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The contribution of epiphytic microbiota in oat and Italian ryegrass to silage fermentation products and bacterial community structure of whole-crop maize

Siran Wang[1], Shiwei Hu[1], Zhihao Dong[1], Junfeng Li[1], Jie Zhao[1], Mudasir Nazar[1], Niaz Ali Kaka[1] and Tao Shao[1*]

Abstract

Background The purpose of this research is to study the contribution of epiphytic microbiota in fresh oat (OT), Italian ryegrass (IR) and whole-crop maize (MZ) to silage fermentation products and bacterial community structure of MZ. After γ-ray irradiation, the sterile MZ was treated via microbiota transplantation method: (1) sterile deionized water (STMZ); (2) microbiota epiphytic on MZ (MZMZ); (3) microbiota epiphytic on OT (MZOT); (4) microbiota epiphytic on IR (MZIR). Triplicate silos of each treatment were tested after 1, 3, 7, 15, 30 and 60 days of ensiling.

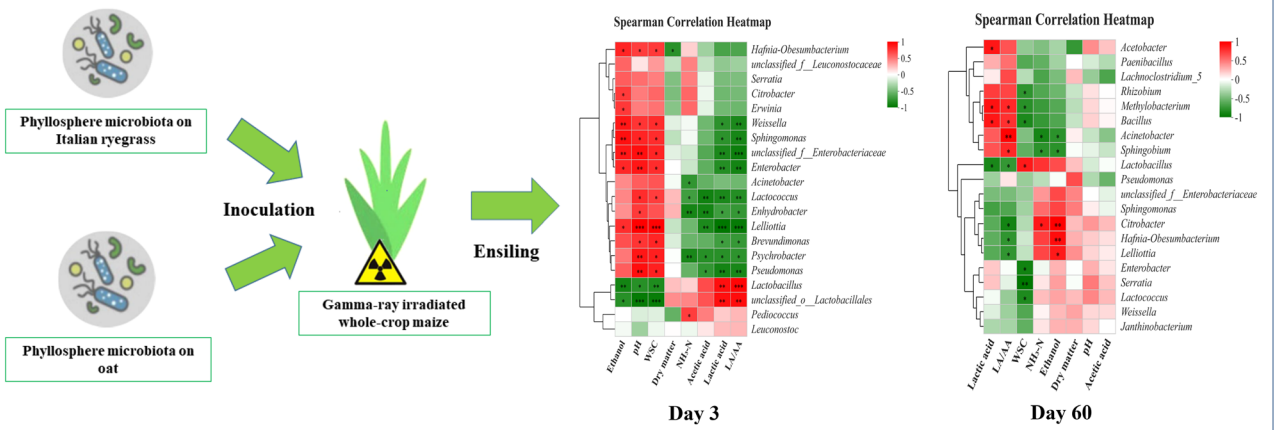
Results MZMZ had higher ($P < 0.05$) lactic acid contents, and lower ($P < 0.05$) ammonia nitrogen and ethanol contents than MZIR and MZOT on day 60. The relative abundance of *Lactobacillus* in MZMZ decreased from 84.0% on day 3 to 44.7% on day 60. MZMZ had higher ($P < 0.05$) abundances of 'Nucleotide metabolism', 'Replication and repair' and 'Membrane transport', and lower ($P < 0.05$) abundance of 'Amino acid metabolism' than MZOT and MZIR on day 3.

Conclusions The silage fermentation products of MZ were highly affected by the activity and compositions of epiphytic microbiota. The Enterobacteriaceae, *Hafnia-Obesumbacterium*, hetero-fermentative and acid-resistant *Lactobacillus* took primary responsibility for the high dry matter loss and ethanol contents and low lactic acid contents in MZ silage.

Keywords Bacterial composition, Fermentation product, Whole-crop maize, Ensiling, Silage

*Correspondence:
Tao Shao
taoshaolan@163.com
Full list of author information is available at the end of the article

Graphical Abstract



Background

Ensiling is a method to conserve fresh plant materials based on the production of lactic acid under anaerobic conditions. It has been applied for several decades to preserve the silages produced from different forages [1]. There are two major categories (C_4 and C_3 plants) as raw materials during silage production. They evolve in the different climate conditions, leading to their unique plant tissue structures, functions, and optimal growth conditions [2]. The high energy and biomass production can be supplied by C_4 forage crops, such as whole-crop maize (MZ, *Zea mays* L.) and sugarcane (*Saccharum officinarum* L.). The high palatability of C_3 forage makes it popular around the world, such as oat (OT, *Avena sativa* L.) and Italian ryegrass (IR, *Lolium multiflorum* Lam.). Moreover, C_4 plants evolved in arid and tropical environment, whereas C_3 plants originated from temperate zone [3].

Large amounts of lactic acid are often accumulated in the quality silage regardless of fermentable substrates. Nevertheless, the major fermentation products in tropical C_4 plants sometimes differ from that in temperate C_3 plants. High contents (> 35.0 g/kg dry matter (DM)) of ethanol and 2,3-butanediol were found in IR silage, whereas > 20.0 g/kg DM of acetic acid was observed in guinea grass (one tropical C_4 plant) silage [4]. Furthermore, high ethanol contents (> 45.0 g/kg DM) and low lactic acid contents (< 16.0 g/kg DM) were noticed in *Lolium perenne* (one temperate C_3 grass) silage [5]. The tendency of tropical C_4 and temperate C_3 forage silage to produce high acetic acid or ethanol contents has confused farmers for a long time. The chemical and microbial compositions in C_4 and C_3 forages both decide the final fermentative products. In chemical components, C_4 forage is characterized by coarse and stemmy structure, resulting in lots of air trapped in silos and incompact [6]. Moreover, C_3 forage contains abundant fructans, fructose, glucose, and sucrose, while C_4 forage is rich in starch [7]. In microbial compositions, there is a big difference in epiphytic bacterial community structure on various forages [8]. Nevertheless, few studies investigated the microbial contributions to fermentative products in tropical C_4 and temperate C_3 forage silages.

In recent years, the effects of epiphytic microbiota on silage fermentative products have been studied by several researchers. Whereas, they only investigated the influences of epiphytic microbiota from various tropical C_4 forages on fermentative products and bacterial compositions [9], or just evaluated the effects of epiphytic microbiota from grass on fermentative products of legume [10]. Furthermore, they often overlooked the functional characteristics of bacterial community in silage. Hence, it is unknown whether the epiphytic microbiota in temperate C_3 forages can be well-adapted and reconstituted in tropical C_4 forages silage.

Currently, the most widely planted tropical C_4 forage for ensiling is MZ due to its desirable features for ensiling, such as optimal DM level, sufficient fermentable substrates, and low buffering capacity [11]. The OT and IR are the primary C_3 grasses in temperate zone, thus accounting for a big proportion in silage production. This research aimed to study the contribution of epiphytic microbiota in fresh OT and IR to silage fermentative products, bacterial community structure and their predicted functionality in MZ silage. The knowledge about the impact of epiphytic microbiota can help us further understand the microbial factors that lead to the

different silage fermentative products between tropical C_4 and temperate C_3 forages.

Materials and methods

Preparing inoculum and making silage

Whole-crop maize (MZ; *Zea mays* L.; Variety: Yayuqingzhu No. 8), Italian ryegrass (IR; *Lolium multiflorum* Lam.; Varitety: Dongmu 70) and oat (OT; *Avena sativa* L.; Variety: Intimidator) were planted in the experimental farm of Nanjing Agricultural University (31° 36′ N, 119° 10′ E; Baima campus, Lishui District, Jiangsu Province, China; average annual temperature 16.0 °C, average elevation 43.1 m, average annual precipitation 1099 mm). During the entire growing season of three forages, there was no additional fertilization, and the weeds were removed every 2 weeks. The MZ was harvested at the dough stage (one-third milk line), and the IR and OT were both harvested at the heading stage. After harvest, three fresh forages were separately cut into 10–20 mm via a paper cutter (93ZT-300; Xingrong Co. Ltd, Guangzhou, China) without wilting, and mixed thoroughly for inoculum preparation and silage making.

On the first day, the epiphytic microbiota inoculum of MZ, OT and IR were first prepared based on the description of Mogodiniyai Kasmaer et al. [12] with some improvements. Considering the microbiota loss (about 10%) during the inoculum preparation, the complete (nearly 100%) epiphytic microbiota on 400 g fresh forage should be eluted from 444 g fresh forage. After shaking (120 rpm, 1 h, 20 °C) in the horizontal shaker, all the eluted liquid in four 1-L plastic bottles (each bottle containing 111 g fresh forage and 850 mL Ringer solution added with 0.5 mL/L Tween-80) was collected to represent the epiphytic microbiota on 400 g fresh forage. After centrifugation (10,000 rpm, 10 min, 4 °C), all the sediment was collected and centrally stored in 5-mL tubes in the fridge (− 20 °C). Hence, the final 5-mL inoculum from 444 g fresh forage was used to represent the complete epiphytic microbiota on 400 g fresh forage.

On the second day, after chopping, the 400 g fresh MZ was packed into the vacuum-packed plastic bag (45 × 32 cm) and sealed by the vacuum sealing machine (DZD-400; Aomitai Technology Co., Ltd, Nanjing, China). Totally, 72 plastic bags (6 ensiling time × 4 treatments × 3 replicates) were prepared. After sealing, all the bags were directly stored in the car fridge (− 20 °C) and immediately transported to the Xiyue Irradiation Technology Company (Wuhu, Anhui Province, China) for irradiation within 1.5 h. The irradiation condition was using ^{60}Co source at 30 kGy for 10 min according to the method of Junges et al. [13]. The prepared epiphytic inoculum of MZ, IR and OT was thawed in 4 °C fridge one night in advance, and then collected and mixed in three separated glass beakers (1 L) at the ambient temperature according to the treatments, and left in the ultra-clean workbench for inoculating. After collecting, the sterilized MZ bags were opened by scissors with a small opening in the ultra-clean workbench. After opening, the irradiated MZ (400 g) was inoculated by the prepared 5-mL epiphytic inoculum eluted from MZ, OT, and IR with the pipette gun (5 mL) and sterilized pipette tip (5 mL) in the workbench according to the experimental design. After inoculation, each bag was gently rubbed by hand for mixing the added inoculum, and then bags were evacuated and sealed again using the vacuum sealing machine (DZD-400; Aomitai Technology Co., Ltd, Nanjing, China). The treatments were as follows: (1) sterile deionized water (STMZ); (2) microbiota epiphytic on MZ (MZMZ); (3) microbiota epiphytic on OT (MZOT); (4) microbiota epiphytic on IR (MZIR). At last, all the bags were stored at ambient temperature (24–27 °C). Triplicate bags of each treatment were opened after 1, 3, 7, 15, 30 and 60 days of ensiling.

Chemical and microbial analyses

When the bags were opened, wet MZ samples were first mixed completely in a container. Then, a part of subsample (25.0 g) was homogenized with deionized water (100 mL), and filtered by two layers of sterile gauze and one filter paper. The filtrates were utilized for the further tests. The pH of samples was tested using a glass electrode pH meter. The buffering capacity of fresh material was analyzed based on the report of Playne and McDonald [14]. The filtrate was also used to analyze the ammonia-nitrogen (NH_3-N), organic acid and ethanol contents following the methods of Broderick and Kang [15] and Wang et al. [10], respectively. A second part of samples (200 g) was tested for the DM content in a forced-draft oven to a constant weight (60 °C for 48 h). The dried pre-ensiled and silage samples were ground in a mill to pass a 1-mm screen, and preserved in plastic bags pending further analysis. The total nitrogen (TN), water soluble carbohydrate (WSC) and fiber compositions were tested according to the descriptions of Krishnamoorthy [16], Thomas [17], and Van Soest et al. [18], respectively. A third part of samples (10.0 g) was mixed and shaken (20 °C for 1 h) with sterilized saline (90 mL) and used for microbial counting. The microbial numbers (including lactic acid bacteria (LAB), Enterobacteriaceae, yeast, aerobic bacteria) of samples were counted based on the report of Wang et al. [19]. The residual liquid was filtered and collected for DNA extraction and sequencing analyses.

Bacterial community structure analysis

The bacterial community compositions change dramatically during the early stage of ensiling, and the fermentation end stage of silage is critical for assessing the silage quality. Thus, the fresh materials (MZFM, IRFM, OTFM), and silage samples on day 3 (MZMZ-3, MZOT-3, MZIR-3) and day 60 (MZMZ-60, MZOT-60, MZIR-60) were selected to sequence their bacterial community compositions and predicted functionality via Next Generation Sequencing method based on the description of Wang et al. [19]. In brief, the bacterial DNA was extracted from the preserved liquid by centrifugation (11,000*g* for 12 min) according to the manufacturer's protocols of the DNA extraction kit (MP Biomedicals, Santa Ana, CA, United States). The V3–V4 regions of bacterial 16S ribosomal RNA were amplified by the primers 338F and 806R. The Illumina platform MiSeq PE300 (San Diego, CA, United States) was used for DNA paired-end sequencing.

The raw reads were checked by FLASH, and the quality was controlled by QIIME (scores > 85). The UPARSE pipeline was used to cluster the OTUs (operational taxonomic units, 98% similarity). The chimeric sequences were removed by UCHIME, and the alpha-diversity parameters were analyzed by Mothur. The bacterial community structure was determined on phylum and genus levels using Silva 138 (confidence, > 75%). The functionality of bacterial community was analyzed using KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway classification and Tax4Fun tool [20]. The raw sequencing data were deposited in NCBI's Sequence Read Archive (SRA) under the accession number PRJNA781143.

Statistical analysis

The microbial numbers were estimated as cfu (colony-forming units)/g on the fresh weight (FW) basis and transformed to $\log_{10}$ cfu/g FW. Log was the denary logarithm of the numbers. The SPSS (Statistical Packages for the Social Sciences) was utilized to examine the differences among treatments. The comparison between sterilized and fresh MZ was conducted by one-way analysis of variance (ANOVA). Data on fermentation parameters and microbial counts was analyzed by two-way ANOVA. Data on abundances of KEGG pathway was analyzed by one-way ANOVA. Tukey's multiple comparison was used to analyze the statistical difference. Differences were considered as significant at $P < 0.05$.

Results

Chemical and microbial compositions of sterile and fresh whole-crop maize

The WSC content of fresh MZ was 163 g/kg DM. Epiphytic LAB count on fresh MZ was 8.23 $\log_{10}$ cfu/g FW, while the undesirable microbes containing yeasts, Enterobacteriaceae, and aerobic bacteria were higher than 7.40 $\log_{10}$ cfu/g FW (Table 1). The chemical components between sterile and fresh MZ were similar ($P > 0.05$). The epiphytic microbial populations were not detected in sterile MZ.

Alpha diversity in fresh forage and silage

With the increase of reads sampled number, all the rarefaction curves showed an increase trend at the early stage, and kept stable at last (Fig. 1A, B). The Shannon curves reached to stable levels at the early stage of detection. Fresh forages had higher alpha diversity than silage samples, especially in indices of Sobs, Ace and Chao1 (Table 2). MZMZ had lower Sobs and Shannon indices, while higher Simpson indices than MZIR and MZOT on day 3. The coverage values for all samples were higher than 99.60%.

Bacterial community structure of fresh forage and silage

Proteobacteria and Firmicutes were dominant in IRFM (51.3%; 47.2%) and OTFM (48.4%; 49.2%), and they accounted for > 97.0% proportions of whole epiphytic bacterial community (Fig. 1C). Proteobacteria was 86.2% in bacterial community in MZFM. After fermentation, the relative abundances of Firmicutes in treated groups

Table 1 Chemical and microbial compositions of fresh and sterile whole-crop maize before ensiling

Items	Fresh whole-crop maize	Sterile whole-crop maize	*P* value
Dry matter (g/kg FW)	270	269	0.662
Water soluble carbohydrates (g/kg DM)	163	161	0.549
Buffering capacity (mEq/kg DM)	65.5	63.7	0.269
Neutral detergent fiber (g/kg DM)	580	577	0.818
Acid detergent fiber (g/kg DM)	291	287	0.831
Crude protein (g/kg DM)	66.6	66.1	0.311
Lactic acid bacteria ($\log_{10}$ cfu/g FW)	8.23	ND	–
Aerobic bacteria ($\log_{10}$ cfu/g FW)	9.24	ND	–
Yeasts ($\log_{10}$ cfu/g FW)	7.41	ND	–
Enterobacteriaceae ($\log_{10}$ cfu/g FW)	9.47	ND	–

DM, dry matter; FW, fresh weight; mEq, milligram equivalent; cfu, colony-forming units; ND, not detected

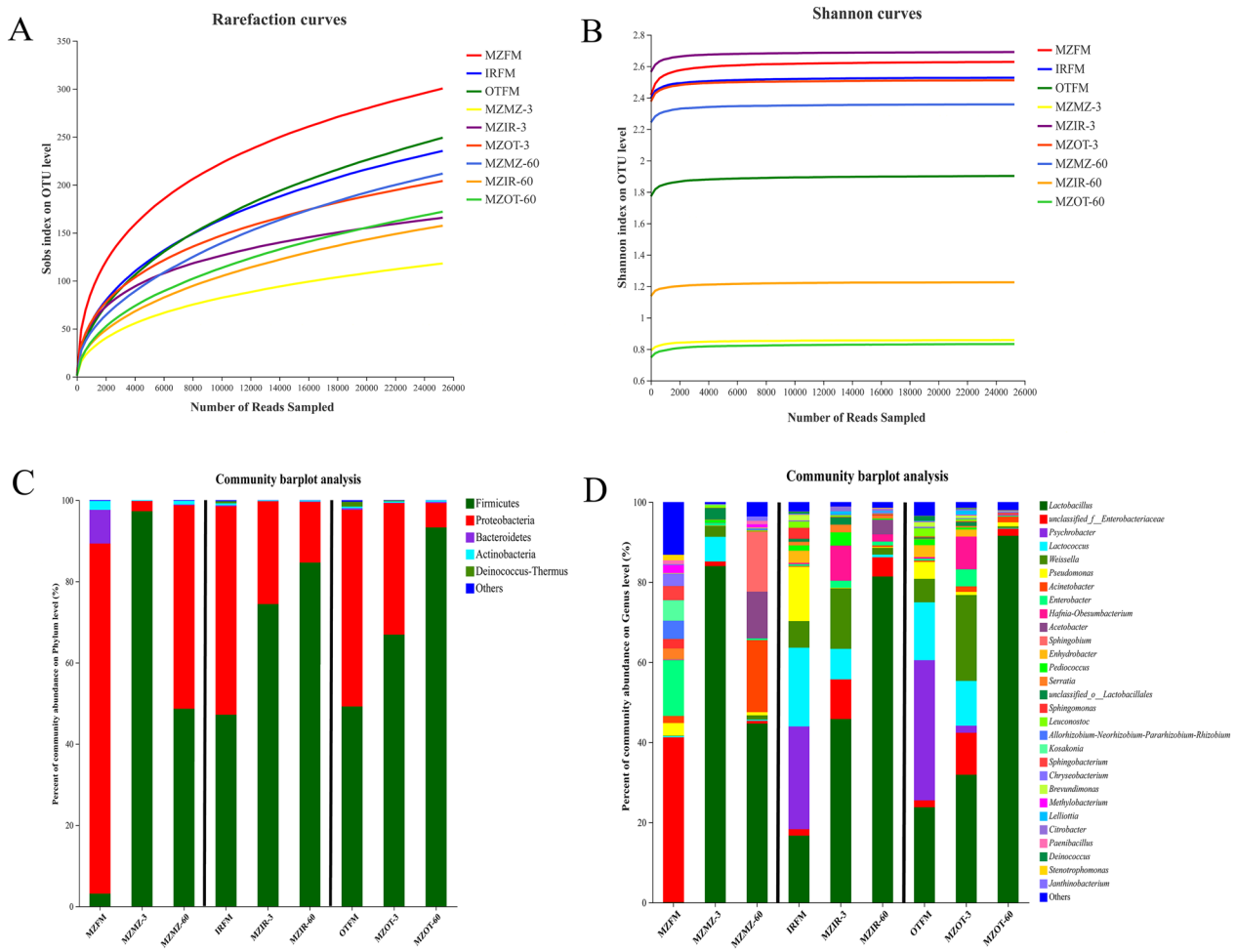


Fig. 1 Rarefaction curves (**A**), Shannon curves (**B**), phylum (**C**) and genus (**D**) level compositions of the bacterial community in fresh materials and whole-crop maize silages. MZFM, fresh material of whole-crop maize; IRFM, fresh material of Italian ryegrass; OTFM, fresh material of oat; MZMZ, sterile whole-crop maize inoculated by epiphytic bacteria from whole-crop maize; MZIR, sterile whole-crop maize inoculated by epiphytic bacteria from Italian ryegrass; MZOT, sterile whole-crop maize inoculated by epiphytic bacteria from oat; 3, 3 days of ensiling; 60, 60 days of ensiling

Table 2 Richness and diversity indices of bacterial communities in fresh materials and whole-crop maize silages on days 3 and 60

Samples	Sequence number	Sobs	Shannon	Simpson	Ace	Chao1	Coverage
MZFM	40,816	300	2.627	0.242	401	403	0.9964
IRFM	53,538	235	2.527	0.151	385	356	0.9966
OTFM	49,809	249	1.902	0.394	500	370	0.9960
MZMZ-3	52,446	118	0.858	0.705	225	210	0.9981
MZIR-3	72,506	166	2.690	0.124	241	233	0.9981
MZOT-3	46,340	204	2.511	0.150	325	299	0.9972
MZMZ-60	48,982	212	2.357	0.157	387	300	0.9966
MZIR-60	49,619	157	1.225	0.583	299	243	0.9974
MZOT-60	62,860	172	0.832	0.747	361	282	0.9971

MZFM, fresh material of maize; IRFM, fresh material of Italian ryegrass; OTFM, fresh material of oat; MZMZ, sterile maize inoculated by epiphytic microbiota from maize; MZIR, sterile maize inoculated by epiphytic microbiota from Italian ryegrass; MZOT, sterile maize inoculated by epiphytic microbiota from oat; 3, 3 days of ensiling; 60, 60 days of ensiling

on days 3 and 60 were enhanced in different proportions. The relative abundance of Firmicutes in MZFM was rapidly increased from 3.14 to 97.3% in MZMZ on day 3.

High abundance of Enterobacteriaceae (41.2%) was found in MZFM, while higher than 25.7% of *Psychrobacter* were observed in OTFM and IRFM (Fig. 1D). During fermentation, the relative abundance of *Lactobacillus* in MZMZ (84.0%) was higher than that in MZIR (45.8%) and MZOT (31.9%) on day 3, while the relative abundance of *Lactobacillus* in MZMZ (44.7%) was lower than that in MZIR (81.4%) and MZOT (91.6%) on day 60. The relative abundances of *Weissella* in MZOT (21.4%) and MZIR (15.1%) were higher than that in MZMZ (2.71%) on day 3. The relative abundances of Enterobacteriaceae in MZIR (9.90%) and MZOT (10.5%) were higher than that in MZMZ (1.16%) on day 3. The high abundances of *Hafnia-Obesumbacterium* were found in MZIR (8.74%) and MZOT (8.17%) on day 3. The high abundances of *Acetobacter* (11.7%), and *Acinetobacter* (18.0%) were found in MZMZ on day 60. The components 1 and 2 explained 37.92% and 22.25% of the total variance, respectively (Fig. 2).

Fermentative products and their relationships with bacterial community structure

The silage in STMZ was not fermented, and had similar chemical components compared with fresh MZ during ensiling (Table 3). At the early stage, lactic acid contents quickly increased and pH decreased rapidly in the fermented groups. On days 3 and 60, MZMZ had higher ($P<0.05$) lactic acid contents and ratios of lactic acid to acetic acid than MZOT and MZIR. In fermented groups, the acetic acid contents were gradually enhanced during fermentation. The butyric acid contents were < 2 g/kg DM in fermented silage samples. MZOT and MZIR had higher ($P<0.05$) ethanol contents than MZMZ on day 60.

The DM contents of silage samples in STMZ were similar with that of fresh MZ (Table 4). The DM contents in fermented silage samples decreased ($P<0.05$) during fermentation. MZMZ had higher ($P<0.05$) DM contents than MZIR and MZOT on day 60. The NH_3-N contents were gradually enhanced during the ensiling of STMZ. The acceptable levels of NH_3-N (< 81.0 g/kg TN) were found in the fermented silage samples. After 60 days, MZMZ had lower ($P<0.05$) NH_3-N contents than MZOT and MZIR. STMZ had a stable level of WSC during fermentation. After 3 days, higher ($P<0.05$) populations of LAB and lower ($P<0.05$) populations of Enterobacteriaceae were observed in MZMZ than MZOT and MZIR.

In fresh whole-crop maize (Fig. 3A), the abundance of Enterobacteriaceae had a positive correlation ($P<0.001$) with acid detergent fiber (ADF) contents, and a negative correlation ($P<0.001$) with crude protein contents. On day 3 (Fig. 3B), the abundance of *Lactobacillus* had a positive correlation ($P<0.01$) with lactic acid contents and ratios of lactic acid to acetic acid, while a negative correlation ($P<0.05$) with pH values. On day 60 (Fig. 3C), the ethanol contents had a positive correlation ($P<0.05$) with the abundance of *Hafnia-Obesumbacterium*. There was a negative correlation ($P<0.05$) between the abundance of *Lactobacillus* and lactic acid contents.

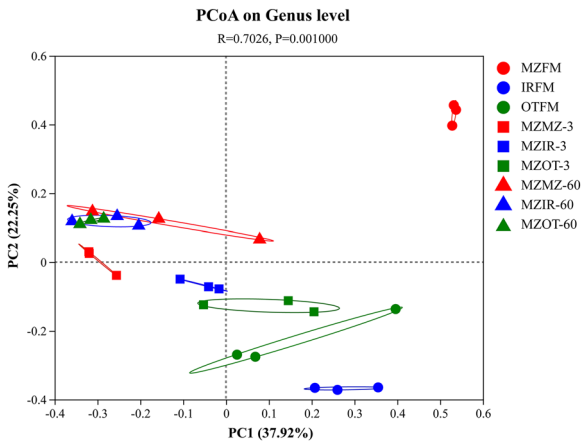


Fig. 2 Principal co-ordinates analysis (PCoA) of bacterial communities on Genus level in fresh materials and whole-crop maize silages. MZFM, fresh material of whole-crop maize; IRFM, fresh material of Italian ryegrass; OTFM, fresh material of oat; MZMZ, sterile whole-crop maize inoculated by epiphytic bacteria from whole-crop maize; MZIR, sterile whole-crop maize inoculated by epiphytic bacteria from Italian ryegrass; MZOT, sterile whole-crop maize inoculated by epiphytic bacteria from oat; 3, 3 days of ensiling; 60, 60 days of ensiling

Functionality of bacterial communities in fresh forage and silage

The pathway levels of epiphytic bacterial community in three fresh forages differed (Fig. 4). After 3 days, MZOT and MZIR had higher ($P<0.05$) abundances of 'Cellular Process', and lower abundances of 'Genetic Information Processing' than MZMZ. After 60 days, MZOT and MZIR had higher ($P<0.05$) abundances of 'Environmental Information Processing' and 'Genetic Information Processing', while lower ($P<0.05$) abundances of 'Metabolism' than MZMZ.

The 'Carbohydrate metabolism' pathways in different groups were promoted after ensiling (Fig. 5). After 3 days, MZMZ had higher ($P<0.05$) abundances of 'Carbohydrate metabolism', 'Nucleotide metabolism', 'Replication and repair' and 'Membrane transport', while lower ($P<0.05$) abundances of 'Amino acid metabolism' and 'Signal transduction' than MZOT and MZIR. After 60 days, MZMZ had lower ($P<0.05$) abundances

Table 3 Effect of inoculating exogenous microbiota on pH value, organic acid and ethanol contents in whole-crop maize silage

Items	Treatments	Ensiling days (d)						SEM	*P* value		
		1	3	7	15	30	60		*T*	*D*	*T*×*D*
pH value	STMZ	5.60^{a}	5.54^{a}	5.49^{a}	5.50^{a}	5.45^{a}	5.46^{a}	0.026	< 0.001	< 0.001	< 0.001
	MZMZ	4.18^{Ac}	3.51^{BCd}	3.57^{Bc}	3.50^{BCc}	3.41^{Cc}	3.44^{BCc}				
	MZIR	5.26^{Ab}	3.88^{Bc}	3.62^{Cc}	3.65^{Cbc}	3.57^{Cb}	3.64^{Cb}				
	MZOT	4.19^{Ac}	4.09^{ABb}	3.95^{ABb}	3.69^{Bb}	3.53^{BCbc}	3.27^{Cd}				
Lactic acid (g/kg DM)	STMZ	0.74^{b}	0.82^{d}	0.64^{d}	0.68^{d}	0.76^{d}	0.76^{d}	1.206	< 0.001	< 0.001	< 0.001
	MZMZ	24.8^{Da}	62.6^{Ca}	74.4^{Ba}	81.1^{ABa}	84.9^{Aa}	86.9^{Aa}				
	MZIR	1.55^{Eb}	36.6^{Db}	63.4^{Cb}	70.9^{ABb}	68.7^{Bb}	74.4^{Ab}				
	MZOT	23.1^{Ea}	28.6^{Ec}	37.2^{Dc}	45.3^{Cc}	61.4^{Bc}	66.9^{Ac}				
Acetic acid (g/kg DM)	STMZ	0.61^{CDd}	0.56^{Dc}	0.75^{BCc}	0.93^{Ac}	0.93^{Ad}	0.81^{ABd}	0.526	< 0.001	< 0.001	< 0.001
	MZMZ	3.42^{Cb}	6.44^{BCa}	7.57^{BCb}	8.34^{Bb}	11.5^{Ab}	10.4^{ABb}				
	MZIR	2.51^{Fc}	6.30^{Ea}	8.39^{Da}	12.2^{Ca}	15.4^{Ba}	17.0^{Aa}				
	MZOT	4.39^{Ea}	5.69^{Db}	7.34^{Cb}	8.55^{Bb}	9.58^{Ac}	9.78^{Ac}				
Lactic acid/Acetic acid	STMZ	1.30^{ABc}	1.47^{Ad}	0.85^{ABd}	0.74^{Bc}	0.82^{Bd}	0.92^{ABd}	0.129	< 0.001	< 0.001	< 0.001
	MZMZ	7.24^{Ca}	9.70^{Aa}	9.84^{Aa}	9.72^{Aa}	7.40^{Ca}	8.33^{Ba}				
	MZIR	0.63^{Dc}	5.81^{Bb}	7.58^{Ab}	5.81^{Bb}	4.47^{Cc}	4.37^{Cc}				
	MZOT	5.28^{Bb}	5.03^{Bc}	5.08^{Bc}	5.30^{Bb}	6.41^{Ab}	6.84^{Ab}				
Butyric acid (g/kg DM)	STMZ	1.01^{a}	1.01^{a}	1.01^{a}	1.00^{a}	1.03^{a}	1.03^{a}	0.225	< 0.001	< 0.001	< 0.001
	MZMZ	1.00^{a}	0.89^{a}	0.81^{b}	0.87^{b}	1.03^{a}	1.04^{a}				
	MZIR	0.40^{b}	0.54^{b}	0.75^{b}	0.68^{bc}	0.64^{b}	0.66^{b}				
	MZOT	0.46^{b}	0.48^{b}	0.45^{c}	0.46^{c}	0.44^{b}	0.44^{b}				
Ethanol (g/kg DM)	STMZ	4.80^{b}	4.63^{b}	4.38^{b}	4.89^{bc}	4.83^{c}	4.61^{c}	1.267	< 0.001	< 0.001	< 0.001
	MZMZ	2.75^{c}	2.42^{c}	2.80^{b}	2.87^{c}	2.84^{c}	2.96^{c}				
	MZIR	2.90^{Ec}	5.24^{Eab}	17.2^{Da}	24.8^{Ca}	35.4^{Ba}	46.1^{Aa}				
	MZOT	5.49^{Da}	5.42^{Da}	5.62^{Db}	7.81^{Cb}	16.8^{Bb}	37.0^{Ab}				

DM, dry matter; d, day; STMZ, sterile maize; MZMZ, sterile maize inoculated by epiphytic microbiota from maize; MZIR, sterile maize inoculated by epiphytic microbiota from Italian ryegrass; MZOT, sterile maize inoculated by epiphytic microbiota from oat; SEM, standard error of means; *T*, microbiota; *D*, ensiling days; *T*×*D*, the interaction between microbiota and ensiling days

Means with different letters in the same row ($^{A-F}$) or column ($^{a-d}$) differ ($P < 0.05$)

of 'Nucleotide metabolism', 'Carbohydrate metabolism', 'Membrane transport' and 'Replication and repair', while higher ($P < 0.05$) abundances of 'Energy metabolism' than MZOT and MZIR.

Discussion

Chemical and microbial compositions of sterile and fresh whole-crop maize

The high-quality silage required > 50 g/kg DM of WSC in raw material [21]. In this study, the WSC contents in fresh MZ was 163 g/kg DM, indicating fresh MZ can be used to evaluate the contribution of epiphytic microbiota to silage fermentation products, because adequate fermentation substrates could be supplied for microbes during fermentation. Besides, sterile MZ had similar chemical components with fresh MZ, indicating our irradiation condition was optimal, because it did not alter the chemical components of raw materials. It is critical for studying the effects of epiphytic microbiota on fermentative products. The microbes were not detected in sterile MZ group, suggesting the γ-ray irradiation can inactivate the epiphytic microorganisms of forages.

Metagenomic analysis and functional sequencing

Metagenomics is defined as culture independent, direct genetic analysis of genomes within environmental samples [22]. Advances in the Next Generation Sequencing (NGS) have revolutionized the field of microbial ecology. Two types of NGS-based metagenomics studies are commonly conducted: (1) single/marker-gene amplification metagenomics, more appropriately called "metaprofiling" or amplicon-based profiling (e.g., 16S rRNA gene in prokaryotes), and (2) whole shotgun metagenomics [23]. These methods allow profiling of the whole microbial community including uncultivable species and can generate an in-depth description of microbial diversity within various ecosystems at a reasonable cost. Marker gene-based metaprofiling generates taxonomic and phylogenetic classification of microorganisms in complex

Table 4 Effect of inoculating exogenous microbiota on chemical and microbial compositions in whole-crop maize silage

Items	Treatments	Ensiling days (d)						SEM	*P* value		
		1	3	7	15	30	60		*T*	*D*	*T*×*D*
Dry matter (g/kg DM)	STMZ	266^{a}	263^{a}	265^{a}	266^{a}	264^{a}	266^{a}	2.401	< 0.001	< 0.001	< 0.001
	MZMZ	262^{Aab}	249^{Bb}	245^{Bb}	237^{BCb}	234^{CDb}	227^{Db}				
	MZIR	262^{Aab}	244^{Bb}	232^{Cc}	224^{CDc}	217^{Dc}	203^{Ec}				
	MZOT	255^{Ab}	244^{Bb}	234^{Cb}	227^{CDc}	221^{Dc}	204^{Ec}				
Water soluble carbohydrates (g/kg DM)	STMZ	161^{a}	163^{a}	162^{a}	163^{a}	161^{a}	160^{a}	1.325	< 0.001	< 0.001	< 0.001
	MZMZ	160^{Aa}	110^{Bc}	56.9^{Cd}	30.2^{Dd}	25.5^{DEc}	20.7^{Eb}				
	MZIR	154^{Ab}	133^{Bb}	115^{Cb}	68.5^{Db}	36.2^{Eb}	21.3^{Fb}				
	MZOT	153^{Ab}	137^{Bb}	75.3^{Cc}	55.9^{Dc}	33.8^{Eb}	23.1^{Fb}				
Ammonia nitrogen (g/kg TN)	STMZ	15.5^{Ec}	22.0^{Dd}	36.2^{Cc}	47.3^{Ba}	47.6^{Bc}	54.0^{Ac}	1.281	< 0.001	< 0.001	< 0.001
	MZMZ	30.2^{Db}	48.4^{Cb}	52.5^{Cb}	61.0^{Bb}	67.6^{ABb}	68.0^{Ab}				
	MZIR	46.6^{Da}	55.1^{Ca}	59.8^{BCa}	63.4^{Bb}	74.7^{Aa}	80.2^{Aa}				
	MZOT	26.6^{Fb}	37.9^{Ec}	48.0^{Db}	58.3^{Cb}	67.0^{Bb}	74.8^{Aa}				
Lactic acid bacteria (log_{10} cfu/g FW)	STMZ	ND	ND	ND	ND	ND	ND	0.123	< 0.001	< 0.001	< 0.001
	MZMZ	10.0^{Ba}	11.3^{Aa}	10.4^{Ba}	9.65^{Bb}	8.51^{Cb}	6.09^{Db}				
	MZIR	6.11^{Eb}	8.37^{Cc}	9.10^{Bb}	11.3^{Aa}	8.67^{BCb}	6.68^{Db}				
	MZOT	5.58^{Ec}	9.68^{Bb}	9.23^{Cb}	10.2^{Ab}	9.49^{BCa}	7.82^{Da}				
Enterobacteriaceae (log_{10} cfu/g FW)	STMZ	ND	ND	ND	ND	ND	ND	0.124	< 0.001	< 0.001	< 0.001
	MZMZ	7.31^{Ab}	5.51^{Bb}	4.37^{Cb}	3.39^{DEb}	3.85^{CDa}	2.60^{Eb}				
	MZIR	8.63^{Aa}	7.04^{Ba}	4.93^{Ca}	4.19^{Da}	3.43^{Eb}	2.71^{Fb}				
	MZOT	7.63^{Ab}	6.46^{Ba}	4.25^{Cb}	4.15^{Ca}	3.98^{CDa}	3.64^{Da}				

DM, dry matter; FW, fresh weight; TN, total nitrogen; cfu, colony-forming units; ND, not detected; STMZ, sterile maize; MZMZ, sterile maize inoculated by epiphytic microbiota from maize; MZIR, sterile maize inoculated by epiphytic microbiota from Italian ryegrass; MZOT, sterile maize inoculated by epiphytic microbiota from oat; SEM, standard error of means; *T*, microbiota; *D*, ensiling days; *T*×*D*, the interaction between microbiota and ensiling days

Means with different letters in the same row ($^{A-F}$) or column ($^{a-d}$) differ ($P < 0.05$)

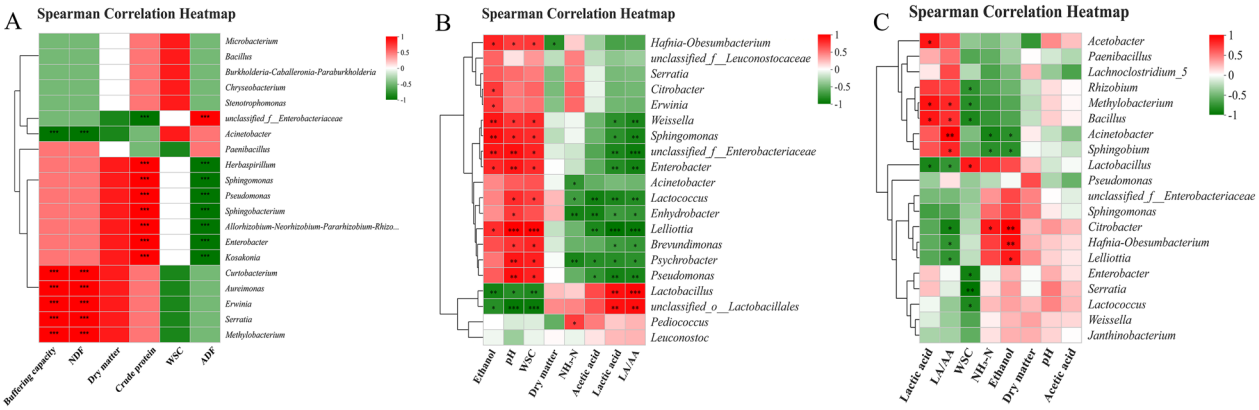


Fig. 3 Spearman correlation heatmap between chemical compositions or fermentation parameters and bacterial community compositions in fresh whole-crop maize (**A**) or whole-crop maize silages on day 3 (**B**) and day 60 (**C**). NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water soluble carbohydrate; NH_3-N, ammonia-nitrogen; LA/AA, ratio of lactic acid to acetic acid

ecosystems with less investment in time and computational power (~ 50,000 reads/sample) than whole shotgun metagenomics. Other advantages of metaprofiling over whole metagenomics include cheaper sequencing costs, and no (eukaryotic) contamination with host DNA as a result of target-specific amplification of conserved regions (e.g., 16S rRNA gene). However, it has certain limitations as primer selection can result in biases toward certain members within microbial communities and resolution is often insufficient to identify bacteria to

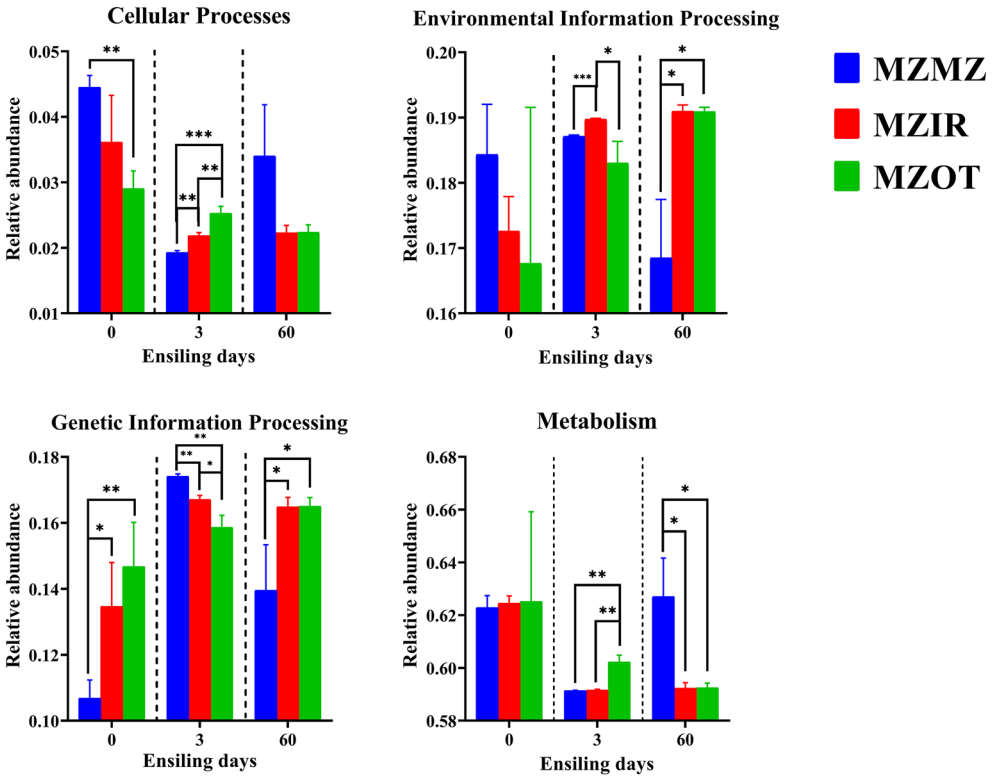


Fig. 4 Changes of KEGG metabolic pathways on the first level obtained with Tax4Fun in fresh materials and whole-crop maize silages. KEGG, Kyoto Encyclopedia of Genes and Genomes; MZMZ, sterile whole-crop maize inoculated by epiphytic bacteria from whole-crop maize; MZIR, sterile whole-crop maize inoculated by epiphytic bacteria from Italian ryegrass; MZOT, sterile whole-crop maize inoculated by epiphytic bacteria from oat. 0, fresh material; 3, 3 days of ensiling; 60, 60 days of ensiling. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$

the strain or even the species level. In addition, different primers are required for multi-domain communities that harbor bacteria, archaea, and eukaryotes, and no marker genes are available to amplify and differentiate members within the virome.

Although marker gene (e.g., 16S rRNA, 18S rRNA, and ITS) sequencing has been widely used to describe microbial communities and programs such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) [24] and Tax4Fun [20] have been used to predict their functionality, such approaches do not generate a complete genetic profile of microbial populations. In contrast, shotgun metagenomics sequencing of the DNA isolated from a sample provides thorough genetic information of microbial communities as well as genomic linkages between the function and phylogeny of uncultured organisms [25]. Shotgun metagenomics avoids primer biases as all microbes in the community including eukaryotes and viruses can be sequenced and identified. In addition to providing community composition, it also helps generate information on the function of the community. However, some of the disadvantages include very high cost of deep sequencing to generate millions of reads, host/site contamination, lack of information on "rare" species due to limitations of deep sequencing, and complex bioinformatics algorithms that require significant computational resources.

The majority of NGS studies have used amplicon sequencing to define the microbial ecology of silage, but shotgun sequences offer advantages in that genes associated with both phylogeny and function are sequenced [23]. Thus, specific genes involved in metabolic pathways can thus be targeted in an effort to understand their functional contribution to the ensiling process. Such approaches may have application in characterizing biochemical pathways involved in the production or degradation of mycotoxins during ensiling. However, to our knowledge, limited metagenomic sequencing studies focusing on functional aspects of the ensiling process have yet to be undertaken.

Alpha diversity in fresh forage and silage

Herein, the changes of rarefaction curves in all samples indicated that the quantity of sequencing was suitable, and could describe the profiles of bacterial community

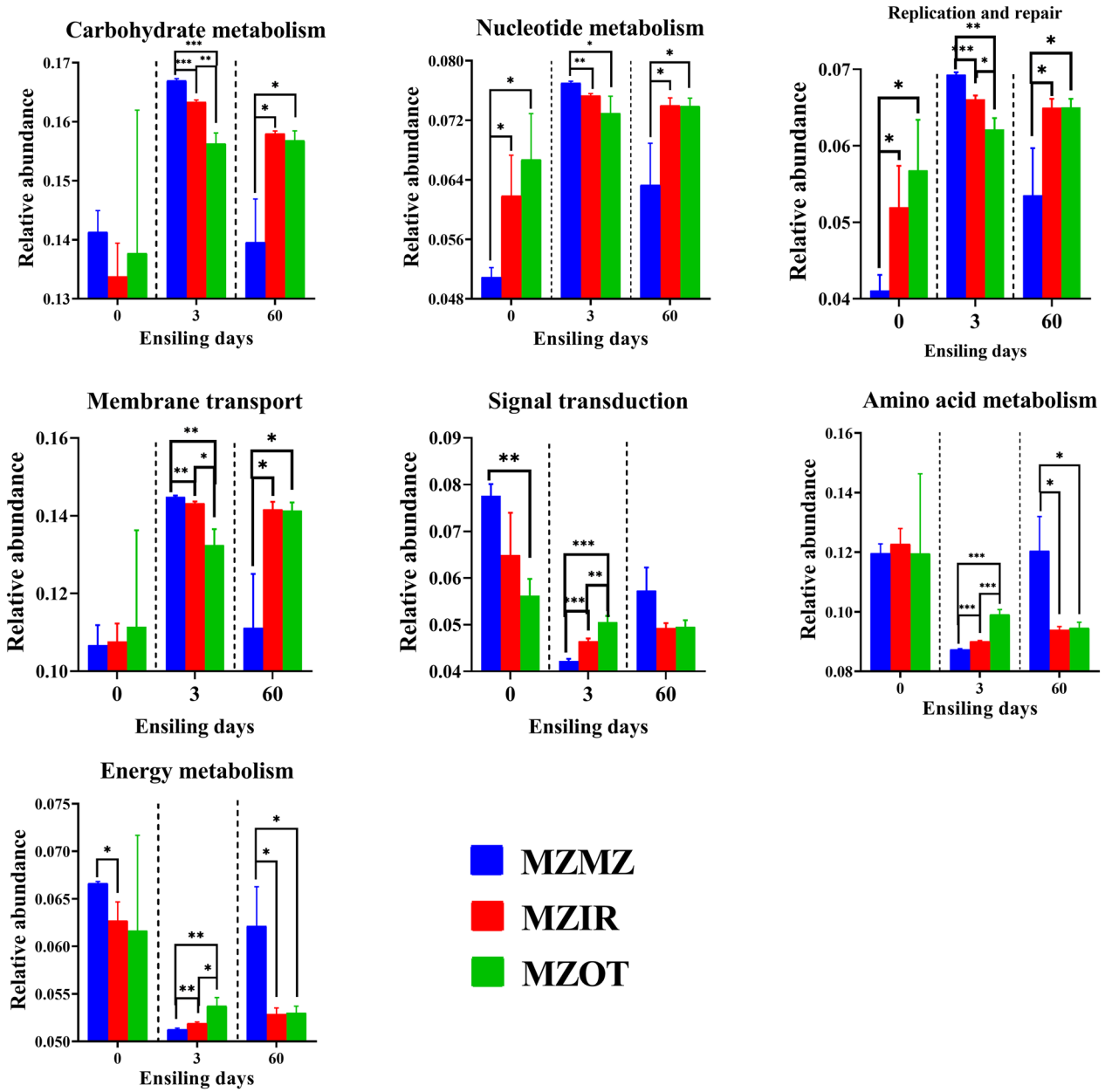


Fig. 5 Changes of KEGG metabolic pathways on the second level obtained with Tax4Fun in fresh materials and whole-crop maize silages. KEGG, Kyoto Encyclopedia of Genes and Genomes; MZMZ, sterile whole-crop maize inoculated by epiphytic bacteria from whole-crop maize; MZIR, sterile whole-crop maize inoculated by epiphytic bacteria from Italian ryegrass; MZOT, sterile whole-crop maize inoculated by epiphytic bacteria from oat. 0, fresh material; 3, 3 days of ensiling; 60, 60 days of ensiling. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$

structure. The stable levels of Shannon curves at the beginning of detection also proved that the sequencing depth was sufficient to reflect the bacterial community in all samples. The higher alpha diversity parameters in fresh forages than silages were mainly due to the anaerobic and acidic environments were quickly achieved after ensiling, resulting in the decline of bacterial community diversity. It could partly explain the lower alpha diversity indices in MZMZ than MZOT and MZIR after 3 days of ensiling. Similarly, Du et al. [26] also reported that the high-quality silage had the lowest alpha diversity indices.

Bacterial community structure of fresh forage and silage

The Firmicutes and Proteobacteria were dominant in fresh IR and OT. Proteobacteria played an important

role in the acceleration of carbon and nitrogen cycles, and degradation of organic matter [27]. In anaerobic condition, Firmicutes can produce various enzymes whereby their acid hydrolytic function [28]. In contrast, the predominant phylum in MZFM was only Proteobacteria, which could be due to the different growth conditions and chemical components of fresh forages [19]. After fermentation, the populations of Firmicutes increased in inoculated silage. This was because the growth of Firmicutes required an acidic and anaerobic condition during fermentation [29].

The high abundance of Enterobacteriaceae in fresh MZ was in accordance with the findings of Wang et al. [19], who described that the most abundant epiphytic microorganisms were molds, Enterobacteriaceae, and yeasts. The most predominant genus *Psychrobacter* in fresh OT and IR was probably because *Psychrobacter* adapted to grow under cold and humid conditions. After 3 days of ensiling, MZMZ had much higher abundances of *Lactobacillus* than MZOT and MZIR. Two molecules of lactic acid can be produced by *Lactobacillus* using one molecule of glucose. Once ensiling, *Lactobacillus* could quickly proliferate and grow, and produce abundant lactic acid to decrease pH of silage, and then the undesirable microbes are inhibited [30]. Hence, the abundant *Lactobacillus* may be responsible for the high lactic acid contents in MZMZ-3. On day 60, the abundance of *Lactobacillus* in MZMZ was reduced by 46.8%, whereas the abundances of *Lactobacillus* in MZIR and MZOT were enhanced by 43.7% and 65.2%, respectively. The similar pH values in three inoculated groups indicated that the *Lactobacillus* in MZMZ-60 had a lower capacity for tolerating the acid environment, thus leading to a decreased tendency of *Lactobacillus* at the end of fermentation.

Interestingly, a higher proportion of *Hafnia-Obesumbacterium* was observed in MZOT and MZIR on day 3. Few studies discussed the role of *Hafnia-Obesumbacterium* in silage. As enterobacteria, *Hafnia-Obesumbacterium* can promote the proteolytic activities during ensilage [28]. Whereas, the excellent LAB additives could not inhibit the growth of *Hafnia-Obesumbacterium* [31]. Thus, more research about *Hafnia-Obesumbacterium* should be conducted. Besides, MZOT and MZIR had higher proportions of Enterobacteriaceae than MZMZ on day 3. Enterobacteriaceae are harmful during ensiling, because they can survive in weak acidic and anaerobic conditions, compete with LAB for limited WSC, and consume the WSC and lactic acid, resulting in loss of nutrients and DM [32]. Thus, the high proportions of Enterobacteriaceae may be responsible for the slow decrease of pH in MZIR-3 and MZOT-3.

After 3 days, MZOT and MZIR had higher proportions of *Weissella* than MZMZ. As obligate hetero-fermentative bacteria, *Weissella* mainly converted WSC to acetic and lactic acids [33]. *Acetobacter* accounted for a high proportion in MZMZ-60. The aerobic spoilage of corn silage may result from *Acetobacter* [34]. A high abundance of *Acinetobacter* was also observed in MZMZ-60. *Acinetobacter* can grow rapidly in the acidic condition, and lead to aerobic spoilage in silage [35].

Fermentative products and their relationships with bacterial community structure

The STMZ remained unfermented state during the entire fermentation period, indicating the conditions of used γ-ray irradiation were optimal and could divide the chemical and microbial factors of fresh forages. High lactic acid content was rapidly produced and pH values decreased in three inoculated groups during the early stage of ensiling. The chopped forages can promote the release of plant juice, and ensure the growth of LAB after ensiling. Higher contents of lactic acid in MZMZ than MZOT and MZIR may be due to the higher proportions of *Lactobacillus* in MZMZ on day 3. Nevertheless, MZMZ with lower proportions of *Lactobacillus* still had higher lactic acid contents than MZOT and MZIR on day 60. It was probably because most of *Lactobacillus* in MZOT and MZIR were hetero-fermentative, which had lower efficiency in producing lactic acid [36]. The increased tendency of acetic acid in fermented groups may result from the metabolism of hetero-fermentative LAB, *Propionibacterium* and enterobacteria during ensiling [37]. It is well-known that butyric acid is undesirable in silage because of the nutritional damage caused by secondary fermentation as a result of clostridial activity. Silages high in butyric acid are usually low in nutritive value, and such silages may also be high in soluble protein contents and contain small protein compounds called amines that have sometimes shown to adversely affect animal performance. Moreover, high concentrations of butyric acid might induce ketosis in lactating cows [38]. In this study, trace amount (< 2 g/kg DM) of butyric acid in all fermented groups could be attributed to the rapid decrease in pH during the early stage of ensiling, thus inhibiting the propagation of undesirable microorganisms (e.g., clostridia).

High ethanol production in silage could lead to the high energy and DM losses during ensiling. The flourishment of yeasts in silage may be mainly responsible for the high ethanol contents (30–40 g/kg DM) [39]. Thus, MZIR had > 40.0 g/kg DM of ethanol contents on day 60, which may be closely linked with action of acid-resistant yeasts, Enterobacteriaceae and hetero-fermentative LAB. Enterobacteriaceae could utilize WSC and lactic acid to

produce ethanol [32], and hetero-fermentative LAB can produce ethanol, CO_2, acetic and lactic acids [36]. It also proved that most of *Lactobacillus* in MZOT and MZIR on day 60 were hetero-fermentative LAB.

STMZ had stable DM contents during the entire ensiling process, indicating the microbes epiphytic on fresh forages can be successfully inactivated by γ-ray irradiation, thus inhibiting the fermentative process. In contrast, the DM contents in fermented groups decreased during ensiling, suggesting the microbial consumption of substrates to water and CO_2. On day 60, the higher DM contents in MZMZ than MZOT and MZIR may be correlated with the rapid decrease of pH and less hetero-fermentative LAB strains in MZMZ, restricting the growth of harmful microbes and conserving more nutrients in silage. The high DM recovery in quality silage was related to the lower ethanol contents, indicating less inefficient secondary fermentation by hetero-fermentative bacteria and yeasts [40]. Furthermore, the production of NH_3-N in STMZ was mainly due to the action of plant enzymes. Low NH_3-N contents (< 100 g/kg TN) were observed in the all fermented groups, indicating good fermentation quality [41]. After 60 days, the lower NH_3-N contents in MZMZ than MZOT and MZIR were mainly due to the rapid production of lactic acid and decrease of pH at the early stage of fermentation in MZMZ, limiting the enzyme activities of microbes and plants. The higher LAB numbers and lower Enterobacteriaceae numbers in MZMZ-3 may be responsible for the rapid production of lactic acid in MZMZ on day 3.

In fresh whole-crop maize, the positive correlation between Enterobacteriaceae and ADF may indicate that Enterobacteriaceae can proliferate extensively in high-fiber forages. After 3 days, the positive correlation between *Lactobacillus* and lactic acid and ratios of lactic acid to acetic acid indicated that species of *Lactobacillus* played important roles in promoting homo-lactic acid fermentation at the early stage. However, *Lactobacillus* had a negative correlation with lactic acid contents on day 60. It was suggested that some species of *Lactobacillus* were hetero-fermentative LAB, and their efficiency of acid production was lower than homo-fermentative LAB. After 60 days, the positive relationships between *Hafnia-Obesumbacterium* and ethanol indicated that *Hafnia-Obesumbacterium* may enhance the production of ethanol during MZ ensiling. It was probably because *Hafnia-Obesumbacterium* belongs to enterobacteria, which can convert lactic acid and WSC to ethanol and other products [28, 32].

Functionality of bacterial communities in fresh forage and silage

KEGG as a bioinformatics resource can be used to understand the utilities and functions of organisms and cells [42]. The information about the function of bacterial community in silage is conducive for us to know the ensiling process. Thus, KEGG was utilized to evaluate the influence of epiphytic microbiota on functional dynamic changes in MZ silage.

On day 3, MZIR and MZOT had higher abundances of 'Cellular Process', while lower abundances of 'Genetic Information Processing' than MZMZ. According to the fermentative products in various groups, it indicated that the epiphytic microbiota from fresh MZ enhanced the fermentation quality of MZ. It may be related to the change of cell characteristics, and inhabitation of membrane transport and signal transduction of harmful bacteria. On day 60, higher proportions of 'Metabolism' in MZMZ suggested that the silage quality of MZMZ may be improved by promoting the metabolism of LAB during ensiling. Moreover, the carbohydrate metabolism primarily included glycolysis and gluconeogenesis metabolism [43]. All the groups enhanced the 'Carbohydrate metabolism' after ensiling, indicating that the microbes in MZ silage mainly *Lactobacillus* had a stronger capacity to consume substrates than other bacteria.

Conclusions

The silage fermentation products of MZ were highly affected by the activity and compositions of epiphytic microbiota. The Enterobacteriaceae, *Hafnia-Obesumbacterium*, hetero-fermentative and acid-resistant *Lactobacillus* took primary responsibility for the high dry matter loss and ethanol contents and low lactic acid contents in MZ silage.

Abbreviations

FW	Fresh weight
ETH	Ethanol
NH_3-N	Ammonia-nitrogen
PA	Propionic acid
AA	Acetic acid
BA	Butyric acid
WSC	Water soluble carbohydrate
DM	Dry matter
LA	Lactic acid
LA/AA	Ratio of lactic acid to acetic acid
MZFM	Fresh material of maize
IRFM	Fresh material of Italian ryegrass
OTFM	Fresh material of oat
STMZ	Sterile maize
MZMZ	Sterile maize inoculated by epiphytic microbiota from maize
MZIR	Sterile maize inoculated by epiphytic microbiota from Italian ryegrass
MZOT	Sterile maize inoculated by epiphytic microbiota from oat

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Author contributions
Designed experiments, TS, and SW; carried out experiments, SW; analyzed experimental results, SW, SH, JZ, ZD and JL; wrote and edited the manuscript, SW, NAK and MN. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

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.

Author details
[1]Institute of Ensiling and Processing of Grass, College of Agro-Grassland Science, Nanjing Agricultural University, Weigang 1, Nanjing 210095, China.

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