half of the dose of nitrogen (i.e., 40 kg ha^{-1}) were applied, aiming to utilize the nitrogen obtained through the biological fixation by legumes. For the *Stylosanthes* cv. Bela monocropped system, only 60 kg ha^{-1} K_2O was applied.

To *Spodoptera frugiperda* and *Dalbulus maidis*, we applied the insecticides Klorpan (active ingredient Chlorpyrifos) and Connect (active ingredient Beta-cyfluthrin and Imidacloprid) at the rate of 0.4 and 0.1 L ha^{-1} of commercial product, respectively. Both applications were performed using knapsack sprayers.

Crop silage

Harvesting of cultures for silage was carried out 93 days after sowing the cultures. During this period, sorghum, Tamani guinea grass, and *Stylosanthes* cv. Bela were harvested at 340.66, 276.16, and 285.04 g kg^{-1} dry matter (DM), respectively. To evaluate the dry mass production and proportion of the ensiled material (Table 1), the material was collected, separated, and weighed to determine the proportions of sorghum, Tamani guinea grass, and *Stylosanthes*. Subsequently, the material was placed in an oven at 55°C until it reached a constant mass, and its dry weight was determined and expressed in kg ha^{-1}.

To make the silage, the material was ground together in the treatments of the intercropping systems, in particles of ~10 mm size. The material was stored in experimental PVC silos measuring

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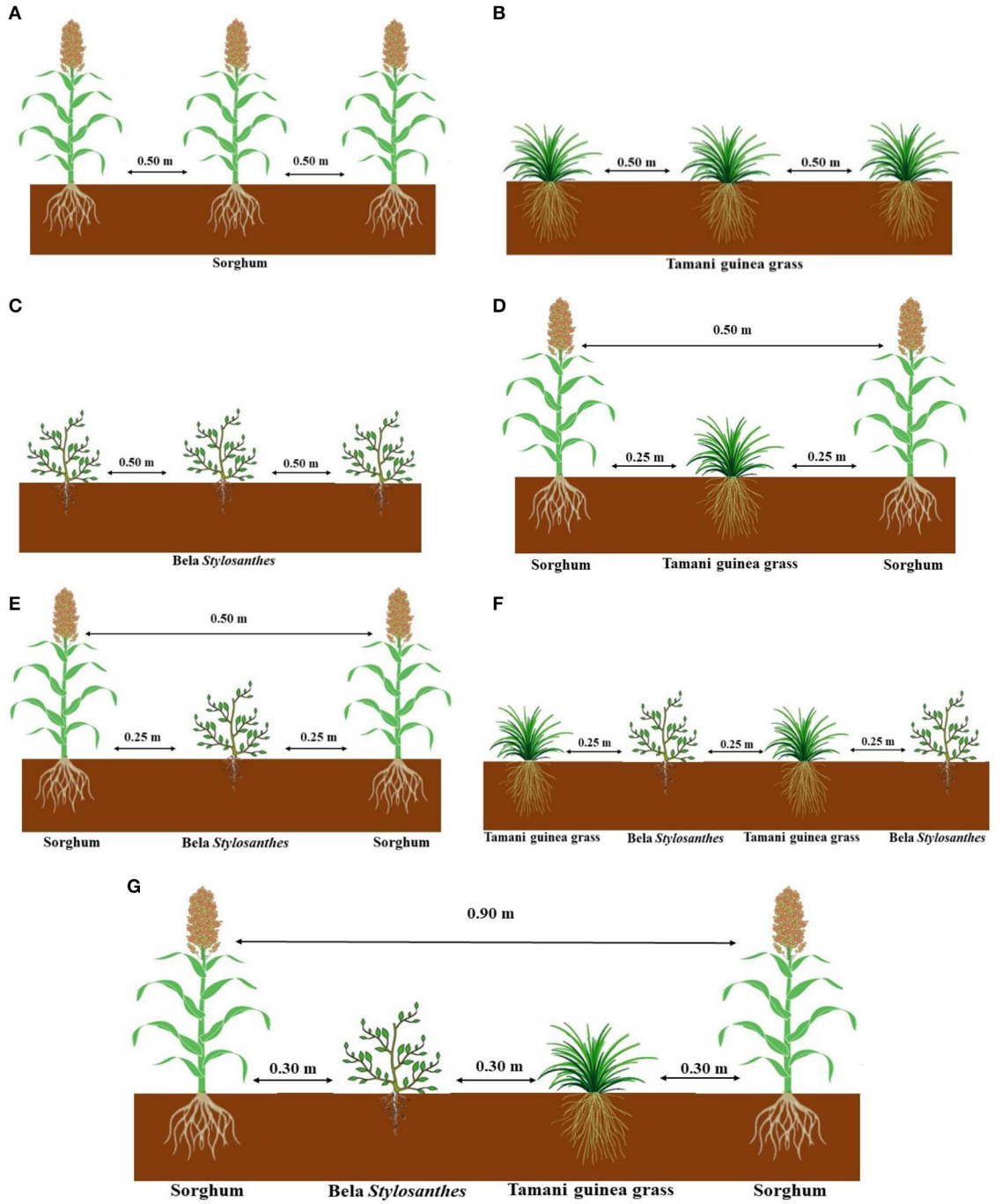

FIGURE 2
Arrangement of crops: Sorghum in monocropped **(A)**, Tamani guinea grass in monocropped **(B)**, *Stylosanthes* cv. Bela in monocropped **(C)**, Sorghum intercropped with Tamani guinea grass **(D)**, Sorghum intercropped with *Stylosanthes* cv. Bela **(E)**, *Stylosanthes* cv. Bela intercropped with Tamani guinea grass **(F)** and Sorghum intercropped with Tamani guinea grass and *Stylosanthes* cv. Bela **(G)**.

10 cm in diameter and 40 cm in length. The material was compacted with an iron pendulum, closed with a PVC lid, and sealed with adhesive tape to prevent the entry of air. They were then stored at room temperature and protected from rain and sunlight.

In the material *in natura* (before ensiling), bromatological analyses were performed (Table 2) to determine the dry matter (DM), method 934.01; crude protein (CP), method 920.87; lignin, method 973.18; ether extract (EE) contents, method 920.85, and mineral matter (MM), method 924.05, according to the methodologies described by the AOAC (1990). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured as described by Mertens (2002). Total digestible nutrients (TDN) were calculated using the equation proposed by Chandler (1990). For the determination of *in vitro* dry matter digestibility (IVDM), the technique described by Tilley and Terry (1963) was used, adapted to the artificial rumen, developed by ANKON®, using the

TABLE 1 Proportion of material ensiled from sorghum intercropped with Tamani guinea grass and *Stylosanthes* cv. Bela.

Cultivation system	Proportion of ensiled material (%)		
	Sorghum	Tamani guinea grass	*Stylo santhes*
Sorghum in monocropped	100	0	0
Tamani guinea grass in monocropped	0	100	0
Stylosanthes cv. Bela in monocropped	0	0	100
Sorghum + Tamani guinea grass	68.3	31.7	0
Sorghum + *Stylosanthes* cv. Bela	70.8	0	29.2
Tamani guinea grass + Bela *Stylosanthes*	0	60.3	39.69
Sorghum + Tamani guinea grass + Bela	56.7	24.4	18.9

TABLE 2 Chemical–bromatological composition (g kg^{-1}) of sorghum, Tamani guinea grass, and *Stylosanthes* cv. Bela in monocropped and intercropped before ensiling.

Cultivation system	DM	CP	MM	EE	IVDMD
Sorghum in monocropped	340.66	76.78	45.92	41.65	613.88
Tamani guinea grass in monocropped	276.16	130.80	65.90	21.89	600.72
Stylosanthes cv. Bela in monocropped	285.04	155.35	62.66	22.41	619.72
Sorghum + Tamani guinea grass	315.74	110.91	54.24	28.72	606.35
Sorghum + *Stylosanthes* cv. Bela	318.58	125.68	53.81	29.11	616.65
Tamani guinea grass + Bela *Stylosanthes*	283.55	141.38	66.56	22.82	615.53
Sorghum + Tamani guinea grass + Bela	323.26	127.57	53.56	29.26	613.69
SEM	2.656	2.283	1.450	1.013	5.879
	TDN	NDF	ADF	Lignin	
Sorghum in monocropped	61.90	612.17	348.70	43.03	
Tamani guinea grass in monocropped	53.15	660.26	375.05	27.69	
Stylosanthes cv. Bela in monocropped	56.93	596.16	352.44	26.59	
Sorghum + Tamani guinea grass	58.38	621.06	359.95	35.52	
Sorghum + *Stylosanthes* cv. Bela	56.83	605.38	360.91	33.13	
Tamani guinea grass + Bela *Stylosanthes*	53.46	636.55	361.59	28.50	
Sorghum + Tamani guinea grass + Bela	57.44	621.07	366.30	38.49	
SEM	1.286	3.988	3.617	0.833	

DM, dry matter; CP, crude protein; MM, mineral matter; EE, ether extract; IVDMD, *in vitro* dry matter digestibility; NDF, neutral detergent fiber; ADF, acid detergent fiber; TDN, total digestible nutrients; SEM, standard error of mean.

instrument "Daisy incubator" by Ankom Technology (*in vitro* true digestibility—IVTD). The ruminal collection was carried out in dairy cattle fed on pasture and receiving corn silage twice a day. The liquid was collected through a ruminal cannula, in the morning, 3 h after feeding the animal. The initial project was approved by the Research Ethics Committee (REC) of the Federal Goiano Institute, protocol 53752405-16.

Analysis of the fermentation and bromatological characteristics of the silages

After 50 days of fermentation, the silos were opened, and the upper and lower portions of each silo were discarded. The central portion of the silo was homogenized and placed in a plastic tray. Part of the *in natura* silage was separated for the analysis of fermentation parameters: buffering capacity, pH, and ammoniacal nitrogen in total nitrogen (N-NH_3/NT), following the method described by Bolsen et al. (1992).

The pH and buffering capacity analyses were performed when the silos were opened to avoid changes in the expected values due to heat and humidity. To determine ammoniacal nitrogen, the silage was frozen to inactivate the activity of anaerobic bacteria, thus avoiding the volatilization of nitrogen, and the samples were later thawed for juice extraction (Bolsen et al., 1992). Total dry matter loss and effluent production were determined according to the methodology proposed by Jobim et al. (2007). Organic acids were determined using high-performance liquid chromatography (HPLC), according to the method described by Kung and Shaver (2001) for the determination of lactic, acetic, propionic, and butyric acids.

The other portion of the material (~0.5 kg) was weighed and dried in a forced ventilation oven at 55°C until it reached a constant mass. The samples were, then, ground in a knife mill with a 1 mm sieve and stored in plastic containers. Subsequently, the chemical–bromatological characteristics of the silage were analyzed following the methodology described above for the *in natura* material.

Statistical analysis

The variables were subjected to an analysis of variance using the R program version R-3.1.1 (R Core Team, 2014) and the ExpDes package (Ferreira et al., 2014). Means were compared using Tukey's test at 5% probability.

To understand the cause-and-effect relationship between the variables, Pearson's correlation analyses (low: $r \leq 0.30$; moderate: $0.30 < r \leq 0.70$: and high $r > 0.70$) and trail analysis were performed, considering pH and IVDMD as dependent variables

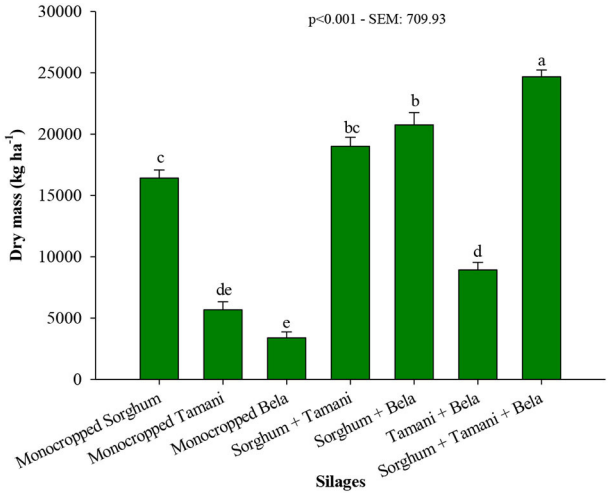

FIGURE 3
Dry mass production of silage material from sorghum, Tamani guinea grass, and *Stylosanthes* cv. Bela in monocropped and intercropping. Means followed by different letters, differ by Tukey's test ($p < 0.05$). The vertical bars represent the standard error of the mean.

due to the importance of these variables for the fermentative profile and nutritional value of silages. To define the causal diagram, multiple linear regression analysis was performed using the "stepwise" procedure with the "backward" option (Coimbra et al., 2005; Charnet et al., 2008). Subsequently, multicollinearity was diagnosed based on the condition factor (ratio between the highest and lowest Eigenvalues), and the number of conditions (NC) < 100 was verified, indicating that multicollinearity is weak and does not constitute a problem for the analysis (Cruz et al., 2014).

The contributions of the direct and indirect effects of the variables were quantified as percentages. Contributions above 50% were considered high direct effect (Botelho et al., 2019; Ribeiro et al., 2019). For the statistical analysis, the "corrplot," "lavaan," and "semPlot" packages of the R development computational program were used.

Results

Dry mass production

The cropping systems had a significant impact ($p < 0.05$) on dry mass production for silage (Figure 3). The highest production was observed in the triple intercropping of sorghum + Tamani guinea grass + Bela, followed by the double intercropping of sorghum + Tamani guinea grass and sorghum + Bela. Monocropped forages exhibited lower silage mass production. The intercropping systems resulted in a 36.20% increase in silage mass in the triple intercrop and a 22.09% increase in the double intercrop compared with monocropped sorghum.

Fermentation characteristics

Fermentation characteristics, including pH, buffering capacity, dry matter (DM), and N-NH_3 content, were influenced by the different silages (Figure 4). Silages derived from Tamani guinea grass and *Stylosanthes* cv. Bela in both the monocropped and intercropping systems exhibited the highest pH values (4.42, 4.30, and 4.28, respectively). In the intercropping systems with sorghum, there was an average reduction of 10.5% in the pH values of the silage for sorghum + Tamani guinea grass + Bela, sorghum + Bela, and sorghum + Tamani guinea grass consortia compared with the Tamani guinea grass and *Stylosanthes* cv. Bela silages in monocropped systems. The sorghum silage displayed the lowest pH value (3.47). Similar results were observed for buffering capacity (Figure 4B), with a reduction of 13.9% for the triple and double intercrop silages with sorghum compared with the monocropped silages of the respective forages.

Sorghum silage exhibited the highest dry matter (DM) content (341.50 g kg^{-1}), followed by sorghum + Tamani guinea grass + Bela silage (326.91 g kg^{-1}; Figure 4C). Silages of sorghum + Tamani guinea grass and sorghum + Bela showed similar results, averaging at 306.64 g kg^{-1} DM. Monocropped silages of the forages displayed the lowest DM contents (279.99 g kg^{-1} for Tamani guinea grass and 282.35 g kg^{-1} for *Stylosanthes* cv. Bela, respectively).

The monocropped silage of *Stylosanthes* cv. Bela exhibited the highest N-NH_3 value (72.74 g kg^{-1} DM), followed by Tamani guinea grass silage in monocropped and Tamani guinea grass + Bela silage, with an average of 63.43 g kg^{-1} DM, which did not differ significantly from the silage of sorghum + Bela and sorghum + Tamani guinea grass + Bela consortia. The silages of sorghum in monocropped and sorghum + Tamani guinea grass had the lowest N-NH_3 values (39.85 and 47.80 g kg^{-1} DM, respectively; Figure 4D).

The silages of *Stylosanthes* cv. Bela in monocropped and Tamani guinea grass + Bela exhibited the highest dry matter losses (26.47 and 27.15 g kg^{-1} DM, respectively), followed by the silages of Tamani guinea grass in monocropped, sorghum + Bela, and sorghum + Tamani guinea grass + Bela, which did not differ significantly from the silage of sorghum + Tamani guinea grass. Sorghum silage showed the lowest DM losses at 13.30 g kg^{-1} DM (Figure 5A).

The highest effluent productions were observed in the silages of Tamani guinea grass (17.89 kg t^{-1} FM), *Stylosanthes* cv. Bela (17.44 kg t^{-1} FM), and *Stylosanthes* cv. Bela + Tamani guinea grass (18.39 kg t^{-1} FM; Figure 5B). Intercropping sorghum with forage crops contributed to an average reduction of 17.16% in effluent production compared with the silages of Tamani guinea grass and *Stylosanthes* cv. Bela in monocropped. Sorghum silage exhibited the lowest effluent production (11.42 kg t^{-1} FM).

Monocropped sorghum silage exhibited the highest lactic acid content (44.43 g kg^{-1} DM) (Figure 5C). In contrast, silages of Tamani guinea grass, monocropped *Stylosanthes*, and *Stylosanthes* cv. Bela + Tamani guinea grass had the lowest values, with an average of 19.31 g kg^{-1} DM. The intercropped systems with sorghum silage showed a 39.36% increase in lactic acid content compared with the silages of grass and legumes in monocropped and the Bela + Tamani guinea grass combination.

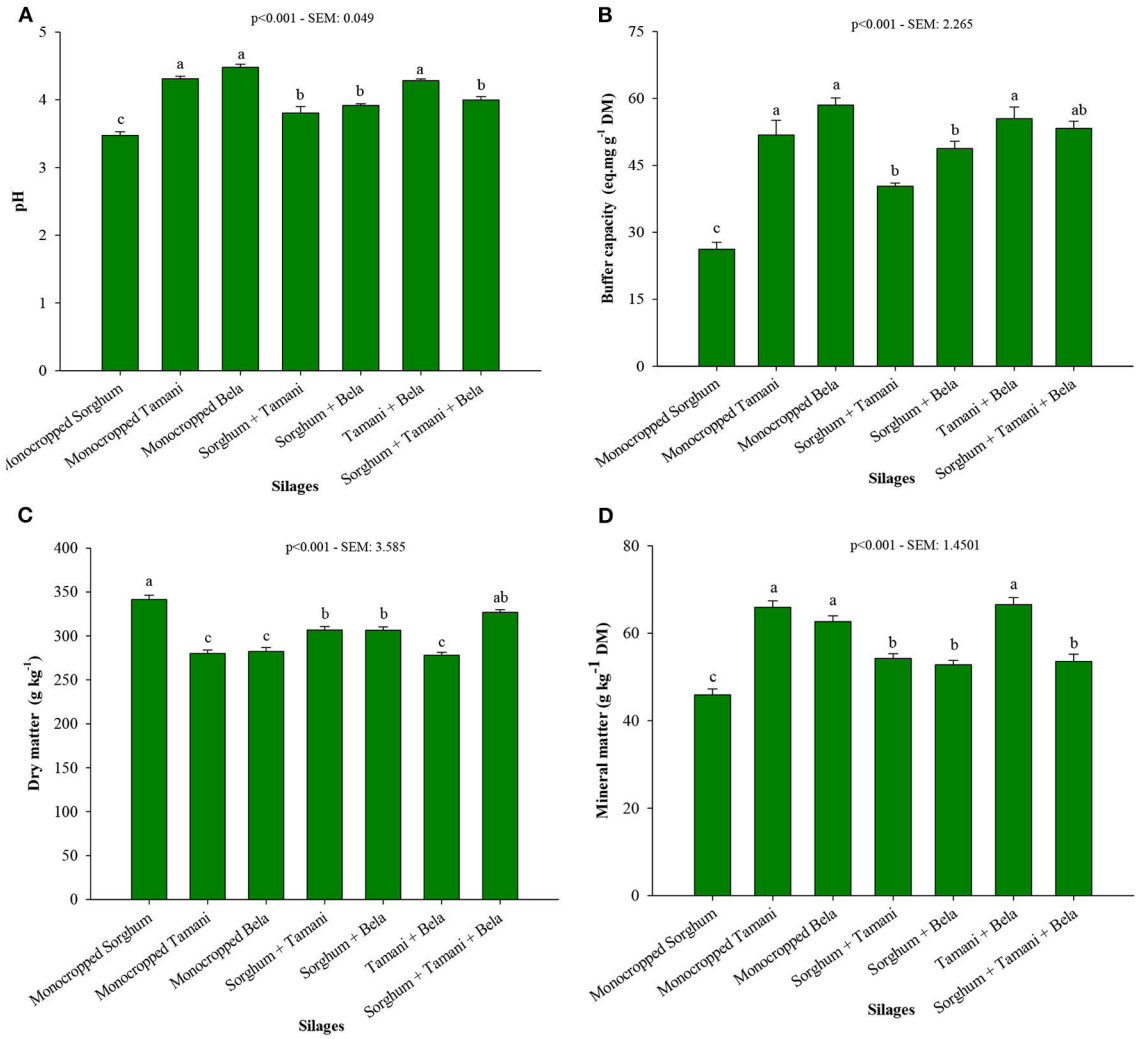

FIGURE 4
pH **(A)**, buffering capacity **(B)**, dry matter **(C)**, and N-NH_3 **(D)** of silage sorghum, Tamani guinea grass and *Stylosanthes* cv. Bela in monocropped and intercropping. Means followed by different letters differ by Tukey's test at 5% probability. Vertical bars represent standard error of mean of each point. SEM, standard error of mean.

Silage of *Stylosanthes* cv. Bela and Tamani guinea grass in monocropped and the Bela + Tamani guinea grass combination exhibited the highest values of acetic acid (Figure 5D), with an average of 8.93 g kg^{-1} DM. On the other hand, silages from intercropped systems containing sorghum in their composition showed a 13.78% reduction in acetic acid. Sorghum silage in monocropped exhibited the lowest values (5.94 g kg^{-1} DM) of acetic acid.

Bromatological characteristics

Bromatological characteristics such as NDF, ADF, lignin, mineral matter, crude protein, ether extract, IVDMD, and TDN were significantly influenced by the different silages (Figures 6, 7). Monocropped sorghum silage exhibited the lowest NDF (542.08 g kg^{-1} DM) and ADF (297.90 g kg^{-1} DM) contents (Figures 6A, B). Conversely, the highest NDF (609.12 g kg^{-1} DM) and ADF (336.20 g kg^{-1} DM) contents were observed in the monocropped Tamani guinea grass silage. Silages from intercropped systems (double and triple-cropped) showed intermediate values.

The monocropped sorghum silage exhibited the highest lignin content (Figure 6C) at 43.02 g kg^{-1} DM. On the other hand, the lowest levels were observed in the silages of *Stylosanthes* cv. Bela (26.59 g kg^{-1} DM) and Tamani guinea grass (27.69 g kg^{-1} DM) in monocropped and double-cropping systems (28.50 g kg^{-1} DM). The intercropping systems effectively reduced lignin concentration, with reductions of 10.52, 17.43, and 23.01% observed in the sorghum + Tamani guinea grass + Bela, sorghum + Tamani guinea grass, and sorghum + Bela silages, respectively, compared with monocropped sorghum silage. Regarding mineral matter (Figure 6D), the highest values were observed in the Tamani guinea grass + Bela (66.55 g kg^{-1}), Tamani guinea grass (65.91 g kg^{-1}), and *Stylosanthes* cv. Bela (62.66 g kg^{-1}) silages in monocropped. The double sorghum + Tamani guinea grass and sorghum + Bela, as well as the triple sorghum + Tamani guinea grass + Bela silages,

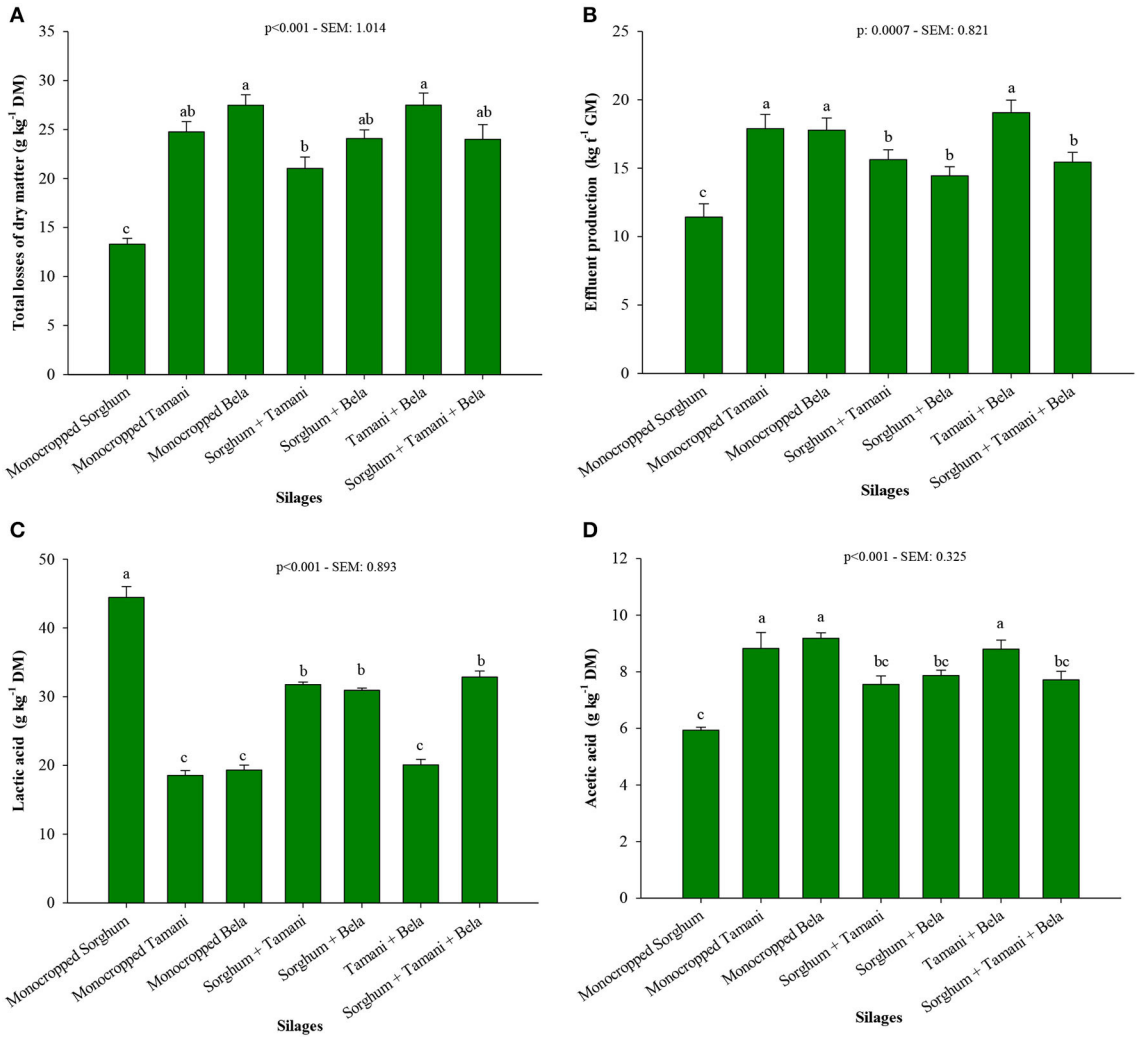

FIGURE 5
Total dry matter losses **(A)**, effluent production **(B)**, lactic acid **(C)**, and acetic acid **(D)** of silage sorghum, Tamani guinea grass, and *Stylosanthes* cv. Bela in monocropped and intercropping. Means followed by different letters differ by Tukey's test at 5% probability. Vertical bars represent standard error of mean of each point. SEM, standard error of mean.

showed a 16.2% reduction in mineral matter compared with the forage silages in monocropped.

Silage of *Stylosanthes* cv. Bela in the monocropped had the highest crude protein content (152.33 g kg^{-1}), followed by *Stylosanthes* cv. Bela + Tamani guinea grass at 140.88 g kg^{-1} DM (Figure 7A). The silages of Tamani guinea grass in the monocropped, sorghum + Tamani guinea + Bela, and sorghum + Bela showed similar results, with an average of 126.43 g kg^{-1} DM. There was 35.32 and 41.88% increase in the crude protein content of the sorghum + Tamani guinea grass and sorghum + Bela double intercrop silages, respectively, and 43.54% for the triple intercrop silage when compared with the silage of sorghum in monocropped, which showed a crude protein content of 71.46 g kg^{-1} DM.

The sorghum silage had the highest ether extract content (40 g kg^{-1} DM; Figure 7B). There was 20.05, 32.4, and 32.4% reduction in the ether extract content for silages of sorghum + Tamani guinea grass + Bela silage, sorghum + Tamani guinea grass, and sorghum + Bela, respectively, compared with silage of sorghum in the monocropped. Silages of *Stylosanthes* cv. Bela + Tamani guinea grass and Tamani guinea grass and *Stylosanthes* cv. Bela in monocropped showed the lowest ether extract values (20.32, 19.89, and 19.81 g kg^{-1} DM, respectively). The content of IVDMD (Figure 7C) was similar among the silages, with an average of 612.36 g kg^{-1} DM.

Silage of sorghum in monocropped showed the highest value of TDN (61.90 g kg^{-1} DM), followed by silage of sorghum + Tamani guinea grass, sorghum + Bela, and sorghum + Tamani guinea grass + Bela, showing increases of 5.8, 3.41, and 4.27%, respectively, for the intercrop silages compared with the silages of the forages in monocropped (Figure 7D).

In the correlation analysis (Figure 8), IVDMD was the only variable that did not show a correlation with any analyzed variable. Moreover, the formation of two groups of variables was observed: Group 1 was composed of NDF, N-NH_3, acetic acid, pH, CP, effluent production, buffering capacity, DM loss, and ADF, whereas Group 2 was composed of DM, lactic acid, EE, lignin, and TDN.

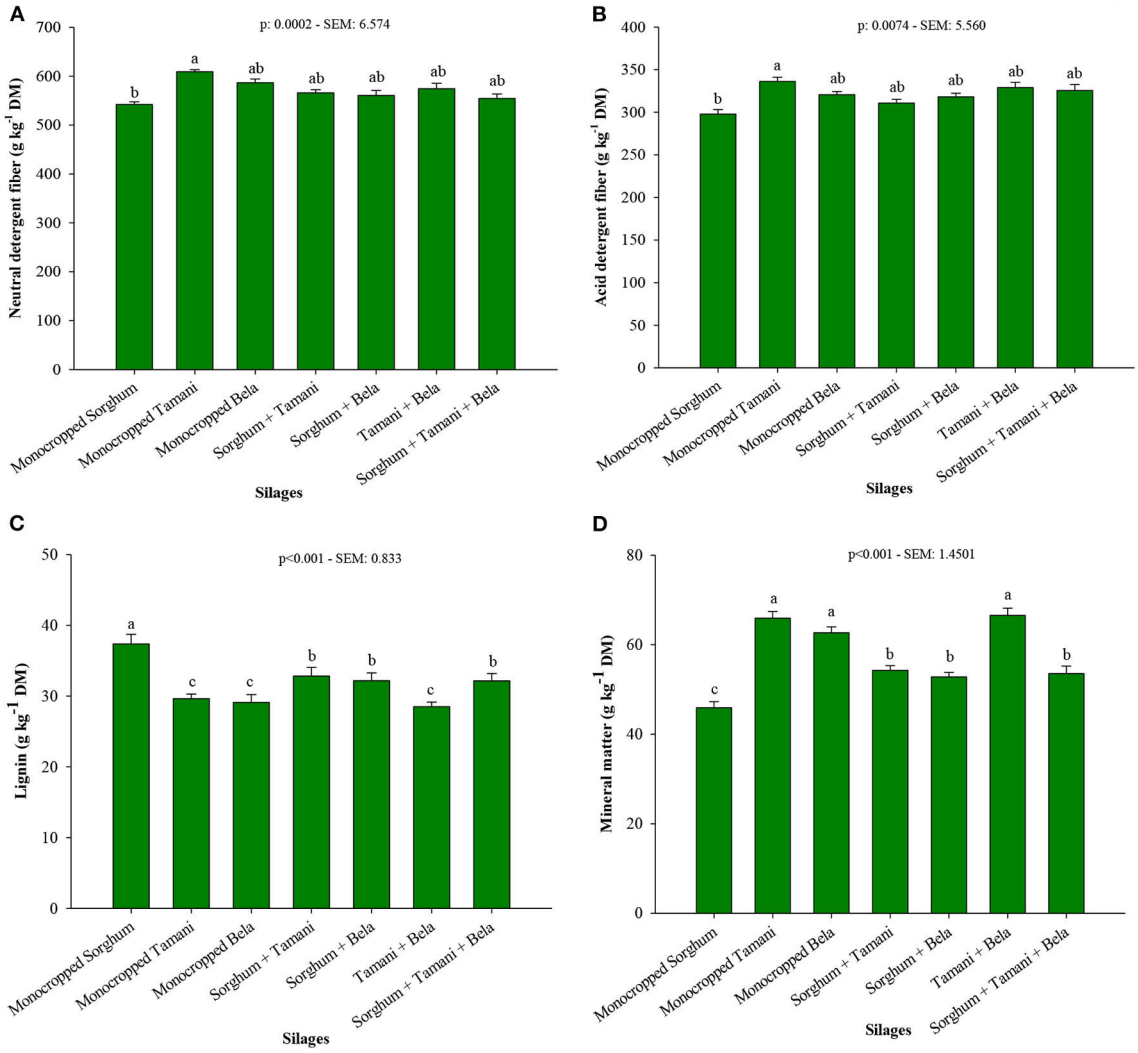

FIGURE 6
Neutral detergent fiber **(A)**, acid detergent fiber **(B)**, lignin **(C)**, and mineral matter **(D)** contents of sorghum, Tamani guinea grass, and *Stylosanthes* cv. Bela silage in monocropped and intercropping. Means followed by different letters differ by Tukey's test at 5% probability. Vertical bars represent standard error of mean of each point. SEM, standard error of mean.

The variables within the same group showed positive correlations and those between distinct groups showed negative correlations.

Through the causal diagram obtained from the multiple regression analysis using the "stepwise" procedure with the "backward" option, it was found that the variables IVDMD, ADF, TDN, CP, buffering capacity, EE, and lactic acid were maintained in the model to explain the pH, with significance for the first four variables and a coefficient of determination of 0.96 (Table 3). The model to explain IVDMD showed a coefficient of determination of 0.72 and was composed of the variables, such as pH, buffering capacity, ADF, NDF, TDN, and CP, all of which were significant in the model.

In the causal diagram of pH (Figure 9), it was verified by the decomposition of correlation that CP, ADF, and IVDMD had direct effects with contributions above 50% of the correlation coefficient and positive values for the first two variables and negative values for IVDMD (Table 3). The other variables showed low direct effects and a high correlation with pH. For the causal diagram of IVDMD (Figure 10), there was a high contribution of direct effects to the correlation for pH, CP, NDF, and ADF, and the correlation was negative for all variables except ADF.

Discussion

Dry mass production

Integrated systems promote diversification of production and food production without the need for clearing new areas, making them a sustainable approach (Costa et al., 2016) that contributes to global food security (Sekaran et al., 2021).

In this study, the triple intercropping system demonstrated the highest dry mass production due to the simultaneous cultivation of three crops, enabling better utilization of the available land and resulting in increased silage yield. These findings highlight the benefits of intercropping annual and tropical forage crops

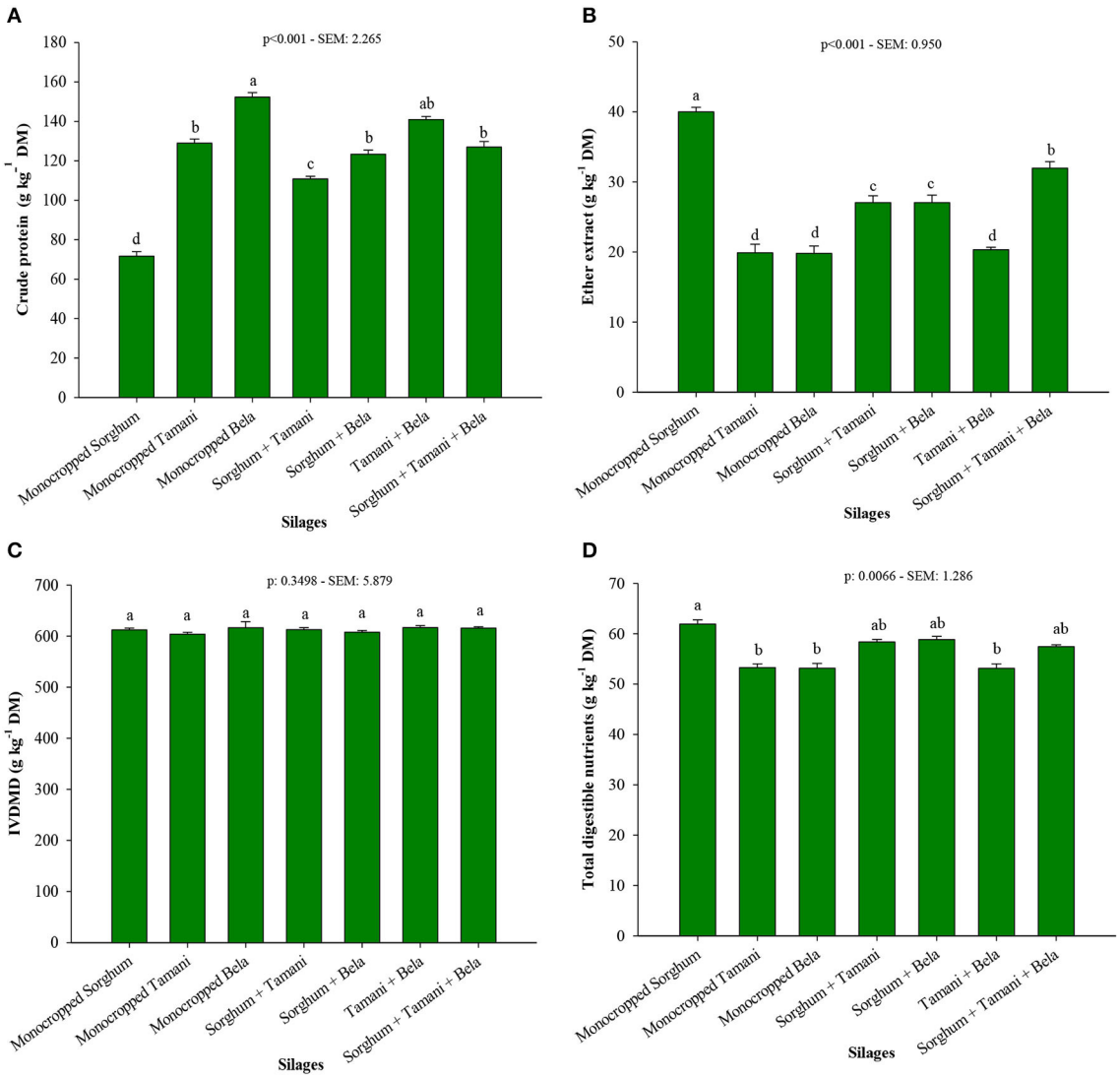

FIGURE 7
Crude protein **(A)**, ether extract **(B)**, IVDMD **(C)**, and TDN **(D)** contents of sorghum, Tamani guinea grass, and *Stylosanthes* cv. Bela silage in monocropped and intercropping. Means followed by different letters differ by Tukey's test at 5% probability. Vertical bars represent standard error of mean of each point. SEM, standard error of mean.

(grasses and legumes) for crop-livestock integration, as it enhances the yield of dry mass for silage compared with monocropped systems. Furthermore, after harvesting crops for silage production, the formed pasture can be utilized for low-cost animal grazing during the off-season when weather conditions are unfavorable for most crops (Santos et al., 2020). Therefore, this cultivation strategy is an effective means to improve land-use efficiency (Costa et al., 2016), particularly in tropical regions (Oliveira et al., 2020).

In addition to these advantages, legumes contribute to integrated systems by reducing the need for nitrogen application in grasses. Through biological nitrogen fixation, legumes increase the availability of this macronutrient in the soil, thereby enhancing the sustainability of cropping systems and reducing the reliance on mineral nitrogen fertilizers in the system (Bolson et al., 2022).

Fermentation characteristics

The findings of this study are highly significant in evaluating the fermentation characteristics and nutritional value of silage produced in integrated systems. Legumes and grasses possess a high buffering capacity, low soluble carbohydrate concentration, and low dry matter content, which makes it challenging to lower the pH during ensiling (Hawu et al., 2022). This explains the observed results for Tamani guinea grass and *Stylosanthes* cv. Bela silages in monocropped and combined (Bela + Tamani guinea grass) systems, where the pH stabilized above 4.2 (Figure 4A). Such pH values promote the growth of undesirable microorganisms during silage fermentation, thereby compromising the final quality (Kung et al., 2018).

On the other hand, the silages from intercropped systems with sorghum were effective in achieving pH reduction, displaying

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values within the recommended range of 3.7–4.2 for good-quality silage classification, as suggested by Mcdonald et al. (1991). Grasses such as sorghum and maize exhibit good stability due to their adequate levels of soluble carbohydrates and lactic acid, which contribute to pH reduction when ensiled with appropriate levels of dry matter (Rodrigues et al., 2020).

The higher buffering capacity of *Stylosanthes* cv. Bela and Tamani guinea grass silages in monocropped and combined systems (*Stylosanthes* cv. Bela + Tamani guinea grass; Figure 4B) can be attributed to the inherent buffering capacity of forage grasses and legumes compared with sorghum silage in

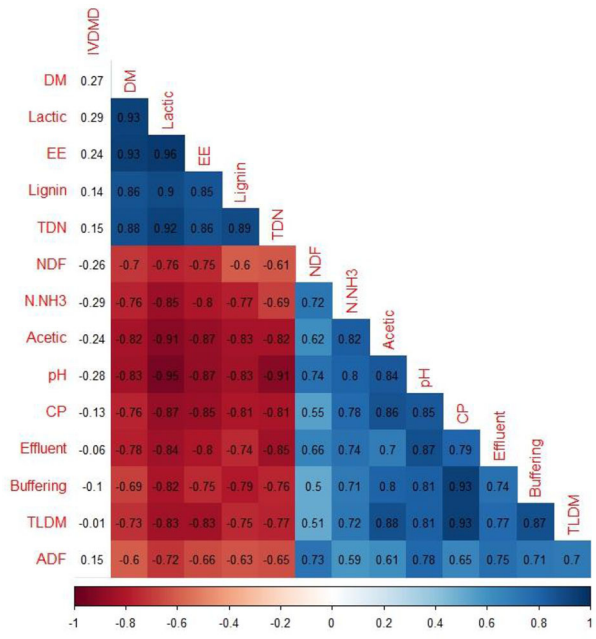

FIGURE 8
Pearson's correlation between the variables of the fermentation profile and nutritional value of silages of sorghum, Tamani guinea grass, and *Stylosanthes* cv. Bela in monocropped and intercropping systems.

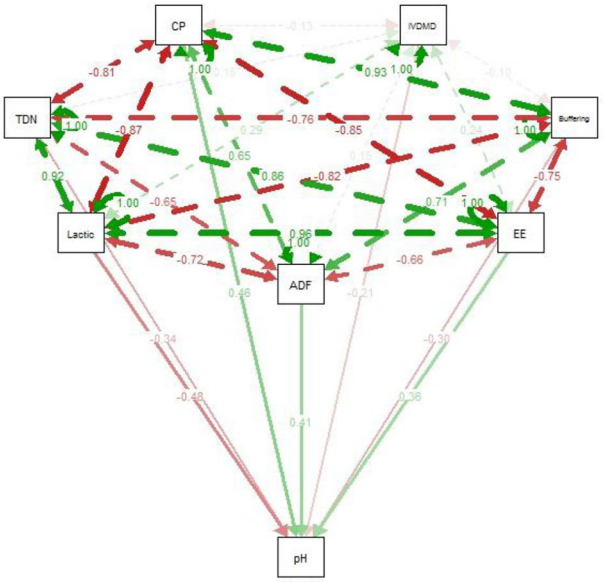

FIGURE 9
Causal diagram showing the correlations between the independent variables and the cause–effect relationships with pH.

TABLE 3 Direct and indirect effects, correlation, and coefficient of determination of the causal models.

Variables dependent	Variables independent	Effect direct		Effect indirect		Correlation	R^2
		Coefficient	%	Coefficient	%		
pH	IVDMD	−0.212*	75.71	−0.0680	24.29	−0.28	0.97
	Buffering	−0.303	21.40	1.1130	78.60	0.81	
	EE	0.365*	22.81	−1.2350	77.19	−0.87	
	ADF	0.408*	52.31	0.3720	47.69	0.78	
	Lactic	−0.475	50.00	−0.4750	50.00	−0.95	
	TDN	−0.340*	37.36	−0.5700	62.64	−0.91	
	CP	0.461*	54.24	0.3890	45.76	0.85	
IVDMD	pH	−1.759*	54.32	1.4790	45.68	−0.28	0.72
	Buffering	−0.788*	53.39	0.6880	46.61	−0.10	
	CP	0.858*	46.48	−0.9880	53.52	−0.13	
	NDF	−0.553*	65.37	0.2930	34.63	−0.26	
	ADF	1.459*	52.71	−1.3090	47.29	0.15	
	TDN	−0.732*	45.35	0.8820	54.65	0.15	

%, percentage of contribution; *significance at 5%.
IVDMD, *in vitro* dry matter digestibility; buffering, buffering capacity; EE, ether extract; ADF, acid detergent fiber; lactic, lactic acid; TDN, total digestible nutrients; CP, crude protein; NDF, neutral detergent fiber.

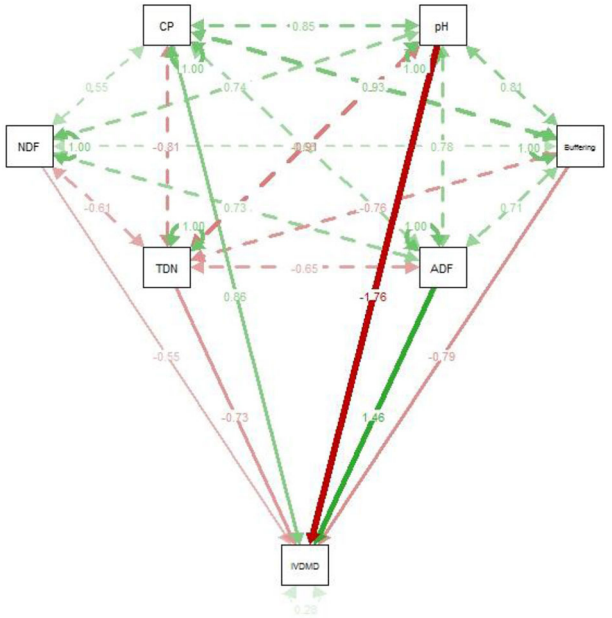

FIGURE 10
Causal diagram showing the correlations between the independent variables and the cause–effect relationships with IVDMD.

monocropped systems (Oliveira et al., 2020). This increased buffering capacity makes them more susceptible to proteolysis during the fermentation process, impeding pH reduction and resulting in inadequate forage preservation (Gomes et al., 2021). The intercropping of sorghum with forage crops contributed to a reduction in buffering capacity due to the higher proportion of sorghum in the ensiled mass (Table 1). Similar findings were reported by Oliveira et al. (2020), who evaluated silage production of sorghum intercropped with Paiaguas palisadegrass and observed that a higher proportion of sorghum effectively reduced the buffering capacity of monocropped grass silage and contributed to pH reduction. Studies by Carvalho et al. (2016) and Aloba et al. (2022) also observed improvements in the fermentation characteristics of monocropped legume silage with the inclusion of sorghum.

The dry matter (DM) content of the ensiled material significantly impacts the final silage quality as it affects material compaction and the fermentation process (Teixeira et al., 2021). Recommended DM values for good-quality silage classification range from 300 to 350 g kg^{-1} DM (Mcdonald et al., 1991), indicating that the fermentation process of silages from intercropping systems with sorghum occurred adequately. However, the silages of monocropped forage crops exhibited DM contents below 300 g kg^{-1} (Figure 4C), leading to increased effluent production and higher activity of *Clostridium* bacteria, compromising silage quality (Muck and Shinners, 2001). Additionally, as previously mentioned, tropical forage silages in monocropped systems without additives can display inadequate fermentation characteristics (Oliveira et al., 2020; Hawu et al., 2022). These results underscore the importance of silage production in integrated systems, as intercropping can reduce inadequate fermentation characteristics and increase the DM concentration of grass and legume silages, ensuring proper preservation of the ensiled material.

$N\text{-}NH_3$ is a parameter used to assess the quality of the silage fermentation process because an increase in its production can neutralize the acids necessary for the proper fermentation of the ensiled material (Veriato et al., 2018). Silages with adequate fermentation, as defined by Kung et al. (2018), should have values below 100 g kg^{-1} of $N\text{-}NH_3$, similar to the results observed in this study. Silages from intercropping systems with sorghum were more effective in reducing $N\text{-}NH_3$ compared with silages of grass and legumes in monocropped and combination systems. Sorghum contains a sufficient amount of soluble carbohydrates and represents the largest proportion in intercropped silages (Table 1), enabling proper fermentation and lower nutrient loss, which aligns with the findings of Ni et al. (2018) and Li et al. (2022).

Higher DM losses were observed in *Stylosanthes* cv. Bela in monocropped and Bela + Tamani guinea grass systems (Figure 4A) due to the higher moisture content and lower DM content of legumes and grasses at the time of cutting for ensiling compared with monocropped sorghum (Table 2). Tropical forages typically have higher moisture content than annual crops at harvest (Bernardes et al., 2018). Additionally, high water activity and low concentrations of soluble carbohydrates can contribute to DM losses during secondary fermentation (Borreani et al., 2018).

Silage production should be optimized to achieve favorable fermentation patterns and preserve the nutritive value of the ensiled material, ultimately providing high-quality feed. One key management strategy to attain this objective is to minimize forage losses (Köhler et al., 2019). In the current study, silages from intercropping systems with sorghum demonstrated the potential in reducing total DM losses in tropical forage silages due to the higher proportion of sorghum (Table 1) incorporated in these silages, along with an appropriate DM content (340.66 g kg^{-1}) at harvest.

Regarding effluent production, monocropped Tamani guinea grass and *Stylosanthes* cv. Bela silages, as well as the intercropping of Bela + Tamani guinea grass, exhibited the highest effluent production (Figure 5B) due to the high moisture content typically present in grasses and legumes at harvest, as previously mentioned. High effluent production leads to nutrient losses through leaching, which can result in nutritional degradation of the feed and potential environmental contamination (Araújo et al., 2020).

The intercropping systems with sorghum exhibited a significant reduction in effluent production, which was directly influenced by the higher proportion of sorghum present in these silages (Table 1). The high DM content of sorghum efficiently absorbed excess moisture from Tamani guinea grass and *Stylosanthes* cv. Bela, highlighting the close relationship between effluent production and the DM content of the ensiled material (Paludo et al., 2020). These results underscore the importance of silage production in intercropping systems for enhancing the fermentation process of tropical forage silages in monocropped systems and ensuring proper conservation of the ensiled material.

Silage production is a microbial fermentation process in which the lactic acid bacterial community plays a crucial role. These bacteria quickly establish dominance by consuming nutrients in the silage, creating a low-pH environment that inhibits the growth of undesirable protease and other bacterial communities, thus

facilitating an adequate fermentation process (Wang et al., 2021). In this context, silages from intercropping systems with sorghum were more effective in increasing lactic acid values compared with silages of tropical forages in monocropped and combination systems, as shown in Figure 5C. Similar findings were reported by Meng et al. (2022), who evaluated the effects of different proportions of soybean and maize in an intercropping system and observed that intercropping enhanced the microbial community, particularly *Lactobacillus* and *Weissella* (co-producers of lactic acid), thereby improving fermentation and silage quality.

The silages of monocropped forages and their combination exhibited higher acetic acid values (Figure 5D). The intercropping systems resulted in a 13.78% reduction in acetic acid compared with the silages from monocropped and combination forages. However, the observed acetic acid values in all produced silages did not exceed 20 g kg^{-1} DM, which is considered suitable for classifying silage quality and ensuring proper preservation of the ensiled material, as reported by Kung et al. (2018). Therefore, the production of acetic acid did not negatively affect the stability of the silages, and the predominant production of lactic acid ensured the adequate preservation of the ensiled material (Oliveira et al., 2020).

Bromatological characteristics

Regarding the fibrous fractions (Figure 6), the NDF content represents the hemicellulose, cellulose, and lignin fractions, while the ADF content represents the cellulose and lignin fractions. These two fractions are important indicators of forage quality, with lower levels indicating better quality forage (Umesh et al., 2022), as they are negatively correlated with ruminant intake and digestibility (Tang et al., 2018). NDF values above 600 g $kg^{-}1$ DM can reduce dry matter consumption due to rumen filling (Paludo et al., 2020). For ADF, values above 400 g kg^{-1} DM reduce fiber digestibility due to the unavailability of degradable structural carbohydrates, and the lignin content in the material hinders the adherence of rumen microbiota and the subsequent enzymatic hydrolysis of components such as cellulose and hemicellulose (Van Soest, 1994). In the present study, the ADF and NDF contents of all silages were lower than those previously reported.

The silages from the intercropping systems demonstrated the potential to reduce the fibrous fractions of the silages due to the lower NDF and ADF levels in sorghum (Table 2), resulting in the production of high-quality feed. Supporting these results, Oliveira et al. (2020) also observed a dilution of NDF and ADF contents when sorghum was intercropped with guava grass and when a higher proportion of sorghum was included in the silage. However, monocropped sorghum silage had the highest lignin content (Figure 6C). This can be attributed to the higher proportion of stalks in the crop, which is responsible for higher lignin accumulation (Oliveira et al., 2021).

The silage of Tamani guinea grass and *Stylosanthes* cv. Bela in monocropped and combination showed the lowest lignin contents due to the higher leaf blade-to-stalk ratio of Tamani guinea grass (Muniz et al., 2022), which contributed to the low accumulation of lignin in the forage and allowed for better digestibility (Paludo et al., 2020). Additionally, legumes such as *Stylosanthes* cv. Bela, which have herbaceous growth habit characterized by soft stems and a high number of leaves, have reduced lignin content (Castro-Montoya and Dickhoefer, 2020).

Thus, in the present study, it was observed that the double consortia of sorghum + Bela and sorghum + Tamani guinea grass, as well as the triple intercropping of sorghum + Tamani guinea grass + Bela, was efficient in diluting the lignin content of monocropped sorghum silage when ensiled. This emphasizes the importance of intercropping in the production of high-quality silage.

Silage production using integrated production systems is of fundamental importance for improving the nutritional characteristics of traditional maize and sorghum silage. The intercropping of cereals, grasses, and/or legumes primarily aims to increase the crude protein content (Ligoski et al., 2020) of the ensiled mass (Oliveira et al., 2020), as well as to enhance nitrogen use efficiency through biological fixation and minimize the use of mineral fertilizers (N), thereby reducing potential environmental impacts (Zhang et al., 2022) and promoting greater sustainability in food production.

The higher mineral matter content observed in the silages of Tamani guinea grass and Bela in monocropped and mixed systems is attributed to their lower DM content, higher pH, buffering capacity, and N-NH_3 levels (Table 2). These factors contribute to an inadequate fermentation process with DM losses during fermentation, resulting in increased mineral matter content (Oliveira et al., 2020). In contrast, the higher proportion of sorghum in the intercropped silages (Table 1) leads to a reduction in mineral matter, as cereals generally have a low mineral matter content (Paula et al., 2016).

In the present study, we observed that the double consortia of sorghum + Tamani guinea grass, sorghum + Bela, and Bela + Tamani guinea grass, as well as the triple intercropping (sorghum + Tamani guinea grass + Bela), were more effective in increasing the crude protein content of the silage compared with sorghum silage in monocropped systems. Intercrops that included legumes had higher crude protein content than those with Tamani guinea grass (Figure 7A). The inclusion of Tamani guinea grass and *Stylosanthes* cv. Bela in the silage resulted in an increase in crude protein levels, with respective values of 130.80 and 155.35 g kg^{-1} DM. Thus, silages from integrated systems serve as not only an efficient alternative to increase silage mass production per unit area (Souza et al., 2019) but also a cost-effective approach as they maximize nutrient production per unit area in a sustainable manner (Umesh et al., 2022).

In addition to the nutritional benefits for animals, intercropping systems of grasses and legumes offer agronomic advantages such as reducing insects and pests, producing biomass for no-till farming systems, and lowering fertilizer costs through nutrient cycling, particularly with the presence of legumes that can supply adequate amount of nutrients to the soil-plant system (Ligoski et al., 2020; Bourscheidt et al., 2023). These systems also contribute to pasture recovery (Santos et al., 2020), providing sufficient high-quality feed for animals during the dry season. After forage regrowth, the established pasture can be utilized by animals (Oliveira et al., 2020), facilitating the sustainable intensification of the production system (Herrera et al., 2023).

The higher ether extract content observed in monocropped sorghum silage is attributed to the higher fat content in sorghum grains (Oliveira et al., 2021). These findings further support the observations of Bueno et al. (2020), who reported that well-preserved silages exhibit similar ether extract levels to those found in the original ensiled material. The IVDMD contents were similar among the studied silages, with an average value of 612.36 g kg^{-1}. IVDMD can be used to assess the nutritional value and animal feed intake (Xie et al., 2022) and is considered one of the main determinants of forage quality (Daniel et al., 2019).

Silages from the intercropping systems demonstrated efficiency in increasing ether extract and TDN contents compared with silages from monocropped and combined forages, primarily due to the higher proportion of sorghum present in the material (Table 1). The increase in TDN content associated with sorghum in the intercrop was also observed by Ribeiro et al. (2017). It is worth noting that TDN content plays a crucial role in ruminant production as it, along with protein, can be a limiting factor (Oliveira et al., 2020).

Positive correlations between variables within the same group indicate a direct relationship, meaning that an increase in one variable leads to an increase in another, either directly or indirectly through other variables. On the other hand, negative correlations between variables from different groups indicate an inverse relationship, where an increase in one variable causes a reduction in the other and vice versa. Therefore, these results contribute to a better understanding of the relationship between the fermentation profile and the nutritional value of silage (Htet et al., 2021).

The regression models obtained for pH and IVDMD showed high coefficients of determination ($R^2 > 0.70$), indicating that the independent variables included in the models are significant in determining pH and IVDMD. It is recommended to use regression models for bromatological variables when R^2 is higher than 0.70 (Paludo et al., 2020; Rodrigues et al., 2020). Previous studies in the literature have also reported trail analyses with R^2 values below 0.7, providing support for conducting this analysis (Crevelari et al., 2020; Ligoski et al., 2020).

The causal diagram of pH revealed high direct effects on CP, ADF, and IVDMD contents, suggesting that these variables are less influenced indirectly by other variables in their correlations with pH. However, despite having the highest percentage of direct effects, IVDMD showed a non-significant correlation with pH. Therefore, the variables with the greatest impact on pH were CP and ADF contents. On the other hand, CP, EE, lactic acid, and TDN exhibited low direct effects but had high correlations with pH, indicating that their effects occur indirectly through other variables in the model. Consequently, their inclusion is of limited importance in determining the effects of independent variables on pH.

The high direct effects of pH, CP, NDF, and ADF, along with their low correlation with IVDMD, indicate that these independent variables can provide significant benefits for estimation purposes when used in analyses together with other independent variables. However, they should not be relied upon solely. On the other hand, CP and TDN exhibited weak direct effects and correlations with IVDMD, suggesting that these variables have limited utility in the causal model involving IVDMD. These findings imply that the bromatological variables studied have minimal interference with IVDMD. Future research should explore other bromatological variables or different crops to determine how IVDMD can be improved, as it is a crucial variable in animal production.

Thus, our study highlights the importance of integrated systems, particularly the triple intercropping of annual and tropical forage crops (grasses and legumes), as a promising technique for silage production and the restoration of degraded areas or establishment of new pastures (Santos et al., 2020). Furthermore, this system ensures sustainable food production (Simões et al., 2023), mitigates greenhouse gas emissions (Eugène et al., 2021), promotes greater soil carbon sequestration, diversifies production (Bourscheidt et al., 2023), and reduces costs, particularly those associated with mineral nitrogen fertilizer inputs in the system (Bolson et al., 2022).

Conclusion

In conclusion, our study supports the use of sorghum intercropped with tropical forages in integrated silage production systems. This approach enhances land-use efficiency by increasing the production of ensiled mass per area and providing pasture after crop harvest. The intercropping improves fermentation characteristics and increases EE and TDN contents in monocropped forage silages. Additionally, the inclusion of tropical forages in sorghum silage reduces the need for protein salts in ruminant feed, leading to cost savings. Overall, the triple intercropping of sorghum + Tamani guinea grass + Bela is recommended for the production of high-quality silage and annual and tropical forage crops.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

LP, KC, and LS wrote the manuscript. LP, LS, JC, and JS collected data in the field and processed the data. KC, AC, EH, and ES conceived and designed the experiments. All authors contributed to the revision of the manuscript and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Silage preparation and sustainable livestock production of natural woody plant

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As the global population increases and the economy grows rapidly, the demand for livestock products such as meat, egg and milk continue to increase. The shortage of feed in livestock production is a worldwide problem restricting the development of the animal industry. Natural woody plants are widely distributed and have a huge biomass yield. The fresh leaves and branches of some woody plants are rich in nutrients such as proteins, amino acids, vitamins and minerals and can be used to produce storage feed such as silage for livestock. Therefore, the development and utilization of natural woody plants for clean fermented feed is important for the sustainable production of livestock product. This paper presents a comprehensive review of the research progress, current status and development prospects of forageable natural woody plant feed resources. The nutritional composition and uses of natural woody plants, the main factors affecting the fermentation of woody plant silage and the interaction mechanism between microbial co-occurrence network and secondary metabolite are reviewed. Various preparation technologies for clean fermentation of woody plant silage were summarized comprehensively, which provided a sustainable production mode for improving the production efficiency of livestock and producing high-quality livestock product. Therefore, woody plants play an increasingly important role as a potential natural feed resource in alleviating feed shortage and promoting sustainable development of livestock product.

KEYWORDS

animal product, natural biomass resource, silage fermentation, sustainable livestock production, woody plant

Highlights

- A review of recent advances in the renewable use of natural woody plant.
- Natural woody plant produces clean feed to alleviate feed shortage.
- Fermented feed of woody plant produces livestock product with added value.
- Woody plant contributes to the sustainable production of livestock product.

1 Introduction

With the rapid development of the global economy, per capita consumption levels and demand for livestock products such as meat, eggs and milk are increasing (Komarek et al., 2021). Along with the development of livestock farming, the demand for quality forage is also increasing (Keeling et al., 2019). Currently, the main factor affecting livestock production is the insufficient supply of livestock forage, which in many countries is derived from feed crops, grasses, crop by-products and cereals (Cai et al., 2020a; Cai et al., 2021). With increasing global population and decreasing per capita arable land, traditional forage production methods cannot meet the demand for livestock feeding, leading to feed shortages and affecting the sustainable production of livestock products worldwide (Du et al., 2021b). Therefore, there is an urgent need to develop new feed resources, such as nutrient-rich natural woody plant resources, to meet the challenges posed by the rapid development of the livestock industry (Owen-Smith and Cooper, 1987; Vandermeulen et al., 2017).

The major woody plant available for feed worldwide are mulberry [*Morus alba* (L.)], moringa [*Moringa oleifera* (L.)], gliricidia [*Gliricidia sepium* (Jacq.)], leucaena [*Kunth ex Walp. leucocephala* (L.) de Wit], and paper mulberry [*Broussonetia papyrifera* (L.)]. These woody plants are deciduous trees or shrubs that are highly adaptable, widely distributed, drought tolerant and thrive in infertile soil, and can grow in a wide range of soil pH conditions, with high growth rates, high biomass yields, and low planting production costs (Anwar et al., 2007; He et al., 2019; Du et al., 2021a; Du et al., 2022c). In addition, the fresh branches and leaves of woody plants have a high crude protein (CP) content and are rich in various nutrients, such as bioactive components, amino acids, vitamins and macro minerals (Phesatcha and Wanapat, 2016; Oliveira et al., 2018; Wen et al., 2018; He et al., 2020; Du et al., 2021b).

Fresh branches and leaves of woody plants usually have a high moisture content, and the use of hay production methods not only increases the lignification of woody plant branches and leaves but also tends to cause leaf abscission during the drying process, resulting in a significant loss of nutrients (Du et al., 2021a). This suggests that hay processing is not suitable for the preparation of woody plant feeds. Silage, which is a fermented feed prepared from fresh forage for long-term storage, is considered a key technology for clean feed production of woody plant (Du et al., 2022a). In order to effectively utilize natural biomass resources, such as woody plant resources, to solve the problem of feed shortage and to improve the production capacity of livestock, this paper provides a comprehensive overview of the chemical composition and uses of feedable natural woody plants, the main factors affecting silage fermentation, the interactions between microbial co-occurrence networks and secondary metabolites, the regulatory mechanisms of silage fermentation, and the production of high-quality livestock products, with a view to providing important research information and technological support for the realization of the sustainable development of the animal husbandry.

2 Distribution and multifunctional utilization of woody plant

Forageable woody plants are characterized by their diversity and versatility, high biomass and rich nutrient content, making them suitable for feeding ruminants (Figure 1). Mulberry belongs to the family *Moraceae* and is native to north-central China. It is widely cultivated in China, Korea, Japan, Mongolia, India, Vietnam, Russia and other central Asian countries, as well as some European countries (Madhav and Carolyn, 2012). Moringa belongs to the family *Moraceae* and is found in the tropics of Africa and Asia, and is cultivated in Guizhou, Guangdong and Taiwan in China (Çelekli et al., 2019; Pagano et al., 2020). Gliricidia belongs to the family *Leguminosae* and is native to the tropical dry forests of Mexico and central America. In addition to its native range, it grows in many tropical and subtropical regions, including the Caribbean, northern parts of south America, central Africa, parts of India and southeast Asia, northern and central America, and central Africa (De Carvalho et al., 2017; Oliveira et al., 2018). Leucaena also belongs to the family *Leguminosae*, is native to southern Mexico and northern central America (Belize and Guatemala), and is cultivated in tropical regions (Rengsirikul et al., 2011). The paper mulberry belongs to the family *Moraceae* and is native to southwest China, but is now widely distributed throughout China, other Asian countries, mainland Europe and the Pacific islands (Peñailillo et al., 2016).

Woody plants have multiple uses (Figure 2), mainly as edible and medicinal products, but also as feed, compost, bioenergy and fiber products (Jitjaicham and Kusuktham, 2016; Wen et al., 2018; Hao et al., 2020; Ajayo et al., 2022). Because the fresh branches and leaves of woody plants are rich in nutrients and functional components, they can be used as a potential feed resource with additional value (Du et al., 2021b). In addition, woody plants provide economic benefits to farmers by reducing feed costs and increasing the productivity of livestock (Franzel et al., 2014). As shown in Table 1, all woody plants can be used as medicinal plants and as a source of feed for cattle, sheep, pigs and poultry. Some of them are used as raw materials for food, rabbit feed, bioenergy, biogas and green manure. In addition, paper mulberry and mulberry are used as a raw material for paper and fibre products, and mulberry leaves are an important feed for silkworm.

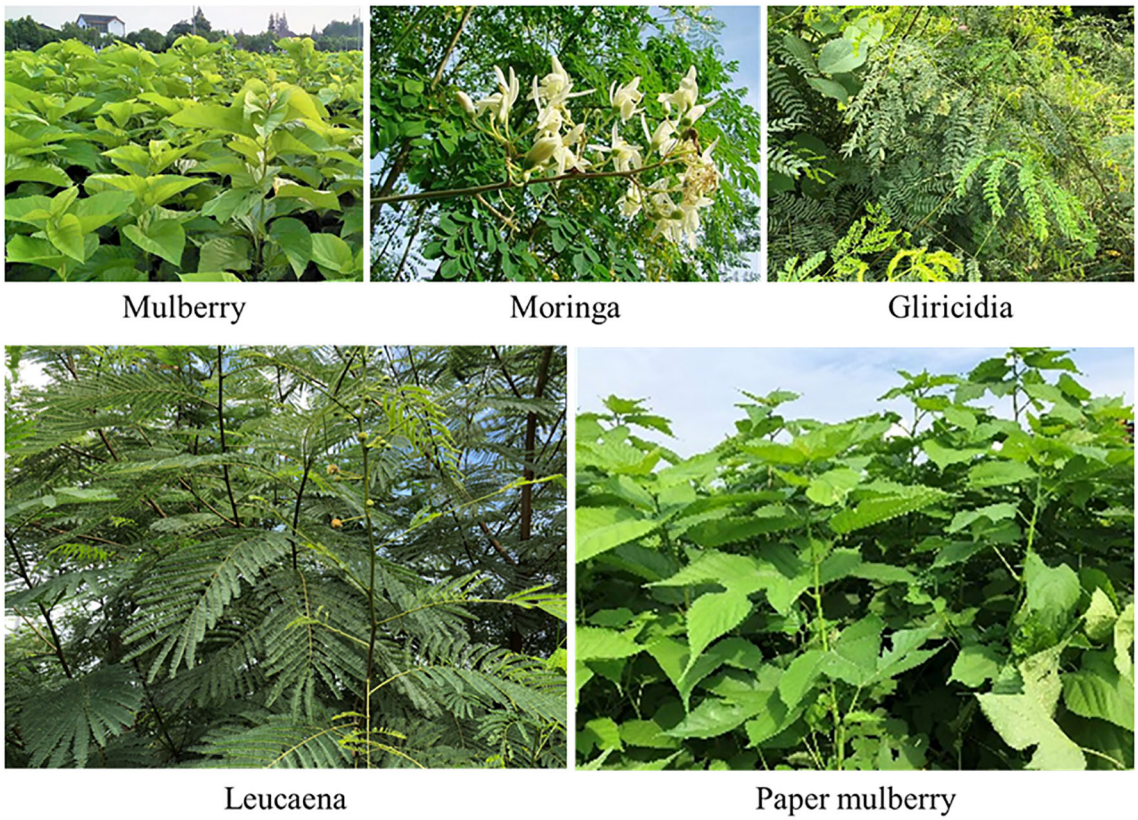

FIGURE 1
Woody plants that can be used as forage.

Some woody plants are rich in biofunctional components, such as polyphenols, carotenoids, alkaloids, terpenoids and sulphur-containing compounds, which have potent effects, including enhanced free radical scavenging and powerful reducing abilities. They also have anti-oxidant, anti-cancer, anti-inflammatory, hepatoprotective, hypotensive, anti-diabetic and hypolipidemic properties, and constitute therefore potential drugs for treating various diseases in humans and animals (Gopalakrishnan et al., 2016; Abd Rani et al., 2018). Gliricidia contains small amounts of coumarin, which can be used as a spice, but is generally not suitable for consumption (Lim, 2014). The leaves of woody plants, such as paper mulberry, mulberry, gliricidia and moringa, are rich in amino acids, fatty acids, vitamin E and beta-carotene, and their calcium and magnesium concentrations are higher than those of many vegetables. Therefore, they are used in many developing countries as leafy greens that provide plant-based protein and play a role in reducing hunger and combating malnutrition (Pakade et al., 2013). The leaves and seeds of woody plants can be eaten raw, cooked or added to food in the form of a dried powder. They are an ingredient in hot pots, teas, edible spices, beverages and yoghurt, and are therefore a popular health food in some Asian countries (Leone et al., 2015). The fruits of some woody plants contain large amounts

FIGURE 2
Multifunctional use of paper mulberry.

TABLE 1 Major use of woody plant.

Item	Feed use							Other use					
	Cow	Sheep	Goat	Pig	Poultry	Rabbit	Silkworm	Edible	Medicinal	Paper making	Bioenergy	Biogas and organic manure	Fabrics
Paper mulberry	○	○	○	○	○			○	○	○	○		○
Mulberry	○	○	○	○	○	○	○	○	○	○	○	○	○
Gliricidia	○	○	○	○	○	○			○		○	○	
Leucaena	○	○	○	○	○	○		○	○		○	○	
Moringa	○	○	○	○	○	○		○	○		○		

Data cited from the following references:
Paper mulberry from Singh et al., 1997; Cheng et al., 2001; Jitjaicham and Kusuktham, 2016; Hong et al., 2017; Si et al., 2018; Chen et al., 2020; Hao et al., 2020; Wang et al., 2020; Dong et al., 2021; Sheng et al., 2021; Tang et al., 2021; Xiong et al., 2021; Ajayo et al., 2022; Ma et al., 2022; Wu et al., 2022.
Mulberry from Janardhan et al., 2008; Kandylis et al., 2009; Dong et al., 2017; Kong et al., 2019; Amna et al., 2021; Ding et al., 2021; Liu et al., 2022; Maqsood et al., 2022; Martínez et al., 2005; Takasaki et al., 2011; Muck et al., 2018; Wang et al., 2018; Song et al., 2021; Tian et al., 2022.
Gliricidia from Nallathambi Gunaseelan, 1988; Mpairwe et al., 1998; Srinivasulu et al., 1999; Phimphachanhvongsod and Ledin, 2002; Kagya-Agyemang et al., 2007; Hariyadi and Hartono, 2018; Olugbenga et al., 2018; Isabel et al., 2019; Aulanni'Am et al., 2021; Marsetyo et al., 2021; Zhang et al., 2022.
Leucaena from Hussain et al., 1991; Maasdorp et al., 1999; Dana et al., 2000; Diaz et al., 2007; Giang et al., 2016; Harun et al., 2017; Chigurupati et al., 2020; Soedarjo and Borthakur, 1996; Santos-Ricalde et al., 2017; Tudsri et al., 2019; Wang et al., 2021.
Moringa from Salem and Makkar, 2008; Mukumbo et al., 2014; Santos-Ricalde et al., 2017; Kholif et al., 2018; Fulvia et al., 2019; Hossam et al., 2019; Yang et al., 2020; Grosshagauer et al., 2021; Wu et al., 2021.

of soluble sugars (Han et al., 2016), which are usually shed at maturity and decay on the ground, resulting in the loss of these resources. Using the fruits of these woody plants as a feedstock, biotechnology has been successfully developed to produce ethanol from their free sugars (Ajayo et al., 2022). The tree trunks of the paper mulberry and mulberry are rich in various chemical constituents, including cellulose, hemicellulose, lignin, waxes and gums, which are widely used in the production of paper and fibre products (Jitjaicham and Kusuktham, 2016). In addition, the leaves of mulberry, gliricidia and leucaena can be used as raw materials for biogas production and green leaf fertilizer (Tambone et al., 2020).

Woody plants are 90–99% organic matter (OM) and consist of 17–27% CP, 3–5% ether extract (EE), 11–21% true protein (TP) and 53–71% total digestible nutrient (TDN), with these ranges being generally higher than in forage crops and grasses (Table 2). The energy content and macro mineral (e.g. calcium, phosphorus, magnesium and potassium) concentrations in woody plant are also higher than in forage. Because woody plants are often utilized as fresh branches and leaves, their fibre and lignin contents are slightly different to those of forage. Woody plants contain the forage components required by livestock and are a high protein feed source. As a result, woody plants are referred to as "woody alfalfa" and their nutritional value is comparable to that of alfalfa (Zhang et al., 2019).

3 The main factor influencing fermentation feed of woody plant

Generally, forage crops and grasses grow well in the summer or tropical rainy season, but in the winter or dry season, cold and dry climatic conditions prevent forage crops from growing, resulting in the demand for feed from livestock far exceeding the production of feed (FAO, 2009). Therefore, woody plants have great potential for development as a feed resource for ruminant livestock, to make up for the shortage of feed. Woody plants are also harvested in large quantities in the summer and early autumn of temperate climates or in the tropical rainy season, and proper preparation and storage techniques after harvesting can effectively increase the self-sufficiency of local feed and the efficiency of livestock production (Su and Chen, 2020). Silage is a common traditional technique for the preparation and storage of forage crops and grasses and is an important means by which woody plants can be used effectively for feed production (Du et al., 2021b). The application of silage fermentation technology for the preparation of woody plant silage can avoid the loss of nutrients and enable long-term storage, thus solving the problem of livestock production during winter or drought seasons when feed is in short supply (Tao et al., 2020).

Silage is a stored feed made from fresh forage crops, grass and crop by-products, and is prepared by microbial fermentation under anaerobic conditions (Muck et al., 2018). Silage is widely used in many countries to make up for the lack of animal feed during the winter or in dry seasons. The fermentation of silage is influenced by various conditions, of which moisture, water-soluble carbohydrate (WSC), buffer energy and the epiphytic microbial community of the material are important factors affecting the fermentation process (McDonald et al., 1991). The appropriate moisture content for silage fermentation is 60–70%. Within this range lactic acid fermentation is promoted and the proliferation of spoilage microorganisms is inhibited. If the silage moisture content is too high, it tends to lead to butyric acid fermentation, dominated by clostridia, which decomposes proteins to produce ammonia nitrogen (NH_3-N), thus reducing the fermentation quality of the silage. If the silage moisture content is too low, lactic acid bacteria (LAB) will be affected by the water activity and their growth will be inhibited, preventing them from producing large amounts of lactic acid to lower the pH of the silage. In addition, low moisture levels result in residual air not being effectively removed from the raw material, thus providing better conditions for harmful microorganisms such as aerobic bacteria and mold to survive,

TABLE 2 Chemical composition, energy, and macro mineral of woody plant and forage.

Material	OM	CP	EE	NDF	ADF	ADL	NPN	TP	ADIP	TDN	NEl	NEm	NEg	Ca	P	Mg	K
	Chemical composition (% DM)						Protein composition (% DM)				Energy (Mcal/kg)			Macro mineral (g/kg DM)			
Woody plant																	
Paper mulberry	91.80	24.65	4.55	37.57	18.52	6.06	2.98	20.22	16.20	70.07	1.69	1.82	1.19	1.80	0.48	0.47	2.33
Mulberry	93.20	17.95	3.76	30.00	21.00	7.49	0.83	17.04	1.61	69.81	1.55	1.68	1.07	1.30	0.24	0.48	2.85
Gliricidia	90.30	25.91	4.02	52.10	34.52	11.09	3.26	18.36	8.94	56.71	1.21	1.29	0.72	1.57	0.27	0.59	2.64
Leucaena	92.62	26.31	3.41	60.62	37.49	13.40	4.14	18.59	12.03	53.19	1.11	1.18	0.62	1.26	0.23	0.42	2.56
Moringa	98.50	20.00	3.13	25.00	17.50	4.49	3.92	16.1	5.51	63.11	1.67	NF	NF	1.40	0.70	0.24	2.59
Forage																	
Corn	93.10	9.11	2.73	52.20	28.70	3.40	1.57	4.50	2.41	46.75	0.81	0.81	0.27	0.32	0.19	0.20	1.66
Sugarcane top	94.65	6.77	1.80	76.10	42.46	5.11	1.50	4.82	3.19	50.78	0.92	0.95	0.41	0.27	0.14	0.18	1.70
Alfalfa	81.30	15.93	4.80	47.30	39.70	7.60	1.48	3.50	10.59	51.70	1.36	1.34	0.72	1.41	0.26	0.26	2.60
Napier grass	85.72	5.56	1.35	66.74	41.53	5.68	1.62	3.43	2.10	41.18	0.64	0.61	0.08	0.41	0.41	0.50	2.06

OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; DM, dry matter; NPN, non-protein nitrogen; TP, true protein; ADIP, acid detergent insoluble protein; TDN, total digestible nutrient; NEm, net energy for maintenance; NEl, net energy for lactation; NEg, net energy for gain; Ca, calcium; P, phosphorous; Mg, magnesium; K, potassium.
Data cited from the following references:
Paper mulberry from Du et al., 2022b; Mulberry from Zhang et al., 2020; Gliricidia and leucaena from Du et al., 2022c; Moringa from He et al., 2020; Corn from Wang et al., 2016; Sugarcane top from Cai et al., 2020a; Alfalfa from Liu et al., 2018; Napier grass from Cai et al., 2020b.

leading to poor quality fermentation (Du et al., 2022a). As shown in Table 3, the moisture content of fresh branches and leaves of woody plants can be as high as 70–80%, which is outside the ideal moisture range for silage fermentation. It is therefore necessary to make moisture adjustments when preparing woody plant silage. To make high quality silage, the material also needs to be above 6% WSC in dry matter (DM) and 10^5 LAB colony-forming unit per gram (cfu/g) in fresh matter (FM). As shown in Table 3, both the WSC content and the LAB count of woody plants were below the theoretical threshold. Moreover, microorganisms harmful to silage fermentation, such as aerobic and coliform bacteria, were present at higher levels than the LAB count. These harmful microorganisms can compete with LAB for nutrients during ensiling and will affect the fermentation quality of silage. The lactic acid buffer capacity (LBC) is also an important factor affecting silage fermentation. Du et al. (2022c) reported that the LBC of woody plants is similar to that of leguminous grasses, generally above 700 mEq/kg of DM. The LBC strength of the silage material is closely related to the mineral composition, as shown in Table 2 above. Woody plants are usually rich in mineral components such as K^+, Ca^{2+} and Mg^{2+}, and these cations neutralize the lactic acid and other organic acids produced by silage fermentation and inhibit the reduction of pH. This allows harmful microorganisms to grow and decompose proteins and produce NH_3-N, resulting in low-quality silage fermentation (Cai et al., 2021). The chemical composition of woody plants in terms of moisture, protein, fiber and minerals varies at different growth stages and can directly affect the fermentation quality of silage (Du et al., 2021b; Du et al., 2022c). In addition, the growth stage and mixing ration of woody plant branches and leaves not only have an important influence on the fermentation quality of silage, but also on the digestibility and productivity of livestock (Anadón et al., 2014).

TABLE 3 Main factor affecting silage fermentation of woody plant.

Material	Moisture (%)	WSC (% DM)	LBC (mEq/kg DM)	Lactic acid bacteria	Harmful microbe
				Lg cfu/g FM	
Paper mulberry	81.25	4.14	892.27	4.66	5.43
Mulberry	71.06	4.92	637.3	4.11	5.68
Gliricidia	75.08	4.62	577.16	4.04	8.28
Leucaena	78.72	4.97	508.18	4.02	8.10
Moringa	80.20	4.88	506.71	3.56	3.87

WSC, water-soluble carbohydrate; LBC, lactic acid buffer capacity; DM, dry matter; cfu, colony-forming unit; FM, fresh matter. Harmful microbibe including aerobic bacteria, coliform bacteria, yeast and mold.
Data cited from the following references:
Paper mulberry from Du et al., 2022b; Mulberry from Zhang et al., 2020; Gliricidia and leucaena from Du et al., 2022c; Moringa from He et al., 2020.

4 Characterization of fermentation feed prepared with woody plant

Woody plants in cultivation or in the native state are usually harvested by harvesters or by hand and then used for silage preparation. In recent years, woody plants have been grown in greenhouses by tissue culture and mechanically harvested after cultivation at a growth height of about 1–1.5 m. They can be harvested 4–5 times a year in tropical or subtropical regions. The fresh branches and leaves of harvested woody plants can have a moisture content of up to 80%. When they are prepared and stored as hay the leaves will fall off, causing the nutrients to be lost during the drying and storage process. Furthermore, the drying process increases the lignin content and reduces the nutritional value and palatability of woody plants to livestock (Du et al., 2021b). Silage fermentation is therefore a good way of preserving the nutrients of woody plants and ensuring a year-round supply of feed for livestock through storage (Du et al., 2021b). Woody plant silage is prepared in the same way as forage crops and grasses, i.e. the woody plants are harvested at a suitable growth stage and the material is cut directly into 1–2 cm. To make good-quality silage, agricultural by-products such as rice bran, wheat bran and corn bran are usually added at 10-15% to regulate the moisture and then packed into silos or drums, sealed and covered for a period of time (Du et al., 2021c). Silage prepared in this way is usually well fermented and can store the nutrients of the silage for a long time.

To investigate the natural fermentation characteristics of woody plant silage, fresh branches and leaves of woody plants were used as the raw material for silage preparation using a drumcan silo. Naturally fermented woody plant silage typically has a high pH, butyric acid and NH_3-N content and a low lactic acid content (Figure 3). This is due to the high moisture content in the woody plant and the competitive use of WSC by the harmful epiphytic microorganisms, which prevents LAB from producing enough lactic acid to lower the pH during silage fermentation. This leads to butyric acid fermentation and the degradation of proteins to produce more NH_3-N (Cai et al., 1999).

To verify the natural fermentation characteristics of woody plant silage, a comprehensive analysis of the dynamic changes in microbial diversity and community structure during woody plant silage fermentation was conducted using the PacBio single molecule real-time (SMRT) sequencing technology. The Venn diagrams in Figures 4A, B clearly show the dynamics of the common and special microbial communities of woody plants before and after silage fermentation. Before ensiling, high moisture content woody plants under aerobic conditions are more suitable for aerobic microbial growth and, therefore, display high operational taxonomic unit (OTU) numbers and a rich microbial diversity. After ensiling, the double stress of the anaerobic and acidic environment created as fermentation progresses leads to the rapid death of some Gram-negative bacteria with thin cell walls. In the final stage of fermentation, Gram-positive bacteria that can adapt to an anaerobic and acidic environment, such as LAB, become the dominant microbial community and carry out lactic acid fermentation, lowering the pH and inhibiting the growth of harmful microorganisms, resulting in a significant decrease in the number and microbial diversity of OTUs (Méndez-García et al., 2015).

Among the microorganisms epiphytic to the fresh branches and leaves of woody plants, the relative abundance of LAB beneficial to silage fermentation is low, while the relative abundance of Gram-negative bacteria harmful to silage fermentation, such as *Pantoea*, *Enterobacter* and *Pseudomonas*, is high (Figure 4C). *Pantoea*

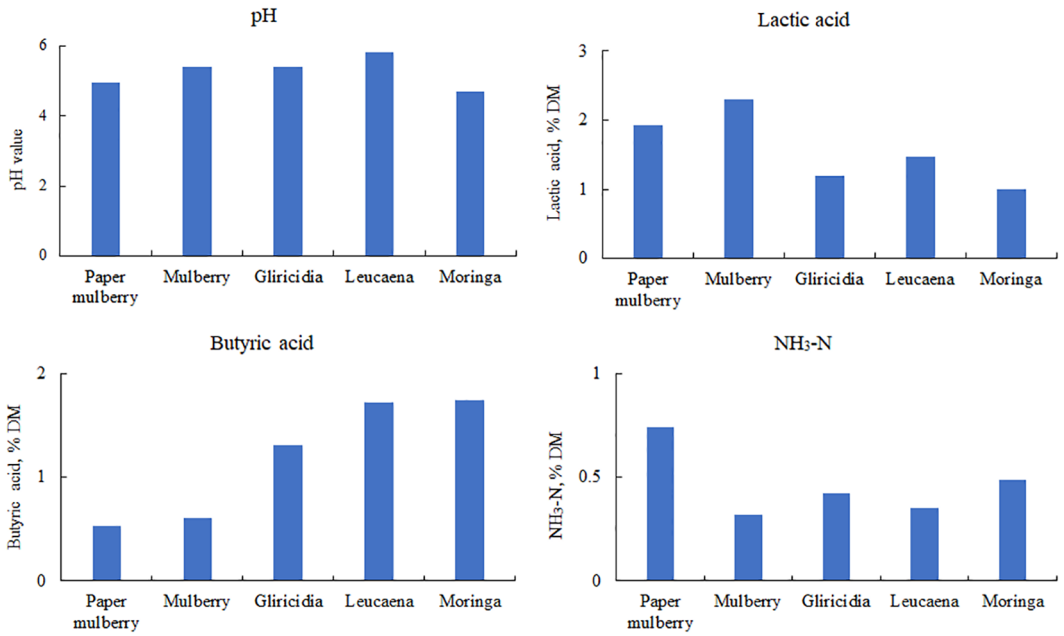

FIGURE 3
Fermentation characteristics of woody plant silage. DM, dry matter; NH_3-N, ammonia nitrogen. Data cited from the following references: paper mulberry from Du et al., 2022b; Mulberry from Zhang et al., 2020; Gliricidia and leucaena from Du et al., 2022c; Moringa from He et al., 2020.

agglomerans, the dominant bacterial community of woody plants, is a parthenogenic anaerobic Gram-negative pathogenic bacteria that is usually found on the surface of plants and is suitable for growth in neutral environments (Jacek et al., 2016). This kind of bacteria can compete with LAB for nutrients in the early stages of silage fermentation, breaking down glucose or other sugars and producing acids (Li and Nishino, 2013). *Enterobacter* constitute an aerobic or facultative anaerobic Gram-negative bacteria with a wide distribution and a large host range, which can be parasitic or symbiotic, epiphytic, saprophytic on humans, animals and plants, and can also survive in soil or water. If grown on plants, it can easily lead to blight (Si et al., 2023). *Pseudomonas* is a common aerobic or facultative anaerobic pathogen that prefers to live in moist environments, usually on soil, water, air and plants. It is designated as low pathogenic but is highly resistant to medication (Roberson and Firestone, 1992).

During ensiling, LAB can proliferate and use the WSC in the plant material to produce lactic acid and lower the pH. The anaerobic acidic environment that forms can play an important role in inhibiting the growth of harmful bacteria in the silage (Cai et al., 2020a). Figure 4C also confirms a significant increase in the relative abundance of LAB in woody plant silage, with *Lactiplantibacillus plantarum* becoming the main dominant species. This species of bacteria is able to respond rapidly to the dual stress of anaerobic and acidic ensiling environments, to carry out lactic acid fermentation and improve the fermentation quality of the silage. In addition, *Enterobacter* and *Citrobacter* have a proportional relative abundance in naturally fermented woody plant silage. *Citrobacter* constitute Gram-negative bacteria that utilize citrate as a sole source of carbon and may be associated with the production of citric acid aroma components in woody plant silage (Janda et al., 1994). This species of bacteria has a low acid tolerance and therefore has a low relative abundance in acidic silage environments. *Enterobacter* constitute some harmful silage bacteria that break down proteins in the early stages of silage fermentation, causing protein deamination and decarboxylation, which produce toxic compounds such as amines and branching fatty acids, resulting in foul-smelling silage (Ávila and Carvalho, 2020). This not only reduces the nutritional value of the silage and the palatability to the animal but can also have an impact on the hygiene and safety of the feed. Therefore, in terms of microbial community dynamics, naturally fermented woody plant silage does not reach a suitable level of quality and it is necessary to regulate the microbial community structure of the silage fermentation and promote lactic acid fermentation.

5 Regulating the anaerobic fermentation of woody plant

5.1 Fermentation regulation of woody plant silage by multiple preparation methods

To address the factors that hinder the fermentation of high-quality silage (i.e. high moisture content, strong LBC, low LAB count and low WSC content) in woody plants, rice bran and wheat bran have been added to adjust the moisture content and increase the fermentation substrate (Du et al., 2021a). Microbial additives, such as LAB, and cellulolytic enzymes have also been applied to improve the microbial community and increase the WSC content (Du et al., 2021b). In addition, hays including Napier grass and rice straw have been used to optimize the silage fermentation process of woody plants (Du et al., 2022a). The PacBio SMRT sequencing technology was applied to conduct an in-depth study of microbial diversity, co-occurrence microbial networks, metabolic pathways, final metabolites and the fermentation regulation mechanism of woody plant silage.

The moisture content of the rice bran and wheat bran used in the study was less than 10%. When mixed with woody plants at a rate of 10–30% of the FM, the moisture was adjusted to exactly 60–70% (Du et al., 2021a), which is the ideal moisture range for silage preparation. Woody plants have a similar epiphytic microbial community structure to forage crops and grasses, i.e. they have a higher relative abundance of harmful microorganisms and a lower relative abundance of LAB in the fresh material. The relative abundance of epiphytic *L. plantarum* from the woody plant material was below the detection level, but increased significantly after ensiling (Figure 5A). Thus, the aerobic environment created by woody plants prior to ensiling was not beneficial for the competitive growth of LAB, while aerobic microorganisms were at an advantage. Figure 5B shows that the relative abundance of clostridia in the mixed silage of woody plants and wheat bran was below the detection level, but the mixed silage of woody plants with rice bran increased the relative abundance of clostridia. Wheat bran effectively regulated the moisture and increased the fermentation substrate of woody plants, and the anaerobic conditions created by silage fermentation accelerated the succession of LAB, which became the dominant community and inhibited the proliferation of clostridia. The silage prepared with 30% wheat bran and 70% woody plants had the best microbial community structure. The addition of rice bran also served to adjust the moisture and fermentation substrate, but rice bran can sometimes be enriched with *Clostridium* spp., which is a strictly anaerobic Gram-positive bacteria whose spores tolerate the acidic and anaerobic environmental conditions of silage (Li et al., 2020). Clostridia breaks down sugars, organic acids and proteins during the silage process, producing butyric acid, NH_3-N, carbon dioxide (CO_2) and hydrogen (H_2), thereby reducing the fermentation quality of the silage (Cassir et al., 2016). In addition, some proportion of clostridia is pathogenic and can be harmful to the health of livestock (Uzal et al., 2018). The microbial community dynamics in Figure 5C also confirmed that *L. plantarum* and *Clostridium typhimurium* were the two dominant bacteria in woody plant silage mixed with rice bran and wheat bran. There were significant differences in the relative abundance of these two species compared to other silage bacterial communities. Therefore, compared to rice bran, wheat bran is a more suitable exogenous addition to the silage fermentation of woody plants, not only to regulate the fermentation conditions but also to improve the microbial community structure, thus promoting woody plant silage fermentation.

In addition, the fermentation characteristics of woody plant silage were explored by applying additions such as LAB inoculant

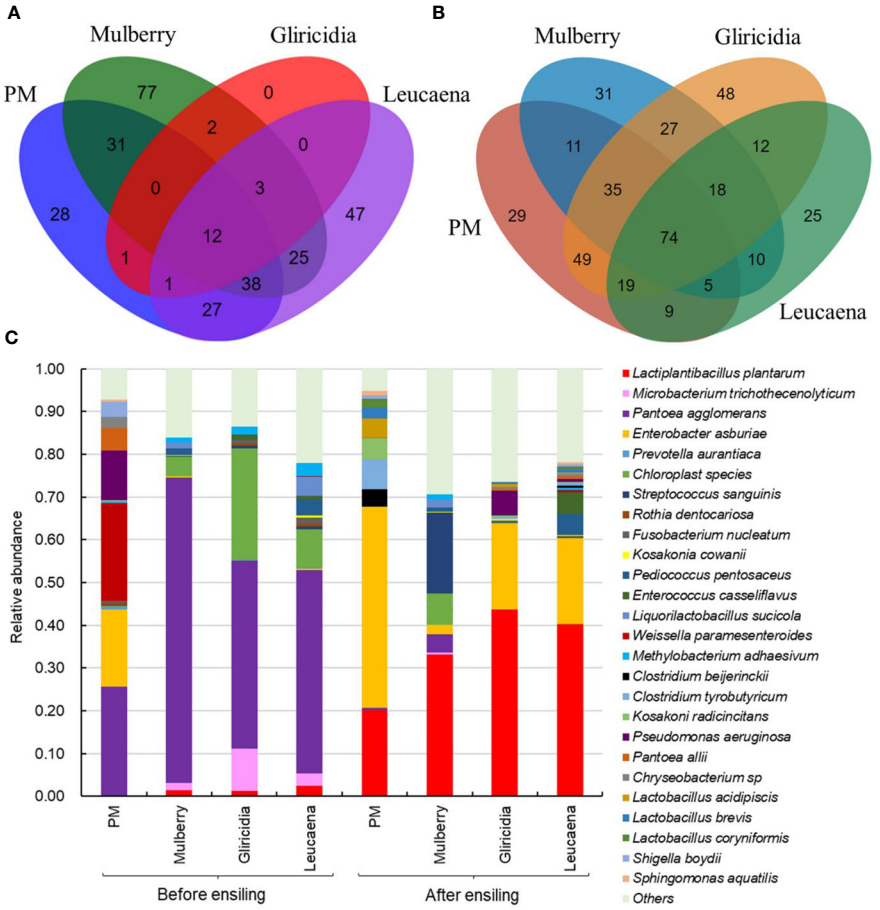

FIGURE 4
Microbial diversity and community structure of woody plant. **(A)** Venn diagram depicting unique or shared bacterial OTU of 97% sequence identity before ensiling; **(B)** Venn diagram after ensiling; **(C)** Relative abundances of the bacterial communities at the species levels. PM, paper mulberry. Data cited from the following references: Paper mulberry from Du et al., 2022b; Mulberry from Zhang et al., 2020; Gliricidia and leucaena from Du et al., 2022c.

and cellulolytic enzymes (Du et al., 2021b). Microbial additives can cause a significant decrease in microbial diversity in woody plant silage and the microbial community rapidly completes a dynamic succession process from Gram-negative to Gram-positive bacteria. In the anaerobic and acidic environment created by silage fermentation, Gram-negative bacteria such as *Enterobacter asburiae* die off rapidly, while LAB respond rapidly to the anaerobic conditions of silage and become the dominant community, dominating the fermentation process. Thus, microbial additives influence silage fermentation by improving the microbial community. In addition, the use of locally available low-cost crop straw and hay mixed with woody plants for silage making is a viable option for improving the silage fermentation quality. The results showed that the addition of Napier grass and rice straw allowed *L. plantarum* and *Lactococcus cellulosus* to act synergistically with each other, enabling LAB rapidly to become the dominant community for woody plant silage, and that the addition of 10–30% hay was effective in improving the fermentation quality of woody plant silage.

5.2 Mechanism of interaction between co-occurrence microbial networks and secondary metabolites during silage fermentation

The metabolites produced by silage microorganisms during fermentation have a strong influence on the fermentation quality, flavor and aerobic spoilage of silage (Ávila and Carvalho, 2020). Silage fermentation forms a microbial co-occurrence network system, which includes a complex process of dynamic succession of the microbial community and changes in their metabolites, which vary greatly from the different microorganisms through their metabolic pathways (Du et al., 2022c). The LAB produce

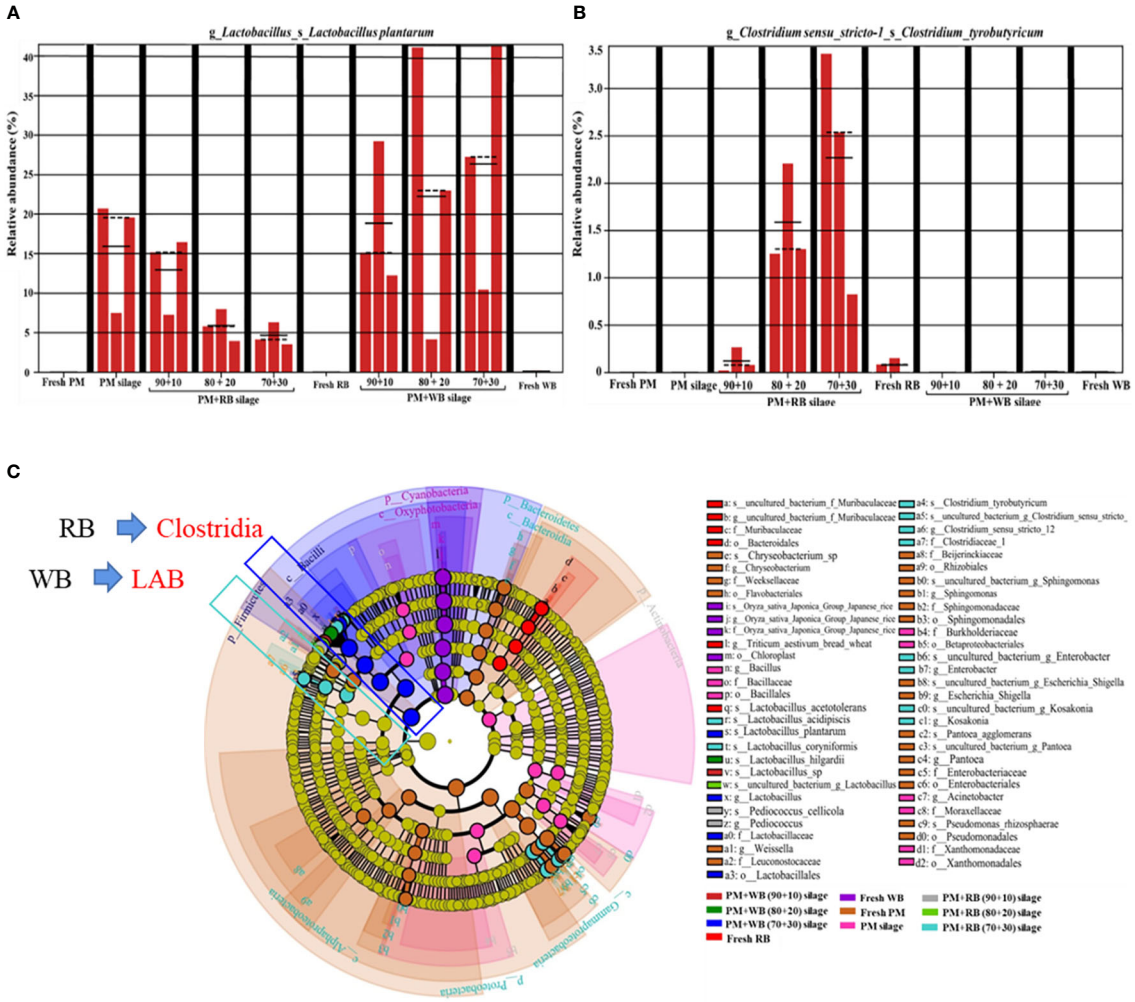

FIGURE 5
The community dynamics of lactic acid bacteria and clostridia in PM prepared with RB and WB before and after ensiling. **(A)** metaStat analysis of *Lactobacillus plantarum*; **(B)** metaStat analysis of *Clostridium tyrobutyricum*; **(C)** cladogram comparison of bacterial community. PM, paper mulberry; RB, rice bran; WB, wheat bran. The figure cited from Du et al., 2021a.

metabolites, such as organic acids, ethanol, 1,2 propylene glycol and biogenic amines during silage fermentation, some of which play an important role in inhibiting the growth of harmful bacteria and improving aerobic stability (Guo et al., 2018). The LAB usually use WSC to produce lactic acid and lower the pH, which can improve the fermentation quality of silage (Okoye et al., 2022). In contrast, *Enterobater* species can ferment glucose to produce succinic acid, lactic acid, acetic acid, formic acid and ethanol, as well as producing CO_2 and H_2 gas, and increasing the DM and energy loss (Thompson et al., 2008). In addition, some metabolites such as lactic and acetic acid reduce the pH and inhibit the growth of aerobic bacteria and molds, which can improve the fermentation quality and aerobic stability of silage (Cai et al., 2020a). Thus, the microbial community structure and metabolites interact and influence the fermentation quality of silage.

The abundance levels of harmful microorganisms such as *Enterobacter* spp. and *Clostridium tyrobutyricum* were positively correlated, which may be related to the low-quality fermentation of the silage (Figure 6A). As silage fermentation progressed, *L. plantarum* rapidly formed the dominant community, which in turn replaced the dominant population of the harmful bacteria *P. agglomerans* in the early stages of fermentation.

A positive correlation was found between the moisture content and *P. agglomerans*, *Sphingomonas* spp., *Aureimonas* spp. and *Methylobacterium* spp. and between WSC and LAB (Figure 6B). This suggests that a high moisture content silage environment encourages the proliferation of these harmful bacteria and that WSC can promote the growth of LAB. In addition, LAB produce lactic acid during ensiling, which inhibits the growth of these harmful bacteria, thereby lowering pH and reducing the production of NH_3-N. *Clostridium beijerinckii* form a nutrient-competitive interrelationship with LAB, i.e. *C. beijerinckii* undergo butyric acid fermentation, which promotes the production of propionic and butyric acids and hinders the proliferation of LAB. *Acidovorax* spp. are aerobic or facultative anaerobic Gram-negative bacteria that can use residual oxygen to produce acetic acid in the

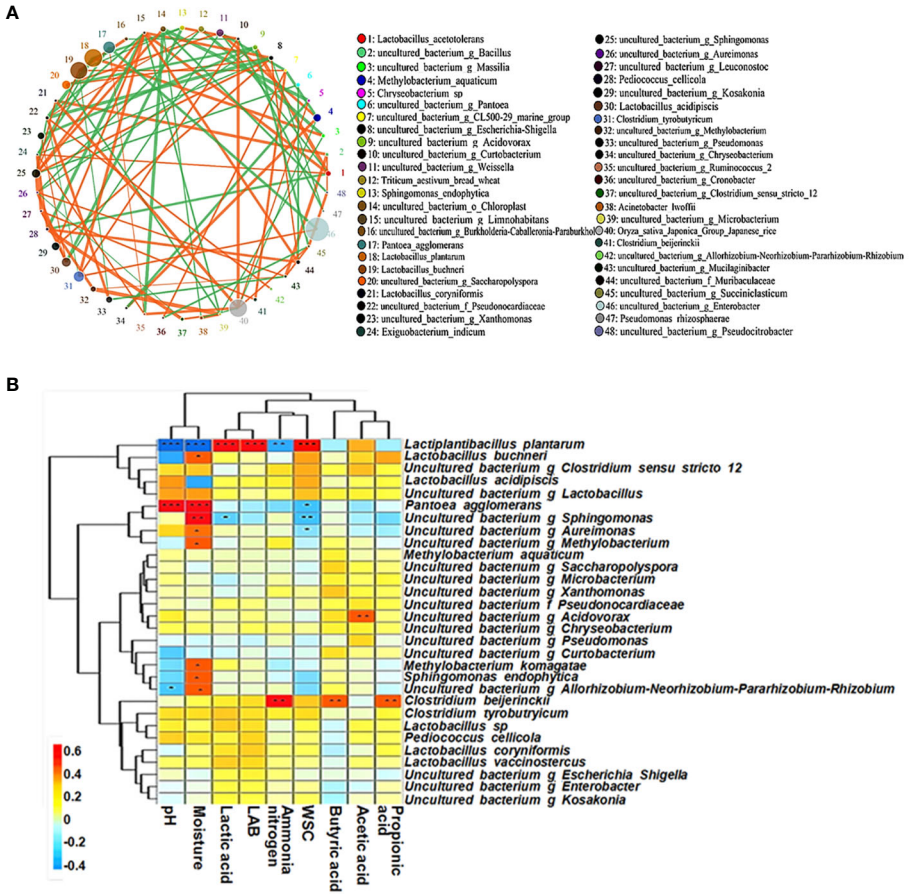

FIGURE 6
The microbial co-occurrence network **(A)** and correlation analyses between bacterial community and terminal fermentation product **(B)** at species level in paper mulberry silage. The figure cited from Du et al., 2021a.

early stages of silage fermentation (Du et al., 2021a). Therefore, Gram-positive and Gram-negative bacteria (e.g. *L. plantarum*) form a mutually constraining opposition during silage fermentation; their community structure and metabolites interact with each other and influence the fermentation quality of the silage.

To explore the fermentation regulation mechanism of woody plant silage, non-targeted metabolomics techniques were used to study the mechanisms of interaction between the co-occurrence microbial networks and secondary metabolites of woody plant silage prepared with bran (Du et al., 2022a). A Spearman correlation analysis of the main bacterial community and metabolites of silage showed that the aromatic compound-like metabolites of silage were positively correlated with LAB, such as *L. plantarum* and *Weissella paramesenteroides*, and negatively correlated with other bacterial community members, such as *C. tyrobutyricum* and *E. asburiae* (Figure 7). Among the aromatic compounds, citric acid and L-malic acid are key intermediates in the tricarboxylic acid cycle metabolic pathway, which is produced by most LAB. Citric acid is formed by the carboxylation of acetyl coenzyme A and oxaloacetate in the tricarboxylic acid cycle and is involved in the metabolism of sugars, fats and proteins (Ke et al., 2017). During silage fermentation, citric acid can play a role in lowering silage pH and inhibiting the activity of undesirable fermentation fungi, such as yeasts and moulds. In addition, LAB in the silage fermentation process can metabolize citric acid to produce diacetyl and acetic acids, and other substances with flavor, which can improve the flavor of silage and the palatability to livestock. When animals are fed silage containing citric acid the proliferation of pathogens can be reduced and the production of toxic metabolites can be inhibited, which can improve the stress capacity of livestock. L-malic acid is an important natural organic acid with antioxidant properties that regulates silage pH and promotes the growth of LAB (Wang et al., 2009). During silage fermentation, LAB with the function of saccharifying starch can use starch directly for fermentation, thus producing L-malic acid, which will play a role in improving the silage fermentation quality. In addition, LAB can use D-(+)-cellobiose as a substrate for energy production, accelerating growth and inhibiting the proliferation of yeast, mold, clostridia and enterobacter. These findings suggest that silage microbiomics are closely linked to metabolomics. The microbial community structure can influence the types of final and secondary metabolites, which in turn are important factors influencing the fermentation quality of silage.

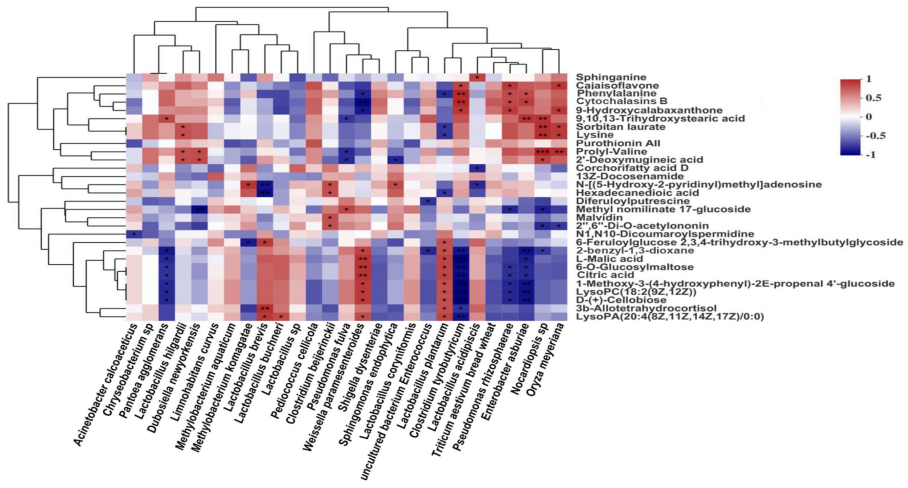

FIGURE 7
Correlations between microbial community and metabolites in silage. Microbial community, main bacterial species; metabolites, differentially presented metabolites. The figure cited from Du et al., 2022b.

6 Woody plant and livestock product

To popularize the use of woody feed resources in livestock production, feeding experiments have been conducted using a variety of livestock, including cattle, sheep and poultry.

In experiments with fed cattle, Holstein cows fed total mixed fermentation (TMR) formulated with 10–15% paper mulberry instead of maize silage, alfalfa hay and oat hay had a similar DM intake and milk production to those fed with conventional TMR. However, the addition of paper mulberry to TMR diets significantly increased the serum levels of immunoglobulin A (IgA), immunoglobulin (IgG), catalase and superoxide dismutase and the total antioxidant capacity, but decreased the levels of 8-hydroxy-2′-deoxyguanosine. In addition, paper mulberry TMR can cause a significant decrease in the milk somatic cell count, enhance the immune and antioxidant function of cows and increase the polyunsaturated fatty acid content of milk (Si et al., 2018). The fresh branches and leaves of mulberry are rich in flavonoids and feeding them to weaned Holstein calves promotes the average daily gain and feed efficiency (Kong et al., 2019). Gliricidia leaves and elephant ear pods are rich in tannins and saponins. When a mixture of the two was fed to beef cattle, it increased the digestible CP intake, inhibited methanogenic bacteria in the rumen and reduced methane emissions (Isabel et al., 2019). Leucaena silage improves the DM intake, feed digestibility and rumen end fermentation products in dairy cows and reduces methane production (Giang et al., 2016). A TMR including moringa improves the milk fat content and levels of secondary metabolites, regulates rumen

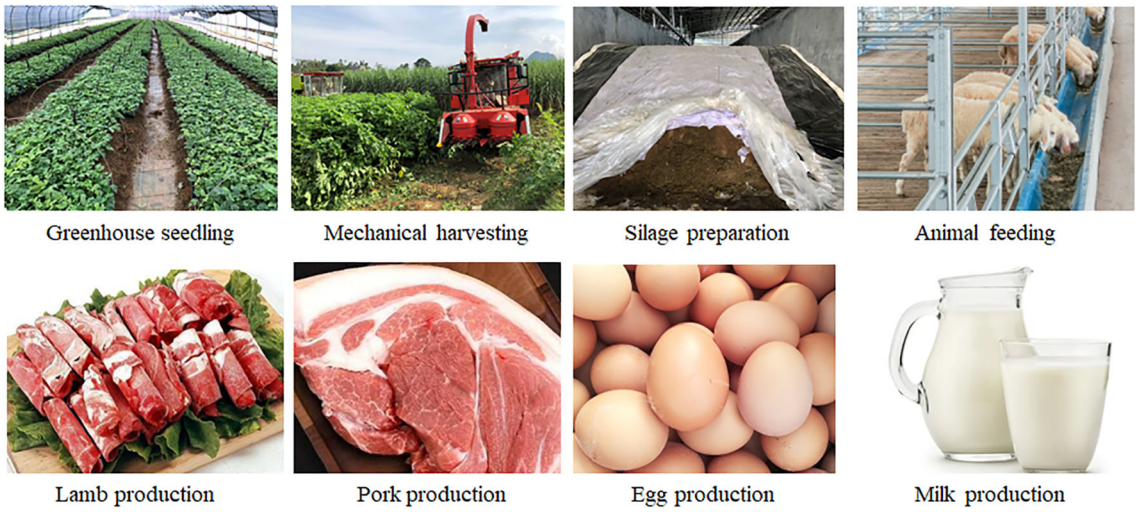

FIGURE 8
Production potential of feed and livestock product from woody plant.

fermentation and inhibits methane production by dairy cattle (Dong et al., 2019).

In sheep feeding trials, replacing some of the soybean meal and corn stover with paper mulberry silage reduced total volatile fatty acids in the rumen, and increased DM intake and average daily gain of lambs (Xiong et al., 2021). When some of the hay and concentrate in the TMR was replaced by mulberry leaves, there was no significant difference in the digestibility of DM, CP, and crude fibre in sheep compared to the conventional TMR (Kandylis et al., 2009). In addition, silage prepared from a mixture of gliricidia with cassava improves growth performance, digestibility, feeding behavior and the carcass characteristics of lambs, and increases the yield of key commercial meats, such as loin and ham, as well as typical foodstuffs, such as lamb (Oliveira et al., 2018). Feed supplementation with moringaleaf extract increased milk production, reduced saturated fatty acids and increased the levels of unsaturated fatty acids and conjugated linoleic acid in goats (Kholif et al., 2018).

In poultry feeding trials, the use of woody plants, such as paper mulberry and mulberry, in place of some of the commercial feeds maintained good indicators in terms of the egg production rate, egg weight, fertilized egg hatching rate, growth performance and meat quality (Yang et al., 2020). There is great future potential for seedling production and breeding of woody plants for healthy livestock feeding with antibiotic substitutes.

7 Conclusion

To develop and utilize new woody feed resources to produce high-quality livestock products, this review provided a comprehensive overview of the composition and uses of natural woody plants that can be fed to animals, the dominant factors affecting woody plant silage fermentation, microbial succession patterns and the mechanisms of interaction between microbial co-occurrence networks and secondary metabolites. Woody plants are rich in nutrients and can be used for the preparation of fermented feeds and the production of value-added livestock products (Figure 8). This has important implications for alleviating feed shortages and promoting sustainable development of animal husbandry.

Author contributions

ZD and YC: Writing - original draft, Investigation. ZD, YC, and FY: Writing–original draft, Formal analysis. ZD and JF: Validation, Writing–review & editing; SY: Conceptualization, Visualization. TO, DN, and HK: Supervision, Resources. YC and FY: Resources, Writing–review & editing, Supervision, Funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Microbiomics and volatile metabolomics-based investigation of changes in quality and flavor of oat (*Avena sativa* L.) silage at different stages

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Objective: This study aimed to analyze the fermentation quality, microbial community, and volatile metabolites of oat silage harvested at two different stages, while examining the correlation between microorganisms and volatile metabolites.

Methods: Oats were harvested at two growth stages (pre-heading [PRH] and post-heading [POH] stages), followed by 90 days of natural fermentation, with 6 replicates per treatment. Pre- and post-silage samples were randomly selected for nutrient composition, fermentation parameters, microbial population, and high-throughput sequencing analysis. Volatile metabolomics analysis was also performed on samples after 90 days of fermentation to detect differences in flavor quality after silage.

Results: The effect of growth stage on the nutrient content of oats was significant, with pre-heading oats having higher crude protein and post-heading oats having higher water soluble carbohydrates content ($p < 0.05$). Following a 90-day fermentation period, the pH and ammonia nitrogen/total nitrogen levels in the PRH-90 (silage from pre-heading oats after 90 days of fermentation) group demonstrated a significant decrease ($p < 0.05$), whereas the lactic acid content was notably higher compared to the POH-90 (silage from post-heading oats after 90 days of fermentation) group ($p < 0.05$). *Lactiplantibacillus* dominated in the PRH-90 group and *Enterococcus* dominated in the POH-90 group, with abundances of (> 86%) and (> 87%), respectively. The differential volatile metabolites of the two treatment groups were dominated by esters and terpenoids, and the differences in flavor were mainly concentrated in sweet, green, and fruity odors. The results of Kyoto encyclopedia of genes and genomes pathway enrichment analysis demonstrated three major metabolic pathways: phenylpropanoid biosynthesis, phenylalanine metabolism, and biosynthesis of secondary metabolites. Specific microorganisms were significantly correlated with flavor indicators and flavor metabolites. *Lactiplantibacillus* was significantly positively correlated with flavor substances indicating sweet and fruity flavors, contributing to good flavor, while

Enterococcus was significantly and positively correlated with flavor substances indicating bad flavors.

Conclusion: In summary, growth stage had significant effects on nutritional components, fermentation parameters and flavor quality of oats, with the fermentation process dominated by *Lactiplantibacillus* leading to good flavor, while the fermentation process dominated by *Enterococcus* led to the development of poor flavor.

KEYWORDS

oats, microbial community, volatile metabolites, flavor, fermentation quality

1 Introduction

As an important grain-feed crop for livestock, oats (*Avena sativa* L.) are widely cultivated worldwide and have the advantages of high nutritional value, high grass yield, and good palatability (Zhou et al., 1999). Ensiling oats, a common preservation method, can be preserve oats for a long period of time, and also improves palatability and the organoleptic quality of oats (Nilsson and Rydin, 1963).

Ensiling refers to a technique of reducing the pH value of raw materials through microbial fermentation, inhibiting the growth and reproduction of detrimental microorganisms, in order to maximize the preservation of its nutrients and extend the shelf life of the raw material (Mcdonald et al., 1991). As a crop rich in protein, fiber, and trace elements, oats can be better preserved and utilized for its nutrient content after ensiling. However, microbial activity and metabolites during ensiling may have an impact on the quality and flavor of oats (Limin et al., 2018).

In recent years, microbiomics and volatile metabolomics have emerged as important branches of modern biotechnology, finding extensive applications in the fields of food science and nutrition (Shi et al., 2022; Zhao et al., 2022). Microbiomics provides insight into the species, abundance and functions of different microorganisms through the study of microbial communities, which in turn reveals their association with the quality and flavor of fermented products (Paraskevi et al., 2020). Volatile metabolomics, on the other hand, can explore the flavor characteristics of fermented products and their relationship with microbial metabolic activities by analyzing the composition and variation of volatile compounds in food (Iijima, 2014). The investigation of microbial communities and volatile metabolites during oat ensiling is essential to understand the complex biochemical processes involved in the ensiling process. Microorganisms, particularly lactic acid bacteria, assume a crucial role in the fermentation process, producing organic acids and other metabolites that contribute to the preservation and flavor development of silage oats (Liu M. et al., 2022). In addition, the composition and abundance of volatile compounds may vary depending on silage conditions, oat fertility and microbial interactions, ultimately affecting the sensory characteristics of the final product. Previous studies have focused on oat fertility, moisture content, and exogenous additives (Gardner and Wiggans, 1961; Mustafa and Seguin, 2003; Jian et al., 2015; Gomes et al., 2019; Xu et al., 2022), with fewer studies on oat silage odor after oat silage fermentation, and no in-depth studies on the relationship between microbial activity and flavor development during the fermentation process.

The objective of this study is to investigate the alterations in quality and flavor of oats following the silage process at different stages using microbiomics and volatile metabolomics. Through the analysis of microbial communities and volatile metabolites, we aim to identify the patterns and dynamics of microorganisms and volatile compounds during oat ensiling, which can lay a scientific foundation for the improvement of oat ensiling techniques and enhancing silage quality.

2 Methods and materials

2.1 Silage preparation

The oats were harvested on August 22, 2022 (pre-heading stage) and September 6, 2022 (post-heading stage), respectively, in Ar Horqin Banner, Chifeng City, Inner Mongolia Autonomous Region, China (43°21′43″-45°24′20″N, 119°02′15″-121°01′E). A part of the harvested oat material was directly returned to the laboratory for the determination of fresh oat material, while the other part was naturally air-dried to the substance with rough 70% moisture content, and then the sample was cut into 2-3cm in length. The experiment was divided into four groups with six bags (250×360mm polythene plastic bag) per group, each bag containing 400 g without any additives. The bags vacuum sealed for storage (Type: DZ-500/2E; Hefei Hanjie Packaging Machinery Inkjet Co., Ltd., Hefei, China) and opened and sampled after 90 d of storage at room temperature for the subsequent analysis. The different treatment groups are named as follows: PRH-fm, fresh oat material at pre-heading stage; POH-fm, fresh oat material at post-heading stage; PRH-90, silage from pre-heading oats after 90 days of fermentation; POH-90, silage from post-heading oats after 90 days of fermentation.

2.2 Laboratory analysis

Samples of 10 g each were collected from each treatment group at 0 days and 90 days. Subsequently, 90 mL of distilled water was added, and the mixture was homogenized for two minutes by a homogenizer (Model: HX-4, Shanghai Huxi Industrial Co., Ltd., China). After filtration of the resulting extract, the pH value was measured using an acidity meter (Model: S400-B, Mettler-Toledo, LLC, America). The remaining materials were placed in an oven at 115°C for 15 minutes and then dried at 65°C for 48 hours for weighing dry matter (DM) content. The dried materials were ground into powder and stored separately. The Kjeldahl method was employed to analyze crude protein (CP) content (Sun et al., 2021). An ANKOM fiber analyzer was utilized to quantify neutral detergent fiber (NDF) and the acid detergent fiber (ADF) (Model: A2000i; Beijing Anke Borui Technology Co., Ltd., China), and the measurement of soluble carbohydrates (WSC) was performed through anthrone-sulfuric acid colorimetry (Murphy, 1958). Determination of lactic acid (LA) and acetic acid (AA) in silage after 90 days of fermentation was accomplished by high performance liquid chromatography (Fu et al., 2022). Determination of ammonia nitrogen (NH_3-N) concentration used the phenol hypochlorite method according to Broderick and Kang (1980).

2.3 Enumeration of microbial community

Ten g of fresh and silage oat samples were collected, and 90 mL of sterile water was added. The mixture was homogenized for 2 minutes using a homogenizer (Model: HX-4, Shanghai Huxi Industrial Co., Ltd., China), and the resulting bacterial solution was obtained after filtration. Culture media (Guangzhou Huankai Microbial Science and Technology Co., Ltd., Guangzhou, China) were used to isolate and enumerate various microorganisms. The culture medium for lactic acid bacteria was De Man Rogosa Sharpe agar culture medium, while nutrient agar culture medium was used for aerobic bacteria, iron-methylene blue agar culture medium for coliform bacteria, and potato glucose agar culture medium for mold and yeast. The quantification of microbial communities was performed using the plate counting method. The number of colonies was the number of viable microorganisms in the colony forming unit (cfu)/g fresh substance (FM). The number of viable microorganisms in colony forming unit (cfu)/g of fresh matter (FM) was determined by counting the colonies.

2.4 DNA extraction and PCR amplification and sequencing

Total microbial genomic DNA was extracted from homogenized experimental fresh oat samples and silage oat samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer's instructions. The quality and concentration of DNA were determined by 1.0% agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (Thermo Scientific, United States). The hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 799F and 1193R (Liu et al., 2016) by T100 Thermal Cycler PCR thermocycler (BIO-RAD, USA). The PCR reaction mixture consisted of 4 μL of 5× Fast Pfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of Fast Pfu polymerase, 0.2 μL of BSA, 10 ng of template DNA, and ddH2O, resulting in a final volume of 20 μL. Following the manufacturer's instructions, the PCR product was extracted and purified from a 2% agarose gel using the PCR Clean-Up Kit (YuHua, Shanghai, China). Subsequently, the purified product was quantified using the Qubit 4.0 system, as per the manufacturer's protocol (Thermo Fisher Scientific, USA). Sequencing data for the 16S rRNA gene sequence of the oat samples in the two treatment groups were uploaded and stored in NCBI BioProject, and the accession number can be found under PRJNA1005624.

2.5 Analysis of volatile metabolites present in oat silage samples

2.5.1 Sample preparation and treatment

The oat materials were harvested, weighed, and promptly frozen in liquid nitrogen for preservation. Subsequently, they were stored at -80°C until required for analysis. To prepare the samples, the frozen oat materials were ground into a fine powder using liquid nitrogen.

For each analysis, 500 mg (1 mL) of the powdered oat sample was immediately transferred into a 20 mL headspace vial (Agilent, Palo Alto, CA, USA). The vial was supplemented with 10μL of saturated NaCl solution (50μg/mL) to prevent enzyme reactions. The vials were tightly sealed using crimp-top caps equipped with TFE-silicone headspace septa (Agilent). Prior to solid-phase microextraction (SPME) analysis, each vial was placed in an oven set at 60°C for 5 minutes. Subsequently, a 120 μm DVB/CWR/PDMS fiber (Agilent) was exposed to the headspace of the sample for 15 minutes at 60°C.

2.5.2 GC-MS conditions

The identification and quantification of volatile organic compounds (VOCs) were carried out using an Agilent Model 8890 gas chromatograph coupled with a 7000D mass spectrometer (Agilent). The GC system was equipped with a DB-5MS (5% phenyl-polydimethylsiloxane) capillary column measuring 30 m × 0.25 mm × 0.25 μm. Helium gas was utilized as the carrier gas, flowing at a linear velocity of 1.2 mL/min. The injector temperature was maintained at 250°C, while the detector temperature was set at 280°C. To achieve separation and analysis, the oven temperature was programmed as follows: initial temperature of 40°C for 3.5 minutes, followed by a ramp of 10°C/min to 100°C, then a ramp of 7°C/min to 180°C, and finally a ramp of 25°C/min to 280°C. The temperature was held at 280°C for 5 minutes.

2.6 Statistical analysis

Significant differences in the test materials were analyzed using SAS 9.2, and the 0.05 level was considered to be the least significance level between the treatment groups. Unsupervised principal component analysis (PCA) was conducted using the prcomp statistical function in R (www.r-project.org). The hierarchical cluster analysis (HCA) results for samples and metabolites were generated using the Complex Heatmap R package. Microbiota and metabolome data were performed using an online platform of Majorbio Cloud Platform (https://cloud.majorbio.com/page/tools/).

3 Results

3.1 Characteristics of fresh and silage oats at different stages

The nutritional components, fermentation products, and microbial populations of whole oats before and after silage at different stages are shown in Table 1. Distinct variations were observed in the nutritional components, fermentation products, and microbial population of oats between the pre-heading and post-heading stages. The NDF (628.27 ± 8.56), ADF (381.98 ± 17.10), and WSC (50.63 ± 1.40) of fresh oats at the post-heading stage significantly increased compared to the pre-heading stage, while CP (10.38 ± 0.41) significantly decreased ($p < 0.05$). Following a 90-day fermentation period, there was a significant decrease in the content of the CP, NDF, ADF, and WSC in silage oats harvested at both pre-heading and post-heading stages ($p < 0.05$). Silage oats at pre-heading stage had lower NDF (464.71 ± 11.83), ADF (296.53 ± 0.55), WSC (17.54 ± 0.51), but higher CP (10.80 ± 0.31) than at post-heading stage ($p < 0.05$). Comparing the fermentation products of the two stages, pH, AA, and NH3-N were significantly lower in pre-heading oats after ensiling compared to post-heading oats, and LA and LA/AA were found to be significantly higher in pre-heading oats compared to post-heading oats ($p < 0.05$). The results of microbial plate count analysis revealed no statistically significant differences in the populations of aerobic bacteria and coliform bacteria attached to fresh oats at the two stages ($p < 0.05$), while lactic acid bacteria and yeast attached to fresh oats at the pre-heading stage were lower than those attached to fresh oats at post-heading stage ($p < 0.05$). After ensiling, the

TABLE 1 Nutritional components, fermentation products, and microbial populations of whole oats harvested at different stages before and after ensiling.

Parameters analyzed	PRH		POH	
	PRH-fm	PRH-90	POH-fm	POH-90
Nutritional Components				
DM, g/kg	136.40 ± 15.47d	284.43 ± 6.89b	205.72 ± 4.69c	332.93 ± 2.95a
CP, g/kg DM	12.91 ± 0.45a	10.80 ± 0.31b	10.38 ± 0.41b	8.74 ± 0.44c
NDF, g/kg DM	501.54 ± 11.89c	464.71 ± 11.83d	628.27 ± 8.56a	553.45 ± 4.95b
ADF, g/kg DM	308.04 ± 22.58c	296.53 ± 0.55c	381.98 ± 17.10a	340.74 ± 6.08b
WSC, g/kg DM	30.32 ± 1.32c	17.54 ± 0.51d	50.63 ± 1.40a	35.33 ± 1.14b
Fermentation Products				
pH	6.26 ± 0.06a	4.73 ± 0.06d	5.95 ± 0.20b	5.57 ± 0.11c
LA, g/kg DM	/	53.66 ± 10.74a	/	30.43 ± 4.39b
AA, g/kg DM	/	8.86 ± 1.74b	/	14.41 ± 0.83a
LA/AA	/	6.34 ± 2.29a	/	2.13 ± 0.42b
AN/TN	/	6.22 ± 0.45b	/	9.06 ± 0.35a
Microbial population				
Lactic acid bacteria (log_{10}cfu/g FM)	3.86 ± 0.48c	5.90 ± 0.29a	5.10 ± 0.26b	4.66 ± 0.55b
Aerobic bacteria (log_{10}cfu/g FM)	5.89 ± 0.21a	6.20 ± 0.86a	6.63 ± 0.40a	4.77 ± 0.31b
Coliform bacteria (log_{10}cfu/g FM)	4.77 ± 0.68a	ND	5.28 ± 0.80a	ND
Yeast (log_{10}cfu/g FM)	2.37 ± 0.32d	6.10 ± 0.09a	4.11 ± 0.07c	4.71 ± 0.24b
Molds (log_{10}cfu/g FM)	ND	ND	ND	ND

DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water soluble carbohydrates; pH, potential of hydrogen; LA, lactic acid; AA, acetic acid; AN, ammonia nitrogen; TN, total nitrogen; cfu, colony forming unit; FM, fresh material;/, no value; ND, not detected. The data are expressed as the mean ± SEM (n= 3). Different letters a, b, c, d within a row indicates statistically significant differences.

populations of lactic acid bacteria and aerobic bacteria increased significantly in oats at the pre-heading stage, while the opposite was observed in oats at the post-heading stage ($p < 0.05$). No presence of molds was detected in any of the treatment groups.

3.2 Microbial community of fresh oats and oat silage

Figure 1 presents the microbiota composition at the phylum and genus levels in both fresh oats and oat silage. At the phylum level, Proteobacteria dominated the fresh oat samples harvested at both two stages, with abundances of (> 68%) and (> 92%). Firmicutes and unclassified d Bacteria were the next most dominant phylum found in the fresh oat samples. It was noteworthy that the abundances of Bacteroidota and Actinobacteria in the fresh oat samples were (> 11%) and (> 1%) at the pre-heading stage, while they were less abundant or not detected in fresh oat samples at the post-heading stage. After 90 days of fermentation, the most abundant phylum was Firmicutes, and almost all 16S sequences belonged to the phylum of Firmicutes in both stages (PRH-90 – 99.48% vs POH-90 – 99.43%).

Genus level compositions of the bacterial community in fresh oats and oat silage are described in Figure 1B. The most abundant genus in fresh oats was *Pantoea* at both stages, but there was a difference in its abundance (PRH-fm – 36.11% vs POH-fm – 88.74%). *Chryseobacterium* was detected in fresh oats at the pre-heading stage, while it was less abundant or not detected in fresh oats at the post-heading stage. Several other major genera detected in fresh oats were *Shigella*, *Exiguobacterium*, and *Pseudomonas*. Following a 90-day fermentation period, silages from raw materials harvested at different stages presented distinct microbial abundances. In pre-heading oat silage, the abundance of *Lactiplantibacillus* was the highest and dominant (> 86%), followed by *Pediococcus* (9.62%) and *Enterococcus* (3.37%), while in post-heading oat silage, the abundance of *Enterococcus* was the highest and dominant (> 87%), followed by *Exiguobacterium* (6.63%), *Weisella* (2.87%), and *Lactiplantibacillus* (0.46%).

As shown in Figures 1C, D, the relative abundance comparison bar chart of species was tested through the Kruskal-Wallis rank sum test. Following the ensiling process, there was a significant increase observed in the abundance of Firmicutes, whereas the abundance of Proteobacteria and unclassified d Bacteria exhibited a significant decrease compared to fresh oats ($p < 0.05$). Among the genera analyzed, *Pantoea*, *Enterococcus*, and *Lactiplantibacillus* exhibited the most significant variations in abundance at the genus level in Figure 1D. The abundance of *Pantoea* exhibited a significant increase following the ensiling process ($p < 0.05$). *Enterococcus*, *Lactiplantibacillus*, and *Pediococcus* significantly increased in pre-heading oat silage, and *Enterococcus*, *Weisella*, and *Streptococcus* significantly increased in post-heading oat silage ($p < 0.05$).

To identify key biomarkers in oat silage, bacterial communities in oat silage were subjected to LEfSe analysis with a linear discriminant analysis threshold of 2.0. Among the bacterial

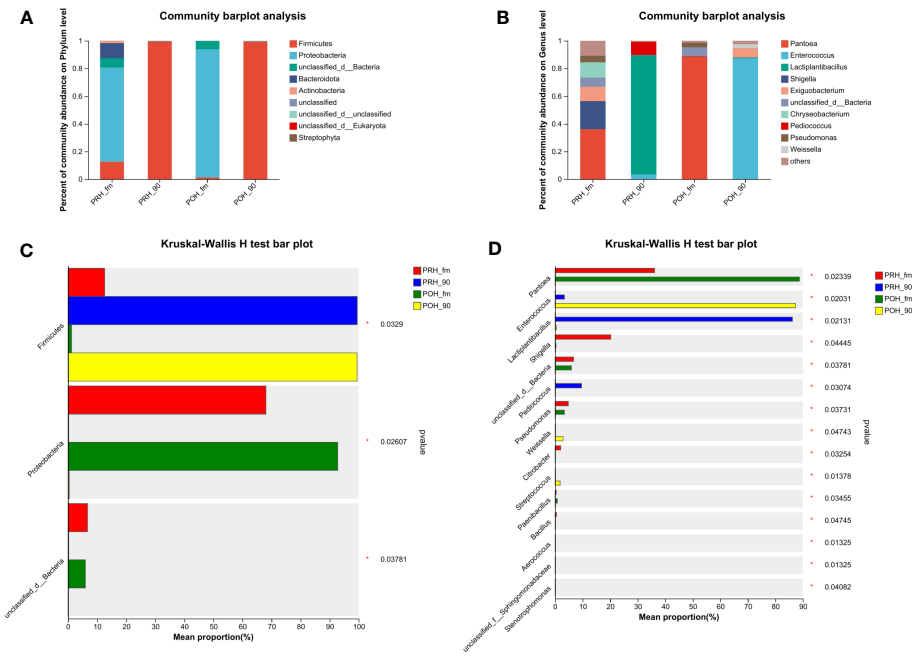

FIGURE 1
Relative abundance **(A, B)** and statistical comparison of relative abundance **(C, D)** of fresh and silage oat bacterial community at the phylum and genus levels. **(A)** Relative abundance at the phylum level. **(B)** Relative abundance at the genus level. **(C)** Statistical comparison of the relative abundance at the phylum level. **(D)** Statistical comparison of the relative abundance at the genus level. PRH-fm, the epiphytic microbiota of fresh oats at the pre-heading stage; PRH-90, the epiphytic microbiota of pre-heading oats after 90 days of fermentation; POH-fm, the epiphytic microbiota of fresh oats at the post-heading stage; POH-90, the epiphytic microbiota of post-heading oats after 90 days of fermentation.

community (Figures 2A, B), a total of 29 bacterial species, including notable genera such as *Bacillus*, *Lactiplantibacillus*, and *Enterococcus*, were identified as significantly different between the four groups. Three genera (*Bacillus*, *Kineococcus*, and *Pseudomonas*) in the PRH-fm group exhibited significant differences compared to the other three groups; two genera (*Pantoea* and *Paenibacillus*) in the POH-fm group exhibited significant differences compared to the other three groups; and two genera (*Lactiplantibacillus* and *Pediococcus*) in the PRE-90 group and (*Enterococcus* and *Weissella*) in the POH-90 group exhibited significant differences compared to the other three groups.

3.3 Relationships between nutritional components, fermentation products, and bacterial community

The Spearman correlation heatmap which reveals the associations between fermentative metabolites and the microbial composition in oat silage is presented in Figure 3. The results show that the primary microorganisms engaged in the fermentation process were *Curtobacterium*, *Planomicrobium*, *Aerococcus*, *Streptococcus*, *Exiguobacterium*, *Pediococcus*, and *Lactiplantibacillus*. The CP content exhibited a significant negative correlation with

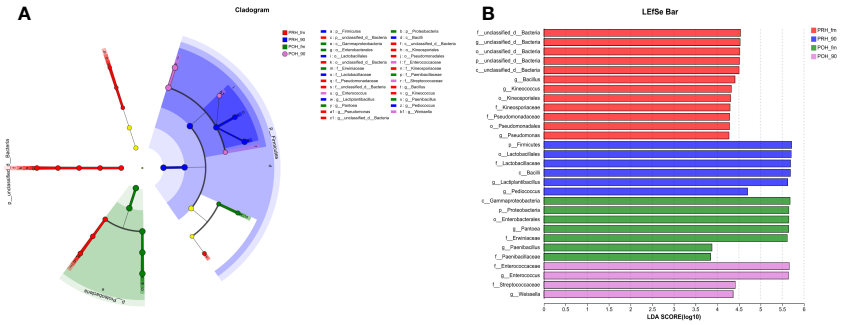

FIGURE 2
Evolutionary branch and LDA value distribution in fresh and silage oats harvested at different stages at the genus level OTU tables. **(A)** Evolutionary branch diagram of various species. The circles radiating from the inside to the outside represent classification levels ranging from phylum to genus. Different colored nodes indicate microbial taxa that are significantly enriched in the corresponding groups and that significantly affect the differences between groups. Light yellow nodes indicate microbial taxa that are not significantly different in any of the different groups or have no significant effect on the differences between groups. **(B)** LDA value distribution of different species (default score = 4). For LDA scores obtained by LDA analysis (linear regression analysis), the larger the LDA score, the greater the effect of species abundance on the differential effect. PRH-fm, the epiphytic microbiota of fresh oats at the pre-heading stage; PRH-90, the epiphytic microbiota of pre-heading oats after 90 days of fermentation; POH-fm, the epiphytic microbiota of fresh oats at the post-heading stage; POH-90, the epiphytic microbiota of post-heading oats after 90 days of fermentation.

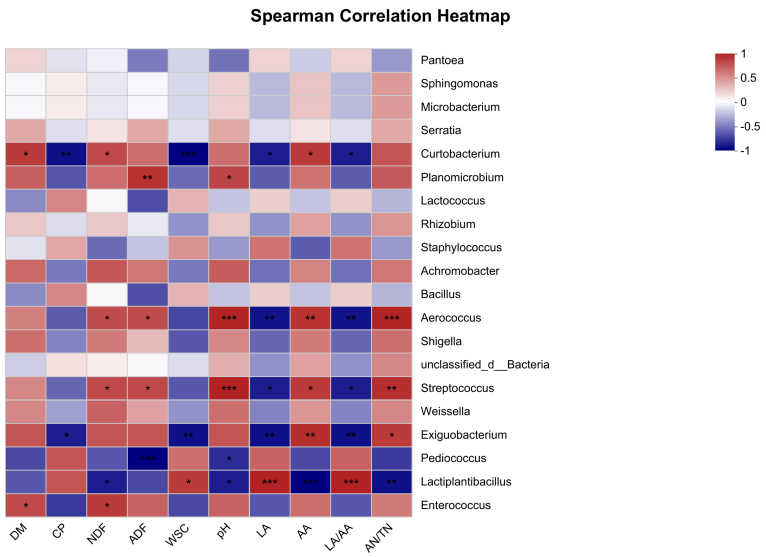

FIGURE 3
Spearman correlation heatmap of fermentative metabolites and bacterial community (top 20 genera) in oat silage fermented for 90 days. R-values are shown in different colors in the graph, with red indicating positive correlation ($0 < r < 1$) and blue indicating negative correlation ($-1 < R < 0$). The p-values are marked with * if $0.01 < p \leq 0.05$; **, $0.001 < p \leq 0.01$; ***, $p \leq 0.001$.

Curtobacterium at $p < 0.01$ and *Exiguobacterium* at $p < 0.05$. The NDF content showed a positive correlation with *Curtobacterium*, *Aerococcus*, *Streptococcus*, and *Enterococcus*, while a negative correlation was observed with *Lactiplantibacillus* ($p < 0.05$). At a significance level of $p < 0.001$, the ADF content exhibited positive correlations with *Planomicrobium*, *Aerococcus*, and *Streptococcus*, while showing a negative correlation with *Pediococcus*. The WSC content was negatively correlated with *Curtobacterium* at $p < 0.001$ and *Exiguobacterium* at $p < 0.01$, while a positive correlation was observed with *Lactiplantibacillus* at $p < 0.05$. *Aerococcus* and *Streptococcus* showed positive associations with pH ($p < 0.001$), AA ($p < 0.05$), and AN/TN ($p < 0.01$), while exhibiting negative associations with LA ($p < 0.05$). *Exiguobacterium* was negatively associated with LA ($p < 0.01$), while a positive correlation was observed with AA at $p < 0.01$ and AN/TN at $p < 0.05$. *Lactiplantibacillus* exhibited a negative association with pH at $p < 0.05$, AA at $p < 0.001$, and AN/TN at $p < 0.01$, while demonstrating a positive association with LA and LA/AA ($p < 0.001$). It was noteworthy that *Planomicrobium* exhibited a positive correlation with pH ($p < 0.05$), AA ($p > 0.05$), and AN/TN ($p > 0.05$), but was negatively associated with LA and LA/AA ($p > 0.05$), while the opposite was true for *Pediococcus*.

3.4 Changes in volatile metabolites of oat silage at pre-heading and post-heading stages

In the current study, an extensive volatile metabolomics analysis was performed on oat silage samples collected at both pre-heading and post-heading stages, leading to the detection of a total of 662 volatile metabolites (Supplementary Table 1), including 30 acids, 50 alcohols, 44 aldehydes, 15 amines, 31 aromatics, 122 esters, 2 ethers, 3 halogenated hydrocarbons, 98 heterocyclic compounds, 60 hydrocarbons, 60 ketone, 7 nitrogen compounds, 14 phenols, 5 sulfur compounds, 119 terpenoids, and 2 others.

Unsupervised PCA was used to assess differences in volatile metabolites after fermentation of oats harvested at different stages. PCA effectively distinguished the PRH-90 and POH-90 samples from the QC samples, as depicted in Figure 4C. Additionally, the data points of the QC group exhibited a high concentration, suggesting a high level of repeatability in the sample collection process. Moreover, the oat silage samples were categorized into distinct regions based on the first principal component (PC1) and the second principal component (PC2), underscoring the impact of fermentation. PC1 (57.49%) and PC2 (13.78%) collectively accounted for 71.27% of the total variance (Figure 4A). Notably, the PRH-90 and POH-90 groups were significantly separated, indicating the substantial alteration of volatile metabolites in silage oats induced by fermentation.

Hierarchical cluster analysis (HCA) was conducted on the accumulation patterns of metabolites across different samples, resulting in the generation of an overall clustering map of the samples (Figure 4B). The heatmap clearly illustrated the distinct profiles of PRH-90 and POH-90, emphasizing the significant influence of microorganism activities during fermentation on the metabolites of oat silage.

Figure 5A shows the radar chart of the top 10 sensory flavors selected for the highest number of annotations for the differential volatile metabolites and the annotated sensory flavor profiles obtained based on the screening criteria identified in the PRH-90 and POH-90 groups. The differential flavor metabolites were mainly enriched in sweet, green and fruity odors, with the most flavor substances indicating sweet odor reaching 56. Figure 5B shows the association network diagram of the top 10 sensory flavors with the

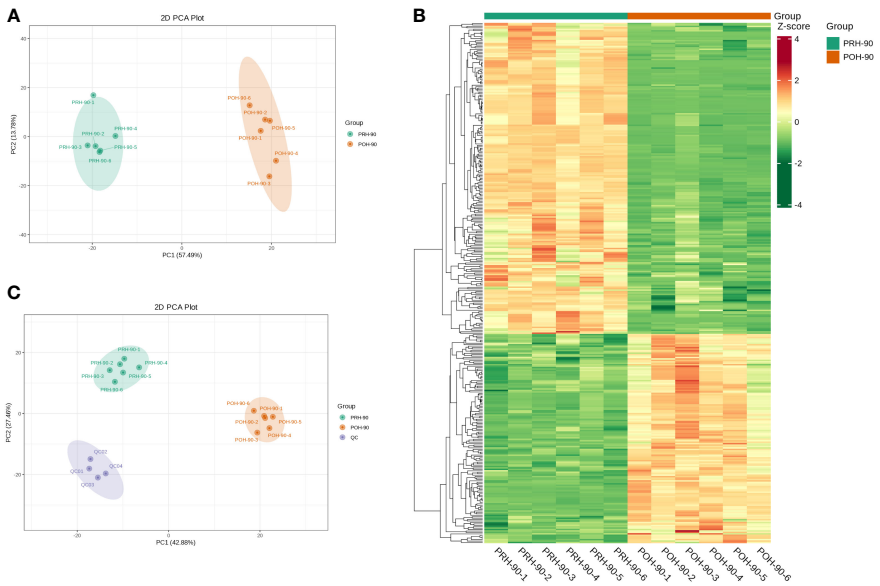

FIGURE 4
Multivariate statistical analysis of volatile metabolites from silage oats (PRH - 90 and POH - 90). **(A)** PCA scored plot of all silage oat samples. **(B)** Hierarchical cluster diagram of all silage oat samples. **(C)** PCA scored plot of all silage oat samples and the QC sample. PRH-90, volatile metabolites of pre-heading oats after 90 days of fermentation; POH-90, volatile metabolites of post-heading oats after 90 days of fermentation.

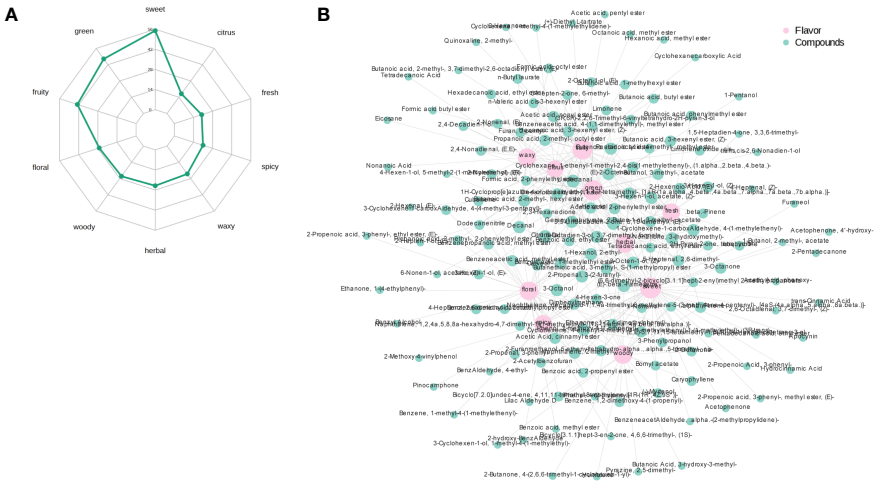

FIGURE 5
Radar chart of the sensory flavor profile of differential volatile metabolites and network chart of sensory flavor association with differential volatile metabolites. **(A)** Radar chart of the sensory flavor profile of differential volatile metabolites. The numbers corresponding to green dots are the number of differential metabolites annotated to that sensory flavor profile. **(B)** Network chart of sensory flavor association with differential volatile metabolites. The pink circle indicates the sensory flavor profile, the green circle indicates the differential metabolite, and the line between the two colored circles represents the differential metabolite annotated to that sensory flavor profile. When the number of annotated differential metabolites exceeds 50, the top 50 differential metabolites with the largest VIP values are displayed.

corresponding differential flavor metabolites. Sweet odor was associated with Benzeneacetic acid, 2-methylpropyl ester, 2-Propenoic acid, 3-phenyl-, 2-Buten-1-ol, 3-methyl-, acetate, etc. The odor of green was associated with 2-Nonenal, (E)-, 2-Propenal, 3-(2-furanyl)-, Butanoic acid, 2-methyl-, hexyl ester, etc. The odor of fruity was associated with 2-Buten-1-ol, 3-methyl-, acetate, Butanoic acid, 2-methyl-, hexyl ester, Formic acid, octyl ester, etc. The above were only the top few differential volatile metabolites with the highest VIP value annotated to a sensory flavor profile.

In order to elucidate the disparities in volatile metabolites between oats at different fermentation stages, orthogonal partial least squares discrimination analysis (OPLS-DA) was conducted. This analysis aimed to identify the different volatile metabolites in PRH-90 and POH-90 samples using the criteria of VIP > 1 and a *P*-value < 0.05. A total of 363 differential volatile metabolites were screened between the PRH-90 and POH-90 samples (Supplementary Table 2). Among them, 146 volatile metabolites were up-regulated while 217 volatile metabolites were down-regulated, and these differentially expressed metabolites were visualized in a volcano plot to depict the distribution of volatile metabolites with significant changes (Figure 6A). Figure 6B showed the grouping ring of differential metabolite classes between PRH-90 and POH-90. The classes that account for a relatively high proportion of the differential volatile metabolites were ester (19.28%), terpenoids (17.36%), heterocyclic compound (13.77%), ketone (9.64%), hydrocarbons (7.71%), alcohol (7.16%), aldehyde (7.16%), and acid (5.79%).

3.5 Changes in metabolic pathways during fermentation

Pathway enrichment analysis of 363 differential volatile metabolites was performed using the KEGG database. A total of 32 differential volatile metabolites were identified, distributed in 17 metabolic pathways. Subsequently, we performed KEGG pathway enrichment analysis (Figures 6C, D) to determine the differences in metabolic pathways between the two groups of samples. The results of KEGG pathway enrichment analysis demonstrated three major metabolic pathways: phenylpropanoid biosynthesis, phenylalanine metabolism, and biosynthesis of secondary metabolites.

3.6 Relationships between bacterial community and volatile metabolites

To clarify the effect of microbial activity on the flavor development of oats throughout fermentation, we screened the top 50 major differential volatile metabolites (VIP > 1 and *P*-value < 0.05) to obtain 28 flavor metabolites (Supplementary Table 3). Subsequently, Spearman correlation analysis was conducted to examine the correlation between the top 20 microorganisms in abundance and flavor metabolites (Figure 7). The results show that a total of 11 genera were related to the first 10 sensory flavor characteristics with higher annotations. In addition, 8 genera were associated with more than 9 flavor metabolites and 3 genera were associated with more than 4 flavor metabolites of less than 9 species.

Figure 7A illustrates the correlations between bacterial genus levels and the top 10 sensory flavor characteristics with extensive annotations. *Enterococcus* was positively correlated with green, herbal, waxy, and fresh odors ($p > 0.05$), while demonstrating a significant negative correlation with floral and spicy odors ($p < 0.05$). *Lactiplantibacillus* showed a positive correlation with spicy odor, but was negatively correlated with waxy and fresh odors ($p < 0.05$). *Pediococcus* showed a negative correlation with green ($p < 0.01$) and herbal ($p < 0.001$) odors. *Exiguobacterium* showed a positive correlation with green, waxy, and fresh odors ($p < 0.05$).

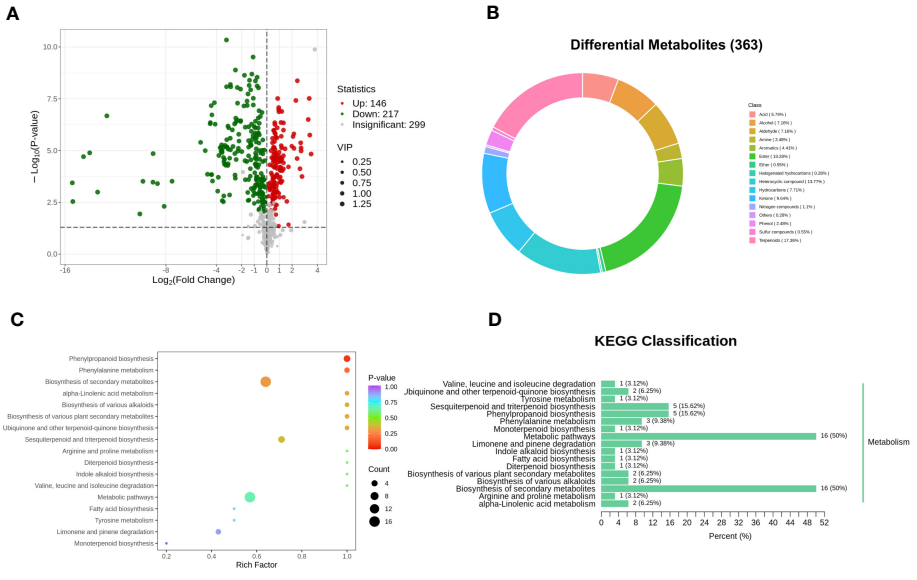

FIGURE 6
Differential volatile metabolites and metabolic pathways associated with differential volatile metabolites between PRH-90 and POH-90. **(A)** Volcano plot of differential volatile metabolites. **(B)** Grouping rings of differential volatile metabolites in terms of the number of classes. **(C)** Overview of KEGG pathway of the differential volatile metabolites. **(D)** Differential volatile metabolite KEGG classification plot.

Streptococcus showed a positive correlation with herbal and waxy odors, but was negatively correlated with floral odor ($p < 0.05$).

The correlations between bacterial genus levels and 28 flavor metabolites are shown in Figure 7B. *Enterococcus* showed a negative correlation with Pyrazine, 2,5-dimethyl-, 3-Octanol, 2-Propenal, 3-(2-furanyl)-, Butanethioic acid, 3-methyl-, S-(1-methylpropyl) ester, 2-Propenoic acid, 3-phenyl-, ethyl ester, (E)-, and 2-Buten-1-ol, 3-methyl-, acetate, but was positively correlated with Phenol, 4-(2-propenyl)-, Benzeneacetaldehyde,.alpha.-(2-methylpropylidene)-, 3-Hexen-1-ol, (Z)-, and Pyrazine, trimethyl- ($p < 0.05$). *Lactiplantibacillus* was positively correlated with TetraSulfur compounds, dimethyl, Butanoic acid, 2-methyl-, hexyl ester, Pyrazine, 2,5-dimethyl-, Isoquinoline, Butanoic Acid, 3-methyl-, and 2-Nonenal, (E)-, but negatively correlated with 2-Undecanone, 6,10-dimethyl-, Phenol, 4-(2-propenyl)-, and Ethanone, 1-(2,4-dimethylphenyl)- ($p < 0.05$). *Pediococcus* showed a positive correlation with Butanethioic acid, 3-methyl-, S-(1-methylpropyl) ester, 3-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, Phenol, 4-ethyl-2-methoxy-, 2-Propenoic acid, 3-phenyl-, ethyl ester, (E)-, and 2-Buten-1-ol, 3-methyl-, acetate, but was negatively correlated with 2-Undecanone, 6,10-dimethyl-, Benzeneacetaldehyde,.alpha.-(2-methylpropylidene)-, and 3-Hexen-1-ol, (Z)- ($p < 0.05$). *Exiguobacterium* was negatively correlated with 2-Acetylbenzofuran, Ethanone, 1-(4-ethylphenyl)-, Isoquinoline, Butanoic acid, 3-methyl-, 2-Nonenal, (E)-, and 3-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-, but negatively correlated with 2-Undecanone, 6,10-dimethyl-, Ethanone, 1-(2,4-dimethylphenyl)-, and 2,3-Dimethyl-5-ethylpyrazine ($p < 0.05$).

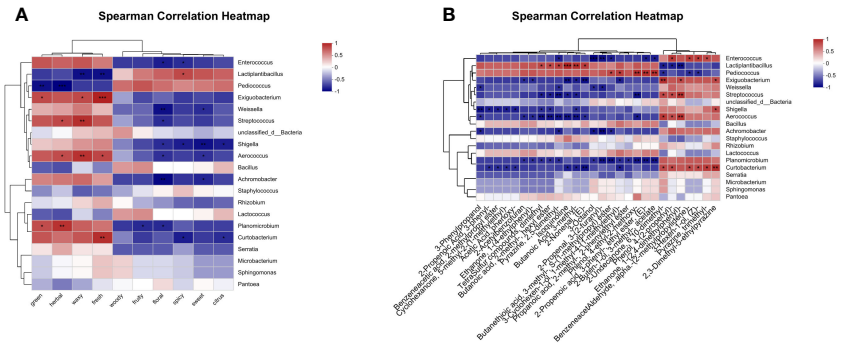

FIGURE 7
Correlation heatmap of bacterial community (top 20 genera) and differential metabolites after 90 days of fermentation. **(A)** Correlation between sweet, green, fruity, floral, woody, herbal, waxy, spicy, fresh, and citrus, as well as microorganisms. **(B)** Correlation between 28 flavor metabolites and microorganisms.

Streptococcus was negatively correlated with Tetrasulfur compounds, dimethyl, Butanoic acid, 2-methyl-, hexyl ester, Pyrazine, 2,5-dimethyl-, Isoquinoline, Butanoic acid, 3-methyl-, and Phenol, 4-ethyl-2-methoxy-, but positively correlated with 2-Undecanone, 6,10-dimethyl-, Phenol, 4-(2-propenyl)-, and Ethanone, 1-(2,4-dimethylphenyl)- ($p < 0.05$).

4 Discussion

Nutritional components, fermentation products, and microbial population differed in oats harvested at pre-heading and post-heading stages. As the fertility of oats increased, water content decreased and dry matter accumulation increased, which led to a gradual decrease in nutritional components, as indicated by the decrease in CP, NDF, and ADF contents. The increase of WSC content in post-heading oats may be due to the gradual accumulation of starch in the seeds as the plant matures (David et al., 2010). However, the WSC content in fresh oats at both stages was lower than the value suggested by Amer et al. (2012), and may not provide sufficient fermentation substrate for lactic acid bacteria, leading to slow growth and inability of lactic acid bacteria to dominate fermentation process. The microbial community attached to the raw material plays a crucial role in determining the quality of silage (Lin et al., 1992). In this study, a low number of lactic acid bacteria were found to be attached to fresh oats, but the count of aerobic bacteria was high, and a large number of coliform bacteria were attached to fresh oats, which are adverse for silage fermentation. Epiphytic populations of yeasts and molds were within the range of values typically reported before ensiling (Pahlow et al., 2003).

After ensiling, CP, NDF, ADF and WSC contents showed a decrease. The decrease in CP content is due to the hydrolysis of true proteins into peptide nitrogen, free amino acid nitrogen, NH3-N and non-protein nitrogen such as amines through the synergistic action of plant proteases and microbial enzymes (Bommert and Whipple, 2018). The decrease in NDF, ADF and WSC content was due to the decomposition of some cellulose and hemicellulose during silage fermentation, which were consumed as substrates together with WSC by microorganisms (Dean et al., 2005). The pH value serves as a crucial indicator of silage quality, providing insights into the preservation status and the degree of decomposition caused by undesirable microorganisms (Ren et al., 2022). In the present study, the pH level of pre-heading oat silage was significantly lower than that of post-heading oat silage (4.73 vs 5.57), which may be due to the high content of unfermentable components such as NDF and ADF in oats at the post-heading stage, which in turn affects the fermentation of oats by microorganisms. Fresh oats at the post-heading stage contained more miscellaneous bacteria, which led to the inability of lactic acid bacteria to dominate in the fermentation and high pH due to poor microbial fermentation. Correspondingly, compared to oats ensiled at the pre-heading stage, oat silage at the post-heading stage contained lower LA (30.43 g/kg DM) and higher AN/TN (9.06). The ratio of AN/TN in silage indicated the extent of protein and amino acid decomposition, and lower values of this ratio indicate less protein decomposition and better silage quality (Yuan et al., 2012). The differences in LA, AA, and AN/TN among the oat silages of both two stages in this study may be related to the type and number of microorganisms attached to the oats at the time of harvesting.

Further microbial sequencing analysis confirmed the experimental results. Throughout the fermentation process of 90 days, there was a shift in the dominant bacterial phylum from Proteobacteria and Bacteroidota to Firmicutes, which is a common occurrence in silage (Siran et al., 2020; Bai et al., 2023). This shift could be attributed to the acidic and anaerobic conditions during the ensiling process, which created a more favorable environment for the proliferation of Firmicutes (Keshri et al., 2018). The bacterial community composition at the genus level was significantly different between the two treatment groups, with *Lactiplantibacillus* dominating the PRH-90 group and *Enterococcus* dominating the POH-90 group. The reason for this finding may be due to the different raw materials and the different microorganisms attached to the raw materials. The PRH-90 group exhibited successful fermentation due to the significant role of *Lactiplantibacillus* in inhibiting the growth of undesirable microorganisms, reducing the ammoniacal nitrogen content, and enhancing the quality of the silage (Du et al., 2022). The POH-90 group contained more undesirable microorganisms on the raw materials and the pH dropped slowly, failing to provide the acidic environment needed for a well-fermented silage. Wang et al. (2019) reported that the naturally fermented treatment group exhibited a higher abundance of *Enterococcus*, which resulted in increased AA and NH_3-N content, while negatively impacting silage quality. Similarly, in this study, the POH-90 group had poorer silage quality due to the dominance of *Enterococcus* in the fermentation, resulting in lower LA content, and higher pH, AA content and NH_3-N/TN compared to the PRH-90 group.

Because the main process of silage fermentation is due to the activities of microorganisms, investigating the relationship between nutritional components, fermentation products, and microorganisms is vital for comprehending their role in the fermentation process. In the present study, a notable association was identified between eight genera and nutritional components, as well as fermentation products. The highest abundance of *Lactiplantibacillus* was found in the PRH-90 group and was positively correlated with WSC and LA, and negatively correlated with pH, AA and AN/TN. This showed that *Lactiplantibacillus* could better preserve WSC, increase LA, and lower pH and AN/TN, thus achieving good nutrient preservation and improving silage quality (Dong et al., 2022). Lower pH and AN/TN are usually better, as low pH could inhibit harmful microorganisms, and AN/TN could reflect the degree of protein degradation during silage process. Well-fermented silage feed generally has low pH and AN/TN (Filya, 2003). *Pediococcus* was the second most abundant genera detected in the PRH-90 group and also showed a negative correlation with pH, probably due to its synergistic effect with *Lactiplantibacillus*. Liu L. et al. (2022) reported that the dominance of *Lactiplantibacillus* on day 31 of the fermentation process was accompanied by a significant rise in the abundance of *Pediococcus*. In the POH-90 group, *Enterococcus* was the most

abundant genera, but there was no significant association with indicators of silage quality. Cai (1999) inoculated samples with *Enterococcus* as an additive and found that *Enterococcus* did not improve the fermentation quality of the silage. The detection of *Exiguobacterium*, *Streptococcus*, and *Aerococcus* in the POH-90 group samples showed a positive correlation with the level of AN/TN, while *Streptococcus* and *Aerococcus* also showed a positive correlation with pH, which may explain the higher levels of pH and AN/TN in the POH-90 group compared to the PRH-90 group.

The odor of silage could reflect the quality of silage more directly, and volatile metabolomics has been widely used to detect flavor chemistry (Qualley and Dudareva, 2009). Ester and terpenoids were found to be the main volatiles of silage in this study, which is consistent with other studies (Di Cagno et al., 2017; Weiss, 2017). Analysis of differential volatiles in the PRH-90 and POH-90 groups detected a total of 363 differential volatiles, indicating that the flavors of the PRH-90 and POH-90 groups were significantly different, which is consistent with the PCA results, where the two groups were clearly separated, implying that the two groups were significantly different. Compared with the PRH-90 group, the content of many esters in the POH-90 group decreased significantly, while flavor substances indicating sweetness and fruitiness, such as Benzoic acid, ethyl ester, 1-Butanol, 3-methyl-, acetate, Pentanoic acid, 4-methyl-, methyl ester, Formic acid, octyl ester, and Benzeneacetic acid, 2-methylpropyl ester, which could make the fermentation more intense in terms of fruity and floral odors, were the main contributing components to the aroma of the silage (Shukui et al., 2007; Fernando et al., 2008; Sympoura et al., 2009; José, 2014). Terpenoids have been previously associated with citrus and pine odors, and an increase in the content of these compounds may enhance the fragrance (Beaulieu et al., 2015). The content of terpenoids with citrus and pine odors (2,6-Octadienal, 3,7-dimethyl-, (Z)-, Limonene oxide, cis-, 2,7-Octadien-4-ol, 2-methyl-6-methylene-, (S)-, etc.) in the POH-90 group also decreased. In addition, there was a decrease in flavor substances among acids, alcohols, ketones, aldehydes and heterocyclic compounds contributing components to the aroma. KEGG pathway enrichment analysis demonstrated metabolic pathways that may be associated with differential metabolite synthesis. The most significant metabolic pathways included phenylpropanoid biosynthesis, phenylalanine metabolism, and biosynthesis of secondary metabolites. The phenylpropanoid biosynthesis pathway is related to phenolic acid substances, and phenylalanine metabolism is an important metabolic pathway in the winemaking process and is connected to the generation of aromatic compounds (Fairbairn et al., 2017; Wang et al., 2022). In this experiment, the content of substances such as Phenol, 2-methoxy-4-(1-propenyl)-, trans-Cinnamic acid, and 2-Propenoic acid, 3-phenyl-, which represent sweet and floral odors, decreased in the POH-90 group. These substances are usually influenced by the phenylpropanoid biosynthesis and phenylalanine metabolism pathway. The biosynthesis of secondary metabolites pathway was related to the synthesis of terpenoids, and the large number of terpenoids detected in this experiment may have been influenced by the biosynthesis pathway of secondary metabolites (De et al., 2011). These findings could potentially elucidate the variation in odors observed between the PRH-90 and POH-90 groups following fermentation.

The formation of metabolites in silage is intricately linked to the presence and activities of microorganisms, and this experiment further analyzed the relationship between the dominant microorganisms (top 20 genera) and odors as well as the main differential flavor substances. The dominant genera (*Lactiplantibacillus* and *Pediococcus*) in the well-fermented PRH-90 group were positively correlated with odors that mostly indicated good flavor such as sweet, fruity, floral, and citrus, while the dominant genera (*Enterococcus*, *Exiguobacterium*, *Weisella*, and *Streptococcus*) in the poorly fermented POH-90 group were negatively correlated with odors that indicated poor flavor such as green, herbal, waxy and fresh, indicating that microorganisms play a pivotal role in shaping the final flavor of silage through their activities during the fermentation process. Butanoic acid, 2-methyl-, hexyl ester, 2-Nonenal, (E)- and 2-Propenoic acid, 3-phenyl-, ethyl ester, (E)- have been reported as aroma substances with a large impact on flavor (Palma-Harris et al., 2001; Young et al., 2004; Pu et al., 2021), and in this experiment they may have been influenced by the fermentation of *Lactiplantibacillus* and *Pediococcus*, and had an important effect on the formation of fruit odor in silage. 2-Propenoic acid, 3-phenyl-, ethyl ester, (E)- and 2-Buten-1-ol, 3-methyl-, acetate have been reported as aroma substances that, contribute significantly to the fermentation consequent aroma, and their synthesis may have been promoted by the fermentation of *Pediococcus* in this experiment, while their synthesis may have been inhibited by the fermentation of *Enterococcus*. Previous studies have shown that *Lactiplantibacillus* and *Pediococcus* contribute to the synthesis of aroma substances during fermentation, suppress undesirable odors and improve the flavor of fermented products, while the fermentation process dominated by *Enterococcus* could produce undesirable odors (Miller et al., 2018; Huipeng et al., 2020; Zhen et al., 2020). However, our results could not fully elucidate the role of microorganisms in the metabolism of flavor substances, and the metabolic mechanisms of flavor substances and the role of microorganisms in them need to be further explored and elucidated.

5 Conclusion

To investigate the changes in silage quality and flavor of oats harvested at pre-heading and post-heading stages, microbial diversity and volatile metabolites were analyzed throughout the oat fermentation process. The results indicated that PRH-90 group was dominated by *Lactiplantibacillus* with lower pH and AN/TN and better fermentation quality compared to POH-90 group, while POH-90 group was dominated by *Enterococcus* with higher pH and AN/TN and poorer fermentation quality compared to PRH-90 group. Esters and terpenoids were compounds that significantly contributed to flavor during oat fermentation. The differences in flavor between the two groups of oats after ensiling were mainly concentrated in sweet, green, and fruity odors. Changes in the relative abundance of major flavor metabolites were highly correlated with microorganisms. *Lactiplantibacillus* and *Enterococcus*, as the predominant genera in the oat fermentation process, are considered to contribute significantly to the flavor quality of silage oats. The results of this study provide insights into

the relationship between microorganisms and flavor development during oat fermentation, and help to further understand the regulatory mechanisms of silage flavor formation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: SRA data: PRJNA1005624 (including 12 SRA (sequence read archive) accession numbers: SRX21369993- SRX21370004).

Author contributions

XD: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, Visualization, Writing – original draft. YJ: Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. GG: Methodology, Project administration, Supervision, Writing – review & editing. ZW: Methodology, Writing – review & editing. ML: Methodology, Writing – review & editing, Conceptualization, Resources, Supervision. JB: Methodology, Writing – review & editing. MZ: Methodology, Writing – review & editing. QS: Methodology, Writing – review & editing. YL: Methodology, Writing – review & editing. WZ: Methodology, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1278715/full#supplementary-material

SUPPLEMENTARY TABLE 1
Volatile metabolites detected by GC-MS.

SUPPLEMENTARY TABLE 2
Differential volatile metabolites detected by GC-MS.

SUPPLEMENTARY TABLE 3
The flavor substances in the top 50 differential metabolites identified by VIP (VIP > 1.0) and P-value (P-value < 0.05).

SUPPLEMENTARY TABLE 4
Flavor substances contained in the top 10 highest number of sensory flavor profiles annotated to differential metabolites identified by the comparison group based on screening criteria.

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The performance of plant essential oils against lactic acid bacteria and adverse microorganisms in silage production

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Plant essential oils have played an important role in the field of antibiotic alternatives because of their efficient bacteriostatic and fungistatic activity. As plant essential oils are widely used, their activity to improve the quality of plant silage has also been explored. This review expounds on the active ingredients of essential oils, their bacteriostatic and fungistatic activity, and mechanisms, as well as discusses the application of plant essential oils in plant silage fermentation, to provide a reference for the development and application of plant essential oils as silage additives in plant silage fermentation feed.

KEYWORDS

essential oil, active ingredient, bacteriostatic, fungistatic, silage

1 Introduction

Considerable attention has been paid to the application of plant extracts in livestock and poultry production as alternatives to banned additives such as antibiotics. Plant extracts is a mixture of natural compounds or components extracted from plant materials. Due to the presence of numerous bioactive compounds with pharmacological properties, they have great potential for research. Moreover, they are considered a sustainable and eco-friendly choice due to their natural, biodegradable nature, and their ability to reduce reliance on synthetic chemicals. Huge scientific studies regarding the application of plant extracts in silage preservation have reported the potential antifungal agents from this enriched flora (Cock and Van Vuuren, 2015), Aloe vera extract has a wide range of microbial growth inhibition activities, and it has been reported to have a significant inhibitory effect on the mycelial growth and spore germination of *Penicillium italiana* (Zapata et al., 2013). The organic easy extract of tea plant contains a variety of natural non-ionic surfactants, which can cooperate with some antibacterial agents to antagonize fungi (Hao et al., 2010). Some studies have reported that the ethanol extract of *Ficus hirta Vahl*

exhibits a fungistatic effect against *Penicillium tilikum* (Wan et al., 2017). As an important class of plant extracts, an increasing number of studies have shown that plant essential oils have significant antibacterial activity, which makes them more attractive to researchers. Commonly used plant essential oils include thyme essential oil, clove essential oil, cinnamon essential oil, oregano essential oil, mint essential oil, and curcumin essential oil. At present, plant essential oils are primarily used to maintain animal health, improve animal performance, and enhance the quality of livestock products in the breeding industry. With the deepening of research on essential oils, the function of plant essential oils in improving the fermentation quality of feed silage has been explored (Matté et al., 2023).

2 Main active component of plant essential oils

Plant essential oil is a chemical substance extracted from the bark, peel, leaves, buds, seeds, flowers, and other parts of plants by steam distillation, solvent-assisted extraction, hydrogenation distillation, ultrasonic-assisted extraction, supercritical fluid extraction, and solvent-free microwave extraction (Kant and Kumar, 2022). The active components of plant essential oil are divided into four categories in accordance with their structure: terpene compounds, aromatic compounds, aliphatic compounds, and sulfur-containing and nitrogen-containing compounds (Shao et al., 2020).

Terpene compounds are the most common chemical components in essential oils. They are generally chain or cyclic olefins with the general formula $(C_5H_8)_n$ Figure 1; PubChem, such as menthol (a), menthone, neomenthol, and isomenthone in peppermint essential oil (Zhao et al., 2022); α-terpinene (b) and myrcene in rosin essential oil (Couladis et al., 2003); carvacrol (c) and eugenol (d) in oregano and clove essential oil (Milovanović et al., 2009); and eucalyptol in rosemary essential oil (Jiang et al., 2011). The second common chemical component is aromatic compounds, which are a class of compounds with a benzene ring structure related to the fragrance of essential oils, such as cinnamaldehyde (e) and cinnamic alcohol (f) in cinnamon essential oil (Vasconcelos et al., 2018), as well as thymol (g) in thyme essential oil (Kim et al., 2022). Aliphatic compounds, which have the smallest relative molecular mass but are widely present in plant essential oils, are organic compounds composed of hydrocarbon chains or their derivatives, such as n-decyl alcohol (h) and leaf alcohol (i). Sulfur and nitrogen-containing compounds, such as allitride (j) and indole (k), are present in small amounts, but t hese compounds have extremely strong odors and characteristic aromas (Saad et al., 2013).

3 Bacteriostatic and fungistatic activity and mechanism of plant essential oils

The bacteriostatic and fungistatic effect of common plant essential oils such as thyme, clove, cinnamon, oregano, peppermint, and curcumin is related to their active ingredients. Each active ingredient exerts its bacteriostatic and fungistatic effect through its functional groups independently or synergistically. Therefore, the bacteriostatic and fungistatic mechanisms of different types of essential oils are distinct (Evangelista et al., 2022).

3.1 Bacteriostatic and fungistatic activity of common essential oils

3.1.1 Main bacteriostatic and fungistatic components and activities of essential oils

The common bacteriostatic and fungistatic active ingredient in thyme essential oil is thymol, chemically known as 5-methyl-2-

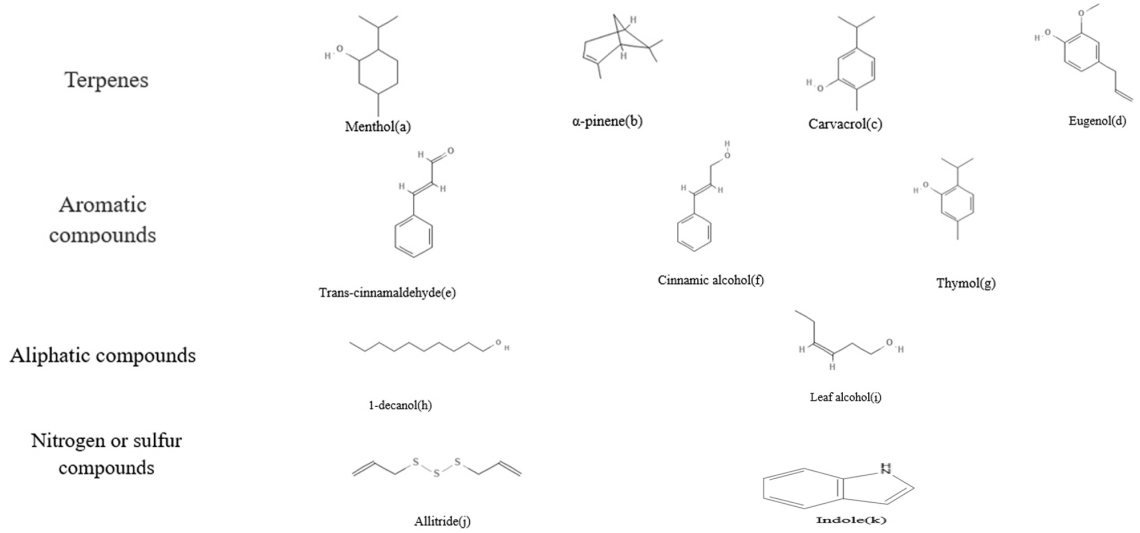

FIGURE 1
Chemical structure formula of plant essential oils.

isopropylphenol, which is a natural monoterpene. Thymol has the strongest inhibitory effect against molds, and 0.01–0.05 mg/mL of thymol is found to be effective against molds such as *Aspergillus niger*, *Aspergillus flavus*, and *Aspergillus fumigatus*. In addition, thymol can effectively inhibit Gram-positive pathogens such as *Bacillus subtilis* and *Staphylococcus aureus* between 0.06 and 0.2 mg/mL, while the inhibitory concentration for lactic acid bacteria must reach 0.1–0.5 mg/mL (Marchese et al., 2016). Another highly effective bacteriostatic and fungistatic active substance in thyme essential oil is terpinene, which can exhibit an bacteriostatic and fungistatic effect equivalent to 70% of streptomycin at a concentration of 0.07 mg/mL against *Phytophthora capsici* (Yang et al., 2022). Thyme essential oil (*Thymus kotschyanus*), as a mixture of multiple bacteriostatic and fungistatic active substances, has a minimum inhibitory concentration of 0.0625 mg/mL against common molds such as *A. niger*, *A. flavus*, and *Fusarium oxysporum* in silage feed or raw materials (Ownagh et al., 2010), and the minimum inhibitory concentration for yeast is 0.5 mg/mL. The inhibitory concentration for some lactic acid bacteria such as *Lactobacillus brevis*, *Lactobacillus plantarum*, and *Lactococcus lactis* must reach at least 3–10 mg/mL (*Thymus vulgaris ct.linalol*, *Thymus serrulatus* and *Thymus schimperi Ronniger*) (Gutierrez et al., 2008; Damtie and Mekonnen, 2020; de Oliveira Carvalho et al., 2020). Thus, whether the active ingredient is single or a mixture in thyme essential oil, it shows low inhibitory concentration against pathogens and high inhibitory concentration against lactic acid bacteria. This selective inhibition laid the foundation for its use in silage feed (Damtie and Mekonnen, 2020).

The main active ingredient in clove essential oil is eugenol, chemically known as 4-allyl-2-methoxyphenol, which belongs to the terpene class of compounds and has a broad-spectrum antibacterial activity against Gram-negative and Gram-positive bacteria (Khalil et al., 2017). The minimum inhibitory concentration of eugenol for Gram-positive bacteria such as *S. mutans* and *S. aureus* is generally between 0.1 and 0.2 mg/mL. Its minimal inhibitory concentrations against Fusarium species commonly found in silage, such as *Fusarium avenaceum*, *Fusarium graminearum* and *Aspergillus nidulans*, ranged from 0.1 mg/mL to 0.14 mg/mL. The minimum inhibitory concentration against *Saccharomyces cerevisiae* is 0.25 mg/mL, whereas the minimum inhibitory concentration for lactic acid bacteria such as *Lactobacillus casei* must reach 1 mg/mL (Marchese et al., 2017). Low levels of eugenol (below the minimum inhibitory concentration) show strong inhibitory activity against the expression of the physiological functions of microorganisms and the production of toxins such as ochratoxin (Jiang et al., 2022). Clove essential oil also exhibits a remarkable bacteriostatic and fungistatic activity, with a minimum inhibitory concentration of 0.2–0.3 mg/mL for yeast and a minimum inhibitory concentration of 0.031, 0.031, and 0.25 mg/mL for fungi such as *F. graminearum*, *Rhizopus stolonifer*, and *Penicillium crustosum*, respectively, which is lower than its minimum inhibitory concentration for lactic acid bacteria such as *Lactobacillus fermentum* and *Lactobacillus lactis* (*Syzygium aromaticum (L.) Merr. and L. M. Perry*) (Sameza et al., 2016; Sharma et al., 2017; Chan et al., 2018). The low inhibitory concentration of clove essential oil against harmful microorganisms and high inhibitory concentration against lactic acid bacteria determines its use in later production.

The bacteriostatic and fungistatic activity of cinnamon essential oil is derived from cinnamaldehyde, which has the strongest bacteriostatic and fungistatic activity among other secondary metabolites. Cinnamaldehyde has shown antifungal activity against *Candida* (Choonharuangdej et al., 2021), *A. flavus* (Achar et al., 2020), *F. graminearum* (Gwiazdowska et al., 2022), *Aspergillus ochraceus* (Wang et al., 2018), and other fungi, as well as broad-spectrum antifungal activity against *Escherichia coli* and *S. aureus* (Zhang et al., 2016). Compared with other active components of plant essential oil, cinnamaldehyde has the strongest inhibitory activity on the synthesis of aflatoxin and ochratoxin. The minimal inhibitory concentration of cinnamaldehyde against *F. oxysporum* and *F. gramineum* is 0.8 mg/mL, and its minimal inhibitory concentration against spoilage yeast is 0.31–1.25 mg/mL (Muñoz-González et al., 2022). However, its minimal inhibitory concentration against lactic acid bacteria varies, with a minimal inhibitory concentration of 5 mg/mL against *Lactobacillus sakei* and a minimum inhibitory concentration of 50 mg/mL against *Lactobacillus brucei* (Sameza et al., 2016; Sharma et al., 2017; Muñoz-González et al., 2022). Cinnamon essential oil also shows good application effects. Cinnamon essential oil inhibits *E. coli*, *S. aureus*, and *Listeria* at a concentration of 0.3–0.5 mg/mL (*Cinnamomum zeylanicum*) (Nematollahi et al., 2020), and its minimum inhibitory concentration against *Penicillium citrinosus* and *Penicillium expandatum* is between 0.4 and 0.5 mg/mL (*Cinnamomum cassia*) (Lucas-Gonzalez et al., 2023). However, the minimum inhibitory concentration against lactic acid bacteria is approximately 10 mg/mL (*C. zeylanicum*) (de Oliveira Carvalho et al., 2020).

The main active ingredients in oregano essential oil are carvacrol and thymol. Thymol has been elaborated in the previous section, and carvacrol is also a class of phenolic compounds with strong bacteriostatic and fungistatic activity. The minimum inhibitory concentration of carvacrol against *S. aureus* is 0.3 mg/mL; the minimum inhibitory concentration of carvacrol against *Streptococcus* is 2.5 mg/mL, and its minimum inhibitory concentration against Gram-negative bacteria such as *E. coli* and *Salmonella typhimurium* is less than 0.25 mg/mL (Marinelli et al., 2018; Rathod et al., 2021). The minimum inhibitory concentration against a variety of yeasts is approximately 0.2 mg/mL. On the contrary, oregano essential oil containing a mixture of carvacrol and thymol shows considerable synergistic bacteriostatic and fungistatic effects. Its minimum inhibitory concentration against a variety of *Fusarium species* is less than 0.8 mg/mL, and its antifungal activity is mostly between 0.2 and 0.3 mg/mL, whereas its minimum inhibitory concentration against a variety of lactic acid bacteria is between 5.5 and 13 mg/mL (*Origanum vulgare L.*) (Gutierrez et al., 2008; Konuk and Ergüden, 2017; Sharma et al., 2017). The bacteriostatic and fungistatic activity of oregano essential oil against lactic acid bacteria is lower than that against pathogenic fungi. Furthermore, oregano essential oil can inhibit the biosynthesis of aflatoxin after it acts on aflatoxin (*Origanum majorana L.*) (Chaudhari et al., 2020).

Peppermint essential oil has been used as a preservative in the food industry for a long time, and its bioactive substances have good

effects on inhibiting pathogenic bacteria and spoilage microorganisms, such as menthol, menthone, and neomenthol (Kazem et al., 2011). A number of reports have shown that peppermint essential oil inhibits the cell activity of *S. aureus* (Kang et al., 2019), *Candida albicans*, *Pseudomonas aeruginosa* (Mahboubi and Kazempour, 2014), *Helicobacter pylori*, and *Salmonella enteritidis* (Imai et al., 2001). The *in vitro* anti-aflatoxin concentration of menthol is 1.0 mg/mL, while menthol stereoisomers and menthone do not show evident antitoxin function, which is related to their structural types (Ownagh et al., 2010; Muñoz-González et al., 2022). Peppermint essential oil has weaker bacteriostatic and fungistatic activity than menthol. Peppermint essential oil has a minimum inhibitory concentration of 62 mg/mL against a variety of mold species and more than 150 mg/mL against lactic acid bacteria, but it has considerable bacteriostatic and fungistatic activity against *S. cerevisiae*, with a minimum inhibitory concentration of 1 mg/mL. The results show that antifungal sensitivity to mold and yeast is higher than sensitivity to lactic acid bacteria. (*Mentha Piperita*) (de Oliveira Carvalho et al., 2020).

Turmerone is a glycosides active substance derived from turmeric essential oil. It mainly includes ar-turmerone and β-turmerone. Given its special structure, it has strong antibacterial and antifungal activity. At a concentration of 0.1% ar-turmerone, this purified compound reduced the growth of *Fusarium semitectum*, *Aspergillus ochraceous*, and *Calletotrichum musae* by 70%, 55%, and 68%, respectively (Dhingra et al., 2007). At 1 mg/disk, ar-turmerone strongly inhibited the growthl of C perfringens and moderately inhibited the growth of *E. coli* without any adverse effects on the growth of four lactic acid bacteria(Lee, 2006). Given the synergistic effect of various bacteriostatic and fungistatic active substances in turmeric essential oil, the bacteriostatic and fungistatic activity of the mixture is remarkably enhanced. The minimum inhibitory concentration of turmeric essential oil to various yeasts is between 0.01 and 0.03 mg/mL (*Curcuma longa L.*) (Konuk and Ergüden, 2017), and the concentration of turmeric essential oil has a remarkable inhibitory effect on Fusarium verticillium at 0.072 mg/mL (*C. longa*) (Avanço et al., 2017). However, the minimum inhibitory concentration against Lactobacillus is as high as 11–14.5 mg/mL (*C. longa*) (Khorsandi et al., 2018).

All of the above mentioned essential oils show strong inhibition against yeast, fusarium, and other pathogenic bacteria but relatively weak inhibition against lactic acid bacteria. This unique bacteriostatic and fungistatic property of essential oil contributes to the inhibition of spoilage bacteria in silage and to the improvement of silage quality.

3.1.2 Synergistic bacteriostatic and fungistatic activity of essential oils

The synergistic bacteriostatic and fungistatic effects of plant oils have been gradually recognized with the exploration of their bacteriostatic and fungistatic properties. The bacteriostatic and fungistatic activity of plant essential oils results from interactions among different components, often by terpenoid compounds with the strongest internal bacteriostatic and fungistatic properties, as well as by a variety of compounds of different classes,For instance, there are interactions between compounds such as citral and thujone, camphor, ethyl acetate of borneol, and citronellal. Similarly, interactions between α-pinene and thujone, camphor, citral, citronellal, and geraniol occur. Moreover, the interaction of cineole oxide with compounds like thujone, hexanal, and hinokitiol also demonstrates a synergistic enhancement of cytotoxicity(Wright et al., 2007), and the fractional inhibitory concentration of each component against bacteria is markedly reduced. The mixed use of eugenol and linalool in basil essential oil shows higher bacteriostatic, fungistatic and antioxidant activity to some fungi and bacteria than each component itself (Juliani et al., 2009). The bacteriostatic and fungistatic active components of cinnamon essential oil, cinnamaldehyde and cinnamic acid, also show bacteriostatic and fungistatic properties when combined. The combination of components derived from different essential oils also shows synergistic effects, such as the chimerism of cinnamaldehyde and carotenoids to form a mixture of solid and liquid fats to cause defects in the structure of the lipid matrix and improve the bioavailability and retention ability of active ingredients (Procopio et al., 2022). The combination of cinnamon essential oil and clove essential oil also has synergistic effects, and the enhanced essential oil remarkably inhibits biofilm formation, destroying the cell wall structure and scavenging free radicals (Yen and Chang, 2008). The same study found that the combination of thyme and rosemary as well as the triple combination of thyme, rosemary, and cinnamon show synergistic effects on *Bacillus cinesulata* and *Pseudomonas ectrosp* (Nikkhah et al., 2017). The combination of oregano and thyme essential oils, bay and almond essential oils, and basil and thyme essential oils all showed synergistic bacteriostatic and fungistatic effects, with minimum inhibitory concentrations reduced by up to 128 times.

3.2 Bacteriostatic and fungistatic mechanism of plant essential oils

Most of the current studies have shown that the main bacteriostatic and fungistatic mechanism of plant essential oils is achieved by destroying the integrity of biofilm, destroying and inhibiting cell wall biosynthesis, damaging membrane proteins, and inhibiting mitochondrial function.

3.2.1 Destruction of biofilm integrity

The bacteriostatic and fungistatic effects of essential oils are directly related to their lipophilicity. The six-carbon aromatic phenol group of cinnamaldehyde allows it to cross the phospholipid bilayer of the bacterial cell wall and bind to the inner and outer membrane proteins, thereby preventing them from performing their normal function. Altered membrane permeability and loss of functional proteins that transport molecules and ions perturb microbial cells, which leads to cytoplasmic coagulation, enzymatic denaturation, and loss of proteins, as well as loss of metabolites and ions (Suxia Shen et al., 2015). Cell membrane damage by essential oil is usually manifested as the change in cell membrane surface structure (usually wrinkled

or irregular), the increase of relative electrical conductivity, the decrease of membrane potential, the increase of extracellular nucleic acid and protein concentration, the change of membrane potential and conductivity, and the accumulation of intracellular active components of essential oil, which leads to the acidification of the cell membrane and the damage of the cell membrane caused by protein denaturation (Trinh et al., 2015).

Cellular ion leakage is also an important bacteriostatic and fungistatic mechanism of plant essential oils. The α- and β-unsaturated bonds of various cinnamaldehydes can be conjugated to the plasma membrane calcium ATPase on the fungal plasma membrane, which opens the ion pathway, induces Ca^{2+} efflux, and inhibits fungal activity (Hu et al., 2013). Under ergosterol inhibition, celery essential oil acts on the cell membrane of *A. flavus* to take part in the α-demethylation of lanosterol, and Ca^{2+} leakage is observed. Mg^{2+} influx is enhanced, leading to the depletion of nutrient uptake, inhibition of nucleic acid synthesis, and inhibition of ATPase-dependent respiratory activity, thereby leading to cell lysis (Das et al., 2019). *P. capsici Leonian* and *A. flavus*, which have no ergosterol production ability, and ethylene glycol bis (2-aminoethyl ether) tetraacetic acid elution treatment were used to exclude the influence of exogenous Ca^{2+}. The cells showed Ca^{2+} outflow and ergosterol reduction after treatment with cinnamon essential oil, fenestra essential, oil and peppermint essential oil (Dwivedy et al., 2017). Ca^{2+} is the most prevalent regulator in the whole living system, which is involved in the regulation of the proliferation, differentiation, and apoptosis of organisms. In fungi, Ca^{2+} regulates spore formation, spore germination, hyphal branching, apical growth, and structural differentiation (Shreaz et al., 2016). The disorder of Ca^{2+} may interfere with the amount of circulating calcium ions in the mitochondria and activate the mitochondrial permeability transition pore, thereby leading to the initiation of the fungal apoptosis program (Berridge et al., 2000).

3.2.2 Reduction of quorum sensing

Quorum sensing (QS) is a feedback intercellular communication system of bacteria based on the secretion and sensing of external signaling molecules. The formation of biofilms is highly correlated with density-dependent QS propagation, which affects the production of bacterial secondary metabolites and regulates the secretion of virulence factors. Bacteria in the form of biofilms are different from planktic bacteria because their association with abiotic surfaces generates a three-dimensional organizational structure that is protected from the threat of being killed by fungicides, disinfectants, and antibiotics. Bacterial cells produce extracellular polymeric substances, proteins, and extracellular DNA, which support structural stability and improve substrate exchange and nutrient cycling in biofilms (Tan et al., 2019). The inhibition of intercellular communication between bacteria and biofilm formation is considered as an bacteriostatic and fungistatic pathway of plant essential oils (Bo et al., 2023). After applying cinnamaldehyde to the biofilm, the hydrophobicity of the cell membrane decreases, resulting in the reduced adhesion of the hydrophobic surface to the biofilm surface. Aggregation also decreased, whereas self-aggregation formed the complete biological structure of several different biofilm strains, which played an important role in biofilm stability (Yu et al., 2020). Molecular docking analysis showed that cinnamaldehyde downregulated the expression of cellulose synthase (BcsA) and transcription activator protein (luxR) receptor genes, thereby inhibiting the synthesis of signaling molecules in QS (Liu et al., 2021). Diallyl disulfide in garlic essential oil inhibits the virulence factors of *P. aeruginosa* at MIC concentrations by affecting the transcription of key genes in three different QS systems (Li W.-R. et al., 2018). Carvacrol binds to homoserine lactone synthase (Expl) and transcriptional regulators (Khan et al., 2017), which in turn inhibits the production of QS signaling molecules and the expression of QS-controlled genes (Joshi et al., 2016). Eugenol inhibits protease, pyocyanin, pyranan biosynthesis, extracellular polysaccharide, and rhamnolipid and closely binds to the synthesis of carbonyl N-coa acylation regulatory protein (LasR) by *P. aeruginosa*, thereby leading to the inhibition of QS (Rathinam et al., 2017). Cyclic diguanosine monophosphate (C-di-GMP) is considered as a key cytoplasmic signal and second messenger that controls bacterial virulence, cell cycle reproduction, motility, and other behaviors, such as the biofilm life cycle in several bacteria. Cinnamaldehyde carbon can affect the level of nitric oxide by regulating C-di-GMP, thereby inducing biofilm dispersion (Topa et al., 2018), whereas the ginger essential oil test found that cinnamaldehyde carbon promoted the degradation of proteins with EAL (*Glu-Ala-Leu*) or HD-GUP (*His-Asp-Gly-Tyr-Pro*) domains. This domain is directly associated with C-di-GMP levels, thereby inhibiting biofilm formation (Kim and Park, 2013).

3.2.3 Inhibition of cell wall formation

The cell wall is an important structure in fungi, indispensable for maintaining shape integrity and fluidity, for interacting with its surroundings, and for regulating the fungal membrane. Chitin, as a scaffold for the cell wall, imparts integrity and strength to the cell wall, thereby offers protection against external stresses, mechanical damage, and immune responses from hosts (Baker et al., 2007). Glucans attached to chitin serve as attachment points for other structures, constituting amorphous outer and intermediate fillers of the fungal cell wall (Shenghui Shen et al., 2019). Glycosylated proteins anchor β (1,6)-glucan chains through glycosylphosphatidylinositol (GPI). Mannosyl proteins account for 40% of the cell wall structure. Damage to mannosyl proteins will lead to the decreased activity of proteins synthesizing the cell wall, thereby affecting the strength and integrity of the cell wall, which is of great importance to the dynamic system of fungal cell wall (Schmidt et al., 2005). The chitin–glucan complex or chitosan complex is the main component of fungal cell wall, accounting for 60% of the dry weight of the cell (Araújo et al., 2020).

The active substances in many plant essential oils are mixed inhibitors of 1, 3-β-glucan synthase (FKS-1) and chitin synthase in fungi. Diallyl disulfide downregulates the expression of the chitin synthase gene, and the treated cells have reduced chitin, damaged epidermis, and changed morphology and physiological activity (Shah et al., 2020). Previous studies found that cinnamaldehyde and eugenol could bind to the active sites of these two enzyme proteins through different amino acid residues (Ju et al., 2022). *Artemisia monosperma*

Del., *Callistemon viminals* G. Don, *Citrus aurantifolia* Swingle, and *Cupressus macrocarpa* Hartw. ex Gordon essential oils also inhibited chitin production (Abdelgaleil and El-Sabrout, 2018). Some experiments have found that a sub-inhibitory concentration of cinnamaldehyde can directly or indirectly attack the target of azole, polyene, and echinocandin through the accumulation of reactive oxygen species to induce moderate downregulation of the transcription of ERG-2, ERG-3, ERG-4, and ERG-11, which is consistent with the change trend of ergosterol (Parks and Casey, 1995). The upregulation of sterol influx transporters (such as AUS-1 and TIR-3) and sterol metabolism regulators (such as SUT-1 and UPC-2) inhibits ergosterol in a dose-dependent manner and then affects cell membrane integrity (Li Q. Q. et al., 2018). Estragole and linalool were found to inhibit ergosterol in a similar way as azole antifungal agents, both of which showed an inhibitory effect on 14α-demethylase, a key enzyme in ergosterol synthesis (Khan et al., 2010).

3.2.4 Damage to mitochondrial function

ATPases are involved in most biological and physiological activities in the cell through energy coupling. When plant oils disrupt the permeability and fluidity of plasma membranes, the membrane potential of organelles is reduced; proton pumping is disrupted, and the synthesis of H-ATPase, the key enzyme in ATP production, is inhibited. Some polyphenolic compounds block the activity of ATP synthase by binding to some chemical binding cavities of the enzyme, and the functional groups of inhibitors have established interactions with key amino acid residues of the enzyme through hydrogen bonding, hydrophobic interaction, etc. (Issa et al., 2019). After the inhibition of ATPase, related dependent enzymes are also affected. The decrease of protease and phospholipase activities can be attributed to the degradation of ATPase mediated by cinnamaldehyde (Pootong et al., 2017), and the expression level of fatty acid synthases such as acetyl-CoA carboxylase and fatty acid biosynthetic enzymes (fasI, fasH, and fasF) is inhibited. In addition, glycerophospho acyltransferase (plsX, plsY, plsC), cytidine diphosphate-diacylglycerol synthase A (cdsA), phosphatidylglycerol synthase A (pgsA), cardiolipin synthase (cls), and polypeptide resistance factor (mprF) glycerophospholipid biosynthesis pathways are inhibited; thus, the degree of damage of bacterial biofilm is aggravated (Pang et al., 2021). After cinnamaldehyde treatment, the ATP permeability of biofilms was increased; the ATP level in biofilms was remarkable decreased, and the intracellular ATP was rapidly consumed to maintain pH. After eugenol and citral treatment, *Pseudomonas roqueforti* was found to have an altered mitochondrial morphology and membrane potential, which further damaged the metabolic pathways of cellular energy and finally induced apoptosis (Ju et al., 2020).

3.2.5 Inhibition of filamentous temperature-sensitive proteins

The filamentous temperature-sensitive protein Z (FtsZ) is a GTPase with weak a sequence homology with tubulin, which plays an active role in guiding the binary division of bacteria. The FtsZ is assembled into a Z-ring structure at the future cell division site. The Z ring can promote cytodivision and recruit more than a dozen other division proteins into the Z ring. As the Z ring contraction leads to membrane closure, the cell divides to form two daughter cells (Osawa et al., 2008). The inhibition of the FtsZ by most plant oils is related to GTPase activity, FtsZ binding capacity, Z-ring assembly, and contraction. The majority of FtsZ inhibitory compounds in essential oils are phenylpropanoids and polyphenols. Cinnamaldehyde can bind to the T7 loop in the C-terminal region of the FtsZ monomer, which interferes with the formation of the Z ring and disrupts its morphology *in vivo*. The formation of the cell membrane is incomplete, and it is in a filamentous structure that is not completely divided (Domadia et al., 2007). By interacting with the GTPase binding cavity, curcumin enhanced the GTPase activity and interfered with the assembly and polymerization of the FtsZ, thereby shortening the steady-state duration of polymer assembly (Duggirala et al., 2014). Totarol is an bacteriostatic and fungistatic active ingredient derived from the plant *Rhamnosus pinus*. After its treatment, the cell GTPase activity and FtsZ polymerization are inhibited; the cells are filamentous, and the Z-ring assembly is misaligned (Jaiswal et al., 2007). Germarene and gemmarene D-4ol in pine needle essential oil can also bind to the hydrophobic cavity of the FtsZ (Anderson et al., 2012).

3.2.6 Interference with microbial gene expression

Nucleic acid is the material in which genetic information is stored, copied, and transmitted. The expression of genetic material controls the synthesis and metabolism of cellular proteins. Therefore, nucleic acid and gene expression are also important antimicrobial pathways. *Listeria monocytogenes* were treated with lemongrass essential oil to observe their transcriptome response, and the virulence genes *hly* and *inlj* were downregulated in a dose-dependent manner, whereas the fatty acid biosynthesis gene *accP* was upregulated (Hadjilouka et al., 2017). Fennel essential oil downregulates genes related to ochratoxin A (Nowotarska et al., 2017) biosynthesis in *A. niger*, thereby reducing OTA levels, but no changes in fungal spore morphology were observed, so fennel essential oil may only inhibit OTA production by reducing the expression of virulence-related genes (El Khoury et al., 2016). The test of citrus essential oil on *S. aureus* also showed that the expression of cytotoxic genes (*comC, comD, gtfB, gffC, and gbpB*) was remarkably downregulated, and its main active components, namely, linalool and limonene, indirectly inhibited the production of glucans required for biofilm formation (Benzaid et al., 2021). Transcriptome analysis showed that the indirect exposure of essential oil could affect the expression of *Penicillium rubens* genes, and essential oil could inhibit the activity of *P. rubens* by affecting polysaccharide, carbohydrate, fatty acid, nucleotide, and nucleoside metabolism (Kisová et al., 2020).

4 Plant essential oil regulates the fermentation quality of feed silage

The quality of silage feed largely depends on the ability to preserve the nutritional components of the silage raw material.

After the plant has been harvested, microbial and plant cell respiration are the main sources of nutrient loss. Therefore, silage should be carried out as soon as possible after the plant is harvested, using lactic acid bacteria to produce organic acids, lower the pH of the silage environment, inhibit the growth of harmful microorganisms, and reduce the loss of raw material nutrients. However, in the early and late stages of plant silage and after the silage feed is opened, the quality of silage feed is often affected by harmful microorganisms such as mold. The characteristic of essential oils that have strong inhibitory effects on mold at equal concentrations but are not remarkably inhibitory to lactic acid bacteria meets the needs of silage. Therefore, plant essential oils can be applied to silage feed to improve silage quality. (Bolsen et al., 1996).

4.1 Inhibition of growth and reproduction of adverse microorganisms in silage

The quality of silage depends on the microbial community of the silage raw material and the succession of microbial colonies during fermentation. However, adverse factors such as loose sealing measures and low sugar content in silage production may lead to changes in microbial community structure in silage affected by Clostridium, yeast, mold and other spoilage microorganisms, thereby reducing the quality of silage (Dunière et al., 2013; Xin et al., 2021). Clostridia can grow in an anaerobic environment, especially in high-moisture forage, and can compete with LAB. It has been reported that *Bacteroidetes* such as *Palidibacter propionicigenes* and *Prevotella ruminicola* have better ability to degrade small molecular substances than *Firmicutes*. It is an important factor in the loss and degradation of small molecular substances such as monosaccharides, disaccharides and non-cellulosic polysaccharides in silage (Klang et al., 2015). Spoilage microorganisms, such as mold and yeast, usually multiply in large numbers during the secondary fermentation of silage, degrading the nutritional quality of silage, affecting the palatability and producing a series of harmful substances: aflatoxins, ochratoxins, trichothecenes, fumonisins, and mycophenolic acids (Haq et al., 2021). In reducing the growth and reproduction of harmful microorganisms in early silage, adding appropriate amount of plant essential oil to inhibit the growth and reproduction of harmful microorganisms can improve the quality of silage. Several experiments have shown that cinnamon essential oil (*C. zeylanicum*), thyme essential oil (*Thymus mongolicus*), oregano essential oil (*Origanum minutiflorum*) (Foskolos et al., 2016; Çayıroğlu et al., 2020), cumin essential oil (*Cuminum cyminum*) (Turan and Önenç, 2018), lemon seed essential oil(*Citrus limon*) (Besharati and Niazifar, 2020), lemongrass essential oil (*C. citratus*) (Júnior et al., 2020), flaxseed essential oil (*Linum usitatissimum*), *Amomum villosum* Lour essential oil (Li et al., 2022), and sweet orange essential oil (*Amomum villosum Lour.*) (Hodjatpanah-Montazeri et al., 2016; Chaves et al., 2021) significantly inhibited the growth of Proteobacteria, Bacteroidetes and Actinobacteria, which provided a favorable environment for the growth of Firmicutes. The addition of cinnamon essential oil in silage reduced the relative abundance of unfavorable silage bacteria, such as Proteobacteria, Bacteroidetes and Actinobacteria, and increased the relative abundance of Firmicutes (Sheng-tan et al., 2011). After the silage was opened and fermented, the number of fungus and yeast colonies in the silage supplemented with cumin essential oil (Turan and Önenç, 2018), cinnamon essential oil (*C. zeylanicum*), oregano essential oil (*Origanum minutiflorum*) and sweet orange essential oil (*Citrus sinensis*) (Chaves et al., 2012) decreased significantly. The results showed that the addition of plant essential oil inhibited the proliferation of mold and yeast and prevented aerobic decomposition.

4.2 Inhibition of mycotoxin production in silage

Mycotoxins are a group of secondary metabolites secreted by fungal organisms belonging to the genera *Aspergillus*, *Fusarium*, *Alternaria*, and *Penicillium*. The ingestion of mycotoxins by animals affects feed intake, livestock product production, neurological activity, hormone levels, and immune capacity (Ogunade et al., 2018). Although ruminal microflora of ruminants has a certain mycotoxin degradation capacity, intake of feed containing high levels of mycotoxins will also have adverse effects on animal health and livestock product production because of the saturation of ruminal detoxification capacity (Cheli et al., 2013). Studies have shown that plant essential oils can reduce the content of mycotoxins in silage, and the use of lemongrass (*C. citratus*), turmeric (*C. longa*), mint (*Mentha canadensis Linnaeus*), rosemary (*Rosmarinus officinalis*), rose grass (*Cymbopogon martini*), and other essential oil extracts can inhibit the production of a variety of mycotoxins at a certain concentration (Bryła et al., 2022). *Hedychium spicatum* L. essential oil can remarkably reduce the enol concentration (DON) and zearalenone (ZEA) content of *F. deoxynivalene* (Kalagatur et al., 2018a). Ylang-ylang essential oil (*Cananga odorata (Lam.) Hook. F. and Thomson*) at 3.9 mg/g can completely inhibit the production of DON and ZEA mycotoxins in corn containing *F. graminearum* (Kalagatur et al., 2018b). Further research has found that some plant essential oils can directly reduce mycotoxins and destroy their toxicity. Of these, *Cinnamomum cassia* is the most efficient at degrading Fumonisin B1, followed by citral (*C. limon*), *S. aromaticum*, eucalyptus (*Eucalyptus* spp.), and camphor (*Cinnamomum camphora L.*) essential oils. However, lemon, grapefruit, eucalyptus, and palm essential oils had the highest degradation efficiency of ZEA (Perczak et al., 2019). The levels of ochratoxin A (Nowotarska et al., 2017) were below the limit of detection and the levels of ZEA, DON and T-2 toxins (Trichothecenes) were significantly reduced in corn silage treated with 3 mg/kg of oregano (*O. vulgare.*) ethanol extract and 6 mg/kg of thyme (*T. vulgaris*) ethanol extract, respectively (Vaičiulienė et al., 2020). When the ethanolic extract mixture of oregano (*O. vulgare*) and thyme (*T. vulgaris*) was involved in the silage of whole corn, the levels of ZEA and DON mycotoxins, as well as T-2 toxins, in the two above mentioned plant extract mixtures were remarkably lower than those in the other experimental groups (Vaičiulienė et al., 2022).

4.3 Improving the feeding quality of silage

Reducing the loss of nutrients from plant raw materials is an important role of silage. During silage fermentation, plant nutrients such as protein and starch are partially degraded by plant enzymes into soluble nitrogen and soluble carbohydrates, and this reaction continues until the pH drops below 4.0. Enzymes are key factors in the degradation of nutrients such as protein and starch during silage, among which bacterial enzymes are the main cause of proteolysis (60%), followed by plant proteases (30%); fungal enzymes and fermentation products contribute about 5% of proteolysis during fermentation (Junges et al., 2017). The use of essential oils as a silage additive can indirectly reduce nutrient loss during silage. After adding 200 mg/kg of cumin (*C. cyminum*) essential oil, the colony structure of wild oat silage changes considerably, and the lactic acid bacteria multiply in large quantities to produce acid, reduce the pH value, and then inhibit the activity of protein degradation enzymes, thereby reducing the degradation of proteins (Akinci and Önenç, 2021). In addition, the results showed that plant oils directly inhibit the activity of bacterial amylases and proteases. Oregano essential oil shows strong inhibitory properties on enzymes that degrade nutrients, such as tyrosinase, α-amylase, and α-glucosidase (Sarikurkcu et al., 2015). Phenylalanine ammonia-lyase can also catalyze the deamination of L-phenylalanine, and this enzyme is an important factor in plant browning. The research results show that phenolic essential oils such as cinnamaldehyde and carvacrol can inhibit the activity of phenylalanine ammonia lyase through hydrogen bridges and ionic or hydrophobic interactions, thereby reducing the loss of feed nutrients (Foskolos et al., 2016). The silage with 50 mL/kg of cinnamon (*C. cassia*) essential oil showed the lowest ammonia nitrogen level (Hodjatpanah-Montazeri et al., 2016). Cumin (*C. cyminum*) essential oil, lemongrass (*Cymbopogon citratus*) essential oil with a low concentration level, lemon seed (*C. limon*) essential oil, oregano (*O. vulgare*) essential oil, and flaxseed (*L. usitatissimum*) essential oil inhibited the production of ammonia nitrogen, propionic acid, and butyric acid during silage (Turan and Önenç, 2018; Çayıroğlu et al., 2020; Besharati and Niazifar, 2020; Besharati et al., 2020; Júnior et al., 2020). Plant essential oil extracts such as cinnamon (*Cinnamomum cassia L.*) essential oil, lemongrass (*C. citratus*) essential oil, flaxseed (*L. usitatissimum*) essential oil, clove (*E. caryophyllata Thunb.*) essential oil, thyme (*T. mongolicus*) essential oil, *A. villosum* Lour essential oil, lemon (*C. limon*) seed essential oil, and flaxseed (*L. usitatissimum*) essential oil with appropriate content as silage additives improved the evaluation level of silage and reduced the loss of crude protein, crude fat, and soluble sugar (Hodjatpanah-Montazeri et al., 2016; Turan and Önenç, 2018; Çayıroğlu et al., 2020; Besharati and Niazifar, 2020; Besharati et al., 2020; Júnior et al., 2020; Chaves et al., 2021; Li et al., 2022).

4.4 Improving the aerobic stability of silage

Given its wide application, silage can easily produce secondary fermentation in an aerobic environment after opening the bag (open the cellar), and aerobic microorganisms multiply in large numbers, consume nutrients in the feed, and produce a lot of heat. Therefore, the aerobic stability of silage after prolonged opening of the bag (open the cellar) is important (Wilkinson and Davies, 2013). Plant essential oil remarkably extended the aerobic stability of silage because of its excellent bacteriostatic and fungistatic activity. Studies showed that 0.13 mL/cm^2 of oregano(*O. vulgare*) essential oil was sprayed in beet pulp (Çayıroğlu et al., 2020). Alfalfa was supplemented with 60 mg/kg of lemon seed(*C. limon*) essential oil or 60 mg/kg of flaxseed(*L. usitatissimum*), cinnamon(*C. cassia*) seed, and lemon seed(*C. limon*) mixed essential oil (Besharati and Niazifar, 2020). The addition of 120 mg/kg of cinnamon essential oil(*C. cassia*), thyme essential oil(*T. vulgaris*), oregano essential oil(*O. vulgare*), or cumin essential oil(*C. cyminum*) to corn silage can improve the aerobic stability of silage (Hodjatpanah-Montazeri et al., 2016). Cinnamon(*C. cassia*) essential oil, sweet orange(*Citrus sinensis*) essential oil, oregano(*O. vulgare*) essential oil, and thyme(*T. vulgaris*) essential oil at a level of 120 mg/kg can maintain aerobic stability for 2 weeks (Chaves et al., 2021), whereas the blank control group without added essential oil can only maintain aerobic stability for 72 h. Peppermint(*Mentra piperita*) essential oil has also been proven to have an inhibitory effect against *F. oxysporum* and *Costomyces in vitro*, and the aerobic stability time of silage was prolonged by 50 h after adding peppermint essential oil to silage (Moghaddam et al., 2013).

5 Summary

Plant essential oils have strong inhibitory activity against a variety of adverse microorganisms in silage. Plant essential oil achieves its bacteriostatic and fungistatic ability by inhibiting biofilm formation, changing cell membrane permeability, and interfering with cell division and ATPase activity. The difference in bacteriostatic and fungistatic activity of plant essential oil against some fungi and lactic acid bacteria lays a theoretical foundation for its application as feed additives in silage. The use of appropriate levels of plant essential oils in silage production can control changes in colony structure by inhibiting the growth of some adverse microorganisms such as Clostridium, Fusarium and yeast in silage, and indirectly promote lactic acid bacteria to become dominant microorganisms. Reduce nutrient loss in silage, improve fermentation quality, and improve the aerobic stability of silage after secondary fermentation in the later stage.

Author contributions

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Optimizing corn silage quality during hot summer conditions of the tropics: investigating the effect of additives on *in-silo* fermentation characteristics, nutrient profiles, digestibility and post-ensiling stability

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Corn crop grown and ensiled at high temperature have lower water soluble carbohydrates (WSC), epiphytic lactic acid bacteria (LAB) population, lactic acid concentration, fermentation quality and aerobic stability. This study systematically investigated the effects of heterofermentative LAB (hetLAB), homofermentative LAB (homLAB), molasses and their mixture (MIX) on *in-silo* fermentation characteristics, chemical profiles, Cornell Net Carbohydrate and Protein System (CNCPS) carbohydrate subfractions, *in vitro* digestibility (DMD), microbial count, and post-ensiling aerobic stability of whole crop corn silage during hot summer (30 to 45°C) condition. Corn hybrids (P30K08 and DK6789) were ensiled at targeted dry matter (DM) of 330 g/kg for 0, 3, 7, 21, and 150 days in 3 L silos, without additive (CCS) or treated with hetLAB (4×10^6 cfu/g *Lactobacillus buchneri*), homLAB (1×10^6 cfu/g of *L. plantarum*), molasses (3% of fresh forage) or MIX (half of individual doses of homLAB, hetLAB and molasses) additives. The CCS, homLAB, hetLAB, molasses, or MIX treated chopped material of each hybrid were ensiled in 16 replicate silos at a density of 260 kg of DM/m^3. Compared to CCS, the additives significantly improved silage nutritional and fermentation quality, DM digestibility (*in vitro*), count of LAB, DM recovery and aerobic stability, and decreased counts of yeast and mold. Among the inoculants, the homLAB and MIX inoculated silages had greatest improvement in fermentation quality and nutritional value. The homLAB produced corn silage with the highest ($P < 0.05$) content of lactic acid, and soluble carbohydrates, and lowest contents of acetic acid, NH_3-N and pH, demonstrating desirable and restricted *in silo* fermentation. On the other hand, the hetLAB inoculated silages had the greatest ($P < 0.05$) value of acetic acids, highlighting greater aerobic stability. Interestingly, the MIX silages followed the hetLAB in acetic acid value and homLAB in lactic acid value. Notably, without additive stable pH was not

achieved during 21 days, with application of molasses, hetLAB and the MIX inoculants stable pH was achieved during 7 days, and with homLAB stable pH was achieved during the first 3 days of ensiling. The greatest numbers of viable LAB were recorded in homLAB (8.13 log cfu/g) and MIX (7.89 log cfu/g) inoculated silages, while the lowest for CCS (6.29 log cfu/g). The lowest yeast (1.48 log cfu/g) and mold (0.22 log cfu/g) were recorded for hetLAB inoculated silage. The greatest ($P < 0.05$) DM recovery was recorded for hetLAB (97.3%) and MIX (96.9%), and the lowest for the control silage (92.9%). All additives significantly improved the aerobic stability of corn silage, and the greatest value of >72 h was recorded for hetLAB and MIX inoculats, and the lowest for CSC (25 h). In conclusion, additives application can improve fermentation quality, nutritional value, DM recovery and aerobic stability of whole crop corn silage under hot summer conditions of the tropics. The MIX inoculant showed potential to improve in-silo fermentation, and aerobic stability at the same time, however, further investigation are required, particularly with respect of dose rate.

KEYWORDS

silage additives, homofermentative, heterofermentative, corn silage, in silo fermentation quality, CNCPS subfraction, microbial count, aerobic stability

1 Introduction

Feeding good quality silage is a pivotal determinant of the profitability of dairy production (Arriola et al., 2021). Whole plant corn is ideal for good quality silage production, due to its high biomass and grain yields, good ensiling characteristics, high metabolizable energy content, and easy incorporation in total mixed ration (Jiang et al., 2022). Moreover, a meta-analysis has shown that incorporation of corn silage in grass or grass silage based diets significantly increases dry matter (DM) intake, and yields of milk and milk protein in dairy cows (Khan et al., 2015). Due to these advantages, new high yielding corn genotypes have been developed for various climatic conditions (Abeysekara et al., 2018), and also for different seasons (spring and summer/autumn) of the year (Jiang et al., 2022). In the changing climatic conditions, selection of suitable corn genotype for quality silage production is one of the most important factors (Khan et al., 2015; Abeysekara et al., 2018), that markedly influence the biomass and nutrient yields, starch: neutral detergent fiber (NDF) ratio, ruminal NDF degradability, site (rumen vs small intestine) of starch digestion, and starch fermentation rate in the rumen (Bal et al., 2000; Khan et al., 2011; Khan et al., 2015).

Although corn silage has good ensiling characteristics, the hot environmental conditions during tropical summer, too high or too low crop DM content at harvest, limitations in the post-harvest processing-technologies and compaction, can significantly deteriorate corn silage fermentation quality and nutritional value (Jiang et al., 2022). Moreover, prolong in-silo fermentation and aerobic exposure during ensiling or feed-out period can increase DM and nutrient losses. Therefore, various additives are used to control the in-silo fermentation process, by compensating limitations of the process through stimulation of desirable fermentation process, but also through prevention of undesirable types of fermentation, resulting in lower DM losses and higher aerobic stability (Muck et al., 2018). Lactic acid producing bacteria (LAB), fermentable substrates (molasses, glucose) and enzymes are used to stimulate desirable fermentation, while organic acids are used to inhibit fermentation (loss of nutrients) by quickly reducing the pH (Oliveira et al., 2017; Bernardi et al., 2019). Among available additives, LAB represents the most popular additives either of those homofermentative LAB (homLAB) or heterofermentative LAB (hetLAB) strains (Muck et al., 2018).

During ensiling, the natural LAB in plants converts sugars under anaerobic conditions to produce lactic acid and acetic acid, which lowers the pH to preserve silage. Fast initial acidification is the key to control the growth of competing enterobacteria, clostridium, yeasts, and molds, and losses of nutrients. However, when the total population and type of natural LAB is not sufficient for the quick fermentation process, then LAB inoculants are usually used to produce high-quality silage by ensuring rapid fermentation (Avila et al., 2014; Bernardes et al., 2018). The LAB has the ability to suppress the growth of undesirable microorganisms and thus reduces the process of proteolysis, and DM loss during the early fermentation phase (Muck et al., 2018; Guan et al., 2020). Furthermore, bacterial inoculants can minimizes mold and yeast growth and improve aerobic stability of silage (Blajman et al., 2018). However, the effects of these LAB inoculants on silage fermentation quality and aerobic stability is highly dependent on the types and species of bacteria (homLAB vs hetLAB) used during silage fermentation (Oliveira et al., 2017).

The homLAB ferment 6-carbon sugars like glucose and fructose to just lactic acid and rapidly decrease silage pH. Therefore, most silage inoculants contain strains of homLAB (Guan et al., 2020). The preferred strains in homLAB inoculants are *Lactobacillus* spp.

(*L. plantarum*, *L. acidophilus*, *L. lactis*, *L. bulgaricus*) that can produce a high quantity of lactic acid in a short fermentation period, and stabilizes the silage with minimal nutrients and DM losses (Ni et al., 2015; Wu et al., 2019). On the other hand, hetLAB ferment sugars into both lactic acids and acetic acids. Inoculants containing hetLAB alone typically improve the aerobic stability of silages by fermenting water-soluble carbohydrates (WSC) into organic acids (acetic and propionic acids) that inhibit the growth of aerobic fungi which can cause spoilage to silage, but often slightly increase DM losses and silage pH (Oliveira et al., 2017). The primary and preferred strains in hetLAB inoculants are *L. buchneri* and, less commonly *L. brevis* (Muck et al., 2018).

The primary factor for quality silage production is the composition and abundance of microbial communities during ensiling (Kung et al., 2018; Zi et al., 2021). However, most silage LAB can grow at optimum temperatures of 25 to 40 °C. Ensiling at high temperatures reduces LAB population, lactic acid concentration, fermentation quality and aerobic stability of silage (Guan et al., 2020). Various homLAB and hetLAB additives have been reported to improve silage preservation (Muck et al., 2018; Rabelo et al., 2019), however, their efficiency have not been tested for corn silage production during tropical summer conditions. Moreover, during the corn growth and grain ripening stages, the hot temperature can reduce sugar deposition in corn plants by slowing the rate of photosynthesis through the inactivation of ribulose 1, 5-bisphosphate carboxylase/oxygenase (Bernardes et al., 2018). Therefore, supplementation of molasses alone or in combination with LAB is expected to enhance the fermentation quality of corn grown in the hot climatic conditions.

To our knowledge, the effect of silage additives on corn silage fermentation and nutritional quality under hot summer conditions of the tropics has not been investigated. The recent rise in global temperature further provides impetus to test silage additives for their effectiveness at high ambient temperatures. The first objective of this study was to systematically investigate the effect of additives (homLAB, hetLAB, molasses and their mixture), corn genotypes (Dk6789 and P30K08) and ensiling duration (0, 3, 7, 21, and 150 days) on (1) *in-silo* fermentation characteristics and DM losses; and (2) nutrient composition, Cornell Net Carbohydrate and Protein System (CNCPS) carbohydrate subfractions and *in vitro* digestibility of whole crop corn silage during hot summer (30 to 45°C) conditions. The CNCPS is one the most widely used feed protein and carbohydrate evaluation systems for ruminants (Refat et al., 2017; Xin et al., 2020). The second objective was to evaluated microbial count, and post-ensiling aerobic stability and changes in microbial composition after 150 days ensiling.

2 Materials and methods

2.1 Corn crop production

Corn was grown during summer season in irrigated research fields of the University of Agriculture Peshawar (34°02′ North Latitude, 71°48′ East longitude, and 347 m above the sea level), Pakistan. The climatological data for the area is shown in Figure 1. Seeds of two promising summer corn hybrids, Dk6789 from Monsanto (Monsanto Co. Pvt. Pakistan) and P30K08 from pioneer (Pioneer Hi-Bred International Inc., Pakistan), were sown on June 19, 2022 in four replicate fields. The seeds were sown in ridges (row-to-row spacing of 75 cm, and plant to plant space of 20 cm), at a seed rate of 66,000/ha. Standard, uniform fertilization, irrigation, and weeds control practices were followed for all experimental fields. Based on soil nutrient profile (tested before the experiment), the fields were fertilized with 250, 90 and 90 kg/ha of N, P and K, respectively using di-ammonium phosphate, urea, and sulfate of potash, respectively. The K, P and half of the N fertilizers were applied at the time of sowing and the remaining half of the N fertilizer was applied after first irrigation. For complete weeds control, Prime extra Gold 720SC herbicide was used at a rate of 1200 mL/ha after the first irrigation in a wet field. Besides herbicides, manual weed removal was also carried out, when required. Corn is sensitive to water-stress, and requires frequent irrigation for successful vegetative and reproductive growth under the semi-arid tropical condition. Therefore, all the plots were first irrigated on June 25, 2022, and then irrigated after every two growing weeks. Plant growth was monitored weekly by counting the number of leaves on plants from 1 m randomly selected strip of two consecutive rows of each field, excluding the exterior 1 m area. Similarly, the flowering and silking data was also recorded for better estimation of the harvest date.

2.2 Crop harvest, application of inoculant and laboratory-scale silage preparation

The crop was harvested at targeted whole-plant DM content of 33.1%, chopped (with theoretical lengths of cuts ranging from 1.3 to 1.5 cm) with self-propelled forage harvester. The chopped crop of each field was mixed, and a representative samples (100 kg each) were taken. Each sample was treated with the respective inoculant (homLAB, hetLAB, molasses or MIX) or deionized water in case of control corn silage (CCS), and then ensiled in 3 L laboratory silos. Each of the inoculants/deionized water was sprayed on thin layers of chopped corn crop and properly mixed. The homLAB was applied @ 2 mg/kg of fresh forage to supply 1×10^6 cfu/g of *L. plantarum*, hetLAB inoculant was applied @ of 1 mg/kg fresh forage to supply 4×10^6 cfu/g of *L. buchneri*, and molasses was added at a 3% of fresh forage. The homLAB inoculant (@ of 2 mg/L) and hetLAB inoculant (@ of 1 mg/L) was dissolved in deionized water and sprayed in a fine mist on the chopped corn. The same amount of deionized water was sprayed over control, without inoculum. The molasses was first diluted in deionized water to easily spray it in a fine mist on the chopped forage. While the MIX treatment contained half of the individual doses of homLAB, hetLAB and molasses, applied with the respective methods as explained above. For each genotype, four subsamples of the control and each of the four inoculated silages were collected and processed as day-0 samples. For each genotype, the CCS, homLAB, hetLAB, molasses, or MIX treated chopped material were ensiled in 16 replicate silos at the density of 260 kg of DM/m^3. For each corn hybrid, four replicate silos of each treated/control silages were used for each of 3, 7, 21 and 150 days ensiling periods.

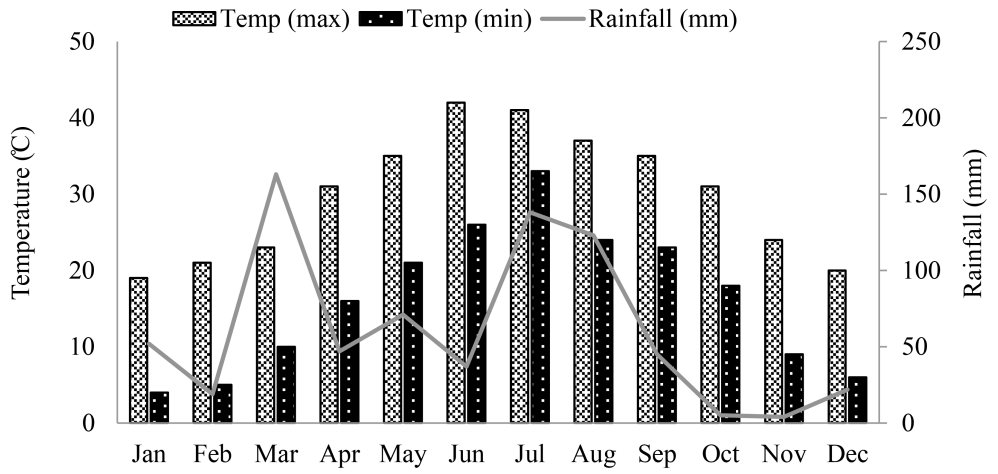

FIGURE 1
Total monthly rainfall (mm), and minimum and maximum temperature (°C) of the experimental area from January to December 2022. .

2.3 Chemical composition

After the specific ensiling durations, the silos containing silage were weighed, opened, and samples were collected for fermentation parameters evaluation, aerobic stability, chemical analysis, carbohydrates subtraction and digestibility. For chemical analysis, subsamples (200 g) of silage from each silo were dried at 70°C in air-dry oven till constant weight. The dried samples were ground in Wiley mill (Thomas Scientific) using 1 mm mesh screen, and analyzed for the contents of DM (method 934.01; AOAC, 2012), ash (method 942.05; AOAC, 2012), ether extract (EE, method 920.39; AOAC International, 2012) and CP (method 981.10; AOAC, 2012). The acid detergent fiber (ADF, method 973.18; AOAC, 2006) and neutral detergent fiber (NDF, method 2002.04; AOAC, 2012) were analyzed without correction for residual CP using Ankom 200 Fiber Analyzer (ANKOM Technology, Macedon, New York). Starch was analyzed using the method of Hall (2015). The neutral detergent-insoluble CP (NDICP) and acid detergent-insoluble CP (ADICP) contents were determined according to the method of Licitra et al. (1996), using ADF and NDF residues. Non-protein nitrogen (NPN) content was analyzed by precipitating the true protein of feed samples with tungstic acid and calculated as the difference between total CP content and CP content of the residues after filtration (Licitra et al., 1996). Soluble CP (SCP) was analyzed by incubating samples with bicarbonate-phosphate buffer for one h at 39°C and filtering the residues through the Whatman #54 filter paper. The SCP content was calculated as the difference between the total CP content and the CP content in the residues. The non-fiber carbohydrate (NFC) was calculated as NFC = 100 – (NDF – NDICP) – EE – CP – ash (NRC, 2001).

2.4 Fermentation quality, microbial composition and aerobic-stability analysis

2.4.1 Fermentation quality

For measurement of fermentation quality after 3, 7, 21, 150 days of ensiling, 50 g of fresh, unground sample form each replicate silo was dissolved in 450 mL of distilled water. The mixture was sealed, blended with high speed blender until complete homogenization. Immediately after blending, the pH was measured by pH meter (AE150 pH Benchtop meter; Thermo Fisher Scientific Inc.). The homogenate was filtered through two layers of cheesecloth. One aliquot of the filtrate was centrifuged at 10,000×g for 10 min at 4°C, and analyzed for ammonia-N (NH_3-N), and organic acids contents. The NH_3-N was analyzed by colorimetry using an auto-analyzer (Technicon; now SEAL Analytical, Hampshire, UK). The contents of organic acids, including lactic acid, acetic acid, propionic acid and butyric acid, was analyzed using high-performance liquid chromatography (Hitachi, L-2400, Ibaraki, Japan), equipped with a UV detector (wavelength 210 nm; Hitachi L-2400), autosampler (Hitachi Autosampler L-2200), and an Aminex HPX87H column (Bio-Rad Laboratories) with 0.015 M sulfuric acid mobile phase, run at a flow rate of 0.7 mL/min at 47°C.

2.4.2 Enumeration of microorganisms

The second aliquot of filtrate of each sample was immediately used for measuring total LAB, yeast, and mold counts by using the pour plate method. The yeast and mold were enumerated after 72 h aerobic incubation at 32°C in malt extract agar (Thermo Fisher Scientific-Oxoid CM0059B). The malt extract agar was acidified (after autoclaving) with 85% lactic acid at rate of 0.5% (vol/vol) of liquid agar medium, to inhibit growth of bacteria. The numbers of viable yeast and mold colonies were counted. The numbers of LAB were determined using the pour plate method with de Man Rogosa Sharpe agar (Thermo Fisher Scientific R01585), and incubated anaerobically at 32°C for 48 h.

2.5 DM recovery and aerobic-stability analysis

The DM recovery after 150 days of ensiling was calculated using the DM content and the weight of the forage placed in the silo before ensiling, and on the day of the opening as follows:

$$DM\ \ recovery = \frac{(FCX\ \ \times DMFX)}{(SSX \times DMSX)}$$

Where FCX is weight of fresh whole crop corn forage placed in the silo X, DMFX is the DM content of the fresh forage placed in the silo; SSX is the weight of silage in the silo X after 150 days, and MDSX is the DM content of the silage in silo X.

In the current study the silages were exposed to air at a high ambient temperature (36 ± 1°C) for determination of aerobic stability after, 150 days ensiling. Aerobic stability was defined as the time (hours) required for increasing the temperature of aerobically exposed silage by 2°C above the ambient temperature. Two kg silage from each replicate silo were placed in 8 L buckets, mixed for complete aeration, and kept in a room with a controlled environment. The temperature was measured every 15 min using two temperature sensors, placed in the geometric center of the silage. Buckets were covered with two layers of sterile cheesecloth to avoid contamination and drying out of the silage, yet allowing infiltration of air to the silage. Three additional sensors were placed in the room to record ambient temperature. The temperature was collected using a multipoint real-time temperature recorder (Mike Sensor Technology Co., LTD., Hangzhou, Zhejiang, China). The numbers of lactic acid bacteria, yeast, aerobic bacteria and mold were also measured as spoilage parameters after aerobic exposure.

2.6 Carbohydrates sub fraction and *in vitro* digestibility

The carbohydrates subtractions were computed using the updated version of the Cornell Net Carbohydrate and Protein System (CNCPS; Higgs et al., 2015; Van Amburgh et al., 2015). The carbohydrates were fractionated into CA1-subfraction (volatile fatty acids), CA2-subfraction (lactic acids), CA3-subfraction (organic acids), CA4-subfraction (soluble sugars), CB1-subfraction (starch), CB2-subfraction (soluble fiber), CB3-subfraction (available NDF), and CC-subfraction (unavailable NDF). The degradation rates (Kd) of the different subfractions in the rumen are: 0/h for CA1, 0.7/h for CA2, 0.5/h for CA3, 0.40–0.60/h for CA4, 0.20–0.40/h for CB1, 0.20–0.40/h for CB2, 0.04–0.09/h for CB3, and CC is non-degradable subfraction.

The two-stage *in vitro* procedure was adopted for determination of the *in vitro* DM digestibility (DMD) as reported earlier by Khan et al. (2022).

2.7 Statistical analysis

The effects of inoculum, genotypes, and ensiling duration on silage fermentation characteristics, chemical composition, carbohydrate subfractions, and DMD were determined by repeated measure analysis of variance using the PROC MIXED procedure of SAS (SAS Inst., Inc., Cary, NC). The different covariance structures of repeated matrices were evaluated according to Littell et al. (2006) and Wang and Goonewardene (2004) using the Akaike information criterion and the Schwarz Bayesian criterion. The following model was used:

$$Yijkl = \mu + Ii + CVj + EDk + Ii \times EDj + \epsilon ijkl$$

Whereas, Yijkl is the response of the treatment, μ is the overall mean, Ii is the fixed effect of inoculum, CVj is the fixed effect of cultivars of silage corn, EDk is the fixed effect of ensiling duration, Ii × EDj is the effect of interaction of inoculums and ensiling duration (only significant and interesting interactions are presented in the results), and ϵijkl is the random error. The effects of inoculum type on DM recovery, aerobic stability and number of microorganisms was determined using Proc Mixed procedure of SAS. Genotype and replication were considered as random effects. *Post-hoc* analyses were carried out using the Tukey–Kramer test to compute pair wise differences in the means. Means with different letters were obtained with "pd mix 800SAS macro".

3 Results

3.1 Chemical profile and digestibility (*in vitro*) of corn silage

Data on the overall effects of silage additives, ensiling duration and corn genotypes on the proximate chemical composition, CP and carbohydrates chemical profiles and DMD (*in vitro*) of whole crop corn silages are summarized in Table 1. Except lignin and starch, the mean contents of all measured chemical components of whole crop corn silage were significantly ($P < 0.01$) altered by the additives. Compared to CCS, all additives treated silages had higher ($P < 0.05$) contents of CP, NDICP, ADICP, NFC and DMD, and lower contents of SCP, ADF and NDF. Notably, among the inoculated groups the variation in contents on CP (6.94-7.13% DM), SCP (24.0-26.3% CP), NDICP (22.2-23.6% CP), ADICP (6.63-7.36% CP), NFC (48.3-49.8% DM) and DMD (63.9-66.4%) were quantitatively small. The homLAB and MIX inoculated silages had the highest ($P < 0.05$) contents of DM, CP, NFC and DMD, and lowest ($P < 0.05$) content of SCP.

The mean contents of all measured chemical components altered ($P < 0.001$) with the length of ensiling period (0-150 days), except ADF, NDF, lignin and starch (Table 1). The contents of DM (35.0 to 32.9%), CP (7.33 to 6.66% DM), NDICP (20.6 to 18.7% CP), ADICP (7.19 to 6.30% CP) and NFC (49.8 to 47.4% DM) consistently decreased ($P < 0.05$) with the increase in ensiling duration from 0 to 150 days. In contrast, the content of SCP (12.6 to 36.9% CP) and DMD (60.9 to 65.8%) increased ($P < 0.05$) during the 150 days ensiling. Among the corn genotypes, silages produced from P30K08 had greater ($P < 0.05$) content of NFC (51.0 vs 45.9% DM), starch (33.9 vs 28.3% DM), and *in vitro* DMD (64.3 vs 63.2%) as compared to DK6789.

3.2 CNCPS carbohydrates subfractions

Data on the overall effect of silage additives, ensiling duration, and corn genotypes on the CNCPS carbohydrates subfraction composition of the corn silage are presented in Table 2. All the reported CNCPS subfractions significantly varied ($P < 0.001$) due to the application of additives, except CB1, CB3 and CC subfractions.

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TABLE 1 Effect of silage additives, ensiling duration and genotypes on chemical profile, protein and carbohydrates chemical profiles and digestibility (*in vitro*) of whole crop corn silages.

	DM (%FM)	CP (%DM)	SCP (%CP)	NDICP (%CP)	ADICP (%CP)	WSC (%DM)	NFC (%DM)	Lignin (%DM)	ADF (%DM)	NDF (%DM)	Starch (%DM)	DMD (%)
Silage additives												
Control	33.1^{c}	6.66^{c}	27.9^{a}	21.2^{d}	5.64^{d}	2.42^{c}	47.4^{c}	2.93	23.3^{a}	42.6^{a}	30.0	63.1^{d}
HomLAB	34.6^{a}	7.13^{a}	24.0^{c}	23.6^{a}	7.36^{a}	3.53^{a}	49.8^{a}	2.88	21.7^{c}	40.9^{c}	30.9	66.4^{a}
HetLAB	33.8^{b}	7.00^{b}	26.3^{b}	22.6^{bc}	6.96^{b}	2.63^{c}	48.3^{b}	3.06	22.5^{b}	41.8^{b}	30.6	63.9^{c}
Molasses	33.7^{b}	6.94^{b}	25.8^{b}	22.2^{c}	6.63^{c}	2.90^{b}	48.7^{b}	2.95	22.8^{b}	41.7^{b}	31.5	65.2^{b}
Mixture	34.2^{ab}	7.06^{ab}	25.9^{b}	22.9^{b}	6.94^{b}	3.29^{ab}	49.4^{ab}	3.01	22.9^{ab}	42.3^{ab}	31.0	65.8^{ab}
SEM	0.18	0.06	0.31	0.21	0.161	0.21	0.39	0.02	0.24	0.38	0.24	0.33
Ensiling duration (days)												
D0	35.0^{a}	7.33^{a}	12.6^{e}	20.6^{a}	7.19^{a}	6.47^{a}	49.8^{a}	2.99	22.7	43.7	32.1	60.9^{d}
D7	34.1^{b}	7.14^{b}	21.5^{d}	19.6^{b}	7.18^{a}	2.71^{b}	49.3^{b}	2.98	22.7	43.6	32.0	62.9^{c}
D21	33.5^{c}	6.95^{cd}	26.3^{c}	19.6^{b}	6.89^{ab}	2.52^{b}	48.7^{c}	2.96	22.7	43.7	32.2^{c}	64.7^{b}
D42	33.2^{cd}	6.68^{d}	31.4^{b}	18.9^{bc}	6.58^{b}	2.19^{c}	48.2^{d}	2.94	22.6	43.8	32.2	65.7^{a}
D150	32.9^{d}	6.66^{d}	36.9^{a}	18.7^{c}	6.30^{b}	2.05^{c}	47.4^{e}	2.91	22.6	43.9	32.3	65.8^{a}
SEM	0.17	0.10	0.360	0.19	0.150	0.19	0.24	0.02	0.254	0.48	0.28	0.13
Corn genotypes												
P30K08	33.4^{b}	6.75^{b}	24.9^{b}	19.3	6.94	3.01	51.0^{a}	2.89^{b}	22.1	39.5^{b}	33.9^{a}	64.3^{a}
Dk6789	34.1^{a}	7.09^{a}	26.6^{a}	19.7	6.72	3.02	45.9^{b}	3.03^{a}	23.1	42.9^{a}	28.3^{b}	63.2^{b}
SEM	0.05	0.015	0.05	0.13	0.10	0.11	0.25	0.01	0.15	0.24	0.14	0.04
Significance												
Inoculums	***	***	***	***	***	***	**	NS	**	**	NS	***
ED	***	***	***	***	***	***	***	NS	NS	NS	NS	***
Genotype	***	***	***	NS	NS	NS	***	***	NS	***	***	*

Mean with different superscription ($^{a\text{-}c}$) in the same column within silage inoculants, ensiling duration or corn genotypes differ at P < 0.05.
homLAB, inoculated with homofermentative lactic acid producing bacteria (LAB) (2 mg/kg fresh forage to supply 1×10^{6} cfu/g of Lactobacillus plantarum); hetLAB, inoculated with heterofermentative LAB (1 mg/kg of fresh forage to supply 4×10^{6} cfu/g of L. buchneri); Mol, inoculated with molasses (3% of fresh forage); MIX, inoculated with combination of half of the individual doses of hetLAB, hetLAB and molasses inoculants; ED, ensiling days; DM, dry matter; CP, crude protein; SCP, Soluble CP; NDICP, neutral detergent insoluble CP; ADICP, Acid detergent insoluble CP; WSC, water soluble carbohydrates; NFC, non-fibrous carbohydrates; ADF, acid detergent fiber; NDF, neutral detergent fiber; DMD, dry matter digestibility; SEM, standard error of mean; NS, non-significant; ***P < 0.001; **P < 0.01; *P < 0.01.

Compared to CCS, all additives increased ($P < 0.05$) of CA1, CA2 and CA4 subfractions, and decreased ($P < 0.05$) CB2 subfraction. Among the additives, the homLAB produced corn silage with the highest ($P < 0.05$) content of CA2 (9.20% DM; Kd 0.7/h) and CA4 (2.82% DM; Kd 0.40–0.60) subfractions, and lowest contents of CA1 (1.70% DM; Kd 0/h) and CB2 (2.73% DM; Kd 0.20–0.40/h) subfractions, demonstrating desirable and restricted in-silo fermentation. On the other hand, the hetLAB inoculated silages had the greatest ($P < 0.05$) value of CA1-subfraction, highlighting greater production of organic acids, required for aerobic stability. Interestingly, the MIX silages followed the hetLAB in CA1 value and homLAB in CA2 value. With the increase in ensiling duration from 3 to 150 days, there were consistent decline in CA1 (2.50 to 1.74% DM), CA4 (2.52 to 2.02% DM) and CB2 (39.5 to 37.9% DM) CNCPS subfractions. In contrast the CA2-subfraction increased from 5.42 to 8.82% DM during the 150 days ensiling period. Among the corn genotypes, P30K08 had greater ($P < 0.05$) CB1 (33.8 vs 28.3% DM), and lower CB3 (36.4 vs 41.3) and CC (2.71 vs 3.23% DM) subfractions as compared to DK6789.

3.3 Fermentation quality of whole crop corn silage

Data in Table 3 and Figure 2 illustrate the fermentation quality whole crop corn silage treated with different additives and ensiled for different durations. The applications of different additives altered the contents of lactic acids ($P < 0.001$), acetic acids ($P < 0.001$), NH_3-N ($P < 0.001$) and pH ($P < 0.01$) of corn silage. Compared to CCS, application of additives increased ($P < 0.05$) the contents of lactic

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TABLE 2 Effect of silage additives and ensiling duration on Cornell Net Carbohydrate and Protein System (CNCPS) subfractions of whole crop corn silage.

	CA1	CA2	CA4	CB1	CB2	CB3	CC
Silage Inoculants							
Control	1.41^{e}	5.92^{e}	2.01^{d}	30.0	8.86^{a}	39.5	3.02
homLAB	1.70^{d}	9.20^{a}	2.82^{a}	30.9	2.73^{d}	39.5	2.92
hetLAB	2.92^{a}	6.21^{d}	2.28^{c}	30.6	7.89^{b}	38.7	3.04
Molasses	2.03^{c}	6.96^{c}	2.57^{b}	31.5	6.01^{c}	38.3	2.93
Mixture	2.45^{b}	7.35^{b}	2.33^{c}	31.0	6.71^{bc}	38.7	2.96
SEM	0.09	0.055	0.030	0.24	0.456	0.39	0.04
Ensiling duration							
ED3	2.50^{a}	5.42^{d}	2.92^{a}	32.1	7.12^{a}	39.5^{a}	3.04
ED7	2.10^{b}	6.74^{c}	2.88^{a}	32.0	6.87^{a}	39.3^{a}	3.03
ED21	2.08^{b}	7.55^{b}	2.19^{b}	32.2	6.36^{ab}	38.5^{b}	2.96
ED150	1.74^{c}	8.82^{a}	2.02^{c}	32.2	5.59^{b}	37.9^{c}	2.86
SEM	0.15	0.55	0.028	0.214	0.40	0.35	0.092
Genotype							
P30K08	2.13	7.10	2.40	33.8^{a}	6.28	36.4^{b}	2.71^{a}
DK6789	2.10	7.15	2.39	28.3^{b}	6.60	41.3^{a}	3.23^{b}
SEM	0.12	0.35	0.08	0.14	0.288	0.248	0.024
Significance							
Inoculums	***	***	***	NS	***	NS	NS
Ensiling duration	***	***	***	NS	*	**	NS
Genotype	NS	NS	NS	***	NS	***	***

Mean with different superscription ($^{a-c}$) within column within silage inoculants, ensiling duration and corn genotypes differ at P < 0.05. homLAB, inoculated with homofermentative lactic acid producing bacteria (LAB; 2 mg/kg fresh forage to supply 1×10^{6} cfu/g of Lactobacillus plantarum); hetLAB, inoculated with heterofermentative LAB (1 mg/kg of fresh forage to supply 4×10^{6} cfu/g of L. buchneri); Mol, inoculated with molasses (3% of fresh forage); Mix, inoculated with MIX (combination of half of the individual doses of hetLAB, hetLAB and molasses) inoculants. CA1, wolatile fatty acids (Kd 0/h); CA2, lactic acid (Kd 0.7/h); CA4, sugar (Kd 0.40–0.60/h); CB1, starch (Kd 0.20–0.40/h); CB2, soluble fiber (Kd 0.20–0.40/h); CB3, digestible fiber (Kd 0.04–0.09/h); CC, indigestible fiber; ED, ensiling days; SEM, standard error of mean; NS, non-significant; *p < 0.05; **p < 0.01; ***p < 0.001.

acids and acetic acids and decreased ($P < 0.05$) the content of NH_3-N and pH. Among the additives, homLAB inoculated silage had the highest ($P < 0.05$) content of lactic acid (9.20% DM), and lowest ($P < 0.05$) content of acetic acid (1.16% DM), NH_3-N (3.46% N) and pH (3.66). The highest ($P < 0.05$) value of acetic acid was observed for hetLAB (2.01% DM), followed by MIX (1.41% DM). With the increase in ensiling duration from 3 to 150 days, the lactic acids (5.42 to 8.82% DM) and NH_3-N (5.42 to 8.82% DM) increased ($P < 0.05$), whereas, acetic acid (1.88 to 1.05% DM) and pH (4.54 to 3.69) decreased ($P < 0.05$). Corn genotypes, did not alter any of the measured silage fermentation characteristics.

Changes in pH (Panel A), NH_3-N (Panel B) and lactic acids (Panel C) contents during 150 days ensiling of whole crop corn, as affected by the different inoculants are depicted in Figure 2. It is evident from Figure 2 that without inoculum stable pH was not achieved even 21 days after ensiling. However, with application of molasses, hetLAB and the MIX inoculants, stable pH was achieved during 7 days of ensiling, and with homLAB stable pH was achieved during the first 3 days of ensiling. The rapid drop in pH with homLAB was associated with the greater increase in lactic acid concentration (0 to 7.20% DM) during the first 3 days of ensiling (Figure 2B). At all ensiling durations, the highest values of lactic acids were recorded for homLAB, followed by MIX (Figure 2B). Moreover, the lowest increase in NH_3-N concentration (0.66 to 4.23% CP) during the 150 days ensiling was recorded for homLAB treated corn silage (Figure 2C). In contrast, the CCS had the greatest values of NH_3-N concentration at all ensiling durations, followed by molasses, MIX and hetLAB inoculated silages.

3.4 Microbial population, dry matter recovery and aerobic stability of whole crop corn silage

Figure 3 show the effects of different inoculants on the total number of viable LAB (Panel A), yeasts (Panel B), mold (Panel C)

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TABLE 3 Effect of silage additives and ensiling duration on fermentation characteristics of whole crop corn silage.

	Lactic acid (% DM)	Acetic acid (% DM)	Propionic Acid (% DM)	NH_3-N (%N)	pH
Silage inoculants					
Control	5.92^{e}	0.91^{e}	0.10	8.08^{a}	4.99^{a}
^{hom}LAB	9.20^{a}	1.16^{c}	0.00	3.46^{e}	3.66^{c}
^{het}LAB	6.21^{d}	2.01^{a}	0.10	4.22^{c}	3.94^{b}
Molasses	6.96^{c}	1.31^{bc}	0.15	6.27^{b}	3.91^{b}
Mix	7.35^{b}	1.41^{b}	0.20	3.89^{d}	3.76^{b}
SEM	0.055	0.09	0.12	0.20	0.133
Ensiling duration (days)					
ED3	5.42^{d}	1.88^{a}	ND	3.76^{d}	4.54^{a}
ED7	6.74^{c}	1.50^{b}	0.10	4.96^{c}	4.12^{b}
ED21	7.55^{b}	1.00^{c}	0.20	5.70^{b}	3.84^{bc}
ED150	8.82^{a}	1.05^{c}	0.35	6.95^{a}	3.69^{c}
SEM	0.055	0.10	0.10	0.12	0.13
Corn genotypes					
P30K08	7.15	1.36	0.20	5.36	4.07
Dk6789	7.10	1.35	0.10	5.31	4.04
SEM	0.35	0.05	0.10	0.06	0.02
Significance					
Inoculums	***	***	NS	***	**
Ensiling time	***	***	NS	***	***
Genotype	NS	NS	NS	NS	NS

Mean with different superscription ($^{a-e}$) in the same column within silage inoculants, ensiling duration or corn genotypes differ at P < 0.05. homLAB, inoculated with homofermentative lactic acid producing bacteria (LAB; 2 mg/kg fresh forage to supply 1×10^6 cfu/g of Lactobacillus plantarum); hetLAB, inoculated with heterofermentative LAB (1 mg/kg of fresh forage to supply 4×10^6 cfu/g of L. buchneri); Mol, inoculated with molasses (3% of fresh forage); Mix, inoculated with MIX (combination of half of the individual doses of hetLAB, hetLAB and molasses) inoculants. NH_3-N; ammonia nitrogen; ED, ensiling days; ND, not determined; DM, dry matter; SEM, standard error of mean; NS, non-significant; **P < 0.01; ***P < 0.001.

and DM recovery (Panel D) of whole crop corn ensiled for 150 days. All the tested inoculants significantly improved ($P < 0.001$) the count of LAB and DM recovery, and decreased ($P < 0.001$) the count of yeast and mold. Notably, the greatest number of viable LAB was ($P < 0.05$) recorded in homLAB (8.13 log cfu/g) and MIX (7.89 log cfu/g) silages, while the lowest ($P < 0.05$) number was recorded for CCS (6.29 log cfu/g). The lowest ($P < 0.05$) yeast counts were recorded in hetLAB (1.48 log cfu/g) inoculated silage, and the highest ($P < 0.05$; 3.77 log cfu/g) for the CCS (Figure 3B). The highest ($P < 0.05$) count of mold was recorded for the CCS (2.10 log cfu/g), and the lowest ($P < 0.05$) for hetLAB (0.22 log cfu/g) and MIX (0.28 log cfu/g). The DM recovery of whole crop corn silage was significantly improved ($P < 0.001$) with the application of the different inoculants (Figure 3D). The greatest ($P < 0.05$) DM recovery (97.3%) was recorded for hetLAB and MIX (96.9%), and the lowest ($P < 0.05$) for the control silage (92.9%).

Figure 4 shows the number of viable LAB (Panel A), yeasts (Panel B), mold (Panel C) and aerobic stability (Panel D) after 72 h exposure to air of the 150 days ensiled whole crop corn. All the tested inoculants significantly improved ($P < 0.001$) the count of LAB and aerobic stability, and decreased ($P < 0.001$) the count of yeast and mold. The highest ($P < 0.05$) numbers of viable LAB were counted for homLAB (7.04 log cfu/g) and MIX (6.94 log cfu/g) inoculated silages after the 72 h exposure to air, and the lowest ($P < 0.05$) for CCS (4.83 log cfu/g). The lowest ($P < 0.05$) number of viable yeasts (2.63 log cfu/g) and mold (0.35 log cfu/g) were counted for hetLAB inoculated silages. The highest aerobic stability of >72 h was recorded for hetLAB and MIX inoculated corn silages. Silages inoculated with homLAB were stable for 31 h and those with molasses were only stable for 33 h. Nevertheless, all the inoculated corn silages had greater aerobic stability than the CSC (25 h).

4 Discussion

High ambient temperature during the grain filling maturity (Bernardes et al., 2018), ensiling (Ali et al., 2015) and feed-out period (Khan et al., 2009; Ferrero et al., 2021) strongly influence the chemical composition, epiphytic microbial population, *in-silo*

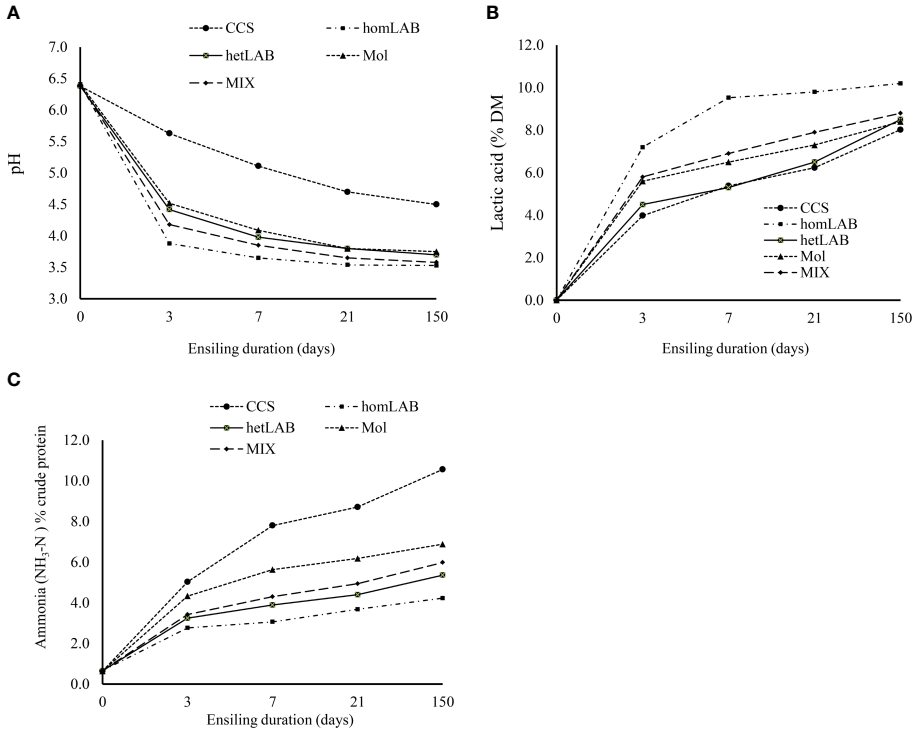

FIGURE 2
Changes in pH **(A)** ammonia [NH_3-N; **(B)**] and lactic acids **(C)**] contents during 150 days ensiling of whole crop corn, as affected by the different inoculants. CCS, control corn silage; homLAB, inoculated with homofermentative lactic acid producing bacteria (LAB; 2 mg/kg fresh forage to supply 1×10^6 cfu/g of *Lactobacillus plantarum)*; hetLAB, inoculated with heterofermentative LAB (1 mg/kg of fresh forage to supply 4×10^6 cfu/g of *L. buchneri*); Mol, inoculated with molasses (3% of fresh forage); MIX, inoculated with a mixture of half of the individual doses of homLAB, hetLAB and molasses inoculants. .

fermentation characteristics, and aerobic stability of corn silage (Guan et al., 2020; Ferrero et al., 2021). Therefore, global warming has been envisaged as a major challenge for future silage production, particularly in the tropics (Guan et al., 2020). The results of this study present the first dataset on the effects of homLAB (*L. plantarum)*, hetLAB (*L. buchneri*), molasses and their mixture on the chemical composition, ensiling quality, DM losses, CNCPS carbohydrate subfractions, *in vitro* digestibility, microbial count, and post-ensiling aerobic stability of corn silage during hot summer conditions of the tropics.

During *in-silo* fermentation, sugars and other easily fermentable carbohydrates are converted to volatile fatty acids, lactic acid, and CO_2 by microbes and plant enzymes (Oliveira et al., 2017; Muck et al., 2018; Bernardi et al., 2019). The process results in DM and energy loss, which reduces the availability of fermentable carbohydrates for ruminal fermentation (Cone et al., 2008). As such, faster LAB fermentation, and establishment of low (< 4.2; McDonald et al., 1991) pH is important for stabilization of the silage, and for avoiding undesirable microbial growth and losses due to prolong and undesirable fermentation (Muck et al., 2018). For a quicker and quality fermentation the content of WSC and epiphytic LAB are very important. However, high ambient temperature can reduce the WSC (Bernardes et al., 2018) and population of epiphytic LAB in corn silage (Muck et al., 2018; Guan et al., 2020), and increase the production of acetic acid, which slow-down pH decline and increase DM and energy loss (Bernardes et al., 2018). Therefore, in current study LAB inoculants, molasses and their mixture were used to stimulate a rapid decline in pH, inhibit the growth undesirable anaerobic microorganisms, and prevent prolonged fermentation, extensive proteolysis and DM loss during the ensiling process (Ferrero et al., 2018; Arriola et al., 2021).

In the current study, DM content of the whole crop corn silages ranged from 33.1 to 34.6%, which is within the optimal range (Khan et al., 2015). All additives improved nutrient profile and digestibility of corn silage. The highest improvement in DM, CP, NDICP, WSC and NFC contents were recorded for homLAB inoculated silages. The homLAB stimulated fast homo-lactic acid fermentation, resulting in a rapid decline in pH and lower losses of fermentable carbohydrates. Moreover, the fast establishment (within 3 days) of lower pH (3.88) prevented the growth undesirable anaerobic microorganism and extensive proteolysis in the homLAB inoculated silage, which can explain the high DM content, and improvement in nutrient profile (Oliveira et al., 2017; Muck et al., 2018). In agreement with our findings earlier studies have reported greater DM content, and better nutritional value for corn silages inoculated with homLAB (Bernardi et al., 2019; Zhang et al., 2022). The lower content of CP, NDICP and WSC, and higher content of SCP in the CCS reflects prolonged/undesirable fermentation and

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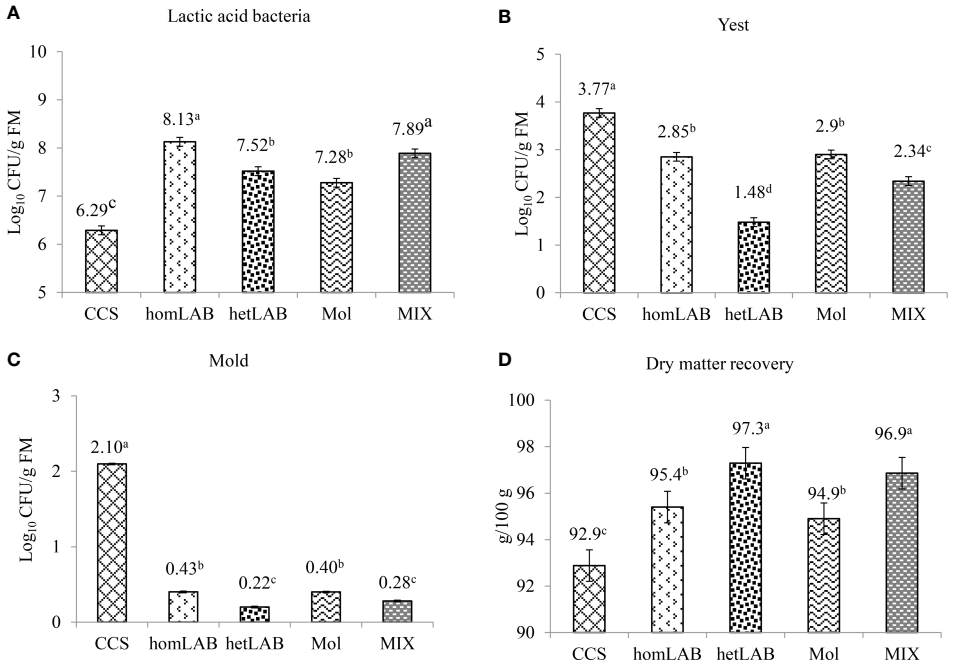

FIGURE 3
Effects of different inoculants on population of lactic acid bacteria **(A)**, yeast **(B)** mold **(C)** and dry matter recovery (Panel **D**) of whole crop corn ensiled for 150 days. CCS, control corn silage; homLAB, inoculated with homofermentative lactic acid producing bacteria (LAB; 2 mg/kg fresh forage to supply $1x10^6$ cfu/g of *Lactobacillus plantarum)*; hetLAB, inoculated with heterofermentative LAB (1 mg/kg of fresh forage to supply $4x10^6$ cfu/g of *L. buchneri*); Mol, inoculated with molasses (3% of fresh forage); MIX, inoculated with a mixture of half of the individual doses of homLAB, hetLAB and molasses inoculants.

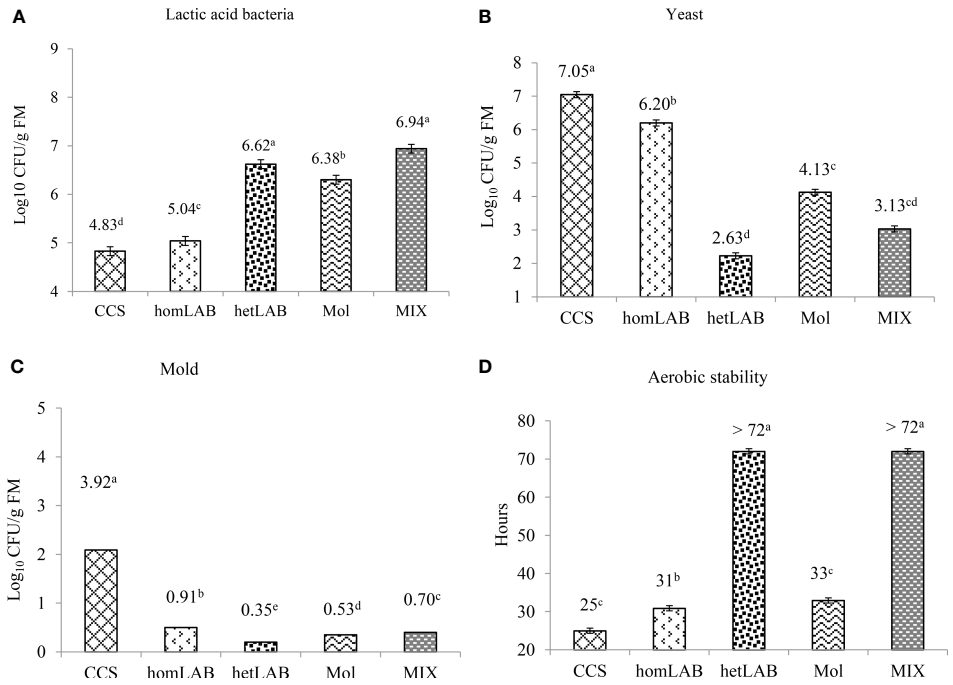

FIGURE 4
Effects of different inoculants on population of lactic acid bacteria **(A)** yeast **(B)** mold **(C)**) and aerobic stability **(D)** after 72 h exposure to air of the 150 days ensiled whole crop corn. CCS, control corn silage; homLAB, inoculated with homofermentative lactic acid producing bacteria (LAB; 2 mg/kg fresh forage to supply $1x10^6$ cfu/g of *Lactobacillus plantarum)*; hetLAB, inoculated with heterofermentative LAB (1 mg/kg of fresh forage to supply $4x10^6$ cfu/g of *L. buchneri*); Mol, inoculated with molasses (3% of fresh forage); MIX, inoculated with a mixture of half of the individual doses of homLAB, hetLAB and molasses inoculants.

extensive proteolysis (Oliveira et al., 2017). The contents of NDF ranged from 41.7 to 42.6% DM, with the lowest value recorded for homLAB-treated silage. In agreement with our findings earlier studies have reported that the application of homLAB reduces the NDF content (by up to 5%) by decreasing cell wall recalcitrance during the fermentation (McDonald et al., 1991; Jalc et al., 2009). For quality silage fermentation, the presence of WSC content (> 5% DM) is necessary (McDonald et al., 1991; Zi et al., 2021). In the present study the WSC of the unensiled whole crop corn was 6.47% DM, which was enough for the ensiling process, and this could have probably diminished the positive effects of molasses additives on corn silage nutritional quality. The homLAB-treated silages had the highest content of WSC as compared to the other groups, highlighting better/restricted fermentation and lower losses. Our results are in line with Huisden et al. (2009), who reported higher content WSC in silage inoculated with homLAB. All additives improved DMD of corn silage. The DMD of homLAB inoculated corn silage was 4.7% greater than the CCS. Similar increases in DMD were found in earlier studies where homLAB was applied to silages, supporting our findings (Huisden et al., 2009). High digestibility of forages is one of the most desirable characteristics for proper diet formulation and animal performance, as digestibility of forages is closely associated with intake, energy and nutrients supply, and animal performance (Khan et al., 2015). Notably, the nutritional value of the MIX silage was not significantly different from that of homLAB inoculated silage. The MIX silage did not exceed the homLAB in terms of silage nutritional value, which may be related to the lower doses of individual additives used in the MIX inoculant. In agreement with our findings, a recent review reported inconsistent effects of MIX inoculants on silage nutritional value (Muck et al., 2018).

Irrespective of the additives application, ensiling duration had a significant effect on the chemical composition, protein, and carbohydrates chemical profiles. The CP content decreased from 7.09 to 6.66% during the 150 days ensiling period. In agreement with our findings, earlier studies reported that the CP content decreased by 2-3% with advancing ensiling duration (Cone et al., 2008; Silva et al., 2015). A large fraction of WSC was consumed by LAB during the fermentation process, which can explain the decrease in its concentration from 6.47 to 2.05% DM during the 150 days ensiling. During the 150 days ensiling, the NDF content decreased by 3%. Gerlach et al. (2013) and Silva et al. (2015) reported a similar decrease (2-5%) in the NDF content with the increase in ensiling duration from 1 to 60 days. During the ensiling and fermentation processes, enzymatic and acid hydrolysis of the highly digestible cell wall portion, hemicellulose, may account for the decrease in NDF content (Junges et al., 2013). The DMD increased by 8.05% with the increase in ensiling duration from 0 to 150 days. Since the easily fermentable carbohydrates fractions of the silages decreased with the increase in ensiling duration, the increase in digestibility could be related to the increase in protein solubility and the positive effects on ensiling on degradability of the insoluble protein and carbohydrates fractions.

The CNCPS (Higgs et al., 2015; Van Amburgh et al., 2015), is widely used for evaluation of feed protein and carbohydrates nutritional value for ruminants, and for diet formulation according to dairy cattle requirements (Refat et al., 2017; Sun et al., 2018). The CNCPS subfractions (CB1, CB2, CB3 and CC) reported in this study were close to the reported values of Jiang et al. (2022). All inoculated silages had higher CA1 (Kd 0/h), CA2 (Kd 0.7/h) and CA4 (Kd 0.40–0.60) subfractions, demonstrating that the use of additives increased the contents of rapidly fermentable carbohydrates in corn silage. In agreement with our findings, earlier studies have reported greater CA-subfractions for LAB inoculated silages (Higgs et al., 2015). It may be noted that the CNCPS subfractions are calculated from the chemical compositions data (Xin et al., 2020b), and as such, the variations observed on the CNCPS are strongly explained from the differences in chemical composition due to the additives, ensiling duration and genotypes. The highest CA2 (9.20% DM) and CA4 (2.82% DM) subfractions, and lowest contents of CA1 (1.70% DM) and CB2 (2.73% DM; Kd 0.20–0.40/h) subfractions in the homLAB silages, are the consequence of faster fermentation and quicker establishment of low pH. On the other hand, the hetLAB inoculated silages had the greatest ($P < 0.05$) value of CA1-subfraction, highlighting greater production of organic acids, required for aerobic stability. Interestingly, the MIX silages followed the hetLAB in CA1 value and homLAB in CA2 value. These findings highlight that there is a potential to get the benefits of desirable in-silo fermentation of homLAB, and aerobic stability of hetLAB from the MIX inoculants. However, more work is needed to develop proper mixture.

A lower pH (< 4.2; McDonald et al., 1991) is an important index of good silage preservation (Rabelo et al., 2019). In the current study, without inoculum the pH was not stable (>4.2), even after 21 days of ensiling. However, with the application of molasses (4.09) and hetLAB (3.87) stable pH was achieved during 7 days of ensiling, and with homLAB (3.88) and MIX (4.18) stable pH was achieved during the first 3 days of ensiling. In agreement with our findings, earlier studies have reported lower pH values for homLAB inoculated silages (Oliveira et al., 2017). The rapid drop in pH was associated with the greater increase in lactic acid concentration in homLAB (0 to 7.20% DM) and MIX (0 to 5.8% DM) during the first 3 days of ensiling (Figure 2B). The rapid decrease in pH inhibits the growth of (spoilage)-microorganisms, thus reducing the proteolysis and butyric acids production, and preserve nutritional value of silage (McDonald et al., 1991; Ogunade et al., 2016). In currents study inoculation with homLAB increased the lactic acid production and reduced acetic acid and other acids which is consistent with literature findings (Oliveira et al., 2017). However, the lower content of acetic acid and other organic acids with homLAB can reduce yeast count and aerobic stability of silage (Oliveira et al., 2017; Muck et al., 2018). The hetLAB are mainly producing hetero fermentative acids, lactic acid, acetic acid, ethanol and CO_2 that reduces pH slowly as compared to homLAB (Oliveira et al., 2017). This could explain the highest content of acetic acid in hetLAB inoculated silages in present study. Moreover, the high

content of acetic acid is favored for increasing aerobic stability due to its potential to inhibit yeasts, responsible for initiating aerobic spoilage (Muck et al., 2018). Notably, like homLAB, inoculation with MIX inoculant significantly improved the rate and extent of pH decline and lactic acid production. On the other hand, MIX inoculant significantly improved acetic acid production. Although, in the current study the positive effects of MIX did not exceed, the individual (hom/het LAB) inoculants, it shows potential to improve in-silo fermentation and aerobic stability at the same time and needs further investigation, particularly with respect of dose rate.

The concentration of NH_3-N, reflects the extent of proteolysis, and amino acid deamination and decarboxylation during ensiling (Oliveira et al., 2017). During in silo fermentation, protein hydrolysis can be inhibited by establishment of low pH environment, which prevent the growth of proteolytic microorganisms and ceases the activity of proteolytic enzymes. All additives significantly decreased the concentration of NH_3-N by accelerating the rate and extent of pH decrease during ensiling. The homLAB inoculated silage had the lowest concentration of NH_3-N, which can be related to the rapid decrease in pH. The NH_3-N concentration consistently increased during the 150 days ensiling. However, in the homLAB, hetLAB and MIX inoculated silages, most of the increase NH_3-N concentration occurred during the first 3 days (Figure 2C), which correspond to the decrease in pH (Figure 2A). After the establishment of stable pH there were minimal increases in NH_3-N. The increase in NH_3-N in the early stages of ensiling may be the result of excessive fermentation and proteolytic activity by microorganisms and plant respiration. A decrease in pH inhibits the growth of undesirable bacteria and microbes, halts the production of NH_3-N, and preserves the forage material for an extended period of time (Muck et al., 2018).

All the tested inoculants significantly improved the count of LAB and DM recovery, and decreased the count of yeast and mold of whole crop corn ensiled for 150 days. Notably, the greatest numbers of viable LAB were observed for homLAB, which improved the in-silo fermentation. Whereas, the lowest number of yeasts, mold and highest DM recovery was observed for hetLAB. The hetLAB decreased yeasts count by 60%, mold by 90% and increase DM recovery by 4.53%. In agreement with our findings *L. buchneri* as most used heterofermentative LAB strain can produce acetic acid to inhibit the growth of yeast and mold, thus improving DM recovery and aerobic silage stability (Guan et al., 2020). The LAB count decreased 72 hour exposure to air, and the maximum decrease (38%) was observed for homLAB, followed by control (23%), and the minimum for hetLAB (12%). In general the yeast count increased after 72 h exposure to air, but the magnitude of increase differed due to the type of inoculation. The maximum increase of 54% was observed for homLAB, followed by control (47%), while the hetLAB had the lowest increase of 27%. The greater spoilage in homLAB after exposure is because of the lower concentration of acetate, which is strongly antifungal, and greater concentration of lactate, which is a growth substrate for spoilage yeasts and mold (Oliveira et al., 2017). The highest aerobic stability of >72 h was recorded for hetLAB and MIX inoculated corn silages and lowest for control. It has been well established that *L. buchneri* increases the concentration acetic acid in silage, which inhibit the growth of yeasts and molds. Yeast is responsible for initiation of aerobic spoilage (Oliveira et al., 2017; Guan et al., 2020). Notably, like hetLAB, the MIX inoculant had lowest count of yeast, mold and highest DM recovery and aerobic stability, which could be related to the increase in acetic acid production as discussed before. Although, in the current study the positive effects of MIX did not exceed, the het LAB, it showed potential to improve aerobic stability and DM recovery.

5 Conclusions

The results of this study revealed that homLAB significantly improved silage fermentation quality and nutritional value, whereas hetLAB significantly improved DM recovery and aerobic stability of whole crop corn silage under hot summer conditions of the tropics. Among the additives, the homLAB inoculated silages had the highest ($P < 0.05$) content of lactic acids (9.20% DM), soluble carbohydrates (3.53% DM), and lowest contents of acetic acid (1.16% DM), NH_3-N (3.46% N) and pH (3.66), demonstrating desirable in silo fermentation. On the other hand, the hetLAB inoculated silages had the greatest ($P < 0.05$) content of acetic acids (2.01% DM), DM recovery (97.3%), and aerobic stability (>72 h). The greatest numbers of viable LAB were recorded in homLAB (8.13 log cfu/g) and MIX (7.89 log cfu/g) inoculated silages, while the lowest for control (6.29 log cfu/g). The lowest yeast (1.48 log cfu/g) and mold (0.22 log cfu/g) were recorded for hetLAB inoculated silage. Notably, like homLAB, inoculation with MIX inoculant significantly improved the rate and extent of pH decline and lactic acid production. On the other hand, hetLAB the MIX inoculant had lowest ($P < 0.05$) count of yeast, mold and highest DM recovery and aerobic stability. Although, in the current study the positive effects of MIX did not exceed, the individual (hom/het LAB) inoculants, it shows potential to improve in-silo fermentation and aerobic stability at the same time and needs further investigation, particularly with respect of dose rate.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The use and care of cannulated animals were approved by Institutional Animal Care and Use Committee of The University of Agriculture (Peshawar, KP, Pakistan).

Author contributions

NAK: Conceptualization, Formal analysis, Methodology, Writing – original draft. NK: Investigation, Methodology, Resources, Writing – review & editing. ST: Conceptualization, Data curation, Formal analysis, Project administration,

Supervision, Writing – review & editing. ZT: Conceptualization, Data curation, Funding acquisition, Investigation, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effect of endogenous sodium and potassium ions in plants on the quality of alfalfa silage and bacterial community stability during fermentation

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This study investigated the impact of endogenous sodium and potassium ions in plants on the quality of alfalfa silage, as well as the stability of bacterial communities during fermentation. Silage was produced from the fermented alfalfa, and the chemical composition, fermentation characteristics, and microbiome were analyzed to understand their interplay and impact on silage fermentation quality. The alfalfa was cultivated under salt stress with the following: (a) soil content of <1‰ (CK); (b) 1‰–2‰ (LP); (c) 2‰–3‰ (MP); (d) 3‰–4‰ (HP). The results revealed that the pH of silage was negatively correlated with the lactic acid content. With the increase of lactic acid (LA) content increased (26.3–51.0 g/kg DM), the pH value decreased (4.9–5.3). With the increase of salt stress, the content of Na^+ in silage increased (2.2–5.4 g/kg DM). The presence of endogenous Na^+ and K^+ ions in plants significantly affected the quality of alfalfa silage and the dynamics of bacterial communities during fermentation. Increased salt stress led to changes in microbial composition, with *Lactococcus* and *Pantoea* showing a gradual increase in abundance, especially under high salt stress. Low pH inhibited the growth of certain bacterial genera, such as *Pantoea* and *Pediococcus*. The abundance of *Escherichia–Shigella* and *Comamonas* negatively correlated with crude protein (CP) content, while *Enterococcus* and *Lactococcus* exhibited a positive correlation. Furthermore, the accumulation of endogenous Na^+ in alfalfa under salt stress suppressed bacterial proliferation, thereby reducing protein degradation during fermentation. The pH of the silage was high, and the LA content was also high. Silages from alfalfa under higher salt stress had higher Na^+ content. The alpha

diversity of bacterial communities in alfalfa silages showed distinct patterns. Desirable genera like *Lactococcus* and Lactobacillus predominated in silages produced from alfalfa under salt stress, resulting in better fermentation quality.

KEYWORDS

salt stress, endogenous ions, anaerobic fermentation, fermentation quality, microbial community

Introduction

According to statistics from the United Nations Educational, Scientific and Cultural Organization (UNESCO), saline–alkali soils are widely distributed worldwide, spanning more than 100 countries and encompassing roughly 25% of the global land area (Yun et al., 2023). In China, the total extent of saline–alkali soils is approximately 33.51 million hectares, accounting for 4.88% of the country's total land area. These soils are predominantly found in the North China, Northeast, and Northwest inland regions, with approximately 30% being agriculturally viable (Li and Wang, 2018). A prominent feature of saline–alkali soils is the excessive accumulation of Na^+ and K^+ ions. The presence of Na^+ has a profound impact on the physical, chemical, and biological properties of the soil (Hanum et al., 2022). Consequently, these factors contribute to soil structural instability, deterioration of soil hydraulic characteristics, nutrient imbalances in plants, and diminished vegetation coverage (Zhao et al., 2018).

Alfalfa (*Medicago sativa* L.) is an excellent leguminous forage characterized by its high nutritional content and palatability to livestock, alfalfa has a deep root system and strong nitrogen-fixing capacity, making it highly valuable for soil improvement (Xie et al., 2023). In saline–alkali soils, due to the superior nutrient quality, alfalfa can provide substantial quantities of high-quality protein, with silage being one of the primary preservation and utilization methods, particularly in environments with limited availability such as the rainy season. In recent years, research has shown that Na^+ and K^+ in plants have a significant impact on the quality of silage and the microbial dynamics during fermentation (Qiang et al., 2021b).

Ensiling is a fermentation process in which lactic acid bacteria (LAB), convert into lactic acid (LA) and other organic acids, thereby reducing the pH of the ensiled feed and inhibiting the growth of harmful bacteria, thus preserving the nutritional components of the silage (Nazar et al., 2020). However, silage fermentation is a complex process involving multiple microbial interactions, and the types and quantities of microorganisms directly influence the quality of silage. Salt is considered to be one of the critical factors affecting microbial growth and metabolism during fermentation. It is worth noting that the toxicity of Na^+ is higher than that of Cl^-, and an increase in Na^+ concentration can inhibit microbial activity and interfere with their metabolism (Ye et al., 2008).

The concentration of salt has been found to influence the microbial community during fermentation (Jeong et al., 2021). According to Yang et al. (2020), their study on the fermentation of Northeast-style sauerkraut at different salt concentrations revealed that the dominant genera were *Lactobacillus* and *Leuconostoc*. In sauerkraut samples with 0.5% salt, Lactobacillus was the most abundant genus, accounting for 88.46% of the total. The population of Lactobacillus gradually increased in samples with 0.5% salinity but showed a decreasing trend in samples with the three salt concentrations (1.5%, 2.5%, and 3.5%). These findings indicate that salt concentration significantly affects the microbial community. According to Sarkar et al. (2020), a low level of NaCl improves hydrolysis and acidification. Huang et al. (2022) conducted a study on acid production during fermentation of high-salt kitchen wastewater. They demonstrated the trend of diminishing acid production by LAB with an upsurge in NaCl concentration from 0 to 8 g/L. While optimal concentrations of Na^+ can indeed enhance enzymatic activities and help maintain osmotic balance, an excess can suppress LAB growth and fermentative function.

While the biotechnological applications of LAB have been extensively investigated in various contexts, their specific roles and potential still require meticulous research. To seek the better fermentation effect as we provided, this study hypothesizes that the endogenous levels of Na^+ and potassium K^+ in alfalfa plants are pivotal in determining the quality of alfalfa silage and the stability of the bacterial community during fermentation. We propose that optimal concentrations of these ions will enhance fermentation quality by supporting the growth and metabolic activity of beneficial lactic acid bacteria. In contrast, deviations from these optimal levels may impair silage quality and disrupt microbial homeostasis. Ultimately, this research may contribute to the development of strategies to enhance the nutritional value and microbial stability of alfalfa silage.

Material and methods

Experimental field and preparation of silage

The experiment was carried out at the Baotou Experimental Station for Forage Processing and High Efficient Utilization, located in Baotou City, Inner Mongolia, China. This region, situated in the Hetao Plain, is known for its high salinity. Geographically, the experimental site spans between 110°37”~110°27” E and 40°05”–40°17” N. The climate of the area is characterized as a north-

temperate continental climate with arid and windy conditions. The prevailing wind direction throughout the year was northwest. The average annual temperature is 6.8 °C, and the frost-free period lasts approximately 165 days. Annual average rainfall measures 330 mm, while the average evaporation rate is 2094 mm.

Description of raw materials and preparation of silage

The field experiments were conducted in 2022 utilizing the ZhongMu No.3 variety of alfalfa, provided by the Beijing Institute of Animal Science and Veterinary Medicine of the Chinese Academy of Agricultural Sciences. This particular variety of alfalfa exhibited strong salt resistance, excellent palatability, high nutritional value, and richness. In May 2022, alfalfa was sown using a drilling method with a row-to-row distance of 10 cm. Four different positions were selected to represent varying levels of salt stress: non-stress (CK), low stress (LP), moderate stress (MP), and high stress (HP). The salt stress contents at the CK, LP, MP, and HP sites were <1‰, 1‰–2‰, 2‰–3‰, and 3‰–4‰, respectively. Each group was replicated three times. The physical properties of the soil are presented in Table 1 for reference.

Alfalfa was harvested in the initial flowering stage. Then, wilted for 5 hours to obtain a targeted dry matter (DM) content, and immediately chopped into 2–3 cm lengths by a fodder chopper. Each material was treated separately to prevent crossing contaminations. 2 kg of the prepared alfalfa were packed in polyethylene plastic bags and sealed with a vacuum sealer in each group. All bags were assigned without additives. To investigate the effect of endogenous Na^+ and K^+ ions in plants on the quality of alfalfa silage and bacterial community stability during fermentation, triplicate samples for each group were prepared. Triplicate for each group was opened after 1, 3, 5, 7, 15, and 30 days of ensiling, respectively.

Chemical composition and organic acid

The DM of the fresh alfalfa and silage was determined by oven drying at 65°C for 72 h. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured according to Van Soest's procedures (Van et al., 1991). Colorimetry after reaction with anthrone reagent was used to determine the starch and water-soluble carbohydrate (WSC) content (Qiang et al., 2021b). Non-structural carbohydrates (NSC) are the sum of WSC and starch. The crude protein (CP = total N × 6.25) was determined using a Kjeldahl apparatus (Gerhart Vapodest 50 s, Germany) and the soluble protein (SP) was performed using the trichloroacetic acid method according to Cunniff (1997). The concentrations of Na^+ and K^+ ions of alfalfa were measured relative to standard solutions using a model 425 flame photometer (Sherwood Scientific Ltd., UK).

To assess the fermentation characteristics of the forage, 10 g of silage was mixed with 90 g of deionized water. The liquid extract was filtered through four layers of cheesecloth and filtered paper. The prepared filtrates were determined for measuring pH, ammonia nitrogen (ammonia-N), and organic acids. The pH was measured immediately with a glass electrode pH meter (LEICI pH S-3C, Shanghai, China). The content of ammonia-N was followed by the phenol-hypochlorite procedure of Broderick and Kang (1980). The concentration of organic acids were determined by high-performance liquid chromatography (HPLC, Waters e2695, Massachusetts USA; column: Waters Symmetry C18; oven temperature, 50°C; mobile phase 3 mmol L^{-1} perchlorate solution; flow rate 1.0 mL min^{-1}; flame photometric detector 210 nm; sample size 5.0 μL) of Ping et al. (2017). Buffering capacity (BC) was determined by the hydrochloric acid sodium hydroxide method (Lin et al., 1992).

Microorganisms enumeration

Microbial enumeration was performed using a 10 g fresh sample or silage. The sample was shaken with 90 mL of sterile distilled water at 120 rpm for 2 hours. From this solution, 1 mL was extracted and subjected to a 10-fold serial dilution for microorganism enumeration. The remaining solution was filtered and stored in a −80°C refrigerator for DNA extraction. Enumeration of LAB colonies was conducted on MRS agar medium (Nissui seiyaku Ltd., Tokyo, Japan). The plates were incubated in an anaerobic incubator (Heal Force Instrument Manufacturing Co., Ltd., Shanghai, China) at 37°C for 48 hours. Aerobic bacteria were cultured and counted on a nutrient agar medium, while yeasts were counted on potato dextrose agar (Nissui-seiyaku Ltd., Tokyo, Japan). Enumeration of *Enterobacteriaceae* was performed on Violet Red Bile Glucose Agar medium under aerobic conditions after 48 hours of incubation at 37°C. Colony-forming units (cfu) were used to express the microbial data, which were further transformed to a logarithmic scale on a fresh matter (FM) basis.

TABLE 1 The physical and chemical properties of soils.

Indictors	Na^+(g/kg)	K^+(g/kg)	pH	EC (mS/cm)
CK	0.11± 0.006c	0.027 ± 0.001d	7.4 ± 0.21b	0.21 ± 0.02a
LP	0.15 ± 0.004b	0.031 ± 0.001c	8.4 ± 0.08a	0.59 ± 0.10b
MP	0.16 ± 0.002b	0.035 ± 0.001b	8.6 ± 0.11a	1.35 ± 0.01c
HP	0.25 ± 0.019a	0.041 ± 0.001a	8.7 ± 0.34a	2.3 ± 0.29d

CK, without salt stress; LP, under light salt stress; MP, under moderate salt stress; HP, under severe salt stress; EC, electrical conductivity. Numbers in a column followed by different lowercase letters differ at $P < 0.05$.

High throughput sequencing of microbial population

The E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) was employed to extract microbial DNA from alfalfa samples, following the manufacturer's protocols. The concentration and purity of the final DNA were assessed using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA), and the quality was verified through 1% agarose gel electrophoresis. To amplify the V3–V4 hypervariable regions of the bacteria 16S rRNA gene, a thermocycler PCR system (GeneAmp 9700, ABI, USA) was utilized with primers 338F (5'-ACTCCTAC GGGAGGCAGCAG-3') and 806R (5'-GGACTACH VGGGTWTCTAAT-3'). The PCR reactions consisted of an initial denaturation at 95°C for 3 minutes, followed by 27 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. Each 20 μL reaction mixture included 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. The PCR reactions were performed in triplicate.

The resulting PCR products were extracted from a 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). Quantification was conducted using the QuantiFluor™-ST (Promega, USA) as per the manufacturer's instructions. The raw fastq files underwent demultiplexing and quality filtering using Trimmomatic. Subsequently, they were merged using FLASH based on the following criteria: (a) reads with an average quality score <20 over a 50 bp sliding window were truncated; (b) primers were allowed up to 2 nucleotide mismatches, and reads containing ambiguous bases were discarded; (c) sequences with an overlap longer than 10 bp were merged. Operational taxonomic units (OTUs) were clustered at a 97% similarity cutoff using UPARSE, while UCHIME was employed for the identification and removal of chimeric sequences. The RDP Classifier algorithm was used to analyze the taxonomy of each 16S rRNA gene sequence against the Silva (SSU123) 16S rRNA database, with a confidence threshold of 70%.

Statistical analysis

The statistical data were analyzed by the procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). Duncan's multiple range tests were used to evaluate differences among groups. High throughput sequencing data were performed using an online platform of Majorbio I-Sanger Cloud Platform (www.i-sanger.com).

Results

The chemical composition of fresh alfalfa

The chemical composition and microbial population of fresh alfalfa under various salinity groups are detailed in Table 2. It is evident that salt stress markedly affected the Na^+ and K^+ content in the alfalfa, with significant differences ($P < 0.05$). Specifically, the LP group resulted in the highest K^+ concentration, at 31.2 g/kg ($P < 0.05$), while the highest Na^+ concentration was recorded in the HP group, at 5.1 g/kg ($P < 0.01$). The DM content varied slightly between groups, ranging from 304.3 to 319.0 g/kg, with the MP group demonstrating the highest DM content. SP content was significantly greater in the MP group (103.7 g/kg) compared to other groups ($P < 0.01$). The WSC content was notably lower in the CK group (366.3 g/kg) than in other groups ($P < 0.01$). Furthermore, the alfalfa subjected to the HP group exhibited the highest NDF content (467.7 g/kg), while the lowest was observed in the LP group (420.3 g/kg), demonstrating a significant effect of salt stress on NDF

TABLE 2 Chemical composition and microbial populations of fresh alfalfa.

Items	CK	LP	MP	HP	SEM	*P*-value
Dry matter (g/kg FM)	304.3	312.7	319.0	305.3	0.25	0.21
Crude protein (g/kg DM)	192.3C	207.8B	234.3A	207.0B	0.11	0.19
Soluble protein (g/kg DM)	66.3C	72.3B	103.7A	68.3BC	0.08	<0.05
Neutral detergent fiber(g/kg DM)	432.0B	420.3C	474.3A	467.7A	0.12	<0.05
Acid detergent fiber (g/kg DM)	353.7C	348.0D	386.3A	381.0B	0.07	<0.05
Water-soluble carbohydrate (g/kg DM)	366.3C	372.3B	394.0A	368.3BC	0.07	<0.05
Lactic acid bacteria (Log_{10} cfu/g FM)	6.59	6.42	6.49	6.41	0.03	0.23
Enterobacteriaceae (Log_{10} cfu/g FM)	4.41A	4.21B	4.30AB	4.19B	0.03	0.06
Mold (Log_{10} cfu/g FM)	5.59	5.53	5.49	5.36	0.08	0.77
Aerobic bacteria (Log_{10} cfu/g FM)	7.31A	7.03BC	6.95C	7.04B	0.01	<0.05
Na^+ (g/kg DM)	1.9D	2.7C	4.8B	5.1A	0.40	<0.05
K^+ (g/kg DM)	26.7D	31.2A	30.9B	29.4C	0.53	<0.05

CK, without salt stress; LP, under light salt stress; MP, under moderate salt stress; HP, under severe salt stress; DM, dry matter; FM, Fresh matter; cfu, colony forming unit; SEM, standard error of mean value. The mean values (a–d) of different letters in the same column were significantly different (P < 0.05).

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($P < 0.01$). Interestingly, the MP group had significantly lower counts of aerobic bacteria, only 6.95 Log_{10} cfu/g ($P < 0.01$), which underscores the influence of salt stress on the microbial populations associated with the plant.

Fermentation characteristics and chemical composition of alfalfa silage

The fermentation characteristics of alfalfa silage at different days of ensiling are presented in Table 3. The results indicated that salt stress (T), ensiling days (D), and their interaction significantly ($P < 0.01$) affected the pH, LA, Butyric acid (BA), Acetic acid (AA), and ammonia nitrogen content. After 30 days of ensiling, the pH of alfalfa silage ranged from 4.93 to 5.12, with a significantly lower pH observed for MP silage compared to other silages ($P < 0.05$). The pH of silage at different fermentation stages within 1–30 days were significantly different ($P < 0.05$), with a gradual decrease in pH as the ensiling time increased, with HP silage decreasing from 6.23 on day 1 to 5.13 on day 30, which was a significant decrease of 1.1 of pH. The rapid decrease in pH (Table 3) led to an 80g/kg reduction in WSC content for 30-day MP silage compared to 1-day silage (384.3 g/kg vs. 304.3 g/kg, respectively, Table 4). The AA contents of the 30-day silage ranged from 31.4g/kg to 46.7g/kg, with significantly lower AA content observed for HP silage compared to other silages ($P < 0.05$). With an increase in salt stress, the accumulated amount of AA in silages for the first 15 days showed an increasing trend across all four groups, but a decreasing trend was observed for MP and HP silages on day 30. BA content ranged from 13.1g/kg to 16.4g/kg, with CK silage having the highest BA content (16.4g/kg) ($P < 0.05$) on day 30 of fermentation and the lowest LA content (26.3 g/kg) ($P < 0.05$). The ammonia nitrogen contents of CK and LP silages were significantly ($P < 0.05$) higher than that of MP and HP silages at 30 days of ensiling.

The results showed that salt stress (T), ensiling days (D), and their interaction significantly ($P < 0.01$) affected the contents of WSC, CP, NDF, and ADF (Table 4). In the study, the NDF content ranged from 377g/kg to 439g/kg, and the SP content ranging from 124.3g/kg to 150.3g/kg. Both T and D significantly influenced the NDF and SP content ($P < 0.05$). After 30 days of ensiling, the SP content of the MP group (124.3 g/kg) was significantly lower than that of the other groups. The CP contents of 30-day silage ranged from 181.3g/kg to 206.3g/kg, with significantly higher CP content observed for MP silage compared to other silages ($P < 0.05$).

TABLE 3 Fermentation characteristics of alfalfa silage on different days of silage.

Items	Group	Ensiling(d)						SEM	*P*-value		
		1d	3d	5d	7d	15d	30d		T	D	T×D
pH	CK	6.20ABa	5.99ABb	5.73Ac	5.18Bd	5.18Ad	5.12Ad	0.01	<0.01	<0.01	<0.01
	LP	6.17BCa	5.90ABb	5.83Ab	4.91Cc	4.67Dd	4.99ABc				
	MP	6.13Ca	5.83Bb	5.24Cc	4.84Cde	4.76Ce	4.93Bd				
	HP	6.23Aa	6.03Ab	5.61Bc	5.43Ad	5.17Ae	5.13Ae				
Ammonia-N (g/kg DM)	CK	10.3Ae	12.4Ad	15.9Ac	18.7Ab	23.6Ba	24.3Ba	0.01	<0.01	<0.01	<0.01
	LP	8.7Bf	12.6Ae	14.7Bd	17.3Bc	24.3Ab	27.3Aa				
	MP	8.7Be	11.7Bd	14.1Cc	16.8Bb	17.1Cab	17.7Ca				
	HP	7.1Ce	10.8Cd	12.4Dc	14.4Cb	14.7Dab	15.4Da				
Lactic acid (g/kg DM)	CK	10.8Ae	15.6Ad	19.7Cc	23.1Bb	25.8Da	26.3Da	0.01	<0.01	<0.01	<0.01
	LP	10.5Af	16.1Ae	20.5Bd	28.1Ac	38.7Ab	45.6Aa				
	MP	10.2Af	14.5Be	21.9Ad	28.9Ac	36.6Bb	43.2Ba				
	HP	8.4Bf	13.6Ce	20.2BCd	23.9Bc	31.4Cb	40.1Ca				
Acetic acid (g/kg DM)	CK	3.3Af	12.4Ae	27.7Ad	31.5Ac	41.8Ab	46.7Aa	0.01	<0.01	<0.01	<0.01
	LP	3.6Af	11.3Be	24.5Bd	30.2Bc	38.4Bb	41.2Ba				
	MP	2.7Bf	11.4Be	23.0Cd	29.3Cc	37.5Ca	32.9Cb				
	HP	1.9Cf	10.5Ce	21.8Dd	29.6BCc	36.6Da	31.4Cb				
Butyric acid (g/kg DM)	CK	2.5Bf	5.6Ae	8.7Ad	12.5Ac	14.4Ab	16.4Aa	0.003	<0.01	<0.01	<0.01
	LP	3.1Af	5.6Ae	7.8Bd	9.6Cc	13.5Bb	15.4Aa				
	MP	201Ce	4.7Bd	6.7Cc	10.3Bb	12.5Ca	12.7Ba				
	HP	1.1Df	2.5Ce	4.7Dd	7.6Dc	09.5Db	13.1Ba				

CK, without salt stress; LP, under light salt stress; MP, under moderate salt stress; HP, under severe salt stress; DM, dry matter; T, salt stress; D, ensiling day; T × D, interaction between salt stress and silage days; SEM, standard error of mean value. The mean values (a–f) of different letters in the same column were significantly different (P < 0.05).

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TABLE 4 Study on chemical composition of alfalfa silage on different days of silage.

Items	Group	Ensiling(d)						SEM	*P*-value		
		1d	3d	5d	7d	15d	30d		T	D	TxD
Dry matter (g/kg FM)	CK	304.7Aa	293.3Aab	281.7Abc	275.7Bc	273.3Ac	270.7Ac	0.1	0.02	<0.01	0.11
	LP	305.3Aa	301.0Aa	298.3Aa	298.7Aa	277.0Ab	267.3Ab				
	MP	314.7Aa	292.0Aab	277.7Ab	283.7ABb	291.0Aab	280.3Ab				
	HP	303.7Aa	301.3Aa	288.0Aab	289.0ABab	288.0Aab	275.3Ab				
Crude protein (g/kg DM)	CK	203.7Ba	197.3Bab	191.7Cb	190.3Bbc	180.7Bd	181.3Ccd	0.05	<0.01	<0.01	<0.01
	LP	204.0Ba	198.0Bab	185.0BCabc	196.0Babc	187.7Bc	193.0Bbc				
	MP	228.7Aa	228.0Aa	230.0Aa	223.3Aa	215.7Ab	206.3Ac				
	HP	204.3Ba	203.3Ba	201.0Bab	196.7Bb	188.7Bc	195.3Bb				
Soluble protein (g/kg DM)	CK	66.7Df	73.7Ce	86.7Dd	99.7Cc	110.7Cb	132.7Ca	0.02	<0.01	<0.01	<0.01
	LP	75.7Bf	80.0Be	92.3Cd	98.7Cc	126.7Ab	150.3Aa				
	MP	104.3Af	108.3Ae	113.3Ad	116.7Ac	122.3Bb	124.3Da				
	HP	71.0Cf	82.3Be	94.7Bd	104.3Bc	126.3Ab	138.3Ba				
Neutral detergent fiber (g/kg DM)	CK	428.0Ca	423.3Cb	419.3Bc	417.7Bc	418.3Bc	407.7Bd	0.03	<0.01	<0.01	<0.01
	LP	421.7Da	417.7Da	408.7Cb	395.3Cc	383.7Cd	377.0Ce				
	MP	469.3Aa	462.3Ab	456.3Ac	447.3Ad	442.3Ae	439.0Af				
	HP	463.3Ba	459.7Bb	455.7Ac	449.3Ad	442.3Ae	436.30Af				
Acid detergent fiber (g/kg DM)	CK	351.3Ca	347.7Cb	341.0Cc	337.3Cd	335.7Ce	327.7Cf	0.02	<0.01	<0.01	<0.01
	LP	347.0Da	342.3Db	337.3Dc	332.3Dd	328.0De	325.3Df				
	MP	381.3Aa	375.3Ab	367.7Bc	361.3Bd	354.3Be	347.3Bf				
	HP	381.3Aa	377.0Ab	370.7Ac	366.7Ad	359.0Ae	350.7Af				
Water-soluble carbohydrates (g/kg DM)	CK	356.7Ca	353.7Ba	346.7Bb	336.3Bc	317.3Ad	312.7Ad	0.04	<0.01	<0.01	<0.01
	LP	365.7Ba	346.7Cb	339.0Bc	328.7Cd	316.7Ae	303.7Bf				
	MP	384.3Aa	378.3Aa	363.3Ab	343.3Ac	312.3ABd	304.3Bd				
	HP	354.3Ca	342.3Cb	334.7Bc	325.3Cd	309.7Be	305.0Be				

CK, without salt stress; LP, under light salt stress; MP, under moderate salt stress; HP, under severe salt stress; FM, Fresh matter; DM, dry matter; T, salt stress; D, ensiling day; T × D, interaction between salt stress and silage days; SEM, standard error of mean value. The mean values (a–f) of different letters in the same column were significantly different ($P < 0.05$).

Analysis of microbial community quantities

Analysis revealed significant effects ($P < 0.01$) of salt stress (T), ensiling days (D), and the interaction between salt stress and ensiling days on the quantities of LAB, *Escherichia coli*, molds, and general aerobic bacteria (Table 5). In terms of ensiling fermentation days, the quantity of LAB in alfalfa silage reached its peak after 3 days of fermentation and then leveled off. The CK silage group had a significantly higher quantity of LAB than the other groups, at 7.66 Log_{10} cfu/g. There were no significant differences ($P > 0.05$) in the quantity of LAB between the 5d, 7d, 15d, and 30d fermentation periods. At 1 day of alfalfa ensiling fermentation, the number of LAB in the CK group was significantly lower than in the other groups ($P < 0.05$), at 6.97 Log_{10} cfu/g. Overall, except for the 3d ensiling, the MP silage group had a significantly higher quantity of LAB than the other group ($P < 0.05$). There were no significant differences ($P > 0.05$) in the quantity of LAB between the 15d and 30d fermentation periods, indicating that the quantity of LAB in salt–alkali alfalfa silage reached a stable state after 30 days of fermentation.

From the perspective of ensiling fermentation days, the quantity of *Escherichia coli* in alfalfa silage reached its peak after 1 day of fermentation, and then gradually decreased. The MP group (6.17 Log_{10} cfu/g) had a significantly higher quantity of *Escherichia coli* than the CK and LP groups. At 3 days of fermentation, the quantity of *Escherichia coli* in the MP silage group was significantly lower than in the other groups, at 2.16 Log_{10} cfu/g. *Escherichia coli* was not detected in the MP and HP groups at 5d of fermentation. *Escherichia coli* was not detected in any of the groups at 7d, 15d, and 30d of fermentation. From the perspective of ensiling fermentation days, the quantity of molds in alfalfa silage gradually decreased after 1d of fermentation, and no molds were detected at 3d, 5d, 7d, 15d, and 30d of fermentation. At 1 day of alfalfa silage fermentation, the quantity of molds in the CK group

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TABLE 5 Study on microbial quantity of alfalfa silage on different days of silage.

Items	Groups	Ensiling(d)							*P*-value		
		1d	3d	5d	7d	15d	30d	SEM	T	D	TxD
Lactic acid bacteria (Log_{10} cfu/g FM)	CK	6.97Bd	7.66Aa	7.58Aab	7.56Aab	7.53Bbc	7.45Ac	0.01	<0.01	<0.01	<0.01
	LP	7.02Bb	7.61Aa	7.61Aa	7.52Aa	7.52Ba	7.57Aa				
	MP	7.19Ab	7.58Aa	7.65Aa	7.61Aa	7.68Aa	7.53Aa				
	HP	7.25Ac	7.45Ab	7.33Bc	7.51Aab	7.51Bab	7.59Aa				
Enterobacteriaceae (Log_{10} cfu/g FM)	CK	4.41Ba	3.10Ab	3.10Ab	ND	ND	ND	0.06	0.2	<0.01	<0.01
	LP	4.43Ba	2.26Ab	3.32Aab	ND	ND	ND				
	MP	6.17Aa	2.16Ab	ND	ND	ND	ND				
	HP	6.14Aa	3.16Ab	ND	ND	ND	ND				
Mold(Log_{10} cfu/g FM)	CK	6.01Aa	ND	ND	ND	ND	ND	0.01	<0.01	<0.01	<0.01
	LP	5.88Ba	ND	ND	ND	ND	ND				
	MP	ND	ND	ND	ND	ND	ND				
	HP	ND	ND	ND	ND	ND	ND				
Aerobic bacteria (Log_{10} cfu/g FM)	CK	7.31Aa	7.15Ab	7.09Ab	6.96Ac	7.05Abc	6.77Bd	0.004	<0.01	<0.01	<0.01
	LP	7.05Cb	7.15Aa	6.94Cc	6.98Ac	6.97Bc	6.82Bd				
	MP	7.15Ba	7.07Bb	7.02Bbc	6.99Ac	6.97Bc	6.78Bd				
	HP	7.01Ca	6.99Ca	6.94Cbc	6.97Aab	6.92Bc	6.92Ac				

CK, without salt stress; LP, under light salt stress; MP, under moderate salt stress; HP, under severe salt stress; ND, not detected; cfu, colony forming unit; T, salt stress; D, ensiling day; T × D, interaction between salt stress and silage days; SEM, standard error of mean value. The mean values (a–d) of different letters in the same column were significantly different (P < 0.05).

was significantly higher than in the LP group ($P < 0.05$), while no molds were detected in the MP and HP groups, indicating better preservation in the later stages.

In terms of ensiling fermentation days, the quantity of general aerobic bacteria in alfalfa silage reached its peak after 1 day of fermentation, and then gradually decreased. The CK group had a significantly higher quantity of general aerobic bacteria than the other groups ($P < 0.05$). There were significant differences ($P < 0.05$) in the quantity of general aerobic bacteria between the 5d, 7d, 15d, and 30d fermentation periods. At 30d of fermentation, the HP group had the highest quantity of general aerobic bacteria, at 6.92 Log_{10} cfu/g. However, the quantity of general aerobic bacteria was the lowest at 1d of fermentation, at 7.01 Log_{10} cfu/g.

Analysis of bacterial community composition

To gain further insights into the dynamic succession of bacterial communities in alfalfa silage under salt stress, we assessed the bacterial communities at the phylum and genus levels (Figure 1). The bacterial communities in fresh alfalfa and silage feed were mainly composed of four phyla (Figure 1A). Before ensiling, the phylum Proteobacteria had the highest abundance, followed by Actinobacteria, Bacteroidetes, and Firmicutes. After ensiling, Firmicutes became the dominant phylum. Compared to the other groups, the MP-30 group showed a higher abundance of Firmicutes in the silage feed. At the genus level, the dominant genera in the pre-ensiling group were *Pediococcus*, *Pseudomonas*, and *Sphingomonas*. In the HP group, the relative abundance of *Pediococcus* and *Pseudomonas* was higher than in the other groups, while *Escherichia–Shigella* had a lower relative abundance. After 30 days of ensiling, the dominant genera in all groups were *Enterococcus*, *Lactococcus*, *Escherichia–Shigella*, and *Sphingomonas* (Figure 1B). *Enterococcus* and *Escherichia–Shigella* were the dominant genera in CK-30 and LP-30 silage feed. *Lactococcus* dominated in the HP-30 group. The lowest concentrations of *Pseudomonas* and *Flavobacterium* were observed in the MP-30-treated silage feed.

With prolonged fermentation time, the relative abundance of *Pantoea* gradually decreased, while *Enterococcus* and *Pseudomonas* gradually became dominant (Figure 1C). In the LP-30 silage, after establishing favorable anaerobic conditions, *Enterococcus* continued to proliferate, inhibiting the growth of LAB, leading to a gradual decrease in the abundance of *Lactococcus* and LAB, and an increase in the abundance of *Enterococcus*. Furthermore, both low and high levels of salt stress were unfavorable for the growth of *Escherichia–Shigella*.

The Non-metric Multidimensional Scaling (NMDS; Figure 2A) and Neutral Community Model (NCM; Figure 2B) analyses performed in this study reveal the variations in bacterial communities during anaerobic fermentation. The stress value of 0.091 indicates a good fit of the data to the model. The NMDS analysis demonstrates similarities and differences between the samples from different groups.

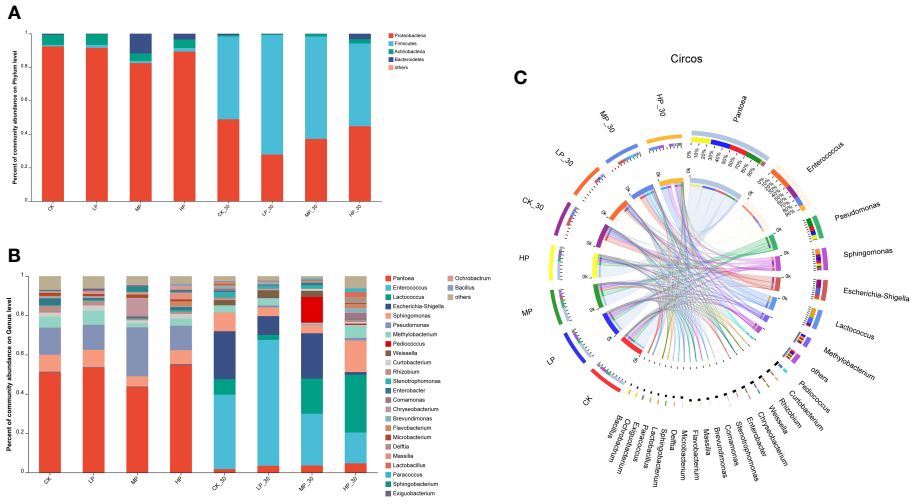

FIGURE 1
Bacterial communities and relative abundance by phylum level **(A, C)** and genus level **(B)** for raw alfalfa and alfalfa silage. CK, without salt stress; LP, under light salt stress; MP, under moderate salt stress; HP, under severe salt stress; 30, 30 days of ensiling.

This diagram shows the relative positions of the samples in each of the eight groups. The HP group shows a significant difference compared to the other groups, with the samples distributed in the central region of the graph. MP-30, LP-30, and CK-30 alternately overlap and are distributed on the left side of the figure, while CK, MP, and HP alternately overlap and are distributed on the right side of the figure. Supported by the NCM model and NMDS analysis provides clear insights into changes in bacterial communities during anaerobic fermentation. The results showed that salt stress had significant effects on the microbial composition of fresh and 30-day-old alfalfa silage. This information helps us understand the dynamics of bacterial communities and helps optimize anaerobic fermentation processes to improve silage quality. This study employed the NCM to analyze the impact of Na^+ and K^+ concentrations on the assembly mechanisms of bacterial communities in alfalfa silage. The degree of community assembly was assessed by calculating the model's goodness-of-fit (R^2).

The relationships among the ions, microbial community, and characteristics products in alfalfa silage

The pH of the silage is positively correlated with the abundance of *Comamonas*, *Stenotrophomonas*, and *Weissella*, and negatively correlated with the abundance of *Pantoea* and *Pediococcus* (Figure 3). The DM content of the silage is significantly negatively correlated with the abundance of *Weissella* and *Enterococcus* ($P < 0.01$). The WSC content of the silage is positively correlated with the abundance of *Escherichia–Shigella* and *Lactococcus* ($P < 0.05$). The Na^+ content of the silage is significantly positively correlated with the abundance of *Pantoea*, and significantly negatively correlated with the abundance of *Escherichia–Shigella*, *Weissella*, *Stenotrophomonas*, *Enterococcus*, and *Methylobacterium* ($P < 0.05$). The LA content of the silage is positively correlated with the abundance of *Lactococcus* ($P < 0.01$).

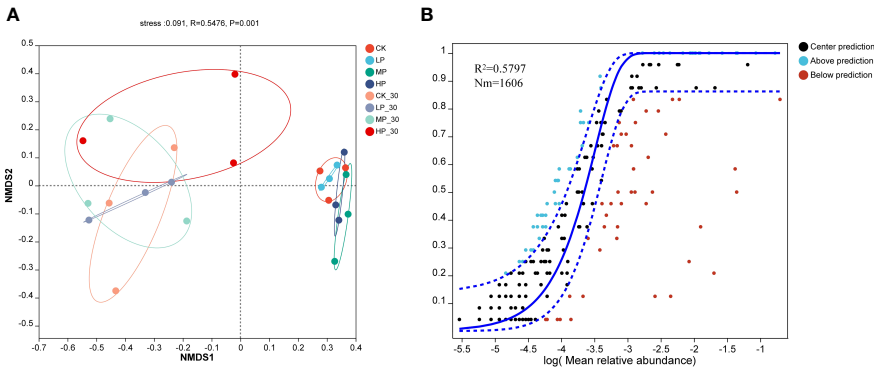

FIGURE 2
The non-metric multidimensional scaling (NMDS) on genus level for raw alfalfa and alfalfa silage **(A)** and the neutral community model (MCN) of silage on genus level **(B)**. CK, without salt stress; LP, under light salt stress; MP, under moderate salt stress; HP, under severe salt stress; 30, 30 days of ensiling.

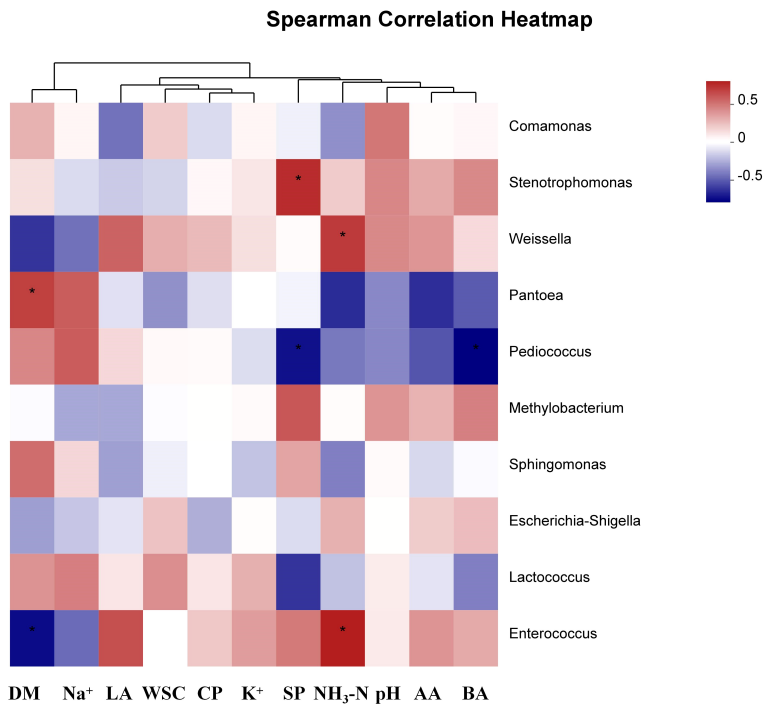

FIGURE 3
Heatmap of prominent bacterial genera (10 most abundant genera) for alfalfa silage. CK, without salt stress; LP, under light salt stress; MP, under moderate salt stress; HP, under severe salt stress; 30, 30 days of ensiling. *, $P < 0.05$.

The BA content of the silage is positively correlated with the abundance of *Weissella*, *Stenotrophomonas*, *Enterococcus*, *Methylobacterium*, and *Escherichia–Shigella* ($P < 0.05$).

The regression equation for Na^+ and the bacterial community in alfalfa silage is y = 0.065x + 0.229 (Figure 4A). As the concentration of Na^+ increases, the beta diversity of the bacterial community in alfalfa silage follows the order CK-30 > MP-30 > LP-30 > HP-30. This study demonstrates a significant positive correlation between Na^+ and the beta diversity of the bacterial community in alfalfa silage, indicating that Na^+ has an impact on the structure of the bacterial community. It can be observed that the regression equation for K^+ and the bacterial community in alfalfa silage is y = 0.2x − 0.572 (Figure 4B). The beta diversity of the bacterial community in alfalfa silage follows the order CK-30 > MP-30 > LP-30 > HP-30. These findings highlight the influence of endogenous Na^+ and K^+ in alfalfa on the structure of the bacterial community in salinity–alkalinity soil. They further confirm the impact of endogenous Na^+ and K^+ in plants on the stability and quality of bacterial communities during silage fermentation.

Co-occurrence network analysis of bacterial communities

Significant changes in microbial populations within silage occurred under different salt stress levels, with evident interactions among different genera (Figure 5). Additionally, the corresponding network analysis revealed that lower salt stress levels resulted in a more complex and interconnected microbial network compared to higher stress levels. Specifically, there was an increase in the number of positive correlations between different bacterial genera under low salt stress, indicating a more diverse and stable microbial community. In contrast, the microbial network complexity observed in the MP group was relatively low, with fewer positive correlations among different genera. These findings suggest that the LP group may induce synergistic effects on the microbial community within the silage, leading to increased diversity and stability within the microbial ecosystem. Furthermore, after 30 days of ensiling, the LP group exhibited a relatively complex microbial community. Overall, based on the topological metrics, the LP group displayed the most complex network with significant microbial interactions.

Discussion

The chemical composition of fresh alfalfa

Salt stress affects the normal growth and development of plants and affects the chemical composition of plants (Monroy and Ericsson, 2023). Most plants are sensitive to salt, and alfalfa is no exception. When plants are subjected to salt stress, both their growth and chemical composition are affected. Significant differences exist in SP content among different groups. Compared to the CK, the low-concentration salt stress groups (LP and MP) exhibited a significant increase in SP content. Among them, the MP group had the highest SP content, reaching 103.7 g/kg. As salt

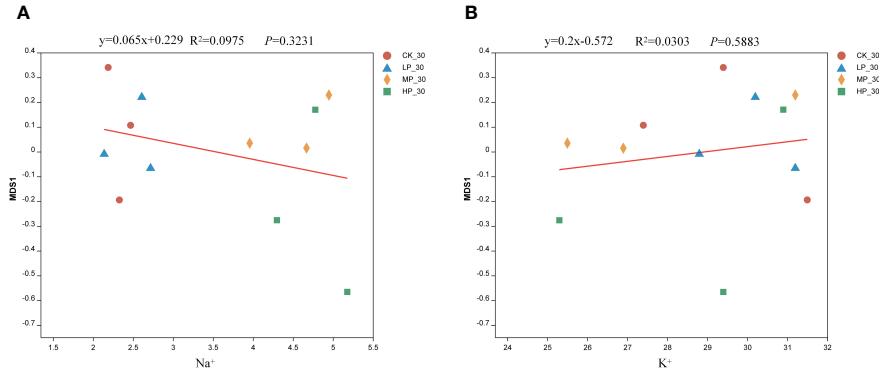

FIGURE 4
Regression analysis of alfalfa microbial community and sodium ions **(A)** and potassium ion **(B)**. CK, without salt stress; LP, under light salt stress; MP, under moderate salt stress; HP, under severe salt stress; 30, 30 days of ensiling.

concentration increased, the SP content in the HP group significantly decreased, consistent with the findings of Horchani et al. (2023), who demonstrated a decrease in SP content under high salt stress conditions. Carbohydrates are typically categorized as non-structural and structural carbohydrates. Non-structural carbohydrates are found within plant cells and mainly include sugars, starch, organic acids, and other storage carbohydrates (Kristin et al., 2023). Under salt stress, plants generally increase their carbohydrate levels, such as sugars and starch, to mitigate the stress (Shao et al., 2022). The WSC content in the MP group was significantly higher than that in the HP group. This indicated that as salt stress intensity increased, the WSC content gradually decreased, suggesting that salt stress was an important factor influencing the chemical composition of alfalfa.

Fermentation characteristics and chemical composition of alfalfa silage

The pH of silage is an important indicator for evaluating the fermentation effect, and a pH of 4.2 is considered as the benchmark for high-quality silage (He et al., 2021). With increasing salt concentration, the pH of the four silage groups continuously decreased. The results of the study showed that the pH of the LP

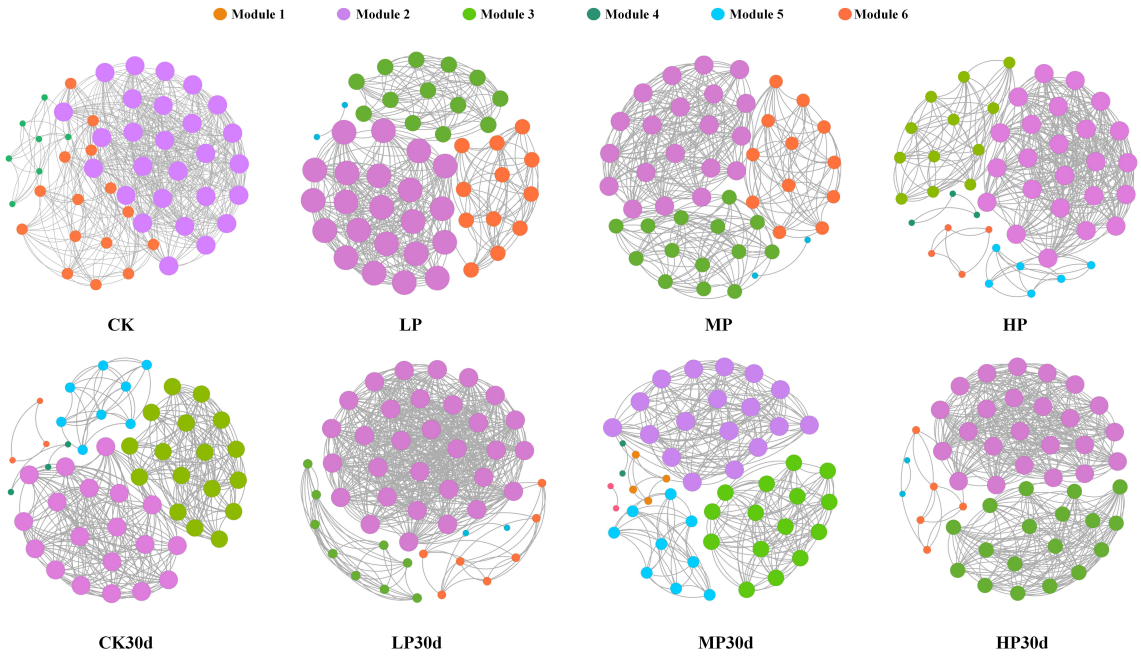

FIGURE 5
Differences of co-occurrence network visualization in each group. CK, without salt stress; LP, under light salt stress; MP, under moderate salt stress; HP, under severe salt stress; 30, 30 days of ensiling.

and MP groups was significantly lower than that of the CK and HP groups from day 1 to day 30. At day 30 of silage fermentation, the HP group had the highest pH value of 5.13, while the MP group had the lowest pH value of 4.93. These results indicate that higher pH values are associated with poorer fermentation quality. The variation in pH may be attributed to different salt concentrations in alfalfa or differences in microbial populations during silage fermentation. The significant differences in the chemical composition of alfalfa samples and silage fermentation characteristics are closely related. The lower pH and higher LA concentration in the MP silage group are due to the rapid metabolism of WSC into LA by LAB, resulting in a decrease in pH and stability of the silage within a short period (Wang et al., 2021).

During the 30-day fermentation period, *Weissella*, *Enterobacter*, and *Pseudomonas* were detected. *Weissella* and *Enterobacter* not only consumed a large amount of WSC content but also exhibited low utilization efficiency of WSC (Blajman et al., 2020). Therefore, with increasing salt concentration and fermentation days, the WSC content in all groups continuously decreased. BA, LA, and AA reflect the efficiency of silage fermentation or secondary fermentation. In addition, as the LA content continuously increased, the BA content gradually increased as well, indicating that the amount of BA depends on the amount of LA. This may be caused by secondary fermentation by heterofermentative LAB and yeast (Qiang et al., 2021a).

The presence of ammonia nitrogen (NH_3-N) during the ensiling process is an important indicator of the protein hydrolysis (He et al., 2020). The inhibitory effects of MP and HP on ammonia accumulation suggest enhanced preservation of protein during the ensiling process. In the ensiling process, protein undergoes extensive degradation and deamination of amino acids (Bachmann et al., 2020), and a typical reason for ammonia nitrogen accumulation is protein hydrolysis enzymes (Tian et al., 2022). After the fermentation of alfalfa, the SP content increased from 66.7–104.3 g/kg to 124.3–150.3 g/kg, which may be related to protein hydrolysis during the fermentation. The degradation of macromolecular proteins into small molecular proteins with water-soluble characteristics could be one of the reasons for the increase in SP content (Liu et al., 2021).

Microbial community of alfalfa silage

In this study, LAB, *Escherichia coli*, and some aerobic bacteria were found to dominate in all alfalfa silages. These findings are consistent with previous research reports on alfalfa silage and even corn silage (Guo et al., 2020). Mold was observed in the CK and LP groups on day 1 of ensiling, but it disappeared as the duration of ensiling and salt concentration increased. This may be attributed to the metabolites produced by LAB, which inhibit the growth of harmful bacteria such as Clostridium botulinum and mold (Qixuan et al., 2021). LAB and *Escherichia coli* were the most abundant microbial types during ensiling. Nazar et al. (2020) reported a transition in the bacterial community from Proteobacteria to Firmicutes, and they found that anaerobic and acidic conditions favored the growth of Firmicutes. Additionally, the quantity of *Escherichia coli* decreased regularly with increasing salt stress.

Biological and abiotic stresses are closely related to the growth and development of alfalfa, with salt stress being a major abiotic stress factor affecting yield and nutritional quality (Shao et al., 2022). Microbes play a significant role in regulating plant growth, stress resistance, and disease resistance (Wang et al., 2021), and the structure of microbial communities is influenced by plant species and growth stages. After experiencing biotic and abiotic stresses, plants can alleviate the harm caused by stress by adjusting the structure of the microbial community (Afridi et al., 2022). Bouzroud et al. (2023) found that plants without symbiotic microorganisms are more susceptible to diseases and less likely to survive in natural environments.

The genus and quantities of microorganisms are closely related to the nutritional and fermentation quality of alfalfa silage (Qiang et al., 2021a). In the MP group, there was a strong positive correlation between general aerobic bacteria, *Escherichia coli*, and CP, WSC, ADF, and NDF. From the perspective of the relationship between the main nutrients and microorganisms in alfalfa silage from saline–alkaline land, a higher quantity of LAB is associated with better nutritional quality of alfalfa, while higher quantities of *Escherichia coli*, mold, and general aerobic bacteria are unfavorable for the nutritional preservation of alfalfa silage from saline–alkaline land. In moderately saline–alkaline land alfalfa silage, there was a negative correlation between general aerobic bacteria, *Escherichia coli*, mold, and SP content. This is consistent with Chauhan et al. (2023) previous research. Based on the above analysis, it can be concluded that LAB is the key factor influencing the nutrition of alfalfa silage from different saline–alkaline lands. The species and quantity of LAB in alfalfa silage raw materials directly determine the quality of alfalfa silage and are crucial for the success of silage fermentation (Qiang et al., 2021b). LAB can establish a dominant microbial population during the silage process when their quantity exceeds 10^5 Log_{10} cfu/g, meeting the requirements for silage (Zhu et al., 2022). In this study, the LAB content in alfalfa silage raw materials under different salt stress ranged from 6.41 Log_{10} cfu/g to 6.59 Log_{10} cfu/g, meeting the requirements for direct silage. In saline–alkaline land alfalfa silage, the quantity of LAB increased with the duration of silage, and the quantity of LAB in the 30-day silage fermentation was significantly higher than that in the silage raw materials. In this study, the quantity of LAB dominated after 3 days of silage, and no molds were found in all groups after 3 days of silage, while no *Escherichia coli* was found in all groups after 7 days of silage. This is consistent with the findings of Dong et al. who reported that in the later stages of silage fermentation, the accumulation of LA produced by LAB and the decrease in pH inhibit the growth of *Escherichia coli* and mold (Dong et al., 2020).

The LAB played a crucial role in the process of ensiling, while soluble carbohydrates serve as fermentation substrates necessary for normal microbial metabolism in silage feed, it is also the main factor affecting the fermentation quality of silage (Stirling et al., 2022). As the ensiling process progresses, soluble carbohydrates are metabolizes by LAB to produce organic acids, resulting in a

decrease in soluble carbohydrate content (Luc and Frédéric, 2020). In this study, pH rapidly decreased starting from the 5th day of fermentation, and this downward trend in pH was closely related to the increase in the population of LAB on the 5th day of fermentation. Meanwhile, the average lactate content gradually increased. The low pH and high lactate content in the MP and HP groups can be attributed to the LAB, which rapidly metabolize soluble carbohydrates into LA through LA fermentation (Nazar et al., 2020). The increased LAB species include Lactobacillus, *Enterococcus*, *Enterobacter*, and *Streptococcus*. This is consistent with the findings of Mariele et al., where LA production by LAB led to a decrease in the pH of the silage feed (Agarussi et al., 2019). The results of this study indicate that the pH variation may be attributed to the differences in the adherent bacterial community of alfalfa from different saline–alkaline lands, which in turn have an impact on the differential lactate and acetate contents observed in this study. These findings further confirm the significant relationship between chemical composition and microbial communities. Understanding the composition and distribution of microorganisms is of great importance for improving silage feed quality, promoting the development of the forage processing industry, and conserving and utilizing microbial resources.

Na^+ and K^+ were likely the cause of microbial community changes in this study, as plant-associated microorganisms exhibit different reactions to varying salt stress levels. In our investigation, both *Lactococcus* and *Pantoea* showed a gradual increase in abundance with increasing salt stress, particularly dominating in the HP-30 group. An anaerobic environment, compared to fresh samples, brings about alterations in the microbial habitat, suppressing aerobic microorganisms during anaerobic fermentation and consequently resulting in noteworthy differences between fresh and ensiled microbial communities (Nazar et al., 2020). Bacterial diversity and richness of CK, LP, MP, and HP groups all decreased after 30 days of fermentation. Similarly, Jie et al. (2020) reported a decrease in bacterial diversity in silage due to the increased abundance of dominant genera such as *Lactococcus* and *Enterococcus*. By the 30th day of ensiling, *Enterococcus*, *Lactococcus*, *Escherichia–Shigella*, and *Sphingomonas* were the dominant genera in all groups. The growth and proliferation of *Pantoea*, *Pseudomonas*, and *Methylobacterium* were inhibited by the lower pH, while the relative abundance of *Pantoea* gradually declined with increasing salt stress. Under anaerobic conditions, *Enterococcus* dominated in CK-30 (37.8%) and LP-30 (64.1%). Similar to other LAB members, *Enterococcus* exhibits the ability to survive, resist, and proliferate under adverse conditions, including low and high pH levels, high temperature, and osmotic stress (Acciarri et al., 2023).

In this study, A relatively low R^2 value (0.5797) was observed, suggesting that the assembly of bacterial communities in alfalfa silage aligns more closely with the neutral model, indicating a greater susceptibility to deterministic processes and less influence from stochastic processes. As the content of Na^+ and K^+ increased, the richness of the bacterial community gradually decreased. This may be attributed to the increase in taxonomic groups within the bacterial community under salt stress, which aligns with the findings of Meng et al. (2019). Their research revealed that the bacterial community exhibits a highly active and sensitive response to Na^+ and K^+. The abundance of *Escherichia–Shigella* and *Comamonas* was negatively correlated with CP in silage, while the abundance of *Enterococcus* and *Lactococcus* showed a positive correlation. With increasing salt stress and ensiling duration, the CP content continued to rise. This may be due to the higher sodium ion concentration in alfalfa under salt stress, which inhibits bacterial proliferation and subsequently reduces protein breakdown (Hong et al., 2023).

Conclusion

The study presented herein examined the presence of endogenous Na^+ and K^+ in plants significantly impacts the quality of alfalfa silage and the stability of bacterial communities during fermentation. The impact of endogenous sodium and potassium ions on alfalfa silage is twofold. Increased salt stress leads to changes in microbial composition, with *Lactococcus* and *Pantoea* exhibiting a gradual increase in abundance, particularly in the highly salt-stressed group. Moreover, low pH inhibits the growth and reproduction of certain bacterial genera, including *Pantoea* and *Pediococcus*. The abundance of *Escherichia–Shigella* negatively correlates with CP, whereas *Enterococcus* and *Lactococcus* exhibit a positive correlation. The accumulation of endogenous ions in alfalfa under salt stress suppresses bacterial proliferation, thereby reducing protein degradation during fermentation. To harness the influence of endogenous sodium and potassium ions on alfalfa silage, it is imperative to develop tailored strategies aimed at optimizing the fermentation process. These valuable findings provide a solid foundation for the refinement of techniques and approaches in silage production and preservation, ultimately enhancing overall silage quality and nutritional value.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA753242.

Author contributions

JS: Writing – original draft. GZ: Data curation, Writing – review & editing. WK: Data curation, Writing – review & editing. YP: Software, Writing – review & editing. MH: Software, Writing – review & editing. CC: Methodology, Writing – review & editing. DS: Formal analysis, Writing – review & editing. ML: Conceptualization, Writing – review & editing. YL: Conceptualization, Writing – review & editing. QL: Conceptualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Effect of calcium and magnesium on starch synthesis in maize kernels and its physiological driving mechanism

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The content of kernel starch (STC), which is a fundamental indicator of the nutritional value of maize, is directly correlated with the grain's taste and aroma. Both calcium (Ca) and magnesium (Mg) are critical nutrients that play a significant role in the growth and development of maize, as well as in the synthesis of STC. To determine the physiological driving mechanisms of Ca and Mg effects on the accumulation of STC synthesis in maize kernels and the characteristics of their effects on endogenous hormones and enzymes of STC synthesis in maize leaves, our study applied foliar Ca and Mg fertilizers at various levels to maize prior to pollination. (1) The levels of Ca, Mg, indole-3-acetic acid (IAA), gibberellin (GA), and zeatin riboside (ZR) in maize leaves increased and then decreased after the supplementation of Ca and Mg. They peaked on the 32nd day after pollination. In contrast, the levels of abscisic acid (ABA) initially decreased and then increased. Ca and Mg had a negative correlation with ABA and a positive correlation with IAA, GA, and ZR. (2) As the levels of Ca and Mg increased, correspondingly rose the activities of enzymes responsible for STC synthesis and the content of STC and its components. Principally influencing the synthesis of STC were ABA, IAA, uridine diphosphate-glucose pyrophosphorylase (UDPG), granule-bound starch synthase (GBSS), and soluble starch synthase (SSS). (3) "IAA–UDPG or GBSS–STC" was the predominant physiological regulation pathway of Ca on kernel STC, whereas "IAA–GBSS–STC" was the dominant physiological regulation pathway of Mg on kernel STC. The regulatory impact of STC by UDPG and GBSS was positive, as were the effects of IAA on UDPG and GBSS. In conclusion, the accumulation of kernel starch was significantly enhanced by Ca and Mg supplementation via the modulation of endogenous hormone levels and key enzyme activities. This research identifies a viable approach to improve the nutritional composition of maize.

KEYWORDS

calcium and magnesium, kernel starch, endogenous hormone, enzymes of STC synthesis, physiological mechanism

1 Introduction

Constantly utilized as a food, feed, and industrial raw material, maize is one of the most essential food crops in the world. In maize kernels, STC is a significant nutrient source (Mancebo et al., 2015). Glucose is supplied to germinating seedlings and developing embryos to maintain their normal metabolic activities and to regulate the kernel's size, texture, and nutritional quality (Wasserman et al., 2021). Furthermore, STC constitutes a significant carbohydrate source in both animal and human food. Ca and Mg are crucial nutrients for the development and growth of crops. Ca and Mg can control kernel STC synthesis in maize via physiological mechanisms, including the regulation of membrane permeability, activation of hormonal components and enzyme systems, and maintenance of cellular osmotic pressure (Abadi et al., 2020; Zhang G. S. et al., 2020). Hence, an investigation into the regulation of STC synthesis and accumulation in maize kernels by Ca and Mg can contribute to a more comprehensive comprehension of the maize growth and development mechanism, as well as furnish a theoretical foundation and technical assistance for the enhancement of maize quality (Buchelt et al., 2020).

Endogenous hormones play a crucial role as signaling molecules within crops, governing a multitude of growth and development processes. Previous research has demonstrated that endogenous hormones can regulate the expression of genes associated with STC synthesis in maize kernels, thereby influencing the synthesis and accumulation of STC (Ahmad et al., 2019; Zhang T., et al., 2020). Such as, GA can increase the rate of STC synthesis by stimulating the expression of genes involved in starch synthesis. Furthermore, the stability and activity of enzymes involved in STC synthesis can be influenced by hormones, which in turn impacts the synthesis and accumulation of STC (Ahammed et al., 2020). STC synthesis in maize kernels involves Ca and Mg in conjunction with endogenous hormones in an interactive manner. Liu et al. (2018) discovered that the synthesis and signaling of endogenous hormones can be influenced by Ca and Mg, which regulate the activity of STC synthase and the expression of genes associated with STC synthesis. Additionally, endogenous hormones can influence the accumulation and distribution of Ca and Mg in maize kernels by regulating their uptake and transport. This finding provides compelling evidence that the interaction between endogenous hormones and Ca and Mg is a significant regulator of STC synthesis in maize kernels (Lando et al., 2020). Hence, conducting a comprehensive investigation into the interplay and mechanisms involving endogenous hormones, Ca, and Mg in the synthesis of STC from maize kernels holds substantial theoretical and practical importance. Such research would unveil the regulatory network governing STC synthesis and contribute to the enhancement of maize quality (Li et al., 2018).

The enzymes responsible for STC synthesis comprise a consortium that functions synergistically throughout the process, cooperating to achieve the formation and retention of STC molecules (Li et al., 2014). Enzymes involved in STC synthesis typically collaborate in a particular sequence to progressively produce STC molecules (Ding et al., 2009). At the outset, amylose is formed when adenosine diphosphate-glucose pyrophosphorylase (ADGP) catalyzes the reaction between glucose-1-phosphate and adenosine diphosphate (ADP) glucose (Li et al., 2021). Starch synthase (SS) then increases the length of existing STC granules by adding glucose molecules. The formation of branches in the STC molecule is the joint effort of GBSS and starch branching enzyme (SBE) (Baranov et al., 2014). Enzymes involved in STC synthesis collaborate synergistically to guarantee the correct formation of the STC molecule. An illustration of this is how the activity of ADGP influences the activities of SS and SBE (Ballicora et al., 2004). SS necessitates the provision of an appropriate quantity of ADP glucose, while SBE relies on the branch initiation sites produced by GBSS to add branches. Enzymes of this nature are crucial in the maize STC biosynthesis pathway, guaranteeing the synthesis and accumulation of STC so that carbon sources and energy can be released from maize when necessary (He et al., 2020). Numerous studies have demonstrated that Ca and Mg can regulate the synthesis and accumulation of STC in maize by influencing the activity and expression of enzymes involved in STC synthesis (Rehman et al., 2018). Ca and Mg, for instance, are regarded as cofactors of enzymes involved in STC synthesis and can bind to the active site of said enzymes to aid in the catalytic process. Enzymes involved in STC synthesis may experience an increase in catalytic activity due to conformational or charge state modifications caused by the binding of Ca and Mg (Lambers and Barrow, 2020). In addition, Ca and Mg are also implicated in the regulation of genes encoding enzymes that facilitate STC synthesis. They influence the binding capacity or activity of transcription factors, which in turn govern the levels of gene expression and promoter activity in enzymes responsible for STC synthesis. This has an impact on the enzyme accumulation during STC synthesis (Liu et al., 2018). Furthermore, metabolic pathways linked to the enzymes responsible for STC synthesis, including sugar synthesis and transport, can be influenced by Ca and Mg. Their potential involvement in enzyme-substrate translocation and metabolism could have an impact on the accessibility of substrates for the enzymes responsible for STC synthesis. This regulates the activity of enzymes involved in STC synthesis and the STC synthesis process as a whole (Tang and Luan, 2017). Nevertheless, the precise regulation of STC synthesis enzyme activity by Ca and Mg may be compromised. An imbalance in the concentrations of Ca and Mg can lead to perturbed enzyme activity during STC synthesis, thereby influencing the overall process (Kong et al., 2020). Meanwhile, phytohormones have a substantial impact on the activity and expression of enzymes involved in STC synthesis. Moreover, the regulation of these enzymes is altered through complex interactions between Ca and Mg supplementations and phytohormones (Rashid et al., 2020; Zhang et al., 2020). To achieve the objective of more precise and efficient regulation of maize STC accumulation, the quantification of Ca and Mg supplements and the mastery of their multiple interactions with phytohormones and enzymes of STC synthesis must be addressed further in the study.

Northwest China's major rain-fed spring maize-producing region is the Loess Plateau. When considering the provision of Ca and Mg for maize absorption, the substitutional states exhibit the highest efficiency. Ca substitutional levels in the farmland soils of

the Loess Plateau are 3,338 mg kg^{-1}, while Mg substitutional levels are 282 mg kg^{-1} (Wang et al., 2020). Nevertheless, for continuous maize growth, it is critical to apply Ca and Mg supplements containing 5000 mg kg^{-1} and 2000 mg kg^{-1}, respectively (Cakmak and White, 2020). This observation suggests that the efficient levels of Ca and Mg in this region have substantially reduced the minimum thresholds necessary for normal maize development and growth. Considering the inadequate levels of Ca and Mg fertility on farmland located on the Loess Plateau, the objective of our research was to examine how Ca and Mg supplementations affect the regulation of STC synthesis and accumulation in maize kernels in rain-fed arid regions of the Loess Plateau. The study primarily focused on the following inquiries: (1) to examine the interplay between Ca and Mg and endogenous hormones, as well as to quantify the impact of varying levels of Ca and Mg on the synthesis and accumulation of STC in maize kernels; (2) to elucidate the function of Ca and Mg in controlling the activity of enzymes responsible for STC synthesis and to identify the mechanism that governs the "Ca and Mg–endogenous hormones–enzymes of STC synthesis–STC level" of the kernels. This provides crucial theoretical support for elucidating the regulatory network underlying STC synthesis in maize kernels and enhancing maize quality.

2 Materials and methods

2.1 Experimental scheme

(1) Experimental site: Yan'an University's College of Life Sciences has designated the field test station for agricultural ecosystems as the study's location. The station is situated at 36°54′21′′N, 109°35′45′′E in the Baota district of Yan'an, Shaanxi Province. It is an average rain-fed dry agricultural area with 540mm of precipitation annually, mostly concentrated in July through September, with an average temperature of 8.7°C, 2421 hours of sunlight annually, and 146–179 days of frost-free time annually. The test region's loess parent material is mostly yellow loamy soil, generally homogenous in kind. It is also extensively exposed on the ground. Sandy loam is a kind of soil (Chen et al., 2015). The pH of 8.6 in the soil layer ranging from 0 to 100 cm, 6.33 g kg^{-1} of organic matter, 0.88 g kg^{-1} of total nitrogen, 0.64 g kg^{-1} of total phosphorus, 19.72 g kg^{-1} of total potassium, 16.45 mg kg^{-1} of effective phosphorus, 145.28 mg kg^{-1} of fast-acting potassium, and uniform soil fertility are the basic physical and chemical properties of the soil.

(2) Single-factor randomized block design was used as the experimental design. Using the reference threshold of deficiency supplementation and the deficit and surplus criteria of maize demand for Ca and Mg, three Ca levels (none: 0.00kg hm^{-2}, low: 17.50kg hm^{-2}, high: 49.00kg hm^{-2}) and three Mg levels (none: 0.00kg hm^{-2}, low: 24.50kg hm^{-2}, high: 35.00kg hm^{-2}) were designed for the entire reproductive period of maize (Piao, 2020). For a total of 15 trial samples, five treatments with varying Ca and Mg levels were established and duplicated three times each. Low calcium (CA1), high calcium (CA2), low magnesium (MG1), high magnesium (MG2), and no calcium and no magnesium (CTL) were the specific treatments. To guarantee the correctness of the results, protection rows were placed on the edges of each test plot at a distance of 5 m, and the sample plots were spaced 4 m apart. The nutrients Ca and Mg were extracted from sugar alcohol chelated Ca (Ca≥180g L^{-1}, 250g/bottle) and Mg (Mg≥120g L^{-1}, 300g/bottle), which were non-toxic, easily absorbed, and beneficial to the environment. As the spring maize variety in testing, "H6281" was chosen because it is disease-resistant, high-yielding, highly adaptable, and quickly dehydrates seeds (Hu et al., 2016).

Ca and Mg supplementation schedule and methodology: As per previous studies (Rhodes et al., 2018), Ca and Mg were supplied during the subsequent phases of maize growth: silking-filling, elongation-tasseling, tasseling-silking, and seedling-elongation by the ratio of 1:2:3:4. From the seedling stage to the filling stage, this generated four nutrient gradients that were utilized to investigate continuously the dynamic characteristics of the physiological process by which Ca and Mg regulate the generation of STC in reproductive-stage maize kernels. Ca and Mg were chosen for uniform foliar spray distribution on the growth sites of above-ground organs, including leaves (including center leaves), stems, and kernels, after 16:00 hours on a windless and sunny day, to ensure that maize would adequately assimilate the nutrients.

(3) Field management: The experimental design comprised 15 sample sites, each measuring 6 m×7 m. Each sample plot was cultivated using full mulching technology, in which maize was applied directly onto the furrow surface. The mulch, composed of oxidized biodegradable ecological material, measured 0.008 mm in thickness. Every individual sample plot was established at a density of 60,000 plants hm^{-2}. The furrow widths were 30 cm, monopoly heights were 15 cm (with a 10cm marginal monopoly width), and monopoly heights were 20 cm. The distance between the maize on the side of each row and the edge of the sample plot is 10 cm. Between rows, the maize was spaced at 50 cm. The seedlings were harvested on September 25, 2022, after being sowed on April 28, 2022. Prior to sowing, basal fertilizer was uniformly distributed in all sample sites in the following proportions: N, 130 kg hm^{-2}; P_2O_5, 120 kg hm^{-2}; K_2O, 38 kg hm^{-2}). This practice adhered to local recommendations that prioritized water conservation, yield stability, and fertilizer efficiency (Zheng et al., 2018). Sugar alcohol Ca and Mg fertilizers were applied topically on May 30th, June 25th, July 10th, and July 29th. Preserved maize kernels were collected and analyzed for indicator purposes on the following days after maize pollination: 8, 16, 24, 32, 40, and 48. Taking into account the impact of precipitation days, the precise dates of sample collection were as follows: August 7th, August 15th, August 21st, September 2nd, September 11th, and September 24th. Four replicates of each treatment were selected at random for each sampling.

2.2 Measurement method of main indicators

Considering the impact of variations in precipitation, 10g of representative leaves and kernels, separately, were collected at

random from four maize plots (specifically, non-marginal plants were chosen to ensure the reliability of the test data) on September 7th, August 15th, August 21st, September 2nd, September 11th, and September 24th. Each sample plot contained healthy, disease-free growth. Individually labeled and sealed in tinfoil, clean gauze, and aluminum foil tape, kernel samples for each treatment were combined. After being promptly frozen in a liquid nitrogen tank, the specimens were returned to the laboratory where they were stored as a backup sample for measurement in an ultra-low temperature refrigerator set to -80°C. Biochemical experiments conducted indoors yielded the subsequent principal physiological and biochemical indicators: (1) endogenous hormones of maize leaf; (2) key enzymes of STC synthesis in maize kernel. Meanwhile, a random sampling technique was employed to collect 5 g of fresh, disease-free leaves from four non-marginal maize plots. Prior to being dried at 100°C to a constant weight, the grains were desiccated naturally after being thoroughly mixed. Save maize leaves that have been ground and crushed with an automatic ball mill and a 60-mesh sieve for Ca and Mg analysis.

Furthermore, 10 g of fresh, normal kernels were collected from four maize plants at the same time. These kernels were mixed and sealed in a preserving apparatus (a refrigerated icebox) before being returned to the laboratory in a timely manner. The samples underwent a natural drying process. Once the moisture content of the samples was reduced to 14% ± 1%, the kernels were dried in an oven set at 60°C until they reached a constant weight. The kernels were then weighed and tested for analysis using the Richards equation to determine the parameters of kernel filling in accordance with the method described by Khan et al. (2019). The kernels underwent a process of crushing, sieving via a 100-micron sieve, and subsequent storage in a desiccator in order to ascertain their respective contents of amylose, amylopectin, and total STC. Each of the aforementioned measures was examined four times for each indicator.

(1) Leaf Ca and Mg levels, using the method of HNO_3-$HClO_4$ ablation.

A single determination was conducted using 1g of maize leaf sample powder (with an accuracy of 0.0001g). Following a series of procedures including hydrochloric acid digestion, chilling, filtration, and HNO_3-$HClO_4$ elimination, the sample powder was transferred to a 100mL volumetric vial and thoroughly mixed. Following the pipetting of 10 mL of the sample solution and the addition of 0.50 mL of the internal standard solution (100 μg L^{-1}) via pipette, the Ca and Mg levels in the leaves were determined using the filtrate in conjunction with an inductively coupled plasma emission spectrometer (ICP, AES-iCAP6300, Thermo Fisher Scientific, MA, USA) (Khan et al., 2010).

(2) Leaf ABA and IAA level, using the method of high-performance liquid chromatography (HPLC).

A 5g (0.001g) sample of crushed maize leaf was precisely weighed in a single determination, transferred to a 100mL volumetric flask, to which 80mL of methanol was added, and subsequently extracted using ultrasonic shaking for 20 minutes, vortexing for 2 minutes, and fixing with methanol as the extract solution. Subsequently, the ethyl acetate layer was dissolved in 10mL water, rotary evaporated to dry at 40°C, and extracted with 50mL ethyl acetate. The pH was adjusted to approximately 2 using a 1mol L^{-1} hydrochloric acid solution. Subsequently, the substance was dissolved in methanol, the volume was adjusted to 1 mL, it was filtered through a 0.45 μm membrane filter, and an analysis was conducted using liquid chromatography (HPLC) (Ahmad et al., 2019).

(3) The determination of maize kernel STC content was referred to the two-wavelength method of Khan et al. (2019).

The absorbance values of the test group samples were determined at 460 nm, 550 nm, 630 nm, and 740 nm, respectively, using the sample blank as a control. △A (amylose) = A630-A460, △A (amylopectin) = A550-A740, and the contents of amylose and amylopectin in the samples were calculated by the standard curves of amylose and amylopectin. Total starch content = amylose content + amylopectin content.

(4) For the determination of key enzyme activities of STC synthesis, 5 ml of Hepes-NaOH buffer (pH 7.5) was added to the kernel samples and ground in an ice bath. 30 μl of homogenate was taken, 1.8 ml of buffer was added, microcentrifuged, and the precipitate was suspended in buffer and used for the determination of GBSS activity. The rest of the homogenate was centrifuged at 10,000 g for 15 min, and the supernatant was used for the determination of SS, ADPG, SBE, and DBE activities (Dai, 2010). UDPG and SSS were determined according to the method of (Teng et al., 2015). All parameters were measured four times.

2.3 Analysis software and methods

SPSS 19.0, Origin Pro 2021, R 3.5.2, and Matlab 7.0 were utilized as scientific software for the statistical analysis of the experimental data. After maize pollination, the differences between all treatments in leaf Ca, Mg, endogenous hormones, enzymes of STC synthesis, STC, and its components were compared using one-way analysis of variance (ANOVA) followed by Tukey's test. Using Pearson's correlation analysis, the degree of correlation between Ca, Mg, and endogenous hormones for each treatment was determined at a significance level of P0.05. The redundancy analysis was employed to ascertain the extent to which Ca, Mg, endogenous hormones, and enzymes involved in STC synthesis impacted STC and its components. By employing the structural equation model, the primary driving pathway of STC accumulation and synthesis was identified. Using the least squares method and partial least squares method, linear regression and dominant regression models between STC and key driving indicators were constructed.

3 Results and analysis

3.1 Levels of Ca and Mg, endogenous hormones and their relationship in maize leaves

The levels of Ca and Mg in maize leaves exhibited a cyclical pattern of elevation and subsequent decline after the application of

Ca and Mg, peaked on the 32nd day following pollination, and subsequently declined substantially as the reproductive period progressed. The levels of both Ca and Mg in CTL exhibited a persistent downward trajectory. The leaf Ca levels were most pronounced in CA2 and differed significantly from the other treatments ($P<0.05$) (Figure 1). MG1 and MG2 exhibited comparable Ca levels with no statistically significant distinctions; in contrast, CTL demonstrated the lowest Ca levels and differed significantly from the remaining four treatments ($P<0.01$). Between days 8 and 48 post-pollination, leaf Mg levels were found to be maximum in MG2 and significantly different from the other treatments ($P<0.05$); they were lowest in CA1. CTL exhibited the lowest level of Mg, which was notably reduced in comparison to the other treatments ($P<0.01$) (Figure 1).

Since the pollination, the IAA, GA, and ZR concentrations in maize leaves have exhibited a general pattern of initial increase followed by subsequent decrease. The highest value was attained on the 32nd day following pollination, while the lowest value was attained on the 48th day. The leaf ABA content exhibited a declining and then ascending trend, with its minimum value occurring 32 days following pollination. In contrast, the trend of ABA was observed to be precisely opposite that of IAA, GA, and ZR (Figure 2).

Following the results obtained regarding the effects of Ca and Mg supplementation levels on endogenous hormones, Ca and Mg significantly increased leaf IAA, GA, and ZR levels while decreasing leaf ABA levels. An elevation in the levels of IAA, GA, and ZR would substantially stimulate the division and elongation of cells in the root stem and leaf, augment the rate of pollination, and facilitate the growth and development of the kernel, in addition to the synthesis of nutritional indices. Conversely, a reduction in the level of ABA would attenuate the inhibitory impact on maize growth and postpone the start of maize senescence (Figure 2).

In terms of endogenous hormone variability among treatments, MG2 had the highest levels of endogenous hormones ZR, IAA, and GA in its leaves, followed by CA2. In contrast, MG1 and CA1 had the lowest levels of these hormones in their leaves, and the differences between them were statistically significant ($P<0.05$). The variations in ABA levels between the treatments supplemented with Ca and Mg were insignificant (Figure 2). The levels of endogenous hormones in CA1, CA2, MG1, and MG2 were all significantly different ($P<0.001$) with CTL.

Following Ca and Mg supplementation, the levels of these two elements in leaves exhibited a highly significant positive correlation, as determined by the correlation analysis. Concurrently, noteworthy associations were observed between Ca and Mg and endogenous hormones; specifically, they exhibited substantial positive correlations with IAA, GA, and ZR and highly significant negative correlations with ABA. No statistically significant correlation was observed between Ca and Mg and endogenous hormones in CTL (Figure 3).

3.2 Characterization of changes in STC and enzymes of STC synthesis in maize kernels

Generally, the kernel STC and its component content continued to increase with the advancement of the reproductive stage after Ca and Mg supplementation. Notably, these values were significantly ($P<0.001$) greater than those of the CTL (Figure 4). Concerning the variability of STC throughout treatments, CA2 and MG2 exhibited the highest levels of kernel STC, amylose, and amylopectin, followed by CA1 and MG1. This finding suggests that the addition of Ca and Mg supplements resulted in a significant and moderate increase in the level of kernel STC. Furthermore, as the level of Ca and Mg added increased, so did the content of STC (Figure 4).

The enzyme activities involved in STC synthesis, encompassing SS, UDPG, ADPG, SSS, GBSS, SBE, and DBE, exhibited a pattern of initial increase followed by subsequent decrease in response to Ca and Mg supplementation. The enzyme activities peaked on the 24th and 32nd day following maize pollination (Figure 5). Treatments CA1, CA2, MG1, and MG2 exhibited significantly higher enzyme activities for STC synthesis than CTL. This suggests that the addition of Ca and Mg significantly increased the activity of enzymes involved in STC synthesis. Based on the observed

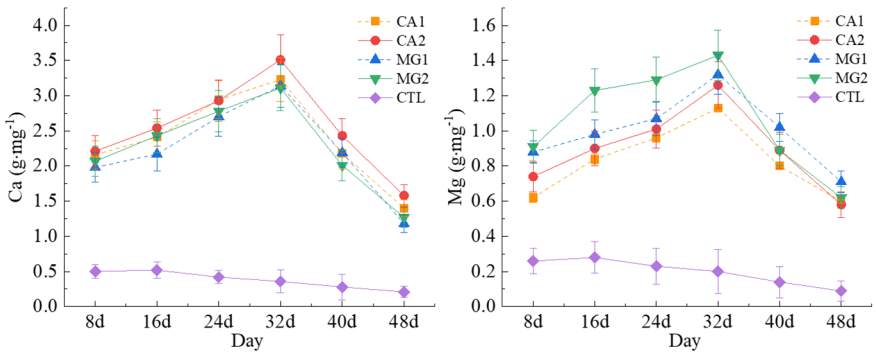

FIGURE 1
Characteristics of changes in leaf Ca and Mg levels after pollination of maize. Data represent mean ± standard deviation (n=4). CA1, CA2, MG1, MG2, and CTL represent the treatment of low Ca, high Ca, low Mg, high Mg, and no Ca and no Mg (the same below).

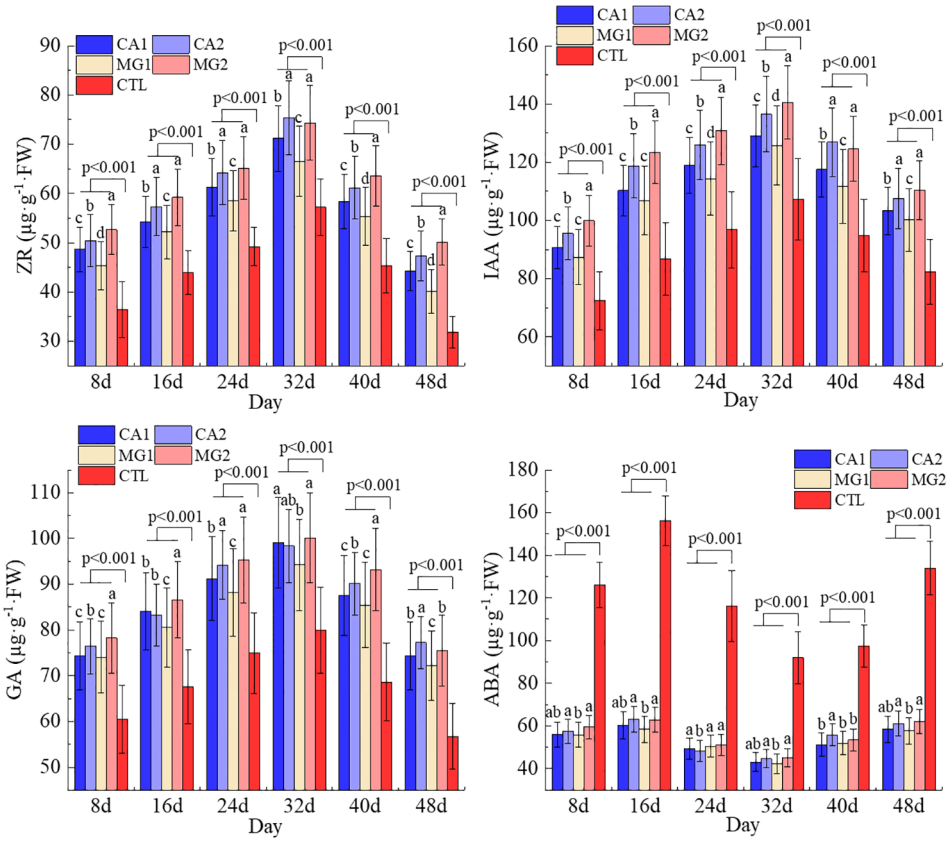

FIGURE 2
Characterization of endogenous hormone changes in leaves of maize after pollination. Data represent mean ± standard deviation (n=4). Lowercase letters a, b, c, d, and e represent the significance of the difference on the same day after maize pollination for each indicator at the P<0.05 level according to ANOVA followed by Tukey's test. P<0.001 indicates that the treatment with added calcium and magnesium differed significantly from the control group. ABA, IAA, GA, and ZR represent abscisic acid, indole-3-acetic acid, gibberellin, and zeatin riboside, respectively.

variation in enzyme activities during STC synthesis in all treatments, it was determined that MG2 contained the highest activities of UDPG, ADPG, SSS, and SBE; CA2 had the highest activities of SS and GBSS; and MG1 had the lowest activities of all the aforementioned enzymes (Figure 5). In summary, the enzyme activities involved in STC synthesis exhibited a greater magnitude in treatments supplemented with Ca and Mg, with MG2 and CA2 exhibiting the highest value, followed by CA1, and MG1.

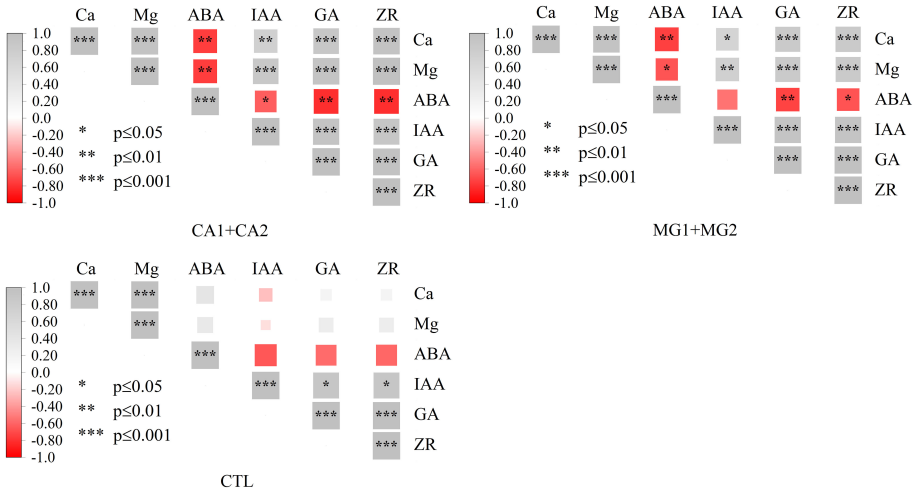

FIGURE 3
Correlation of Ca and Mg and endogenous hormones in maize leaves. *, **, *** represent P ≤ 0.05, P ≤ 0.01, P ≤ 0.001, respectively.

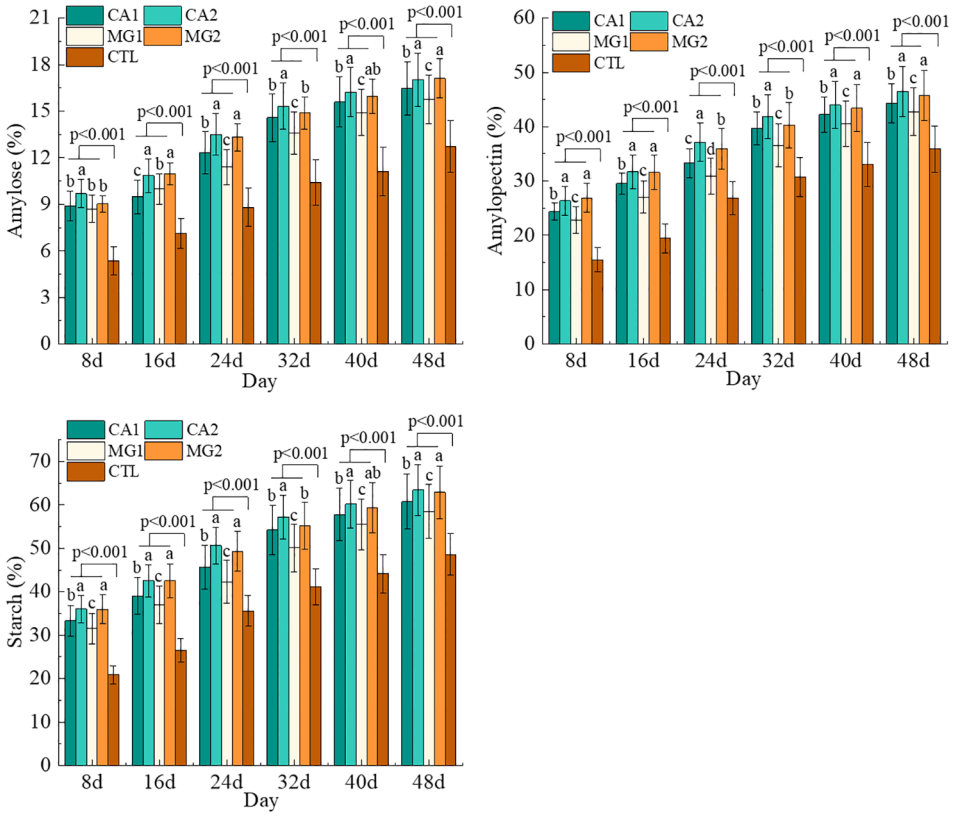

FIGURE 4
Characteristics of changes in the content of STC and its components in maize kernels after pollination. Data represent mean ± standard deviation (n=4). Lowercase letters a, b, c, d, and e represent the significance of the difference on the same day after maize pollination for each indicator at the P<0.05 level according to ANOVA followed by Tukey's test. P<0.001 indicates that the treatment with added calcium and magnesium differed significantly from the control group.

3.3 Physiological mechanisms of Ca and Mg regulation of STC synthesis

After conducting a redundancy analysis, it was determined that the primary endogenous hormone factors influencing the synthesis of STC and its components in the kernel subsequent to Ca and Mg supplementation were IAA and ABA. The principal synthase enzymes controlling the synthesis of STC were UDPG, GBSS, and SSS. It is worth noting that the impact of the key enzymes of STC synthesis was more pronounced than that of the endogenous hormones on the STC and its components (Figure 6). Furthermore, it was observed that STC, AML, AMP, and ABA exhibited negative correlations with these primary drivers, whereas positive correlations were found with all other significant factors. STC synthesis in CTL was significantly more influenced by leaf Ca and Mg levels than by endogenous hormones and key enzymes of STC synthesis; however, STC, AML, and AMP exhibited negative correlations with STC (Figure 6).

Overall, Ca and Mg supplementation attenuated the extent to which leaf Ca and Mg levels affected kernel STC, while contributing to the more pronounced driving effect of IAA and ABA on STC, and also to the significant positive regulation of STC synthesis by UDPG and GBSS.

Based on the structural model of physiological regulation of kernel STC, "IAA–UDPG or GBSS–STC" (Figure 7) was the predominant physiological regulatory pathway by which Ca supplementation affected STC synthesis. Regarding path coefficients, it was observed that IAA had a highly significant positive impact on both UDPG and GBSS. Furthermore, both UDPG and GBSS exhibited highly significant positive influence relationships on STC. "IAA–GBSS–STC" was the predominant physiological regulation pathway of STC synthesis in reaction to Mg supplementation. Positive effects were observed for both the regulation of STC by GBSS and the impact of IAA on GBSS; this pathway was identical to that of the treatment CTL (Figure 7). According to the aforementioned findings, IAA and GBSS were two fundamental physiological indicators that controlled the accumulation of STC synthesis in kernels.

By integrating the results of redundancy analysis and employing partial least squares estimation to simulate the accumulation of kernel STC, the dominant driving indicators of STC synthesis were utilized to assess the level of accumulation. The multiple regression function between these variables was assessed, and it was observed that all fitted equations had R^2 values exceeding 0.80. This finding suggests that the aforementioned core driving indicators adequately characterized the accumulation and synthesis of kernel STC (Figure 8).

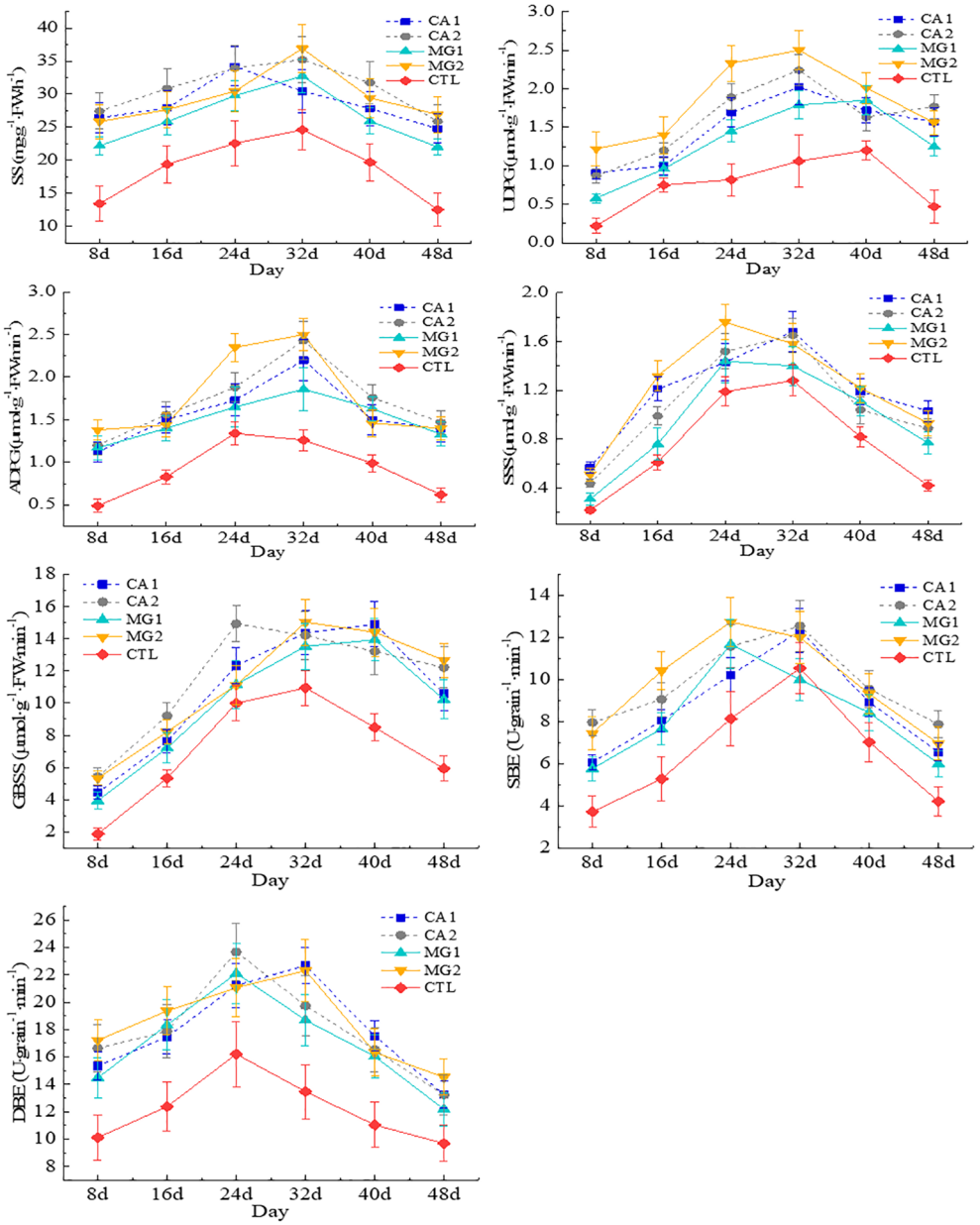

FIGURE 5
Characterization of changes in kernel enzymes of STC synthesis after pollination in maize. Data represent mean ± standard deviation (n=4). SS, UDPG, ADPG, SSS, GBSS, SBE, DBE represent sucrose synthase, uridine diphosphate-glucose pyrophosphorylase, adenosine diphosphate-glucose pyrophosphorylase, soluble starch synthase, granule-bound starch synthase, starch branching enzyme, and starch debranching enzyme, respectively (the same below).

4 Discussion

4.1 Effects of Ca and Mg supplementation on Ca and Mg levels and endogenous hormones in maize leaves

Ca and Mg are crucial nutrients for the development and growth of crops. The supplementation of Ca and Mg into maize leaves resulted in an initial increase in Ca and Mg levels, followed by a decrease as pollination progressed fertility-wise (Figure 1). This may be because maize requires more nutrients to support the development of flower kernels and ovules following pollination, therefore, Ca and Mg levels will increase initially. The maturation of the flower kernels and ovules during the reproductive period leads to a reduction in the maize's demand for Ca and Mg, consequently causing levels to decline (Cakmak and White, 2020). Both Mg and Ca exhibit highly significant positive correlations in maize, indicating that they have an interactive relationship. The increase in Ca levels leads to a corresponding rise in Mg levels in maize leaves, primarily as a result of the reciprocal influence between Ca and Mg (Ciampitti and Vyn, 2013). Ca is a structural and functional element that maintains the stability of maize cells and participates

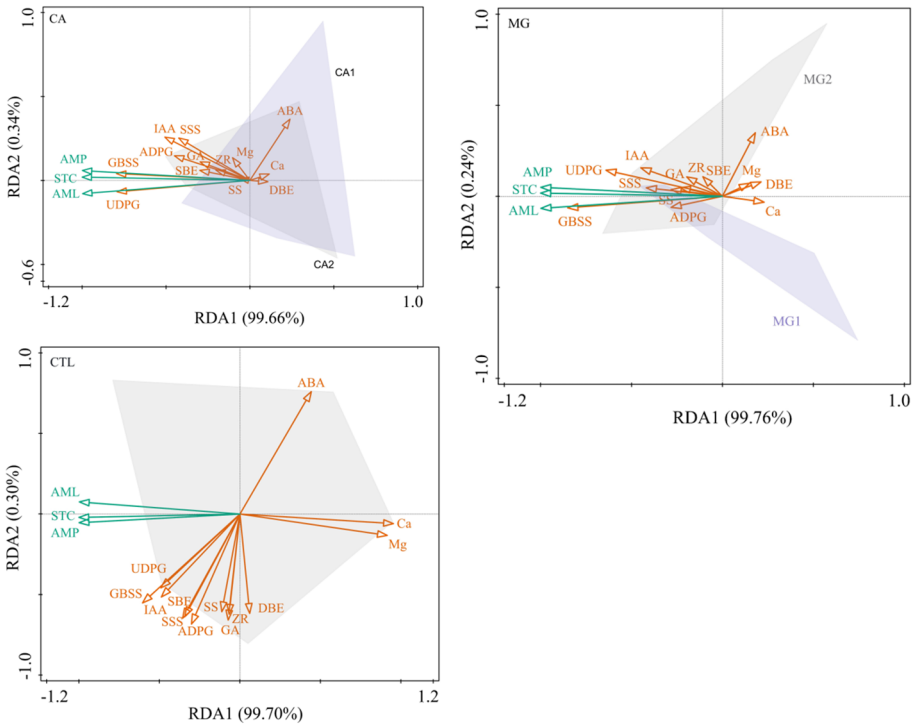

FIGURE 6
Redundancy analysis of physiological indicators regulating the accumulation of STC synthesis in maize kernels. STC, AML, and AMP represent starch, amylose, and amylopectin, respectively (the same below).

in cell wall synthesis. Elevated Ca concentrations stimulate the development of cell walls and bolster cellular mechanical integrity, leading to the production of maize leaves that are more robust and stable (Tang and Luan, 2017). Additionally, Ca regulates the transport and absorption of Mg^{2+} in maize. The formation of complexes between Ca^{2+} and specific proteins within the cell can facilitate the absorption and transport of Mg^{2+}. By controlling the activity of these complexes, the efficiency of their uptake and utilization by the maize can be enhanced (Collignon et al., 2011). Ca levels increase concurrently with Mg. This is because Ca is typically present in maize in ionic form and is transported across the cell membrane via Ca channels (Rhodes et al., 2018). By influencing the activity of these Ca channels, Mg can increase both the rate and quantity of Ca translocation. Additionally, Ca is deposited as Ca in the interstitial spaces and cell walls to form a Ca matrix. Ca deposition can be facilitated by the influence of Mg on the formation and stability of the Ca matrix (Ertiftik and Zengin, 2017). Additionally, numerous enzymes in maize utilize Mg as a cofactor, which regulates the activity and function of these enzymes. Among these enzymes are those that facilitate the transport, utilization, and absorption of Ca. Consequently, elevated Mg concentrations may facilitate the uptake and utilization of Ca, resulting in a concomitant rise in Ca levels (Farhat et al., 2016a).

The synthesis of nutritional quality in maize is significantly impacted by endogenous hormones, which can also regulate physiological processes, growth and development, nutrient uptake and transport, and maize metabolism (Malaga et al., 2020). Ca and Mg supplementation substantially increased the leaf concentrations of IAA, GA, and ZR while decreasing the leaf concentrations of ABA. In leaves, Ca and Mg exhibited a positive correlation with IAA, GA, and ZR, while they demonstrated a negative correlation with ABA (Figures 2, 3). This can be attributed to their distinct impacts on the growth and development of maize. Ca and Mg are integral components in maize internal signaling and are crucial nutrients for maize development and growth. According to Modareszadeh et al. (2020), the administration of Ca and Mg supplements improves the maize's receptivity to phytohormones, including IAA, GA, and ZR, which stimulate the growth and development of maize. In maize, these hormones govern cellular processes including elongation, differentiation, and cell division. The growth of maize is stimulated by the activity of these hormones, which is enhanced by the presence of Ca and Mg (Naeem et al., 2020). Conversely, ABA is a hormone that primarily regulates the maize plant's response to stress and adversity and inhibits maize growth. The inhibitory impact of ABA on maize growth is mitigated through the reduction in synthesis and accumulation of ABA due to the availability of Ca and Mg (Piao, 2020). This implies that Ca and Mg exert a beneficial influence on the control of maize development and growth.

4.2 Effect of Ca and Mg supplementation on STC synthesis in maize kernels and its driving mechanism

The percentage of STC and components in maize kernels continued to rise following Ca and Mg supplementation. This is

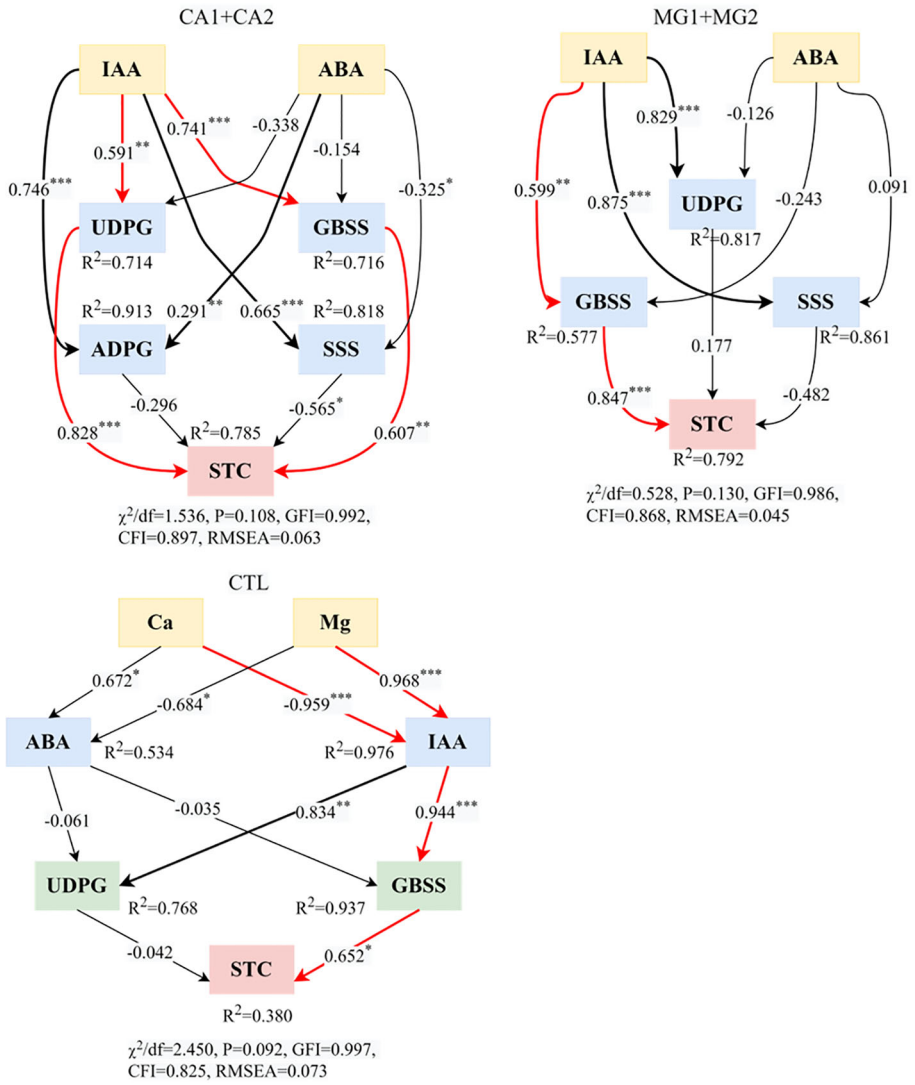

FIGURE 7
Structural equation modeling of Ca and Mg-regulated physiological processes of STC synthesis in maize kernels. * represents P<0.05, ** represents P<0.01, *** represents P<0.001. χ^2 represents the chi-square; df represents the degree of freedom; GFI represents the goodness of fit index; CFI represents the comparative fit index; RMSEA represents the root mean square error of approximation.

because supplementation with Ca and Mg can influence the process of carbon metabolism in maize. More precisely, the involvement of Ca and Mg in the synthesis and transportation of sucrose in maize has been observed to impact the synthesis and accumulation of STC (Farhat et al., 2016b). Ca and Mg regulate the synthesis and translocation of sucrose, which is a precursor to STC. Sucrose synthesis and translocation are enhanced in maize kernels when Ca and Mg are abundant, thus facilitating STC accumulation and synthesis (Ferreira et al., 1999). Supplementation with Ca and Mg increased the activity of STC synthesis enzymes (Figure 5). The reason for this is that Ca and Mg function as coenzymes, facilitating enzyme activity within maize cells. Both substances can form enzyme-metal ion complexes with STC synthesis enzymes, consequently augmenting the enzyme's catalytic activity (Kaya et al., 2018). This phenomenon occurs due to the interaction between the positively charged residues in the enzyme and the negatively charged Ca^{2+} and Mg^{2+}. This interaction facilitates the formation of the active site and enhances the stability of the enzyme's steric structure (Piao, 2020). Furthermore, the catalytic efficiency of the enzyme can be enhanced by regulating the substrate binding and release processes with the assistance of Ca and Mg (Rosanoff and Kumssa, 2020). Therefore, Ca and Mg supplementation can promote the synthesis and accumulation of STC by increasing the activity of enzymes involved in STC synthesis in maize kernel.

The primary endogenous hormonal factors that influence STC synthesis are IAA and ABA (Figure 6). By influencing the gene expression and activity of STC synthesis enzymes, IAA primarily participates in the regulation of STC synthesis (Yang et al., 2019). IAA can stimulate the post-transcriptional and transcriptional regulation of genes encoding enzymes responsible for STC synthesis, thereby increasing STC synthase expression. Furthermore, IAA can enhance the catalytic efficiency of STC synthesis by directly influencing the activity of enzymes (Zakari

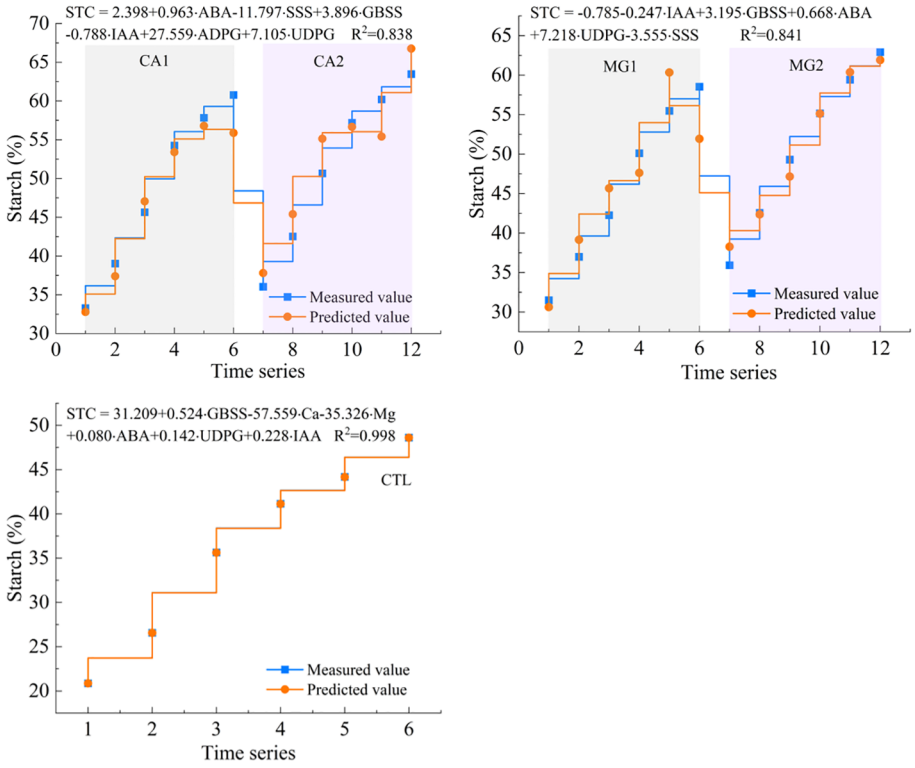

FIGURE 8
Curve fitting of dominant drivers to kernel STC levels.

et al., 2020). Consequently, IAA significantly and positively regulated the synthesis of kernel STC (Figure 7). Conversely, ABA functions as an inhibitory hormone. The primary mechanism by which ABA regulates STC synthesis in maize is through the inhibition of transcriptional and post-transcriptional regulation of the gene-encoding enzymes involved in STC synthesis. By inhibiting and decreasing the expression of the genes encoding the enzymes involved in STC synthesis, ABA is capable of reducing STC synthesis. As a result, the catalytic efficiency of STC is diminished as ABA inhibits the activity of enzymes involved in its synthesis (Singh et al., 2020). The principal synthetic enzymes that control STC synthesis are UDPG, GBSS, and SSS (Figure 6). UDPG serves as the precursor material for STC synthesis and is the primary substrate for the process. Following the conversion of UDPG to glucose-1-phosphate, a sequence of enzyme-catalyzed reactions completes the synthesis of the STC molecule (Rahim et al., 2020). It catalyzes GBSS, one of the essential enzymes for STC synthesis. STC synthesis is catalyzed by GBSS, an essential enzyme that facilitates the polymerization of glucose molecules into STC chains. The primary structure of the STC molecule is formed of α-1,4-glucose chains, which are produced by GBSS from G1P (Han et al., 2022). An additional crucial enzyme in the synthesis of STC is SSS, which facilitates the polymerization of G1P into branched α-1,6-glucose chains. The degree of branching of the STC molecule is determined by the activity of SSS, this, in turn, influences the structure and properties of the STC (Guo et al., 2021). To summarize, the primary synthases that govern STC synthesis are UDPG, GBSS, and SSS. These enzymes participate in distinct stages of STC synthesis and collectively control their rate, structure, and properties (Jiang et al., 2013).

"IAA–UDPG or GBSS–STC" was the predominant physiological regulatory pathway for STC synthesis by Ca. IAA had a highly significant positive effect on both UDPG and GBSS, and GBSS and UDPG, in turn, had a highly significant positive effect on STC (Figure 7). This could potentially be attributed to the regulatory function of Ca^{2+} in plant cells, which includes signaling for hormones (Rashid et al., 2020). An increase in Ca^{2+} concentration induces interactions with intracellular proteins, resulting in alterations to the conformation and activity of said proteins. Ca^{2+} can enhance the beneficial regulatory effects of IAA on UDPG and GBSS when it interacts with the IAA signaling pathway (Lando et al., 2020). One possible mechanism by which Ca^{2+} enhances the ability of IAA to regulate the expression of target genes is by interacting with proteins in the IAA signaling pathway and facilitating the transmission of the signal transduction chain (Wei et al., 2015). In addition, an increase in Ca^{2+} can influence the subcellular localization and intracellular activity of enzymes. By controlling the translation, post-translational modification, or subcellular localization of UDPG and GBSS, it may augment the beneficial regulatory impact of IAA (Wang et al., 2014). The process of STC synthesis is intricate, requiring the involvement of numerous enzymes and regulatory factors. UDPG, which functions as a glucose-forming enzyme in the precursor of the amylopectin gene, is crucial for STC synthesis. GBSS plays a pivotal role in the biosynthesis of STC granules as an enzyme (Corbi et al., 2011). In the STC synthesis pathway, Ca^{2+} interacts with UDPG

and GBSS to alter their conformation and activity, thereby promoting the STC synthesis process and enhancing the enzymes' catalytic capability (Guo et al., 2023).

"IAA–GBSS–STC" was the predominant physiological regulatory pathway governing STC synthesis by Mg. Positive effects were observed, specifically in the regulation of STC by GBSS and the impact of IAA on GBSS (Figure 7). The beneficial impact of IAA on GBSS subsequent to Mg supplementation could potentially be attributed to the function of Mg as a crucial cofactor in the modulation of GBSS enzyme activity. A gigantic interaction between Mg^{2+} and the GBSS enzyme can regulate the enzyme's stability and conformation (Roy et al., 2019). These structural alterations could potentially increase the GBSS enzyme's vulnerability to interaction with IAA and amplify the impact of IAA on its activity. Furthermore, intracellular signaling pathways can have their ion concentrations and enzyme activities regulated by Mg^{2+}, an essential participant in signaling (Peng et al., 2020). By, among other mechanisms, influencing the synthesis, degradation, or transport of the signaling molecule IAA, Mg^{2+} may augment the positive regulatory effect of IAA on GBSS. One potential explanation for the positive regulation of STC synthesis by GBSS via Mg^{2+} is that Mg^{2+} can interact with the GBSS enzyme to preserve the enzyme's three-dimensional structure stability and proper folding state, thereby influencing the activity and conformation of the enzyme (Rehman et al., 2018). Mg^{2+} has the potential to regulate the catalytic efficiency and substrate binding affinity of GBSS enzymes through its binding to these enzymes. This, in turn, could enhance the activity of GBSS enzymes and facilitate the synthesis of STC. The level of GBSS gene expression may also be regulated by Mg^{2+}, either directly or indirectly (Zhao et al., 2012). Elevated Mg^{2+} availability has the potential to stimulate or facilitate the transcription and translation of GBSS genes, consequently augmenting both GBSS expression and STC synthesis.

5 Conclusion

After Ca and Mg supplementation, the levels of these elements in maize leaves initially rose and subsequently fell. The peak levels of leaf IAA, GA, and ZR were observed on the 32nd day afterwards pollination, followed by a gradual decline. The lowest levels of leaf ABA were observed on day 32 following pollination. Supplemental Ca and Mg increased leaf IAA, GA, and ZR levels significantly while decreasing leaf ABA levels. Ca and Mg were correlated positively with leaf IAA, GA, and ZR, and negatively with ABA.

The supplementation of Ca and Mg resulted in a sustained increase in the level of STC and its components in maize kernels. Enzymes engaged in the synthesis of STC exhibited a substantial increase in activity, which was positively correlated with the gradient of Ca and Mg levels. The endogenous hormone factors IAA and ABA exhibited the greatest impact on STC synthesis, while the synthase enzymes UDPG, GBSS, and SSS demonstrated the most influence on STC formation. Regarding STC synthesis, "IAA–UDPG or GBSS–STC" is the principal regulatory pathway for Ca, whereas "IAA–GBSS–STC" is the principal regulatory pathway for Mg. Both UDPG and GBSS were positively influenced by IAA, and the regulation of STC by UDPG and GBSS was also positive.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

ZH: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Writing – original draft, Writing – review & editing. XS: Investigation, Methodology, Validation, Visualization, Writing – review & editing. TZ: Conceptualization, Project administration, Software, Supervision, Validation, Writing – review & editing. JY: Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Bacillus velezensis promotes the proliferation of lactic acid bacteria and influences the fermentation quality of whole-plant corn silage

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Objective: This study aimed to investigate the promoting effect of a *Bacillus velezensis* (BV) strain on lactic acid bacteria (LAB) and determine its influence on the fermentation quality and aerobic stability of silage.

Methods: Flat colony counting method was used to evaluate the effect of BV on the growth of LAB. Freshly harvested whole-plant corn was inoculated separately with BV and L. plantarum (LP), along with an uninoculated control group (CK), and assessed at 1, 3, 5, 7, 15, and 30 days of ensiling.

Results: The results indicated that BV exhibited a proliferative effect on *Weissella confusa, Lactobacillus plantarum* L-2, and Pediococcus pentosaceus. And exhibited a more rapid pH reduction in BV-inoculated silage compared with that in CK and LP-inoculated silage during the initial stage of ensiling. Throughout ensiling, the BV and LP experimental groups showed enhanced silage fermentation quality over CK. Additionally, relative to LP-inoculated silage, BV-inoculated silage displayed reduced pH and propionic acid. BV also prolonged aerobic stability under aerobic conditions. The microbial community in BV-inoculated silage showed greater stability than that in LP-inoculated silage. Additionally, *Firmicutes* and *Lactobacillus* exhibited more rapid elevation initially in BV versus LP-inoculated silage, but reached comparable levels between the two inoculation groups in the later stage.

Conclusion: In summary, BV enhanced the efficacy and aerobic stability of whole-plant corn silage fermentation by stimulating LAB proliferation.

KEYWORDS

***Bacillus velezensis*, proliferation, lactic acid bacteria, whole-plant corn, silage**

1 Introduction

Whole-plant corn silage is one of the most important roughage sources worldwide (Zhang et al., 2022a). It is characterized by high yield, rich nutrient content, good palatability and digestibility, making it an indispensable basic feed source for ruminants, especially dairy cattle, in Europe and the United States, whole-plant corn silage has become a vital and widely used feed in dairy production (Khan et al., 2015; Ferraretto et al., 2018; Reed et al., 2022; Shi et al., 2022a). The application of whole-plant corn silage solves the problem of insufficient feed supply for livestock, reduces farming costs to some degree, ensures stable agricultural development, and promotes increased production and yield of ruminant livestock such as cattle and sheep (Shi et al., 2022a). Zhao et al. (2020) reported that the use of whole-plant corn silage as the only roughage in a complete mixed diet improves the growth performance and meat quality of beef sheep. Cui et al. (2022) demonstrated that whole-plant corn silage enhances rumen flora in beef cattle, which in turn improves rumen fermentation and growth in beef cattle. Silva et al. (2021) found that whole-plant corn silage increases dry matter intake and milk yield in dairy cows. Zhang et al. (2022a) showed that meat quality of beef cattle fed whole corn silage improves. The development of high-quality whole-plant corn silage is vital to increasing the proportion of grass-fed livestock production in China.

Whole-plant corn silage is a method of preserving whole corn under anaerobic conditions based on fermentation by lactic acid bacteria (LAB). LAB utilize soluble sugars and other substances in whole corn as substrates, metabolizing them to produce organic acids and, creating an acidic environment that inhibits the growth of harmful microorganisms and preserves the whole-plant corn silage for an extended period to avoid deterioration and spoilage (Pahlow et al., 2003; Wilkinson et al., 2003). Nevertheless, the production of superior quality whole-plant corn silage in practice is impeded by several factors, especially the brief stabilization period under aerobic conditions post-exposure, its tendency to undergo, secondary fermentation is one of the most critical impediments impacting the efficacy of whole-plant corn silage utilization (McDonald et al., 2010; Shi et al., 2022b). Secondary fermentation is attributed to the activation of aerobic microbes including molds, *Clostridia*, and *Enterobacteriaceae* upon exposure to oxygen, eliciting aerobic degradation and subsequent elevation of silage pH and internal temperature (Haq et al., 2021). Deleterious microorganisms present during the pre-ensiling and post-exposure periods lead to diminished whole corn silage quality, elevated mycotoxin levels in whole-plant corn silage, and adverse effects on feed intake, productivity, reproduction, livestock product quality, and mortality in livestock after feeding (Binder, 2007; Richard, 2007; Drouin et al., 2021; Chen et al., 2022). Mitigating the detrimental impacts of harmful microflora on whole-plant corn silage and ameliorating silage quality through silage additives have elicited profound research interest. Common additives comprise microbial inoculants (e.g., *Lactobacillus* and *Bacillus* spp.), enzymes (e.g., cellulase and hemicellulose), and chemical additives (e.g., formic acid and benzoic acid), among others (Muck et al., 2018). Of all the additives, microbial inoculants are the most extensively utilized primarily by LAB. These inoculants can swiftly reduce silage pH by rapid proliferation during the initial ensiling phase, suppressing deleterious acid-intolerant microbes and yielding superior quality silage (Carvalho et al., 2021; Chen et al., 2023). Nascimento Agarussi et al. (2019) used *Lactobacillus plantarum* and *Pediococcus pentosaceus* to ensile alfalfa and found that both LAB were able to enhance the fermentation quality of the silage feed. Li et al. (2021) discovered that inoculating with *L. plantarum* and *Lactobacillus buchneri* could mitigate the adverse effects of fungi on ensiled corn by changing the bacterial and fungal communities, thereby improving the fermentation quality of the corn silage. Presently, *Bacillus* spp. also garnering interest because of their capacity to hydrolyze plant cell walls, releasing soluble sugars via cellulase and hemicellulase production and facilitating LAB (Ning et al., 2017; Li et al., 2018). Concurrently, *Bacillus* can suppress undesirable microbes like molds and yeasts in the initial and post-exposure phases by generating bacteriocins. Several studies have indicated that using *B.* spp. as a silage additive can improve the fermentation quality and aerobic stability of silage feed, and have positive effects on the microbial community (Bai et al., 2020; Bonaldi et al., 2021; Zhu et al., 2022). In this study, *Bacillus velezensis* (BV) was utilized, which has demonstrated an ability to restrain harmful microbes including molds, *Escherichia* coli and yeasts in prior studies. Theoretically, BV as an inoculant can ameliorate the fermentation quality of whole corn silage, although its effects in whole corn silage application remain uninvestigated. Therefore, this study aimed to examine the impacts of this BV strain on whole-plant corn silage quality when utilized as a silage inoculant.

2 Materials and methods

2.1 Effect of BV on LAB proliferation

Flat colony counting method was used to evaluate the effect of BV on the growth of LAB. The experiment was divided into three groups: the blank group (inoculated with 2% BV at a concentration of 1.0×10^6 cfu (colony forming units)/g in De Man, Rogosa and Sharpe (MRS) liquid medium), the control groups (inoculated with 1% LAB at a concentration of 1.0×10^6 cfu/g in MRS liquid medium), and the experimental groups (inoculated with 2% BV at a concentration of 1.0×10^6 cfu/g and 1% LAB at a concentration of 1.0×10^6 cfu/g in MRS liquid medium). After each group was cultured for 12 h, equal amounts of bacterial suspensions were taken and diluted to 10^{-5}. Then, 15 μL of the diluted suspensions was spread onto the MRS solid medium and incubated for 12 h. The bacterial colony counts in the culture dishes were used to calculate the cfu/g of MRS medium. For this experiment, three types of LAB were selected: *Weissella confusa*, *L. plantarum* L-2, and *P. pentosaceus*. These LABs were isolated, identified, and preserved from whole-plant corn silage material. The BV used in the experiment was isolated, identified, and preserved from the environment in the laboratory. The MRS, Luria-Bertani culture media, and agar were purchased from HaiBo Biotechnology Co., Ltd (Qingdao).

2.2 Preparation of silage

After whole-plant corn (National High tech Agricultural Park of Anhui Agricultural University, 31° 58′ N, 117° 24′ E) was harvested at the milk-ripe stage, it was chopped into 2–3 cm lengths and thoroughly mixed before being randomly sampled for ensiling. The experiment included one control group and two treatment groups: control (CK) group (1% saline added), BV-inoculated silage, with the addition of 1% BV at a concentration of 1.0×10^6 cfu/g FM, and LP-inoculated silage, with the addition of 1% *L. plantarum* (LP) at a concentration of 1.0×10^6 cfu/g FM. Each group had 3 replicates. Silage was carried out using silage bags (250 × 300 mm, Hefei Xi Yue Biological Co., Ltd., Hefei), with 400 g of material per bag. The bags were stored at room temperature in darkness. Samples were collected at room temperature (25 ± 2°C) for analysis on days 1, 3, 5, 7, 15, and 30 of ensiling (Each days had 3 replicates). The LP, a commercial *L. plantarum* strain (Guangzhou Weiyuan Biotech Co., Ltd, Guangzhou, China), has a viable count of 50 billion cfu/g FM.

2.3 Chemical composition analysis

Ten grams of ensiled sample was mixed with 90 mL distilled water and extracted at 4°C for 12 hours. The pH of the filtrate was measured using a pH meter (Mettler Toledo). Approximately 10 mL of the filtrate was centrifuged (4500× g, 15 minutes, 4°C), and the supernatant was analyzed for lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA) contents by using high-performance liquid chromatography (HPLC). An Agilent TC-C18 column (250 nm × 4.6 nm, 5 μm) was used, with acetonitrile as mobile phase A and 0.01 mol/L potassium dihydrogen phosphate (pH 2.70) as mobile phase B at a ratio of 3:97. The flow rate was set to 0.6 mL/min, and UV detection was performed at a wavelength of 210 nm. The column temperature was maintained at room temperature (Wang et al., 2019). Ammonia nitrogen was determined following the method of Broderick and Kang (1980).

The content of dry matter (DM) were analyzed following the AOAC method. Water-soluble carbohydrates (WSCs) were measured using anthrone-sulfuric acid colorimetriy to determine soluble sugars, in accordance with AOAC (1990). Crude protein (CP) was determined using the Kjeldahl method with an automated nitrogen analyzer. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using a fiber analyzer on the basis of the analysis system outlined by Van Soest et al. (1991).

2.4 Aerobic stability

After 30 days of ensiling whole-plant corn, the silage bags were opened. The contents were transferred into plastic containers (500 mL) and placed in a room maintained at 25°C, and the containers were left uncovered. At intervals of 30 minutes, the temperature at the center of the silage was measured using a digital temperature probe until the sample temperature exceeded the ambient temperature by 2°C (Chen et al., 2016).

2.5 Bacterial community analysis

Total genomic DNA of bacteria on the surface of fresh and silage maize feeds at time points 1, 3, 5, 7, 15, and 30 days of fermentation was extracted utilizing a DNA isolation kit (D4015, Omega, Inc., USA). Polymerase Chain Reaction (PCR) amplification of the full-length 16S rRNA gene of bacteria was conducted using forward primer 343F (TACGGRAGGCAGCAG) and reverse primer 798R (AGGGTATCTAATCCT).

For PCR amplification, the total reaction volume was 25 μL, comprising 25 ng of template DNA, 12.5 μL of PCR premix, 2.5 μL of each primer, and PCR-grade water to adjust the volume. The PCR amplicons were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified using Qubit (Invitrogen, USA). Amplicon pools were prepared for sequencing. The size and quantity of the libraries were assessed on Agilent 2100 Bioanalyzer (Agilent, USA) and an Illumina library quantification kit (Kapa Biosciences, Woburn, MA, USA), respectively. Sequencing was performed on an Illumina NovaSeq PE250 platform. The samples were sequenced on an Illumina NovaSeq platform. Paired-end reads were assigned based on unique sample barcodes and truncated by cutting off barcode and primer sequences. Paired-end reads were merged using FLASH. Raw reads were subjected to quality filtering under specific conditions by fqtrim (v0.94) to obtain high-quality, clean sequences. Chimeric sequences were filtered using Vsearch software (v2.3.4). Feature tables and feature sequences were obtained after iterative processing with DADA2. Alpha diversity and beta diversity were calculated by QIIME2, and the same number of sequences was randomly selected by reducing the number of sequences to the minimum of some samples. Bacterial taxa were classified based on relative abundance (X number of bacteria/total number). Species-annotated sequence comparisons were performed using Blast software, and the comparative databases utilized were SILVA and NT-16S. Linear discriminant analysis effect size analysis (LEfSe) was employed to identify communities or species with significant differences among the three groups. The sequence data from this study have been deposited in the NCBI database under Accession No. PRJNA1013177.

2.6 Statistical analyses

Single-factor ANOVA was employed to analyze the microbial populations, chemical composition, and fermentation quality data of fresh and ensiled whole-plant corns to assess the efficacy of LAB inoculants. Duncan's multiple range test was utilized to assess differences among means. $P < 0.05$ was considered statistically significant. The analysis was conducted using IBM SPSS Statistics 26.0 (SPSS, Inc., Chicago, IL).

3 Results

3.1 Effect of BV on LAB proliferation

In this experiment, BV was cultured in the MRS medium as a blank group, but it did not grow after 12 hours in MRS medium (Figure 1A-1). However, it exhibited normal growth on the Luria-Bertani agar media (Figure 1A-2). BV was mixed cultured with different LABs for 12 hours, followed by subsequent cultivation on MRS solid medium for an additional 12 hours (Figure 1B). The results indicated that this bacterium exhibited a promotive effect on the proliferation of *L. plantarum* L-2 (Figure 1C), *P. pentosaceus* (Figure 1D), and *W. confusa* (Figure 1E). In Particular, a significant enhancement was found on the growth of *L. plantarum* L-2 and *W. confusa* ($P < 0.05$), whereas the promotion of *P. pentosaceus* was not significant ($P > 0.05$).

3.2 Chemical composition and fermentation characteristic analysis

As shown in Table 1, the DM content of the whole-plant corn material was 27.49%. On the basis of DM, the CP, EE, crude ash, NDF, ADF, and WSC contents were 10.60%, 7.85%, 3.62%, 26.57%, 14.60%, and 8.15%, respectively.

Table 2 shows that ensiling time significantly reduced the DM content ($P < 0.05$), and the additives did not ($P > 0.05$). Moreover, the additives and ensiling time did not significantly affect the CP content ($P > 0.05$). Ensilage time did not have a significant effect on the NDF and ADF contents ($P > 0.05$). The NDF content in BV- and LP-inoculated silages was lower than that of CK at days 1, 3, and 30 ($P < 0.05$), with no significant difference between the two test groups ($P > 0.05$). At day 5, the LP-inoculated silage had a lower NDF content than CK ($P < 0.05$), whereas the BV-inoculated silage did not significantly differ from the other groups ($P > 0.05$). The ADF content in the BV-inoculated silage was lower than those in CK and LP-inoculated silage at days 1, 5, 7, and 15 ($P < 0.05$).

With the increase in ensiling days, the NH_3-N content in all groups increased ($P < 0.05$). Compared with CK, the LP-inoculated silage had reduced NH_3-N content on days 1, 7, 15, and 30 ($P < 0.05$), whereas the BV-inoculated silage showed a decrease in NH_3-N content in all ensiling days ($P < 0.05$). No significant difference was found in the NH_3-N content between the LP and BV-inoculated silages ($P > 0.05$).

As the ensiling time progressed, the WSC content exhibited a decreasing trend in all groups ($P < 0.05$). LP and BV inoculations reduced the losses of WSC in the silage compared with CK ($P < 0.05$). The BV-inoculated silage had the highest WSC content, higher than the LP-inoculated silage on days 3, 5, 7, and 15 ($P < 0.05$).

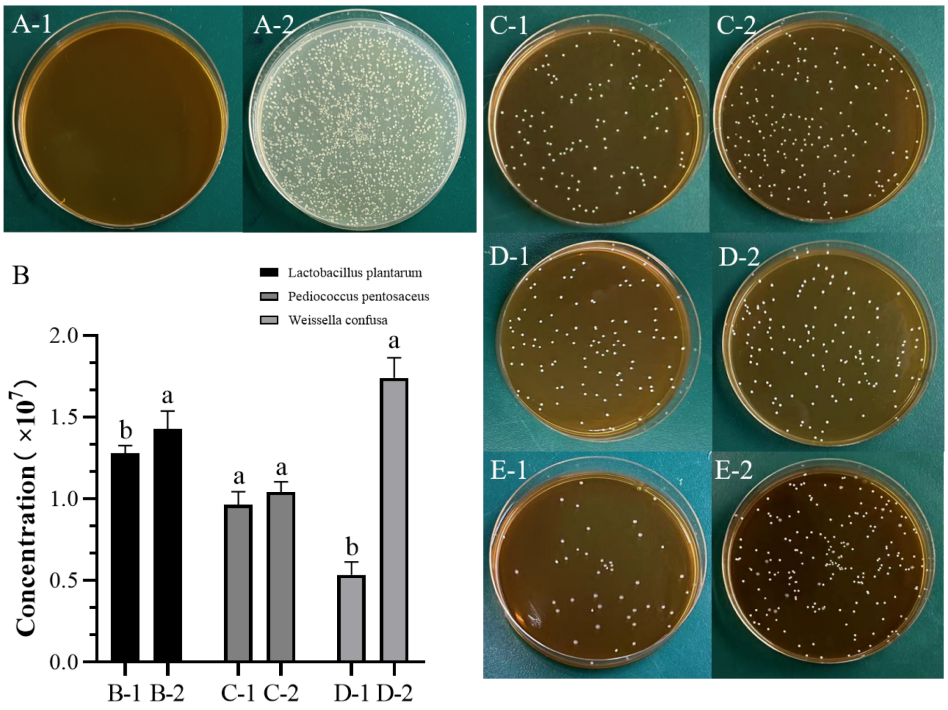

FIGURE 1
Promoting effect of *Bacillus velezensis* on lactic acid bacteria. Different lowercase letters indicate significant differences ($P < 0.05$). **(A)** Picture of *B. velezensis* cultured on Luria-Bertani and MRS plates for 12 hours. (A-1) MRS medium. (A-2) Luria-Bertani medium. **(B)** Concentration column chart of each milliliter of bacterial liquid after 12 hours of cultivation. **(C)** *L. plantarum* L-2 group. (C-1) Control group without *B.velezensis*. (C-2) Experimental group with *B. velezensis* added. **(D)** *P. pentosaceus* group. (D-1) Control group without *B. velezensis*. (D-2) Experimental group with *B. velezensis* added. **(E)** *W. confusa* group. (E-1) Control group without *B. velezensis*. (E-2) Experimental group with *B. velezensis* added. MRS, De Man, Rogosa and Sharpe. (*L. plantarum* L-2 and LP are two different strains of *L. plantarum*.).

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TABLE 1 Chemical composition of fresh samples.

Items	Fresh matter
DM, % FM	27.49±0.30
Ash, % DM	3.62±0.06
CP, % DM	10.60±0.04
EE, % DM	7.85 ± 0.37
WSC, % DM	8.15 ± 0.15
NDF, % DM	26.57±0.54
ADF, % DM	14.60±0.40

DM, dry matter; WSC, water-soluble carbohydrates; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber.

In Table 3, the pH values of the three groups rapidly decreased during the ensiling process and exhibited a significantly decreasing trend ($P < 0.05$). The pH values of the BV-inoculated silage were lower than those of the control at all observed time points ($P < 0.05$). Specifically, the pH values of the BV-inoculated silage were lower than those of the LP-inoculated silage on days 1, 3, 5, 7, and 30. Meanwhile, the pH values of the LP-inoculated silage were lower than those of CK on days 1, 3, 7, and 15 ($P < 0.05$). The LP-inoculated silage exhibited the highest LA content, significantly surpassing that of the CK group throughout the entire ensiling period ($P < 0.05$). In the BV-inoculated silage, the LA content was notably higher than that of the CK group during the initial 7 days of ensiling, subsequently dropping on day 15 ($P < 0.05$) and showing no significant difference from that of the CK group on day 30 ($P > 0.05$). The AA content in the LP-inoculated silage was lower than that of CK during the first 3 days of ensiling, but it increased as the ensiling time progressed ($P < 0.05$). By contrast, the BV-inoculated silage exhibited lower AA content than CK on day 1 ($P < 0.05$), followed by higher levels from day 3 to day 15 ($P < 0.05$), with no significant difference from the control on day 30 ($P > 0.05$). For PA content, the LP and BV-inoculated silages maintained lower levels than CK at all observed time points ($P < 0.05$). Moreover, the BV-inoculated silage had the lowest PA content. Overall, inoculation with exogenous silage inoculants increased the LA and AA contents while reducing the PA content in the ensiled maize feed. No detectable BA was observed in any of the groups during the entire ensiling period.

3.3 Aerobic stability

The aerobic stability of whole-plant corn silage is depicted in Figure 2. The aerobic stability times for CK, the BV-inoculated silage, and the LP-inoculated silage were 45, 118, and 73 hours, respectively. The BV-inoculated silage exhibited the longest aerobic stability time,

TABLE 2 Effect of *Bacillus velezensis* on the chemical composition of whole-plant corn silage.

	Treatments(T)	Storage period (D)						SEM	P-value		
		1	3	5	7	15	30		T	D	T×D
DM, % FM	CK	33.60a	33.59a	32.22b	31.39c	30.87d	27.71e	0.003	0.992	<0.001	0.986
	BV	33.63a	33.56a	32.31b	31.43c	30.75d	27.69e				
	LP	33.61a	33.62a	32.30b	31.41c	30.35d	27.68e				
CP, % DM	CK	8.21	7.67	7.54	7.49	7.48	7.42	0.007	0.315	0.225	0.969
	BV	8.57	7.92	7.8	7.78	7.74	7.68				
	LP	8.51	7.93	7.92	7.84	7.75	7.64				
NDF, % DM	CK	27.98A	27.89A	27.14A	27.26	27.10	27.66A	0.008	<0.001	0.403	0.894
	BV	26.63B	26.26B	26.34AB	26.43	26.06	26.12B				
	LP	26.77aB	26.44abB	26.15abB	25.99b	26.08ab	26.27abB				
ADF, % DM	CK	17.95A	17.84	17.94A	17.61A	17.66A	17.63	0.006	<0.001	0.631	0.775
	BV	16.97B	16.9	16.4B	16.43B	16.30B	16.87				
	LP	17.77A	17.69	17.49A	17.33A	17.41A	17.42				
Ammonia-N % TN	CK	2.19dA	3.37cA	3.97bcA	3.37cA	4.04abA	4.27aA	<0.001	0.001	<0.001	0.476
	BV	1.88cB	2.65bB	2.85bB	2.58bB	2.86bB	3.48aB				
	LP	1.99dB	2.74bcAB	2.98bcAB	2.58cB	3.08abB	3.46aB				
WSC, % DM	CK	5.08a	2.93bB	1.35cC	1.32cdC	1.28cdC	1.17dB	0.002	0.381	<0.001	0.847
	BV	5.09a	4.39bA	2.49cA	2.13dA	1.94eA	1.70fA				
	LP	5.04a	2.70bC	2.11cB	1.86dB	1.72eB	1.60fA				

Differences marked with different uppercase letters in the same column are significant ($P < 0.05$), and differences marked with different lowercase letters in the same row are significant ($P < 0.05$). BV, *Bacillus velezensis* added; LP, Lactobacillus plantarum added.

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TABLE 3 Effect of *Bacillus velezensis* on the quality of whole-plant corn silage.

	Treatments(T)	Storage period (D)						SEM	P-value		
		1	3	5	7	15	30		T	D	T×D
pH	CK	5.06aA	4.51bA	4.34cA	4.20dA	4.00eA	3.89fA	0.052	0.122	<0.001	0.687
	BV	4.66aC	4.30bC	4.00cB	3.80dC	3.86dB	3.70eB				
	LP	4.96aB	4.38bB	4.03cA	3.99cB	3.89dB	3.80dA				
LA% DM	CK	1.98fB	3.08eC	3.81dB	4.99cC	8.49bB	9.24aB	0.004	0.021	<0.001	0.350
	BV	2.17eA	5.59dA	5.59dA	8.52bA	8.23cC	9.29aB				
	LP	2.13eA	3.88dB	5.52cA	7.81bB	10.01aA	10.00aA				
AA, % DM	CK	1.21bA	1.05cB	0.41fB	0.56eC	0.70dC	1.86aB	<0.001	0.008	<0.001	<0.001
	BV	1.11cdB	1.15cA	1.03dA	1.38bA	0.77eB	1.96aB				
	LP	1.12bB	0.99cC	0.99cA	0.80dB	1.12bA	3.06aA				
PA, % DM	CK	2.56fA	3.53dA	4.44cA	3.29eB	5.98bA	9.11aA	0.003	<0.001	<0.001	<0.001
	BV	1.92eC	2.94dB	3.26cC	3.79bA	3.99bC	7.06aC				
	LP	2.30eB	2.91dB	3.59cB	3.55cB	4.95bB	7.63aB				
BA, % DM	CK	ND	ND	ND	ND	ND	ND				
	BV	ND	ND	ND	ND	ND	ND				
	LP	ND	ND	ND	ND	ND	ND				

Differences marked with different uppercase letters in the same column are significant (P < 0.05), and differences marked with different lowercase letters in the same row are significant (P < 0.05). LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid. BV, *Bacillus velezensis* added; LP, Lactobacillus plantarum added.

which was higher than CK ($P < 0.05$). Moreover, the LP-inoculated silage had higher aerobic stability time than CK ($P < 0.05$).

3.4 Bacterial community composition and diversity in whole-plant corn silage

The bacterial community diversity of whole-plant corn silage is illustrated in Figure 3. During the ensiling process, the alpha diversities (Shannon index) of the BV and LP experimental groups was lower than that of CK (Figure 3A). In accordance with beta diversity (principal coordinate analysis, PCoA), significant differences and consistent changes in bacterial community composition were observed in whole-plant corn silage at different fermentation stages (Figure 3B). BV and LP did not exhibit clear separation from CK on day 1 of ensiling. However, on other ensiling days, both experimental groups were distinct from CK, whereas no clear separation was observed between the two experimental groups.

The bacterial community composition of whole-plant corn silage at the phylum and genus levels is presented in Figure 4. As shown in Figure 4A, the main epiphytic bacteria at the phylum level before ensiling were *Proteobacteria* and *Bacteroidetes*, whereas *Firmicutes* had a relatively lower proportion. After ensiling, the proportions of *Proteobacteria* and *Bacteroidetes* decreased, whereas that of *Firmicutes* increased. On different ensiling days, the abundance of *Firmicutes* in the BV-inoculated silage was higher than that in CK, whereas the abundance of *Bacteroidetes* was lower in the experimental groups than in CK. In the LP-inoculated silage, the proportion of *Firmicutes* showed no significant change compared with that in CK on day 3, but it was higher on other ensiling days. Meanwhile, the abundance of *Bacteroidetes* was lower than that in CK on all ensiling days.

As shown in Figure 4B, the predominant genus in fresh samples at the genus level was *Pantoea*. On day 1 of ensiling, the dominant genus in the CK and experimental groups were *Lactococcus*,

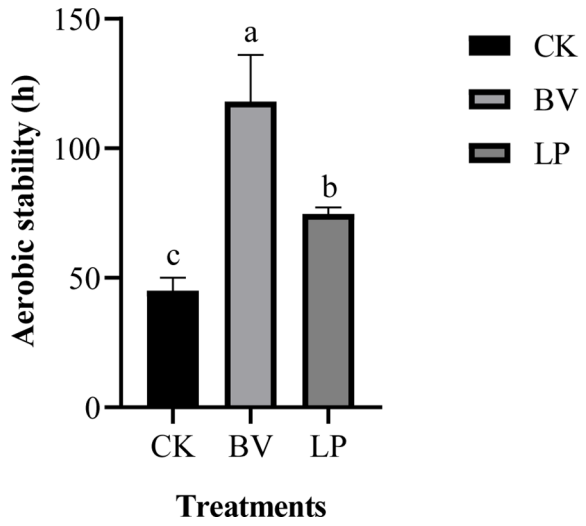

FIGURE 2
Aerobic stability of whole-plant corn silage. Different lowercase letters indicate significant differences ($P < 0.05$). CK: control group; BV, *Bacillus velezensis* added; LP, *Lactobacillus plantarum* added.

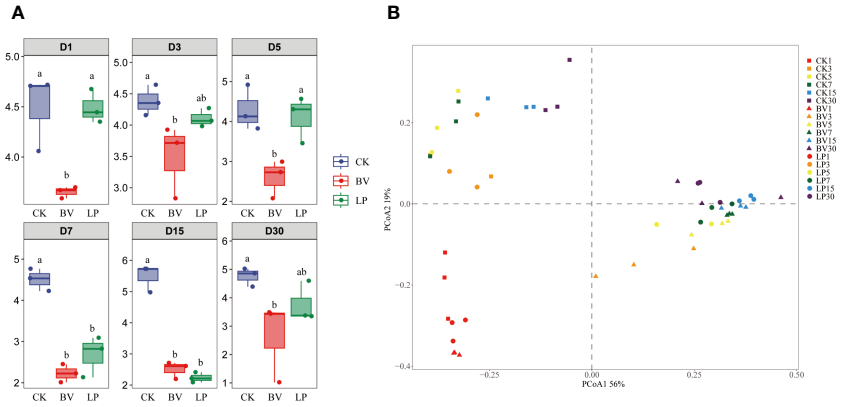

FIGURE 3
Bacterial community diversities and compositions in whole-plant corn silage during ensiling. Different lowercase letters indicate significant differences ($P < 0.05$). **(A)** Variations in community alpha-diversities (Shannon index). **(B)** Community dissimilarities in different groups and fermentation times, calculated using principal coordinate analysis (PCoA). BV, *Bacillus velezensis* added; LP, *Lactobacillus plantarum* added.

Weissella, and *Klebsiella*. As fermentation progressed, the abundance of *Lactobacillus* gradually increased and became dominant, whereas the proportions of *Weissella*, *Lactococcus*, and *Klebsiella* decreased. After 3 days of ensiling, *Lactobacillus* was higher in the two experimental groups than in CK. Moreover, it was lower in the LP-inoculated silage than in the BV-inoculated silage from day 1 to day 5 and higher from day 7 to day 30.

LEfSe analysis was performed to explore the differences in bacterial communities among the three groups, as shown in Figure 5. On day 1 of ensiling (Figure 5A), *Proteobacteria*, *Lactococcus*, and *Enterobacter* were higher in CK, *Weissella* was higher in the BV-inoculated silage, and *Klebsiella* was higher in the LP-inoculated silage. On day 3 of ensiling (Figure 5B), *Lactobacillus* and *Lactococcus* were higher in CK, *Weissella* was higher in the BV-inoculated silage, and *Bacteroides* and *Sphingobacterium* were higher in the LP-inoculated silage. On day 5 of ensiling (Figure 5C), *Lactobacillus*, *Lactococcus*, and *Streptococcus* were more abundant in CK, *Weissella* was significantly higher in the BV-inoculated silage, and *Lactobacillus* was higher in the LP-inoculated silage. On day 7 of ensiling (Figure 5D), CK remained to have higher *Lactobacillus brevis*, *Lactococcus* and demonstrated an increase in *Proteus*. *Pediococcus* was higher in the LP-inoculated silage, whereas *Bacillus* and *Lactobacillus* were enriched in the BV-inoculated silage. On day 15 of ensiling (Figure 5E), *Proteus* was higher in CK, *Serratia* was higher in the BV-inoculated silage, and *Lactobacillus* and *Lactococcus* were higher in the LP-inoculated silage. On day 30 of ensiling (Figure 5F), *Flavobacterium* and *Sphingobacterium* were higher in CK, *Lactobacillus* and *Bacteroides* were enriched in the LP-inoculated silage, and *Serratia* and *Acinetobacter* were higher in the BV-inoculated silage.

4 Discussion

In general, LAB are common inoculants used for ensiling feedstock (Muck et al., 2018; Mu et al., 2020; Yang et al., 2021). They can rapidly lower the pH of ensiled materials and improve fermentation quality (Chen et al., 2023). However, in recent years, studies found that due to their ability to produce cellulases, hemicellulases, and amylases, *B.* spp. were used as exogenous inoculants for silage fermentation (Bonaldi et al., 2021), to enhance the fermentation efficiency of plant materials in ensiling. Several reports (Bai et al., 2020; Bonaldi et al., 2021; Bai et al., 2022a) indicated that the use of *B.* spp. as silage inoculants can improve the fermentation quality and reduce the loss of nutritional value in silage. As one of the strains of *Bacillus*, *B. velezensis* possesses potential probiotic properties (Khalid et al., 2021). It can promote the growth and reproduction of *W. confusa*, *P. pentosaceus*, and *L. plantarum* L-2 in ensiled whole-plant corn feedstock. Furthermore, the whole-plant corn ensiled with BV treatment exhibited significantly lower pH values, especially in the initial 7 days of ensiling, than CK and the LP-inoculated silage. This finding indicated that BV has a rapid pH-lowering effect on whole-plant-corn-ensiled feedstock due to its promotion of LAB proliferation. This result indicated that BV rapidly reduces the pH of whole-plant corn silage in the early stage of ensiling by promoting the proliferation of LAB. LP also contributes to lowering pH during corn ensiling, but its pH-lowering effect, especially during the first 7 days of ensiling, is weaker than that of BV at the same dosage, further confirming the role of BV in promoting the growth of LAB. In the early stage of ensiling, the pH of silage inoculated with LP was significantly lower than CK. As ensiling progressed, the pH of CK decreased rapidly, and by day 30 of ensiling, the difference in pH between CK and the LP-inoculated silage became insignificant. This finding can be attributed to the higher initial population of exogenous LP in the whole corn plant during the early ensiling stages in comparison to CK. As ensiling progressed, the epiphytic LAB in CK gradually proliferate and produce organic acids that lower the pH of the ensiling environment. In the late fermentation stage, microbial activity was inhibited in all groups due to low pH values, and finally, pH reached a stable state on day 30.

During ensiling fermentation, LABs utilize carbohydrates to produce LA. In this study, the silage inoculated with LP exhibited lower LA concentration than the BV-inoculated silage during the first 7 days, and then it reached its highest concentration on days 15 and 30. This finding suggested that the LP originating from exogenous environment require some time to adapt to the

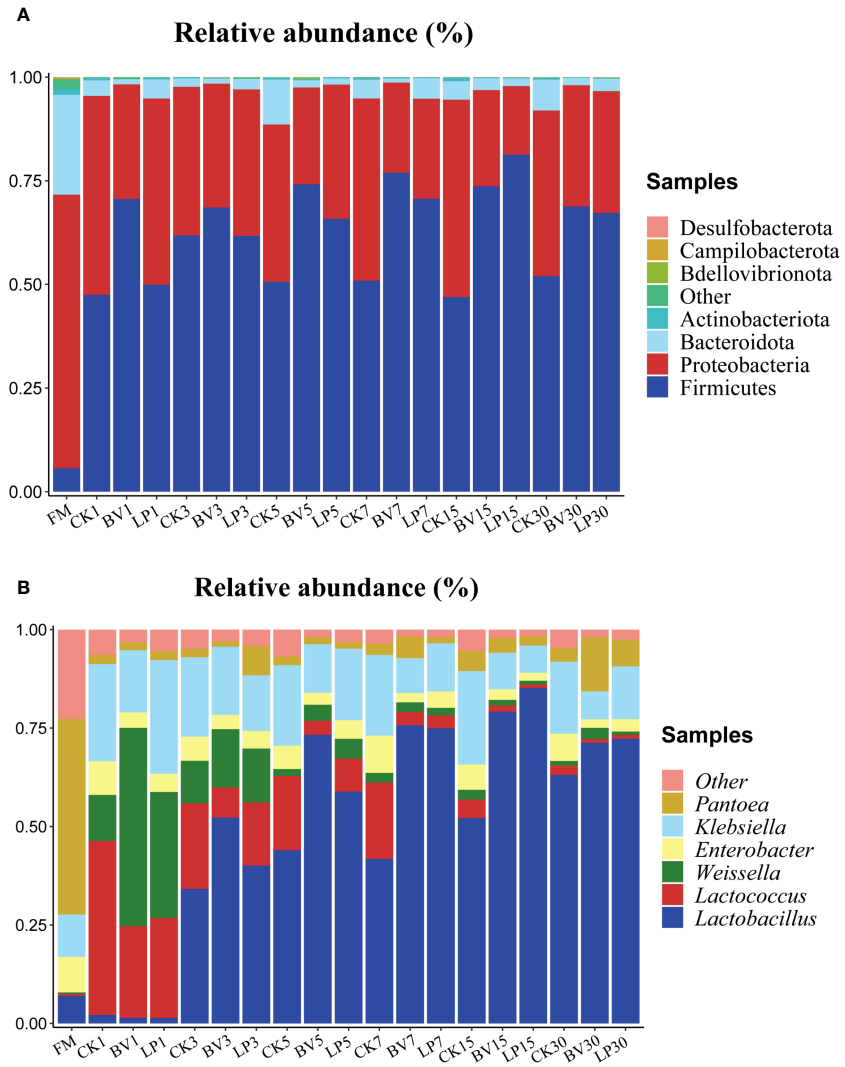

FIGURE 4
Bacterial community composition and succession of whole-plant corn silage. **(A)** Phylum level. **(B)** Genus level. BV, *Bacillus velezensis* added; LP, *Lactobacillus plantarum* added.

ensiling substrate in the early stages of fermentation. As the ensiling time was extended, LP gradually acclimated to the environment of whole-plant corn silage. Meanwhile, the LA concentration in the BV-inoculated silage was higher than that in CK on days 1, 3, 5, 7, and 30. This finding may be attributed to the rapid depletion of oxygen by BV during the fermentation process, thus creating an anaerobic environment that favors the growth of LABs and promoting their proliferation. In this experiment, the use of LP suppressed the generation of AA in the early stage, but in the later stages, the AA was significantly higher than that in CK. *L. plantarum* has traditionally been considered a homofermentative LAB, that mainly produces LA. However, recent studies suggested that *L. plantarum* is a facultative heterofermentative LAB. In addition to utilizing glucose to produce LA, *L. plantarum* produces phosphoketolase, an enzyme that can utilize pentose to produce LA and some AAs (Muck et al., 2018). Furthermore, some researchers pointed out that when using *L. plantarum* for ensiling, a low AA content in the early stages may result in weakened inhibition of yeast activity, leading to aerobic spoilage and subsequently causing an increase in AA content in the later stages (Lynch et al., 2012). Both possibilities mentioned above could potentially lead to increased AA content. However, the specific reason for the increased AA content in the LP group in the present experiment warrants further investigation. The inoculation of BV resulted in higher AA content than that in CK during 3–30 days of ensiling. This finding is in line with the findings of Bai et al. (2022b) and Xu et al. (2018), that is inoculation of *Bacillus* is beneficial for increasing the AA content in silage. Similar results suggested that a moderate amount of AA can enhance the aerobic stability of silage (Danner et al., 2003). In addition, the BV-inoculated silage showed a higher AA content than the LP-inoculated silage in the first 7 days of ensiling, possibly due to the faster adaptation of BV to the ensiling environment than the exogenous LP. Moreover, in terms of aerobic stability, the BV-inoculated silage had the significantly

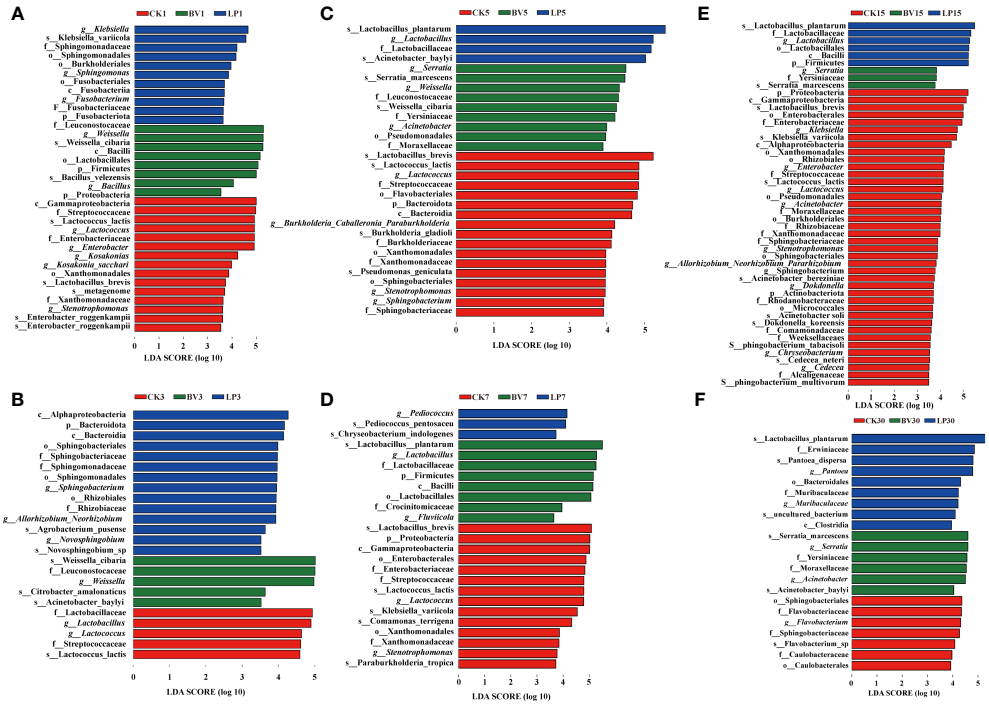

FIGURE 5
Identification of the communities or species with significant differences among three groups. BV, *Bacillus velezensis* added; LP, *Lactobacillus plantarum* added. **(A)** after 1 d of ensiling; **(B)** after 3 d of ensiling; **(C)** after 5 d of ensiling; **(D)** after 7 d of ensiling; **(E)** after 15 d of ensiling; **(F)** after 30d of ensiling.

longest time (118 hours) before a temperature increase of 2°C. This finding can be attributed to the antimicrobial capabilities of BV. Tabbene et al. (2009) indicated that *B. velezensis* can produce various antifungal compounds that have antibacterial activity against *E. coli*, *Listeria monocytogenes*, and *Fusarium oxysporum*. Fazle Rabbee and Baek (2020) indicated that genome sequencing of *B. velezensis* revealed the presence of numerous biosynthetic gene clusters that encode enzymes responsible for synthesizing various antifungal compounds. Cao et al. (2018) found that two strains of *B. velezensis* exhibited antagonistic effects against *F. oxysporum*. The inhibitory effect of BV on molds can reduce the occurrence of aerobic spoilage. In LP group, LP may potentially achieve this by preserving the abundance of *Lactobacilli* in the late stages of ensiling, thereby maximizing DM and CP retention, minimizing protein loss, and maintaining an acidic environment in the late fermentation stage to suppress the growth of acid-intolerant bacteria, thereby improving aerobic stability. Some studies showed that *L. plantarum* can enhance the aerobic stability of silage (Mu et al., 2020; Mu et al., 2021). However, contrasting findings suggested that it failed to improve aerobic stability and may have even reduce it, causing aerobic spoilage (Keshri et al., 2018; Wu et al., 2022). The mechanism behind LP increasing the aerobic stability remains to be explored.

In this experiment, the use of BV resulted in decreased the ADF and NDF contents compared with those in CK, along with a reduction in the ammonia nitrogen content in the feed. This finding may be due to the antibacterial ability of BV, which can inhibit the decomposition of protein by spoilage microorganisms, thereby reducing ammonia nitrogen and aerobic spoilage. Meanwhile, the ammonia nitrogen content in the LP group was also lower than that in CK, demonstrating that the LP used in this experiment has the ability to inhibit aerobic spoilage of corn silage and reduce protein decomposition and further confirming why LP can improve aerobic stability. Soluble sugars are one of the major components of plant silage fermentation (Liu et al., 2023). In the present study, the soluble sugar content in silage feed inoculated with BV and LP was higher than that in CK. Inoculation with LP reduced the loss of soluble sugars, whereas the BV-inoculated silage demonstrated an increase. This phenomenon is possibly due to the enzymatic action of cellulases, hemicellulases, and amylases, which all hydrolyze carbohydrates from plant cell walls, thereby releasing soluble sugars for fermentation (Bonaldi et al., 2021).

Silage feeds with higher fermentation quality typically exhibit lower α-diversity (Bai et al., 2021). In the present study, inoculation with BV and LP reduced α-diversity at various stages of silage fermentation. The PCoA plot revealed that the BV-inoculated silage significantly separated from CK after day 3 of fermentation, whereas separation from the LP-inoculated silage was not observed until day 5. This finding indicated that the effect of BV inoculation on bacterial community was similar and superior to those of LP and CK, respectively. This finding aligns with the results of Bai et al. (2022a) and Xu et al. (2021). *Lactococcus*, *Weissella*, and *Klebsiella* are commonly found in the early stages of silage fermentation. As the fermentation progressed and the pH of the feed decreased, *Klebsiella*, *Lactococcus*, and *Weissella* gradually decreased in all groups, whereas *Lactobacillus* became the dominant genus. This trend is consistent with the findings of Zhao et al. (2021); Zhang et al. (2022b), and Xu et al. (2021). The presence of microorganisms

in silage feed serves as a crucial indicator of its quality. Inoculating BV can optimize microbial community within corn silage, thereby enhancing the production of high-quality silage. LEfSe analysis was employed to further investigate the differences in bacterial composition among CK, the LP-inoculated silage, and the BV-inoculated silage. The results revealed that on days 1 and 7 of silage fermentation, the abundance of *Lactobacillus*, *Bacillus*, and on day 5, the abundance of *Weissella* was higher in the BV-inoculated silage than in the LP and CK groups. In addition, the increased in LAB, especially *Weissella*, in the BV-inoculated silage suggested that BV promotes the proliferation of LAB not only in liquid media but also during the fermentation process. This phenomenon was previously observed in various studies without substantial attention (Bai et al., 2020; Zhu et al., 2022; Wang et al., 2023).

5 Conclusion

BV demonstrates superior effects in rapidly lowering the pH of silage feed by promoting the proliferation of LAB, enhancing the aerobic stability of the silage feed under aerobic stress conditions, and leading to a more stable microbial community structure. BV enhances the fermentation quality of whole-plant corn silage.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject accession number: PRJNA1013177.

Author contributions

YW: Writing – original draft, Writing – review & editing. GY: Writing – original draft. ZZ: Writing – review & editing. YT: Writing – review & editing. YZ: Writing – review & editing. LC: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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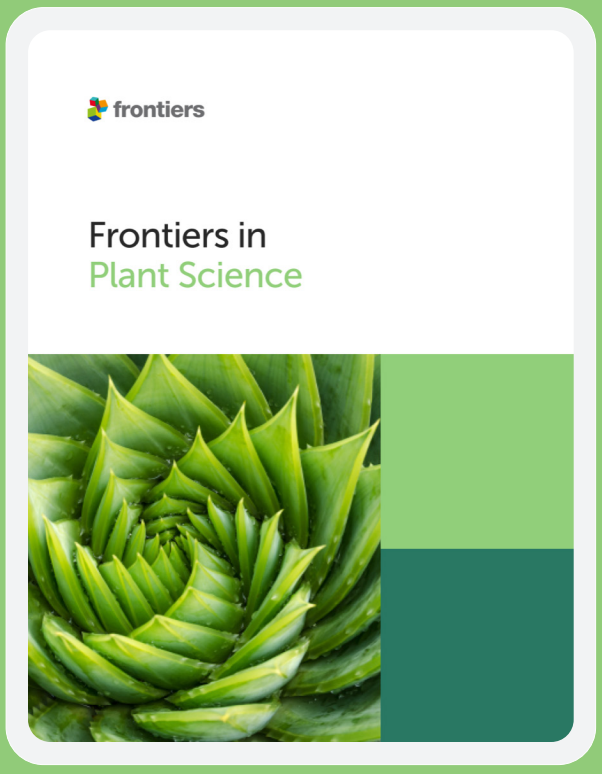

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