



Unraveling microbial community diversity and succession of Chinese Sichuan sausages during spontaneous fermentation by high-throughput sequencing

Xinhui Wang^{1,2} · Songhu Wang¹ · Hai Zhao¹

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Abstract The microbial community diversity and succession of Chinese Sichuan sausages during the spontaneous fermentation were demonstrated using high-throughput sequencing technology. The bacterial diversity was abundant and the succession of bacterial community along the direction of *Lactobacillus* spp. increased and *Weissella* spp. decreased. While fungal diversity was single and trace fungal population was detected. The core functional microbiota were lactic acid bacteria, including *Lactobacillus* spp., *Weissella* spp. and *Pediococcus* spp. In initial fermentation, *Weissella* spp. was the dominant bacteria and its relative abundance was 49.84%, but then its relative abundance decreased to 11.96% during fermentation before recovering to 26.74% at the end of fermentation. Meanwhile, *Lactobacillus* spp. rose from 24.70 to 55.74% and became the dominant genus. Moreover, *Pediococcus* spp. increased from 0.06 to 18.05% on day 20 but then decreased to 1.89% on day 30. These results revealed that the primary microorganisms contributing to spontaneous fermentation of Chinese Sichuan sausages were bacteria, while eukaryotic microorganisms such as yeast scarcely contributed to fermentation.

Keywords Chinese Sichuan sausages · Core microorganisms · Microbial community diversity · High-throughput sequencing

Introduction

Chinese Sichuan sausages are Chinese traditional meat products with a long history in China, which are fermented spontaneously for 4–5 weeks at 8–15 °C for the development of distinctive flavors (Chen et al. 2016; Liu et al. 2010; Wang et al. 2013). On the basis of their production process, they are considered as fermented sausages and involve various microorganisms which usually determine their quality and flavors (Chen et al. 2016; Rebecchi et al. 2015). Therefore, it is essential to investigate microbial community characteristics to identify the primary microorganisms and core functional microorganisms for improvement of fermentation management and exploring microbial resource as starter cultures.

In the past several years, the analyses of microbial community in Chinese fermented sausages, mainly focused on bacterial community, especially for lactic acid bacteria, using culture-dependent methods, PCR-temporal temperature gradient gel electrophoresis (PCR-TTGE) and PCR-denaturing gradient gel electrophoresis (PCR-DGGE), but little attention has been paid to fungal diversity (Gao et al. 2014; Wang et al. 2016; Mauriello et al. 2004). Accordingly, a complete understanding of the composition, diversity and succession of microbial community remains elusive, and the core functional microorganisms shift in Chinese Sichuan sausages fermented spontaneously remains unclear.

Nowadays, high-throughput sequencing (HTS) technology conducted by Illumina MiSeqTM analysis has been

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✉ Xinhui Wang
wangxinhui19820319@163.com

¹ Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu 610041, Sichuan Province, China

² Meat-Processing Application Key Laboratory of Sichuan Province, Chengdu University, Chengdu 610106, Sichuan Province, China

considered as a powerful tool for exploring complex microbial ecosystems regarding its potentiality to overcome classical microbiology deficiency because it is rapidly improving in quality, speed and cost (Quigley et al. 2012; Dalmaso et al. 2016; Carmen and Mas 2016). Therefore, it is becoming widely used to investigate whole microbial communities in food matrixes, such as Italian salami (Wang et al. 2018; Połka et al. 2015), Paocai (Liang et al. 2018) and Douchi (Yang et al. 2016). However, until now this approach has not been applied to research the microbial community in Chinese Sichuan sausages fermented spontaneously. Thus, in this study, microbial community diversity and succession of Chinese Sichuan sausages during spontaneous fermentation was unraveled by HTS approach. These results will enhance the understanding of the characteristics of microbial community and microbial ecological function of Chinese Sichuan sausages from the point of core functional microorganisms.

Materials and methods

Samples preparation and sampling

Chinese Sichuan sausages formulation includes 750 g/kg lean pork meat, 250 g/kg pork back fat, 25 g/kg NaCl, 2 g/kg sucrose, 5 g/kg chili powder, 50 mg/kg seed powder of Chinese prickly ash, 10 g/kg Spice powder and 100 mg/kg NaNO₂. Then the sausages were made and fermented spontaneously according to the methods as described by Wang et al. (2013). Sausages (50 g) were sampled in triplicate from fermentation on day 1, 10, 20 and 30. Then samples were immediately stored at −80 °C for subsequent analysis.

The pH, aw, total lactic acid and total volatile base nitrogen measurement

The pH, aw, total lactic acid and total volatile base nitrogen (TVB-N) were measured in triplicate according to the methods as described by Wang et al. (2018).

DNA extraction

The microbial DNA of samples was extracted using the E.Z.N.ATM Mag-Bind Soil DNA Kit (OMEGA, USA) and DNA quality was monitored using agarose gel electrophoresis according to the methods as described by Wang et al. (2018).

Illumina high-throughput sequencing

Extracted DNA was used as the template and the V4 region of bacterial 16S rRNA and fungal 18S rRNA were amplified by PCR using the universal bacterial primers 515F and 806R with Illumina barcoded adapters (Caporaso et al. 2010; Wang et al. 2018), and the universal fungal primers V43NDF and Euk_V4_R (Wang et al. 2018), respectively. The two step-PCR technology was conducted in which the first PCR step worked 25 cycles using untagged primers, and the second PCR step worked 5 cycles with tagged primers and the first step products used as template. The PCR amplification was carried out according to the methods as described by Wang et al. (2018). Then, the purified PCR products was sequenced with a MiSeq Illumina instrument (Illumina, USA) at Sangon Biotech Co., Ltd (Shanghai, China) (Caporaso et al. 2010; Edgar 2010; Mago et al. 2011).

Data analysis

The operational taxonomic unit (OTU) at an identity threshold of 97% with UPARSE software (Edgar 2010) and the taxonomy based approaches at a set confidence threshold of 80% using Rv 3.0.0 (R Development Core Team 2011) with the Vegan package (Dixon 2003) were performed for the sequence data analysis. Shannon index and Chao1 estimator values were calculated in RDP at 97% sequence similarity (<http://pyro.cme.msu.edu/>). Alpha diversity was evaluated by Rarefaction curves, Good's coverage, Simpson and Shannon diversity indices and Chao 1 richness (Grice et al. 2009). Principal component analysis (PCA) was performed to depict the microbial communities composition of samples (Jiang et al. 2013). Venn diagram was used to assess the similarity and difference in microbial communities among all samples (Liu et al. 2017).

Results and discussion

The pH, aw, total lactic acid values and TVB-N concentration

The analyses of pH, aw, total lactic acid values and TVB-N concentration in the Chinese Sichuan sausages during spontaneous fermentation are shown in supplemental Table 1. A slight increase in pH was found in sausages through the whole fermentation course and pH increased from initial 5.49 to 5.73, which may be because of peptides, amino acids, and amines generated by microbial proteolytic activity, resulting in a buffering effect on the organic acids (Ruiz-Moyano et al. 2011). These findings

are in agreement with those reported by other authors (Essid and Hassouna 2013; Lorenzo et al. 2014). Interestingly, a sharp increase in total lactic acid values was detected in sausages. The total lactic acid values increased from initial 23.2 to 68.7 mmol/kg on day 10, but then gradually reduced to 48.7 mmol/kg on day 30. These results are in agreement with microbial community structure variety, by which time *Lactobacillus* spp., which possesses strong acid producing ability and acid resistance (Wang et al. 2013), underwent a sharp increase from 24.70% (day 1) to 63.14% (day 20), then decreased to 55.74% on day 30. The a_w value was 0.992 in the initial fermentation, and then gradually decreased to 0.815 along with the fermentation. Such low water activity has some advantages including the inhibition of food-borne pathogenic and spoilage bacteria (Wang et al. 2018). A slight increase reduction in TVB-N concentration was observed from day 1 to day 20, but then increased to 2.73 mg/kg on day 30. The variation trend of TVB-N content as a useful index for spoilage (Hu et al. 2007) was opposite to the change in proportion of core functional microorganisms, including *Lactobacillus* spp., *Weissella* spp. and *Pediococcus* spp. These results indicate that core functional microorganisms becoming dominant microorganisms can conducive to improve the hygienic quality of fermented sausages.

Abundance and diversity of the bacterial and fungal microorganisms

To understand microbial communities composition of samples, OTU reads were performed to depict the bacterial and fungal microorganisms in Chinese Sichuan sausage during spontaneous fermentation. Based on the sequencing results, a total of 259,423 high quality bacterial tags and a total of 283,690 high quality 18S rDNA reads were generated from 4 examined sample sets across the entire sausage fermentation process, respectively. Accordingly, the bacterial sequence reads ranged from 109,844 to 46,110, and fungal sequence reads ranged from 78,592 to 65,637. These sequence reads were clustered into 7609 OTUs at a 97% similarity level, in which 1363 OTUs belonged to bacteria and 6246 OTUs was attributed to eukaryotic microorganism. Moreover, the average number of OTUs was 340 for bacteria and 1561 for eukaryotic microorganisms, respectively. Furthermore, the analyses of the alpha-diversity indices, including the Chao1, Shannon and Simpson diversity indices, are presented in Table 1. The Good's coverage values were 100% for bacterial OTUs and 99% for fungal OTUs in all samples, suggesting that major bacterial and fungal OTUs had been captured (Grice et al. 2009). The bacterial Chao1 richness estimator decreased from 1 to 20 days fermentation with a range from 560.24 to

Table 1 The operational taxonomic units (OTUs) for Chinese Sichuan sausages during spontaneous fermentation

Fermentation time (d)	Reads		Observed OTUs		ACE index		Chao1 index		Good's coverage		Shannon index		Simpson	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
1	109,844	65,637	468	1462	608.25	3628.37	560.24	2549.81	1.00	0.99	2.31	1.52	0.24	0.65
10	52,191	68,545	290	1299	516.13	3249.97	420.80	2416.96	1.00	0.99	2.20	1.59	0.19	0.61
20	46,110	70,916	209	1374	385.99	3700.21	304.29	2571.26	1.00	0.99	1.46	1.36	0.40	0.66
30	51,278	78,592	396	2111	761.24	5843.78	580.08	3730.50	1.00	0.99	2.57	1.94	0.17	0.52

Richness estimators (Chao and Ace) and diversity indices (Shannon and Simpson) of the bacterial 16S rRNA and fungal 18S rRNA for each sample were determined using OTU based analysis

304.29, whereas Chao1 richness estimator increased to 580.08 at 30 days fermentation. The fungal Chao1 richness estimator changed little from 1 to 20 days fermentation, except for 20 days fermentation which the Chao1 richness estimator increased to 3730.5. According to the estimated Chao1, the bacterial diversity was clearly tended to decline during the early and mid-term stages of fermentation, and gradually recover at the end of fermentation. Eukaryotic diversity was fluctuating during the early and mid-term fermentation, then a dramatic increase of eukaryotic diversity was observed at the end of fermentation. These results showed that bacteria were prolific at initial stage of fermentation, but suffered along with the fermentation, presumably due to interspecific competition and the decrease of a_w during the ripening process, which may seriously inhibit the growth of bacteria, while fungal diversity remained stable during fermentation. In addition, the both bacterial and fungal Simpson indices were the lowest in sample CWC4 which was sampled at the end of fermentation. These results indicated that microbial species increased in the sausages at the end fermentation due to attenuation of core functional microbiota.

Microbial community composition

Bacterial community

Rarefaction analysis based on OTUs at 97% similarity showed approximation to an asymptote as shown in Fig. 1a, which indicated that the sequences commendably represented the bacterial diversity in the 4 samples from different fermentation stages.

The Venn diagram was performed to evaluate the distribution of OTUs among the different samples as shown in Fig. 1b. In total, 468, 290, 209 and 396 OTUs were generated from CWC1, CWC2, CWC3 and CWC4 sample, respectively. A total of 46 OTUs were shared by the four samples, which indicated that a low level similarity of bacteria diversity was presented in the four samples. These results suggest that the succession of bacterial communities changed greatly during the fermentation. The shared bacterial OTUs mainly belonged to Firmicutes and Proteobacteria at phylum level, and *Lactobacillus* spp., *Weissella* spp., *Pediococcus* spp. and *Staphylococcus* spp. at the genus level.

A total of 17 phyla, 35 classes, 69 orders, 111 families and 121 genera were received from bacterial sequences using the RDP classifier. The bacterial population of the samples was investigated at both the phylum (Fig. 2a) and genus levels (Fig. 2b), and a more precise percentage of the bacterial composition at the genus level among four samples is presented in supplemental Table 2. Five bacterial phyla, including Firmicutes, Proteobacteria,

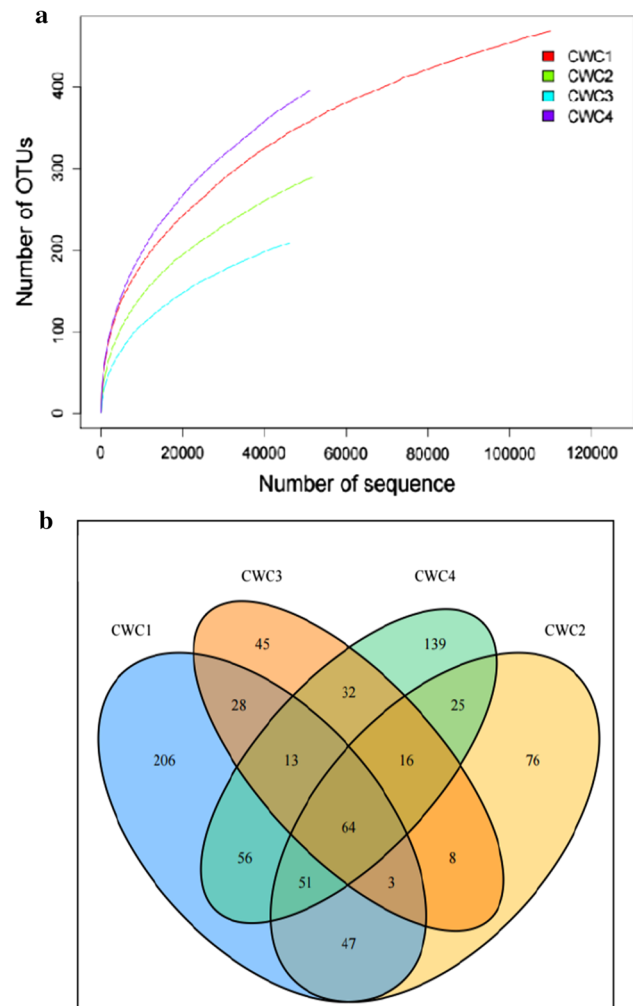


Fig. 1 Richness rarefaction curves (a) and Venn diagrams (b) of the four samples based on OTUs of bacterial sequences (3% distance). CWC1: sample fermented for 1 day, CWC2: sample fermented for 10 days, CWC3: sample fermented for 20 days, CWC4: sample fermented for 30 days

Actinobacteria, Bacteroidetes and Chloroflexi, were identified in the initial fermentation, in which Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes were detected through the whole fermentation process. Furthermore, Firmicutes and Proteobacteria were the predominant phyla, which ranged from 85.65 to 93.96% and from 5.59 to 13.95%, respectively.

In terms of genus, 31, 30, 30 and 30 genera were determined in CWC1, CWC2, CWC3 and CWC4 sample, respectively. In the initial fermentation (sample CWC1), *Weissella* spp. was the most abundant bacterial genus, comprising about 49.4% of bacteria, followed by *Lactobacillus* spp., accounting for 24.70% of bacteria. A drastic reduction in bacterial relative abundance of *Weissella* spp. were detected from day 1 to day 20 during fermentation, and the proportion of *Weissella* spp. declined from 49.41 to

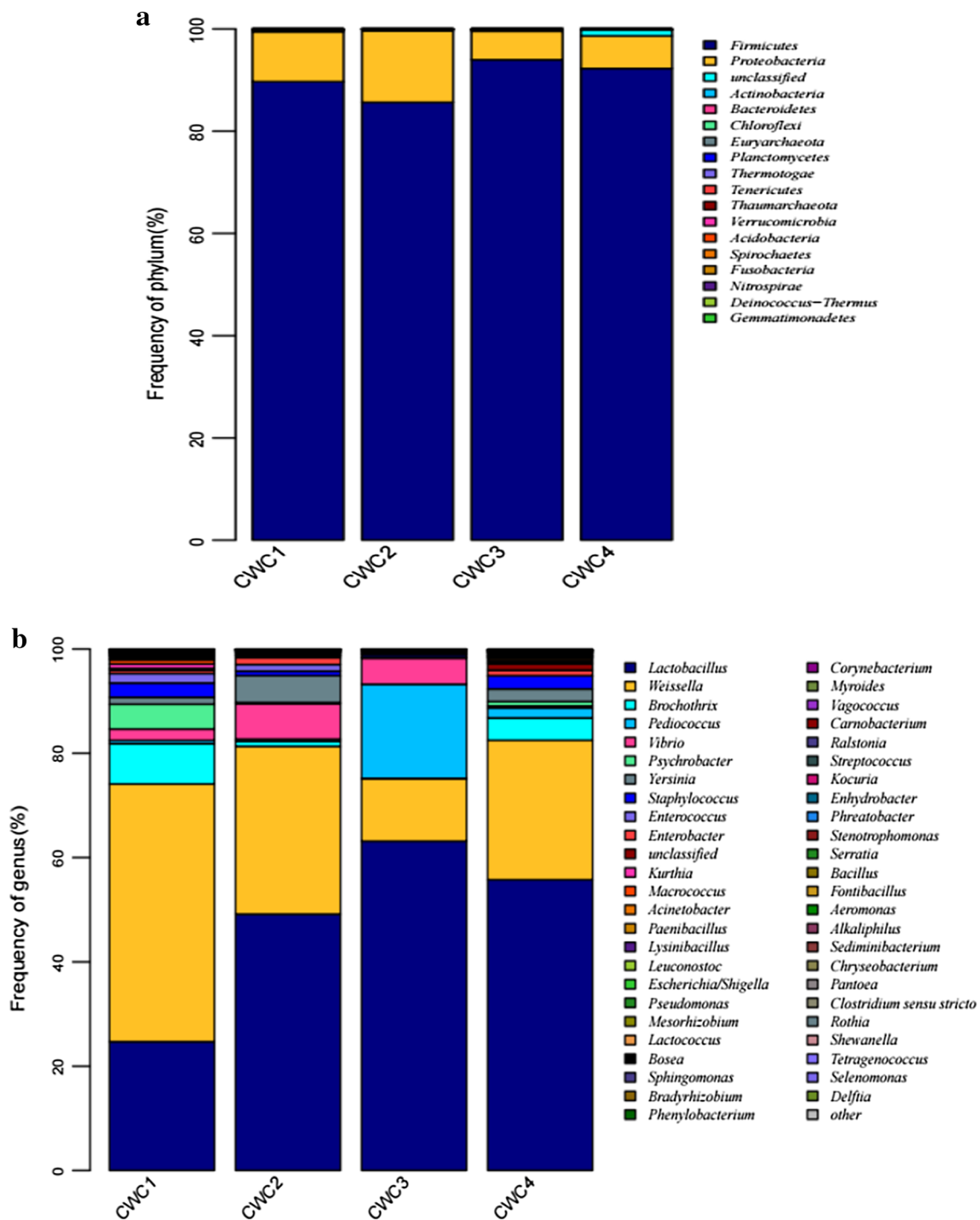


Fig. 2 Relative abundance of bacteria community proportions at phylum (a) and genus (b) level. Phyla and genera occurred at < 1% abundance in all the samples are defined as “Others”. Taxonomic classification of 97% sequence identity clusters demonstrated at

genera classification levels. CWC1: sample fermented for 1 days, CWC2: sample fermented for 10 days, CWC3: sample fermented for 20 days, CWC4: sample fermented for 30 days

11.96%. But then *Weissella* spp. recovered rapidly to reach 26.74% at the end of fermentation (day 30). The decrease of *Weissella* spp. might be due to interspecific competition with *Lactobacillus* spp., by which time *Lactobacillus* spp. underwent a sharp increase from 24.70 to 63.14% and

become the most dominant genus, substituting for *Weissella* spp.. Interestingly, in the initial fermentation, *Pediococcus* spp. was barely detectable in sample CWC1, but during the subsequent fermentation, this genus increased sharply from 0.06% (day 1) to 18.05% (day 20), then

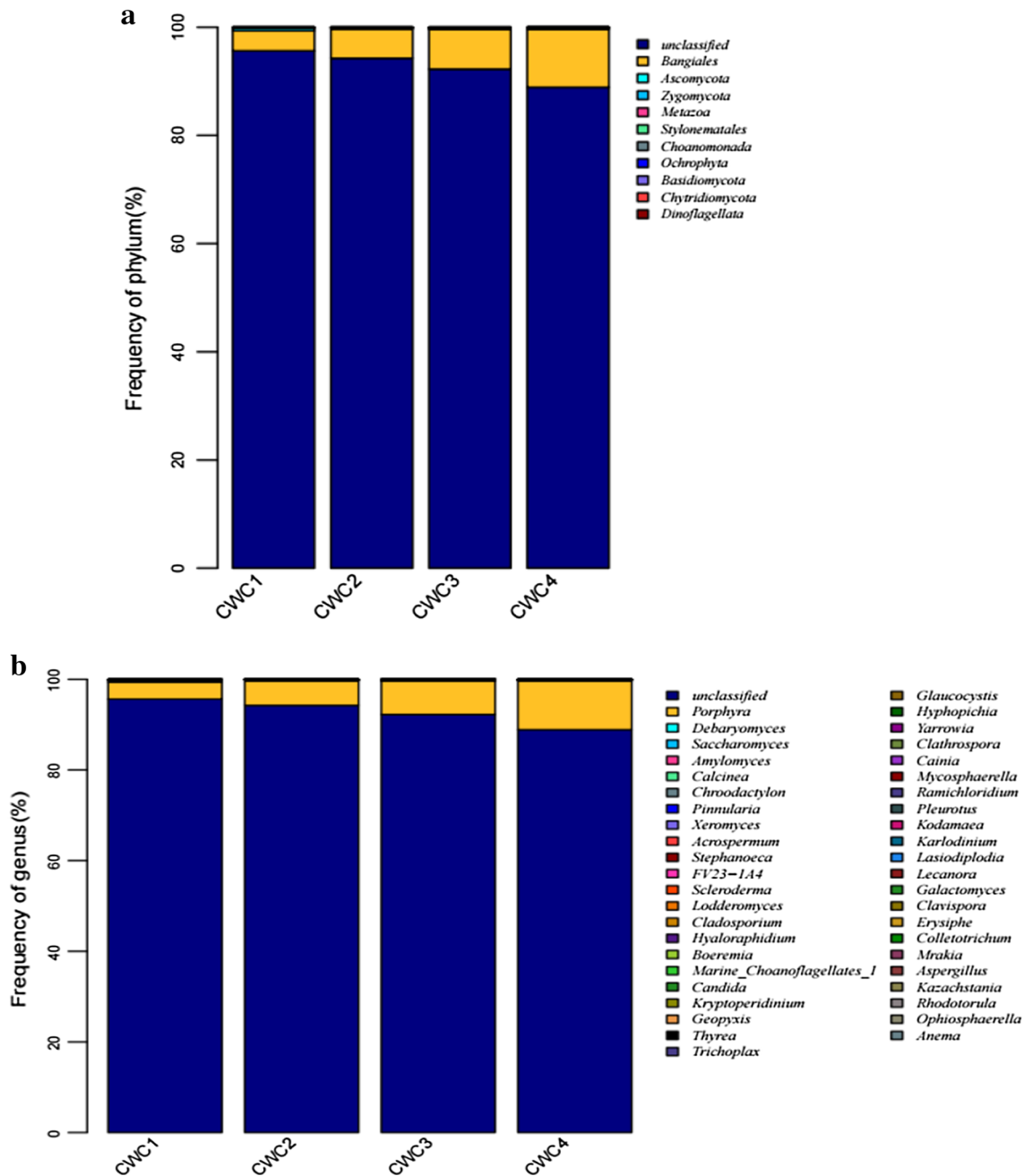


Fig. 3 Relative abundance of fungal community proportions at phylum (a) and genus (b) level. Phyla and genera occurred at < 1% abundance in all the samples are defined as “Others”. Taxonomic classification of 97% sequence identity clusters demonstrated at

genera classification levels. CWC1: sample fermented for 1 days, CWC2: sample fermented for 10 days, CWC3: sample fermented for 20 days, CWC4: sample fermented for 30 days

declined dramatically again to 1.89% on day 30. Moreover, other major genera present in the initial fermentation included *Brochothrix* spp., *Psychrobacter* spp., *Staphylococcus* spp., *Enterococcus* spp., *Vibrio* spp. and *Yersinia* spp., each constituting more than 1% of total bacteria. Then, *Psychrobacter* spp., *Enterococcus* spp. and *Vibrio* spp. declined dramatically during the subsequent fermentation and these genera were not detected on fermentation

day 30. *Brochothrix* spp. which belongs to LAB declined dramatically from 7.77% (day 1) to 0.09% (day 20), then recovered rapidly to 4.30% on day 30. *Staphylococcus* spp. exhibited a similar trend to *Brochothrix* spp., with the bacterial relative abundance decreased dramatically from 2.74% (day 1) to 0.56% (day 20), then recovered rapidly to 2.52% on day 30.

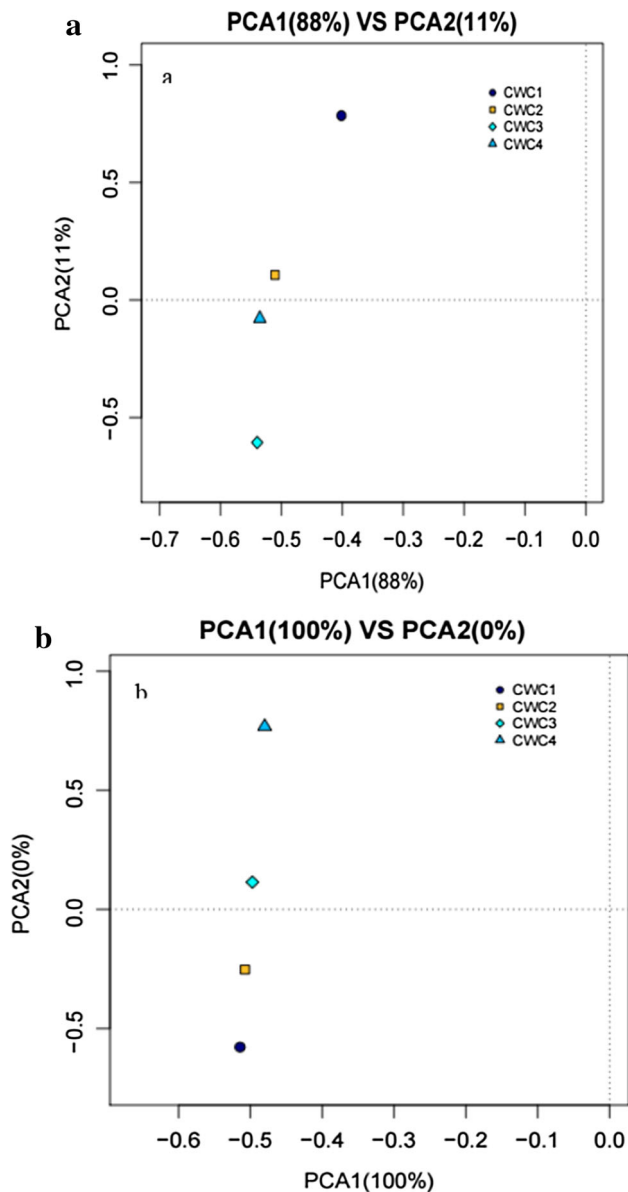


Fig. 4 Multiple samples of PCA analysis according to the bacterial (a) and fungal (b) diversity. CWC1: sample fermented for 1 days, CWC2: sample fermented for 10 days, CWC3: sample fermented for 20 days, CWC4: sample fermented for 30 days

Fungal community

Fungal diversity of the samples investigated at both the phylum and genus levels is shown in Fig. 3. Most of the reads, occupying for 90% of the total fungal sequences, belonged to unclassified reads. Compared with bacteria, the fungal diversity in sausages was lower during fermentation. At fungal phylum level, only Bangiales and Ascomycota was presented higher than 0.1% throughout the entire fermentation process. At the genus level, *Porphyra* spp. dominated throughout the entire fermentation process, increasing gradually from 3.75 to 10.75%. *Debaryomyces*

spp. and *Saccharomyces* spp. were detected with a small amount of quantity, lower than 0.1%.

Microbial succession analysis

The similarities in microbial communities in sausages during fermentation process were evaluated by PCA analysis as shown in Fig. 4, showing an obvious separation of the bacterial communities among the four different samples. The first and second axes showed values of cumulative percentage variance of species equal to 88% and 11%, respectively (Fig. 4a). In total, 99% variances of species were explained by the two axes. According to PCA analysis, bacterial communities fluctuated wildly during fermentation from day 1 to 30. However, the fungal communities were relatively stable during fermentation (Fig. 4b).

The heat map analysis was performed to compare the differences and similarity in the microbial community structures of sausages during fermentation as shown in Fig. 5 (Rundell et al. 2014). Bacterial community structure of Chinese Sichuan sausages fluctuated greatly during fermentation. In the initial fermentation, *Weissella* spp. was the most dominant genus, but then decreased from 49.84 to 11.96% during fermentation before recovering to 26.74% at the end of fermentation which remained the predominant genera. The members of *Weissella* spp. have been regarded as the functional lactic acid bacteria because of their useful metabolites, particularly for acids and bacteriocins, which not only could be conducive to the formation of flavour compounds, but also inhibit the growth of pathogens. During fermentation, *Lactobacillus* spp. increased from 24.70% (day 1) to 55.74% (day 30) and became the most dominant genus, replacing the dominant position of *Weissella* spp.. The members of *Lactobacillus* spp. have been considered to be responsible for development of the distinctive sour taste and rich flavour in sausages due to their capacities for the breakdown of proteins to peptides and amino acids (Georgieva et al. 2009; Luckow and Delahunty 2004). Besides, The proportion of *Pediococcus* spp. which have been believed to be response for the proteolysis and release of free amino acids increased sharply from 0.06% (day 1) to 18.05% (day 20) but then decreased to 1.89% on day 30 become a minority genus. These findings indicate that *Lactobacillus* spp., *Weissella* spp., and *Pediococcus* spp. are core functional microorganisms in Chinese Sichuan sausages during spontaneous fermentation, which play an impotent role in the formation of the maturity and flavor of the sausages. While low abundance of *Staphylococcus* spp. and *Micrococcus* spp. which have been developed as commercial starter cultures for the manufacture of fermented sausages (Leroy et al. 2006; Corbière Morot-Bizot et al. 2007) were found in Chinese Sichuan

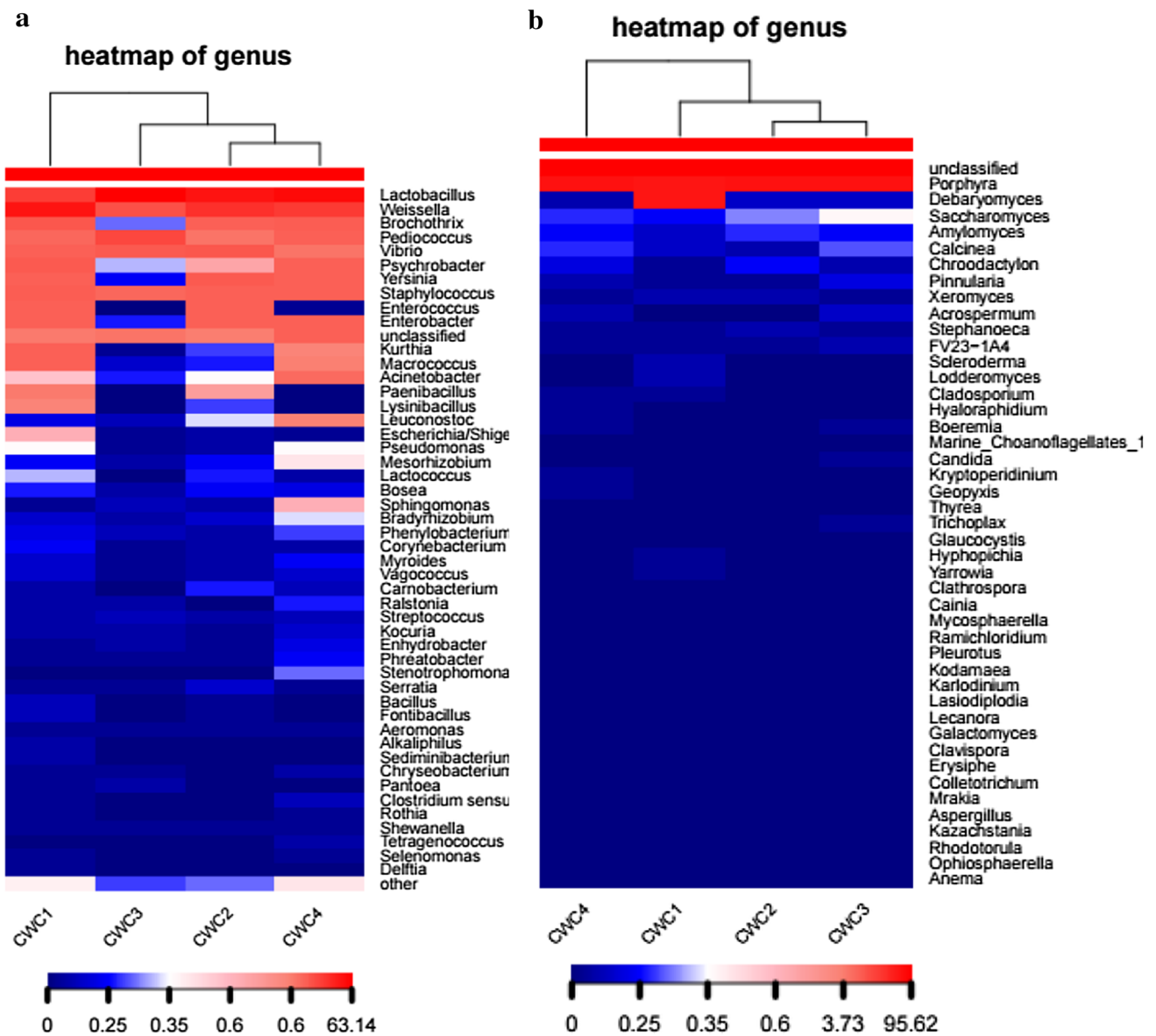


Fig. 5 Bacterial (a) and fungal (b) community heatmap analysis of Chinese Sichuan sausages during the spontaneous fermentation. CWC1: sample fermented for 1 days, CWC2: sample fermented for

10 days, CWC3: sample fermented for 20 days, CWC4: sample fermented for 30 days

sausages during spontaneous fermentation. These findings are in disagreement with those reported by other authors (Zhang et al. 2009) who reported that predominant microorganisms in Chinese traditional dry-cured sausages were *Staphylococcus* spp., *Micrococcus* spp. and *Lactobacillus* spp. with culture-dependant method and Random amplified polymorphic DNA (RADP) molecular marker technology. Moreover, the bacterial distribution of sample CWC1 (at start stage of fermentation) was most abundant, while microbial diversity gradually became lower along with the fermentation (sample CWC2 and CWC3).

It is more suitable for undesirable microbial reproduction in spontaneous fermentation than inoculated

fermentation. In this study, the *Enterococcus* spp. as one of important pathogens in Gram-positive bacteria with relative abundance of 1.84% was checked in CWC1. The most common clinical infections caused by *Enterococcus* spp. is urinary tract infection, followed by bacteremia, bacterial endocarditis, diverticulitis and meningitis (Ubeda et al. 2010). Moreover, Gram-negative bacteria with relative abundance of 18.07%, including *Brochothrix* spp., *Pseudomonas* spp. and *Acinetobacter* spp. were found in CWC1. Many members of Gram-negative bacteria are involved as pathogenic and spoilage bacteria. Thus, they are frequently considered as undesirable bacteria in fermented meaty products and a hazardous source of

transmissible antibiotic resistance (Greppi et al. 2015). Interestingly, when *Lactobacillus* spp., *Weissella* spp., and *Pediococcus* spp. gradually became the predominant strains, the growth of these undesirable microorganisms was inhibited. Compared with bacteria, trace fungal population except from *porphyra* spp. was detected in among all the samples. Just low abundance of *Debaryomyces* spp. (0.39%) was found in CWC1(at start stage of fermentation). Subsequently, the abundance of *Debaryomyces* spp. gradually decreased along with fermentation. *Debaryomyces* spp. were found in fermentation sausages by culture-dependant method and *Debaryomyces* spp., such as *Debaryomyces hansenii* has been considered that plays an important role in fermentation sausages, which has been widely inoculated in fermented sausages as a starter (Corral et al. 2014; Cano-García et al. 2013; Núñez et al. 2014). However, in the present study, it was showed that *Debaryomyces* spp. was very rare in Chinese Sichuan sausages fermented spontaneously and did not become a core microorganism. Microbial community diversity and succession of Chinese Sichuan sausages during fermentation indicated that bacteria are the dominant microbial communities while eukaryotic microorganisms are seldom, suggesting that bacteria, especially for lactic acid bacteria including *Lactobacillus* spp., *Weissella* spp. and *Pediococcus* spp., play a leading role in the sausage fermentation process. These results revealed that *Lactobacillus* spp., *Weissella* spp. and *Pediococcus* spp. can be developed as starter cultures.

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