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Characterizing the microbial diversity and major metabolites of Sichuan bran vinegar augmented by *Monascus purpureus*

Mei Ai[a,b], Xue Qiu[a,b], Jun Huang[a], Chongde Wu[a,b], Yao Jin[a,b], Rongqing Zhou[a,b,c,*]

[a] *College of Light Industry, Textile & Food Engineering, Sichuan University, Chengdu 610065, China*
[b] *Key Laboratory for Leather and Engineering of the Education Ministry, Sichuan University, Chengdu 610065, China*
[c] *National Engineering Research Centre of Solid-state Brewing, Luzhou 646000, China*

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ABSTRACT

This research aimed to evaluate the impacts of *Monascus purpureus* on the microbial community and major metabolites of *Cupei* and vinegar of Sichuan bran vinegar (SBV). *Cupei* is the mixture of fermented materials and vinegar is the liquid leached from *Cupei*. The characteristics of microbial community were revealed by Illumina-MiSeq. The result suggested that inoculation of *M. purpureus* decreased the microbial diversities and inhibited several pathogens related microbes including *Erwinia*, *Proteus* and *Ignatzschineria* of *Cupei*. The dominant genera of SBV were *Lactobacillus*, *Acetobacter*, *Trichoderma* and *Candida*. With addition of *M. purpureus*, the total relative abundance of *Lactobacillus* and *Acetobacter* was increased from 75.14% to 99.79%. Furthermore, the major metabolites in corresponding vinegar were investigated by HPLC and HS-SPME-GC–MS. The result indicated that the addition of *M. purpureus* significantly promoted the accumulation of organic acids, aromatic esters and alcohols, whose contents were increased by 1.95, 2.30 and 3.55 times, respectively. Meanwhile acetic acid, lactic acid, phenethyl acetate and β-phenethyl alcohol were the dominant components in organic acids, esters and alcohols, respectively. In addition, the relationship between dominant microbes and major metabolites explored by redundancy analysis displayed that *Lactobacillus*, *Acetobacter*, *Candida* and *Monascus* were closely related with seven volatiles and five organic acids. This study provided an insight on regulation of microbial community and metabolic function of traditional fermented foods by bioaugmentation.

1. Introduction

Sichuan bran vinegar (SBV) is the most famous Chinese vinegar with Shanxi aged vinegar, Zhenjiang aromatic vinegar and Fujian *Monascus* vinegar. They are featured by spontaneous fermentation with solid-state pattern in an open environment compared to pure-cultured white vinegar. Besides, the long history and special technologies shaped the special tastes and flavor of different types of Chinese vinegar (Liu et al., 2004). The general process of Chinese vinegar includes hydrolysis of macromolecules, alcoholic and acetic fermentations and the production of aromatic components, each stage was highly related with coexistence of various microbes. In the last two decades, more and more researches focused on microbial diversity and microbial evolution during the fermentation process of Chinese typical vinegar including Shanxi aged vinegar, Zhenjiang aromatic vinegar and Tianjin duliu mature vinegar. Lactic acid bacteria (LAB), including *Lactobacillus*, *Leuconostoc*, *Weissella* and *Pediococcus*, and acetic acid bacteria (AAB), such as *Acetobacter* and *Gluconacetobacter*, were the dominant microbes and had important influences on the unique flavor and tastes of vinegar, besides, *Saccharomyces*, *Aspergillus*, *Pichia* and *Saccharomycopsis* were also dominant (Nie et al., 2017; Srianta et al., 2014; Xu et al., 2011b; Zhang et al., 2017). In addition, *Cuqu*, the starter of Chinese traditional vinegar, is not only an important source of microbes, but also an indispensable crude enzyme preparation. The quality and characteristics of *Cuqu* is closely related to the unique style of different vinegars (Chen et al., 2009). As development of metagenomic sequencing, researches explored that *Mucor* sp., *Rhizopus* sp., *Aspergillus* sp. and *Monascus* sp. were the dominant microbes of *Cuqu* for Shanxi aged vinegar, Zhenjiang aromatic vinegar and Fujian *Monascus* vinegar (Liu et al., 2004). Nowadays, bioaugmentation was proved to be feasible to regulate the microbial diversities and their metabolism for Chinese traditional solid-state fermentation. It was conducted by inoculating functional microorganism to the original fermentation system. For examples, *A. pasteurianus* and *Lactobacillus brevis* were inoculated to fermentation of Zhenjiang aromatic vinegar and enhanced content of acetoin (Lu et al., 2016), and *Bacillus amyloliquefaciens* was inoculated into *Cuqu* of SBV

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[*] Corresponding author at: College of Light Industry, Textile and Food Engineering, Sichuan University, Chengdu 610065, China.
E-mail address: zhourqing@scu.edu.cn (R. Zhou).

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and increased contents of acetoin and tetramethylpyrazine (Zhang et al., 2017). Furthermore, similar pattern was also applied to other traditional fermented foods to improve quality or prolong shelf life, such as sausages (Kargozari et al., 2014) and Kimchi (Chang and Chang, 2010).

Monascus purpureus, a well-known functional fungi, produces a variety of valuable compounds such as c-aminobutyric acid, monacolin K, red pigments (Srianta et al., 2014) and has been applied in Fujian *Monascus* vinegar. It was reported that the contents of monacolin K and γ-aminobutyric acid of Zhenjiang aromatic vinegar was enhanced by inoculating pure-cultured microbes (Yu et al., 2010).

This research aims to provide a way to regulate the microbiota and their metabolisms with bio-perturbation technology. The effects resulted from bio-perturbation were evaluated from alterations of microbial community and major metabolites. Besides, a further analysis based on redundancy analysis (RDA) simply revealed the relationships between dominant microbes and major metabolites.

2. Materials and methods

2.1. Strain and culture conditions

Monascus purpureus (CGMCC 3972) was purchased from China General Microbiological Culture Collection Center (CGMCC). The strain was cultured on potato dextrose agar slants at 30 °C for 72 h and stored at 4 °C until inoculation. Brown rice, white middling and white bran were purchased from local farmers' markets (Chengdu, China). *Herb qu* was supplied by Langzhong vinegar industry Co., Ltd (Langzhong City, Sichuan Province, China).

2.2. Vinegar fermentation and sampling

The *M. purpureus*-augmented SBV fermentation included three parts. The first part was *Hongqu* preparation (Fig. S1A). *Hongqu* was the culture of *M. purpureus*. Firstly, the spore suspension of *M. purpureus* (2.3×10^5 cfu/g) was inoculated to the steamed glutinous rice (moisture > 45% w/w), and then cultured at 32 °C with moisture > 95%. After fermentation for 10 days, the *Hongqu* was dried at a lower temperature (≤ 45 °C) and smashed before use. The second part was *Herb qu* pretreatment (Fig. S1B). The third part is vinegar fermentation process (Fig. S1C), in which all materials including wheat bran, wheat middling, *Huipei* (leached *Cupei*), water, *Hongqu* and *Herb qu* were mixed well. The ratios of the former four substances of each experiment are kept same of 100:40:200:90 (w/w/w/w) and the ratios of *Hongqu* and *Herb qu* is presented in Table 1.

Table 1
The ratio of *Herb qu* and *Hongqu* added to different fermentation experiments.

Starters	H0	H2	H3	H4
Herb qu	10%	8%	7%	6%
Hongqu	0	2%	3%	4%

The percentage of each starter was based on the weight of wheat bran and wheat middling.

Vinegar fermentation was carried out under ambient temperature in ceramic jar (50 L) in triplicate. Each ceramic jar contained a mixture of 30 kg. During this period, the mixture went through a consecutive and spontaneous fermentation (Fig. S1C) including saccharification, alcoholic and acetic fermentation and it would often be turned over to maintain the temperature below 45 °C. After fermented for 30 days, fermented mixture turned to be fresh *Cupei* and it would be covered with salt for ripening, subsequently, fresh vinegar obtained by soaking and leaching, was boiled and bottled. Then certain ratio of the leached *Cupei* would be remixed to a new batch of vinegar fermentation. At third batch before adding salt, 600 g *Cupei* were equally taken from the top, middle and bottom layer, and mixed well. Samples were stored at −20 °C till analysis.

2.3. Extracting of DNA in Cupei and sequencing

Total genomic DNA samples were extracted with the Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions, and stored at −20 °C prior to further analysis. The quantity and quality of extracted DNAs were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. For bacteria, the V3–V4 domains of the 16S rRNA genes were amplified using primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). For fungi, the internal transcribed spacer ITS regions were amplified with primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS1 (5′-GCTGCGTTCTTC ATCGATGC-3′). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The detailed PCR procedures were conducted according to a previous method (Li et al., 2014). PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 300 bp sequencing was performed using the Illumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). The Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) pipeline was employed to process the sequencing data, as previously described (Caporaso et al., 2010). Briefly, raw sequencing reads with exact matches to the barcodes were assigned to respective samples and identified as valid sequences. The low-quality sequences (length below 150 bp, average Phred scores < 20, mononucleotide repeats over 8 bp, and with ambiguous bases) were removed (Chen and Jiang, 2014; Gill et al., 2006).

After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by UCLUST (Edgar, 2010). A representative sequence was selected from each OTU using default parameters. OTU taxonomic classification was conducted by BLAST searching the representative sequences set against the Greengenes Database (DeSantis et al., 2006). An OTU table was further generated to record the abundance of each OTU in each sample and the taxonomy of these OTUs.

2.4. Nucleotide sequence accession number

All sequencing data have been deposited at the Sequence Read Archive of the National Center for Biotechnology Information, the accession number is SRP135847. Bio-Sample accessions of fungal sequences: SRX3802381 (H0), SRX3802382 (H3), SRX3802383 (H4), SRX3802384 (H2); Bio-Sample accessions of bacterial sequences: SRX3802076 (H4), SRX3802077 (H2), SRX3802078 (H0), SRX3802079 (H3).

2.5. Determination of organic acids and volatile components

Contents of free organic acids were analyzed by HPLC equipped with C18E column (Agilent 1260, Agilent technologies, Palo Alto, CA) according to the method described by Zhang et al. (2017). While contents of volatiles were mainly determined with HS-SPME-GC/MS according to Zhang et al. (2017) with some modifications. Volatiles of the raw vinegar were extracted by HS-SPME with 50/30 μm DVB/CAR/PDMS fiber (Supelco, Inc., Bellefonte, PA). Methyl octanoate (0.870 mg/mL in methanol) was used as internal standard instead. Volatiles were analyzed by GC–MS (Trace GC Ultra gas chromatograph-DSQ II mass spectrometer (Thermo Electron Corporation, Waltham, USA), which equipped with a HP-INNOWAX capillary column (30.0 m × 0.25 mm × 0.25 mm, Agilent Technology, Santa USA).

Table 2
OTU distribution on difference level of different samples.

Samples	No. of sequences				Proportions (%)	
	Effective sequences		High quality sequences			
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
H0	43,625	149,436	35,573	142,383	81.54	95.28
H2	53,261	103,239	50,214	94,559	94.28	91.59
H3	49,118	103,349	47,932	100,319	97.59	97.07
H4	54,482	106,819	51,373	86,783	94.29	81.24

Operation condition of injector temperature, carrier gas flow, parameter of mass spectrum and temperature of ion source, quadrupole mass filter and transfer line were same with Zhang et al. (2017), and splitless mode was used. The oven temperature was programmed as follows: 40 °C holding 5 min, then ramped at 4 °C/min to 100 °C, followed by an increase to 230 °C at 5 °C/min and hold for 5 min. The identification of each volatile were obtained by comparing their mass spectrum with those in the NIST05 library database (Finnigan Co., California, USA) and their retention indexes were obtained according to previous method, which was based on C_7–C_{30} alkanes (van Den Dool and Kratz, 1963). All tests were carried out in triplicate.

2.6. Ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

2.7. Data analysis

Alpha and beta diversity were used to evaluate the microbial community diversity within and among samples. Chao1, ACE, Shannon and Simpson were indexes of alpha diversity, Chao1 and ACE emphasized on species richness while Shannon and Simpson took both species richness and individual abundance into account (Crist et al., 2003). Non-metric multidimensional scaling (NMDS) analysis was used to evaluate the beta diversity. It visually depicted the alteration of microbial communities (genus with relative abundance over 0.01%) with distances conducted by unconstrained ordination method and when the stress value is between 0 and 0.05, it would be considered as a good representation of the original data (Kenkel and Orloci, 1986). In our study, we conducted weight- and unweighted-NMDS, the former took both species richness and individual abundance in account, while the later only took species richness into consideration. Heatmap simultaneously reveals row and column hierarchical cluster structure in a data matrix, it represent the abundance of the dominant genus (abundance over 0.1%) with color-scaled tiles (Wilkinson and Friendly, 2009). Alpha indexes were conducted with QIIME (Caporaso et al., 2010). Both NMDS and heatmap were performed in R environment (https://www.r-project.org/) with vegan and ggplot2, and pheatmap packages, respectively.

Significant differences of organic acids and volatile components were represented by the analysis of variance (ANOVA) ($p < 0.05$) with SPSS 19.0 software (SPSS Inc. Chicago, IL, USA).

Redundancy analysis (RDA) was used to assess the correlation between dominant microbes and major metabolites in a multifactorial analysis-of-variance model. The cos between genera and variables showed a positive ($\cos > 0$) or negative ($\cos < 0$) relationship among them, while projector distance of variables on the direction of genus represents their quantitative relation (Lee et al., 2015). It was conducted with canoco for windows 4.50. PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) is a computational approach to predict the functional composition of a metagenome using marker gene data and a database of reference genomes (Langille et al., 2013).

3. Result and discussion

3.1. Microbial diversity of different *Cupei*

After the removal of the low quality, chimera reads and the trimming of PCR primers, the effective bacterial reads ranged from 43, 625 to 54, 482, while ratios of high-quality sequences ranged from 81.54% to 97.59%. Meanwhile, the effective fungal reads ranged from 103, 239 to 149, 436, and ratios of high-quality sequences ranged from 81.24% to 95.28%. The amount of sequences in our reads was similar with those of related researches, such as Chinese liquor and daqu starter (Li et al., 2017; Wang et al., 2017). Besides, the lowest sequences of bacteria and fungi were found in H0 (35,573 sequences) and H4 (86,783 sequences), respectively. Thus, alpha and beta diversity of bacterial and fungal community were conducted with normalized sequences of 31,900 and 78,000, respectively (Table 2). The rarefaction curves were approaching the saturation plateau (Fig. S2), which suggested that sequencing depth in this research covered situation of bacteria and fungi well.

As shown in Table 3, α-diversity of microbial community was conducted with normalized sequences. The result suggested the effect of *M. purpureus* on bacterial community was stronger than that on fungal community, but they shared the same tendency. Addition of *M. purpureus* significantly decreased the bacterial and fungal species richness and diversity of augmented *Cupei*, furthermore the values of the four indexes decreased with the quantity of added *Hongqu*. All the highest values were found in H0, while all the lowest values were found in H4.

Heatmap analyzed the changes in abundance of dominant genera among samples. According to heatmap analysis (Fig. 1), augmented and non-augmented samples were clustered into separated group. As a result of higher amount of inoculated *M. purpureus*, H4 held higher abundance of *Acetobacter*, *Monascus*, *Trichoderma* and lower abundance of *Lactobacillus* and *Candida* than H2 and H3, thus it was clustered into a subgroup.

NMDS analysis further unclosed the difference in microbial community among samples (Fig. 2). The result suggested that the bacterial (Fig. 2A & B) and fungal (Fig. 2C & D) species richness and abundance were affected by *Hongqu*, the most significant difference was found

Table 3
α diversity difference of the microbial community index among samples.

No.	Abundance index				Diversity index			
	Chao 1		ACE		Shannon		Simpson	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
H0	559.00	99.00	559.00	105.77	3.09	2.12	0.58	0.64
H2	347.00	71.00	347.00	76.44	1.45	1.98	0.47	0.64
H3	209.00	69.00	209.00	76.54	1.44	1.85	0.33	0.62
H4	128.00	65.00	128.00	71.31	0.61	1.90	0.17	0.61

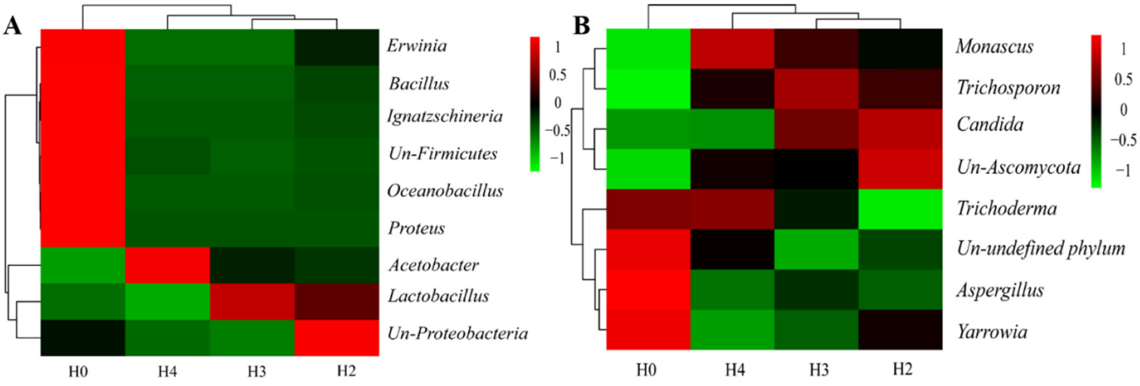

Fig. 1. Heatmap of dominant microbes in different *Cupei*. Bacteria (A), fungi (B).

between H0 than H4. But there was a little difference between bacterial (Fig. 2B) and fungal (Fig. 2D) species richness, that fungal species richness in augmented samples was more homogenous than the bacterial. The difference may result from that bacterial and fungal species had different sensibility to *Hongqu*.

3.2. Microbial community structure of different Cupei

At phylum level, a total of 10 phyla were detected in all samples, including 6 bacterial phyla (*Thermi*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria*) (Table S1) and 4 fungal phyla (*Anthophyta*, *Ascomycota*, *Basidiomycota*, *Zygomycota* and unidentified phyla) (Table S2). According to the relative abundance of each phylum, *Firmicutes* and *Proteobacteria* were the predominant bacterial phyla in the four samples, and they counted for over 99% relative abundance of bacterial phyla. While *Ascomycota* was the most predominant fungal phylum followed by *Basidiomycota* in the four samples. *Ascomycota* held relatively stable abundance around 96% in each sample, while the total relative abundance of *Basidiomycota* and other three fungal phyla were below 4%.

A total of 63 bacterial genera (Table S1) and 41 fungal genera (Table S2) were detected. Most bacterial genera belonged to *Firmicutes* (26 genera) and *Proteobacteria* (22 genera), while most fungal genera were *Ascomycota* (30 genera) and *Basidiomycota* (8 genera).

The differences of dominant genera (relative abundance ≥ 0.5%) among different samples were observed. 8 bacterial genera were dominant in H0, including *Lactobacillus*, *Oceanobacillus*, *Bacillus*, *Erwinia*, *Proteus*, *Ignatzschineria*, undefined genus of *Firmicutes* and *Proteobacteria* (Fig. 3A), 4 genera were dominant in H2 including *Lactobacillus*, *Acetobacter*, *Erwinia* and undefined genus of *Proteobacteria*,

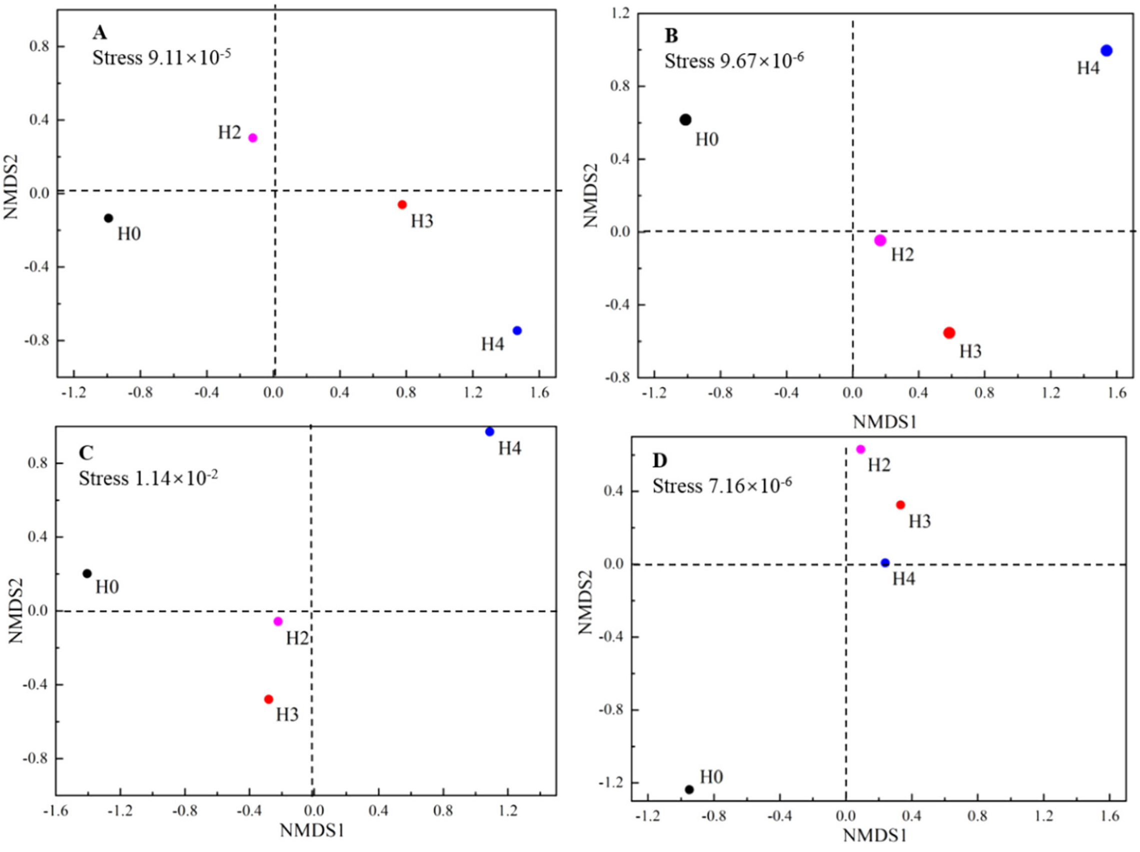

Fig. 2. NMDS map of microbial community on different *Cupei*. Weighted and unweighted-NMDS of bacterial community (A and B), weighted and unweighted-NMDS of fungal community (C and D).

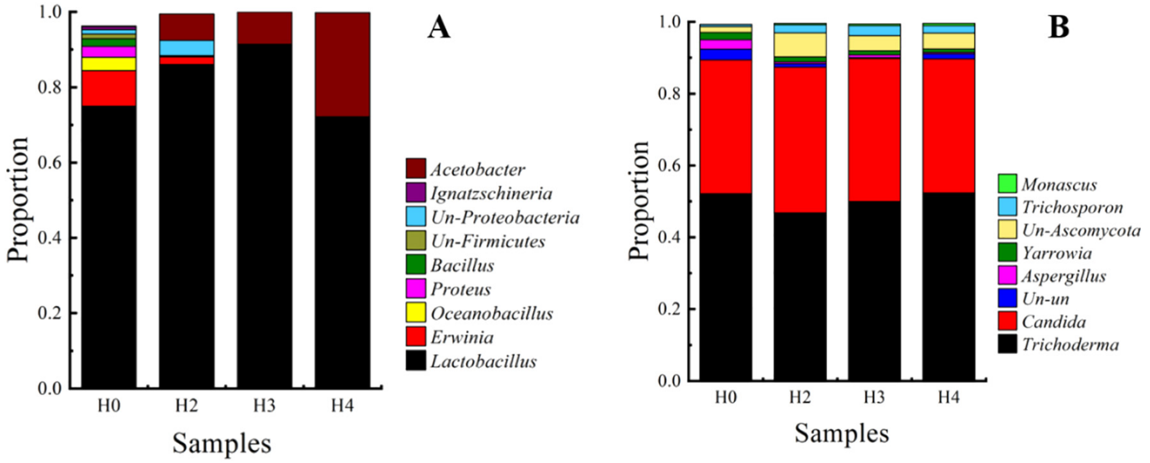

Fig. 3. Dominant microbes of different *Cupei*. Bacteria (A), fungi (B).

and 2 genera were dominant in H3 and H4, including *Lactobacillus* and *Acetobacter*. It was notable that, *Erwinia* and *Proteus* were *Enterobacteriaceae*, which were frequently reported in the ecosystem of fermented sausages and were usually considered as pathogens control in food fermentation industries (Ducic et al., 2014). Besides, *Ignatzschineria* was usually found in adult flies, which was reported to associated with human wound infections (Barker et al., 2014; Mejias et al., 2016; Roudiere et al., 2007). As a result, relative abundance of those possible-risk microbes decreased by the addition of *M. purpureus*. The effect may result from bacteriocin produced by *Lactobacillus* and increasing contents of organic acids such as lactic acid and acetic acid (Schnürer and Magnusson, 2005). In addition, *Lactobacillus* was the most predominant bacterial genus of the four samples. The relative abundance of *Lactobacillus* was 74.97% in H0, and it was increased in H2 (86.02%) and H3 (91.30%), but it was lightly decreased in H4 (71.76%) comparing to H0. Furthermore, with addition of *M. purpureus*, relative abundance of *Acetobacter* was increased from 0.17% (H0) to 27.64% (H4), the increased *Acetobacter* was proportional to the quantity of added *Hongqu*. It was reported that high ethanol concentration and rich oxygen would stimulate the growth of *Acetobacter* (Matsutani et al., 2013). Thus, the accumulation of alcohols might contribute to increase of *Acetobacter*. While the slightly decreased relative abundance of *Lactobacillus* in H4 might be associated with the high relative abundance of *Acetobacter*. It was notable that, the total relative abundance of *Lactobacillus* and *Acetobacter* in H0, H2, H3 and H4 was 75.14%, 93.05%, 99.79% and 99.40%, respectively. The result suggested that the addition of *M. purpureus* would be in favor of acids producing microbes. Furthermore, *Lactobacillus* and *Acetobacter* were the dominant bacterial genera in Chinese typical vinegar (Peng et al., 2015; Wu et al., 2017; Zhu et al., 2018). *Lactobacillus* was facultative anaerobic and was reported as dominant in the late stage of alcoholic fermentation and early stage of acetic fermentation in Zhenjiang aromatic vinegar, while *Acetobacter* mainly promoted the stage of acetic acid fermentation (Xu et al., 2011a).

For the fungal genera, there were 8 dominant genera in the four samples (Fig. 3B), including *Aspergillus*, *Candida*, *Monascus*, *Trichoderma*, *Yarrowia*, *Trichosporon*, unidentified genus of *Ascomycota* and unidentified phylum. Among them, relative abundance of *Aspergillus* and *Yarrowia* decreased by the addition of *M. purpureus*. It would be result from high contents of acids and some inhibitory metabolites from *Monascus* (Kim et al., 2006). Besides, *Candida* and *Trichoderma* were the most dominant genera in the four samples, and they remained stable relative abundance around 39% and 49% in each sample. What is more, this was the first report that *Candida* and *Trichoderma* were dominant fungal genera in Chinese vinegar fermentation. *Candida* was reported as a dominant genus in other cereal fermented foods, such as sour dough bread, which was responsible for the formation of CO_2, lactate, acetate and ethanol (Brandt et al., 2004; Sugihara et al., 1971). Instead *Trichoderma* had a preference to habitat in the cellulose/hemicellulose-rich medium. This might be explained by the fact that cellulose was an important inducer for *Trichoderma* to produce cellulase, and cellulase was highly important for the degradation of cellulose and hemicellulose (Kumar et al., 2008; Schuster and Schmoll, 2010). It was worth elaborating that, relative abundance of detected *Monascus* in H2, H3 and H4 were proportion to added quantity of *Hongqu*.

3.3. The difference of organic acids and major volatiles of raw vinegar

Effect of *M. purpureus* on the accumulation of organic acids was investigated (Fig. 4). A total of six organic acids were determined, including lactic acid, acetic acid, L-pyroglutamic acid, oxalic acid, citric acid and L-malic acid (Fig. 4A). The average concentration of total organic acids in augmented vinegar was 2.00 times of that in non-augmented. Furthermore, lactic acid, acetic acid and L-pyroglutamic acid were the dominant organic acids of the four vinegar. It was reported that lactic acid and acetic acid were the dominant organic acids in Chinese typical vinegar like Shanxi aged vinegar, Zhenjiang aromatic vinegar and Tianjin duliu mature vinegar (Chen et al., 2013; Nie et al., 2013; Xu et al., 2011b), while L-pyroglutamic acid was one of the specific dominant components in SBV and it was derived from the glutamic acid of cereal-materials (Zhang et al., 2017). As a result, the addition of *M. purpureus* increased the contents of lactic acid and acetic acid with an average of 2.12 and 1.84 times.

As for volatile components, total 59 volatile components were detected (data not shown), meanwhile eleven volatile components were considered as major volatiles according to their odor activity value (Table S3). The eleven components included 3-furanmethanol, propionic acid, 3-methyl butyric acid, phenylacetic acid, β-phenethyl alcohol, phenylacetaldehyde, phenylethyl acetate, benzaldehyde, 2,3-butylene glycol diacetate, 4-methyl guaiacol and 4-vinyl guaiacol. As a result, augmentation with *M. purpureus* had significant influences on volatiles. Concentration of esters and alcohols increased with an average of 1.30 and 2.55 times, respectively (Fig. 3B). In detail, β-phenethyl alcohol and phenylethyl acetate were the dominant components for alcohols and esters, respectively, the average contents of them in augmented vinegar was 4.23 and 2.80 times of that in non-augmented vinegar, respectively. According to Table S3, the first dominant volatile component in non-augmented vinegar was phenylacetaldehyde while it became β-phenethyl alcohol in augmented vinegar. It was notable that the concentration of phenylacetaldehyde in vinegar remained almost unchanged by augmentation. β-Phenethyl alcohol and phenylethyl acetate were widely used as rose-flavoring agent in food industry

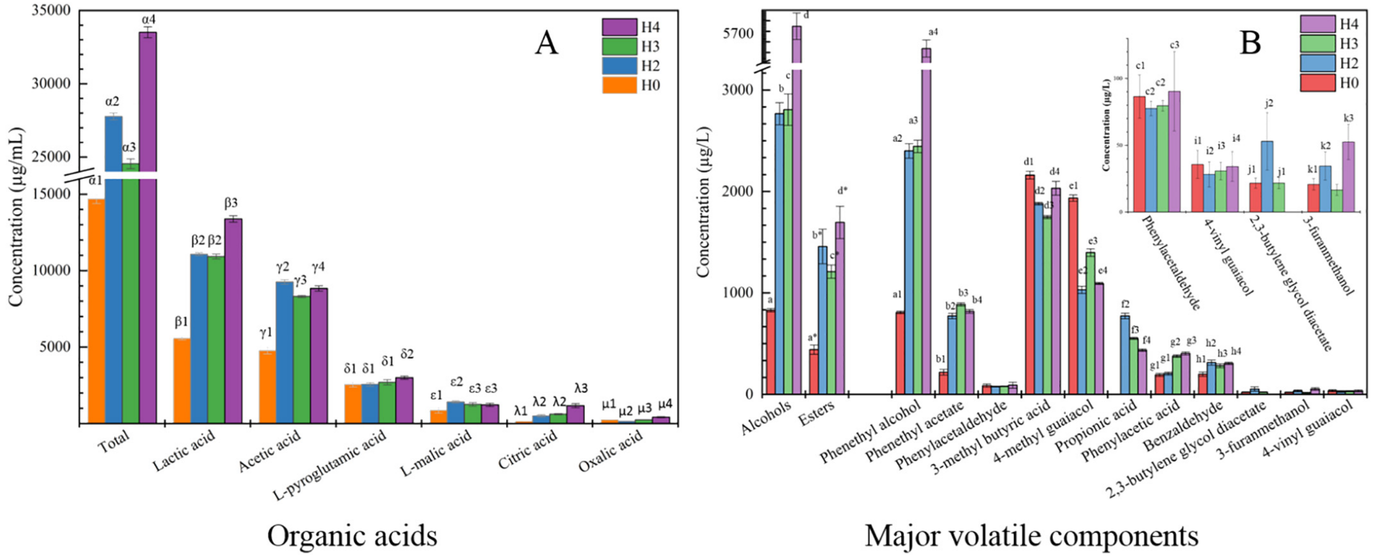

Fig. 4. Significant analysis of organic acids (A) and major volatiles (B) of different raw vinegar. Error bars indicate RSD (n = 3). Lowercase letters α, β, γ on the graph obtained by ANOVA indicate significant difference ($p < 0.05$, n = 3).

(de Carvalho et al., 2017; Lee and Ahn, 2009), and phenylacetaldehyde has intense aromas of sweet flower and chocolate. The result suggested that the addition of *M. purpureus* significantly increased the contents of rose-like volatile components and did not significantly influence the content of original dominant volatile component in SBV. More information about the sensory properties of augmented and non-augmented vinegar was appended in Tables C1 and C2 of Supplementary material.

3.4. Relationship between main microbes and major metabolites

According to the result of RDA (Fig. 5), *Lactobacillus*, *Acetobacter*, *Candida* and *Monascus* were closely related with phenethyl acetate, 2,3-butylene glycol diacetate, β-phenethyl alcohol, 3-furanmethanol, benzaldehyde, propionic acid, phenylacetic acid, citric acid, L-malic acid, lactic acid, acetic acid and L-pyroglutamic acid. The correlations between the dominant microbes and major metabolites explored by RDA were consistent with the changes of some organic acids and volatile components (Fig. 4). For example, lactic acid and acetic acid were the main metabolites of *Lactobacillus* and *Acetobacter* in vinegar

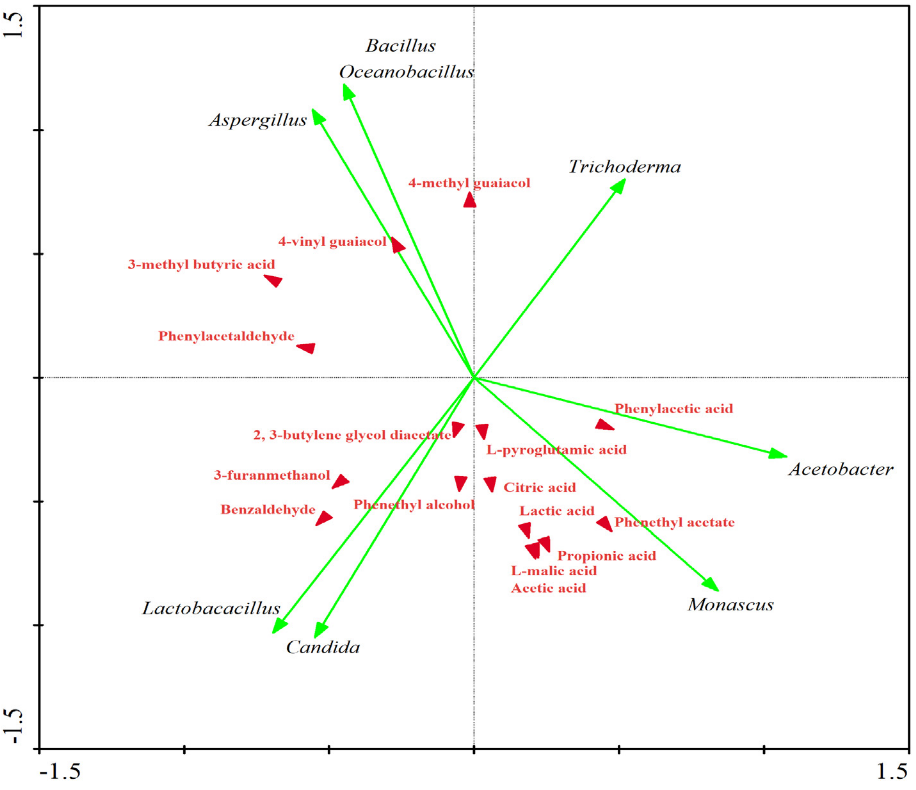

Fig. 5. RDA of dominant microbes and major metabolites.

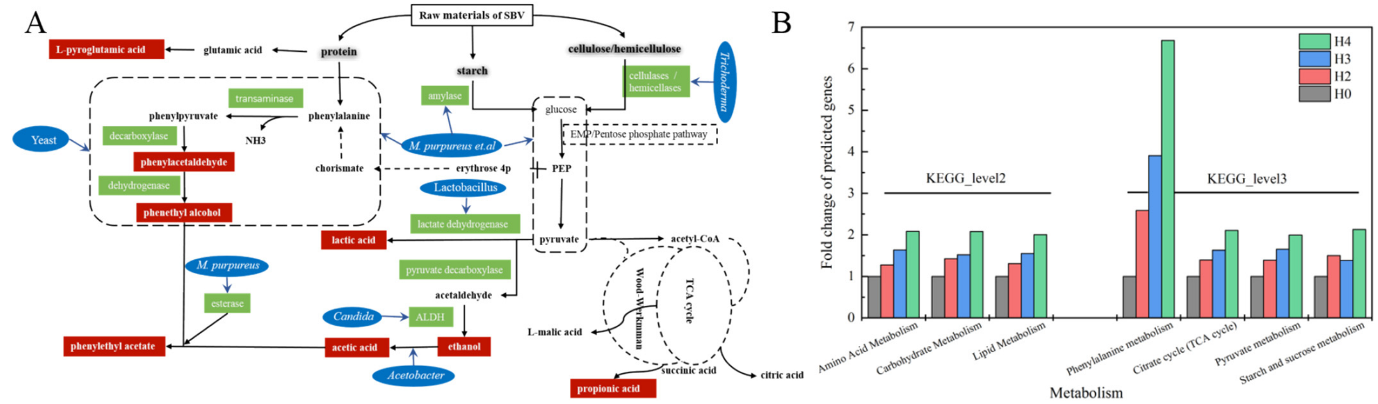

Fig. 6. Metabolic map of dominant genera during vinegar fermentation (A), changes of predicted functional genes by PICRUSt (B).

fermentation (Corsetti et al., 1998; Hammes and Vogel, 1995), and a positive correlation either between *Lactobacillus* and lactic acid or between *Acetobacter* and acetic acid was exhibited.

Moreover, *Oceanobacillus*, *Bacillus* and *Aspergillus* were closely related to four volatiles, including 4-methyl guaiacol, 4-vinyl guaiacol, 3-methy butyric acid and phenylacetaldehyde. Thus, the decrease in proportion of *Oceanobacillus*, *Bacillus* and *Aspergillus* led to the decrease in content of 4-methyl guaiacol, 4-vinyl guaiacol, 3-methy butyric acid and phenylacetaldehyde (Fig. 4B). It was reported that 4-methyl guaiacol and 4-vinyl guaiacol were positive with *Bacillus* (Lee et al., 2017). *Aspergillus* sp. was related with the metabolites of aldehydes (Yoshizaki et al., 2010). With inhibition of *Aspergillus*, the concentration of aldehydes decreased in augmented vinegar.

According to metabolic map of dominant genera in SBV (Fig. 6A), *Trichoderma* promoted the degradation of celluloses and hemicellulose and transformed them into fermentable monosaccharides. Consecutively, fermentable substances were transformed to pyruvate, which was the key metabolite leading to lactic and alcoholic fermentation. At the same time, *Lactobacillus* and *Candida* were the main microbes responsible for the lactic and alcoholic fermentation, respectively. Furthermore high content of ethanol would stimulate the proliferation of *Acetobacter* (Matsutani et al., 2013) and metabolism of β-phenylethyl alcohol was highly related with *M. purpureus* and phenylalanine metabolism (Hazelwood et al., 2008; Rahayu et al., 2017). Phenylethyl acetate was esterified from β-phenylethyl alcohol and acetic acid. According to metabolic map and present literatures (Chen et al., 2011; Srianta et al., 2014), *M. purpureus* widely involved in the hydrolysis of raw materials with α-amylase, lipase, peptidases and chitinase. From the result of PICUSt (Fig. 6B), abundance of predicted functional genes involving in metabolisms of amino acid, carbohydrate (especially for starch and sucrose), lipid, pyruvate, and phenylalanine also increased by over 1.28 to 2.00 times in augmented samples. Especially for the metabolism of phenylalanine, the abundance of predicted genes of which was increased by 2.59, 3.91 and 6.68 times in H2, H3 and H4, respectively (Fig. 6B). Besides, several organic acids including citric acid, succinic acid, L-malic acid and propionic acid could be synthesized through TCA cycle and Wood-Werkmman pathway. Meanwhile the abundance of predicted genes involving in TCA cycle also increased (Fig. 6B).

Those analyses revealed the impacts of *M. purpureus* on microbial community from metabolites and estimated abundance of predicted functional genes. As the microbial community was modified, metabolisms of amino acid, carbohydrate and lipid were also enhanced, the phenotypic result was the accumulation of corresponding metabolites like β-phenylethyl alcohol, phenylethyl acetate, citric acid and L-malic acid.

4. Conclusions

This research investigated that the effect of *M. purpureus* on microbial community and metabolites in SBV, results suggested that bio-perturbation with specific functional species could be used to shift microbial community and regulate metabolic function of SBV. For SBV, the results indicated that, the microbial diversity significantly decreased and several pathogens related microbes were inhibited by addition of *M. purpureus*. Besides, *Lactobacillus*, *Acetobacter*, *Trichoderma* and *Candida* were the dominant microbes of fermentation process. A proper amount of *M. purpureus* would promote the growth of *Acetobacter* and *Lactobacillus*. As a result of influences on microbial community, the contents of organic acids, aromatic alcohols and esters significantly increased, especially for lactic acid, acetic acid, β-phenylethyl alcohol and phenylethyl acetate. Furthermore, RDA suggested that dominant microbes as well as *M. purpureus* were positively related with the major metabolites. This study revealed the effects of bio-perturbation on SBV and provided an approach to regulate microbial community and metabolic function of traditional fermented foods.

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Conflict of interest

No conflict of interest was declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.12.008.

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