Changes in the taxonomic and functional structure of microbial communities during vegetable waste mixed silage fermentation

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Complete List of Authors:	Xiang, Quanju; Sichuan Agricultural University - Chengdu Campus, College of Resource Zhang, Juntao; Sichuan Agricultural University, Department of Microbiology Huang, Xiying; Sichuan Agricultural University, Department of Microbiology Ma, Menggen; Sichuan Agricultural University - Chengdu Campus, College of Resource Zhao, Ke; Sichuan Agricultural University Yu, Xiumei; Sichuan Agricultural University Chen, Qiang; Sichuan Agricultural University - Chengdu Campus, College of Resource Zhang, Xiaoping; Sichuan Agricultural University Penttinen, Petri; Sichuan Agricultural University, Department of Microbiology Gu, Yunfu; Sichuan Agricultural University - Chengdu Campus, College of Resource
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Changes in the taxonomic and functional structure of microbial communities during vegetable waste mixed silage fermentation

Running title: Mixed silage fermentation of vegetable waste

Quanju Xiang[†], Juntao Zhang[†], Xiying Huang[†], Menggen Ma, Ke Zhao, Xiumei Yu, Qiang Chen, Xiaoping Zhang, Petri Penttinen, Yunfu Gu*

Department of Microbiology, College of Resources, Sichuan Agricultural University, Chengdu 611130, China

[†]These authors contributed equally to this work

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For correspondence. E-mail: guyf@sicau.edu.cn; Tel.: +86-028-86290982; Fax: +86-028-86290983

Abstract: Silage fermentation, a sustainable way to use vegetable waste resources, is a complex process driven by a variety of microorganisms. We used lettuce waste as the main raw material for silage, analyzed changes in the physico-chemical characteristics and bacterial community composition of silage over a 60-day fermentation, identified differentially abundant taxa, predicted the functional profiles of bacterial communities, and determined the associated effects on the quality of silage. The biggest changes occurred in the early stage of silage fermentation. Changes in the physico-chemical characteristics included a decrease in pH and increases in ammonia nitrogen to total nitrogen ratio and lactic acid content. The numbers of lactic acid bacteria (LAB) increased and molds, yeasts and aerobic bacteria decreased. The bacterial communities and their predicted functions on day 0 were clearly different from those on day 7 to day 60. The relative abundances of phylum *Firmicutes* and genus *Lactobacillus* increased. Nitrite ammonification and nitrate ammonification were more prevalent after day 0. The differences in the predicted functions were associated with differences in pH and amino acid, protein, carbohydrate, $\text{NH}_3\text{-N}$, ether extract and crude ash contents.

Keywords: Vegetable waste; Silage; Bacterial communities; Functional prediction

Introduction

Vegetable waste refers to withered, residual leaves of vegetables after harvest, derived from the production, transportation, storage, distribution and consumption of vegetables (Ji *et al.* 2017). With the rapid economic development and structural adjustment of agriculture in China, the vegetable industry has expanded quickly, and needs to dispose of large amounts of vegetable waste. In China, approximately 1.3 million tons of vegetable waste is produced per day (Shen *et al.* 2013). Vegetable waste is mostly discarded randomly instead of recycling, causing huge waste of resources and serious environmental problems. In the fields, vegetable waste with high moisture and organic matter content will easily rot, attract mosquitoes and flies, and promote the spread of plant diseases and pests (Edwiges *et al.* 2018; Li *et al.* 2018). Dumping the vegetable waste into water bodies pollutes rivers, lakes and groundwater systems, and its incineration decreases air quality; all these affect the environment and human health (Zhang *et al.* 2011).

Making silage is a sustainable way to use vegetable waste resources (Cao *et al.* 2011). Silage fermentation relies on lactic acid bacteria (LAB) to ferment water soluble carbohydrates (WSC) and lignocellulolytic material anaerobically to produce lactic acid (LA); LA lowers pH that further inhibits the growth of molds, aerobic bacteria, yeasts and other unfavorable microorganisms, thus improving the quality and the storage stability of silage (Duniere *et al.* 2013; Yuan *et al.* 2015). Usually, the epiphytic LAB on the ensiled material are not abundant or efficient enough to lower the pH to values required for good quality silage (Puntillo *et al.* 2020). Among the strains isolated from various vegetable waste in Sichuan, less than 10 LAB strains showed good acid-tolerant and acid-producing ability (Zhang *et al.* 2019). Therefore, exogenous LAB inoculants are commonly applied to improve silage fermentation. Previously, silage was mainly fermented using corn and alfalfa (Guan *et al.* 2018; Ni *et al.* 2020). Vegetable waste is also suitable raw material for silage, due to its abundant nutrient content including vitamins, minerals and vegetable fiber (Cao *et al.* 2011; Yang *et al.* 2010). However, the fermentation of silage with vegetable waste as the raw

material has received limited attention, and more knowledge is needed to develop vegetable waste fermentation practices.

Water content and soluble sugar content of silage raw materials are important factors affecting the quality of silage. The high water and low soluble sugar contents of leaf vegetables results in low quality silage. Mixing high moisture content raw materials with high dry matter content materials such as bran, rice bran or straw is an effective way to reduce water drainage and avoid nutrient loss (Denek *et al.* 2006; Cummins *et al.* 2007). Nutritional additives, such as molasses and urea, can increase the nutritional value of silage. Baytok *et al.* (2014) reported that adding molasses can increase the dry matter and crude protein content of corn silage. Molasses also improved the fermentation quality of mixed rice straw, vegetable by-product and alfalfa silage as indicated by higher lactic acid and lower pH and ammonia-N contents (Wang *et al.*, 2017).

Silage fermentation depends on the microbial communities during ensiling. In a high-throughput sequencing analysis of microbial communities in the fermentation of soybean, Ni *et al.* (2017) showed that *Lactobacillus* was the dominant genus in the fermentation process. Ogunade *et al.* (2018) examined the effects of adding *Escherichia coli* O157:H7 with or without chemical and microbial additives on the bacterial diversity and composition of alfalfa silage, and found that *Lactobacillus*, *Weissella* and *Pediococcus* were the predominant genera. LAB have been found to be the dominant group in the fermentation of different silage materials and to affect the quality of silage. However, the functions of LAB and other microorganisms in silage have not received attention. Functional Annotation of Prokaryotic Taxa (FAPROTAX) has been successfully used to predict microbial functions in ecosystems like soil and waste water (Gu *et al.* 2019; Li *et al.* 2019; Louca *et al.* 2016). Studying the functional variation could elucidate the microbial functions during silage fermentation, and how microbial communities affect the whole silage ecosystem.

In this study, we used lettuce waste as the main raw material for silage, analyzed changes in the physico-chemical characteristics and bacterial community composition of silage, identified differentially abundant taxa, and predicted the functional profiles of

bacterial communities during silage fermentation via amplicon sequencing of the 16S rRNA gene, and determined the associated effects on the quality of silage.

Material and methods

Ensiling material and silage preparation

Lactuca sativa var. *asparagina* Bailey (lettuce) waste, dry rice straw, wheat bran and molasses were collected from local farms in Chengdu, Sichuan, China, and their nutrient compositions were analyzed (Table S1). The lettuce waste and rice straw were chopped into 1-2 cm pieces, and the molasses was diluted with water in a ratio of 1:2. Silage samples, composed of 200 g lettuce waste, 30 g dry rice straw, 30 g wheat bran, and 40 ml molasses per sample, were inoculated with *Lactobacillus plantarum* C20, isolated from vegetable waste in our laboratory, at a rate of 10^5 colony forming units g^{-1} of fresh silage. The silage was packed into plastic bags that were sealed using a vacuum sealer and kept at room temperature (approximately 26 °C to 28 °C). Three bags per day were sampled on day 0, 7, 15, 30, 45 and 60.

Chemical and nutritional composition analysis

Ten gram of silage was suspended in 90 mL of sterilized distilled water and kept at 4 °C for 24 h. The pH was measured using a glass electrode pH meter (FiveGo, Mettler Toledo, Greifensee, Switzerland). Ammonia nitrogen (AN) content was determined using a spectrophotometer (UV/VIS Spectrometer, PG Instruments Ltd., London, UK) according to Fawcett and Scott (1960). The organic acid (lactic acid, LA; acetic acid, AA; propionic acid, PA; butyric acid, BA) and water-soluble carbohydrate (WSC) contents were measured using HPLC as described by Cai (2004) using a SC1011 column (8.0 mm × 30 cm; Shoko, Tokyo, Japan), 80 °C oven temperature, water as the mobile phase at 1.0 mL/min, and RI-1530 detector (Jasco Corp., Tokyo, Japan). The dry matter (DM), total nitrogen (TN), ether extract (EE) and crude ash (CA) contents were analyzed according to the standard procedures by the Association of Official Analytical Chemists (AOAC 1989). Crude protein (CP) content was calculated by multiplying TN by 6.25. The neutral detergent fiber (NDF) and acid detergent

fiber (ADF) were analyzed as described by Van Soest *et al* (1991). Total amino acid (TAA) content was analyzed using an automatic amino acid analyzer (L-8800, Hitachi, Japan).

Determining numbers of culturable micro-organisms

The numbers of colony forming units (cfu) in fresh silage were determined by mixing 10 g of silage and 90 mL of sterilized water, followed by serial dilution from 10^{-1} to 10^{-9} in sterilized water. To quantify LAB, serial dilutions were inoculated on de Man, Rogosa, and Sharpe (MRS) agar and incubated at 37 °C for 48 h under anaerobic conditions. Yeasts and molds were quantified on Potato Dextrose Agar incubated at 28 °C for 24 h. The yeasts were distinguished from molds and bacteria by colony appearance and cell morphology. Aerobic bacteria were quantified on Nutrient Agar incubated at 30 °C for 24 h under aerobic conditions.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from 10 g of silage. The silage was mixed with 90 mL sterile 0.85% NaCl, shaken at 120 rpm for 2 h, filtered through three layers of gauze cloth, and centrifuged at 10,000 rpm for 10 min at 4 °C. The pellet was suspended in 3 mL sterile 0.85% NaCl solution, centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was discarded. DNA was extracted from the pellet using a Fast DNA™ SPIN Kit (MP Biomedicals, Solon, OH, USA) and eluted in a final volume of 70 µL. The quantity and quality of DNA was assessed by agarose gel electrophoresis. DNA samples were stored at -20 °C.

The 16S rRNA gene V4 hypervariable region was amplified with primers 515F (5'-GTGCC-AGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3') with adapter and barcode sequences (Caporaso *et al.* 2012). The reaction mixture contained 3 U Ex Taq HS DNA polymerase (TaKARA Shuzo Co., Shiga, Japan), 5.0 mM dNTP mixture (TaKARA), 2.0 mM MgCl₂, 5.0 µL of 10× Ex Taq Buffer (TaKARA), 0.6 µM of each primer, 4.0 ng of DNA, and ddH₂O to 50 µL. The PCR procedure included initial denaturation at 94 °C for 4 min, 30 cycles of 15 s at 94 °C, 15 s at 55 °C and 30 s at 72 °C, and a final extension at 72 °C for 10 min. PCR products were purified using PCR Clean-up Purification Kit (MP

Biomedicals, Solon, OH, USA) and quantified using Qubit 2.0 fluorimeter (Invitrogen, Carlsbad, CA, USA). Purified amplicons were pooled in equimolar concentrations and sequenced using MiSeq Reagent Kit V2 on an Illumina MiSeq platform (Paisennuo, Shanghai, China). The sequence data were submitted to NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra/>) with accession number PRJNA637532.

Bioinformatics analysis

The sequencing reads were assembled using FLASH V1.2.7 (Magoc and Salzberg 2011). Low quality reads were removed according to the QIIME quality control process (V1.7.0) (Caporaso *et al.* 2010). Chimeric sequences were removed by using both UCHIME denovo and UCHIME reference (Edgar 2013). Sequences were assigned into operational taxonomic units (OTUs) at 97% similarity cutoff using Uparse v7.0.1001 (Edgar 2013). The representative sequences of the OTUs were aligned to the Greengenes Database (DeSantis *et al.* 2006) based on the RDP classifier algorithm V2.2 to annotate taxonomic information.

Statistical analysis

Alpha diversity indices (Chao1, Shannon, Simpson and ACE) were calculated with QIIME software (Version 1.7.0) using the Statistical Package for the Social Sciences (SPSS Version 19.0, SPSS Inc., Chicago, IL, USA). One-way ANOVA was done to test for differences in the nutrient composition, microbial population and alpha diversity. Differences were considered statistically significant at $p < 0.05$. Relative abundances of taxa at the phylum and genus levels were visualized using the package in R v3.4 (Pollard *et al.* 2015). Distance decay analysis was applied to test the rate of decay of microbial community similarity (Bray-Curtis) along the silage fermentation process. Non-metric multidimensional scaling (NMDS) based on the unweighted UniFrac distance was done using the Vegan package in R (Oksanen *et al.* 2017). Differential abundance of the OTUs were tested using DESeq2 as described previously (Gu *et al.* 2018; Love *et al.* 2014). Briefly, relative abundances of OTUs at T0 (day 0, initial stage), T1T2 (day 7 and day 15, early stage), and T3T4T5 (day 30, day 45 and day 60, middle and late stages) were compared to each other, and OTUs with twofold higher or lower relative abundance ($p < 0.05$) were considered differentially abundant.

Functional prediction

The functional profiles of the microbial communities were predicted using FAPROTAX (Louca *et al.* 2016). Correlation between the nutrient compositions and predicted functions were analyzed using distance based redundancy analysis (dbRDA) in R package vegan (Oksanen *et al.* 2017). The relative importance of each variable in the dbRDA model was estimated using the envfit function. Differential functions were tested using Kruskal-Wallis rank sum test ($p < 0.05$), and visualized in a heatmap. The contributions of taxa to differences in functional profiles were estimated based on relative abundances using Python and R scripts (Oksanen *et al.* 2017).

Results

Changes in silage characteristics and culturable micro-organisms during ensiling

Silage pH decreased from 5.93 to 3.98 from day 0 to day 45 (Table 1). Ammonia nitrogen (AN) to total nitrogen (TN) ratio and lactic acid (LA) and butyric acid (BA) contents increased from 1.87 to 5.03%, 1.24 to 2.46% and 0.04 to 0.27%, respectively, during the 60 day fermentation ($p < 0.05$). Acetic acid (AA), propionic acid (PA) and total amino acid (TAA) contents decreased from 0.25 to 0.15%, 0.52 to 0.22% and 8.95 to 6.41%, respectively ($p < 0.05$). Dry matter (DM), crude protein (CP), acid detergent fiber (ADF), and water soluble carbohydrate (WSC) contents decreased within 60 days of silage, and ether extract (EE) and crude ash (CA) contents increased ($p < 0.05$) within 45 days of ensiling. The neutral detergent fiber (NDF) content fluctuated slightly with no clear trend during the ensiling. For most of the characteristics, the biggest changes were detected after 7 or 15 days.

The number of culturable LAB increased after the first 7 days ($p < 0.05$) and remained relatively stable thereafter (Table 2). The number of molds decreased from day 0 to day 7 ($p < 0.05$) and was below detection level afterwards. The number of yeasts and aerobic bacteria decreased from day 0 to day 7 ($p < 0.05$) and remained relatively stable later on.

Bacterial diversity during ensiling

A total of 614151 sequences from the 18 samples passed the quality criteria, with an average of 34119 sequences per sample. The sequences were assigned to 3311 OTUs at 97%

similarity level. The rarefaction curves (Fig. S1) indicated that the OTUs represented well the sampled populations. The Shannon index decreased slightly from day 0 to day 45 ($p < 0.05$). The Chao1 index was lowest on day 0 ($p < 0.05$) (Table 3).

Proteobacteria (96.25%) was the dominant phyla on day 0 (Fig. 1A). The relative abundance of *Firmicutes* was highest on day 7 (81.86%), after which the relative abundances of *Firmicutes* decreased gradually and that of *Proteobacteria* increased. At the genus level, the relative abundances of *Pseudomonas* (59.27%) and *Pantoea* (24.93%) were highest on day 0 (Fig. 1B). From day 7 to day 60, *Lactobacillus* (58.38-73.70%) and *Kluyvera* (5.83-18.08%) dominated the populations. The relative abundance of *Pediococcus* was high on day 15 (9.28%).

In the NMDS based on the unweighted UniFrac distances, the bacterial communities on day 0 were clearly separated from those on day 7 to day 60 (Fig. 2). Communities on day 7 and day 15 (the early stage of silage fermentation) and day 30 to day 60 (the middle and late stages of silage fermentation) grouped separately. Distance decay analysis based on Bray-Curtis similarity confirmed that the communities on day 0 were clearly different from those on later sampling days ($R = -0.45$, $p < 0.001$), and the within sampling day similarities were approximately on the same level ($R = 0.33$, $p > 0.05$) (Fig. 3).

Differential abundance analysis

Altogether 1164 (35.16%) differentially abundant OTUs were detected between day 0 and the early stage of silage fermentation (day 7 and day 15), 1290 (38.96%) between day 0 and the middle and late stages of silage fermentation (day 30 to day 60), and 645 (19.48%) between the early stage of silage fermentation and the middle and late stages of silage fermentation (Fig. 4). At the phylum level (Fig. S2A and Table S2), the relative abundances of *Firmicutes* were higher and those of *Actinobacteria* lower at the early, middle and late stages of silage fermentation than at day 0. Compared to the early stage of fermentation, the relative abundances of *Bacteroidetes* were higher at day 0 and at the middle and late stages, and those of *Proteobacteria* and *Actinobacteria* were higher at the middle and late stages. The relative abundances of thirteen genera were higher and those of 36 genera lower at the early

stage of silage fermentation than at day 0 (Fig. S2B and Table S3): the more abundant genera included *Lactobacillus*, *Weissella*, *Lactobacillaceae* *unidentified*, *Pediococcus* and *Kluyvera*, and the less abundant genera included *Pseudomonas*, *Samsonia*, *Franconibacter*, *Sphingobacterium* and *Curtobacterium*. The relative abundances of fourteen genera were higher and those of 38 genera lower at the middle and late stages of silage fermentation than at day 0, including *Kluyvera*, *Lactobacillus*, *Citrobacter*, *Pediococcus*, *Leuconostoc* and *Pseudomonas*, and *Samsonia*, *Curtobacterium*, *Franconibacter* and *Pantoea*, respectively. The relative abundances of eight genera were higher and those of four lower at the middle and late stages than at the early stage, including *Allorhizobium*, *Sphingobacterium*, *Brachybacterium* and *Ochrobactrum*, and *Pediococcus*, *Lactobacillaceae* *unidentified*, *Weissella* and *Timonella*, respectively.

Functional prediction

In line with the NMDS (Fig. 2), the predicted functions on day 0 were separated from those on day 7 to day 60. The differences in the predicted functions were associated with differences in pH and AA, TAA, CP, WSC, $\text{NH}_3\text{-N}$, EE and CA contents ($p < 0.001$) (Fig. 5). Among the predicted functions, nitrite ammonification and nitrate ammonification were more prevalent after day 0 (Fig. 6A), and denitrification, invertebrate parasites, cellulolysis, aromatic hydrocarbon degradation, hydrocarbon degradation, aromatic compound degradation, plastic degradation, dark thiosulfate oxidation and dark oxidation of sulfur compounds were less prevalent after day 0. Chemoheterotrophy was prevalent during the whole ensiling period.

The contribution of taxa to the predicted functions was estimated based on relative abundances. Five taxa contributed 100% to a single function: *Curtobacterium* to aerobic chemoheterotrophy, and *Erwinia*, *Leuconostoc*, *Pediococcus* and *Weissella* to fermentation (Fig. 6B). The 100% contribution of *Bromus tectorum* to chloroplasts was probably an artefact created by the FAPROTAX function prediction program. Eight genera contributed to at least five functions.

Discussion

Silage fermentation is a complex process where various bacteria affect the chemical composition and nutritional quality of the silage (Hu *et al.* 2009). Elucidating the community composition and variation, and functional roles of the bacteria in silage will increase our understanding of vegetable waste silage fermentation. Previous studies have mainly focused on the diversity, abundance and composition of the bacterial communities in alfalfa or corn silage (Guan *et al.* 2018; Ogunade *et al.* 2018), and the potential functional profiles in vegetable waste silage have not been addressed.

Fermentation products and physicochemical characteristics, such as pH and lactic acid (LA), acetic acid (AA) and ammonium nitrogen (AN) contents, indicate the nutritional quality of silage (Liu *et al.* 2019; Wang *et al.* 2019). In our study, the pH value of the lettuce based vegetable waste silage was less than 4.0 after 60 days of fermentation with lactic acid bacteria (LAB) inoculation, indicating that the ensiling had resulted in good quality silage. Throughout the ensiling, the biggest changes in fermentation products and physicochemical characteristics occurred at the early stage of silage fermentation. Possibly the rapid increase in LAB in the early stage of the fermentation allowed them to convert a large amount of water soluble carbohydrates (WSC) into organic acids, which led to a decrease in pH and made the silage a stable, anaerobic and acidic environment (Duniere *et al.* 2013; McDonald 1981). At the same time, crude protein (CP) decomposition by microorganisms led to an increase in AN content. The AN concentration in silage is an indicator of proteolysis during ensiling, and it is usually driven by plant proteases and proteolytic *Clostridia* (Pahlow *et al.*, 2003). During the middle and late stages of silage fermentation, the physicochemical characteristics and the numbers and community composition of microorganisms remained relatively stable, indicating that the early stage of silage fermentation is the key stage in determining the final quality of silage.

Silage is a complex microbial system. A variety of microorganisms participates in the silage fermentation, including microorganisms attached to the raw materials, fermenting microorganisms and spoilage microorganisms (Muck 2013). Thus, it is valuable to further clarify bacterial diversity, composition and variation during the fermentation. Barnett (1954)

studied the fermentation process and concluded that, at the first stage, carbon dioxide is released by respiration of the microorganisms attached to plants, and that consumes some of the nutrients in the ensiled material. At the second, relatively short stage, cocci-type bacteria and fungi produce acetic acid. At the third stage after approximately three days of fermentation, LAB produce lactic acid and the lactic fermentation begins. At the fourth stage that lasts until approximately 17-21 days, lactic acid content reaches a peak and pH decreases to 4.2 or lower. At the fifth stage water-soluble carbohydrates are exhausted and pH does not decrease, and the butyric acid bacteria produce butyric acid. Based on the changes in microbial communities, the fermentation could be separated into three stages. Hu *et al.* (2018) divided the microbial communities in maize silage fermentation into an early-ensiling cluster, a midterm-ensiling cluster, a late-ensiling cluster and an aerobic exposure cluster. In our study, the communities were divided into an initial community at day 0 dominated by *Proteobacteria*, early stage communities and middle and late stage communities. This is consistent with that of Yuan *et al.* (2019) and Ali *et al.* (2020), who demonstrated that *Proteobacteria* was the most prevalent phylum in fresh napier grass and rapidly replaced by *Firmicutes* during ensiling.

Firmicutes and *Proteobacteria* have been found as the predominant phyla in grass (Eikmeyer *et al.* 2013) and alfalfa silages (McGarvey *et al.* 2013). The two phyla were also the most abundant phyla in barley silages with or without LAB inoculation; at the end of fermentation, LAB accounted for 99% of the total bacterial abundance in the inoculated silage (Liu *et al.* 2019). Different materials have different epiphytic bacteria (Wang *et al.* 2019), and *Proteobacteria* is the dominant phyla in lettuce (96.25%). Consistent with the finding that *Proteobacteria* was the most prevalent phylum in fresh napier grass and rapidly replaced by *Firmicutes* during ensiling (Yuan *et al.* 2019), the *Firmicutes* increased to over 80% on day 7. The transition from *Proteobacteria* to *Firmicutes* during the ensiling is probably due to change from aerobic to anaerobic condition (Yuan *et al.* 2019; Ali *et al.* 2020).

LAB have an important role in silage fermentation (Eikmeyer *et al.* 2013; Langston and Bouma 1960). The majority of silage LAB involved in lactic acid fermentation belong to the

genera *Lactobacillus*, *Pediococcus*, *Lactococcus* and *Weissella*. Inoculation of LAB can result in a rapid drop in pH and high LA production (Wang *et al.* 2018). Consistent with this, inoculation of *L. plantarum* enhanced the LA production and decreased the pH rapidly in the early stage of ensiling. A recent meta-analysis concluded that inoculation with *L. buchneri* increased the concentration of acetate, reduced yeast counts and improved aerobic stability (Arriola *et al.* 2021). However, the effects of LAB inoculation on ensiling different raw materials are not necessarily similar. Inoculation with *Lactobacillus plantarum* and *Enterococcus faecium* did not effectively delay aerobic spoilage of barley silage (Inglis *et al.* 1999). Compared with ensiling corn, LAB inoculation affected more the fermentation and aerobic stability of barley silage (Addah *et al.* 2011).

Lactococcus, *Leuconostoc*, *Pediococcus* and *Weissella* are often detected in the early stage of silage fermentation (Gharechahi *et al.* 2017; Zheng *et al.* 2017). In agreement, *Lactobacillus*, *Weissella* and *Pediococcus* were abundant in all except the initial stage of vegetable waste ensiling. Generally, *Pediococcus* are the dominant LAB during the early stage of ensiling and initiate lactic acid fermentation; *Lactobacillus* become dominant as the pH decreases (Liu *et al.* 2019; Zhou *et al.* 2016). In this study, the relative abundance of *Pediococcus* was higher in the early stage of the silage fermentation, but *Lactobacillus* was the dominant genus during the whole process, possibly due to using *Lactobacillus plantarum* as an inoculant. The relative abundances of *Pseudomonas* and *Pantoea* were high on day 0 and those of *Lactobacillus* and *Kluyvera* from day 7 onwards. Ogunade *et al.* (2018) reported that *Pantoea* correlated negatively with AN concentration in silage, and suggested that *Pantoea* may have inhibited proteolytic bacteria. *Pseudomonas* that can thrive in aerobic and anaerobic conditions (Arai H. 2011) were detected in smaller relative abundances on day 7. In alfalfa silage, *Pseudomonas* correlated negatively with AN concentration and yeast count, suggesting that *Pseudomonas* may contribute to protein preservation and yeast inhibition (Ogunade *et al.* 2018). In wastewater treatment, *Pseudomonas* species were more abundant toward the onset of nitrification where ammonia is oxidized to nitrite and then to nitrate (Krishna *et al.* 2013), which may occur in silage fermentation as well. *Kluyvera* sp. is a type

of bacteria involved in amine production, which is reported to be positively correlated to all metabolic functional features (Zhang et al. 2016), and the increase in the abundance of *Kluyvera* was accompanied with an increase in nitrogen content in the middle and late stages of ensiling.

The proteobacterial *Enterobacter* has been frequently detected in the silage fermentation. *Enterobacter* strains are among the principal competitors of LAB for WSC in silage and produce acetic acid, succinic acid, 2,3-butanediol and endotoxins, which can decrease the nutritional quality of silage (Muck and E. 2010). However, Östling and Lindgren (2006) concluded that *Enterobacter* might account for the rapid decrease in undesirable microorganisms during the ensiling process and increase the storage stability of silage.

FAPROTAX provides insights into the functions of microorganisms based on functional data of culturable bacteria in databases, applicable to a variety of environmental samples (Galand et al. 2018; Louca et al. 2016). Several abundant taxa, including *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Weissella*, *Buttiauxella*, *Citrobacter*, *Enterobacter* and *Klebsiella* were predicted to participate in fermentation function, suggesting that the organic matter was decomposed by these microorganisms and various metabolites, e.g. LA, PA and BA, were produced under aerobic or anaerobic conditions. Nitrite ammonification and nitrate ammonification increased when the silage fermentation started. Microorganisms, including *Enterobacter*, *Escherichia*, *Shigella* and *Lactobacillus*, mediate the reduction of nitrite or nitrate to ammonium, referred to as dissimilatory nitrate reduction to ammonium (DNRA) (Rutting et al. 2011). DNRA is prevalent in ecosystems like soil and fresh water (Yang et al. 2017). The increase in DNRA was accompanied with an increase in AN and a decrease in pH, and may improve the quality of silage. FAPROTAX has its limitations, thus the sugar metabolism in fermentation requires further study.

In summary, *Firmicutes* and *Lactobacillus* were the dominant taxa during vegetable waste silage fermentation. The microbial communities and the predicted functions changed along the silage fermentation, and the changes were accompanied with changes in the

physico-chemical characteristics, especially with a decrease in pH and increases in AN to TN ratio and LA content.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1 Bacterial community composition at (A) phylum and (B) genus level in the vegetable waste silage during ensiling. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

Figure 2 Non-metric multidimensional scaling (NMDS) of the bacterial communities in the vegetable waste silage during ensiling, based on the Bray-Curtis dissimilarity between samples. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

Figure 3 Distance decay analysis of the bacterial communities in the vegetable waste silage during ensiling, based on the Bray-Curtis similarity between samples. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

Figure 4 Differential abundance analysis of operational taxonomic units (OTUs) in the vegetable waste silage during ensiling. OTUs with twofold higher (green) or lower (red) relative abundance ($p < 0.05$) were considered differentially abundant. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

Figure 5 Redundancy analysis (RDA) of predicted functional profiles in the vegetable waste silage during ensiling. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. DM, dry matter; AN, ammonia nitrogen; TN, total nitrogen; LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid; TAA, total amino acid; FM, fresh material; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water soluble carbohydrate; CA, crude ash. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

Figure 6 (A) The differential functions among the predicted functional profiles in the vegetable waste silage during ensiling, and (B) the estimated contributions of bacterial genera to the functions based on their relative abundances. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

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Tables

Table 1 Physico-chemical characteristics of the vegetable waste silage during ensiling

Time	DM (%)	pH	AN/TN (%)	LA (%)	AA (%)	PA (%)	BA (%)	TAA (%)	CP (%)	EE (%)	ADF (%)	NDF (%)	WSC (%)	CA (%)
T0	28.9±0.03b	5.9±0.03d	1.9±0.04a	1.2±0.02a	0.23±0.01 c	0.5±0.01d	0.04±0.00 b	8.9±0.21d	13.5±0.12f	3.0±0.08a	18.7±0.09 d	34.3±0.05 c	19.9±0.11 a	9.3±0.15a
T1	28.5±0.21a b	4.5±0.00c	4.4±0.08b	1.6±0.02b	0.2±0.01b	0.4±0.00c	0.02±0.00 ab	7.7±0.06c	12.1±0.18 e	3.4±0.01b	18.5±0.20 cd	34.5±0.07 c	13.3±0.05 b	10.1±0.14 b
T2	28.1±0.16a	4.1±0.01b	4.8±0.34c	1.7±0.01c	0.2±0.00b	0.3±0.01b	0.02±0.00 a	7.5±0.06c	11.6±0.15 d	3.5±0.08b c	18.2±0.07 abc	34.1±0.03 b	12.3±0.05 b	11.4±0.24 c
T3	28.2±0.14a	4.0±0.01ab	4.7±0.04c	2.1±0.15d	0.2±0.01b	0.2±0.00a	0.13±0.01 c	7.3±0.10b c	11.2±0.05 c	3.7±0.07c d	18.4±0.05 bc	35.2±0.01 d	10.7±0.13 c	11.6±0.13 c
T4	28.0±0.11a	4.0±0.01a	4.9±0.07c	2.5±0.02c	0.2±0.01b	0.2±0.00a	0.20±0.00 d	7.1±0.08b	10.5±0.09 b	3.8±0.06d	18.1±0.24 ab	34.4±0.04 c	10.5±0.06 d	11.7±0.10 c
T5	28.15±0.18 a	3.99±0.01a	5.03±0.04 d	2.14±0.00 d	0.15±0.00 a	0.22±0.01 a	0.27±0.01 c	6.41±0.18 a	10.06±0.0 7a	3.59±0.08 bc	17.87±0.1 1a	33.90±0.0 1a	10.22±0.0 5e	10.16±0.0 4b

Data are average ± SEM (n = 3). Different letters in a column denote statistically significant differences ($p < 0.05$). Nutrient contents are percentages of the dry matter. DM, dry matter; AN, ammonia nitrogen; TN, total nitrogen; LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid;

TAA, total amino acid; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water soluble carbohydrate; CA, crude ash. T0, day 0 (after the inoculation); T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

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Table 2 Culturable micro-organisms in the vegetable waste silage during ensiling

Time	LAB	Mold	Yeast	Aerobic bacteria
	(lg CFU/g FM)	(lg CFU/g FM)	(lg CFU/g FM)	(lg CFU/g FM)
T0	4.2±0.49a	2.45±0.33b	5.8±0.38c	7.3±0.16c
T1	8.2±0.24bc	1.49±0.49a	4.0±0.32b	4.2±2.13b
T2	8.4±0.27bc	BLD	3.7±0.22ab	3.6±0.29ab
T3	8.9±0.13c	BLD	3.2±0.13ab	3.2±0.27a
T4	8.2±0.20bc	BLD	3.2±0.66ab	3.0±0.46a
T5	7.8±0.11b	BLD	2.9±0.16a	3.1±0.23a

Data are expressed as the average ± SEM. Different letters in a column denote statistically significant differences at $p < 0.05$ ($n=3$). LAB, lactic acid bacteria; FM, fresh material. T0, day 0 (after the inoculation); T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day. BLD, below the detection level.

Table 3 Alpha diversity of the bacterial communities in the vegetable waste silage during ensiling

	Number of sequences	Simpson	Shannon	Chao1	ACE
T0	36564±474a	0.964±0.007a	6.89±0.18ab	721.3±14.1c	727.8±11.7c
T1	30713±842a	0.965±0.006a	7.29±0.07a	1671.4±196.3a	1584.9±121.6a
T2	34947±601a	0.945±0.004ab	6.57±0.13bc	1168.6±16.4b	1145.7±13.3b
T3	36639±3672a	0.949±0.009ab	6.70±0.17abc	1638.1±106.1a	1438.8±47.5b
T4	34202±476a	0.934±0.008b	6.21±0.35c	1238.2±181.6ab	1146.0±153.5ab
T5	31651±1984a	0.946±0.004ab	6.59±0.02bc	1405.9±168.7ab	1273.9±105.7b

Data are expressed as the average ± SEM. Different letters in a column denote statistically significant differences at $p < 0.05$ (n=3). LAB, lactic acid bacteria; FM, fresh material. T0, day 0 (after the inoculation); T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

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Figure 1 Bacterial community composition at (A) phylum and (B) genus level in the vegetable waste silage during ensiling. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

185x189mm (300 x 300 DPI)

Figure 2 Non-metric multidimensional scaling (NMDS) of the bacterial communities in the vegetable waste silage during ensiling, based on the Bray-Curtis dissimilarity between samples. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

149x136mm (300 x 300 DPI)

Figure 3 Distance decay analysis of the bacterial communities in the vegetable waste silage during ensiling, based on the Bray-Curtis similarity between samples. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

231x127mm (300 x 300 DPI)

Figure 4 Differential abundance analysis of operational taxonomic units (OTUs) in the vegetable waste silage during ensiling. OTUs with twofold higher (green) or lower (red) relative abundance ($p < 0.05$) were considered differentially abundant. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

245x91mm (300 x 300 DPI)

Figure 5 Redundancy analysis (RDA) of predicted functional profiles in the vegetable waste silage during ensiling. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. DM, dry matter; AN, ammonia nitrogen; TN, total nitrogen; LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid; TAA, total amino acid; FM, fresh material; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water soluble carbohydrate; CA, crude ash. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 60.

256x219mm (300 x 300 DPI)

Figure 6 (A) The differential functions among the predicted functional profiles in the vegetable waste silage during ensiling, and (B) the estimated contributions of bacterial genera to the functions based on their relative abundances. T0, day 0; T1, day 7; T2, day 15; T3, day 30; T4, day 45; T5, day 6

187x202mm (300 x 300 DPI)