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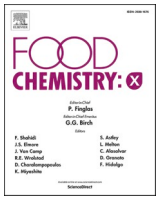

Use of *Lactiplantibacillus plantarum* ZJ316 as a starter culture for nitrite degradation, foodborne pathogens inhibition and microbial community modulation in pickled mustard fermentation

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ABSTRACT

The potential of *Lactiplantibacillus plantarum* ZJ316 (ZJ316) as a starter culture for quality improvement and microbial community regulation in pickled mustard fermentation was elucidated in this study. Our results show that ZJ316 can deter the occurrence of nitrite peaks and maintain the nitrite content of pickled mustard at a low level (0.34 mg/kg). The headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry results indicate that ZJ316 gives a good flavor to pickled mustard. According to the 16S rDNA results, Firmicutes were the predominant microbiota after inoculation with ZJ316, and the abundances of *Citrobacter*, *Enterobacter*, and *Proteus* decreased simultaneously. In addition, antibacterial activity analysis showed that the supernatant of pickled mustard inoculated with ZJ316 had a significant inhibitory effect on *Staphylococcus aureus* D48, *Escherichia coli* DH5α, and *Listeria monocytogenes* LM1. In conclusion, *L. plantarum* ZJ316 has potential for use as an ideal starter in the process of vegetable fermentation.

1. Introduction

Pickled mustard is a kind of semi-dry pickled vegetable product using the root as raw material (Chen et al., 2013). It is known as one of the three most famous pickles in the world, together with European pickles and west German sweet and sour cabbage. Due to the high contents of proteins, amino acids, fiber and minerals, pickled mustard could provide multiple health advantages for humans, including prevention of constipation and cancer, reduction of serum cholesterol and antioxidant effect (Li, 2003). Pickled mustard is usually made through a process of dehydration, salting and microbial fermentation. However, various issues arise throughout the fermenting processes, such as high content of salt and nitrite, which may be accompanied by the contamination of pathogenic bacteria, including *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli*. Therefore, it is of vital importance to control the nitrite content and microbial composition in the pickled mustard to maintain food safety.

Lactic acid bacteria (LAB), a type of probiotic, are generally recognized as safe and often used in fermented foods. LAB strains could produce several metabolites (organic acids, hydrogen peroxide and bacteriocins, etc.) to inhibit the growth of foodborne pathogens and degrade nitrite (Stoianova et al., 2012). Kim et al. (2021) showed that inoculation of *Limosilactobacillus fermentum* J2 increased ferulic acid content in fermented rice bran and no visible growth of foodborne pathogens were found during fermentation. Coelho et al. (2014) revealed that two bacteriocin-producing LAB strains significantly controlled *L. monocytogenes* in cheese, reducing by roughly 5 log units after 7 days when compared with that absence of these LAB. In addition, many studies proved that LAB can promote the formation of a low-pH environment and inhibit the growth of nitrate-reducing bacteria, thereby reducing the content of nitrite in fermented pickle (Xia et al., 2017; Yan et al., 2008).

Inoculation of LAB also facilitates the formation of flavor substances. *Lactiplantibacillus plantarum* (Zheng et al., 2020), *Leuconostoc mesenteroides* and *Pediococcus ethanolidurans* are the most common microbiota in pao cai products. Yun et al. (2021) reported that *Lactiplantibacillus*

Abbreviations: *L. plantarum* ZJ316, *Lactiplantibacillus plantarum* ZJ316; LAB, Lactic acid bacteria; SCFAs, Short-chain fatty acids; HS-SPME/GC/MS, Headspace solid-phase micro-extraction/gas chromatography/mass spectrometry.

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plantarum improved sourness and sweetness in pao cai, whereas *Leuconostoc mesenteroides* and *Pediococcus ethanolidurans* made the paocai with prominent umami taste. Ma et al. (2021) used LAB to ferment black garlic and found that the contents of components with unpleasant baking flavors (e.g., furfural, 2-acetyl furan, and 5-methyl furfural) were reduced, whereas that of green grass, floral and fruit aromas increased apparently.

Inoculation of LAB has great potential to improve the quality of fermented vegetables. Nevertheless, limited studies have investigated the dynamics of microbial community during fermentation after LAB's inoculation. Furthermore, the field has not yet identified a kind of LAB strain for pickles mustard that can simultaneously reduce nitrite content, modulate microbial composition and generate specific flavors.

L. plantarum ZJ316 was isolated from healthy newborn infant fecal samples by our laboratory. In previous studies, we purified antimicrobial components such as plantaricin NC8 (class IIb LAB bacteriocin) (Jiang et al., 2018) and phenyl lactate from *L. plantarum* ZJ316 (Zhou et al., 2020), which exhibited significant antibacterial efficacy against common foodborne pathogens. The capacity of *L. plantarum* ZJ316 to grow in a low-pH environment with excellent acid tolerance further indicated its potential to break down nitrite (Zhou et al., 2021). The goal of this study was to compare *L. plantarum* ZJ316 inoculation, commercially available bacterial inoculation, and natural fermentation during pickled mustard fermentation, as well as to investigate for the first time the effect of *L. plantarum* ZJ316 as a starter on pH, LAB content, nitrite content, volatile compounds, and bacterial community during different fermentation periods of pickled mustard. These findings could help the field of fermented vegetables better understand *L. plantarum* ZJ316 as a potential starter for pickled mustard.

2. Materials and methods

2.1. Preparation of starter culture

The *L. plantarum* ZJ316 strain was isolated from the feces of healthy newborns and deposited at the China Center for Type Culture Collection (CCTCC) under the strain number CCTCC no. M 208077. The strain was stored in 50% (*v/v*) glycerol at −80 °C, cultured in de Man, Rogosa, and Sharpe (MRS) broth (Hopebio, Qingdao, China), Static culture at 37 °C for 24 h, and propagated twice in MRS broth at 37 °C for 18 h. The cells were washed 3 times and resuspended in 0.9% (*w/v*) sodium chloride solution to produce LAB suspension for starter inoculation. Finally, the strain density of ZJ316 was 1.5×10^9 CFU/ mL.

2.2. Fermentation of pickled mustard and sampling

In this experiment, pickled mustard samples were obtained from Tongqianqiao Food and Vegetable Co., Ltd (Ningbo, China). The commercial *L. plantarum* starter (composed of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Leuconostoc mesenteroides*) was purchased from Pinyue workshop technology development Co., Ltd. Pickled mustards were washed, cut, and put into three jars for sampling. Afterward, the material was added to pure water with 8% salt and submerged, and then starters were added to the samples. In group ZJ316 and group C (with commercial starter), 1.5×10^6 CFU/mL of starters were inoculated, and no starter was added in the natural fermentation group (group N). The jars were sealed with water to exclude air during fermentation. All samples were fermented at room temperature for 29 days, and the fermentation supernatant and tubers were collected on day 0, day 1, day 5, day 9, day 13, day 17, day 21, day 25 and day 29, respectively.

2.3. Measurement of pH value and LAB enumeration

The pH values of pickled mustard supernatant were measured by a pH meter (METTLER TOLEDO, Shanghai, China) on day 0, day 1, day 5, day 9, day 13, day 17, day 21, day 25, and day 29, respectively. The supernatant of the fermented sample was diluted with normal saline and spread on MRS solid medium. Each sample was incubated at 37 °C for 48 h, and the number of LAB in the pickled mustard samples was calculated by counting the colony-forming units (CFU) per gram in each sample.

2.4. Measurement of nitrite residue

Pickled mustard samples (5 g) were collected on day 0, day 1, day 5, day 9, day 13, day 17, day 21, day 25, and day 29, respectively. All samples were gently rinsed twice and finely pulverized in 10 mL of sterile water until no solid particles could be seen. The nitrite residue in pickled mustard was measured by the N-(1-naphthyl)-ethylenediamine dihydrochloride spectrophotometric method according to GB 5009.33-2016.

2.5. Analysis of volatile flavor substances by HS-SPME/GC–MS

After 29 days of fermentation, the influence of *L. plantarum* ZJ316 starter on the volatile flavor components of pickled mustard was assessed by HS-SPME/GC–MS (7890A/5975C, Agilent, USA). Samples were homogenized, filtered, and deposited in headspace injection vials with a volume of 20 mL. Prior to analysis, the headspace injection vials were put in a water bath at 50 °C for 30 min. With a sample injection volume of 1L, a DB-WAX (60 m × 0.25 mm × 0.5 m) column was employed. The temperature was set at 35 °C for 3 min, then escalated to 220 °C at a rate of 3 °C per minute for 10 min. An EI source with a 230 °C ion source temperature and a 150 °C quadrupole temperature was used for mass spectrometry. An EI source with a 230 °C ion source temperature and a 150 °C quadrupole temperature was used for mass spectrometry. The mass number scan range was 33–500 amu, and a computerized mass spectrometry instrument was used to analyze the results of the search for undiscovered chemicals (NSIT14 database). The results of volatile compounds were expressed as relative peak area by normalizing the peak area, as reported by Galvan-Lima et al. (2021) and Xue et al. (2020).

2.6. 16S rDNA gene amplification and high-throughput sequencing analysis

The pickled mustard samples and supernatant during fermentation were collected on day 5, day 18, and day 29, respectively. According to the manufacturer's instructions, the total genomic DNA of different samples was extracted by Omega kit (M5635-02, Omega Bio-Tek, USA). The quality of DNA was determined by ultraviolet spectrophotometer and evaluated by 0.8% agarose gel electrophoresis at 120 V for 40 min. Then DNA was then kept at −80 °C until it was ready to be processed. Illumina high-throughput sequencing was used to examine the extracted DNA template. The V3-V4 hypervariable region of the bacterial 16S rDNA genes was amplified by PCR using the following primers: 341F: 5′-CCTAYGGGRBGCASCAG-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′. The thermal cycling conditions were as follows: 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s, 72 °C for 7 min. According to the preliminary results of electrophoresis, the PCR products were quantitated by fluorescent reagent Quant-iT PicoGreen dsDNA assay kit and pooled with equal amounts. Pair-end sequenced using the Illumina HiSeq 2500 platform at Parsono Biotechnology Co., Ltd., Shanghai, China.

The original amplicons are grouped based on barcode sequences using QIIME2 software, then the paired gene sequences were combined to obtain the initial amplicon, the barcodes and primers were removed, and low-quality amplicons were removed through a quality-control step (Bolyen et al., 2019). Using the "Analysis pipeline" script, several dozen software programs were combined in a particular order to complete the amplicon analysis task (Liu et al., 2019). DADA2 was used to denoise and obtain ASV feature tables, and QIIME2 was used to calculate Alpha-

diversity (Callahan et al., 2016). Beta-diversity analysis based on UniFrac evaluates the differences between samples, usually combined with the dimensionality reduction method Principal Coordinate Analysis (PCoA), to obtain a visual representation. Finally, linear discriminant analysis (LDA) effect sizes (LEfSe) were performed to analyze species with significantly different abundances between groups.

2.7. Analysis of anti-microbial activity

The anti-microbial activities of the group ZJ316, group C and group N fermentation supernatants were tested by the 96-well plate inhibition test method using *Staphylococcus aureus* D48, *Escherichia coli* DH5α, and *Listeria monocytogenes* LM1 as the indicator strains, respectively. On the last day of fermentation, the treated supernatants were collected and kept at 4 °C until use. *S. aureus* D48, *E. coli* DH5, and *L. monocytogenes* LM1 were inoculated at 0.5% in 10 mL of LB broth and incubated at 37 °C for 12 h. The 96-well plate was then filled with 180 μL of bacterial solution and 20 μL of fermented supernatant, which was cultured for 10 h at 37 °C. As a control, LB broth was used. Finally, using a microplate reader (Tecan, Co. Switzerland), the optical density values were measured at 600 nm and expressed as antimicrobial activity.

2.8. Statistical analysis

All the data are presented as the mean ± standard deviations (SD). The difference among groups was analyzed by one-way analysis of variance (ANOVA) and the least significant difference test using SPSS 25.0 software. $p < 0.05$ was defined as statistically significant.

3. Results and discussion

3.1. Dynamics of LAB counts and pH values during pickled mustard fermentation

The changes in the LAB counts in the pickled mustard supernatant during fermentation are shown in Fig. 1A. The LAB counts of both the ZJ316 and C groups showed an increasing and then decreasing trend. The LAB counts of the ZJ316 group increased sharply from the initial value of 6.30 (day 0) to 7.76 Log_{10} CFU/mL (day 1), and then gradually increased and reached a maximum of 8.74 Log_{10} CFU/mL on day 9, after which they continuously decreased, reaching the lowest value of 6.74 Log_{10} CFU/mL. Similarly, the LAB counts of the C group increased significantly from an initial value of 6.17 Log_{10} CFU/mL (day 0) to 7.76 Log_{10} CFU/mL (day 5), reaching a peak of 8.02 Log_{10} CFU/mL on day 9, then decreasing continuously to 5.91 Log_{10} CFU/mL. The LAB counts of the N group only increased from 0 (day 0) to 6.49 Log_{10} CFU/mL till the end (day 29). This might be due to LAB entering the exponential phase and increasing significantly at the early fermentation stage. The later fermentation stage showed a decreasing trend due to the decrease in nutrients and the decrease in LAB. LAB play a crucial role in the late fermentation of pickles (Jeong et al., 2013). Therefore, an increase in LAB number can accelerate the completion of this stage.

The pH changes of the pickled mustard supernatant are also displayed in Fig. 1B. Overall, the pH values of the three fermentation modes decreased rapidly in the early fermentation stage, and the pH value reduction rates were similar. After approximately 13 days, the pH value became relatively stable. Among the groups, the ZJ316 group had the most significant pH reduction compared to that of the C and N groups, and the pH value reached 3.79 at the end of the fermentation. These results show that *L. plantarum* ZJ316 has a strong acid production ability in the fermentation environment of pickled mustard, and can metabolize various organic acids through fermentation. In addition, after nine days of cultivation, the pH of the ZJ316 group was below 4, which could effectively inhibit the formation of nitrite and the growth of harmful microorganisms (Rhee et al., 2011), so as to ensure the safety of the pickled mustard ripening stage.

3.2. Dynamics of nitrite concentration during pickled mustard fermentation

The changes in nitrite concentration during fermentation of pickled mustard are shown in Fig. 1C. Notably, a nitrite peak was formed in the initial stage of group N on day 9, with a maximum value of 10.28 mg/kg, followed by a gradual decrease until the end of day 29. However, the nitrite content of the ZJ316 and C groups with LAB inoculation increased slowly compared to the N group. The nitrite content of the C group showed a slight upward trend, from 1.50 (day 0) to 4.39 mg/kg (day 9), and then decreased and remained relatively stable. The ZJ316 inoculation could further reduce the nitrite content of pickled mustard, and the concentration of the ZJ316 group decreased to almost 0.34 mg/kg by the end of the fermentation. These results show that *L. plantarum* ZJ316 is more effective in inhibiting the abnormal accumulation of nitrite during vegetable fermentation. This phenomenon may be due to the fact that, at the early stage of fermentation, the low salinity environment does not effectively restrain the increase of nitrate-reducing bacteria, which can reduce nitrate to nitrite under anaerobic conditions. As the fermentation process proceeds, the LAB proliferate rapidly, and they become the dominant bacteria inhibiting the growth of nitrate-reducing microorganisms and effectively degrading nitrite (Xia et al., 2017). It has been speculated that LAB might indirectly reduce the content of *N*-nitro-sodimethylamine (NDMA) content in kimchi by inhibiting the formation of the formation NDMA precursors originating from kimchi, thereby improving the nitrite-scavenging ability (Kim et al., 2017). Therefore, it is suggested that *L. plantarum* ZJ316 can be used to improve food quality, especially in nitrite degradation.

3.3. Effect of L. plantarum ZJ316 on the volatile flavor components of pickled mustard during fermentation

After inoculation with different ferments, the volatile components of pickle mustard were examined by HS-SPME/GC–MS, and the retention times (RT), relative peak areas, and CAS numbers are presented in Table 1. By comparing mass spectral quality >80 with NIST14, a total of 81 volatile chemicals were discovered in these three samples, 18 of which have been reported in pickle mustard fermentation. (Lee et al., 2021). Alcohols (15), aldehydes (3), acids (4), esters (13), alkanes and their derivatives (3), olefins and their derivatives (1), benzene and its derivatives (8), sulfates (7), furans (2), and others were found in group 316 (57), which was split into 10 categories. There was no difference between groups of chemicals with peak area percentages more than 5% (Akbar et al., 2020), which were all thiocyanates (allyl isothiocyanate, phenethyl isothiocyanate, and 3-butenyl isothiocyanate), and these volatiles dominated the majority of the samples. Isothiocyanates are breakdown products of the cruciferous vegetable secondary metabolite thioglucoside (GSL), and allyl isothiocyanate (AITC) possesses anti-inflammatory and anti-cancer properties (Jeong et al., 2004; Wagner et al., 2012; Xiao et al., 2003). During the vacuum extraction of volatile oils from cabbage and cauliflower, 3-butenyl isothiocyanates were discovered (Buttery et al., 1976). Because they have a low odor threshold and a distinct odor, they are classified as distinctive aromas.

Alcohols, aldehydes, esters, acids, and other volatiles are generated by the oxidative breakdown of fatty acids under the action of lipoxygenases in processed vegetables (Condurso et al., 2016). 3-hexen-1-ol, 1-octanol, and 1-nonanol were identified during the fermentation of group 316. 3-Hexen-1-ol has been linked to sweetness, and Cheng et al. discovered that increasing the concentration of sugars and volatiles improved sweetness perception (Cheng et al., 2020). The main fragrant active ingredient in Jasmine rice has been discovered as 1-octanol, which affects the sweetness and freshness of stored rice (Buttery et al., 1976). 1-nonanol was found to be crucial in distinguishing spoiled hazelnuts (Stilo et al., 2021). As a result, it was feasible to provide the pickled mustard with a better and outstanding fermentation flavor for the *L.plantarum* ZJ316 inoculation fermentation, and the esters

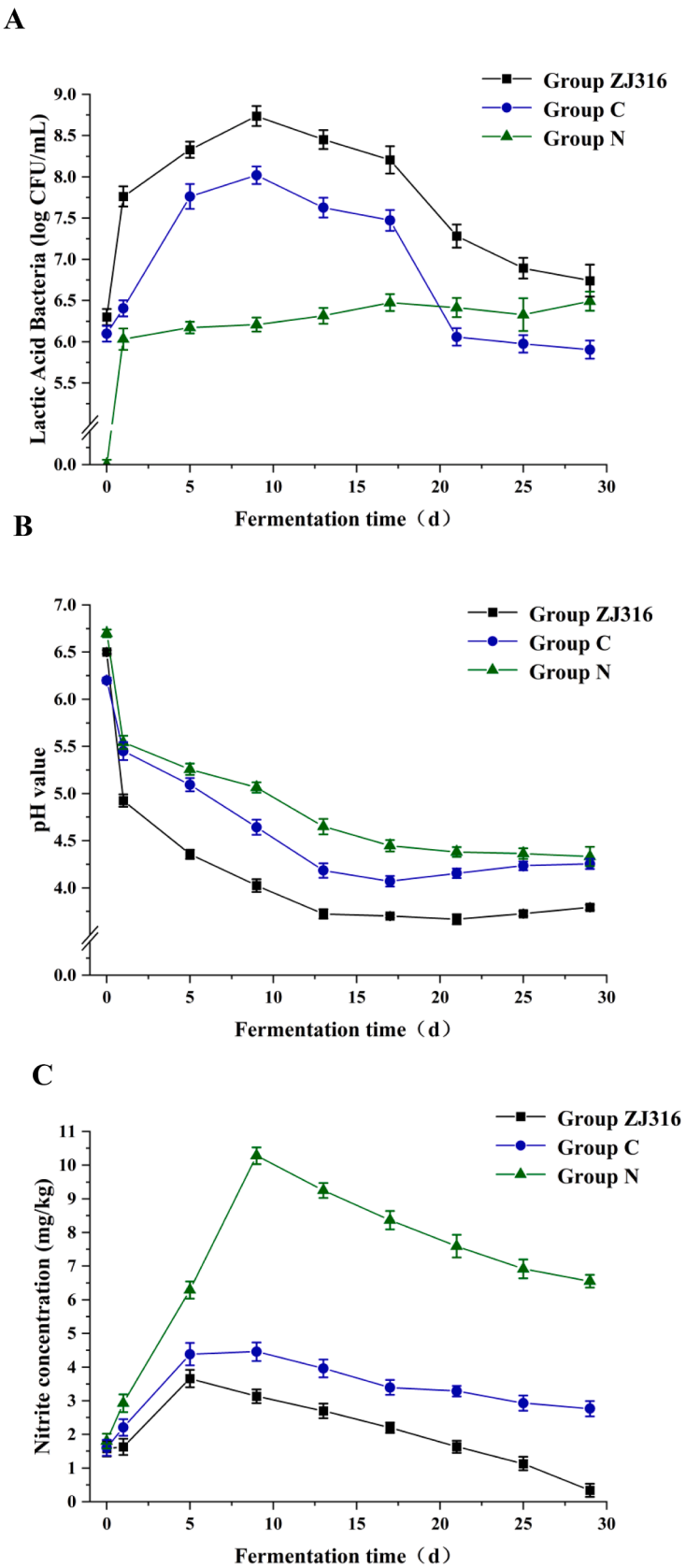


Fig. 1. Effects of ZJ316 as a starter on the LAB counts (A) pH value (B) and nitrite concentrations (C) of pickled mustard during fermentation. (■) Group ZJ316 indicated the fermentation group with *L. plantarum* ZJ316 starter; (●) Group C indicated the fermentation group with commercial starter; (▲) Group N indicated the natural fermentation group (n = 3).

Table 1
The contents of volatile flavor components in fermented pickled mustard by HS-SPME/GC–MS.

Class	[a]RT (min)	Library/ID	CAS	Group ZJ316		Group N		Group C	
				relative peak area(%)	[b]qual	relative peak area(%)	[b]qual	relative peak area(%)	[b]qual
Alcohols	9.74	Ethanol	000064-17-5	0.56	78	0.35	83	0.41	78
	50.61	Phenylethyl Alcohol	000060-12-8	0.22	94	0.24	90	–	–
	28.26	1-Hexanol	000111-27-3	0.20	83	0.25	86	0.12	90
	36.94	3-Hexen-1-ol, (E)-	000928-97-2	0.20	83	–	–	–	–
	37.00	1-Octanol	000111-87-5	0.19	74	0.30	90	–	–
	35.35	2-Nonanol	000628-99-9	0.17	83	0.13	83	0.06	83
	41.08	1-Nonanol	000143-08-8	0.09	87	–	–	0.04	58
	56.24	2,4,7,9-Tetramethyl-5-decyn-4,7-diol	000126-86-3	0.06	91	–	–	–	–
	56.06	2,4,7,9-Tetramethyl-5-decyn-4,7-diol	000126-86-3	0.05	91	–	–	–	–
	21.52	1-Butanol, 3-methyl-	000123-51-3	0.05	83	–	–	0.02	86
	40.49	Silanediol, dimethyl-	001066-42-8	0.04	90	–	–	0.03	72
	32.38	1-Octen-3-ol	003391-86-4	0.04	80	–	–	–	–
	3.83	Methanethiol	000074-93-1	0.02	91	–	–	–	–
	44.96	1-Decanol	000112-30-1	0.02	90	–	–	–	–
	47.00	Benzenemethanol,.alpha.-methyl-, (S)-	001445-91-6	0.02	93	–	–	–	–
	52.19	1-Hexadecanol	036653-82-4	–	–	–	–	0.02	91
Aldehydes	47.30	Benzaldehyde, 2,4-dimethyl-	015764-16-6	0.10	91	–	–	–	–
	35.85	Benzaldehyde	000100-52-7	0.04	96	0.09	91	0.07	96
	34.51	2,4-Heptadienal, (E,E)-	004313-03-5	0.02	87	–	–	0.15	95
	42.94	2,4-Nonadienal, (E,E)-	005910-87-2	–	–	–	–	–	93
	15.48	Hexanal	000066-25-1	–	–	–	–	0.03	90
	22.00	2-Hexenal, (E)-	006728-26-3	–	–	–	–	0.04	98
Acids	32.55	Acetic acid	000064-19-7	0.67	86	2.96	91	–	–
	55.61	Octanoic acid	000124-07-2	3.88	97	1.22	94	0.14	72
	61.92	Berteroin	004430-42-6	0.00	89	0.74	90	–	–
	69.61	Dodecanoic acid	000143-07-7	0.03	93	0.44	95	–	–
Esters	28.65	Allyl Isothiocyanate	000057-06-7	42.61	96	42.26	95	41.48	96
	60.93	Benzene, (2-isothiocyanatoethyl)-	002257-09-2	20.52	97	20.43	97	21.05	91
	32.84	1-Butene, 4-isothiocyanato-	003386-97-8	10.74	94	7.14	95	9.88	94
	24.36	Butane, 2-isothiocyanato-	004426-79-3	1.25	96	0.55	94	0.88	76
	53.21	Propane, 1-isothiocyanato-3-(methylthio)-	000505-79-3	0.85	97	0.80	97	0.89	97
	26.67	Isobutyl isothiocyanate	000591-82-2	0.43	91	0.18	90	0.32	91
	61.20	aldeh	000628-97-7	0.35	90	0.41	83	0.54	93
	31.41	2-Methylbutyl isothiocyanate		0.34	59	–	–	1.54	93

(*continued on next page*)

Table 1 (*continued*)

Class	[a]RT (min)	Library/ID	CAS	Group ZJ316		Group N		Group C	
				relative peak area(%)	[b]qual	relative peak area(%)	[b]qual	relative peak area(%)	[b]qual
			004404-51-7						
	34.17	*n*-Pentyl isothiocyanate	000629-12-9	0.12	72	–	–	1.30	95
	25.12	Cyclopropane, isothiocyanato-	056601-42-4	0.11	72	1.42	90	0.32	56
	11.03	Allyl isocyanate	001476-23-9	0.07	38	–	–	0.03	91
	58.03	Erucin	004430-36-8	0.06	98	–	–	0.05	98
	20.29	Isopropyl isothiocyanate	002253-73-8	0.05	90	–	–	0.04	87
	60.15	Hexadecanoic acid, methyl ester	000112-39-0	–	–	–	–	0.03	91
	71.66	Phthalic acid, isobutyl tra–hex-3-enyl ester	1000360-48-1	–	–	–	–	0.08	90
	46.64	Carbonotrithioic acid, dimethyl ester	002314-48-9	–	–	–	–	0.01	94
	57.00	Benzene, (isothiocyanatomethyl)-	000622-78-6	–	–	–	–	0.03	80
	37.73	3,5-Octadien-2-one, (E,E)-	030086-02-3	–	–	–	–	0.04	90
Alkanes and derivatives	10.90	Cyclotetrasiloxane, octamethyl-	000556-67-2	0.21	91	–	–	–	–
	17.36	Cyclopentasiloxane, decamethyl-	000541-02-6	0.13	91	–	–	–	–
	60.69	Methane, di-p-tolyl-	004957-14-6	0.02	86	–	–	–	–
Olefins and derivatives	52.18	1-Decene	000872-05-9	0.04	94	–	–	–	–
	36.94	4-Methyl-1,3-pentadiene	000926-56-7	–	–	–	–	0.14	80
Benzene derivatives	54.99	Benzenepropanenitrile	000645-59-0	0.84	91	0.78	90	0.60	91
	53.57	Phenol	000108-95-2	0.13	95	–	–	–	–
	44.64	Oxime-, methoxy-phenyl-_	1000222-86-6	0.10	80	–	–	–	–
	54.51	Phenol, 4-ethyl-2-methoxy-	002785-89-9	0.04	91	0.26	91	0.13	91
	57.25	2,2′-Dimethylbiphenyl	000605-39-0	0.03	95	–	–	0.04	95
	59.80	1,1′-Biphenyl, 2,2′,5,5′-tetramethyl-	003075-84-1	0.02	91	–	–	–	–
	60.02	Benzene, 1-methyl-3-[(4-methylphenyl) methyl]-	021895-16-9	0.02	96	–	–	–	–
	56.89	1,1′-Biphenyl, 4-methyl-	000644-08-6	0.02	95	–	–	0.02	87
	43.31	Benzaldehyde, 3-ethyl-	034246-54-3	–	–	–	–	0.04	92
	57.79	1,1′-Biphenyl, 2,3′-dimethyl-	000611-43-8	–	–	–	–	0.02	87
	47.31	Benzaldehyde, 3,5-dimethyl-	005779-95-3	–	–	–	–	1.35	95
	59.80	9,9-Dimethyl-9-silafluorene	013688-68-1	–	–	–	–	0.03	87
	60.03	Benzene, 1,1′-methylenebis[3-methyl-	021895-14-7	–	–	–	–	0.03	96
	59.00	Phenol, 2-ethyl-	000090-00-6	–	–	0.19	81	–	–
	47.31	Benzaldehyde, 3,4-dimethyl-	005973-71-7	–	–	2.43	91	–	–
Sulfides	29.81	Dimethyl trisulfide	003658-80-8	0.90	98	–	–	–	–
	24.98	Disulfide, methyl 2-propenyl	002179-58-0	0.85	95	–	–	–	–
	15.21	Disulfide, dimethyl		0.84	97	0.10	95	0.20	97

(*continued on next page*)

Table 1 (*continued*)

Class	[a]RT (min)	Library/ID	CAS	Group ZJ316		Group N		Group C	
				relative peak area(%)	[b]qual	relative peak area(%)	[b]qual	relative peak area(%)	[b]qual
			000624-92-0						
	33.97	Diallyl disulphide	002179-57-9	0.19	93	0.43	95	0.71	93
	38.76	Trisulfide, methyl 2-propenyl	034135-85-8	0.13	96	–	–	–	–
	10.39	Sulfide, allyl methyl	010152-76-8	0.08	97	–	–	–	–
	18.56	Diallyl sulfide	000592-88-1	0.04	95	–	–	0.05	97
	24.98	Disulfide, methyl 2-propenyl	002179-58-0	–	–	–	–	0.52	91
Furan	25.74	tra–2-(2-Pentenyl)furan	070424-14-5	0.09	98	–	–	–	–
	10.25	Furan, 2-ethyl-	003208-16-0	0.08	91	–	–	0.03	91
	25.75	*cis*-2-(2-Pentenyl)furan	070424-13-4	–	–	–	–	0.09	97
	25.74	tra–2-(2-Pentenyl)furan	070424-14-5	–	–	0.11	89	–	–
Others	19.94	2-Butenenitrile	004786-20-3	0.47	93	0.16	91	0.31	94
	48.78	Naphthalene, 2-methyl-	000091-57-6	–	–	–	–	0.02	94
	59.61	Naphthalene, 2,3,6-trimethyl-	000829-26-5	–	–	0.09	46	0.03	97
	52.62	Naphthalene, 2,7-dimethyl-	000582-16-1	–	–	–	–	0.03	97
	9.74	Pyrazine, 2-methoxy-3-(1-methylpropyl)-	024168-70-5	–	–	–	–	0.02	91

NOTE: Group N indicated the natural fermentation group; Group C indicated the commercial starter fermentation group; Group ZJ316 indicated the fermentation of *L. plantarum* ZJ316 starter group. [a]RT: retention time; [b]qual: Matching score based on literature and NIST14 database, https://www.nist.gov/publications/nist-14-nist-mixture-property-database.

generated by the combination of different alcohols produced by organic acids in the fermentation process may have a distinctive complex flavor.

3.4. Effect of L. plantarum ZJ316 on the phylogeny and bacterial community of fermented pickled mustard

3.4.1. Analysis of microbial diversity

At different fermentation stages, the microbiota communities in pickled mustard were assessed by 16S rRNA genes (V3-V4 region) sequencing. Alpha diversity indices are shown in Fig. 2A. ACE index, Chao1 index, Richness index, and Simpson index were used to estimate the abundances and diversities of bacterial communities in the pickled mustard tubers. From the individual indices of Alpha diversity (Fig. 2A (a–d)), similar conclusions could be obtained: the diversity of community richness in group N was the highest, while that in the C and ZJ316 groups was relatively low, and that in the ZJ316 group was the lowest. This might be because inoculation of *L. plantarum* ZJ316 inhibited the growth of spoilage microorganisms in pickled mustard tubers and reduced community diversity.

Principal coordinates analysis (PCoA) was performed to elucidate the overall composition of the pickled mustard microbial community at the OTU level, with the first two standard axes explaining 49.16% and 26.84% of the variation, respectively. As shown in Fig. 2B, the sampling point of the ZJ316 group always remained in the upper right of the graph, indicating a stable microbial community composition. After being inoculated with ZJ316 for fermentation, the relative abundance of *Lactiplantibacillus* increased, which significantly inhibited the growth of Gram-negative bacteria, and the quality of the fermented pickled mustard products was stable. Compared to the ZJ316 group, the sampling points in the N group moved from the lower right to the upper left of the graph during the early (day 5) to the middle (day 18) periods of fermentation, indicating a significant difference in microbial community composition ($p = 0.001$). No significant differences were observed in the N group from the middle (day 18) to the end of fermentation (day 28). During the half stage of fermentation (days 5–18), the sampling point of the C group moved from the lower right to the upper left, and in the second half of fermentation (days 18–28), the sampling point stabilized near the origin.

3.4.2. Analysis of microbial community composition

The most prevalent phyla in the pickled mustard tuber samples were *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* (Fig. 3A (a)). Analogous results were also observed in the fermentation supernatant of pickled mustard (Fig. S1A). In the ZJ316 group, *Firmicutes* (Fig. 3A (b)) occupied 41% (day 5) to 52% (day 18) in the middle fermentation stage, and nearly 65% at the late. In contrast, the main phylum of group C was *Proteobacteria* (Fig. 3A (c)), whose relative abundance was 80% at the beginning of fermentation, declined to 60% at the mid-fermentation (18 d), and then accumulated to 72% by the end of fermentation. Similar results were observed in the N group; the phylum with the highest abundance was *Proteobacteria*, which were as high as 94% in the N group in the early stage of fermentation, decreased to 57% in the middle stage, and showed some degree of increase in the later stage. A certain amount of *Actinobacteria* phylum (Fig. 3A (d)) was also present in these groups, with the most significant percentage of the phylum *Actinomycetes* in group N, which accounted for approximately 6% at the end of fermentation, but almost none detected in the ZJ316 group. As the fermentation process proceeded, the *Bacteroidetes* phylum (Fig. 3A (e)) also appeared

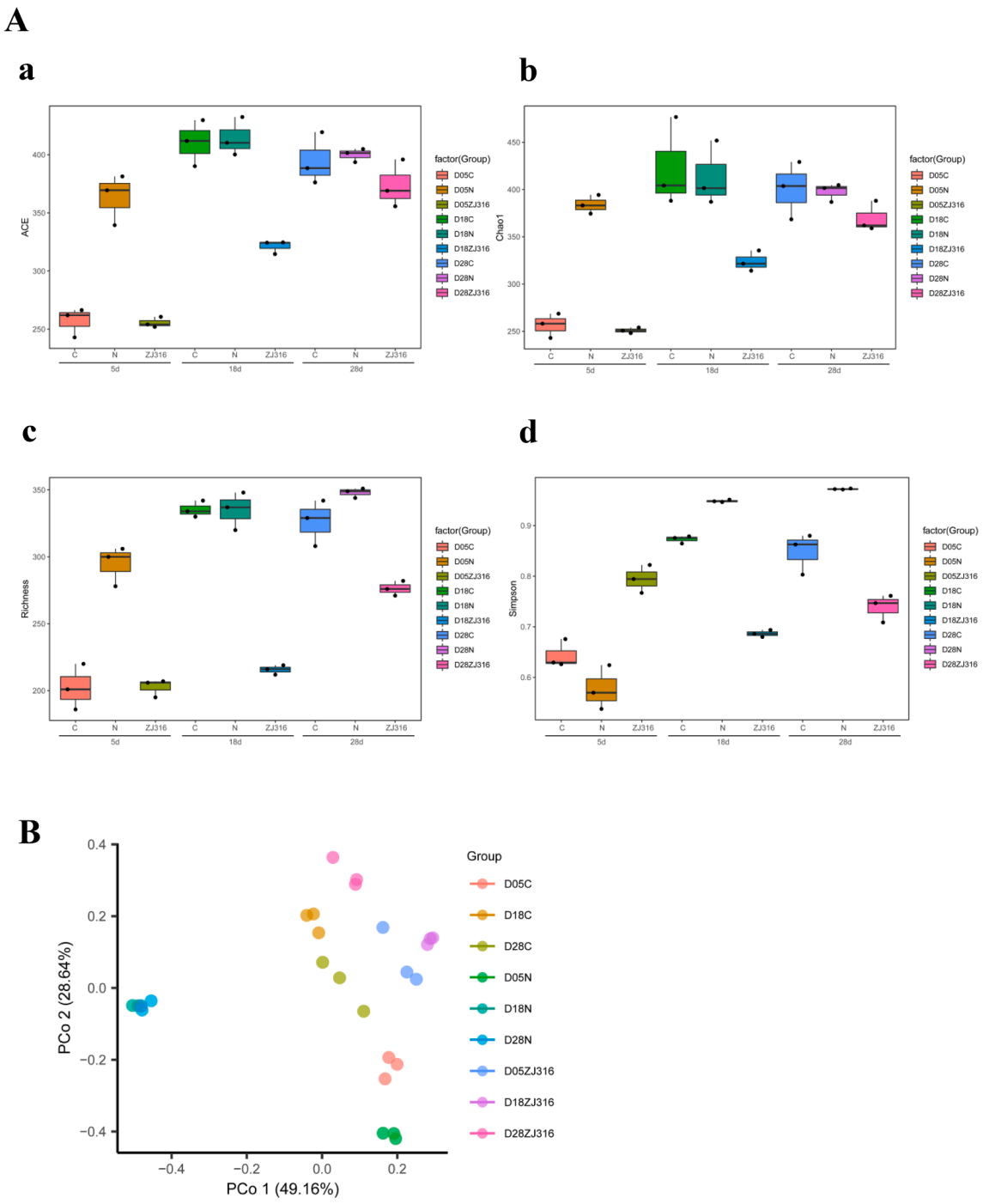
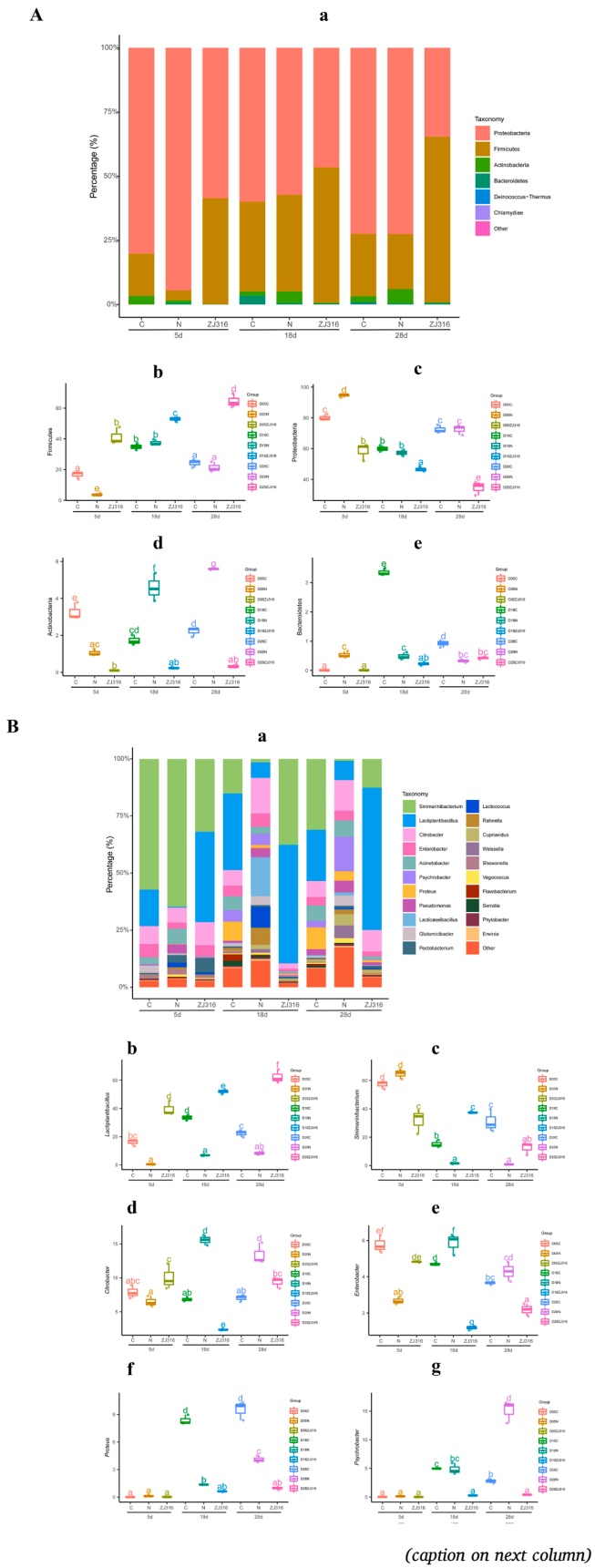


Fig. 2. Bacterial community diversities and structures of pickled mustard tuber microbiota at the OTU level after different treatments. (A) Alpha diversity index of bacteria, including the ACE (a), Chao1 (b), Richness (c), and Simpson diversity (d). (B) PCoA of all bacterial samples based on the unweighted Unifrac distance at 5 d, 18 d, and 28d. ($p = 0.001$, $R^2 = 0.999$). The tops and bottoms of the boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers extend to data no more than 1.5× the interquartile range from the upper edge and lower edge of the box, respectively.

in group C, reaching a peak of approximately 3% in the middle stage of fermentation (18 d) and decreasing to a relatively low level in the late period (28 d). Among them, *Firmicutes* include *Bacillus* and *Lactiplantibacillus*; the latter plays an essential role as a probiotic in maintaining the intestinal health of animals. Some researchers have shown that *L. plantarum* showed anti-inflammatory activity against inflammation-related diseases, especially inflammatory bowel disease (Satish Kumar et al., 2015). Studies have shown that *Proteobacteria* are the largest bacterial group and include pathogenic bacteria, such as *E. coli, Salmonella, Vibrio cholerae,* and *Helicobacter pylori,* which are prone to cause diarrhea in animals (Tenaillon et al., 2010). *Actinobacteria* were mostly trophozoites, and are widely distributed in the soil; relevant studies have illustrated that the *Actinobacteria* phylum is crucial in maintaining intestinal homeostasis (Binda et al., 2018). Most of the major pathogenic bacteria in the intestine are of the phylum *Bacteroidetes*.

At the genus level, the most prevalent genuses in pickled mustard tubers samples were *Sinimarinibacterium*, *Lactiplantibacillus*, *Citrobacter*, *Enterobacter*, *Acinetobacter*, and *Psychrobacter* (Fig. 3B (a)), consistent with those observed in the pickled mustard supernatant (Fig. S1B). The most prevalent genus detected in the ZJ316 group was *Lactiplantibacillus*



(caption on next column)

Fig. 3. (A) Composition of pickled mustard tuber bacterial community after different treatments. Each bar represents the relative abundance of OTUs at the phylum and levels in the pickled mustard tuber microbiota. (a) Bacterial taxonomic profiling at the phylum level of pickled mustard tuber microbiota in each group. Relative abundances of selected bacteria (b) *Firmicutes* (c) *Proteobacteria* (d) *Actinobacteria*, and (e) *Bacteroidetes*, in the pickled mustard tuber microbiota at the phylum level. (B) Composition of pickled mustard tuber bacterial community after different treatments. Each bar represents the relative abundance of OTUs at the genus levels in the pickled mustard tuber microbiota. (a) Bacterial taxonomic profiling at the genus level of colonic microbiota in each group. Relative abundances of selected bacteria (b) *Lactiplantibacillus*, (c) *Sinimarinibacterium*, (d) *Citrobacter*, (e) *Enterobacter*, (f) *Proteus* and (g) *Psychrobacter* in the pickled mustard microbiota at the genus level. Different letters indicate significantly different groups ($p < 0.05$, ANOVA, Tukey HSD). The horizontal bars within boxes represent medians. The tops and bottoms of the boxes represent the 75th and 25th percentiles, respectively. The upper and lower whiskers extend to data no more than 1.5× the interquartile range from the upper edge and lower edge of the box, respectively.

(Fig. 3B (c)), with relative abundance ranging from 40% (day 5) to 52% (day 18) and 62% at the end of fermentation. The proportion of reads assigned to the *Sinimarinibacterium* genus (Fig. 3B (d)) was more significant in the C group than in the ZJ316 group, with 57% at the beginning of fermentation, a sharp decline during fermentation, and a maintenance of 31% by the end of fermentation. The prevalence of *Citrobacter* and *Enterobacter* (Fig. 3B (e, f)) in the N group, and *Proteus* (Fig. 3B (g)) in the C group were significantly different. The percentage of "other genera" (bacteria with an abundance of less than 0.5%) in the N group was higher than 15%, indicating that there were more miscellaneous bacteria in the N group. In addition, samples from the N group had a higher relative abundance of pathogenic bacteria, with *Sinimarinibacterium* being the dominant genus at day 5 and *Psychrobacter* (Fig. 3B (h)) became the new dominant homogeneous genus by the end of fermentation (day 28). *Psychrobacter* was a diverse and complex genus of bacteria, represented by *Pseudomonas syringae* as an essential group of phytopathogenic bacteria (Lalucat et al., 2020). *Lactiplantibacillus* was the dominant genus in the ZJ316 group, maintaining high abundance throughout. The relative abundance of pathogenic bacteria in the ZJ316 group was lower compared to the N group. These results indicated that the mustard inoculated with ZJ316 fermentation had a stable flora during the fermentation process, which was conducted to improve mustard safety and maintain intestinal health. Related studies have shown that *Lactiplantibacillus* could interact with the microbiota through competition for nutrients, antagonism, cross-feeding, and support of microbiota stability. Many probiotic strains are antagonistic to other microorganisms, partly due to the production of organic acids from sugar catabolism and bacteriocins (Hegarty et al., 2016). Additional relevant studies have shown that *Lactiplantibacillus* produces extracellular polysaccharides with antitumor and antibacterial properties. Therefore, we hypothesized that *L. plantarum* ZJ316 produces acid during the fermentation process as well as extracellular polysaccharides and synthesizes bacteriocins in various ways to inhibit the growth of pathogenic bacteria.

3.4.3. Species significant difference analysis

Linear discriminant analysis Effect Size (LEfSe) analysis was performed to determine significant differences in the abundance of taxonomic units between the three groups, classified by different fermentation times (Fig. 4). The corresponding nodes indicated significantly enriched bacterial groups, and their taxonomic levels are shown on the right side of Fig. 4A and 4B. LEfSe analysis (LDA threshold of 5.5) was used to compare the relative abundance of bacteria in tuber samples of pickled mustard (Fig. 4) and fermentation supernatants (Fig. S2) from the ZJ316, N, and C groups. On the 5th day of fermentation (Fig. 4A), *Lactobacillaceae*, *Lactiplantibacillus*, and *Firmicutes* were significantly dominant in the ZJ316 group. *Gammaproteobacteria*, *Nevskiaceae*,

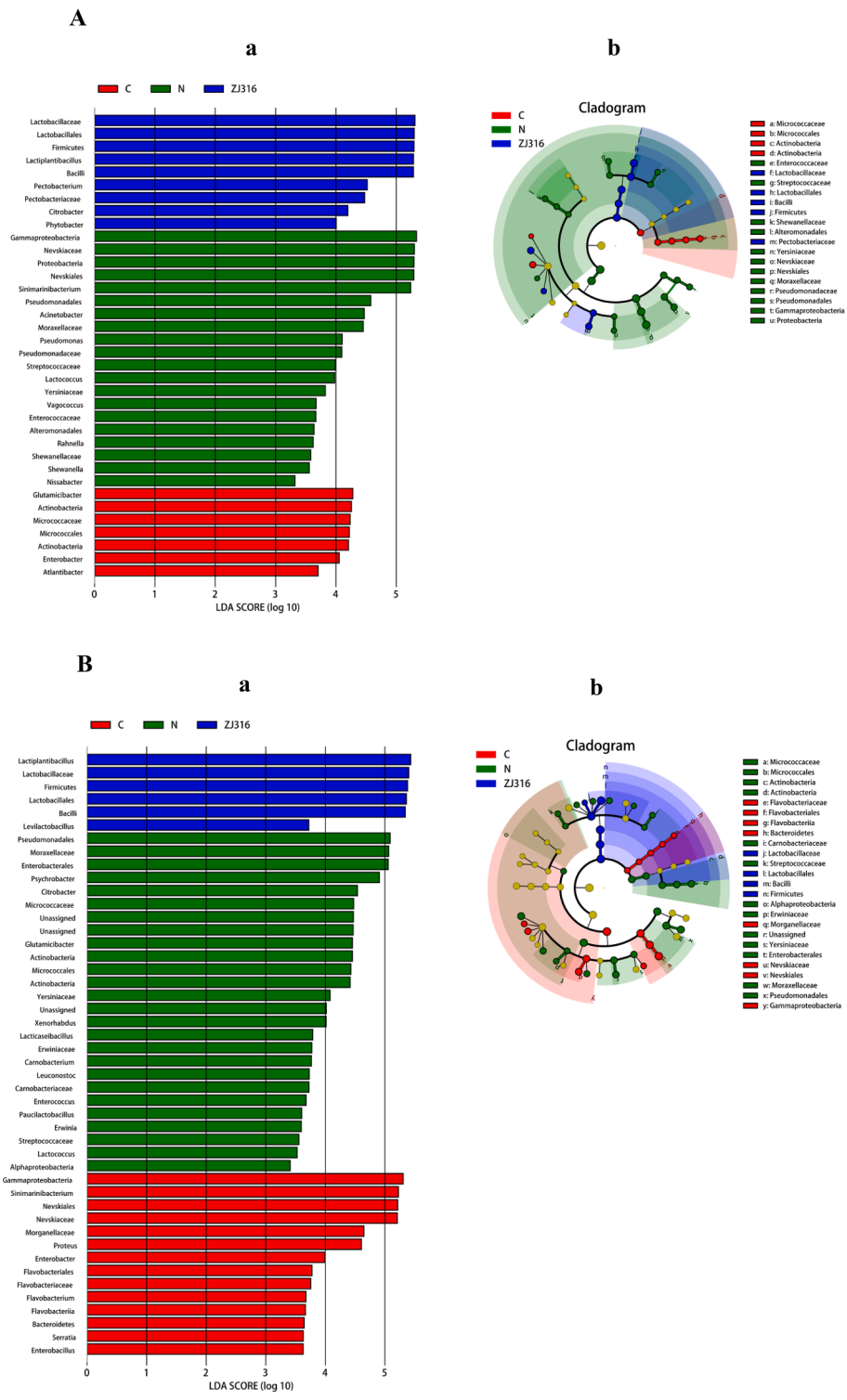


Fig. 4. (A) Pickled mustard bacterial community composition and abundance in groups C, N, and ZJ316 at the early fermentation stage (day 5). (a) LEfSe revealed the microbiomes with significantly different abundances in C (red), N (green), and ZJ316 (blue) groups. (b) LEfSe clearly distinguished bacterial taxa in the microbiota of pickled mustard from groups C, N, and ZJ316. (B) Pickled mustard bacterial community composition and abundance in groups C, N, and ZJ316 at the end of fermentation (day 28). (a) LEfSe revealed the microbiomes with significantly different abundances in C (red), N(green), and ZJ316 (blue) groups. (b) LEfSe clearly distinguished bacterial taxa in the microbiota of pickled mustard from groups C, N, and ZJ316. $^{**}p < 0.01$ and $^{***}p < 0.001$ indicated no statistical significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Proteobacteria, *Nevskiales*, and *Sinimarinibacterium* were significantly different in the N group. At the end of fermentation (day 28) (Fig. 4B), *Lactiplantibacillus* and *Lactobacillales* were newly emerged differential genera in the ZJ316 group, and the presence of these probiotic bacteria indicated that ZJ316 inhibited the growth of pathogenic bacteria by producing organic acids and bacteriocins, and provided an environment for the growth of other probiotics. However, some potentially pathogenic bacteria such as *Gammaproteobacteria*, *Nevskiaceae*, and *Nevskiales* in the C group, *Pseudomonadales*, *Moraxellaceae*, and *Enterobacterales* in the N group, and fermentation supernatant in the LN group (Fig. S2C) were observed, which could increase the food safety risk.

3.5. *Antimicrobial activity of fermented pickled mustard supernatant against pathogenic bacteria*

According to the microbial diversity analysis, it is observed that there are several harmful microorganisms in the fermentation supernatants of the C and N groups after 28 days of fermentation, which might cause pollution. Therefore, the possible foodborne pathogens microorganisms, such as *S. aureus* D48 (Fig. 5A), *E. coli* DH5α (Fig. 5B), and

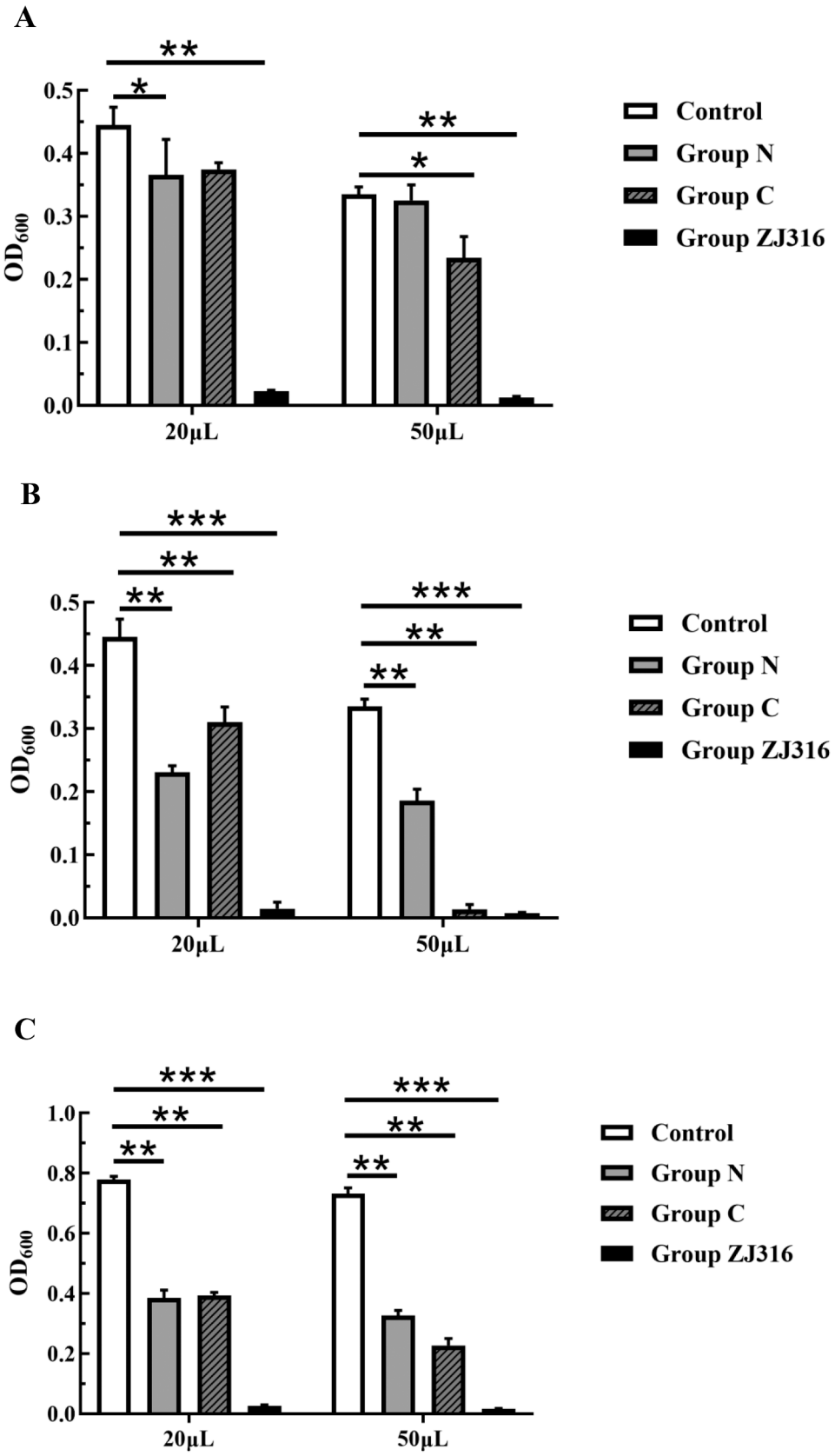


Fig. 5. Antibacterial effect of pickled mustard fermented supernatant against *Staphylococcus aureus* D48 (A), *Escherichia coli* DH5α (B) and *Listeria monocytogenes* LM1 (C). The data are presented as mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

L. monocytogenes LM1 (Fig. 5C), were selected as sensitive indicator bacteria to study the *in vitro* antibacterial activity of the pickled mustard supernatant using the 96-well plate method. When the supernatant concentration of the three groups was 20 μL, the growth of different indicator bacteria was inhibited to varying degrees. Among them, the supernatant of the N group had the same bacteriostatic effect as that of the C group, there was no significant inhibition on *S. aureus* D48 in the N and C groups, while the indicator bacteria hardly grew under the ZJ316 group. When the pickled mustard supernatant was increased to 50 μL, the antibacterial effect of the N group supernatant on three indicator bacteria had no significant change. The inhibition of the supernatant of the C group on *S. aureus* D48, *E. coli* DH5α, and *L. monocytogenes* LM1 significantly increased, and the ZJ316 group further increased the antibacterial activity of three indicator bacteria compared to the C group. Combined with LEfSe analysis (Fig. S2C), the results showed that inoculation with *L. plantarum* ZJ316 as a starter could significantly inhibit *Enterobacterales* spp. represented by *E. coli* DH5α, which remained at low levels in both pickled mustard tubers and fermentation supernatant. Our previous results showed that the culture supernatant of *L. plantarum* ZJ316 exerted significant inhibitory effects on *Salmonella*, *E. coli*, and *L. monocytogenes*, and inoculation with *L. plantarum* ZJ316 could significantly enhance the quality of Landrace-Yorkshire pork (Hang & Zeng, 2022; Suo et al., 2012). Chen et al. also proved that *L. plantarum* ZJ316 had a good antibacterial effect and could inhibit the growth of miscellaneous bacteria in the fermentation process of mustard (Chen et al., 2018). Therefore, it is suggested that *L. plantarum* ZJ316 as a starter has a significant inhibitory effect on harmful microorganisms in pickled mustard and improves food safety.

4. Conclusion

The high lactic acid generation capacity of *L. plantarum* ZJ316 allowed it to swiftly reduce the pH value and nitrite content in the fermentation environment of pickled mustard, yielding a distinct taste. The nitrite residual in the mustard after stabilization was just 0.34 mg/kg. Microbial diversity studies showed that *L. plantarum* ZJ316 could not only raise the relative abundance of probiotics (such as *Lactiplantibacillus*) in the fermentation environment, but also prevent the growth of harmful bacteria (such as *Pseudomonas*, *Proteus*, *Enterobacter*, and other pathogenic bacteria). Moreover, the supernatant of pickled mustard inoculated with *L. plantarum* ZJ316 inhibited *S. aureus* D48 and *E. coli* DH5α, ensuring the safety of pickled mustard. On that basis, it is possible to conclude that *L. plantarum* ZJ316 has industrial potential for use as an ideal starter in the process of vegetable fermentation.

CRediT authorship contribution statement

Xiazhu Zhang: Methodology, Data curation, Visualization, Writing – original draft. **Jiarun Han:** Writing – review & editing. **Xiaogu Zheng:** Writing – original draft, Writing – review & editing. **Jiaqian Yan:** Writing – original draft, Writing – review & editing. **Xiaozhen Chen:** Writing – original draft, Writing – review & editing. **Qingqing Zhou:** Writing – original draft. **Xiaodan Zhao:** Writing – review & editing, Supervision. **Qing Gu:** Writing – review & editing, Supervision. **Ping Li:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100344.

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