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Producing high-quality and safe whole-plant quinoa silage through selecting variety and harvest time

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Abstract

The great potential of whole-plant quinoa (WPQ) as a forage crop has been recognized in recent years. In this study, we investigated the effects of variety and harvest time on the fermentation characteristics, bacterial community, and hygienic quality of WPQ silage. Five varieties (Hongxin, Mengli1, SL577, SL2860, SL923) were grown across five separate experimental fields, with harvest occurring after 90 days (H1), 105 days (H2), or 120 days (H3). The samples were ensiled to evaluate their fermentation characteristics and bacterial composition. Hygienic quality was assessed using the Tax4fun2 and BugBase tools for potential pathogenicity and antimicrobial resistance prediction. The variety significantly influenced ($P < 0.05$) all fermentation variables (including pH, lactic acid, acetic acid, propionic acid, ethanol, and ammonia nitrogen), while harvest time affected pH and the contents of acetic acid, propionic acid, and NH_3-N ($P < 0.05$). An interaction between variety and harvest time was detected ($P < 0.05$) for all fermentation variables. Based on the flieg' score index, silage quality increased for Mengli1 (5.20–54.8), SL577 (36.7–71.5), and SL923 (34.9–77.0) with delayed harvest time, while silage quality decreased for Hongxin (52.1–41.4) and SL2860 (78.4–63.6). Compared to other silages, Hongxin silages exhibited greater differences in bacterial community composition between harvest times (indicated by higher PERMANOVA R^2-value). Tax4fun2 and BugBase analyses revealed that delaying harvest time significantly increased ($P < 0.05$) the relative abundances of pathogenic and antibiotic-resistant KEGG pathways ("Infectious disease: bacterial invasion" and "Drug resistance") and harmful microbes associated with potential pathogenicity and antimicrobial resistance in Hongxin silages. This study highlights the importance of variety and harvest time in producing high-quality, safe WPQ silage, which is beneficial for ensuring the safety in our food supply chain.

Keywords Whole-plant quinoa, Variety, Harvest time, Bacterial community, Pathogenicity, Antimicrobial resistance

Introduction

The scarcity and high cost of conventional feedstuffs have compelled farmers to seek alternative, non-traditional food sources. Quinoa (*Chenopodium quinoa* Wild.), a member of the Amaranthaceae family originating from South America's Andes region, stands out as a prominent non-traditional forage that has garnered significant attention in recent years. As an annual herbaceous plant, Quinoa exhibits remarkable resilience to frost, salinity, and drought, enabling its cultivation across diverse soil

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and climate conditions [1]. Beyond its potential for grain production, the utilization of whole-plant quinoa (WPQ) as a forage crop has been increasingly recognized due to its high protein content and diverse minerals and vitamins [2]. WPQ is also notable for its high concentrations of essential amino acids such as lysine, threonine, and methionine, coupled with significant phenolic and flavonoid levels [3]. These characteristics make WPQ a viable alternative to conventional feeds [4]. Similar to other forage crops, WPQ biomass is seasonally accumulated [5]. Consequently, proper preservation methods are required to achieve the goal of maintaining the original nutritive quality of the moist plant as much as possible.

Ensiling has been identified as the most effective approach for preserving WPQ. This process involves an anaerobic fermentation driven by epiphytic lactic acid bacteria (LAB), which primarily convert plant sugars into lactic acid and acetic acid. The resulting low-pH environment effectively inhibits pathogenic microorganisms and preserves nutrients. The variety and the harvest time of forage plant are important factors for producing high-quality WPQ silage due to variations in material characteristics such as buffering capacity, water-soluble carbohydrate (WSC), dry matter (DM), and epiphytic microorganisms. Ertekin et al. [6] demonstrated that these factors have a pronounced impact on the pH, ammonia nitrogen (NH_3-N) content, and LAB population of WPQ silage. Although ensiling of forage is a well-established microbial-driven process, there remains limited knowledge about how bacterial communities of WPQ silage respond to the changes in variety and harvest time. Moreover, the safety (hygiene) of silage has garnered increasing attention due to its potential impact on animal performance and health [7]. Pathogens present in silage can arise from contamination via manure or irrigation water [8]. Additionally, forage plants naturally contain epiphytic microorganisms, some of which harbor antibiotic resistance. These bacteria may thrive during silage fermentation, contributing to the microbial risk in our food chain [9, 10]. Recent development of 16S rRNA gene sequencing-based tools, such as Tax4fun2 and BugBase, offer a cost-effective means to investigate the potential pathogenicity and antimicrobial resistance within microbial communities [11, 12]. These innovative approaches have successfully elucidated the temporal dynamics of pathogens and antimicrobial resistance during ensiling [13].

In this study, we hypothesized that variety and harvest time would affect silage microbiomes and subsequently affect the microbial risk associated with WPQ silage. To test the hypothesis, we investigated the effects of these factors on the fermentation characteristics, bacterial community composition, and hygienic quality of WPQ silage. Advanced tools including Tax4fun2 and BugBase were employed to evaluate the hygiene of silage by predicting the potential pathogenicity and antimicrobial resistance of the microbial communities. The findings from this study will provide valuable insights into developing beneficial strategies for producing high-quality WPQ silage while minimizing microbial risks at silage production level.

Materials and methods

Plant materials and silage preparation

Five varieties of quinoa (Hongxin, Mengli1, SL577, SL2860, SL923) were planted in February 2023 at Xinyang Agricultural Experiment Station of Yancheng City (N33°52′E120°44′, Yancheng, China). The experimental site lies in the transition zone between the subtropical and warm and humid zones, with saline-alkali soil characteristics. These five varieties were selected for their good adaptability to the soil and climate conditions of the research site while being bred for differing purposes: one for forage production (Hongxin), one for dual-use of grain and forage production (Mengli1), and three for gain production (SL577, SL2860, SL923).

The experiment was set up in a randomized complete block design with four replicates. Five main plots treatments were assigned to five varieties (Hongxin, Mengli1, SL577, SL2860, SL923), and three sub-plots treatments were applied across three growth periods (90 d, 105 d and 120 d). Each sub-plot consisted of 20 rows spaced at 25 cm intervals with a total row length of 10 m. All sub-plots had the same tillage, irrigation, and fertilization practices. The fertilizer rates used in the trial were N 120 kg/ha, P 34.9 kg/ha and K 0 [14]. The quinoa plants were harvested above 5 cm soil level, chopped into a length of 1 to 2 cm, and thoroughly mixed before being packed into polyethylene plastic bags (30×40 cm). These bags were then vacuum-packed using the vacuum packing machine (Aomitai DZD-400/SD, Nanjing, China), with a vacuum time of 10 s and a pressure of −0.09 MPa. The vacuum-packed silage bags were stored at ambient conditions (temperature: 20°C-25°C; relative humidity: 45%−65%) for 90 days. In total, 60 silage bags were prepared (5 varieties×3 growth stages×4 replicates). After ensiling, silage was collected for further analysis.

Experimental analysis

The raw material and silage samples underwent oven drying for 48 h at 60°C to determine the dry matter (DM) content, adjusting for volatiles lost during drying [15]. The dried samples were ground using a laboratory pulverizer (FW100; Taisei Instrument Co., Ltd., Tianjin, China) to pass through a 1-mm screen for analyzing total nitrogen (TN), water-soluble carbohydrates (WSC),

neutral detergent fiber (NDF), and acid detergent fiber (ADF) [6]. The crude protein (CP) content was calculated by multiplying TN by 6.25 [16].

Microbial counts, including lactic acid bacteria (LAB) and enterobacteriaceae, were determined in the raw materials [17]. Thirty-five grams of silage sample was blended with 60 mL distilled water and macerated for 24 h at 4 °C for measuring fermentation variables. The pH was measured with a HANNA HI 2221 pH meter (Hanna Instruments Italia Srl, Villafranca Padovana, Italy). The NH_3-N was determined using the phenol-hypochlorite reaction method [18]. The organic acids (including lactic, acetic, propionic, and butyric acids) and ethanol were quantified using an Agilent 1260 HPLC system equipped with a refractive index detector (Carbomix® H-NP5 column, 2.5 mM H_2SO_4, 0.5 mL/min). Silage fermentation quality was assessed based on Flieg's score using the formula: $220+(2\times\%DM-15)-40\times pH$ [19]. According to the index, silage was classified as very inferior (< 20), inferior (21–40), medium (41–60), good (61–80), or very good (81–100).

Bacterial community analysis

Microbial community DNA extraction from raw material and silage samples was performed using the FastDNA SPIN Kit (MP Biomedicals, Santa Ana, CA). The quantity and quality of obtained DNA were determined by the NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA). The V3-V4 hypervariable regions of bacterial 16S ribosomal RNA genes were amplified using universal primers (341F and 806R) [19]. The PCR products were purified using the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA) and quantified according to the manufacturer's protocol using QuantiFluor™-ST (Promega, USA). The DNA sequences were paired-end sequenced on an Illumina NovaSeq 6000PE250 platform at BIOZERON Technology Co., Ltd., Shanghai, China.

Raw sequences were processed using UCHIME to eliminate sequences with quality scores below 20. Sequences with a minimum length of 200 bp were clustered into operational taxonomic units (OTUs) at a 97% similarity cutoff using UPARSE. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by uclust algorithm (https://github.com/topics/uclust) against the silva 16S rRNA database (SSU138.1). To reveal diversity indices, including the Chao1 and Shannon diversity indices, the rarefaction analysis was conducted based on Mothur v.1.21.1. The beta diversity analysis using UniFrac distance matrices was performed to compare the results of the principal co-ordinates analysis (PCoA). The PCoA was tested for significance by multiple-response permutation procedure (MRPP) and analysis of permutational multivariate analysis of variance (PERMANOVA) in the R package Vegan [20].

Functional and phenotype predictions

Tax4Fun2 and BugBase were respectively employed to predict bacterial community functional profiles and phenotypes [10, 19]. Tax4Fun2 integrates user-defined, habitat-specific genomic information, offering higher accuracy and robustness in predictions compared to PICRUSt and Tax4Fun. BugBase is a novel algorithm that leverages pre-existing databases, annotations and frameworks, along with manual curation, to provide interpretable biological traits such as "Gram Staining," "Biofilm Formation," "Oxidative Stress Tolerance," "Pathogenicity," and "Presence of Mobile Elements" at the organism level [12].

Statistical analysis

Microbial data were $\log_{10}$-normalized to address variability in measurement scales. All data were subjected to 2-way ANOVA with the fixed effects of variety (Hongxin, Mengli1, SL577, SL2860, SL923), harvest time (90 d, 105 d and 120 d), and variety × harvest time using the GLM procedure of Statistical Package for Social Science 22.0 (SPSS, Inc., Chicago, IL, USA). Post-hoc comparisons were performed using Tukey's honestly significant difference test to identify significant differences ($P < 0.05$).

Results

Chemical characteristics and microbial counts of fresh WPQ

The chemical characteristics and microbial counts of fresh WPQ are shown in Table 1. Dry matter (DM) contents varied significantly among the five WPQ varieties, ranging from 146 to 242 g/kg fresh weight (FW). The WSC contents varied from 27.1 to 48.6 g/kg DM; NDF contents varied from 46.1 to 56.4% DM; ADF contents varied from 24.8 to 36.9% DM; CP contents varied from 9.75 to 20.3 g/kg DM; LAB counts varied from 4.64 to 6.36 lg cfu/g FW; enterobacteriaceae counts varied from 6.13 to 7.54 lg cfu/g FW.

The overall trends observed during the harvest period indicated that the WPQ varieties generally exhibited increasing trends in NDF, ADF, and WSC contents with the delay of harvest time, while a decreasing trend in CP content was observed across the varieties. Notably, Hongxin showed the largest decrease in CP contents (from 17.2% to 9.75% of DM) and the largest increase in NDF (from 46.1% to 56.4% of DM) and ADF contents (from 24.8% to 36.9% of DM) compared to the other varieties. Among the varieties, the SL577 showed the largest decrease in WSC content (from 48.5 g/kg DM to 32.4 g/kg DM), while SL2860 recorded the most notable increase in DM content (from 15.1% to 23.0% of FW). In

Table 1 Effects of variety and harvest time on the chemical compositions and microbial counts of fresh WPQ

Item	Variety	Harvest time			SEM	*P*-value		
		H1	H2	H3		V	H	V*H
Dry matter (g/kg FW)	Hongxin	161^{Bb}	159^{Bb}	194^{Ca}	0.902	***	***	***
	Mengli1	146^{Cc}	169^{ABb}	195^{Ca}				
	SL577	180^{Ab}	176^{ABb}	242^{Aa}				
	SL2860	151^{BCc}	187^{Ab}	230^{ABa}				
	SL923	147^{C}	167^{B}	218^{B}				
WSC (g/kg DM)	Hongxin	46.9	41.9^{A}	48.6^{A}	0.518	***	***	***
	Mengli1	39.4^{a}	33.4^{Bab}	30.0^{Cb}				
	SL577	48.5^{a}	33.8^{Bb}	32.4^{Cb}				
	SL2860	42.3	41.7^{A}	40.5^{B}				
	SL923	46.5^{a}	27.1^{Bb}	42.0^{ABa}				
NDF (g/kg DM)	Hongxin	461^{b}	560^{a}	564^{a}	4.805	ns	***	ns
	Mengli1	490	524	551				
	SL577	497	541	529				
	SL2860	472^{b}	556^{a}	561^{a}				
	SL923	491	547	512				
ADF (g/kg DM)	Hongxin	248^{c}	326^{b}	369^{Aa}	3.943	ns	***	*
	Mengli1	269	312	301^{AB}				
	SL577	281^{b}	315a	288^{Bab}				
	SL2860	277	318	310^{AB}				
	SL923	272	325	299^{AB}				
CP (g/kg TN)	Hongxin	172^{a}	142^{Bb}	975^{Bc}	1.772	***	***	***
	Mengli1	203	188^{A}	174^{A}				
	SL577	169	188^{A}	178^{A}				
	SL2860	192^{a}	164^{ABb}	156^{Ab}				
	SL923	191^{a}	182^{Aab}	159^{Ab}				
LAB (cfu/g FW)	Hongxin	5.33^{b}	6.43^{a}	5.81^{Aab}	0.043	**	***	ns
	Mengli1	5.15^{b}	6.33^{a}	5.66^{Aab}				
	SL577	4.70^{c}	6.33^{a}	5.43^{ABb}				
	SL2860	4.92^{b}	6.36^{a}	4.90^{Bb}				
	SL923	4.64^{b}	6.27^{a}	5.26^{ABb}				
EN (cfu/g FW)	Hongxin	6.20^{b}	7.54^{Aa}	6.51^{ABb}	0.039	ns	***	ns
	Mengli1	6.47^{b}	7.39^{ABa}	6.59^{ABb}				
	SL577	6.37^{b}	7.53^{Aa}	6.68^{Ab}				
	SL2860	6.51^{b}	7.30^{ABa}	6.13^{Bb}				
	SL923	6.26^{b}	7.21^{Ba}	6.56^{ABb}				

Means with different letters in the same column (A–D) or row (a–c) differ ($P < 0.05$)

DM dry matter, *FW* fresh weight, *WSC* water-soluble carbohydrates, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *CP* crude protein, *LAB* lactic acid bacteria, *EN* Enterobacteriaceae, *H1* WPQ harvested after 90 d of growth, *H2* WPQ harvested after 105 d of growth, *WPQ* harvested after 120 d of growth, *SEM* standard error of mean, *V* variety, *H* harvest time, *V*×*H* the interaction between variety and harvest time

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns $P > 0.05$

terms of microbial counts, WPQ at H2 showed higher levels of LAB and enterobacteriaceae compared to those at H1 and H3, regardless of variety (Fig. 1B).

Fermentation characteristics of WPQ silage

The fermentation characteristics of WPQ silages after 60 days of ensiling is presented in Table 2. All fermentation variables were significantly affected ($P < 0.05$) by the variety, and harvest time influenced ($P < 0.05$) variables including pH, acetic acid, propionic acid, and NH_3-N. A significant interaction between variety and harvest time was observed for all fermentation variables ($P < 0.05$). The pH values in different WPQ silages showed distinct trends based on the harvest time. Specifically, the pH

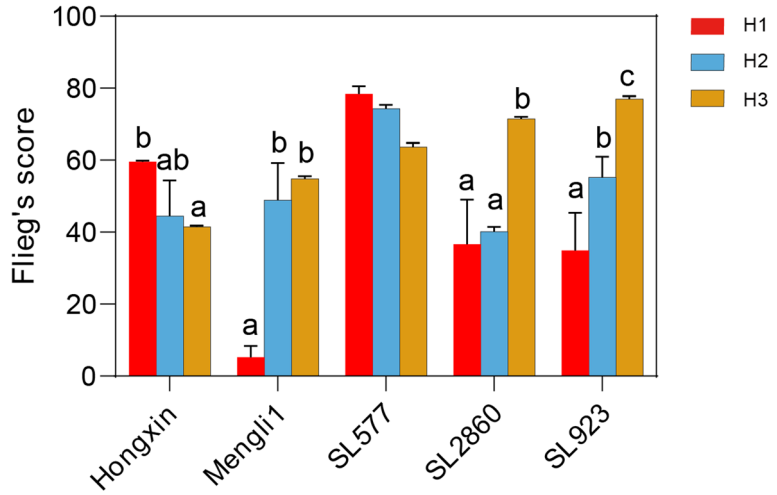

Fig. 1 The flieg' scores of WPQ silages produced from different varieties. H1, WPQ silage harvested after 90 d of growth; H2, WPQ silage harvested after 105 d of growth; WPQ silage harvested after 120 d of growth. [a–c]Means with different lower case were significant at $P < 0.05$

values for Hongxin silages were H1 (4.62) < H2 (4.88) < H3 (5.03); for Mengli1 silages were H1 (5.71) > H2 (4.71) ≈ H3 (4.70); for SL2860 silages were H1 (4.94) ≈ H2 (4.99) > H3 (4.40); for SL923 silages were H1 (4.98) > H2 (4.63) > H3 (4.25). Additionally, pH values in these silages were negatively correlated with lactic acid contents and positively correlated with NH_3-N contents across all varieties. Greater increases ($P < 0.05$) in propionic acid (from 4.84% to 17.8% of DM) and ethanol (from 10.9% to 16.0% of DM) contents were detected in Hongxin silages from H2 to H3 than in other silages.

As shown in Fig. 1, the flieg's scores of silages from Hongxin, Mengli1, SL577, SL2860, and SL923 were respectively recorded within the ranges of 41.4–52.1, 5.20–54.8, 63.6–78.4, 36.7–71.5, and 34.9–77.0. Silages from SL577 and those harvested at H3 from SL2860 and SL923 achieved Flieg's scores above 60. According to the Flieg's score index, the fermentation qualities of WPQ silages produced from Hongxin and SL577 decreased with later harvest times. In contrast, silages from Mengli1, SL2860, and SL923 exhibited improved fermentation quality as delayed harvest time.

Bacterial community of fresh WPQ and WPQ silage

After quality control, a total of 7,503,297 high-quality sequences were identified from the fresh and silage samples. These sequences were grouped into 10,786 operational taxonomic units (OTUs) based on a 97% sequence similarity threshold. Good's coverage values exceeded 0.99 for all samples, indicating robust species representation at sequencing depth. Species richness estimates (Chao1 index) and diversity indices for fresh WPQ and WPQ silages were presented in Fig. 2A and B, respectively. Except for Hongxin, the species richness of fresh WPQ exhibited minimal differences between harvest times. For silage samples, Hongxin, SL2680, and SL923 silages showed increased species richness with the delay of harvest time, while Mengli1 and SL577 silages exhibited lower ($P < 0.05$) species richness at H2 compared to at H1 and H3. Fresh WPQ samples exhibited the lowest bacterial diversity at H2 across all varieties. Delaying harvest time decreased bacterial diversity in Mengli1 silages while increased it in Hongxin and SL2860 silages.

The beta diversity was visualized using PCoA plots and confirmed by PERMANOVA analysis (all *P*-values = 0.001, Fig. 2C-F). Among the varieties, Hongxin silages showed greater differences in bacterial community composition across harvest times, indicated by higher PERMANOVA R^2-value (Fig. 2F). All samples were classified at genus level (Fig. 3A). The dominant genera in fresh WPQ were *Pantoea*, *Saccharibacillus*, *Enterobacter*, and *Pseudomonas*. In silage, the dominant genera were primarily *Lactiplantibacillus*, *Levilactobacillus*, *Companilactobacillus*, *Enterobacter*, and *Pantoea*. The effect of harvest time on bacterial community composition varied by variety (Fig. 3B). For fresh WPQ, delayed harvest times resulted in decreased ($P < 0.05$) relative abundance of *Pantoea* and increased ($P < 0.05$) abundance of *Enterobacter* in SL577, SL2860, and SL923 silages. Additionally, these varieties exhibited higher ($P < 0.05$) relative abundances of *Saccharibacillus* at H2 and *Pseudomonas* at H1 compared to other harvest times. Compared to silages produced from other varieties, Hongxin silages showed greater differences in bacterial community composition across harvest times; delayed harvest time increased ($P < 0.05$) the relative abundance of *Enterobacter* while

Table 2 Effects of variety and harvest time on the fermentation characteristics of WPQ silage

Item	Variety	Harvest time			SEM	*P*-value		
		H1	H2	H3		V	H	V*H
pH	Hongxin	4.62^{BCb}	4.88^{Aab}	5.03^{Aa}	0.077	**	*	***
	Mengli1	5.71^{Aa}	4.71^{ABb}	4.70^{Bb}				
	SL577	4.06^{Cb}	4.15^{Bb}	4.67^{Ba}				
	SL2860	4.94^{ABCa}	4.99^{Aa}	4.40^{Cb}				
	SL923	4.98^{ABa}	4.63^{ABab}	4.25^{Db}				
LA (g/kg DM)	Hongxin	26.6^{ABa}	22.4^{a}	1.59^{Cb}	1.211	***	ns	***
	Mengli1	5.86^{Bb}	26.6^{a}	23.6^{Ba}				
	SL577	39.1^{A}	33.9	30.4^{AB}				
	SL2860	15.2^{AB}	21.7	25.5^{B}				
	SL923	17.5^{ABb}	23.5^{b}	45.4^{Aa}				
AA (g/kg DM)	Hongxin	10.1^{BC}	9.02^{C}	9.06^{B}	0.140	***	***	***
	Mengli1	15.1^{Aa}	11.5^{ABb}	11.9^{Ab}				
	SL577	8.83^{Cb}	8.92^{Cb}	10.2^{Ba}				
	SL2860	13.5^{Aa}	13.4^{Aa}	8.92^{Bb}				
	SL923	12.9^{ABa}	9.89^{BCb}	9.29^{Bb}				
	SL923	1.48^{Bb}	2.43^{ABb}	4.89^{Aa}				
PA (g/kg DM)	Hongxin	5.19^{Cb}	4.84^{Bb}	17.8^{Aa}	0.160	***	***	***
	Mengli1	6.00^{Ab}	5.15^{Bc}	9.29^{Ba}				
	SL577	4.74^{Db}	4.74^{Bb}	8.37^{Ba}				
	SL2860	5.69^{Bb}	9.05^{Aa}	6.55^{Bab}				
	SL923	5.68^{Bb}	10.2^{Aa}	5.42^{Bb}				
ETH (g/kg DM)	Hongxin	7.48^{b}	10.9^{ab}	16.0^{Aa}	0.229	***	ns	***
	Mengli1	7.58^{b}	8.73^{a}	6.93^{Bb}				
	SL577	7.80^{a}	7.09^{ab}	6.61^{Bb}				
	SL2860	7.22	7.64	6.75^{B}				
	SL923	8.06	7.46	7.91^{B}				
NH_3-N (g/kg TN)	Hongxin	126^{BC}	151^{B}	205^{AB}	6.363	***	*	***
	Mengli1	316^{Aa}	183^{ABb}	240^{Aab}				
	SL577	83.4^{Cb}	99.6^{Bb}	252^{Aa}				
	SL2860	235^{AB}	290^{A}	182^{B}				
	SL923	200^{ABC}	131^{B}	177^{B}				

Means with different letters in the same column (A–D) or row (a–c) differ ($P < 0.05$)

DM dry matter, *LA* lactic acid, *AA* acetic acid, *PA* propionic acid, *ETH* ethanol, NH_3-*N* ammonia nitrogen, *TN* total nitrogen, *H1* WPQ harvested after 90 d of growth, *H2* WPQ harvested after 105 d of growth, *WPQ* harvested after 120 d of growth, *SEM* standard error of mean, *V* variety, *H* harvest time, *V*×*H* the interaction between variety and harvest time

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns $P > 0.05$

decreasing ($P < 0.05$) the relative abundances of *Lactiplantibacillus* and *Levilactobacillus*.

Pathogenicity and antimicrobial resistance of bacterial community

For hygiene analysis of bacterial community, the KEGG pathways associated with pathogenicity and antimicrobial resistance were predicted using Tax4Fun2 in fresh and ensiled WPQ samples (Fig. 4A). Compared to their corresponding fresh materials, all WPQ silages exhibited comparable or lower relative abundances of "Infectious disease: bacterial invasion" and "Drug resistance" pathways. Notably, for Hongxin samples, ensiling reduced the relative abundances of these two pathways in H1 silages whereas increased ($P < 0.05$) them significantly in H3 silages. The BugBase phenotypic analysis also revealed that, excluding Hongxin, the relative abundances of bacterial phenotypes associated with "Potential Pathogenicity" and "Contains Mobile Elements" were much lower ($P < 0.05$) in WPQ silages compared to fresh materials (Fig. 5A). However, for Hongxin samples, ensiling only reduced ($P < 0.05$) the abundances of "Potential

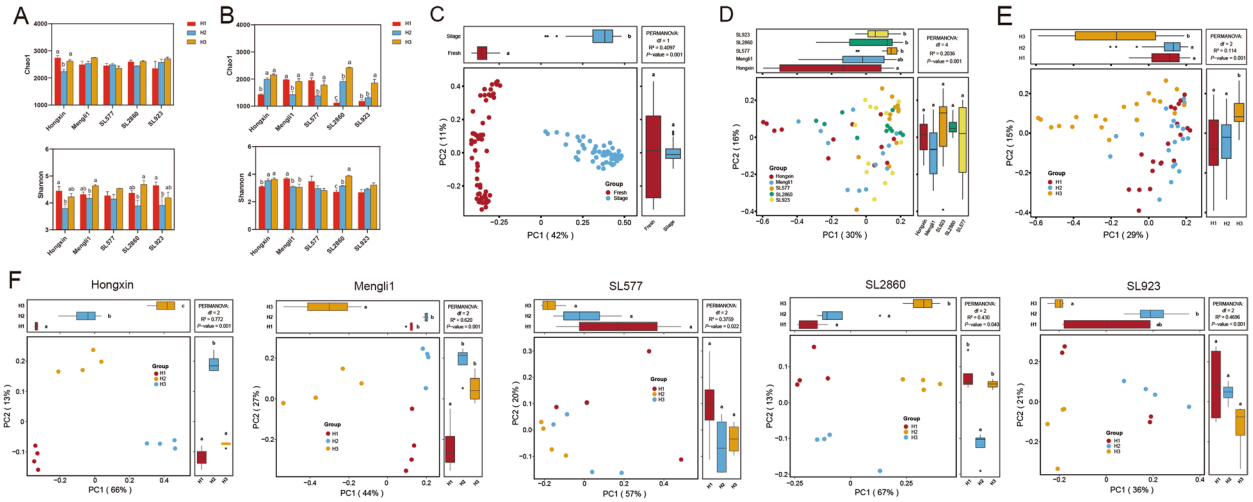

Fig. 2 Alpha- and beta-diversity of bacterial communities in fresh WPQ and WPQ silage. **A** Chao1 and Shannon indexes of bacterial communities in fresh WPQ. **B** Chao1 and Shannon indexes of bacterial community in WPQ silage. **C** Principal component coordinate analysis (PCoA) of bacterial communities of fresh and silage samples. **D** PCoA of bacterial communities of silage samples grouped by variety. **E** PCoA of bacterial communities of silage samples grouped by harvest time. **F** PCoA of bacterial communities of silage samples grouped by harvest time for each variety. Bacterial communities are compared using the unweighted UniFrac distance metric. H1, WPQ silage harvested after 90 d of growth; H2, WPQ silage harvested after 105 d of growth; WPQ silage harvested after 120 d of growth. $^{a-c}$Means with different lower case were significant at $P < 0.05$

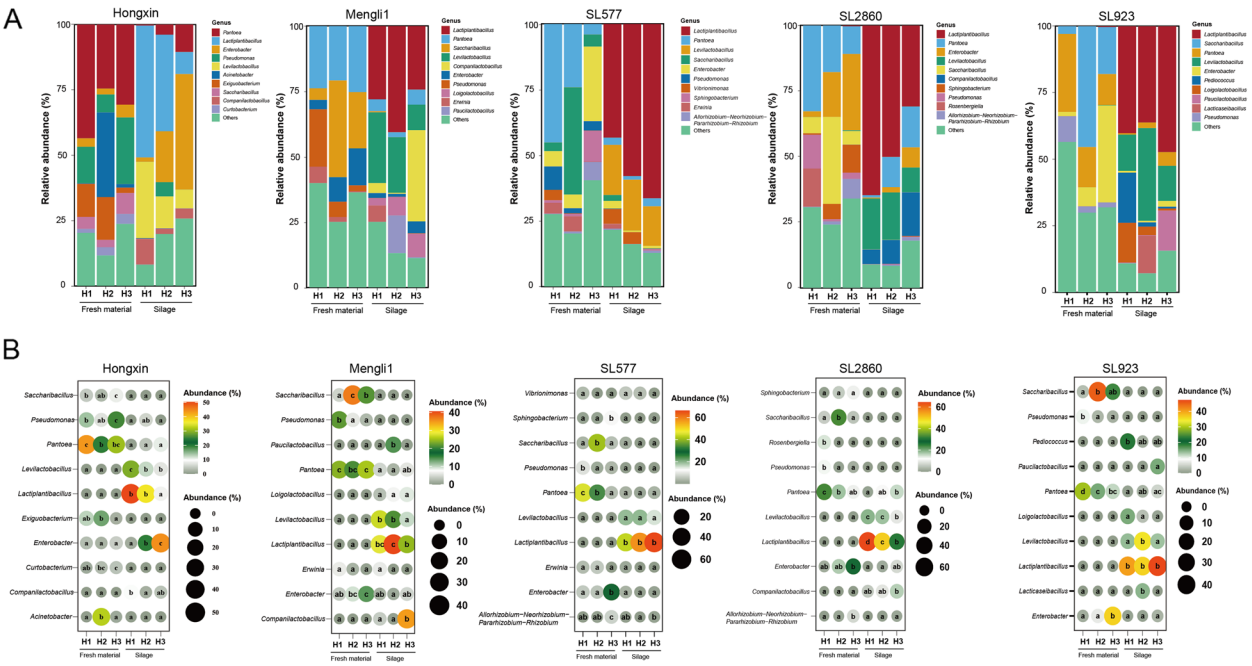

Fig. 3 The bacterial community compositions of fresh WPQ and WPQ silage. **A** Bacterial community composition on genus level in fresh WPQ and WPQ silage harvested from different varieties. **B** Statistical comparison of bacterial communities on genus level between fresh WPQ and WPQ silage harvested from different varieties. H1, WPQ silage harvested after 90 d of growth; H2, WPQ silage harvested after 105 d of growth; H3, WPQ silage harvested after 120 d of growth. $^{a-c}$Means with different lower case were significant at $P < 0.05$

Pathogenicity" in H1 and H2 silages and decreased ($P < 0.05$) "Contains Mobile Elements" in H1 silages. While the relative abundances of these two phenotypes were significantly increased ($P < 0.05$) in Hongxin silages harvested at H3. The bacterial OTUs contributing to "Potentially Pathogenic" and "Contains Mobile Elements"

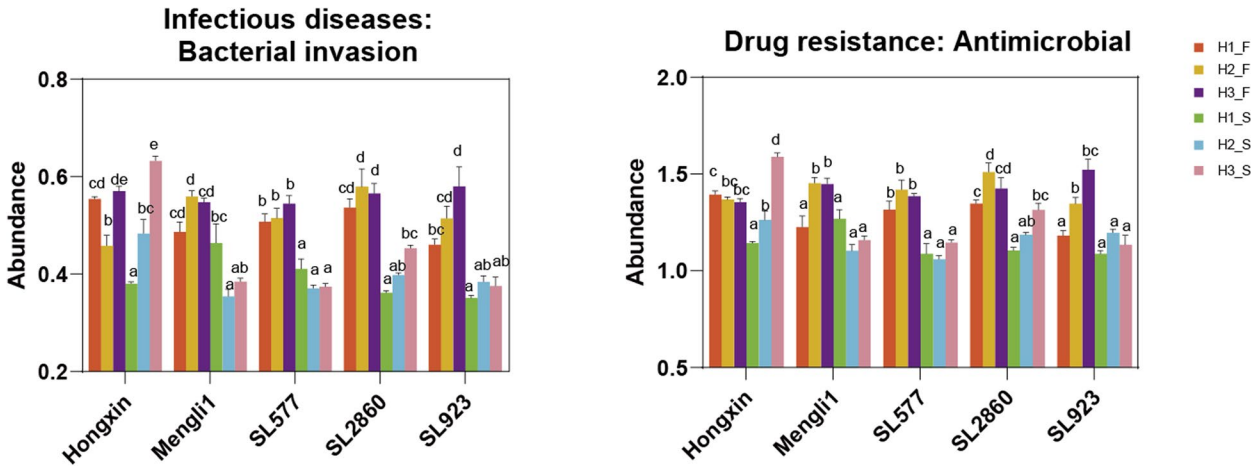

Fig. 4 The functional pathways associated with pathogenicity and antimicrobial resistance in fresh WPQ and WPQ silage. H1_F, fresh WPQ harvested at H1; H2_F, fresh WPQ harvested at H2; H3_F, fresh WPQ harvested at H3; H1_S, WPQ silage harvested at H1; H2_S, WPQ silage harvested at H2; H3_S, WPQ silage harvested at H3. $^{a-c}$Means with different lower case were significant at $P < 0.05$

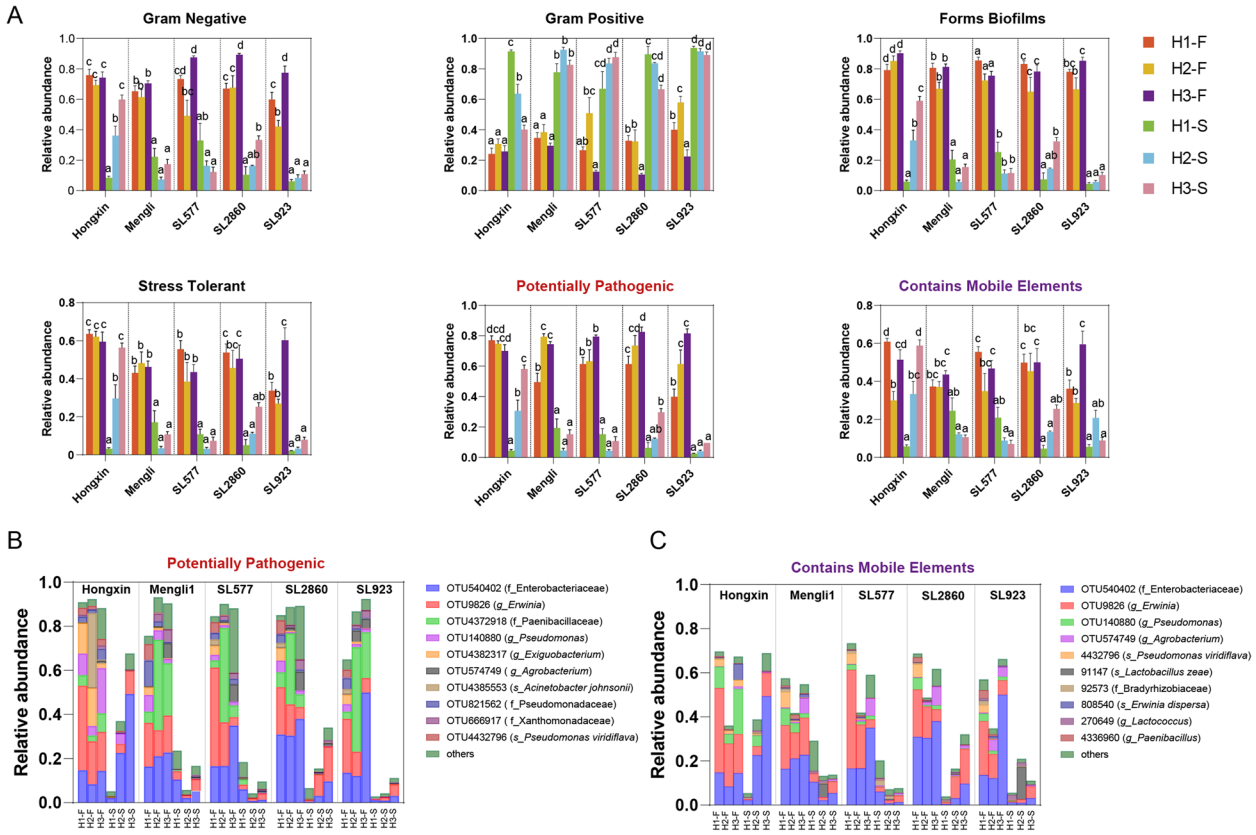

Fig. 5 Bacterial phenotype predictions of fresh WPQ and WPQ silage. **A** The bacterial phenotype predictions in terms of "Gram Negative", "Gram Positive", "Forms Biofilms", "Stress Tolerant", "Potentially Pathogenic" and "Contains Mobile Elements". **B** Bar plot of bacterial OTUs contributing to "Potentially Pathogenic". **C** Bar plot of bacterial OTUs contributing to "Contains Mobile Elements". H1_F, fresh WPQ harvested at H1; H2_F, fresh WPQ harvested at H2; H3_F, fresh WPQ harvested at H3; H1_S, WPQ silage harvested at H1; H2_S, WPQ silage harvested at H2; H3_S, WPQ silage harvested at H3. $^{a-c}$Means with different lower case were significant at $P < 0.05$

were visualized in bar plots (Fig. 5B and C). The results indicated that the bacterial phenotypes in Hongxin silages were predominantly associated with enriched OTUs from *unclassified_f_Enterobacteriaceae* and *Erwinia*.

Discussion

Chemical characteristics and microbial counts of fresh WPQ

Quinoa exhibits an extremely wide genetic diversity and significant variation in agricultural traits [21]. Among the varieties, Hongxin exhibited greater increases in NDF and ADF contents than other varieties during the harvest. This differential increase may reflect underlying differences in biomass allocation patterns among varieties [22]. The Hongxin is specifically developed for forage purpose, and thus exhibiting a superior ability to accumulate cell wall fractions. The CP contents in Hongxin decreased significantly as the plant matured, potentially associated with a dilution effect of fiber fractions on crude protein (CP) content. Similar trends have been reported in other forage crops [23, 24]. The WSC contents decreased and DM contents increased in most varieties with the delay of harvest time. It could be attributed to the accumulation of starch in grains [25].

Fermentation characteristics of WPQ silage

The DM and WSC contents are critical factors influencing the fermentation characteristics of silages. Zhang et al. [26] proposed that a DM range of 300–400 g/kg FW with at least 60 g/kg DM of WSC is required for successful fermentation in forage crops. However, all WPQ varieties examined in this study failed to meet these requirements, regardless of variety or harvest time. Consequently, most silages exhibited poor fermentation qualities, characterized by high pH values (>4.20) and high ammonia nitrogen (NH_3-N) contents (>10%−15% of TN). These findings were corroborated by the low Flieg scores (<60) observed across most silages. The Hongxin and SL577 silages demonstrated higher Flieg's scores during early harvest time (H1), while Mengli1, SL2860, and SL923 silages exhibited higher scores at later harvest times (H2–H3). This discrepancy highlights the substantial variation in fermentation properties among WPQ varieties, which may be attributed to differences in their chemical composition and epiphytic microbial communities.

Lactic acid is a desirable fermentation product for maintaining silage quality due to its energy-saving metabolism. In contrast, undesirable products such as acetic acid, propionic acid, and ethanol are detrimental because their production represents an energy-waste process [24]. Our previous study indicated that naturally fermented WPQ is prone to acetic acid-type fermentation due to high enterobacterial activity [27]. In this study, most WPQ silages exhibited predominant lactic acid, suggesting acceptable enterobacterial activity in the silage. Notably, substantial increases in propionic acid and ethanol were observed in Hongxin silages during H2 to H3. The elevated ethanol production could result from the action of microorganisms such as heterolactic acid bacteria, enterobacteria, and yeasts [28]. The NH_3-N content is a key indicator of protein degradation in silages. Generally, NH_3-N levels exceeding 100–150 g/kg TN indicate extensive protein breakdown [28]. According to this criterion, only a few silages achieved satisfactory levels. This finding aligns with our previous study demonstrating that WPQ proteins were poorly preserved during ensiling process [27]. However, it is noteworthy that Hongxin silages at H1 and SL577 silages at H1 and H2 exhibited NH_3-N contents below 10–15% TN. The low levels of NH_3-N production indicated effective inhibition of plant and microbial proteases during the fermentation process.

Bacterial community of fresh WPQ and WPQ silage

Ensiling is a process driven by bacteria, where the types and abundances of bacteria involved are crucial for the quality of fermentation [29]. The species richness of fresh WPQ exhibited less differences between harvest times, suggesting that epiphytic microbiota is highly conserved during plant growth. This agrees with our previous observation in whole crop oat [24]. Muraro et al. [30] concluded that quality silages typically have low bacterial diversity because complex epiphytic bacterial community will be replaced by LAB strains during ensiling. However, this finding was inconsistent with our observations of SL2860 silages, suggesting that bacterial diversity indices cannot solely be used to assess the quality of silage. Similarly, Wang et al. [30] also found no significant correlation between silage bacterial diversity and fermentation quality. The Principal Coordinate Analysis (PCoA) was employed to evaluate the microbial community similarities across different samples. Clearly separated PCoA plots indicated a significant shift in microbial communities resulting from the fermentation process. Silage samples were grouped into distinct clusters based on their variety or harvest time, revealing significant impacts of these factors on the structure of the silage microbiota. The greater differentiation observed in Hongxin silages between harvest times highlighted a stronger influence of harvest time on the composition of silage bacterial communities.

Epiphytic microbiota plays a critical role in influencing silage quality by directing the initial fermentation process and shaping fermentation dynamics during ensiling [31]. In fresh WPQ, the dominant epiphytic

bacteria included *Pantoea*, *Saccharibacillus*, *Enterobacter*, and *Pseudomonas*. These findings align with similar results obtained from whole crop corn and wheat studies, where *Pantoea*, *Enterobacter*, and *Pseudomonas* were identified as key epiphytic bacterial species [32, 33]. The provision of habitats by plants supports the growth of bacterial communities, and host factors such as leaf age and developmental stage significantly influence the structure of epiphytic bacterial communities [34]. The results revealed that in SL577, SL2860, and SL923 the abundance of *Pantoea* decreased while *Enterobacter* increased with delayed harvest time. This shift could be attributed to altered plant status encompassing hormonal and physiological changes as plant matured [24]. These findings are consistent with those observed in other forages [17], highlighting the dynamic nature of epiphytic bacterial communities during plant maturation.

Silage fermentation depends on the competitive interactions between different microbial groups. The LAB species are the desirable bacteria contributing to silage fermentation quality. Typically, high abundance levels of *Lactiplantibacillus*, *Levilactobacillus*, *Companilactobacillus* were observed in silage due to their inherent acid-resistance properties [35]. Among the LABs, *Lactiplantibacillus* are stands out as particularly crucial for silage fermentation quality because they are able to rapidly ferment a wide range of substrates leading to the large amount of lactic acid production and rapid pH decline [27]. In contrast, *Enterobacter* species are classified as major undesirable bacteria in silage production. These organisms compete with LABs for available sugars and can degrade plant proteins, resulting in the synthesis of toxic compounds such as biogenic amines and branched-chain fatty acids [36]. Compared with other silages, Hongxin silages had notably greater decreases in abundances of *Lactiplantibacillus* and increases in abundances of *Enterobacter* with the delay of harvest time. Given that WSC exhibited no significant differences among different harvest times, a possible explanation is that the thickening of the cell wall affected the release of juice during ensiling, thereby influencing the competition between different microbes. Studies by Greenhill [37] have highlighted that plant cell breakdown and the release of plant juices by plasmolysis is a prerequisite for the development of the LAB during the early stage of ensiling.

Pathogenicity and antimicrobial resistance of bacterial community

The incidence and prevalence of antimicrobial-resistant-bacterial infections have reached incongruous levels during the twenty-first century, posing significant threats to global public health. Forage is a vital livestock feed for producing green animal products but it inevitably comes into contact with various contaminants such as soil, water, and manure [7]. Consequently, ensuring the biosafety of forage is crucial for safeguarding the safety of livestock products. Recent advancements in 16S rRNA gene sequencing-based prediction tools provide a cost-effective approach to assess the potential pathogenicity and antimicrobial resistance within microbial communities. Particularly, Bugbase provides new insights into the phenotype of microbial species at the organismal level. These unique advantages have facilitated its application in microbiome datasets across diverse fields, including precision medicine, agriculture, and environmental researches [12].

Our previous study indicated that the ensiling process can effectively eliminate antibiotic-resistant and potentially pathogenic bacterial communities by suppressing harmful microbes through rapid acidification and antimicrobial metabolites (including organic acids and bacteriocins) [38]. The Tax4Fun2 and BugBase revealed that most WPQ silages exhibited lower relative abundances of pathogenic and antibiotic-resistant KEGG pathways and bacterial phenotypes associated with potential pathogenicity and antimicrobial resistance compared to their fresh counterparts. This demonstrates the feasibility of ensiling in mitigating pathogenicity and antimicrobial resistance risks in WPQ. However, caution is necessary when applying ensiling to Hongxin, as the impact of ensiling on reducing the microbial risk decreases with later harvest times. Especially, Tax4Fun2 and BugBase highlighted that Hongxin silages harvested at H3 had higher relative abundances of pathogenic and antibiotic-resistant KEGG pathways and phenotypes than their fresh counterparts. It suggests that the effects of ensiling process on bacterial pathogenicity and antimicrobial resistance could vary greatly depending on variety and harvest time. The BugBase further revealed that the increased relative abundances of bacterial phenotypes of "Potential Pathogenic" and "Contains Mobile Elements" were predominantly associated with *unclassified_f_Enterobacteriaceae* and *Erwinia*. This well explained the greater microbial risk in Hongxin silages with later harvest times, considering that enterobacterial species are often major pathogens of foodborne enteritis and zoonotic infections, and they also contain numerous mobile genetic elements (such as conjugative plasmids and integrons) contributing to the dissemination of antimicrobial resistance genes [39].

Conclusions

The findings of this study demonstrate that, the variety, harvest time and their interaction, significantly influenced the fermentation quality as well as the bacterial

community composition and hygienic quality of WPQ silage. The Hongxin and SL577 silages exhibited superior fermentation qualities when harvested early, whereas silages produced from Mengli1, SL2860, and SL923 had better fermentation characteristics at later harvest times. Compared to other varieties, Hongxin silages exhibited greater variability in bacterial community composition and potential microbial risk across different harvest times. Early harvest is recommended for Hongxin to minimize the abundances of potentially pathogenic and antimicrobial-resistant bacteria, thereby improving the safety of silage. This study highlights that the choice of variety and the timing of harvest are pivotal factors in ensuring the safety and quality of WPQ silage, which is beneficial for ensuring the safety in our food supply chain.

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Clinical trial number
Not applicable.

Authors' contributions
Z.D. and D.F. designed the experiment and wrote the manuscript. H.C., S.H., Z.J. and D.W. performed the experiment. W.W. helped in data collection. T.S. supervised the study. All authors contributed to the article and approved the submitted version.

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Data availability
Sequence data that support the findings of this study have been deposited in NCBI SRA under accession number PRJNA1172928.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare no competing interests.

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