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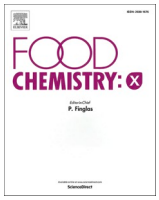

Characterization of the physicochemical Properties, bacterial community and non-volatile profiles of fermented Yu jiangsuan by *Weissella cibaria* and *Lactobacillus plantarum*

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ABSTRACT

Yu jiangsuan (YJS) is a unique traditional fermented condiment in China. Physicochemical, bacterial communities, and non-volatile properties were examined in inoculation Autochthonous Weissella cibaria and Lactobacillus plantarum. The results indicated that inoculation samples did well in shortening fermentation time; amino acid nitrogen (AN) and TCA-soluble peptide contents of fermented YJS were 10.8% and 17.4% higher than those of naturally fermented YJS, respectively. However, its total volatile base nitrogen (TVB-N), thiobarbituric acid (TBARS), and nitrite were only 74.3%, 87.2% and 83.6% of those of naturally fermented YJS. In addition, the dominant bacterial genera were Lactobacillus, Weissella and Pectobacterium, whose contributions were 41.2%, 20.3% and 5.5%, respectively. Moreover, 26 significantly differential metabolites were identified, and involved in 10 metabolic pathways. The decomposition of substrates and the formation of differential metabolites in YJS were primarily centered on the TCA cycle and the metabolism of carbohydrates. Therefore, this study is conducive to discovering the bacterial community structure and metabolite composition of probiotic inoculated YJS fermentation, as well as the potential value of core functional bacteria genera in controlling YJS production in industry.

1. Introduction

Chinese YJS is a unique traditional Miao original fermented condiment in Qian dongnan, Guizhou Province, China, which is made by traditional sealed fermentation. It is the product of a complex series of biochemical reactions using microorganisms carried by itself or in the environment under natural conditions and is rich in essential and non-essential amino acids, lactic acid, citric acid, acetic acid, tartaric acid and other organic acids required by human, and it also contains many mineral elements such as phosphorus, calcium, iron, zinc and other active substances such as capsaicin (Zhou, et al., 2020). Up to now, the traditional YJS has been fermented under natural fermentation conditions based on previous empirical knowledge, which often results in inconsistent quality and flavor. In reality, due to a variety of microorganisms in the natural fermentation process inducing complex biochemical changes, coupled with the long fermentation period (Alkema, et al., 2016 ; Smid and Kleerebezem, 2014), the fermentation process is extremely uncontrollable, and the flavor of fermented condiments is unstable.

The microbial community of YJS plays a decisive role in the quality, flavor, and safety. Inoculated fermentation was not only beneficial for improving the quality, flavor and nutritional properties of fermented products, but also shortened the fermentation time, rapidly produced organic acid, and enhanced products' safety. In our previous study, *Weissella cibaria* and *Lactobacillus plantarum* were isolated from YJS as the most appropriate strains, according to their fermentation properties, antimicrobial characteristics and γ-aminobutyric acid (GABA) production capacity. There were studies revealed that products fermented with starter cultures had better flavor, safety and overall acceptability, rather than products without starter cultures (Kasankala, et al., 2011).

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Furthermore, autochthonous dominant strains, with stronger environmental suitability and safety compared to other strains were screened as starter culture. Many scholars used autochthonous strains for product fermentation. Zeng et al. (2016) used *Staphylococcus xylosus*, *Pentose lactococcus*, *Saccharomyces cerevisiae*, and *Lactobacillus plantarum* to perform single-strain fermentation and mixed fermentation of Suanyu, respectively, and then found that the coordinated interaction of mixed fermentation strains could significantly shorten the fermentation time of the products, whereas Sun et al. (2014) adopted an autochthonous *Lb. plantarum* strain of SGJ-24 that enhanced the global aromatic intensity. In addition, metabolomics technology could be adopted to obtain the metabolic fingerprint of fermented food at different times during the production process, effectively determine the changes in the metabolic group during the fermentation process, and realize the monitoring of fermented food production. Jung et al (2013) studied changes of microbial flora and metabolites during fermentation of Kimchi, and the results showed that *Leuconostoc Mesenteroides* as a starter would increase the Ming string during fermentation, decrease lactobacillus proportion, shorten fermentation time, as well as produce more organic acids and mannitol, which provided direction for kimchi's fermentation improvement.

However, research on YJS has remained in the areas of physical and chemical composition, nutritional value of the product. Effect of inoculated fermentation on YJS fermentation, the physicochemical

Table 1
Isolation and Fermentation Characteristics of γ-Aminobutyric Acid-producing Lactic Acid Bacteria from YJS.

Strain	The strain with the highest similarity in GenBank	GenBank ID	Indentity %	GABA content (48 h)[a]	Acid-resistant ability[b]	Bile salts-resistant ability (0.3 %)[b]	Amino acid decarboxylase activity	Antibacterial diameter of bacteria solution[c] *S. saureus*	*E. coli*	Antibacterial diameter of the supernatant [c]	Eliminate acid and hydrogen peroxide and inhibit bacteria [c]	Protease sensitivity
Y113	*Weissella cibaria strain*	KP137384.1	99.86	+++	+	+++	ND	+	+	ND	ND	ND
Y123		MT613468.1	100.00	++	+	+	ND	+	+	ND	ND	ND
Y133	AT22	NR-136437.1	99.65	++	+	+	ND	+	+	ND	ND	ND
Y341	*Weissella cibaria strain*	MG754544.1	99.93	+	++	++	ND	++	+++	+	+	ND
Y343			100.00	+	++	++	ND	++	+++	+	+	ND
Y346	3057	KY584253.1	100.00	+	++	++	ND	++	+++	+	+	trypsin
Y61	*Weissella bombi strain*	MT645511.1	99.93	+	++	++	ND	++	++	+	+	pepsin
Y63		MT626074.1	99.93	+	++	++	ND	++	+++	+	+	trypsin
Y64	R-53094	MT604709.1	100.00	+++	++	+++	ND	++	++	+	+	and
Y271	*Lactobacillus plantarum strain*	MT613640.1	99.93	++	++	+++	ND	+++	+++	+	+	pepsin
Y272		MT613529.1	100.00	++	++	+++	ND	+++	+++	+	+	ND
Y273		MT473393.1	100.00	+	++	++	ND	++	+++	+	+	neutral
Y274	Sourdough-B12	MN420788.1	99.93	+	++	++	ND	+++	++	+	+	protease
Y278		MT538471.1	100.00	++	++	++	ND	+++	++	+	+	pepsin
Y279	*Lactobacillus plantarum strain* AZZ7 *Lactobacillus plantarum strain* 7232 *Lactobacillus plantarum strain* 1797 *Lactobacillus plantarum strain* 2211 *Lactobacillus plantarum strain* 3356 *Lactobacillus plantarum strain* 3156 *Lactobacillus plantarum strain* MLG5-9 *Lactobacillus pentosus strain* Z2-2 *Lactobacillus plantarum strain* 3596 *Lactobacillus plantarum strain* 3304 *Lactobacillus plantarum strain* 3072	MT613605.1 MT613479.1	100.00	+++	++	+++	ND	+++	++	+	+	neutral protease pepsin pepsin ND

[a] GABA content(48 h):GABA content of 15 strains after fermentation for 48 h, + 0.10 mg/mL < GABA content, ++ 0.10 mg/mL≦GABA content < 0.20 mg/mL, +++ 0.20 mg/mL≦GABA conten.

[b] Acid-resistant ability: Survival of 15 strains after culturing in pH 2.5 for 3 h; Bile salts-resistant ability:OD600 of 15 strains cultured in medium with different concentration of bile salts for 24 h, + low; ++ middle; +++ high.

[c] The inhibition zone size (Y): + Y < 8 mm; ++, 8 mm≦Y < 10 mm; +++, 10 mm≦Y.

properties, bacterial community and non-volatile profiles were still unclear. Therefore, this study aimed to explore the effects of fermentation with *W. cibaria* and *Lb. fermentum* of the products. In order to better improve the YJS fermentation process, improve the quality, inhibit the growth of other putrefying microorganisms, and enhance the product unique flavor and nutrition.

2. Materials and methods

2.1. Bacterial culture

In this study, *W. cibaria* and *Lb. plantarum* were isolated from YJS as the most suitable strains for fermentation based on their fermentation characteristics, antimicrobial properties and GABA production capacity. They accelerated the formation of organic acids and their potential health benefits could improve the quality of the product (Table 1), which were with stronger environmental suitability and safety compared to other strains.

At the beginning, the traditional fermented YJS samples were taken on the 1st, 3rd, 5th, 9th, 15th, 21st and 30th days under aseptic conditions, made into sample solution, and then diluted in gradient. Then, the LAB strains were isolated and purified on an MRS(Bo Microbiology, Shanghai, China) fluid medium when being incubated at 37 ℃ for 48 h. A total of 387 strains in the LAB were isolated and purified from the fermented YJS, and their probiotic potential and fermentation adaptability were evaluated by performing various tests concerning the ability of GABA-producing, acid tolerance, bile salt tolerance, amino acid decarboxylase activity, antibacterial activity, the growth curve, pH value, and the ability of acid-producing (data was shown as Table 1). *W. cibaria* and *Lb. Plantarum were* the dominant bacteria in early and late fermentation respectively. After that, *W. cibaria* and *Lb. plantarum* were experienced complete genome sequencing, and the accession number of *W. cibaria* and *Lb. plantarum* used with the highest similarity in GenBank were *Weissella cibaria strain* AT22 and *Lactobacillus plantarum strain* 3072, as well as GenBank ID were KP137384.1 and MT613479.1, respectively.

2.2. Sample preparation and collection

YJS fermented with starter culture (HY) and without starter culture (ZY) was prepared following the traditional method. The ratio of starter culture was *W. cibaria*: *Lb. plantarum* = 1:1, m/m, 7×10^{10} CFU/g. First, fresh red chili peppers and small scaleless loach were washed, drained and chopped, and then they were mixed in a ratio of 10:1. After that, 3 % white wine, 4 % ginger and 7 % salt were added, followed by HY and ZY being transferred respectively to fermentation jars and fermented 16 days at 25–26 °C. Afterwards, HY and ZY samples were collected randomly under aseptic conditions from day 0, 4, 8, 12 and 16 for physicochemical property determination, identified by Illumina sequencing for microbial diversity and used GC-TOF-MS to determine for non-volatile compounds. Finally, all samples were stored at −80 °C for analysis.

2.3. Microbiological analysis

By referring to the methods for microbiological analysis described by Zeng et al. (2013), 25 g of samples were taken at a sterile operating table, added to 225 mL of sterile saline, and homogenized for 1 min. Then, the 10^{-1} sample solution was diluted in a gradient, and 0.1 mL of the suitable diluted sample solution was taken into PCA, MRS, PDA, MSA and VRBA agar mediums, and counted after incubation at appropriate temperature, respectively. Besides, the results were expressed with logarithms of the number of colony forming units per gram (log CFU/g).Plate Count Agar (PCA), DeMan Rogosa Sharpe (MRS), Manitol Salt Agar (MSA), Potato Dextrose Agar (PDA), and Violet Red Bile Agar (VRBA) agar media (Bo Microbiology, Shanghai, China).

2.4. pH, TA, TCA-Soluble peptides and AN

Wang's method was adopted to determine pH, with 10 g of the sample, 90 mL of deionized water, being homogenized at 12,000 r/min for 1 min, and PHS-3E pH meter (Inesa, Shanghai, China) (Zeng et al., 2013). The TA was measured by titration with 0.1 M NaOH and described as percentage of lactic acid, whereas TCA-soluble peptide was extracted following the method of (Cai et al., 2015) that 27 mL of 5 % (w/v) trichloroacetic acid was mixed with 3 g of samples, homogenized in the water bath at 40 ℃ for 1 h, and centrifuged at 12,000 r/min for 5 min at 40 ℃. Then, its content was confirmed by adopting Lowry's method of protein measurement, when the standard solution was prepared using bovine serum albumin (BSA), and the standard curve was plotted. After that, the content was calculated according to the standard curve, and the results were expressed as mg tyrosine/kg samples (Wang, 2000). On the basis of the end of the total acid titration, add 10 m L formaldehyde solution (38 %), turn on the magnetic stirrer, and continue the titration with 0.05 mol/L titrate containing 0.05 mol/L sodium hydroxide standard solution until pH 9.2. Afterwards, the amount of sodium hydroxide sodium consumption was recorded and the content of AN was calculated.

2.5. TVB-N, TBARS, and nitrite

TVB-N and TBARS were tested by using the method of Zeng et al. (2013), TVB-N, referring to the microdiffusion method, volatile nitrogen substances were released in the alkaline solution, volatilized in the diffusion dish at 37℃ and absorbed in the absorption solution, titrated with standard acid to calculate the content, the results were expressed as mg/100 g samples. TBARS, 25 mL TBARS (0.375 %TBA, 15 %TCA, 0.25 mol /L HCl) were added to 5 g samples for homogenization and heated in a water bath at 95–100 °C for 10 min until the solution turned pink. After cooling, centrifuge at the speed of 5500 g for 25 min. Finally, taking appropriate amount of supernatant at 532 nm spectrophotometer to measure absorbance, and the results were expressed as mg/kg samples. Nitrite was detected with the naphthalene ethylenediamine hydrochloride method, the results were expressed as mg/kg samples.

2.6. DNA Extraction, PCR amplification and Illumina sequencing

Total genomic DNA was extracted from 10 groups of samples (ZY1, ZY2, ZY3, ZY4, ZY5, HY1, HY2, HY3, HY4 and HY5.HY refers to samples with starter cultures, ZY refers to samples without starter cultures, 1–5 indicate different fermentation stages, 0,4,8,12,16 days.) with a Power Soil DNA Isolation Kit (MO BIO Laboratories, USA) according to the manufacturer's protocol. Then, DNA quality and quantity were assessed by the ratios of 260 nm/280 nm and 260 nm/230 nm. At the same time, the V3-V4 region of the bacterial 16S rRNA gene was amplified with the universal primers 338F(5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Zhang et al., 2015). Besides, PCR amplification was performed in a total volume of 50 μL that contained 10 μL Buffer, 0.2 μL Q5 High-Fidelity DNA Polymerase, 10 μL High GC Enhancer, 1 μL dNTP, 10 μ M of each primer and 60 ng genomic DNA. Furthermore, thermal cycling conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 15 cycles at 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 7 min. After that, the PCR products from the first step were purified through VAHTSTM DNA Clean Beads (Vazyme Biotech Co., Ltd., China), while a second round PCR was conducted in a 40 μL reaction which contained 20 μL 2 × Phμsion HF MM, 8 μL ddH2O, 10 μ M of each primer and 10 μL PCR products from the first step. Thermal cycling conditions were as follows: an initial denaturation at 98 °C for 30 s, followed by 10 cycles at 98 °C for 10 s, 65 °C for 30 s min and 72 °C for 30 s, with a final extension at 72 °C for 5 min. Then, the amplified products were purified and recovered using a 1.8 % agarose gel electrophoresis method. In the end, all of the PCR products were pooled, purified by gel extraction and

quantified using the Nanodrop 2000 and then mixed at a mass ratio of 1:1 for sequencing on an Illumina Novaseq6000 PE250 system (Illumina Corporation, USA) by Biomarker Technologies Co, LTD.

2.7. Extraction of metabolites and gas chromatography Time-of-Flight mass spectrometry analysis

2.7.1. Metabolites extraction

Precooled extract, 1000:5 Adonitol and mixed standard were dissolved in 75 % methanol. Methoxy amination hydrochloride, methoxy amination hydrochloride were dissolved in pyridine until the final concentration is 20 mg/mL. BSTFA, contains 1 % TMCS, (v/v). FAMEs, dissolve in chloroform.

Place 50 ± 1 mg YJS samples into a 2 mL EP tube and add 1000 μL precooled extract. Then, put in steel balls, process with a 35 Hz grinding machine for 4 min, as well as ultrasonic treatment with the ice water bath for 5 min (repeat for 3 times). After that, let it stand at −40℃ for one hour. After centrifugation at 4 ℃ for 15 min at 12000 rpm, 200 μL supernatant was transferred to a fresh 1.5 mL EP tube. To prepare the QC (Quality control) samples, 40 μL of each sample was taken out and combined together. Following evaporation in a vacuum concentrator, 40 μL of methoxy amination hydrochloride was added and then incubated at 80 ℃ for 30 min, followed by being derivatized by 60 μL of BSTFA regent at 70 ℃ for 1.5 h. Gradually cooling samples to room temperature, 5 μL of FAMEs were added to the QC sample. All samples were then analyzed by gas chromatograph coupled with a time-of-flight mass spectrometer (GC-TOF-MS).

2.7.2. GC-TOF-MS conditions

GC-TOF-MS analysis was performed using an Agilent 7890 gas chromatograph coupled with a time-of-flight mass spectrometer. Besides, a DB-5MS capillary column was utilized by the system. Furthermore, 1 μL aliquot of sample was injected in the splitless mode, while helium was taken as the carrier gas, with the front inlet purge flow of 3 mL/min, and the gas flow rate through the column of 1 mL/min. Beyond that, the initial temperature was kept at 50 °C for 1 min, raised to 310 °C at a rate of 10 °C min^{-1}, and then maintained for 8 min at 310 °C. In addition, the injection, transfer line, and ion source temperatures were 280, 280 and 250 °C, respectively. Other than that, the energy was −70 eV in the electron impact mode, whereas the mass spectrometry data were acquired in the full-scan mode with the *m/z* range of 50–500 at a rate of 12.5 spectra per second after a solvent delay of 6.25 min. The extracts of each sample were obtained by uninterrupted chromatography.

2.7.3. Data preprocessing and annotation

Raw data analysis, including peak extraction, baseline adjustment, deconvolution, alignment and integration (Kind et al., 2009), was finished with Chroma TOF (V 4.3x, LECO) software, and LECO-Fiehn Rt × 5 database was employed for metabolite identification by matching the mass spectrum and the retention index. Finally, the peak detected in less than half of QC samples or RSD > 30 % in QC samples was removed (Dunn et al., 2011).

2.8. Statistical analysis

All results are the averages of three biological replicates. One-way analysis of variance (ANOVA) was performed using SPSS-25.0 software (IBM, Armonk, USA), while principal component analysis (PCA) was made to present the profiles of the volatile compounds using origin 2018. The data (sample name, standardized data of normalized peak area) were input into SIMCA 14.1 software (V14.1, MKS Data Analytics Solutions) for multivariate statistical analysis. PCA showed the distribution of raw data. The OPLS-DA model was used to confirm the differential metabolites by combining the VIP projection value (VIP ≥ 1.0) and Student's *t*-test ($p < 0.05$).Finally, MetaboAnalyst was used for pathway analysis (https://www.metaboanalyst.ca).

3. Results and discussion

3.1. Physicochemical discussion

3.1.1. Changes in the main microorganisms of YJS during fermentation

The microbial changes of YJS during fermentation were shown in Table 2. The differences in total aerobic bacteria and LAB at the beginning of fermentation were mainly due to the addition of starter culture, which indicates that the addition of starter cultures would affect the number of the microbial community in the fermented product. Besides, HY and ZY samples showed a rapid increase in the number of LAB since the early stage of fermentation, and gradually became the dominant microorganisms as expected, while they reached peak levels on the 4th and 8th days of fermentation with 8.61 log CFU/g and 7.62log CFU/g, respectively. Actually, LAB made full use of raw materials in the fermentation process and produced many metabolites through a series of metabolic reactions, at that time the growth of LAB started to become slow due to the gradual reduction of nutrients (Park et al., 2012).

The amount of yeast in HY samples was essentially unchanged during the initial stage of fermentation, mainly because of the stronger competitive ability of LAB in HY samples to inhibit the growth of other microorganisms, including yeast, by producing organic acids and bacteriocins. Then, on day 8 of the fermentation, the number of LAB, as well as pH value of the fermentation system were basically stable, then a small amount of acid-resistant yeast started to grow and reproduce.

The number of *Staphylococci* and *Enterobacteriaceae* in HY and ZY samples started to decrease rapidly by day 4 of fermentation, and it was less than 1 log CFU/g, which might be related to the rapid increase of LAB and decreased in pH or the production of bacteriocins, and they could inhibit the growth of *Staphylococcal* and *Enterobacteriaceae* (Essid and Hassouna, 2013 ; Xu et al., 2010).

3.1.2. pH and Titratable acid (TA)

Changes of pH and TA during fermentation were shown in Fig. 1, pH of sample HY and ZY showed a rapid decrease followed by a slow increase. To be specific, the rapid decrease in pH was caused by the growth of LAB in this system, while pH showed a slight increase in the middle and late stages of fermentation, probably associated with the accumulation of ethanol, the decomposition of macromolecules and the obvious increase of weakly alkaline buffering substances, such as basic amino acids, nucleotides and higher alcohols (Minoru et al., 2016). In addition, the rapid decrease of pH was conducive to inhibiting the growth and reproduction of harmful microorganisms in YJS. LAB produced a large amount of organic acid during fermentation, while TA increased rapidly and then gradually stabilized or slightly increased, which was basically consistent with pH change.

3.1.3. Changes in TCA-Soluble Peptides, AN, TVB-N, TBARS, and nitrite in the fermentation of YJS

Amino acid nitrogen (AN) could reflect the degree of protein hydrolysis and the amount of α-amino, which was one of the important indexes to evaluate the nutritional value and flavor of fermented foods. Microbial fermentation not only improved the utilization of protein in raw materials, but also produced a wide variety of amino acids (Enujiugha and Badejo, 2017). Amino acid nitrogen(AN) content could reflect the overall level of peptides and amino acids, and to a certain extent characterize the freshness of YJS. Changes of AN was shown in Fig. 2(a).The amino acid peptide content of HY samples increased faster than that of ZY samples at the early stage of fermentation. Subsequently, the increase of AN began to slow down, probably due to the faster hydrolysis of the protein at pH 5–6 that became slow when the protein was in the lower pH (Kasankala et al., 2011). Besides, as shown in Fig. 2(b), with the continuous extension of the fermentation period, HY samples had a higher content of TCA-soluble peptides and increased at a greater

Table 2
Microbial changes (log CFU/g) in YJS during fermentation.

Sample ID	Fermentation Time (days)	Microbiological counts Total aerobic bacteria	Lactic acid bacteria	Yeast	Staphylococci	Enterobacteriaceae
HY1	0	7.95 ± 0.14^{b}	7.87 ± 0.19^{b}	4.41 ± 0.40^{b}	3.26 ± 0.17^{a}	4.39 ± 0.18^{a}
HY2	4	8.60 ± 0.03^{a}	8.61 ± 0.27^{a}	3.84 ± 0.14^{b}	< 1	< 1
HY3	8	6.72 ± 0.01^{c}	6.78 ± 0.18^{c}	2.69 ± 0.21^{c}	< 1	< 1
HY4	12	6.74 ± 0.07^{c}	6.67 ± 0.23^{c}	5.52 ± 0.38^{a}	< 1	< 1
HY5	16	6.38 ± 0.15^{d}	6.36 ± 0.08^{c}	5.41 ± 0.32^{a}	< 1	< 1
ZY1	0	5.43 ± 0.09^{c}	5.24 ± 0.04^{d}	4.44 ± 0.32^{b}	2.22 ± 0.09^{a}	4.74 ± 0.17^{a}
ZY2	4	7.70 ± 0.02^{a}	7.52 ± 0.01^{a}	3.65 ± 0.08^{c}	< 1	< 1
ZY3	8	7.82 ± 0.13^{a}	7.62 ± 0.10^{a}	$3.91 \pm 0.06b^{c}$	< 1	< 1
ZY4	1216	6.74 ± 0.03^{b}	6.78 ± 0.14^{b}	5.51 ± 0.30^{a}	< 1	< 1
ZY5		6.49 ± 0.24^{b}	6.24 ± 0.15^{c}	5.55 ± 0.41^{a}	< 1	< 1

Results are expressed as means with standard deviation. Means in the same group and different letters in thesame column are followed by significant differences are significantly different($P < 0.05$).HY refers to samples with starter cultures; ZY refers to samples without starter cultures; 1–5 indicate different fermentation stages (0,4,8,12,16 days).

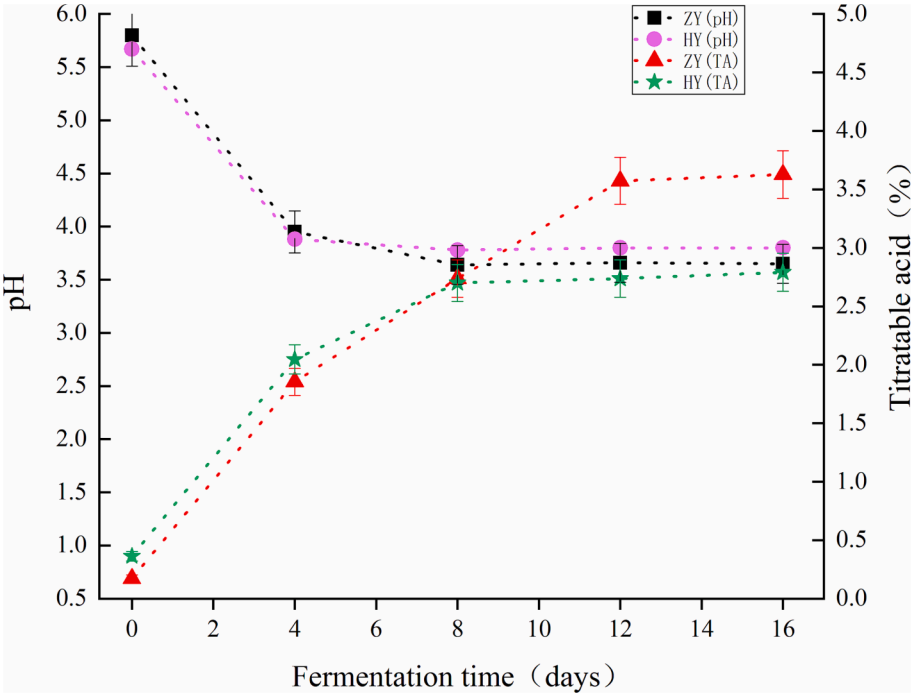

Fig. 1. Changes of pH and TA of YJS during fermentation. HY refers to samples with starter cultures; ZY refers to samples without starter cultures.

rate than ZY samples, which was consistent with the results of changes in the content of AN. Siriporn Riebroy et al. found a great increase in the content of TCA-soluble peptides and α-amino acids during the fermentation of Som-fug (Siriporn Riebroy et al., 2008). The content of TCA-soluble peptides varied considerably between the two fermentation conditions, depending on the raw materials and the activity of acid proteases (Molly et al., 1997). In the later stages of fermentation, the growth trend flattened out because the enzymatic activity was inhibited, made the protein degradation also inhibited, thus showed a slow growth trend.

Usually, TVB-N was generated by enzymatic degradation of nitrogenous compounds, and the higher content, the more amino acids were destroyed, especially methionine and tyrosine. Most countries in the world used TVB-N as an indicator of the degree of spoilage of aquatic products. TVB-N of both groups showed an increasing trend with the extension of fermentation as shown in Fig. 2(c). As fermentation progressed, fish proteins of YJS were broken down and further decomposed to produce basic nitrogen compounds such as nitrogen, ammonia and amines. It could be seen that there was a difference in the rate of change of TVB-N values between inoculated and natural fermentation conditions. The TVB-N content of HY was significantly lower than that of ZY ($P < 0.05$), and finally reached 16.75 mg/100 g and 22.57 mg/100 g, respectively. The addition of *W. cibaria* and *Lb. plantarum* produced a large amount of organic acids through various metabolic pathways, which neutralized TVB-N and contributed to the reduction of TVB-N in the system, thus enhancing the safety of YJS.

As shown in Fig. 2(d), the TBARS content of both groups was low in the prior period of fermentation, and then increased as the fermentation proceeded, while that of ZY samples increased significantly faster than that of HY samples ($P < 0.05$). At the later stage of fermentation, HY and ZY had reached 0.148 mg/kg and 0.129 mg/kg, respectively, which indicated that the addition of *W. cibaria* and *Lb. plantarum* for fermentation could inhibit the oxidation of unsaturated fatty acids, probably resulting from their strong anti-oxidant activity (Zeng et al., 2013). However, the growth of LAB was inhibited in the late stage of fermentation, resulting in a tendency to still increase the malondialdehyde content of YJS.

The nitrite content of both groups during fermentation was at a low level below the limit of the national standard (20 mg/kg) as shown in Fig. 2(e). LAB has the ability to degrade nitrite, mainly through the

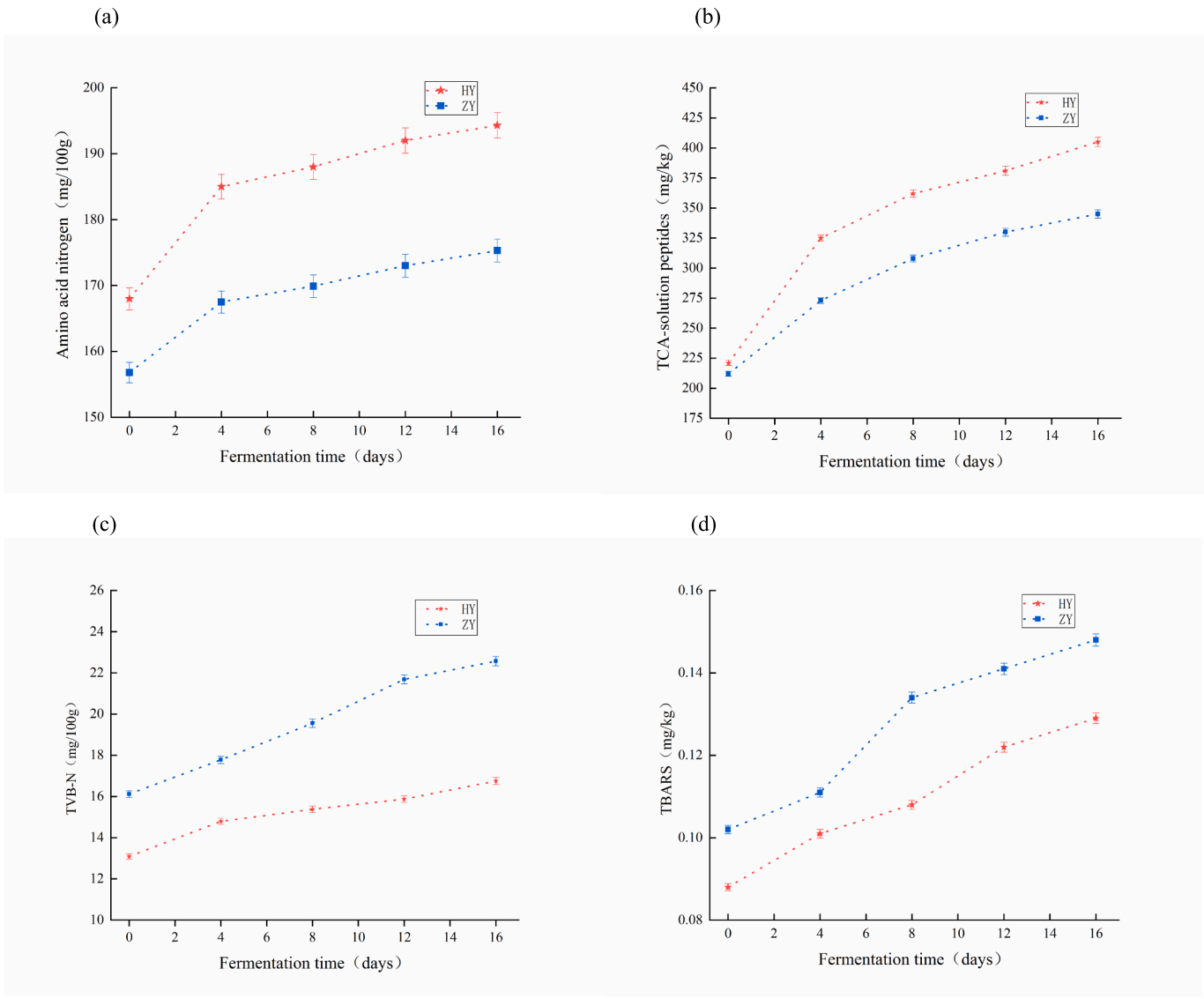

Fig. 2. Changes in AN(a), TCA-Soluble peptides(b), TVB-N(c), TBARS(d), and Nitrite (e)during fermentation, HY refers to samples with starter cultures; ZY refers to samples without starter cultures.

production of nitrite reductase, which acted on nitrite and aroused the reduction reaction, converting nitrite to NO, NO_2 or N_2O (Collins-Thompson and Lopez, 1981). At the same time, the consumption of nitrite would accelerate at lower pH (Oh et al., 2004). In addition, co-inoculation of *W. cibaria* with *Lb.plantarum* for fermentation could play a synergistic role in the inhibition of nitrite formation (Xiang et al., 2019), which might be the reason why the nitrite content of HY samples was lower than that of ZY samples.

3.2. Abundance and members of the bacterial microbiota

Illumina NovaSeq Sequencing generated a total of 800,429 V1-V3 16S rRNA raw sequence reads from ten samples that were then spliced and filtered while chimeras were removed to obtain 795,572 high-quality bacterial tags. Besides, an average of 79,557 bacterial tags was covered in each sample (Table 3). Apart from that, the sequences were clustered based on 97 % sequence similarity and filtered with 0.005 % of the number of all sequences as a threshold to further obtain the total number of operational taxonomic units (OTUs) for bacteria that was 385. Furthermore, biodiversity richness statistics, the biodiversity index and Good's coverage could be found in Table 3, whereas OTUs reflected the species richness and Chao 1 index showed the community abundance, with higher values indicating greater community richness. In addition to that, the Shannon and Simpson indices both presented estimators of sampling diversity and represented the approximated number of species and the uniformity of their distributions in the sample (Soto, M.L.D, Dalmasso, & Civera, 2017). As shown in Table 2, the largest OTU in YJS samples of the two groups appeared in the fifth stage of fermentation, and the bacteria species in the last fermentation stage was the richest. Besides that, it should be noted that bacteria in the initial system may from the raw material itself and the processing environment. As for the bacterial diversity of HY and ZY samples, it showed a significant decreasing trend as the fermentation proceeded, while the bacterial richness gradually increased during the first 12 days of fermentation, and then the increasing trend slowed down, maybe due to the gradual change of the system to an acidic environment, where exogenous microorganisms had difficulty in adaptation and growth (Ma et al., 2016). Furthermore, the coverage of high-quality sequences in each sample was higher than 99 %, indicating that the sequencing depth can cover microbial diversity.

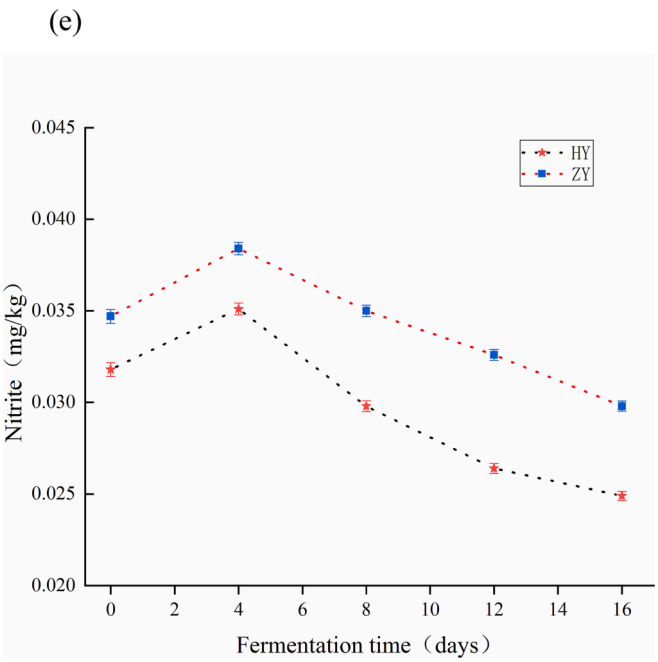

Fig. 2. (*continued*).

Table 3
Sequence abundance and microbial diversity of bacteriain YJS during fermentation with or without starter cultures.

Sample ID	Raw read	Clean tags	Number of OTU	ACE	Chao 1 index	Simpson index	Shannon index	Good's coverage
HY1	79,986	79,524	156	201.648	205.422	0.638	2.075	0.9994
HY2	80,210	79,740	194	233.260	236.249	0.698	2.520	0.9994
HY3	80,081	79,594	249	268.097	272.470	0.794	3.040	0.9996
HY4	80,044	79,512	353	358.574	368.375	0.781	3.583	0.9998
HY5	80,012	79,504	360	365.093	371.911	0.786	3.735	0.9998
ZY1	79,967	79,527	147	192.712	189.480	0.615	2.002	0.9995
ZY2	79,877	79,410	160	252.826	238.368	0.692	2.394	0.9993
ZY3	79,919	79,443	324	356.711	351.857	0.750	2.672	0.9993
ZY4	80,232	79,705	359	364.310	367.875	0.818	3.385	0.9998
ZY5	80,101	79,613	367	371.032	372.192	0.843	3.716	0.9998

HY refers to samples with starter cultures; ZY refers to samples without starter cultures; 1–5 indicate different fermentation stages(0,4,8,12,16 days).

The proportions of bacteria at the genus level during the fermentation of YJS were displayed in Fig. 3. During the initial period of fermentation, in the inoculation samples of YJS, in the beginning, *Weissella* was the first dominant bacterium, followed by *Pectobacterium* and *lactobacillus* with the average abundance of 76.1 %, 2.32 %, and3.07 %, respectively. As the fermentation continued, *Lactobacillus* gradually dominated as the first dominant bacterium, followed by *Weisseria* and *Pectobacterium*, with the average abundance of 41.2 %, 20.3 % and 5.5 %, respectively. In natural fermented samples, *Lactobacillus* didn't appear as the main dominant bacterium until the day 4 of fermentation with an average abundance of 17.6 %, after which it gradually developed into the first dominant bacterium. In addition, *fructobacillus* was detected in the samples, belonging to LAB, with a gradually increasing proportion in the natural fermented samples and a smaller proportion in the inoculated samples. Based on the changes in the abundance of bacteria, it could be seen that *Lactobacillus* and *Weisseria* worked as the dominant microorganisms in the initial and late periods of YJS fermentation, respectively. Especially, *Lactobacillus* has the ability of facultative anaerobic and acid resistance, and could grow well in the fermentation environment of low oxygen and low pH. Moreover, *Lactobacillus* could produce lactic acid and a variety of antimicrobial substances, such as bacteriocin, to inhibit the growth of pathogens and spoilage microorganisms during the fermentation.And it was found in previous studies that *Lactobacillus*, such as *Lb. plantarum*, at the late stage of YJS fermentation had antibacterial activity, which grew and multiplied rapidly, then could better guarantee the safety of YJS. Besides, it should be mentioned that during the fermentation process of YJS, fermentation time, the content of organic acids and other conditions affected the growth of microorganisms in the system. Among them, organic acids had a great influence on the growth of microorganisms, and with the increase of organic acid content, acid-intolerant microorganisms were inhibited, with the inability to adapt to the highly acidic fermentation environment, thus reducing the abundance and diversity of microorganisms. Both inoculated fermentation and natural fermentation showed *Weisseria* had decreased rapidly, which indicated the growth of *Weisseria* was inhibited, as the pH in the fermentation environment decreased. In this case, acid-tolerant LAB became the dominant bacteria (Zang et al.,2018). The better acid resistance of *Lb. plantarum*, the later dominant bacteria screened out, also confirmed that acid-tolerant LAB became the dominant bacteria of YJS late fermentation (Table 1).

3.3. Analysis on non-volatile metabolites

3.3.1. Multivariate statistical analysis

The similarity degree of samples on the principal component analysis diagram (PCA) can be judged by clustering. There was a positive correlation between the degree of similarity of metabolic conditions among

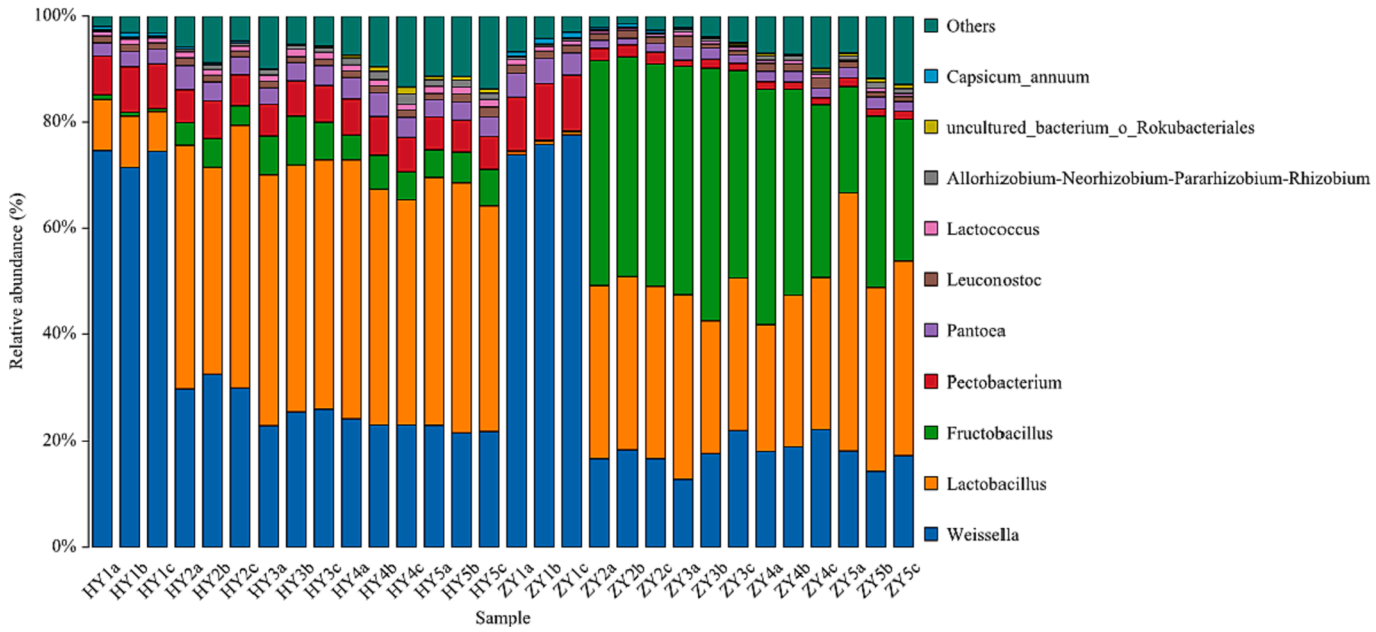

Fig. 3. Relative abundance of bacteria at the genus level in YJS samples. HY refers to samples with starter cultures; ZY refers to samples without starter cultures; 1–5 indicate different fermentation stages(0,4,8,12,16 days).

samples and the degree of proximity in PCA diagram (Cebollero et al., 2010). The two groups of samples fermented for 0, 4 and 8 days could be clearly separated in the PCA plot (Fig. 4), when YJS was undergoing rapid fermentation. Besides, microorganisms such as LAB in the system were actively growing and the metabolites were highly variable. With the proceeding of fermentation, different metabolites interacted with each other and formed a variety of flavor substances through different metabolic pathways, which are specifically shown in the PCA plot as low similarity and obvious separation between the metabolic situations of samples on fermentation day 0, 4 and 8. Moreover, metabolism of samples on day 12 and 16 of fermentation was more similar at this time, and was closer in the PCA plot, probably because of the fact that microorganisms were continuously consuming carbohydrates of the raw materials, producing a large amount of organic acids and lowering pH of the system. Then, the carbohydrates that could be utilized became less and the fermentation efficiency of LAB weakened, resulting in little difference in the changes of metabolites, after entering the late stage of fermentation.

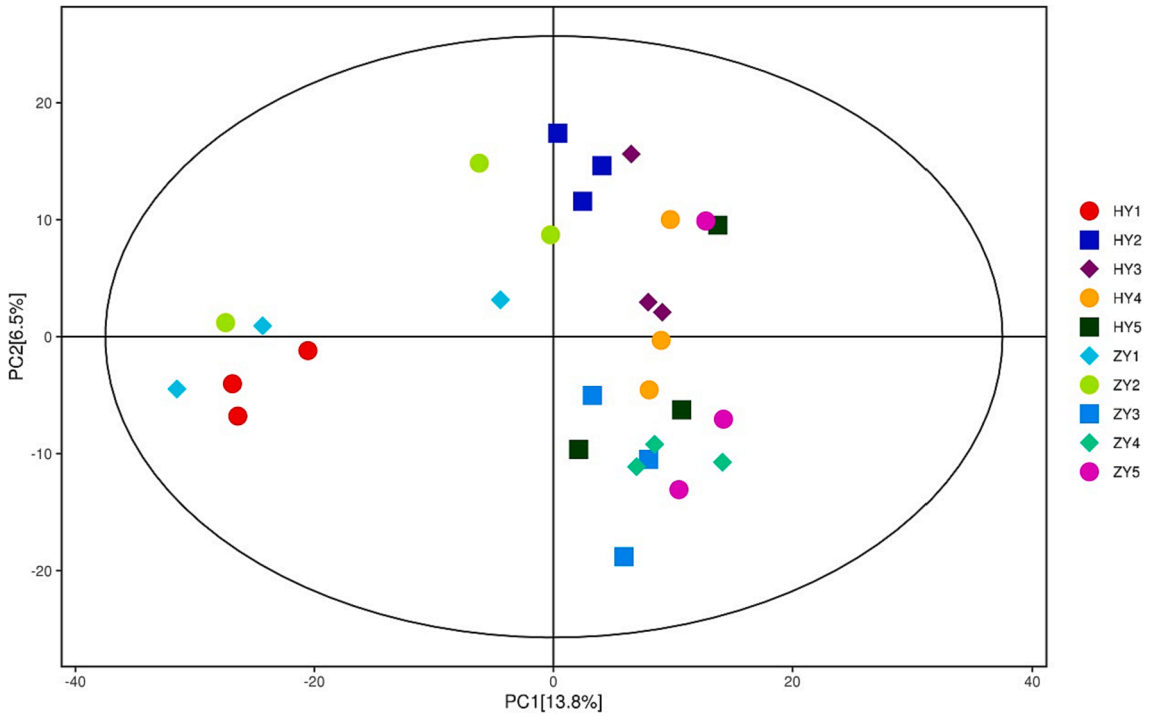

Fig. 4. Principal component analysis of YJS during fermentation. HY refers to samples with starter cultures; ZY refers to samples without starter cultures; 1–5 indicate different fermentation stages (0,4,8,12,16 days).

3.3.2. Differential metabolite analysis

The non-volatile metabolites of samples were determined by GC-TOF-MS, and 579 peaks were obtained by quality control. The retention time (RT) value of the measured substance differs from the RT value of the database within ± 5,000, so it is considered that the measured peak is significant for the substance (Parker et al., 2015). Other than that, 60 reliable metabolites were identified based on the degree of match(similarity ≥ 800) between the substances of the qualitative analysis and those in the standard library (Wu et al., 2012). To further analyze the metabolic differences of the samples, based on their VIP scores > 1.0 of OPLS-DA and $p < 0.05$ of ANOVA, and 26 differential metabolites, including organic acids, sugars, and amino acids were obtained, as shown in Table 4. In addition, there were two metabolites L-Malic acid andmyo-inositol with different fold changes over 5, and myo-inositol was the metabolites with the most dramatically different fold change 35,804,474. During the fermentation of samples, there were 9 metabolites that were significantly up-regulated and 17 metabolites that were down-regulated at the same time to a great extent.

The Euclidean distance matrix was calculated by using the quantitative values of the differential metabolites that were further clustered with the complete chain method and presented as a heat map to identify their group differences (Fig. 5). During the fermentation, metabolites related to the metabolism of organic acids, sugars, amino acids, peptides or vitamins were produced by the action of microorganisms. To be specific, organic acids were derived from the metabolism of microorganisms, and featured with the function of presenting flavor and taste and inhibiting the reproduction of spoilage microorganisms. Here, it should be noticed that different organic acids had different flavors, which could produce a unique and excellent flavor when the content of various organic acids was appropriate in the system. However, the differential metabolites concentrated in the day 0 of fermentation were mainly organic acids such as succinic acid, malic acid and pyruvic acid. Besides, the inoculated group showed high expression of succinic acid and malic acid and low expression of pyruvic acid, maybe because LAB was inoculated, since the rapid proliferation of LAB at this time facilitated an accelerated microbial metabolic reaction through the Embden-Meyerhof-Parnas pathway (EMP pathway).LAB degraded the glucose in the system so as to form pyruvic acid, while the system was gradually decarboxylated, dehydrogenated and completely oxidized through the Tricarboxylic acid cycle (TCA cycle) under aerobic conditions, in which the intermediate metabolites succinic acid and malic acid were produced. On the day 4 of fermentation, the differential metabolites were mainly glyceric acid, xylose and linolenic acid, when content of glyceric acid and xylose in sample was significantly reduced at that time in the inoculated and natural fermentation groups, and then showed a continuous trend of reduction. As could be seen in Fig. 5, the natural fermentation group had a high content of glyceric acid, while the content of pyruvic acid was also increasing, because glyceric acid underwent phosphorylation for producing 3-phosphoglyceric acid, which could further participate in the EMP pathway and then produce pyruvic acid. Xylose, as the unit linking serine to the sugar chain in glycoproteins, decreased with the degradation of serine. At the same time, xylose was also one of the sources of sweetness in the product. The glyceric acid and xylose content of the inoculated fermentation group was obviously lower than those of the natural fermentation group at this stage, which might be related to the raw materials, microbial species and fermentation time. Besides, the linolenic acid content of the inoculated fermentation group started to show an increasing trend, which enhanced the nutritional and probiotic properties of the product. Apart from that, differential metabolites, such as alanine, asparagine, aspartic acid, phenylalanine, allothreonine, tyrosine, ascorbate, pyruvate, gluconate, itaconic acid, quinic acid, propanetriol, 1,2,4-butanetriol, palmitic acid, stearic acid, ribose, uracil and myo-inositol were enriched to a great extent during the day 4–12 of fermentation. Furthermore, the rich taste of fermented products was brought by the large number of different kinds of amino acids produced in the system since they would produce sour, sweet, bitter, salty and fresh flavors. Thus, microorganisms would continue to synthesize amino acids during the fermentation, which would play a positive role in enhancing the taste and flavor of the product (Xiao et al., 2015).

The differentially metabolized amino acids screened from YJS included alanine, asparagine, aspartic acid, phenylalanine, and tyrosine. Among them, alanine, asparagine, and aspartic acid showed different contents under different fermentation conditions. In terms of aspartic acid, it was one of the 20 protein amino acids and an acidic amino acid. Besides, the presence of aspartic acid was detected under both fermentation conditions, showing an overall increasing trend during the fermentation period of day 4 to day 16. Unlike that, phenylalanine

Table 4
Differential metabolites of GC-TOF-MS in the fermentation of YJS.

Name of metabolite	RT (min)	Similarity	Mass (*m/z*)	VIP	P value	Fold Change (HY/ZY)
succinic acid	21.32	945	117	1.65	0.0323	1.72
D-Glyceric acid	11.30	903	133	1.74	0.0140	0.51
gluconic acid	19.00	886	333	1.73	0.0293	2.67
L-Malic acid	13.38	875	147	1.70	0.0444	5.37
Itaconic acid	11.49	860	147	1.93	0.0030	0.12
Pyruvic acid	7.36	859	174	2.32	0.0439	< 0.01
maleic acid	10.97	843	245	1.67	0.0069	0.15
stearic acid	11.12	946	147	2.29	0.0013	2.16
quinic acid	17.60	841	345	1.85	0.0050	0.36
ascorbate	18.53	868	322	1.98	0.0013	< 0.01
palmitic acid	19.53	955	117	1.79	0.0114	0.55
myo-inositol	19.89	925	73	2.00	0.0077	35804473.71
ribose	15.61	895	103	1.85	0.0027	0.44
xylose	15.36	892	307	1.72	0.0037	0.69
Sophorose	25.41	847	73	1.85	0.0430	1.37
Alanine	8.14	958	116	1.74	0.0309	0.63
Asparagine	15.56	949	116	1.63	0.0351	0.61
phenylalanine	15.10	939	218	1.69	0.0362	2.45
L-Allothreonine	12.06	936	117	1.63	0.0271	0.57
aspartic acid	13.80	935	100	1.91	0.0035	0.82
tyrosine	18.48	904	218	2.00	0.0077	0.44
glycerol	10.55	855	117	1.44	0.0388	0.24
2-Deoxyerythritol	10.76	846	117	1.85	0.0082	0.66
linolenic acid	21.09	843	79	1.55	0.0441	2.94
uracilflavin adenine degrad product	11.43	837	99	1.95	0.0044	4.09
	16.31	806	117	1.82	0.0410	0.72

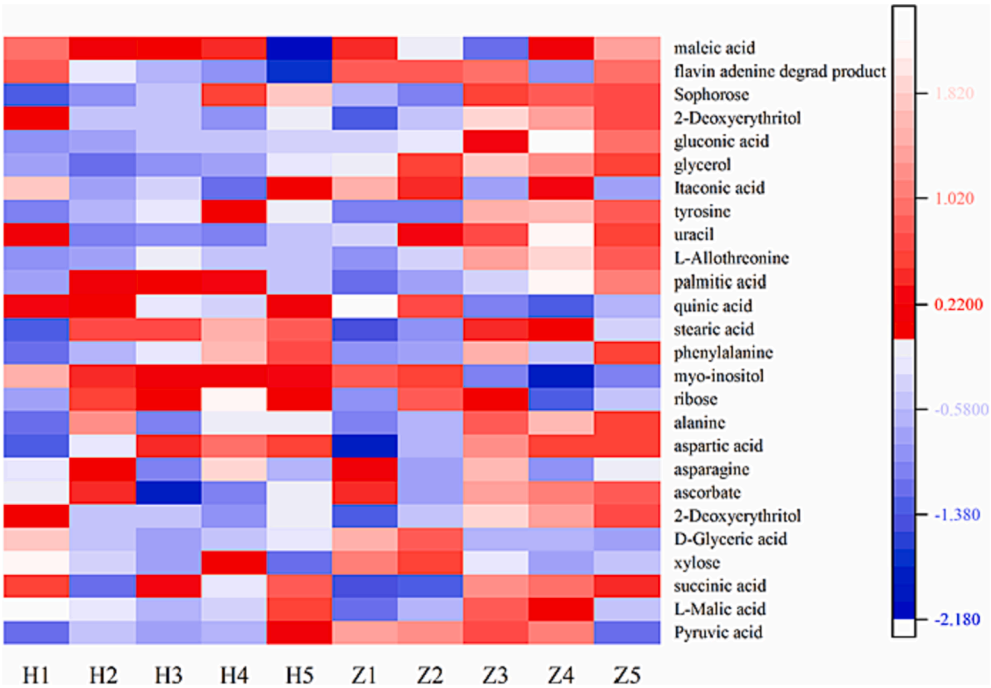

Fig. 5. Heat map of differential metabolites during fermentation of YJS. H refers to samples with starter cultures; Z refers to samples without starter cultures; 1–5 indicate different fermentation stages(0,4,8,12,16 days).

belonged to the aromatic amino acids and was also an essential amino acid that was highest in the inoculated samples on day 12 of fermentation, while under the natural fermentation condition, it reached the highest level on day 8 of fermentation. Here, it should be noted that most phenylalanine of YJS in the system underwent oxidation catalyzed by phenylalanine hydroxylase, which converted it into tyrosine, and also participated in the reaction with tyrosine to synthesize other metabolites such as neurotransmitters and hormones, which functioned in other metabolisms. Both palmitic acid and stearic acid were fatty acids, and had been found to play an important role in flavor formation of fermented products. In addition to that, the content of glycerol and palmitic acid decreased in the inoculated fermentation samples during the late period of fermentation, probably due to the formation of neutral lipids by esterification of glycerol and palmitic acid. Inositol, also known as cyclohexanhexol, was not only a growth factor for animals and microorganisms, but also had a facilitative effect on cell membrane phospholipid homeostasis. In the inoculated fermentation samples, myo-inositol consistently showed high expression, contributing to myo-inositol being the metabolite with the largest difference in multiplicity, while inositol could help to lose weight. Furthermore, it also had antioxidant, anti-aging and anti-inflammatory functions and possessed higher antioxidant activity mainly against superoxide anions, hydrogen peroxide and hydroxyl radicals, which implied that YJS fermented by inoculating might have higher antioxidant activity (Elvia et al., 2010). On day 16 of fermentation, a large number of differential metabolites decreased in species and content, while only malic acid, maleic acid, sophorose and flavin adenine degrading products were enriched at this time.

3.4. Construction of the metabolic network

The screening of major metabolic pathways was performed according to the condition of Impact > 0.01 (Chen et al., 2018), and 10 metabolic pathways related to the fermentation of YJS were obtained, when glycolysis or gluconeo genesis, pyruvate metabolism, TCA cycle, glycerolipid metabolism, phenylalanine metabolism, alanine, aspartate and glutamate metabolism, ascorbate and aldarate metabolism, galactose metabolism, tryptophan metabolism, β-alanine metabolism, pantothenate and CoA biosynthesis were included (Fig. 6). The initial period of fermentation began with the hydrolysis of polysaccharides in the raw material into glucose, xylose and sucrose. Then, sucrose was hydrolyzed into fructose and glucose that entered glycolysis or gluconeo genesis and was converted to glucose 6-phosphate catalyzed by glucokinase, which then catalyzed the cleavage of fructose 1,6-diphosphate by fructokinase 1,6-diphosphate to produce glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. After that, glyceraldehyde 3-phosphate was transformed by phosphoenolpyruvate under the action of pyruvate kinase to produce pyruvate, which could either be synthesized into alanine via the alanine, aspartate, and glutamate metabolism or enter the TCA cycle under the presence of acetyl CoA to generate organic acids such as α-ketoglutarate, succinic acid and malic acid. Among them, α-ketoglutaric acid could produce glutamic acid through alanine, aspartic acid and glutamic acid metabolism, when glutamic acid could be further converted to GABA through glutamic acid decarboxylase (GAD) that further changed into succinic acid from GABA through GABA branch. Moreover, the strains accessed by the inoculated fermentation group all had effective ability to synthesize GABA; however, GABA did not become the main differential metabolite, which could be caused by the conversion of GABA synthesized by microorganisms in the system to succinic acid via the GABA branch, thus entering the TCA cycle and producing other metabolites. Aspartic acid could be generated via the conversion of succinic acid to oxaloacetate via the alanine, aspartate and glutamate metabolism, whereas glycerol could be synthesized into glycerol 3-phosphate by the action of glycerol kinase, which in turn was catalyzed by phosphoglycerol dehydrogenase to produce dihydroxyacetone phosphate that was converted into glyceraldehyde 3-phosphate to enter the glycolytic pathway to synthesize pyruvate, thus participating in the TCA cycle (Jiang, et al., 2020). In this context, the flavor of YJS was able to be produced through the intermediate metabolites of the TCA cycle, while amino acids would enter the TCA cycle again under the action of enzymes to promote the synthesis of organic acids, which was also beneficial to the flavor enhancement.

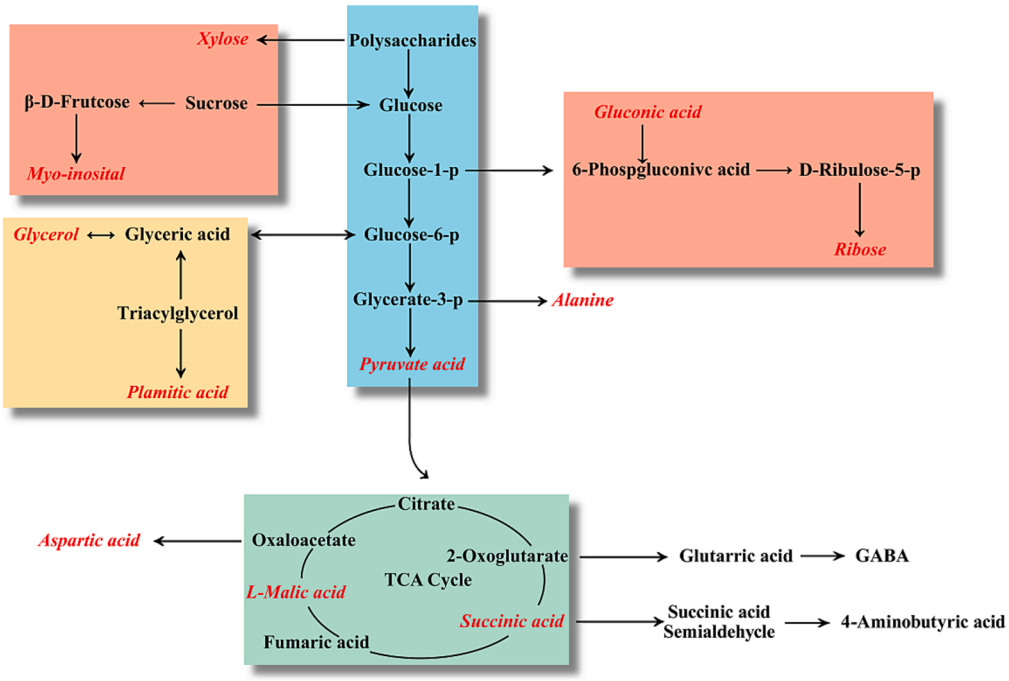

Fig. 6. Metabolic pathway analysis of YJS fermentation process. The red and diagonal font means differential metabolites, Carbohydrate metabolism, TCA cycle, glycolytic or glycoisomeric, synthesis and degradation of fatty acids are shown in pink, green, blue, and yellow, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

In this study, *W. cibaria* and *Lb. plantarum*, as the dominant microorganisms in the initial, as well as the middle and later periods of YJS fermentation, respectively, grew and multiplied rapidly during the fermentation process, thus shortening the fermentation time and inhibiting the growth of spoilage microorganisms to a great extent. Besides, the contents of AN and TCA-soluble peptide were increased, while the contents of nitrite, TVB-N and TBARS were lower than those of naturally fermented YJS. Considering that, the quality and safety had improved. Furthermore, Weissella and Lactobacillus were the first dominant genera in the early as well as the middle and later periods of YJS fermentation, respectively. Throughout the fermentation process, different metabolite species reached the highest level on day 4 ~ 12 of fermentation, mainly involving the TCA cycle, pyruvate metabolism, glycolytic or glycoisomeric, as well as alanine, aspartate and glutamate metabolism. Therefore, *W. cibaria* and *Lb. plantarum* belonged to the key dominant microorganisms during the fermentation of YJS and played a certain role in enhancing the quality and safety of YJS, thus being capable of providing a theoretical reference for further improvement of the YJS fermentation process in the future.

CRediT authorship contribution statement

Lu Liu: Methodology, Formal analysis, Writing – original draft. **Jintao Yang:** Resources, Software. **Hongyan Chen:** Data curation, Visualization. **Lu Jiang:** Data curation, Visualization. **Zhongyue Tang:** . **Xuefeng Zeng:** Methodology, Supervision.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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