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RESEARCH

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Ensilage using *Leuconostoc lactis* and *Weissella confusa* reduces microbial risk and enhances hygienic quality of whole-crop corn

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Abstract

This study combined applied PICRUSt2 and BugBase tools to evaluate the effects of these two strains on the fermentation characteristics, microbial community, potential microbial risk and hygienic quality of whole-crop corn (WCC) silage. Fresh WCC harvested at the dough stage was ensiled with distilled water (CON), *Leuconostoc lactis* (LS) and *Weissella confusa* (WA) for 2, 4, 8, 15 and 30 days. After ensiling, all WCC silages presented desirable fermentation with high lactic acid and *Lactobacillus* proportions, low pH and ammonia nitrogen levels and absent butyric acid. Ensiling decreased the complexity of bacterial co-occurrence networks, and the *Lc. lactis* and *W. confusa* inoculation further decreased the complexity. The inoculation of *W. confusa* suppressed the most pathogenic pathways and related modules associated with zoonosis. In bacterial phenotype predicted analysis, although CON had lower proportions of 'Potentially pathogenic' than fresh material, this undesirable phenotype declined to negligible levels via LS and WA inoculation. Even for well-fermented WCC silages, the risk of pathogens remained after 30 days of ensiling. Therefore, WA could be developed as a promising fast start-up inoculant for reducing pathogenic contamination and improving hygienic quality of silage.

Keywords Whole-crop corn, Fast start-up inoculants, Fermentation quality, Microbial community, Pathogenic risk elimination

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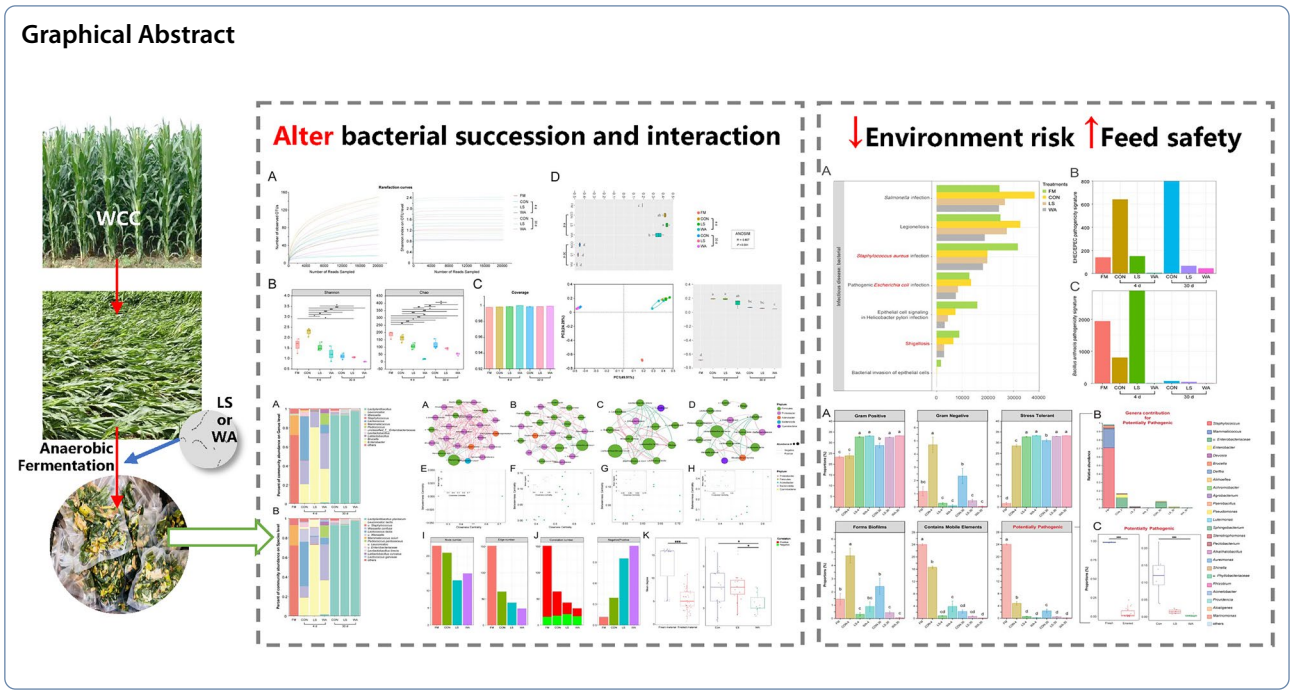

Background

Whole-crop corn (WCC) silage is a commonly utilized roughage in ruminant feeding systems, particularly for dairy cattle, owing to its high energy content and year-round availability [1, 2]. The quality and safety of WCC is the key to the production of green and healthy animal products. Our previous study found that, even if silage fermentation was good, harmful microorganisms such as *Enterobacter* appeared again in large abundance at the late stage of ensiling due to the existence of some acid-resistant strains [3, 4]. Since the proposal of silage technology, additives have played an important role in improving the quality and ensuring the safety of silage [5]. Among various additives, lactic acid bacteria (LAB) inoculants are widely promoted and used because of their safe, stable, effective, low-cost and pollution-free properties. At present, the most studied antibacterial inoculant is *Lactobacillus buchneri*, which has been reported to require 50 days or longer to function [6, 7]. It is common in practical production that dairy producers have to feed silage after a short ensiling period, and in this case, *L. buchneri* will not have enough time to produce antibacterial substances such as acetic acid and 1,2-propanediol [8]. Differently, early colonizers in the anaerobic fermentation process, such as heterofermentative *Weissella* and *Leuconostoc*, have garnered attention for their potential as fast start-up antibacterial inoculants [9].

Although *Leuconostoc lactis* and *Weissella confusa* are relatively new to silage research, they are widely known for their antimicrobial role in the food industry. *W. confusa* isolated by Tenea and Lara [10] has been found to have obvious inhibitory effects on *Escherichia coli*, *Salmonella* and *Shigella*. And the strains of *Lc. lactis* also effectively inhibited the growth of harmful microbes including *Listeria*, *Bacillus*, *Streptophyta*, *Mycospherella*, *Aureobasidium* and *Phoma* [11, 12]. Besides, their probiotic properties have been investigated, as evidenced by excellent gastrointestinal tolerance [13], exopolysaccharide-producing capacity [14], adhesion to intestinal epithelial cells [15], and inhibitory activity against intestinal pathogens [12, 16]. These characteristics of *W. confusa* and *Lc. lactis* make them promising candidates for inhibiting harmful microbes and enhancing silage quality and overall animal performance. However, to our knowledge, there are no studies on the effects of *W. confusa* and *Lc. lactis* on the quality and antimicrobial role of WCC silage.

A comprehensive understanding of the variation of silage microbiota induced by *W. confusa* or *Lc. lactis* inoculation will help the safe and hygienic production process of WCC silage. In recent years, culture-independent techniques represented by single-molecule real-time (SMRT) sequencing technology have assisted researchers in analyzing the bacterial community structure and diversity at species-level precision [17]. In addition to the bio-information mentioned above, functional and phenotypic annotation of microbial communities is increasingly found to be extremely important for microbial ecology research. Ward et al. [18] argued that the analysis of microbial communities should take

into account phenotype, especially pathogenic potential. The phenotype prediction underlying bacterial communities can be used as an effective supplement to SMRT sequencing analysis and will be highly beneficial for silage research.

Therefore, this study aims to investigate the effects of *Lc. lactis* and *W. confusa* as silage inoculants on fermentation characteristics, microbial community dynamics, potential functionality, and hygienic quality in WCC silage.

Methods

Material preparation

Fresh WCC (*Zea mays* L. Heyu 9566) was cultivated in the Baima experimental station (31°60′N, 119°17′E, mean elevation: 24.9 m, Jiangsu, China). At the dough stage (one-third milk line), fresh WCC was harvested at 8:00 am under clear weather conditions leaving a stubble of 15 cm and cut into lengths of about 30 mm by a fodder cutter. The *Lc. lactis* CICC 24759 and *W. confusa* CICC 24453 used in this study were supplied by the China Center of Industrial Culture Collection (CICC) and their inoculant level was 1×10^6 CFU/g fresh weight, respectively. After manual mixing, the chopped material was randomly divided into 3 parts and treated with (i) distilled water (CON), (ii) *Lc. lactis* CICC 24759 (LS), (iii) *W. confusa* CICC 24453 (WA). The applied volume of distilled water or inoculants was 1 mL per 100 g fresh weight. Thereafter, about 0.45 kg of the above material was loaded into a laboratory-scale silo (polythene plastic bag with a size of 30 × 40 cm). A total of 45 laboratory-scale silos (3 treatments × 5 storage lengths × 3 replicates) were prepared, vacuum-sealed and stored at the surrounding temperature (27.5 ± 2.5 °C). On day 2, 4, 8, 15 and 30 of ensilage, triplicate bags per treatment were sampled and mixed completely for the following analyses.

Chemical composition, microbial number and fermentation parameter analyses

Chemical composition and microbial number were assessed using the analytical methods outlined in our prior work [4]. For fermentation quality analysis, raw or fermented material was extracted with deionized water at a 3:1 ratio under 4 °C for 30 min. Thereafter, the pH value was determined by a pH meter after filtration with 4 layers of cheesecloth and filter paper. Ammonia nitrogen concentration was determined using colorimetry after reacting with phenol and hypochloric acid [19] in 96-well plates (H803-96, BDBIO, Hangzhou, China) under thermostatic water bath (DWB20-S, DLAB Scientific Co., Ltd., Beijing, China). Fermentation products including lactic, acetic, propionic, butyric acid, ethanol and 1,2-propanediol of fermented material were determined by an Infinity 1260 HPLC (Agilent Technologies Inc., California, USA) equipped with a Carbomix® H-NP5 column (Sepax Technologies, Inc., Delaware, USA) under the following condition: mobile phase of 2.5 mM H_2SO_4 at a flow rate of 0.5 mL/min and a column run temperature of 55 °C. HPLC internal standard L-lactic acid was purchased from Solarbio Science & Technology Co., LTD. (Beijing, China) and others from Sigma-Aldrich (Shanghai, China).

SMRT sequencing and data analyses

After homogenizing with autoclaved NaCl solution (0.85%) at a ratio of 1:9 and shaken at 37 °C and 120 rpm for 2 min. The above solution was 4 °C centrifuged at 10,000*g* for 15 min to collect bacterial precipitates. Then, bacterial DNA was extracted from bacterial precipitates using the Rapid Bacterial Genomic DNA Isolation Kits (B518225, Sangon Biotech Co., Ltd., Shanghai, China) and tested by 1% agarose gel electrophoresis. The DNA concentration and integrity were checked utilizing the NanoDrop® 2000 ultraviolet–visible spectrophotometer (260/280 nm, Thermo Scientific, Delaware, USA). Based on primers 27F and 1492R [2], the near full length of the bacterial 16S rRNA gene was amplified utilizing the ABI GeneAmp® 9700 PCR amplification instrument (Applied Biosystems, California, USA) with the running program of 95 °C for 3 min, 27 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, with a final extension of 72 °C for 10 min. These amplification products were firstly recovered utilizing 2% agarose gel, then purified by AxyPrep® DNA Gel Extraction Kit (Axygen Biotechnology Co., Ltd., California, USA) and lastly checked by 2% agarose gel electrophoresis. Based on the initial qualitative result of electrophoresis, the purified PCR products were assessed quantitatively using the Quantus™ Fluorometer (Promega Biotechnology Co., Ltd., Wisconsin, USA), in accordance with the sequencing specifications. After library construction using SMRTbell® Express Template Prep Kit 2.0, the purified amplicon was subjected to the Pacbio Sequel II System (Pacific Biosciences of California, Inc, California, USA) for SMRT sequencing.

After receiving the PacBio offline data of subreads, the circular consensus sequencing (CCS) sequence was obtained by SMRTLink 11 software. Subsequently, according to the barcode, the data of each sample were distinguished and the length was filtered. UPARSE (ver. 7.0.0) was used to generate operational taxonomic units (OTUs) through the cluster of the sequences at a 97% identity threshold, and UCHIME (ver. 4.1) was used to identify and remove chimeric sequences. The taxonomic classification of the OTUs was determined by matching against the SILVA database (ver. 138) under the comparison threshold of 70% using the Ribosomal

Database Project (RDP) classifier (ver. 2.11). Before bioinformatic analyses, the sequencing data were normalized to the same number of reads per library to avoid sampling bias effects. In brief, sequencing flattening was conducted according to the minimum number of sample sequences and the number of valid sequences for each sample used for analysis is 20,176. QIIME (ver. 1.9.1) was used to calculate the bacterial α-diversities (Shannon, Chao, Ace, Sobs, Simpson and Coverage indices) and β-diversities presented by the Bray–Curtis metric. Bacterial β-diversities were visualized via principal coordinate analysis (PCoA) plots, which were constructed using the vegan package (ver. 2.6–2) of R software (ver. 4.1.2).

Bacterial co-occurrence network analysis

The Networkx toolkit (ver. 2.6.3, https://networkx.org/) was utilized to calculate multiple abundance correlations and establish the co-occurrence pattern among bacterial taxa based on a matrix at the species level [20]. Only species with a relative abundance of 0.05% were considered. A co-occurrence was considered robust if the coefficient of Spearman's correlation (ρ) was greater than 0.5 and the *P*-value was less than 0.05. Although a wide range of methods and algorithms are available to construct microbial networks, starting with basic Spearman rank correlation-based approaches, it should be noted that the choice of a correlation metric that is insensitive to compositional effects (Spearman) may lead to the identification of spurious correlations [21]. This is a limitation of most current works and need to be addressed in the future. The Gephi interactive platform (ver. 0.9.2) was used to visualize the co-occurrence networks, with the node representing the bacterial species and the edge representing the correlation between nodes [22]. The topological features of co-occurrence networks, including node and edge numbers, negative and positive correlation numbers, etc., were calculated. The identification of keystone taxa was based on a composite score of low betweenness centrality, high closeness centrality and high mean degree [23].

Bacterial functionality and phenotype prediction analyses

The PICRUSt2 tool developed by Douglas et al. [24] and the BugBase tool (https://bugbase.cs.umn.edu/index.html) [18] were utilized to predict KEGG functional profiles and phenotypes of bacterial communities, respectively. In PICRUSt2, the characteristic sequence (16S rRNA) was aligned with the reference sequence of the Integrated Microbial Genomes (IMG) to construct a phylogenetic tree, find the 'nearest species' of the characteristic sequence, and predict the gene information of unknown species according to the gene type and abundance information of known species, so as to predict the pathway of the whole community in combination with the KEGG pathway information of the gene. Compared with PICRUSt, PICRUSt2 can direct predict functionalities for any 16S sequences from OTUs or ASV by taking a sequence placement approach. Default database of reference genomes used for prediction is based on 41,926 bacterial and archaeal genomes from the IMG database, which has been expanded more than 10 times. PICRUSt2 predictions based on several gene family databases including KEGG database, and inference of pathway abundances now relies on MinPath (ver. 1.4), which makes these predictions more stringent. BugBase first normalized the OTU by the predicted 16S rRNA copy number, and then predicted the microbial phenotype using the pre-calculated files provided. BugBase algorithm relies on databases such as IMG, KEGG, and the Pathosystems Resource Integration Center (PATRIC) to predict phenotypes and corresponding microbial contributors at the genus level. The predicted phenotypes include seven categories: Gram positive, Gram negative, biofilm forming, potentially pathogenic, mobile element containing, oxygen utilizing (including aerobic, anaerobic, and facultatively anaerobic), and stress tolerant.

Statistical analysis

The GLM in SAS (ver. 9.2; SAS Institute Inc., North Carolina, USA) was applied to evaluate the effect of cutting height, storage length and their interactions on chemical compositions, fermentative parameters and microbial numbers of fermented WCC. Meanwhile, the Student's *t*-test, one-way ANOVA, ANOSIM with 999, Student's *t*-test and one-way ANOVA permutations and Wilcoxon rank-sum test were employed to determine whether differences occurred between cutting heights in the characteristics of raw material, α-diversity and β-diversity indices, KEGG functional profiles as well as phenotypes of bacterial communities, respectively. $P < 0.05$ was deemed significant.

Results

Chemical and microbial compositions of fresh WCC

The pre-ensiled properties of WCC are shown in Table 1. Fresh WCC had a high water soluble carbohydrate content of 257 g/kg dry matter, moderate dry matter content of 298 g/kg FM and relatively low crude protein content of 75.9 g/kg dry matter. The count of epiphytic LAB was 6.34 $\log_{10}$ CFU/g fresh weight, which was more than 5.0 $\log_{10}$ CFU/g fresh weight, and the counts of aerobic bacteria, yeasts, molds and enterobacteria were 8.00, 4.10, 2.96 and 5.14, respectively.

Chemical and fermentation characteristics of WCC silage

Inoculant had significant ($P < 0.001$) impacts on the pH value, the water soluble carbohydrate content, the

Table 1 The chemical composition and microbial numbers of fresh WCC

Items	WCC	SD
pH	5.61	0.033
Dry matter (g/kg fresh weight)	298	5.521
Water-soluble carbohydrates (g/kg DM)	257	8.078
Neutral detergent fibre (g/kg DM)	513	4.158
Acid detergent fibre (g/kg DM)	260	3.279
Acid detergent lignin (g/kg DM)	115	2.026
Cellulose (g/kg DM)	145	1.882
Hemicellulose (g/kg DM)	253	2.253
Crude protein (g/kg DM)	75.9	2.220
Buffering capacity (mEq/kg DM)	188	6.773
Lactic acid bacteria (Log_{10} CFU/g fresh weight)	6.34	0.065
Aerobic bacteria (Log_{10} CFU/g fresh weight)	8.00	0.056
Yeasts (Log_{10} CFU/g fresh weight)	4.10	0.050
Moulds (Log_{10} CFU/g fresh weight)	2.96	0.083
Enterobacteria (Log_{10} CFU/g fresh weight)	5.14	0.152

FW, fresh weight; DM, dry matter; CFU, colony-forming unit; SEM, standard error of means. WCC, whole-crop corn

concentrations of acetic acid, propionic acid, ethanol and ammonia nitrogen, and the ratio of lactic acid to acetic acid (LA/AA), and fermentation day had significant ($P<0.01$) impacts on the pH value, the content of dry matter and water-soluble carbohydrate, the concentrations of lactic acid, acetic acid, propionic acid, ethanol and ammonia nitrogen as well as the LA/AA of WCC silage (Table 2). The pH value of all treatments decreased sharply within the first 2 days of ensiling and remained at a low level after 4 days of ensiling. The lowest pH value was observed in WA, followed by LS and finally CON. Along with ensiling, the content of dry matter and water-soluble carbohydrate ceaselessly ($P<0.01$) decreased, while the concentrations of lactic acid, acetic acid, propionic acid, ethanol and ammonia nitrogen continuously ($P<0.001$) increased. Although there were no significant ($P>0.05$) differences for dry matter content and lactic acid concentration among treatments, LS and WA showed higher ($P<0.001$) acetic acid concentration and water-soluble carbohydrate content but lower ($P<0.001$) concentrations of ethanol and ammonia nitrogen than CON. Correspondingly, a higher ($P<0.001$) LA/AA value was observed in CON than that in LS and WA silages. Except for 1,2-propanediol detected in LS on day 60, no butyric acid or 1,2-propanediol was found in all WCC silages.

Bacterial community of fresh WCC and WCC silage

The rarefaction curves based on observed OTUs and Shannon index reached near saturation as the number of reads sampled increased (Fig. 1A). The bacterial α-diversity parameters represented by Shannon and Chao indices are shown in Fig. 1B. Compared with FM, except for CON, ensiling process decreased Shannon and Chao indices, and LS and WA inoculation further decreased ($P<0.01$) Chao index (Fig. 1B). Each sequenced sample had a Coverage index exceeding 0.98 (Fig. 1C). A significant difference (ANOSIM, $P=0.001$) in bacterial β-diversity is shown by the PCoA plot in Fig. 1D. The sample points of 0-day, 4-day and 30-day fermented WCC were clearly distributed in different quadrants.

The bacterial community abundance was presented at the genus and species levels (Fig. 2A, B). *Staphylococcus* and *Mammaliicoccus*, unclassified (*u.*) *Staphylococcus* and *Mammaliicoccus sciuri* dominated the bacterial community of raw material at the genus and species level, respectively. After ensiling, the above bacteria were occupied by first cocci LABs and then rod LABs. Concretely, after 4 days of ensiling, *Lactococcus lactis* significantly ($P<0.001$) increased in CON, *Lc. lactis* significantly ($P<0.01$) increased in LS, and *W. confusa* significantly ($P<0.05$) increased in WA (Fig. 2C). After 30 days of ensiling, *Lactiplantibacillus plantarum* emerged as the prevailing species in all bacterial communities, with relative abundance accounting for 93.1%, 92.9% and 97.2%, respectively. Among them, WA treatment had a significantly ($P<0.001$) higher *Lactiplantibacillus plantarum* than CON and LS treatments.

Co-occurrence networks of bacterial community

To investigate the impacts of the ensiling process and inoculants on the relationships between bacterial species, separate 4 bacterial co-occurrence networks were created for fresh and ensiled WCC using Spearman's rank correlation (Fig. 3A–D). *u. Staphylococcus*, *Devosia aurantiaca*, *Sphingobacterium populi*, etc. in FM, *Lactococcus garvieae* and *Weissella oryzae* in CON, *Lactococcus lactis*, *u. Leuconostoc* and *Levilactobacillus brevis* in LS and *Lactococcus lactis*, *Lactococcus garvieae* and *W. confusa* in WA were identified as keystone taxa in the respective co-occurrence networks (Fig. 3E–H). The highest node number was found in the bacterial network of FM, followed by CON, WA, and LS, while the highest edge number was observed in FM, followed by CON, LS, and WA (Fig. 3I). These results suggested that the overall ranking of bacterial networks in terms of nodes and edge number were ranked as follows: FM > CON > LS ≈ WA. The number of positive correlated edge was, respectively, 135, 46, 22, and 15 in FM, CON, LS, and WA and the number of negative correlated edge was, respectively, 16, 18, 21, and 17 in FM, CON, LS, and WA, which implying that except for WA, the correlation edges of all bacterial co-occurrence networks were primarily positive, with negative/

Table 2 Effects of inoculant on fermentation characteristic of WCC silage during ensiling

Items	Treatments	Fermentation days					SEM	*P*-value		
		2	4	8	15	30		T	D	T × D
pH	CON	4.27	3.76	3.71	3.67	3.70	0.026	< 0.001	< 0.001	< 0.001
	LS	3.78	3.75	3.70	3.57	3.56				
	WA	3.68	3.62	3.59	3.61	3.52				
Dry matter (g/kg fresh weight)	CON	274	266	252	236	234	2.408	0.453	0.001	0.262
	LS	255	258	255	239	230				
	WA	257	242	246	250	240				
Lactic acid (g/kg dry matter)	CON	56.5	70.8	94.7	130	143	4.265	0.427	< 0.001	0.217
	LS	81.5	83.4	90.3	98.5	119				
	WA	73.9	91.3	110	124	120				
Acetic acid (g/kg dry matter)	CON	7.03	5.62	7.20	9.59	13.0	1.879	< 0.001	< 0.001	< 0.001
	LS	22.1	28.9	29.7	39.9	50.8				
	WA	13.1	15.0	16.5	18.1	16.9				
Lactic acid/acetic acid	CON	8.07	12.7	13.2	13.5	11.2	0.241	< 0.001	< 0.001	< 0.001
	LS	3.69	2.89	3.02	2.47	2.32				
	WA	5.63	6.15	6.70	6.84	7.05				
Propionic acid (g/kg dry matter)	CON	0.27	0.23	0.29	0.56	4.06	0.241	< 0.001	< 0.001	< 0.001
	LS	0.82	0.68	1.63	3.44	5.85				
	WA	1.08	1.16	1.27	1.16	1.60				
Butyric acid (g/kg dry matter)	CON	ND	ND	ND	ND	ND	–	–	–	–
	LS	ND	ND	ND	ND	ND				
	WA	ND	ND	ND	ND	ND				
1,2-Propanediol (g/kg dry matter)	CON	ND	ND	ND	ND	ND	–	–	–	–
	LS	ND	ND	ND	ND	2.08				
	WA	ND	ND	ND	ND	ND				
Ethanol (g/kg dry matter)	CON	1.93	3.07	8.68	17.5	17.4	0.747	< 0.001	< 0.001	< 0.001
	LS	3.27	2.49	3.29	3.50	4.02				
	WA	2.70	3.45	7.47	8.48	8.49				
Water-soluble carbohydrates (g/kg dry matter)	CON	179	168	157	131	101	3.093	< 0.001	< 0.001	< 0.001
	LS	176	168	158	147	125				
	WA	167	163	152	143	141				
Ammonia nitrogen (g/kg total nitrogen)	CON	41.6	52.6	57.9	70.9	81.4	2.325	< 0.001	< 0.001	0.702
	LS	30.6	42.8	47.0	55.3	64.0				
	WA	23.2	31.8	37.1	45.7	55.5				

SEM, standard error of means; FM, fresh; WCC; CON, control (WCC treated with distilled water); LS, WCC inoculated with *Lc. lactis*; WA, WCC inoculated with *W. confuse*; T, the effect of treatment; D, the effect of fermentation days; T × D, the interaction between treatment and fermentation day

positive ratio of 0.12, 0.39, 0.95, and 1.13 in FM, CON, LS, and WA, respectively (Fig. 3J). Ensiling process and WA inoculation had notable ($P < 0.05$) impacts on the average degree of the bacterial co-occurrence networks (Fig. 3K).

KEGG pathogenic analysis of bacterial community

For KEGG pathway analysis, the KEGG pathogenicity of fresh and ensiled WCC focused on "Infectious disease: bacterial" at pathway level 2, and specifically "*Staphylococcus aureus* infection", "Pathogenic *Escherichia coli* infection" and "Shigellosis" at pathway level 3 (Fig. 4A). The FM had the maximum abundance of "*Staphylococcus aureus* infection" and "Shigellosis", followed by CON and LS and finally WA treatment. The "Pathogenic *Escherichia coli* infection" exhibited a slightly different trend, with the maximum abundance in CON and the minimum relative abundance in WA treatment.

For KEGG module analysis, two pathogenic modules (M00542 and M00860) were closely associated with zoonosis. After ensiling, CON treatment enhanced the abundance of "EHEC/EPEC pathogenicity signature (M00542)" but WA treatment declined its abundance (Fig. 4B). As for "*Bacillus anthracis* pathogenicity

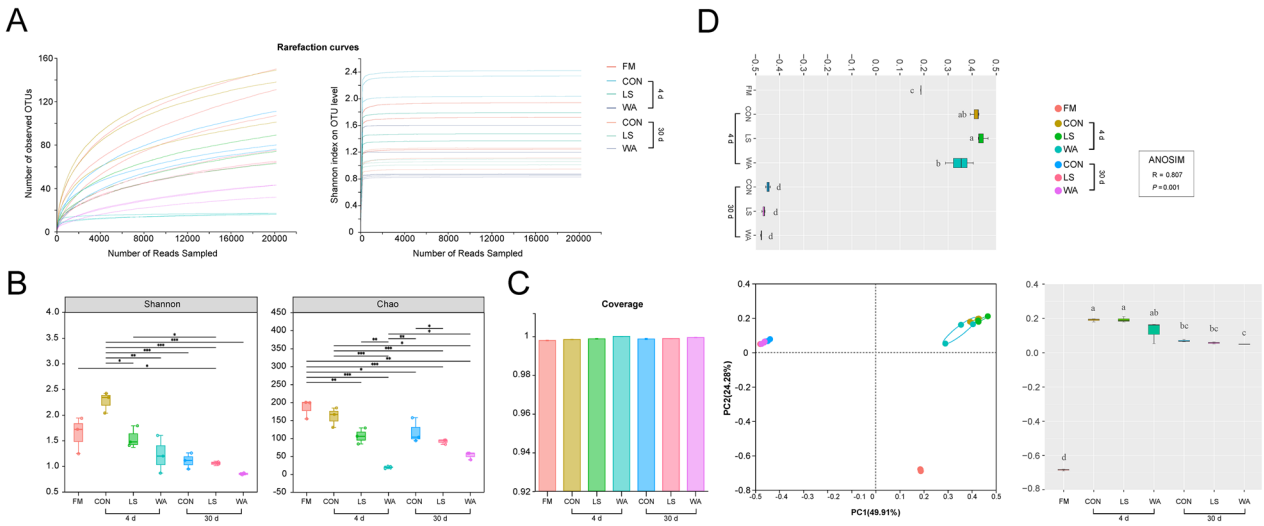

Fig. 1 Bacterial community diversities of fresh WCC and WCC silage. **A** Rarefaction curves based on observed the OTUs number and Shannon index. **B** Alpha diversities (Shannon, Chao) of the bacterial community. **C** Alpha diversities (Coverage indices) of the bacterial community. **D** Beta diversities of the bacterial community, calculated by principal coordinate analysis (PCoA) based on the Bray–Curtis distance metric. FM, fresh WCC; CON, control (WCC treated with distilled water); LS, WCC inoculated with *Lc. lactis*, WA, WCC inoculated with *W. confusa*

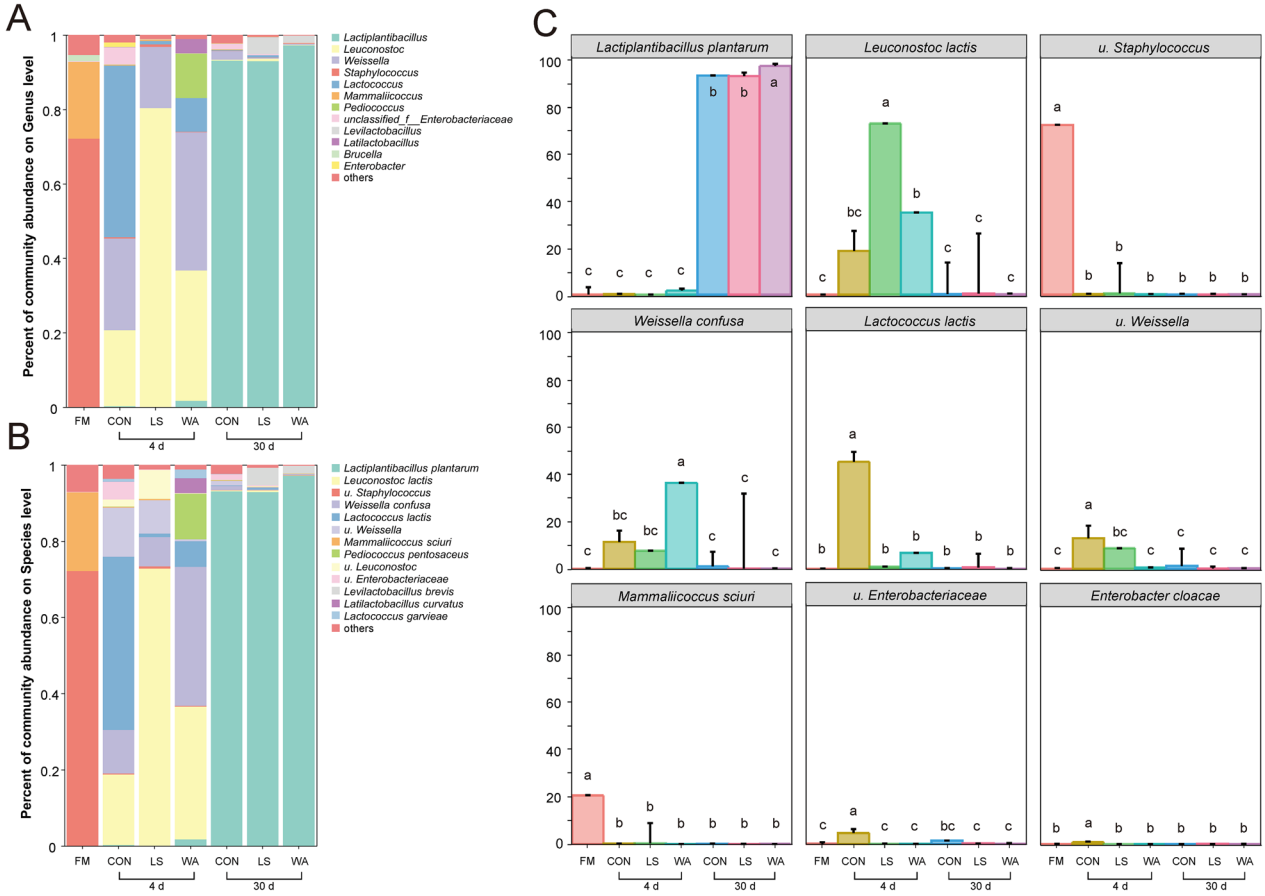

Fig. 2 Bacterial community abundances of fresh WCC and WCC silage. **A** The bar plot of bacterial community abundance from the genus level. **B** The bar plot of bacterial community abundance from the species level. **C** Relative abundance of representative species. FM, fresh WCC; CON, control (WCC treated with distilled water); LS, WCC inoculated with *Lc. lactis*, WA, WCC inoculated with *W. confusa*

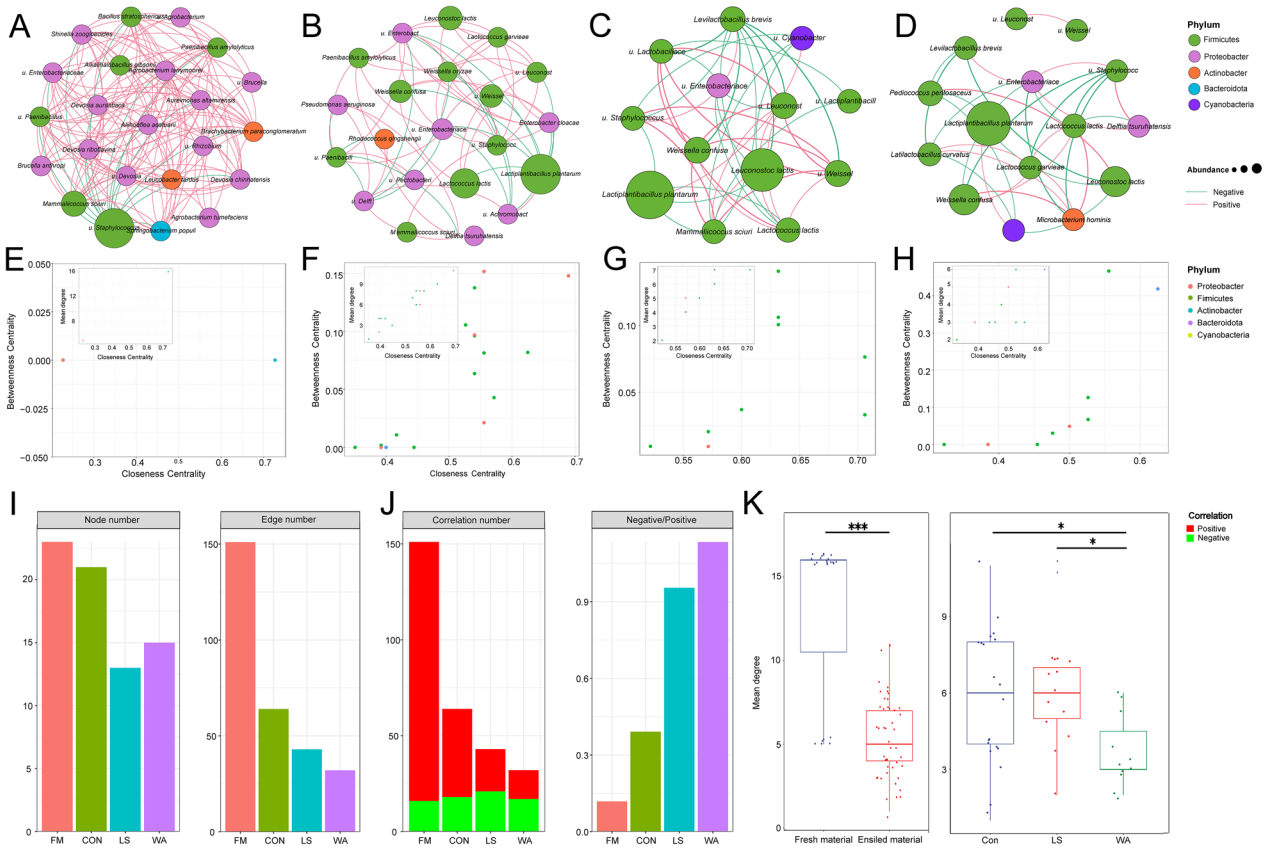

Fig. 3 Bacterial co-occurrence networks of fresh WCC and WCC silage. Bacterial co-occurrence networks of FM (**A**), CON (**B**), LS (**C**), and WA (**D**). Node represents bacterial species, node color represents bacterial phylum, and node size represents relative abundance. Edges are colored according to negative (green) and positive (red) correlations. **E**–**H** Elliptic scatter plots of node-level topological features (closeness centrality and mean degree or closeness centrality and betweenness centrality) in four co-occurrence networks. **I** Bar plots of nude and edge numbers, respectively. **J** Bar plots of correlation number and negative/positive ratio. **K** Overall comparison of the mean degree before and after ensiling or among three treatments. The line within the boxplot represents medians, the tops and bottoms of the boxplot represent 25th and 75th percentiles, and lines extend to 1.5 × inter-quartile ranges. The statistical analysis was conducted using a two-sided *t*-test. *P*-value is indicated by ***$P < 0.001$, and by *$P < 0.05$. FM, fresh WCC; CON, control (WCC treated with distilled water); LS, WCC inoculated with *Lc. lactis*, WA, WCC inoculated with *W. confusa*

signature (M00860)", although the abundance was still high in CON and LS treatments after 4 days of ensiling, the abundance in all treatments, especially LS and WA, dropped to negligible levels after 30 days of ensiling (Fig. 4C).

BugBase phenotypic analysis of bacterial community

Phenotypic prediction results (Fig. 5A) suggested that the ensiling process significantly ($P < 0.05$) reduced the abundance of bacterial phenotypes in terms of "Contains Mobile Elements" and "Potentially Pathogenic". Among all treatments, LS and WA inoculation further ($P < 0.05$) reduced the abundance of the above phenotypes with the lowest level in WA on day 30. Genera contribution for "Potentially Pathogenic" was visualized in a bar plot (Fig. 5B) and showed that *Staphylococcus* and *Mammaliicoccus*, *u. Enterobacteriaceae* and *Enterobacter* might be responsible for the pathogenicity risk of fresh material and CON silage, respectively. Overall, a lower ($P < 0.001$) proportion of "Potentially Pathogenic" was observed in ensiled materials compared to fresh materials, and also in LS and WA treatments compared to CON treatment (Fig. 5C).

Discussion

The pH values of CON, LS and WA after 4 days of ensiling were always < 4.20, indicating that WCC silages could be effectively preserved even without additives [5]. Such a high fermentation rate could be respectively related to the sufficient LAB number, abundant water-soluble carbohydrate content and low BC of raw material, which makes WCC the most popular feedstock for silage making [25]. Even so, LS and WA could still effectively accelerate lactic acid production during the early

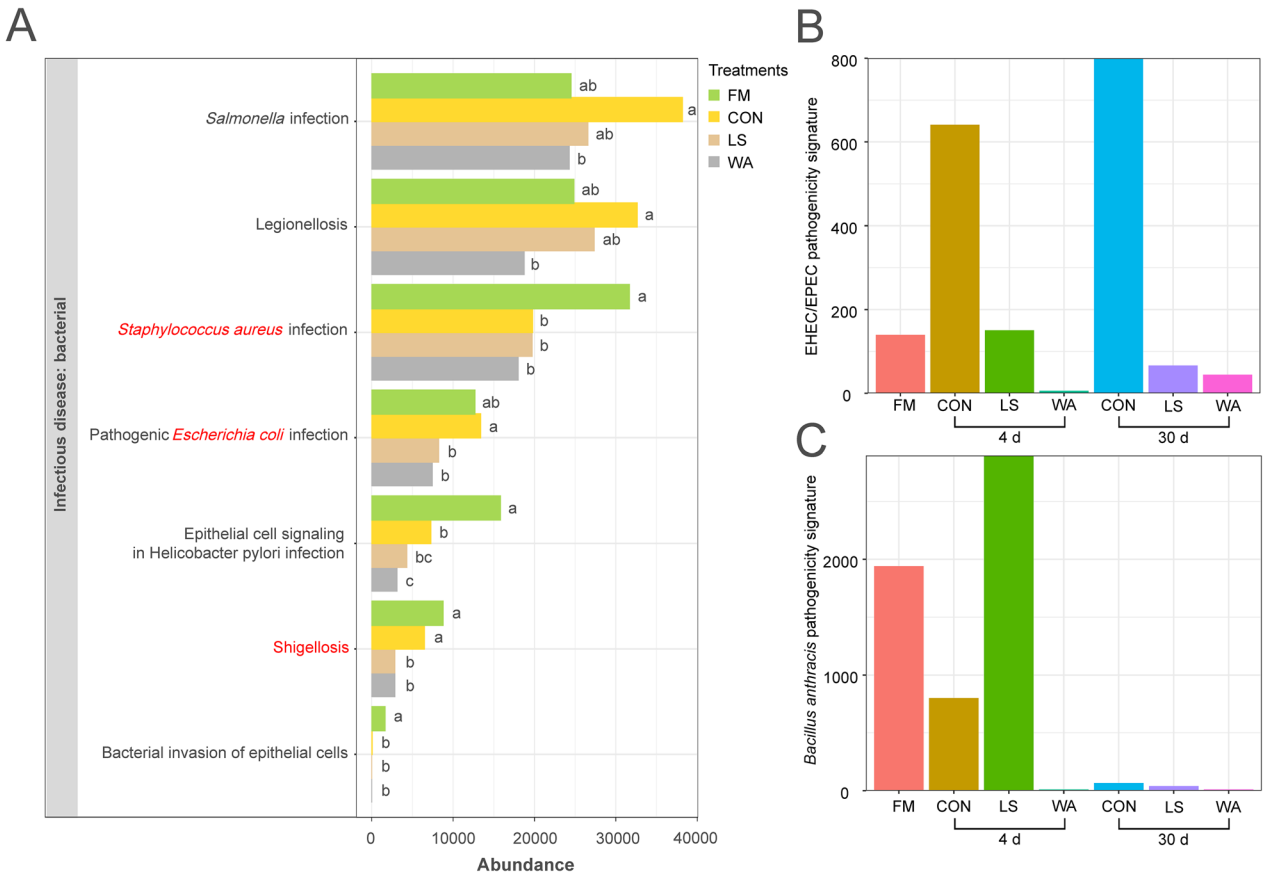

Fig. 4 Bacterial potential pathogenic pathways (**A**) and modules (**B** and **C**) of fresh WCC and WCC silage. EHEC/EPEC: enterohemorrhagic *E. coli*/enteropathogenic *E. coli*. FM, fresh WCC; CON, control (WCC treated with distilled water); LS, WCC inoculated with *Lc. lactis*, WA, WCC inoculated with *W. confusa*

phase of ensiling to further reduce pH value, showing the great potential to function as fast start-up inoculants in silage. Similar to most studies of silage fermentation, with the prolongation of ensiling, acetic acid concentration continuously increased. The relatively high acetic acid concentration after inoculation of LS and WA is to be expected because these two strains are heterofermentative LABs, which also explains the lower LA/AA in LS and WA than in CON silages. Different from LS, WA had relatively high LA/AA (> 3.0) during the whole ensiling, indicating that its fermentation pattern was the homofermentative type [6]. This may be because the addition of WA promoted the growth of homofermentative LABs such as *L. plantarum*, which could also be supported by sequencing data. According to Cai et al. [26], lactic acid-producing cocci initiate lactic acid fermentation and establish a weak-acid environment suitable for lactobacilli. Similar growth-promoting effects on *L. plantarum* were also found in *L. buchneri* [27], *Bacillus amyloliquefaciens* and *B. subtilis* [2]. The presence of butyric acid in silage fermentation is undesirable. The absence of butyric acid in all WCC silages suggested no clostridia, as these microorganisms are mainly responsible for producing butyric acid during ensiling. Similar to butyric acid, high concentrations of ethanol are also commonly associated with undesirable fermentation [5]. The higher ethanol concentrations in CON than that in LS and WA at the end of ensiling could be associated with the non-negligible yeast and enterobacteria number in CON silage. Certain heterofermentative LABs such as *Lentilactobacillus buchneri* are known for the production of 1,2-propanediol by metabolizing lactic acid. So, the presence of 1,2-propanediol in LS at the end of ensiling could be associated with the dominant heterofermentation.

The sufficient water-soluble carbohydrate content in raw material might contribute to the high residual water soluble carbohydrate content (> 100 g/kg dry matter) at the end of ensiling. And the higher residual water soluble carbohydrate content in LS and WA could be attributed to their rapid pH decline and high acetic acid production inhibiting the sugar consumption from undesirable microbes. As an indicator of protein degradation and

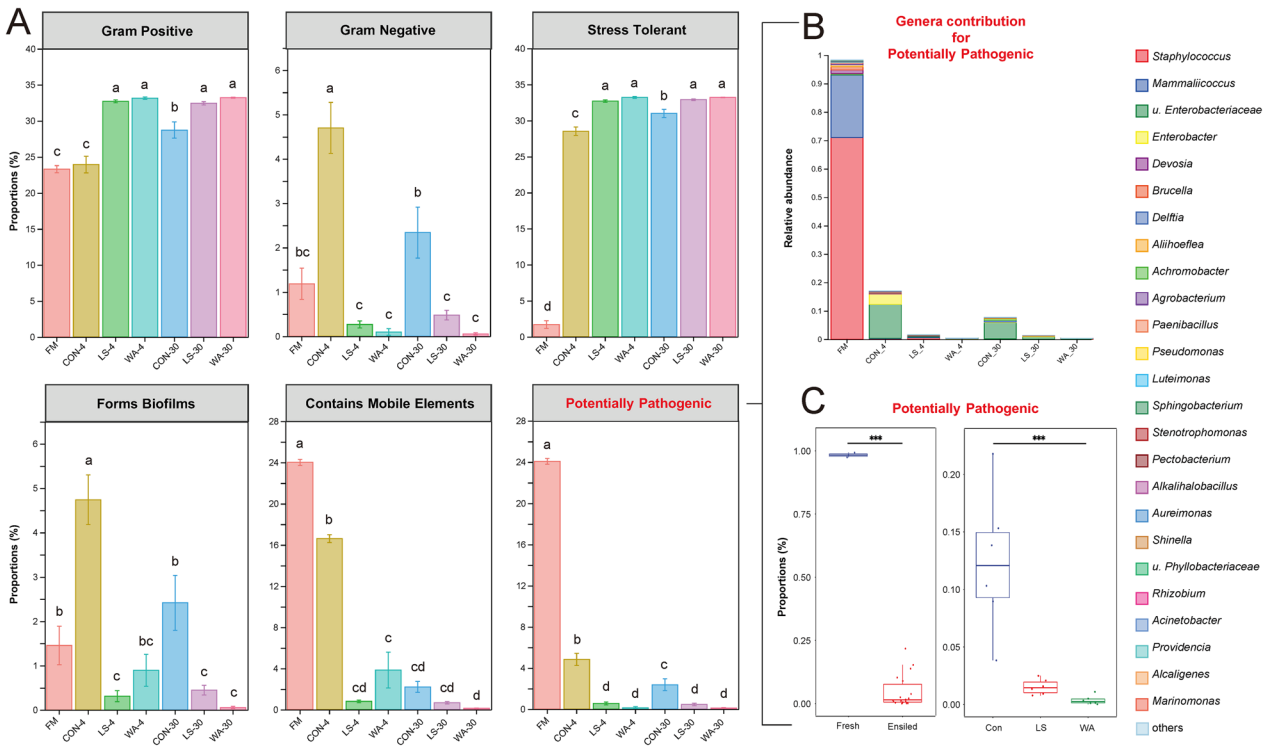

Fig. 5 Bacterial phenotypes annotation of fresh WCC and WCC silage. **A** Phenotypes that reflect bacterial characteristics including "Gram Negative", "Gram Positive" and "Contains Mobile Elements" and bacterial resistance including "Forms Biofilms", "Stress Tolerant" and "Potentially Pathogenic". **B** Bar plot of pathogenic contribution analysis. **C** Overall comparison of the potentially pathogenic before and after ensiling or among three treatments. The statistical analysis was conducted using a two-sided *t*-test. *P*-value is indicated by ***, which represents significant at $P < 0.001$. FM, fresh WCC; CON, control (WCC treated with distilled water); LS, WCC inoculated with *Lc. lactis*, WA, WCC inoculated with *W. confusa*

amino acid deamination, the ammonia nitrogen parameter has been widely used to assess the fermentation quality of silage. Ammonia nitrogen concentrations less than 10% (100 g/kg) of total nitrogen indicate good fermentation [28]. The ammonia nitrogen concentrations of all WCC silages were always below the prescribed threshold, showing no extensive proteolysis and amino acid loss. Protein degradation, amino acid decarboxylation and ammonia formation during anaerobic fermentation are attributed to various factors, including plant protease, clostridia, and enterobacteria activities [29, 30]. Given that no clostridia (reflected by butyric acid concentration and microbial community analysis) was detected in all WCC silages, the lower ammonia nitrogen concentration in LS and WA than that in CON was partly due to the relatively low enterobacteria number in LS and WA. Moreover, the rapid acidification in LS and WA to inhibit ammonia nitrogen production caused by plant proteases is also considered.

The rarefaction curves reached near saturation, indicating the sequencing data were sufficient and reasonable. The high Coverage index (> 99%) implied that the majority of the bacterial community in each sample was successfully captured [31]. The LS and WA inoculation reduced the bacterial α-diversity (diversity and richness) in WCC silage, as evidenced by the lower Shannon and Chao indices in LS and WA than in FM and CON, which could be possibly due to their previously validated antibacterial effects [10–12]. Meanwhile, extensive studies confirmed that pH decline in microenvironments can reduce bacterial diversity. Similar results were also observed in this study, that is, lower pH was associated with lower bacterial α-diversity.

The dissimilarities in bacterial community composition were visualized through distances between symbols in the PCoA plot. The separation among fresh material, 4-day silages, and 30-day silages suggested the obviously different bacterial community compositions of WCC at the various phases of ensiling (pre-ensiling, early and late). These differences could also be ascribed to the inactivation of acid-sensitive bacteria and the succession of acid-tolerant bacteria while fermenting, as mentioned previously. And the cluster among 4-day silages or 30-day silages showed that no bacterial composition differences were found among different treatments on the same ensiling day.

In this study, the opportunistic pathogens e.g. *u. Staphylococcus* and *Mammaliicoccus sciuri* were highly distributed in fresh WCC material, and even *u. Brucella* and *Brucella anthropi* were also detected. These results can be interpreted by the inevitable contamination of microorganisms and pathogens from manure, organic fertilizer, irrigation water, air, or soil during plant growth [32]. Similar to most silage studies, the distinct succession from Proteobacteria to Firmicutes before and after fermentation can largely attributed to the acidic microenvironment which suppressed acid-sensitive bacteria (*u. Staphylococcus*, *Mammaliicoccus sciuri*, *u. Brucella*, *Brucella*, etc.) and promoted LABs. Also, the inoculation of LS and WA respectively increased the proportions of *Lc. lactis* and *W. confusa* in the resulting 4-day WCC silages, which was expected. Cocci LABs such as *Weissella* and *Leuconostoc* are regarded as the early colonizers in silage fermentation because of their ability to adapt to weak acid environments and rapid acid production [33], While, with the progress of ensiling and further decline of pH in silos, cocci LABs are less tolerant to acid than *Lactobacillus*. Thus, the moderately acidic environment created by cocci LABs benefits the growth of rod LABs. Correspondingly, at the end of ensiling, the bacterial community of all WCC silages was ultimately occupied by *Lactobacillus*.

Bacterial co-occurrence networks of fresh WCC or WCC silages were built to comprehensively understand the impacts of LS and WA inoculation on the correlation and interaction of the resulting microbiota. According to node and edge number, the bacterial community complexity of WCC suffered a great reduction after ensiling. The inoculation of LS or WA further reduced the bacterial community complexity resulting in the simplest bacterial correlation structures in WA. These results indicate that (1) ensilage tends to reduce bacterial network complexity; and (2) the antimicrobial properties of LS and WA explain the lower bacterial network complexity.

Banerjee et al. [34] found that the negative correlation in microbial co-occurrence networks suggests a potential competition for resources and shared predators, whereas the positive correlation suggests mutualistic or collaborative associations among microbial taxa. In the current study, the highest positive correlation proportion was observed in the bacterial network of FM. The primarily cooperative nature of the microbiota in fresh WCC implies that the survival of epiphytic bacteria and the stable coexistence of the bacterial community on the plant surface is linked to the collaboration among bacterial cells. It is worth noting that the higher negative correlation proportion in the bacterial network of LS and WA than that in the bacterial network of CON reflects the increased competitive interactions among bacterial species after inoculation. This seems to confirm the competition conjecture between exogenous LABs and herbage epiphytic bacteria proposed by previous studies [35, 36].

According to the method of Berry and Widder [23], *u. Staphylococcus*, *Devosia aurantiaca* and *Sphingobacterium populi* in FM, *Lactococcus garvieae* and *Weissella oryzae* in CON, *Lactococcus lactis*, *u. Leuconostoc* and *Levilactobacillus brevis* in LS, and *Lactococcus lactis*, *Lactococcus garvieae* and *W. confusa* in WA were identified as the keystone taxa, correspondingly. The keystone taxa *Sphingobacterium populi* identified in fresh WCC belongs to Sphingobacteriales, which was consistent with the previous study that Sphingobacteriales was regarded as the keystone taxa in grasslands, forest, or woodlands [37]. Meanwhile, Banerjee et al. [37] found that although keystone taxa have considerable effects on bacterial communities and functions, their abundance is not proportional to their effects. Similarly, the keystone taxa in this work were not necessarily the ones with high relative abundance.

In recent years, emerging tools such as PICRUSt and Tax4Fun have been proposed to assist sequencing analysis for predicting the microbial KEGG functionalities, not only in terms of metabolism but also pathogenicity [24]. Herein, the new PICRUSt2 tool was adopted to reveal the impact of ensilage operation and further LS and WA inoculation on the potential bacterial pathogenicity of WCC silage. Inappropriately managed silage is a source of pathogenic bacteria in ruminants, and feed-borne *Staphylococcus aureus*, *Escherichia coli*, *Shigella* and *Bacillus anthracis* are responsible for mastitis, hemorrhagic colitis, shigellosis and anthrax in dairy cows, respectively [38, 39]. Thus, three pathogenic pathways of *Staphylococcus aureus* infection, pathogenic *E. coli* infection and shigellosis as well as two pathogenic modules of enterohemorrhagic *E. coli*/enteropathogenic *E. coli* (EHEC/EPEC) pathogenicity signature and *Bacillus anthracis* pathogenicity signature are the focus of this study. The spontaneous ensilage process reduced the risk of *Staphylococcus aureus* infection and shigellosis but not pathogenic *E. coli* infection, which might be ascribed to the presence of some acid-resistant strains of *E. coli*. Although most *E. coli* is considered not to be acid-resistant, the presence of some acid-resistant strains in silage, human digestive tract and other extremely acidic environments has still been reported [40–43]. Previous studies have shown that the *E. coli* O157:H7 strain may produce acid resistance by inducing acid-tolerant response, so it can survive in an environment with a pH as low as 3.4 [44, 45]. LS especially WA inoculation further reduced the potential risk of *Staphylococcus aureus* infection, pathogenic *E. coli* infection and shigellosis, which could be attributed to

their aforementioned antibacterial effects. The production of antimicrobial metabolites such as acetic acid may account for their antibacterial effects. While, it is interesting to note that WA has lower acetic acid concentration but higher antibacterial performance than LS. We speculated that the secretion of bioactive compounds such as bacteriocin and extracellular polysaccharides could be the main reason for the antibacterial function of WA [46–49], so further research is needed thereafter. The pathogenic modules showed similar characteristics to the pathogenic pathways, that is, WA showed the best performance in reducing the pathogenicity signature of EHEC/EPEC and *Bacillus anthracis*.

The microbiota of fresh herbage or silage is increasingly being linked to acute and chronic diseases in animals [38, 50]. The foodborne pathogen influences various aspects of animal health, including immune response, metabolism, disease occurrence, and finally body status and behavior [51, 52]. Therefore, it's important to clarify the microbial phenotypes in the microbiome, especially their pathogenic potential [18]. For instance, identifying the ratio of Gram stain can help develop more targeted antibiotic treatment approaches [53]. Furthermore, determining the anaerobic bacteria and pathogen levels in feedstuff can, at sources, prevent potential pathogens from spreading on farms or from animal products to humans. Nevertheless, numerous current tools, including popular PICRUSt, are unable to deduce prevalent microbial phenotypes from sequencing data, such as 'gram negative', 'gram positive', 'forms biofilms', 'contains mobile elements', 'stress tolerant', and 'potentially pathogenic'. To address this, the BugBase tool was adopted to predict bacterial phenotypes relevant to the fresh herbage and silage. BugBase has already been applied to various microbiome datasets, including in precision medicine, agriculture, food and environmental research [18]. It has proven useful in identifying interpretable microbiological discoveries that current tools cannot achieve. When combined with 16S rRNA gene sequencing data, BugBase can provide new insights into the pathogenic risks and safety of silage microbiota, making it a valuable tool for assessing the safety of silages.

In this study, the ensiling process effectively removed the bacterial communities that were mobile-elements-containing and potentially pathogenic, which was due to the effective anaerobic maintenance and fast acidification suppressed a large number of harmful microbes (such as *Staphylococcus*, *Mammaliicoccus* and *Brucella*). The higher relative abundance of gram-positive in LS and WA could be attributed to the promotion of LABs after inoculation since LABs are gram-positive bacteria [54, 55]. After 30 days of ensiling, the negligible relative abundance of bacterial communities that are biofilm-formed, potentially pathogenic and stress-tolerant after LS and WA inoculation showed that, at the bacterial phenotypic level, the inoculation of LS and WA is also able to eliminate pathogenic concerns of WCC silage. The decrease in the relative abundance of gram-negative and biofilm formation accompanied by a decrease in the relative abundance of potential pathogenic is desirable for WCC silage production because gram-negative bacteria with biofilms such as lipopolysaccharide layers are tricky and have been reported to cause serious systemic infections [56, 57]. Ensilage did decrease the proportion and risk of pathogenic bacteria, and pathogens and these negative effects could be further dispelled by LS and WA inoculation.

Given that these pathogenic predictions are based on species assignments of a low-resolution target (97% OTU clustering method), especially pathogenic *Staphylococcus aureus* and nonpathogenic *Staphylococcus epidermidis* share > 98.5% nucleotide sequence identity, the risk of *Staphylococcus aureus* infection are highly speculative and should be examined with caution. The predicted pathogenic pathways, such as abovementioned *Staphylococcus aureus* infection, pathogenic *E. coli* infection and shigellosis, can be further validated by high-resolution approach (e.g. Amplicon Sequence Variant).

Conclusions

Ensiling process and LAB inoculation both had effectively improved the quality of WCC silage in terms of fermentation characteristics, bacterial diversity, co-occurrence networks, predicted functionality and phenotype. Short-term ensilage did reduce the proportion of pathogenic bacteria in WCC material, but was not effective for inhibiting some acid-resistant pathogens, while these risks could be eliminated by inoculation with *W. confuse*. Therefore, *W. confuse* could be developed as a promising fast start-up inoculant for reducing pathogenic contamination and improving hygienic quality of silage.

Abbreviations

WCC	Whole-crop corn
LAB	Lactic acid bacteria
KEGG	Kyoto Encyclopedia of Genes and Genomes
PCR	Polymerase chain reaction
PCoA	Principal coordinate analysis
GLM	General linear model
ANOVA	Analysis of variance
LA/AA	Lactic acid/acetic acid
OTUs	Operational Taxonomic Units
FM	Fresh WCC
CON	Control (WCC treated with distilled water)
LS	WCC inoculated with *Lc. Lactis*
WA	WCC inoculated with *W. confuse*
EHEC/EPEC	Enterohemorrhagic *E. coli*/enteropathogenic *E. coli*

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Author contributions
TS and YJ designed the study, contributed to securing financial support. JZ and ZJ conducted experiment. JZ prepared the manuscript; JL, ZD, and TS revised the manuscript draft; XY performed data collection and statistical analysis. All authors have read and approved the final manuscript.

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Availability of data and materials
The raw sequenced data were deposited in the NCBI Sequence Read Archive (SRA) database under the accession no. PRJNA1073498.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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