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Wang, Yilun

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Investigation of diverse bacteria encoding histidine decarboxylase gene in Sichuan-style sausages by culture-dependent techniques, polymerase chain reaction–denaturing gradient gel electrophoresis, and high-throughput sequencing

Yilun Wang [a], Binbin Li [b], Yuxuan Liu [a], Xiaohong Huang [a], Nan Zhang [a], Yifang Yang [a], Zihan Xiao [a], Qinxin Yu [a], Shujuan Chen [a], Li He [a], Aiping Liu [a], Shuliang Liu [a], Likou Zou [c], Yong Yang [a,*]

[a] College of Food Science, Sichuan Agricultural University, Ya'an, 625014, PR China
[b] Department of Food and Nutrition, University of Helsinki, Helsinki, 00790, Finland
[c] College of Resource, Sichuan Agricultural University, Chengdu, 611130, PR China

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ABSTRACT

The diverse bacteria encoding histidine decarboxylase gene during the fermentation of Sichuan-style sausages were investigated by culture-dependent techniques, polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE), and high-throughput sequencing. All microbial indicators exhibited the advantages of mixed starter culture and the stability of microecosystem was more in the inoculation group than in the control group. DGGE and selected band sequencing were used to investigate the bacterial diversity of these sausages. *Weissella* were the main lactic acid bacteria (LAB) in the initial fermentation stage, whereas *Weissella* and *Lactobacillus* were the dominant bacteria in the later fermentation stage. After sequence alignment analysis, *Enterobacter aerogenes* and *Citrobacter freundi* were the two main bacteria encoding histidine decarboxylase gene and could produce histamine. These findings facilitate the better understanding of bacteria producing histidine decarboxylase during sausage fermentation and provide a theoretical basis for the control of histamine-producing bacteria in the process of fermented sausage processing.

1. Introduction

Sichuan-style sausage is a type of conventional cured fermented meat product increasingly accepted and produced by Chinese people and industries. These sausages are mainly processed with a special Sichuan pepper in the southwest Chinese provinces such as Sichuan, Yunnan, and Kweichow (Sun, Zhou, et al., 2016). Traditionally, the Sichuan-style sausage is fermented and ripened for approximately 30 days at an ambient temperature of 10°C–20 °C until a characteristic flavour, aroma, and taste are achieved. The quality, safety, and properties of fermented sausage are closely related to protein degradation and change in bacterial dynamics (Wang, Zhang, Ren, & Zhan, 2018). Biogenic amines (BAs) are the most common toxic compounds produced in the sausage fermentation process. Histamine is the most toxic and widely studied BAs and formed by decarboxylation of histidine by histidine decarboxylase (HDC) (Aflaki, Ghoulipour, Saemian, & Salahinejad, 2014). A study by Linares et al. indicated the absence of any toxic reaction when the histamine content was 6–25 mg per meal. However, a histamine content of more than 75 mg per meal may cause toxicity (Linares, Martín, Ladero, Alvarez, & Fernández, 2011). Therefore, investigating bacterial diversity and bacteria that encode HDC gene is both meaningful and essential.

In the past few decades, studies about bacterial population have mainly focused on the predominant pathogenic species in fermented sausages, namely LAB, *Staphylococcus*, pathogenic yeast, and *Escherichia coli*, using isolation-based culture-dependent methods; however, these methods do not detect the microorganisms with high accuracy (Hu et al., 2017). Moreover, approximately 99% bacteria in bacterial communities

* Corresponding author. Laboratory of Meat Science, College of Food Science, Sichuan Agricultural University, No. 46, Xinkang Road, Yucheng, Sichuan, 625014, China.
E-mail address: yangyong676@163.com (Y. Yang).

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are uncultured; thus, the results cannot accurately reflect the authentic microecosystem (Iacumin, Comi, Cantoni, & Cocolin, 2006a; b).

Nowadays, molecular techniques combined with databases have been increasingly employed to analyse the bacterial diversity in fermented food (Duru et al., 2018; Jung et al., 2018; Raimondi et al., 2017). The polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) is a molecular fingerprinting technique for the efficient, direct, rapid, and comprehensive study of the microbial community composition and relationship and is a powerful tool for bacterial classification and identification (Chen, Wang, Lin, Shi, & Liu, 2017; Ding, Wu, Huang, Li, & Zhou, 2014). Additionally, high-throughput sequencing is a novel, accurate, and fast molecular biotechnology that can analyse microbial species and their relative quantity in the whole sample, and comprehensively reflect the characteristics of the microbial community (Li et al., 2017; Williams, Foster, & Littlewood, 2014). These two methods have revolutionised the study of food microbial ecosystems and can assist researchers in the investigation of bacteria and intricate bacterial communities in sausages (Nalepa & Markiewicz, 2017). Simultaneously, they also assist in detecting histamine-producing bacteria in fermented sausages. Despite successful food bacterial diversity determination, only a few studies have analysed the bacterial diversity or bacteria encoding for HDC gene in Sichuan-style sausages.

The present study was conducted to assess the bacterial ecology of the Sichuan-style sausage using culture-dependent methods, PCR-DGGE techniques, and high-throughput sequencing to obtain a detailed description of the bacterial diversity and histamine-producing species influenced by mixed starter culture present in the casings.

2. Materials and methods

2.1. Fermented sausage technology and sampling procedures

Fermented sausages were prepared at 16 °C in the meat product processing laboratory of the College of Food Science at the Sichuan Agricultural University. Natural casings were filled with a mixture of 80 kg pork meat, 20 kg lard, 2.5 kg sodium chloride, 1.5 kg sugar, 0.025 kg nitrite, 0.0075 kg nitrate, and 0.02 kg black pepper. The bacteria from the mixed starter culture (*Lactobacillus plantarum, Pediococcus pentosaceus,* and *Weissella confuse*) were isolated from the spontaneously fermented Sichuan-style sausages and identified by previous study. The sausages were of a standard size of 25-cm length and 5-cm diameter. The first stage of ripening consisted of 2 days of drying with a relative humidity (RH) of 80% at 20 °C, followed by 28 days of drying at a decreased temperature of 12 °C and a RH of 65%. The study comprised two groups: the control group (group A) (spontaneous fermentation) and inoculation group (group B) (inoculation of 10^7 CFUg^{-1} *L. plantarum*, *P. pentosaceus*, and *W. confuse*; 1:1:1). Triplicate samples of the meat mixture, prior to filling sausages, were obtained at 0 (A0, B0), 2 (A2, B2), 4 (A4, B4), 6 (A6, B6), 10 (A10, B10), 14 (A14, B14), 18 (A18, B18), 22 (A22, B22), 26 (A26, B26), and 30 (A30, B30) days and were used for culture-dependent and DGGE analyses. The meat mixture was collected at 0 (A0, B0) and 30 (A30, B30) days for high-throughput sequencing analyses.

2.2. Physicochemical parameters analysis

The pH values were determined at room temperature (approximately 22 °C) using a pH meter (PHS–3C, Hangzhou, China). The water activity (Aw) values were obtained at 25 °C using an Aw meter (HD-3A, Huake instrument and meter Co., Ltd, Wuxi, China). The moisture content was determined using the direct drying method prescribed by Chinese standard GB5009.3–2016 (China).

2.3. Microbiological analysis

A total of 25 g of each sample was transferred into a sterile stomacher bag, 225 mL of saline sterile water (8.5 g of NaCl per litre) was added, and the mixture was treated for 3 min in a stomacher machine (Scientz-04, Ningbo, China). Further decimal dilutions were made, and 100 μL of the sample suspension was dispensed on duplicate agar plates for the following analyses: (1) total viable counts on plate count agar (PCA) (Oxoid, HANGWEI, Hangzhou, China) incubated at 30 °C for 72 h; (2) LAB on MRS agar (Oxoid) incubated at 30 °C for 48 h; (3) micrococci and staphylococci on Baird Parker (BP) agar (Oxoid) incubated at 37 °C for 48 h; (4) *Enterobacteriaceae* on violet red bile glucose agar (VRBGA) (Oxoid) incubated at 37 °C for 24 h; and (5) yeasts and moulds on potato dextrose agar (PDA) (Oxoid) incubated at 25 °C for 5 days. Means and standard deviations were calculated after counting.

2.4. Extraction and PCR amplification of DNA from fermented sausage

Triplicate 25-g samples were homogenised at each collection point in a stomacher bag with 225 mL of saline water for 3 min. After each preparation had settled for 1 min, 1 mL of subsamples were placed in 1.5-mL screw-cap tubes containing 0.3 g of glass beads for DNA extraction. The samples were centrifuged at 12,000 *g* at 4 °C for 10 min to pellet the cells thrice. Then, the total genomic DNA was extracted using a DNA kit (Tiangen biochemical technology Co. Ltd, Beijing, China) according to the manual. The DNA concentration and eluate purity were measured through absorbance at 260 nm and by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm and 230 nm using a spectrophotometer UV-1800 (Shimadzu, Kyoto, Japan). Bacterial 16S rRNA genes were amplified using primers GC-U968 (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and L1401 (5′-CGG TGT GTA CAA GAC CC-3′). Each reaction comprised a 25-μL mixture consisting of 12.5 μL of 2 × reaction buffer, 1 μL of primers (10 μM each), 1 μL of template DNA, and 9.5 μL ddH_2O. Denaturation was performed with 35 cycles at 95 °C for 10 min, 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; extension was performed at 72 °C for 5 min. The concentration determination method of PCR products is similar to that of DNA. PCR products were analysed through electrophoresis in a 1.2% agarose gel in 1 × Tris-borate-EDTA buffer at 120 V for 20 min.

2.5. DGGE analysis

The Dcode™ (Bio-Rad, Richmond, California, USA) was used for DGGE analysis. Electrophoreses were performed in a 0.8-mm-thick polyacrylamide gel (8% acrylamide/bisacrylamide at 37.5:1) using a denaturant gradient increasing in the electrophoretic run direction. A denaturant gradient from 40% to 60% (100% corresponded to 7 M urea and 40% formamide) was used for PCR products obtained with primers U968 and L1401. Electrophoresis was performed at a constant temperature of 60 °C in 1 × Tris-acetate-EDTA buffer. After electrophoresis, the gel was stained with silver nitrate (0.5 g $AgNO_3$, 200 μL methanol, and 250 mL ultrapure water) and visualised under UV light. The image was digitally captured and analysed with the Bio Imaging System Gene Genius (Gel Doc™ XR + Imaging system, Bio-RAD, Hercules, CA, USA) for band recognition. The DGGE analyses were performed at least twice. Normalisation of the gel was performed using band ladders.

2.6. DGGE band sequencing and sequence analysis

Polyacrylamide gel blocks containing selected DGGE bands were punched with sterile pipette tips. The blocks were then transferred to 30 μL of sterile water, and the DNA of the bands was allowed to diffuse at 4 °C for 24 h. Reamplification was performed using 2 μL of the water containing the eluted DNA, and the PCR products generated with the GC-clamped primer were analysed through DGGE by using amplified sausage DNA as a control. Only products migrating as a single band and at the same position with respect to the control were amplified with the primer without the GC clamp and sequenced by a commercial facility

Table 1
Microbiological counts (lg $CFUg^{-1}$) and physicochemical parameters during the processing of Sichuan-style sausages.

groups	batch	Microbiological counts					Physicochemical parameters		
		Total viable counts	LAB	*Staphylococcus*	*Enterobacteriaceae*	Moulds and yeasts	pH	Aw	Moisture content
A	A0	6.16 ± 0.17^{f}	5.37 ± 0.12^{f}	5.60 ± 0.00^{h}	3.94 ± 0.34^{e}	4.00 ± 0.00^{g}	6.02 ± 0.08^{a}	0.85 ± 0.00^{a}	0.53 ± 0.06^{ab}
	A2	8.28 ± 0.02^{c}	6.65 ± 0.00^{d}	7.29 ± 0.09^{f}	5.06 ± 0.15^{ab}	5.54 ± 0.02^{d}	5.69 ± 0.05^{b}	0.81 ± 0.01^{b}	0.56 ± 0.05^{a}
	A4	8.37 ± 0.10^{c}	7.26 ± 0.08^{b}	7.36 ± 0.14^{ef}	4.82 ± 0.12^{bc}	5.85 ± 0.03^{c}	5.43 ± 0.01^{d}	0.78 ± 0.02^{c}	0.46 ± 0.08^{bc}
	A6	8.72 ± 0.03^{b}	7.54 ± 0.08^{a}	7.56 ± 0.10^{de}	5.07 ± 0.10^{ab}	6.15 ± 0.08^{a}	5.43 ± 0.02^{d}	0.78 ± 0.02^{cd}	0.40 ± 0.08^{cd}
	A10	8.92 ± 0.02^{a}	7.27 ± 0.23^{b}	7.79 ± 0.06^{bc}	5.27 ± 0.10^{a}	6.09 ± 0.09^{ab}	5.28 ± 0.02^{e}	0.76 ± 0.01^{d}	0.35 ± 0.03^{de}
	A14	8.75 ± 0.02^{b}	7.12 ± 0.03^{bc}	7.95 ± 0.03^{ab}	5.37 ± 0.11^{a}	5.98 ± 0.00^{bc}	5.40 ± 0.02^{d}	0.72 ± 0.02^{e}	0.32 ± 0.02^{def}
	A18	8.61 ± 0.05^{b}	6.91 ± 0.06^{c}	8.11 ± 0.09^{a}	5.05 ± 0.07^{ab}	5.95 ± 0.01^{bc}	5.57 ± 0.05^{c}	0.71 ± 0.00^{e}	0.30 ± 0.04^{efg}
	A22	7.84 ± 0.03^{de}	6.56 ± 0.00^{de}	8.02 ± 0.07^{a}	4.76 ± 0.16^{bc}	5.37 ± 0.09^{e}	5.53 ± 0.04^{c}	0.71 ± 0.01^{e}	0.27 ± 0.02^{efg}
	A26	7.90 ± 0.04^{d}	6.48 ± 0.00^{de}	7.66 ± 0.07^{cd}	4.60 ± 0.03^{cd}	5.18 ± 0.05^{f}	5.44 ± 0.01^{d}	0.71 ± 0.00^{e}	0.25 ± 0.02^{fg}
	A30	7.73 ± 0.02^{e}	6.37 ± 0.06^{e}	7.06 ± 0.19^{g}	4.37 ± 0.10^{c}	5.06 ± 0.09^{f}	5.41 ± 0.03^{d}	0.69 ± 0.01^{e}	0.22 ± 0.02^{g}
B	B0	6.16 ± 0.17^{f}	5.37 ± 0.12^{f}	5.60 ± 0.00^{h}	3.94 ± 0.34^{e}	4.00 ± 0.00^{g}	6.02 ± 0.08^{a}	0.85 ± 0.00^{a}	0.53 ± 0.06^{ab}
	B2	8.11 ± 0.05^{ab}	7.36 ± 0.03^{c}	7.13 ± 0.07^{a}	4.95 ± 0.10^{a}	5.39 ± 0.06^{b}	5.51 ± 0.05^{b}	0.81 ± 0.00^{b}	0.56 ± 0.02^{a}
	B4	8.22 ± 0.14^{a}	7.49 ± 0.02^{c}	7.06 ± 0.22^{a}	4.24 ± 0.34^{b}	5.61 ± 0.09^{a}	5.40 ± 0.03^{c}	0.78 ± 0.00^{c}	0.45 ± 0.07^{b}
	B6	8.10 ± 0.06^{ab}	7.79 ± 0.02^{b}	6.80 ± 0.10^{b}	3.70 ± 0.00^{c}	5.78 ± 0.05^{a}	5.28 ± 0.03^{ef}	0.77 ± 0.01^{cd}	0.36 ± 0.07^{c}
	B10	8.20 ± 0.08^{a}	8.03 ± 0.11^{a}	6.78 ± 0.06^{b}	3.63 ± 0.04^{c}	5.60 ± 0.15^{a}	5.26 ± 0.05^{ef}	0.76 ± 0.00^{d}	0.35 ± 0.01^{cd}
	B14	8.20 ± 0.01^{a}	7.95 ± 0.02^{a}	6.73 ± 0.09^{b}	3.52 ± 0.31^{c}	5.24 ± 0.02^{bc}	5.22 ± 0.03^{f}	0.71 ± 0.02^{e}	0.29 ± 0.02^{cd}
	B18	7.96 ± 0.02^{b}	7.92 ± 0.02^{ab}	6.71 ± 0.03^{b}	3.49 ± 0.22^{c}	5.18 ± 0.00^{cd}	5.37 ± 0.01^{cd}	0.68 ± 0.01^{f}	0.28 ± 0.03^{d}
	B22	7.35 ± 0.07^{c}	7.51 ± 0.11^{c}	6.41 ± 0.09^{c}	3.53 ± 0.18^{c}	5.04 ± 0.15^{de}	5.32 ± 0.01^{de}	0.68 ± 0.02^{fg}	0.21 ± 0.02^{e}
	B26	7.10 ± 0.02^{d}	7.12 ± 0.03^{d}	6.25 ± 0.05^{c}	3.67 ± 0.05^{c}	4.86 ± 0.02^{e}	5.30 ± 0.02^{ef}	0.67 ± 0.00^{fg}	0.12 ± 0.01^{f}
	B30	7.06 ± 0.00^{d}	7.02 ± 0.05^{d}	6.21 ± 0.05^{c}	3.92 ± 0.07^{bc}	4.62 ± 0.11^{f}	5.29 ± 0.04^{ef}	0.66 ± 0.00^{g}	0.13 ± 0.00^{f}

CFU: Colony Forming Unit.
A: Control group; B: Inoculation group (*Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Weissella confuse*; 1:1:1). Ax = x day, Bx = x day.
The values were expressed as mean ± standard deviation (n = 3).
Values in a column with different superscripts are significantly different ($p < 0.05$) according to Duncan's multiple range test.

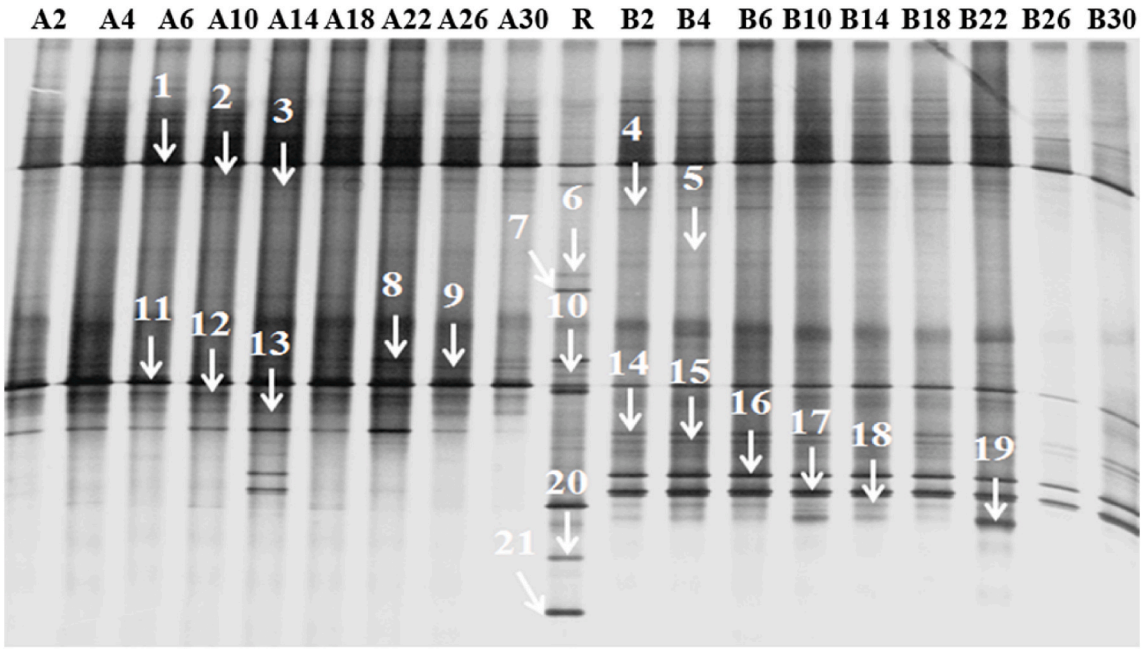

Fig. 1. Bacterial DGGE profile of the PCR products originated with GCU968-L1401 primer obtained from two group of the Sichuan-style sausages.
A: Control group; R: Raw meat sample group; B: Inoculation group (*Lactobacillus plantarum*: *Pediococcus pentosaceus*: *Weissella confuse*; 1:1:1). Ax = x day, Bx = x day.

(Chengdu Qingke Company, Chengdu, Sichuan). GenBank database searches were performed using the basic local alignment search tool programme for determining the closest known relatives of the partial 16S rRNA sequence obtained.

2.7. High-throughput sequencing

The DNA extracted at 0 and 30 days was amplified through PCR by using the universal prokaryotic primers 338F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT) for the V3–V4 region of the 16S rRNA gene. The PCR reactions were performed in 25-μL reaction mixtures consisting of 5 μL of 5 × reaction buffer, 5 μL of 5 × GC buffer, 2 μL of dNTP (2.5 mM), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 2 μL of DNA template, 8.75 μL of ddH_2O, and 0.25 μL of Q5 DNA polymerase. The initial denaturation was performed at 98 °C for 2 min, 25–30 cycles, with denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, final extension at 72 °C for 5 min, and 10 °C hold. The detection method for the concentration and quality of PCR products was similar to that used for the extraction and PCR amplification of DNA. The paired-end sequencing was performed on the Illumina HiSeq 2500 PE250 platform (Novogene Bioinformatics Technology Co., Ltd., Tianjin, China) for 600 cycles

Table 2
Phylogenetic affiliations of bacterial dynamics in Sichuan-style sausages with or without mixed starter culture after 30 days of ripening based on DNA analyses and the corresponding band in the DGGE profile.

Band[a]	Most closely related species	Identity (%)	Accession number[b]
1	*Pediococcus pentosaceus*	99	KX886792.1
2	*Staphylococcus saprophyticus*	99	NR074999.1
3	*Staphylococcus xylosus*	99	NR113350.1
4	*Enterococcus rotai*	98	CP013655.1
5	*Enterococcus moraviensis*	97	NR113937.1
6	*Nissabacter archeti*	98	NR147393.1
7	*Enterobacter aerogenes*	99	CP002824.1
8	*Citrobacter freundi*	98	KM515969.1
9	*Citrobacter sedlakii*	96	KY886230.1
10	*Weissella jogaejeotgali*	98	CP014332.1
11	*Weissella bombi*	99	NR136437.1
12	*Weissella thailandensis*	99	LC097077.1
13	*Weissella hellenica*	100	LC096226.1
14	*Lactobacillus plantarum*	99	NR115605.1
15	*Weissella oryzae*	98	NR036924.1
16	*Weissella cibaria*	99	LC096236.1
17	*Weissella confusa*	100	LC063164.1
18	*Weissella paramesenteroides*	98	LC096224.1
19	*Enterococcus faecium*	99	NR112039.1
20	*Weissella ceti*	99	NR117039.1
21	*Weissella viridescens*	97	LC065037.1

[a] Bands are lettered as indicated on DGGE gel shown in Fig. 1.
[b] Accession numbers of sequences of most closely related species found with Blast search.

Table 3
Statistics of samples sequencing.

Samples	OTUs
A0	65258
B0	64368
A30	70595
B30	76297

A: Control group; B: Inoculation group (*Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Weissella confuse*; 1:1:1). Ax = x day, Bx = x day.

(MiSeq Kit V3). Raw 16S gene sequence data were analysed with Quantitative Insights Into Microbial Ecology (QIIME) software (Caporaso et al., 2010). Pairs of reads from the original DNA fragments were merged using FLASH (Mwadondo, Ghilamicael, Alakonya, & Kasili, 2017) and filtered through QIIME quality filters.

2.8. Bioinformatics and data analysis

The high-throughput sequencing data were sorted into different samples by matching the specific barcodes. The primers, barcodes, and adaptors were then trimmed off using the collection command line tools of FASTX-Toolkits (Liu et al., 2018). Then, the sequence data were filtered, multiplexed, and prepared for statistical analysis (Bassi et al., 2016; Rebecchi et al., 2015). The remaining sequences were called effective sequences. The operational taxonomic units (OTUs) and the taxonomy-based approaches were performed for effective sequences analysis. The effective sequences were clustered into OTUs at the cut-off level of 3% by using Mothurv1.30.0 (Kim, Countway, Yamashita, & Caron, 2012), whereas the taxonomy matrices were obtained at a set confidence threshold of 80% using Rv 3.0.0 with the Vegan package (De Wolf et al., 2018). QIIME was used to evaluate alpha diversity to represent the bacterial biodiversity in a single sample. Rarefaction curves, the Shannon diversity index, Chao 1 richness, and Good's coverage were calculated to evaluate the alpha diversity (Johnston, Park, & Smissen, 2017).

2.9. Statistical analysis

Statistical analysis was performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA). All analyses were run in triplicate. The collected data were subjected to one-way analysis of variance (ANOVA). Duncan's multiple range tests were used to evaluate statistical significance in group means, and $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Microbial analysis and physiochemical parameters

The plate count results, determination of physicochemical parameters, and the ANOVA results of Duncan's multiple range test are presented in Table 1. The total viable counts of *Staphylococcus*, *Enterobacteriaceae*, moulds, and yeasts exhibited the same changing tendency on PCA, BP, VRBGA, and PDA, respectively, with group A exhibiting higher counts than group B during the processing of Sichuan-style sausage. On the other hand, LAB exhibited a higher count in group B than in group A because the LAB inoculated in group B multiplied after fermentation and quickly became the dominant bacteria. This finding is consistent with that of a previous study (Fonseca, Cachaldora, Gómez, Franco, & Carballo, 2013).

From the beginning (6.16 ± 0.17 lg $CFUg^{-1}$ in raw materials) to the end (8.28 ± 0.02 lg $CFUg^{-1}$ in group A and 8.11 ± 0.05 lg $CFUg^{-1}$ in group B) of the fermentation stage (2 days), the total bacterial counts increased significantly, but with a nonsignificant difference, in the two groups. However, in the later stage, the total bacterial count exhibited a significant difference between two groups, with group B always lower than group A. Simultaneously, LAB, *Staphylococcus*, *Enterobacteriaceae,* moulds, and yeasts exhibited the same change tendency as total bacterial counts. The bacterial counts in each instance (except for LAB) were higher in group A than in group B.

Enterobacteriaceae, the main group of microorganisms that produces histamine in sausage, are vital indicators of raw meat hygiene, spoilage,

Table 5
The diversity index of bacterium.

sample	Simpson	Chao 1	Ace	Shannon
A0	0.867985	913	913	4.71
B0	0.767226	938	938	4.02
A30	0.848771	802	802	4.35
B30	0.846813	694	694.69	4.14

A: Control group; B: Inoculation group (*Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Weissella confuse*; 1:1:1). Ax = x day, Bx = x day.

Table 4
Statistics of OTUs classification and taxonomic identification on different levels.

sample	phylum	class	order	family	genus	species	unclassified
A0	911	907	850	799	663	117	0
B0	934	926	864	806	635	112	0
A30	800	798	755	715	616	99	0
B30	693	693	677	664	627	148	0

A: Control group; B: Inoculation group (*Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Weissella confuse*; 1:1:1). Ax = x day, Bx = x day.

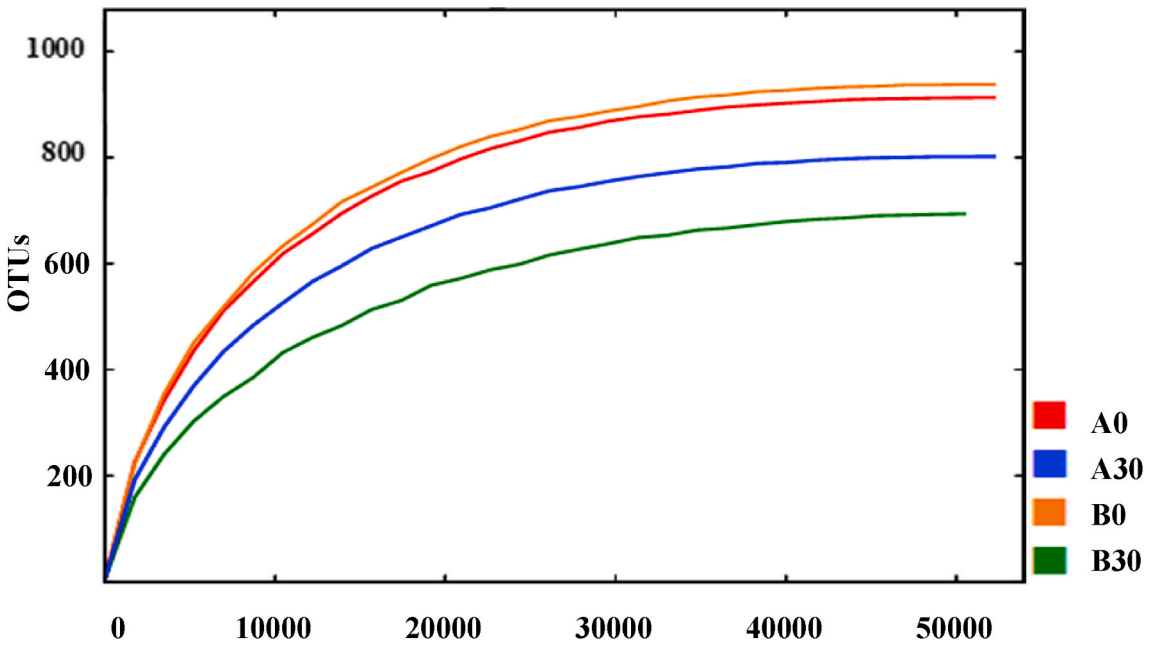

Fig. 2. Rarefaction curve
A: Control group; B: Inoculation group (*Lactobacillus plantarum*: *Pediococcus pentosaceus*: *Weissella confuse*; 1:1:1). Ax = x day, Bx = x day.

and pathogenic microorganisms. The total counts of *Enterobacteriaceae* were in a range between 3.94 ± 0.34 lg $CFUg^{-1}$ and 5.37 ± 0.11 lg $CFUg^{-1}$ in the control group, whereas they were between 3.49 ± 0.22 lg $CFUg^{-1}$ and 4.95 ± 0.10 lg $CFUg^{-1}$ in the inoculation group (Table 1). The total counts of *Enterobacteriaceae* in the sausage were lower in group B than in group A, and the difference between the two groups was statistically significant. The number of *Enterobacteriaceae* in group B increased slightly from 18 to 30 days, which may be due to the neutralisation of acidic substances with protein decomposition and the production of BAs and other alkaline substances in the later stage of processing, which slightly increased the pH value of the sausage and slightly weakened the inhibitory effect on *Enterobacteriaceae* growth (Lorenzo, Gómez, & Fonseca, 2014; Simion, Vizireanu, Alexe, Franco, & Carballo, 2014).

The results of pH measurements are presented in Table 1. The pH value of the two sausage groups exhibited an initial rapid decrease, followed by an increase before being balanced. The pH value of the sausages in group B was always lower than that in group A during entire processing. The initial pH of 6.02 decreased rapidly during the first 2 days of fermentation, followed by a slow decrease from day 2 to day 14, and an increase to 5.57 and 5.37 in group A and group B on day 18, respectively; it later decreased slowly and tended to balance. The presence and metabolic activity of starter cultures are crucial to sausage production, in which LAB produce lactic acid and organic acid through sugar fermentation to reduce the pH value of meat products, thus ensuring the stability and safety of the final products. The pH value of group B was lower than that of the control group because *Lactobacillus* was inoculated in group B and produced lactic acid and organic acid, lowering the pH (Babić et al., 2011). The pH value of the two groups of sausages increased slightly probably because the growth of mixed bacteria led to the production of alkaline substances such as BAs and volatile basic nitrogen, leading to the neutralisation of some acids and increase in the pH value (Olesen, Meyer, & Stahnke, 2004).

Aw is linearly correlated with moisture content in sausages and is a crucial barrier technology factor for measuring the sausage shelf life. The Aw and moisture content of the two groups exhibited a downward trend during the whole fermentation process (Table 1). The Aw of group B was always lower than that of group A, especially in the later stage of processing (14–30 days), which may be due to the loss of free water in the early stage of fermentation, whereas the pH value of group B was lower and the water holding capacity of protein decreased in the later stage of fermentation, lowering the Aw of group B compared with that of group A (Hu, Xia, & Ge, 2007). After 30 days of processing, the Aw of the two groups was lower than 0.70. Studies have demonstrated that when Aw was lower than 0.9, the growth of some bacteria was inhibited. However, yeasts and moulds still grew vigorously, and some moulds with strong adaptability reproduced when the Aw was reduced to 0.80–0.85. Food spoilage significantly slowed down only when Aw was reduced to 0.75 (Sun, Chen, et al., 2016). Both groups ended up with less than 25% water, which is also a sign of maturity.

3.2. DGGE profiles of fermented sausage

The bands at different positions on the DGGE map represented different microbial species. The increased number of bands depicted the abundance of microbial species in the sample, whereas the increased band brightness also depicted the increased number of microorganisms (Miragoli, Patrone, Romaniello, Rebecchi, & Callegari, 2020).

The bacterial DGGE profiles of the two fermentation groups are illustrated in Fig. 1, and the results of band sequencing are presented in Table 2. No differences were detected in the DGGE profiles of the replicates obtained at the same sampling point (data not shown). A comigration was always obtained between the bands cut from the DGGE gel; thus, these sequencing results are not presented in Table 2.

The microbial diversity in the raw material sample was richer than that in the other samples and implied the decrease in bacterial diversity during fermentation. Band 1, band 14, and band 16 were the mixed starter culture species that also appeared at a low level in the raw material and gradually became the predominant bacteria during fermentation. According to the counts and grey value of bands, *P. pentosaceus* (band 1) and *W. bombi* (band 11) were the predominant bacteria in group A, whereas *P. pentosaceus* (band 1), *W. bombi* (band 11), *L. plantarum* (band 14), *W. oryzae* (band 15), *W. cibaria* (band 16), and *W. confuse* (band 17) were the predominant bacteria in group B. The dynamic change in the bacterial system of group B was more stable than that of group A, indicating the controlling ability of the mixed starter culture and the safety of Sichuan-style fermented sausage.

Moreover, according to the result blasted form the National Centre

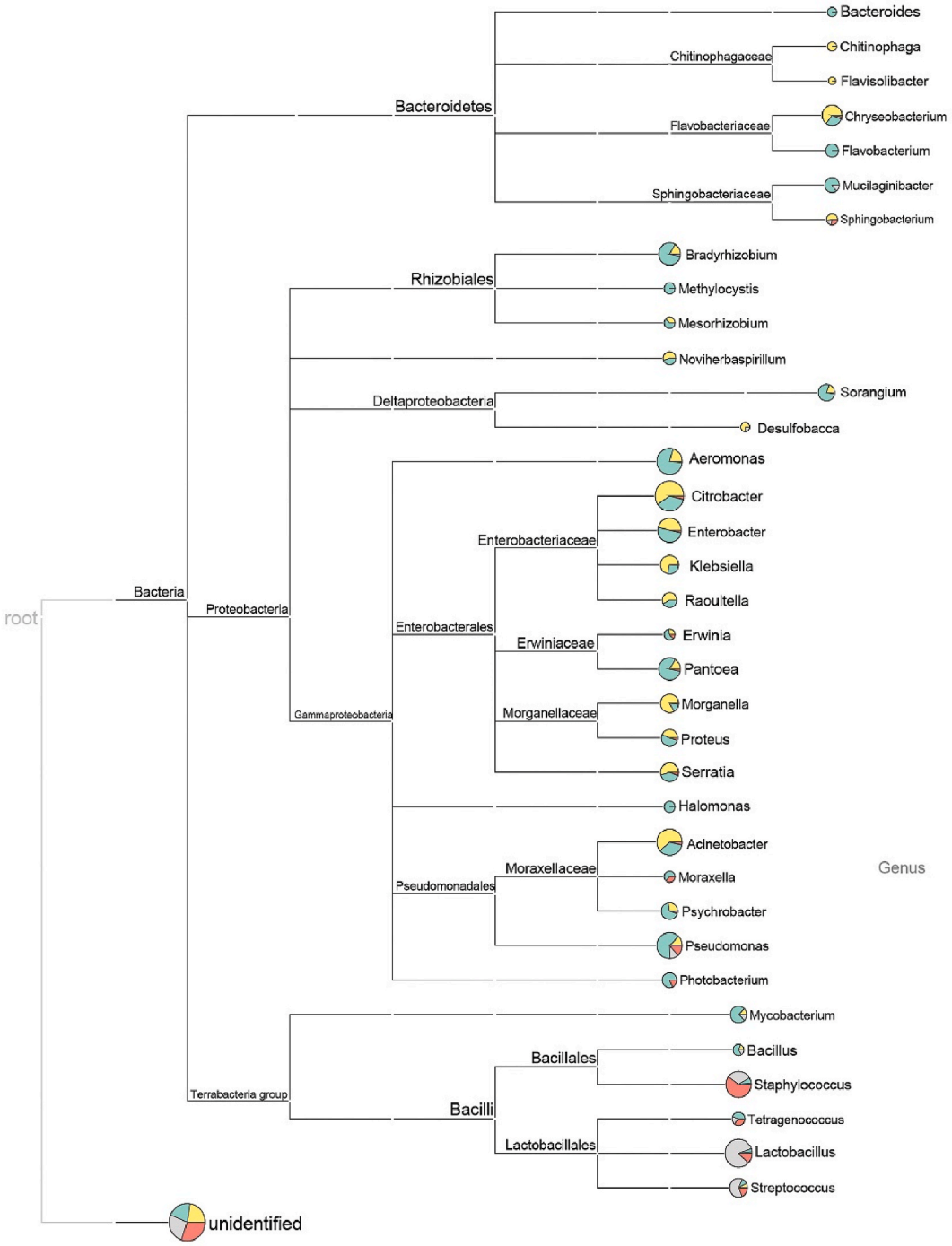

Fig. 3. Phylogenetic tree of 35 kinds of bacteria coding histidine decarboxylase gene.

for Biotechnology Information (NCBI) database, *Enterobacter aerogenes* (band 7) and *Citrobacter freundi* (band 8) were two types of bacteria who encoded HDC gene, indicating the ability to produce histamine. This result was consistent with those of other studies (Durlu-Özkaya, Ayhan, & Vural, 2001; Zaman, Bakar, Selamat, & Bakar, 2010). Different bands were visible in the upper part of DGGE gels in the two groups. They were all determined to be heteroduplexed by sequencing (data not shown).

3.3. Characteristics of the high-throughput sequencing data

After quality control, 276518 high-quality 16S rRNA gene sequences presented in Table 3, ranging from 200 bp to 450 bp, were recovered from four sausage samples that were clustered with the 97% identity level. Statistics of the OTU classification and taxonomic identification on different levels are presented in Table 4.

Common indicators of bacterial diversity are the Chao1 and abundance-based coverage estimator (ACE) indices (which focus on community richness), and Shannon and Simpson indices (which focus on community evenness) (Yu et al., 2019) (Table 5). The Simpson and Shannon diversity indices were the highest in sample A0; the Chao 1 and the ACE estimators were the highest in sample B0; and all indices, except for the Shannon index, were the lowest in B30. The least bacterial biodiversity was present in sample B30, indicating that the bacterial diversity in sausages could be better controlled by the inoculation starter

(Xiao, Liu, Chen, Xie, & Li, 2020). The rarefaction curve evaluated the ability of each sample to reflect the bacterial diversity contained in the sample at the current sequencing depth (Wang et al., 2018). The sparse curves of the four samples all entered the plateau stage, indicating that the sequencing depth was sufficient, and the sequencing results could comprehensively cover the bacterial information contained in the samples (Fig. 2).

The results of high-throughput sequencing exhibited 197 types of bacteria at the genus level, of which 35 bacteria encoded HDC gene (Fig. 3). Statistically, 5 types of bacteria encoded HDC gene among the 10 richest bacteria, namely *Staphylococcus*, *Klebsiella*, *Lactobacillus*, *Citrobacter,* and *Acinetobacter*. Devivilla et al. (2019) also obtained similar results. According to the size of the circles in Fig. 3, *Citrobacter* was the predominant bacteria producing histamine. The results were similar to those obtained through culture-dependent and PCR-DGGE techniques. Most *Enterobacter aerogenes* had HDC activity, and 76% *S. xylosus* isolated from the French dry sausages produced histamine (Hwang, Kung, Lin, Hwang, & Tsai, 2011; Lee et al., 2012; Montel, Masson, & Talon, 1999). Additionally, several studies have reported that *Klebsiella* could produce histamine similar to *Lactobacillus* and *Acinetobacter* (Da Silva, Pinho, Ferreira, Plestilová, & Gibbs, 2002; Hsu et al., 2009). The phylogenetic tree exhibited that *Klebsiella* and *Citrobacter* belong to *Enterobacteriales*, and nine types of bacteria belong to *Enterobacteriales* (Fig. 3). Thus, controlling *Enterobacteriales* would be a feasible method to decrease the content of histamine in Sichuan-style sausages.

4. Conclusion

The present study used culture-dependent techniques, PCR-DGGE techniques, and high-throughput sequencing to investigate the bacterial diversity in Sichuan-style sausages. The results exhibited that the diversity of bacteria in the inoculation group was significantly lower than that in the control group. The bioinformation of bacteria that encoded histidine decarboxylase was obtained with the help of the NCBI database. At the genus level, 5 bacteria that encoded histidine decarboxylase among the 10 richest bacteria in Sichuan-style sausages were *Weissella*, *Staphylococcus*, *Klebsiella*, *Lactobacillus*, *Citrobacter,* and *Acinetobacter*. Approximately 25.7% of the histidine decarboxylase-encoding bacteria belonged to *Enterobacteriales*. Thus, the study revealed poor food hygiene in spontaneously fermented Sichuan-style sausages and exhibited that the mixed starter culture ensured a more stable bacterial diversity and safer Sichuan-style sausages.

CRediT authorship contribution statement

Yilun Wang: Data curation, Writing - original draft, preparation, Software, Validation, Writing - review & editing. **Binbin Li:** Conceptualization, Methodology, Software. **Yuxuan Liu:** Visualization, Investigation. **Xiaohong Huang:** Visualization, Investigation. **Nan Zhang:** Conceptualization, Methodology, Software, Data curation, Writing - original draft, preparation, Software, Validation. **Yifang Yang:** Visualization, Investigation. **Zihan Xiao:** Software, Validation. **Qinxin Yu:** Software, Validation. **Shujuan Chen:** Supervision. **Li He:** Supervision. **Aiping Liu:** Supervision. **Shuliang Liu:** Supervision. **Likou Zou:** Supervision. **Yong Yang:** Supervision.

Declaration of competing interest

The authors declared no conflict of interest in this study.

1: *Pediococcus pentosaceus*, 2: *Staphylococcus saprophyticus*, 3: *Staphylococcus xylosus*, 4: *Enterococcus rotai*, 5: *Enterococcus moraviensis*, 6: *Nissabacter archeti*, 7: *Enterobacter aerogenes*, 8: *Citrobacter freundi*, 9: *Citrobacter sedlakii*, 10: *Weissella jogaejeotgali*, 11: *Weissella bombi*, 12: *Weissella thailandensis*, 13: *Weissella hellenica*, 14: *Lactobacillus plantarum*, 15: *Weissella oryzae*, 16: *Weissella cibaria*, 17: *Weissella confuse*, 18: *Weissella paramesenteroides*, 19: *Enterococcus faecium*, 20: *Weissella ceti*, 21: *Weissella viridescens*.

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