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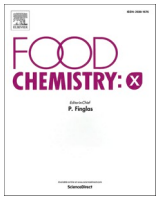

Effect of *L. Plantarum* Y279 and *W. Cibaria* Y113 on microorganism, lipid oxidation and fatty acid metabolites in Yu jiaosuan, A Chinese tradition fermented snack

Hongyan Chen [a,b], Lu Liu [a,d], Lu Jiang [a,b], Wenkang Hu [a,b], Qin Cen [a,b], Rui Zhang [a,b], Fuyi Hui [a,b], Jiamin Li [a,b], Xuefeng Zeng [a,b,c,*]

[a] School of Liquor and Food Engineering, Guizhou University, Guiyang 550025, China
[b] Guizhou Provincial Key Laboratory of Agricultural and Animal Products Storage and Processing, Guiyang, China
[c] Edible Fungus Research Institute Guizhou University, Guiyang, China
[d] Bureau of Agriculture and Rural Affairs of Majiang County, Guizhou Province, China

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ABSTRACT

Fatty acids are one of the main sources of flavour in fermented Yu jiaosuan (YJS) in southwest China. *Bacilli* (50.18 %) and *Oxyphotobacteria* (32.70 %) were the dominant class. *Lactiplantibacillus* (40.51 %) and *Weissella* (20.43 %) were the dominant species in the inoculated fermented group (HY). The peroxide value (ZY: 0.025 g/100 g, HY: 0.016 g/100 g) and lipoxygenase (LOX) (ZY: 5.7654 U/min·g, HY: 3.3856 U/min·g) in the HY group were significantly lower compared with the natural fermentation group (ZY), while acid lipase activity (ZY: 0.3184 U/h·g, HY: 0.7075 U/h·g) and neutral lipase activity (ZY: 12.65443 U/h·g, HY: 20.25142 U/h·g) were significantly higher than the control sample. Totally 40 differential fatty acid metabolites were screened. Arachidonic acid metabolism, unsaturated fatty acid biosynthesis and linoleic acid metabolism were potential metabolic pathways. Seven major bacterial species were closely associated with 15 differential fatty acid. This study contributes to the targeted production of fatty acid functional active substances of YJS.

1. Introduction

Yu jiaosuan (YJS) is one of the ethnic specialties of southwest China with potential non-heritage material culture. Usually, loach and fresh chili are taken as the main ingredients. Ginger, salt, white wine, and other ingredients are also added, endowing the YJS with delicious and unique flavour. Besides, it is rich in fatty acids, organic acids, free amino acids, and other nutrients, and has the effect of promoting digestion, refreshing the mind, and prolonging life. Liu et al. (2023) found that YJS has high amino acid nitrogen and TCA-soluble peptides and that YJS has a variety of metabolites that are beneficial for human health (Jiang, Liu, Chen, Zhang, He, & Zeng, 2022).

Microorganisms exerts a core role in the fermentation of YJS. As traditional YJS is fermented using its own microorganisms, it has problems including the long fermentation period and unstable fermented product quality. However, autochthonous starter cultures can adapt to the growth environment more quickly (Speranza, Bevilacqua, Corbo, & Sinigaglia, 2017), shorten the fermentation cycle, and improve the stability and safety of the product, while giving the product its original colour, aroma and taste as well as improving product quality (Zeng, Xia, Yang, & Jiang, 2013). Then, inoculation with autochthonous starter cultures enhances the competitiveness of the dominant bacteria, inhibits the growth of harmful and spoilage bacteria, promotes the release of free and fatty acids as well as improves the flavour of the product (Xiao, Liu, Chen, Xie, & Li, 2020). Therefore, fermentation with autochthonous starter cultures has become one of the options for the industrialisation of fermented YJS.

During fermentation, many fatty acid metabolites are produced from the microorganisms and their enzymatic activity in the complex fermentation system, and their interaction determines the flavour characteristics of the food (Flores & Toldra, 2011). In the study of fermented dry sausages, it was found that inoculation with autochthonous starter cultures can be more effective in promoting lipid hydrolysis in fermented dry sausages, inhibiting fat auto-oxidation, and improving the flavour of fermented dry sausage (Chen, Q., Kong, Han, Xia, & Xu, 2017). In the fermentation of Suanyu, autochthonous starter cultures

* Corresponding author at: School of Liquor and Food Engineering, Guizhou University, Guiyang 550025, China.
E-mail address: heiniuzxf@163.com (X. Zeng).

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and endogenous lipase exert a vital role in the release of free fatty acids from Suanyu and enhance the flavour in inoculated Suanyu (Xu et al., 2018). In addition, choosing autochthonous starter cultures with high lipase activity for inoculation fermentation can also shorten the fermentation time and promote the development of fermented food flavours (Casaburi, Di Monaco, Cavella, Toldra, Ercolini, & Villani, 2008). Therefore, the use of microbial fermentation enables the fermented product to produce more fatty acid metabolites and obtain a good flavour.

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Although there are numerous studies focusing on the effects of autochthonous starter culture inoculation of fermentation products on fatty acids (Gao, Wang, Xia, Xu, & Jiang, 2016; Jiang, Wu, Wu, Wang, Wu, & Shi, 2022), it is of necessity to investigate the relationship between microorganisms and changes in fatty acid-like metabolites in YJS. However, the effects of autochthonous starter cultures inoculated with fermented YJS on fatty acid oxidation and fatty acid metabolites have rarely been reported. Two strains of *Lactiplantibacillus* with good performance have been screened from the traditional YJS in our laboratory. Therefore, this study concentrated on the microbial community and lipid oxidation in the fermentation of YJS by artificial inoculation with *Lactiplantibacillus*, analyzed the metabolic changes of fatty acid metabolites during the fermentation of YJS with non-targeted metabolomic techniques, and explored the relationship between microorganisms and fatty acid metabolites during the fermentation of YJS. Moreover, this contributes to a better understanding of the role of microorganisms in the inhibition of lipid oxidation and the formation of fatty acid metabolites in YJS, which is also beneficial for regulating the quality of fermented products.

2. Materials and methods

2.1. Preparation of fermenters

Preparation of fermenters was conducted according to the previous study (Zhao et al., 2022). *Lactiplantibacillus plantarum* Y279 and *Weissella cibaria* Y113 were activated by MRS liquid medium for 18 h, twice respectively. The cultured bacterial solution was put into a sterile centrifuge tube, centrifuged at 6000 r/min at 4 ℃ for 1 min, followed by the supernatant medium being discarded, washed twice with sterile saline, and diluted to a bacterial concentration of 10^7 CFU/g.

2.2. Preparation of YJS

The chilies, loaches and other spices used in this experiment were sourced locally (Guiyang, China). After removing its handles, the chilies were washed and chopped up. Preparation of YJS was performed based on the previous study (Jiang, Liu, et al., 2022). Loaches and peppers were mixed at a ratio of 1:10, followed by the addition of 4 % (w/w) ginger, 7 % (w/w) salt and 3 % (w/w) white wine and sufficient mixing. Thereafter, 1 % (v/w) *L. plantarum* Y279 and 1 % (v/w) *W. cibaria* Y113 were placed into the YJS, stirred well, and later put in a sealed glass bottle and a 25 ℃ thermostat for fermentation. The inoculated fermentation group is noted as HY, and naturally fermented YJS is indicated as ZY. Samples were collected from 5 fermentation stages, namely 0 days (ZY1, HY1), 4 days (ZY2, HY2), 8 days (ZY3, HY3), 12 days (ZY4, HY4) and 16 days (ZY5, HY5). These samples were subjected to pyrophosphate sequencing, lipid oxidation analysis, and metabolomics analysis. Three biological replicates were collected at each sampling site and stored at −80 ℃ prior to use.

2.3. DNA extraction and PCR amplification

DNA extraction and PCR amplification were referred to the previous study (Liu et al., 2019) with some minor modifications. DNA was extracted by the Rapid Bacterial Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China). Using primers 338F and 806R, the bacterial 16S rRNA V3-V4 region was amplified. The bacterial DNA amplification was performed with 30 μL reaction solution, containing 10 ng template DNA, 6 μM primers, 15 μL 2 × Phusion Master Mix (New England BioLabs Inc., Ipswich, MA), and 2 μL H_2O. The reaction condition of PCR was initial denaturation at 98 ℃ for 1 min, denaturation at 98 ℃ for 10 s, annealing at 50 ℃ for 30 s, and extension at 72 ℃ for 30 s. After 30 cycles, it was extended for 5 min at 72 ℃. The PCR amplification products were investigated by 2 % agarose electrophoresis and further purified with the AxyPrep DNA Gel Extraction Kit (AxyPrep Biosciences, Union City, CA, USA). Then, the bacterial 16S rRNA gene was sequenced by pyrophosphate.

2.4. Bioinformatic analysis

The FLASH software (version 1.2.11) was employed to splice the middle overlap of each sequence. The original sequences obtained were filtered, and chimeric sequences were removed using Trimmomatic software (version 0.33) and UCHIME software (version 8.1). Then, filtered tags with less than 75 % of the quality control length yield high quality tag data. The obtained high-quality sequences were clustered and intercompartmentalised into operational units by USEARCH software (version 10.0). The sequence similarity was above 97 %. Taxonomic annotation of OUT was based on the Silva (bacteria) taxonomic database.

2.5. Analysis of physical and chemical indicators

2.5.1. pH

In this point, 5 g of YJS samples were weighed, and 25 mL of deionised water was added. Then, it was homogenized at 12000 rpm for 2 min, placed in a freezing centrifuge at 4 ℃, centrifuged at 10,000 r/min for 15 min and measured by the pH meter (PHS-3E, Shanghai, China).

2.5.2. Acid value (AV)

AV values of YJS were determined in accordance with the previous study (Wang et al., 2022) after slight modification. Briefly, 10 g of lipid extract was accurately weighed. Subsequently, 50 mL of an ether-isopropanol mixture (1:1; v/v) and three drops of thymolphthalein indicators were added and shaken gently. Next, the sample was titrated using 0.1 M sodium hydroxide solution (NaOH) until the sample solution began to appear blue and did not fade significantly within 15 s.

2.5.3. Peroxide value (POV)

The POV value was identified in accordance with the previous study (Wang et al., 2022), with slight modification. In addition, 5 g of lipid extract was accurately weighed, and 30 mL of the trichloromethane-glacial acetic acid mixtures (2:3; v/v) were added. Then, the samples

were titrated with a standard solution of sodium thiosulphate (0.01 M). When the solution was pale yellow, a starch indicator was added, continuously titrated and shaken strongly until the blue colour of the solution disappeared.

2.5.4. Thiobarbituric acid reactive substances (TBARS)

The TBARS value was determined using the method described by Wang et al. (2022) after slight adjustment. 2 g of samples were weighed and added with 10 mL of 10 % trichloroacetic acid solution, followed by homogenization for 30 s and then centrifugation in a freezing centrifuged at 10,000 g for 5 min. Afterwards, 5 mL supernatant was collected and added with 5 mL of 0.02 mol/L TBA solution to boil for 20 min. Later, the mixture was cooled in ice water for 20 min to allow for stratification. The absorbance of the supernatant was measured at 532 nm on a spectrophotometer and expressed as mg/kg. Blanks were replaced with 5 mL of trichloroacetic acid solution in place of supernatant removed after centrifugation.

2.6. Measurement of lipase activity

2.6.1. Extraction of crude enzyme solution

The extraction of the crude enzyme solution referred to the previous method (Huang, Li, Huang, Li, & Sun, 2014) with minor modification. 5 g of samples were weighed. In addition, 25 mL 0.05 mol/L phosphate buffer (pH = 7.5, containing 0.005 mol/L EGTA) was added. After homogenisation on ice for 30 s, and centrifugation at 4 ℃ for 20 min at 10,000 g, the enzyme activity of the supernatant was investigated.

2.6.2. Determination of acid lipase activity

The acid lipase activity was identified according to the previous study (Vestergaard, Schivazappa, & Virgili, 2000). 0.1 mL crude enzyme extract was added to 2.8 mL 0.1 mol/L disodium hydrogen phosphate 0.05 mol/L citrate buffer (pH = 5.0, containing 0.05 % (w/v) TritonX-100 and 0.8 mg/mL bovine serum albumin). Then, 0.1 mL of 0.001 mol/L of 4-methylumbelliferyl oleate was taken as a substrate. After holding for 30 min at 37 ℃, the reaction was immediately terminated with 0.5 mL of 1.0 mol/L HCL and the fluorescence was measured by the fluorescence spectrophotometer at λex = 328 nm and λem = 470 nm.

2.6.3. Determination of neutral lipase activity

The neutral lipase activity was confirmed based on the previous study (Vestergaard et al., 2000). 0.1 mL crude enzyme extract was added to 2.8 mL 0.22 mol/L Tris/HCL buffer (pH = 7.5, including 0.05 % (w/v) TritonX-100). Subsequently, 0.1 mL of 0.001 mol/L of 4-methylumbelliferyl oleate was taken as a substrate. After holding for 30 min at 37 ℃, the reaction was immediately terminated with ice baths and the fluorescence was measured by the fluorescence spectrophotometer at λex = 328 nm and λem = 443 nm.

2.6.4. Determination of phospholipase activity

Following the previous study, the phospholipase activity was determined (Vestergaard et al., 2000). 0.1 mL crude enzyme extract was added to 2.8 mL 0.1 mol/L Disodium hydrogen phosphate 0.05 mol/L Citric acid buffer (pH = 5.0, containing 0.15 mol/L Sodium fluoride, 0.05 % (w/v) TritonX-100 and 0.8 mg/mL bovine serum albumin). Later, 0.1 mL of 0.001 mol/L of 4-methylumbelliferyl oleate was taken as a substrate. After holding for 30 min at 37 ℃, the reaction was immediately terminated with 0.5 mL of 1.0 mol/L HCL and the fluorescence was measured by the fluorescence spectrophotometer at λex = 328 nm and λem = 470 nm.

The standard curve was obtained by measuring the fluorescence and calculating the three lipase activities. In addition, 1 g of dry base producing 1 umol of 4-methylumbelliferone in 1 h was defined as 1 unit of enzyme activity (U/h·g).

2.7. Determination of lipoxygenase activity

The determination of lipoxygenase activity was conducted using the method of Zang et al. (2020) with slight modifications. 5 g samples of YJS were weighted, and 35 mL 0.05 mol/L Phosphate buffer (pH = 7.0, containing 0.001 mol/L β-Mercaptoethanol, 0.0005 mol/L PMFS and 0.002 mol/L EDTA) was added. After homogenizing on ice for 30 s, the supernatant was centrifuged at 4 ℃ for 20 min in a freezing centrifuged at 10,000 g and used to investigate the enzyme activity.

Afterwards, 140 mg linoleic acid was dissolved in 5 mL of deionised water. 180 uL of Tween-20 was added, and the pH of the mixed solution was adjusted to 9.0 with 2 mol/L NaOH to dissolve linoleic acid completely. Next, the solution was fixed to 50 mL with deionised water.

Furthermore, 200 uL linoleic acid substrate solution and 0.1 mL crude enzyme extract were added to 2.9 mL 0.05 mol/L Citric acid buffer (pH = 5.5). After mixing in a water bath at 20 ℃, the increment concerning absorbance of the mixture was measured at 234 nm for 1 min. Additionally, a 0.1 increase in the absorbance value in 1 min of 1 g of dry medium was defined as 1 unit of enzyme activity.

2.8. Metabolite extraction of YJS

Metabolite extraction was performed with the method proposed by Doppler et al. (2016) with minor modifications. 50 mg samples were added to 1000 uL extraction solution (methanol: water = 3: 1 (v/v), mixtures including isotopically labelled internal standards). The process is conducted by grinding at 35 Hz for 4 min and sonicating for 5 min in an ice-water bath, which was repeated 2 or 3 times. Then, the samples were placed at −40 ℃ for 1 h. Finally, the samples were centrifuged at 4 ℃ for 15 min at 12,000 rpm. The supernatant was extracted from the injection vial and tested in the machine. To test on the machine, all samples were mixed with equal amounts of supernatant to form QC samples.

2.9. LC-MS/MS analysis

LC-MS/MS analyses were performed with an UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm) coupled to the Q Exactive HFX mass spectrometer (Orbitrap MS, Thermo). The mobile phase consisted of 5 mmol/L ammonium acetate and 5 mmol/L acetic acid in water (A) and acetonitrile (B). The auto-sampler temperature was 4 ℃, with the injection volume being 3 uL.

The QE HFX mass spectrometer was adopted for its ability to obtain MS/MS spectra on the information-dependent acquisition (IDA) mode in the control of the acquisition software (Xcalibur, Thermo). As shown in this mode, the acquisition software continuously evaluates the full scan MS spectrum. The ESI source conditions were set as follows: sheath gas flow rate as 30 Arb, aux gas flow rate as 10 Arb, capillary temperature as 350 ℃, full MS resolution as 60000, MS/MS resolution as 7500, collision energy as 10/30/60 in the NCE mode, and spray voltage as 4.0 kV (positive) or −3.8 kV (negative), respectively.

2.10. Statistical analysis

Statistical analysis of experimental data was made using SPSS 26.0 (IBM Corporation, Armonk, NY, USA) with all samples in triplicate. Screening for differential metabolites was performed based on a P-value < 0.05 for the student's *t*-test, along with a VIP value > 1 for the principal component of the PLS-DA model. The screened differential metabolites were subjected to PCA analysis, volcanograms and thermograms using Origin 2021b (OrginLab, Northampton, MA, USA). In addition, correlations between bacterial species and differential metabolites were calculated using the Spearman correlation coefficient. Interaction networks were constructed using Cytoscape.

3. Results and discussion

3.1. Microbiological analysis of YJS

3.1.1. Dynamic analysis of colony structure

Totally 37 microorganisms were identified at the class level for the five different fermentation stages of the YJS samples. The top 10 classes are indicated in Fig. 1A. The most common microbial groups were *Bacilli* (50.18 %) and *Oxyphotobacteria* (32.70 %), whose relative abundance accounted for 82.88 % of the community composition, suggesting that they might be the dominant class in the fermentation of YJS; the results of the inoculated fermentation were similar to those of Yang et al. (2022). The abundance of *Bacilli* gradually increased and finally stabilized, demonstrating that Bacilli is the dominant class in the fermentation process of YJS, while that of *Oxyphotobacteria* gradually decreased, indicating that this class may mainly exert a role in the early stage of fermentation (Hua, Sun, Xu, Gao, & Xia, 2021).

To investigate the changes in the bacterial community during the fermentation of YJS in a more detailed manner, the relative abundance of the microbial community at the species level is presented in Fig. 1B. Totally 190 species were detected in the bacterial community during the fermentation of YJS. The top 10 bacterial species are displayed in Fig. 1B. After 12 days of fermentation, the most common microbial in the HY group is the *Lactiplantibacillus* (40.51 %) and *Weissella* (20.43 %), and its relative abundance occupies 60.94 % of the community composition. The trend at the species level exhibits a gradual decrease in the abundance of *uncultured_bacterium_o_Chloroplast*, showing that this bacterium is the dominant bacterium in the pre-fermentation stage of YJS. With the progress of the fermentation, the abundance of *Lactiplantibacillus* gradually increased, and the relative abundance of *Lactiplantibacillus* in the ZY and HY groups was 30.46 % and 40.56 % respectively, after 16 days of fermentation, Our results were similar to the study in Chaozhou pickle by Chen et al. (2024), who also reported that *Lactiplantibacillus* was the predominant bacterial genera in Chaozhou pickle. This suggested that inoculated fermentation can promote the growth and reproduction of *Lactiplantibacillus*. Furthermore, it has been found that *Lactiplantibacillus* not only produces various antimicrobial metabolites (Galvez, Abriouel, Lopez, & Ben Omar, 2007), but also uses the carbohydrates in the fermentation system to produce a large number of organic acids, mainly including lactic acid, which can rapidly lower the pH and improve the safety of the food (Wu et al., 2015). This result conforms to the above-described pH change. In the HY group, *Weissella* is the second most dominant species, and studies have indicated that *Weissella* is often found in fermented foods. Besides, it can promote the formation of flavour substances, including short-chain fatty acids, organic acids and esters during food fermentation, and exerts potential probiotic functions (Lynch, McSweeney, Arendt, Uniacke-Lowe, Galle, & Coffey, 2014; Yu, Jang, Lee, & Paik, 2019). After 4 days of fermentation, *Fructobacillus_tropaeoli* grew rapidly as the dominant bacterium in the ZY group, while the growth of *Fructobacillus_tropaeoli* was inhibited by the inoculated fermentation. In addition, studies have suggested that *Fructobacillus_tropaeoli* can produce pectinase (Chen et al., 2021), which can soften peppers and influence the crispness of YJS. After 16 days of fermentation, the relative abundance of *Fructobacillus_tropaeoli* in the ZY group was 20.60 % and that in the HY group was 5.33 %, demonstrated that inoculated fermentation hindered the growth of *Fructobacillus_tropaeoli* and improved the crispness, texture, taste and other qualities of YJS. The dominant bacteria in the ZY group were *Lactiplantibacillus* (25.27 %) and *Fructobacillus_tropaeoli* (25.28 %) during the late stage of YJS fermentation (12–16 days). The dominant bacteria in the HY group were *Lactiplantibacillus* (40.51 %) and *Weissella* (20.43 %). The results of the inoculated fermentation were similar to those of Roh et al. (2010). In summary, inoculated fermentation enables the production of various antimicrobial substances that enhance the safety of YJS, while also promoting the production of fatty acid metabolites.

3.1.2. PCoA analysis

PCoA was used for determining the differences in microbial communities in each YJS sample. As can be seen from the Fig. 2, the samples of YJS on day 4 and day 8 of fermentation can be obviously separated from those on day 0 of fermentation, indicating that the degree of species variation becomes greater at this point. In addition, it is possible that microbial activity is accelerated at this time due to the greater amount of carbon source material available to microorganisms (Wu et al., 2015). On day 12 and day 16, groups ZY4 and ZY5, and HY4 and HY5 were not obviously separated, probably because microorganisms continuously consume carbon source material from the raw material during fermentation, and when they enter the later stages of fermentation, the available nutrients are reduced, while microbial activity weakens and the degree of species differentiation decreases (Jiang, Liu, et al., 2022). By comparing the separation distances of the two groups of YJS samples on the PCoA plot, respectively, it can be found from PCoA1 that group ZY3 is similar in distance to group HY2, and ZY4 and ZY5 are similar in distance to group HY4 in the horizontal coordinates, demonstrating that the microbial composition in the two groups shows many similarities at this time. The results demonstrate that inoculated fermentation can shorten the fermentation time of YJS. Microorganisms in the ZY group were more dispersed during the same time period after the start of fermentation, suggesting that the microbial communities in YJS vary significantly during natural fermentation, while the microorganisms in the HY group were closer, showing a high degree of similarity in the microbial composition of inoculated fermented YJS. Therefore, it can be observed that inoculated fermentation inhibited the microbial diversity in YJS.

3.1.3. Microbial association analysis

The interactions between microorganisms in the fermentation environment are complex, with a variety of interactions such as synergy,

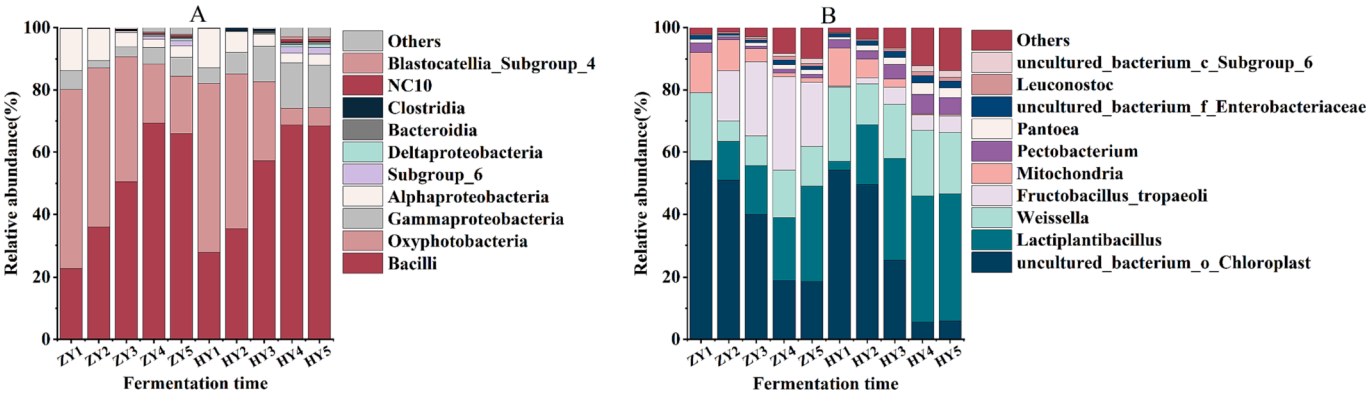


Fig. 1. Changes in bacterial community structure at the class level (A) and species level (B) during the fermentation of YJS.

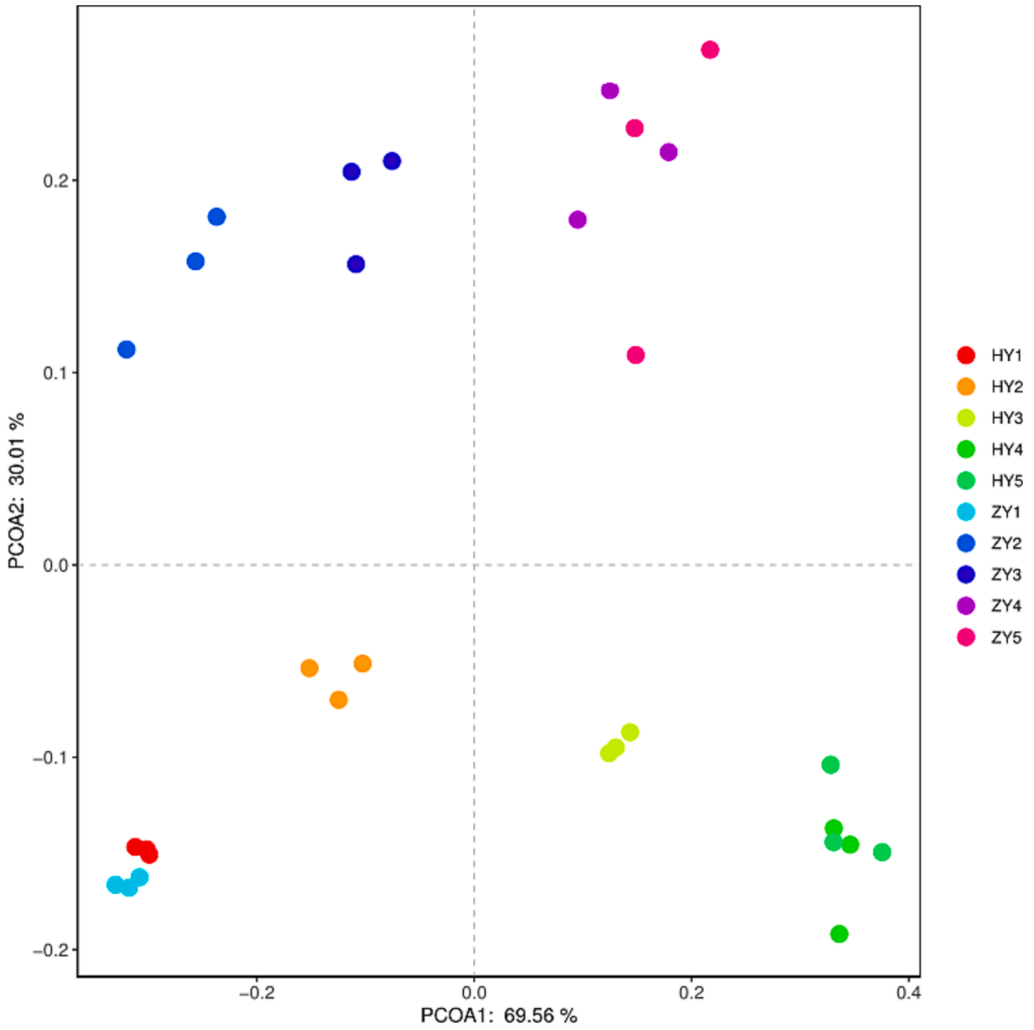


Fig. 2. PCoA scoring of microbial communities during fermentation of YJS.

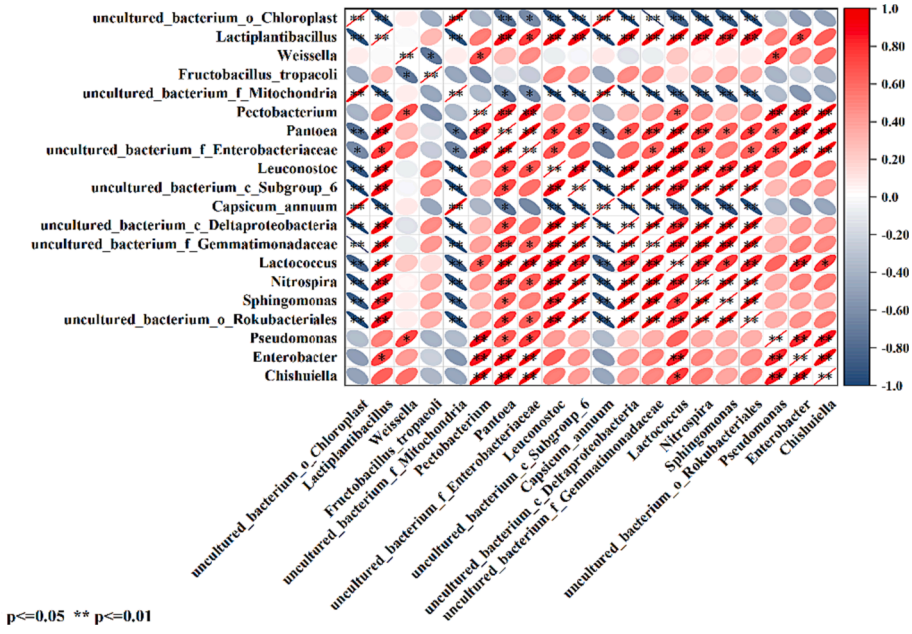


Fig. 3. Heat map of the association of species-level dominant microorganisms ($p < 0.01$, marked with "**", $0.05 < p < 0.01$, marked with "*").

inhibition and mutual inhibition. The microbial communities cooperate with each other to accomplish the fermentation task. Correlation analysis of the dominant microorganisms in the fermentation process of YJS is conducive to understanding whether there is a facilitating or competitive relationship between them. A total of 20 species were analysed (Fig. 3). Among them, *Chloroplast*, *Mitochondria* and *Capsicum_annuum* showed a significant positive correlation with each other, indicating that there is a mutual promotion effect between them, whereas they were negatively correlated with most other species, such as *Lactiplantibacillus*, indicating that *Chloroplast*, *Mitochondria* and *Capsicum_annuum* influenced other microorganisms in the YJS to a great extent. Additionally, there was a positive correlation between *Lactiplantibacillus* and most microorganisms, suggesting a mutual synergistic effect between *Lactiplantibacillus* and most microorganisms in YJS, which is similar to that obtained by Chen et al. (2021). However, *Fructobacillus_tropaeoli*, the dominant bacterium in the ZY group, and *Weissella*, the dominant bacterium in the HY group, were significantly negatively correlated. Thus, the growth of *Fructobacillus_tropaeoli* was hindered in the later stages of fermentation when *Weissella* in the HY group grew and multiplied in large numbers. Therefore, there were interactions between microorganisms in the fermentation of YJS.

3.2. Analysis of physical and chemical indicators

3.2.1. pH

pH is a vital quality parameter influencing the growth of microorganisms and the accumulation of microbial metabolites (Yang, Fan, Li et al., 2022). The change in pH of YJS in the HY and ZY groups is shown in Fig. 4. The pH of YJS reduced with time in both groups. At the beginning of fermentation, the HY group experienced a rapid increase in the number of *Lactiplantibacillus* after accessing the dominant bacteria, causing a significantly lower pH than that of the ZY group ($p < 0.05$). During the later stages of fermentation, the pH dropped slowly and remained stable in the HY group, while it reduced to 3.93 in the ZY group. The rapid reduction in pH effectively inhibits spoilage and pathogenic bacteria in fermented foods, also improving the safety of fermented foods (Zeng, Xia, Jiang, & Yang, 2013). The pH of both groups presented a stable trend in the late stage of fermentation, due to the fact that in the late stage of fermentation, the oxygen in the fermented YJS system is gradually depleted; in addition, the growth of *Lactiplantibacillus* is slow, and the acid production capacity decreases, maintaining the pH in a stable state (Ghanbari, Jami, Domig, & Kneifel, 2013).

3.2.2. Determination of lipid oxidation

AV can be used as an indicator of fat degradation during the fermentation process (Wang, Xie, & Chen, 2021). Fig. 5A shows that the AV gradually increases with the increasing fermentation time. Compared with the HY group, the AV increased more slowly in the ZY group, reaching 2.26 mg/g at the end of fermentation; however, it was 3.64 mg/g in the HY group, which was significantly higher than that of the ZY group ($p < 0.05$). The AV of the two groups of YJS showed an increasing trend, probably due to the production of lactic acid by glycolysis of the glycogen in the YJS, causing an accumulation of acid and an increase in AV (Jiang, Liu, et al., 2022). Therefore, the inoculated fermentation promoted lipid degradation of YJS.

The primary product of the lipid oxidation process is hydroperoxide, and the POV is primarily a measure of the amount of hydrogen peroxide in the system and is often used to characterise the degree of fat and oil oxidation in meat products (Zhou et al., 2019). The POV was characterized by an increasing trend through the fermentation of YJS (Fig. 5B), when it exhibited the fastest increase in the ZY group, having reached 0.29 g/100 g after 16 days of fermentation, while the value in the HY group was 0.16 g/100 g, which was significantly lower than that in the ZY group ($p < 0.05$). This result is similar to that of Sun et al. (2020) in Suanyu. The presence of *Lactiplantibacillus* has been reported to lower fat oxidation in fermented products (Zeng et al., 2013). Therefore, inoculation fermentation reduces the degree of fat oxidation in YJS.

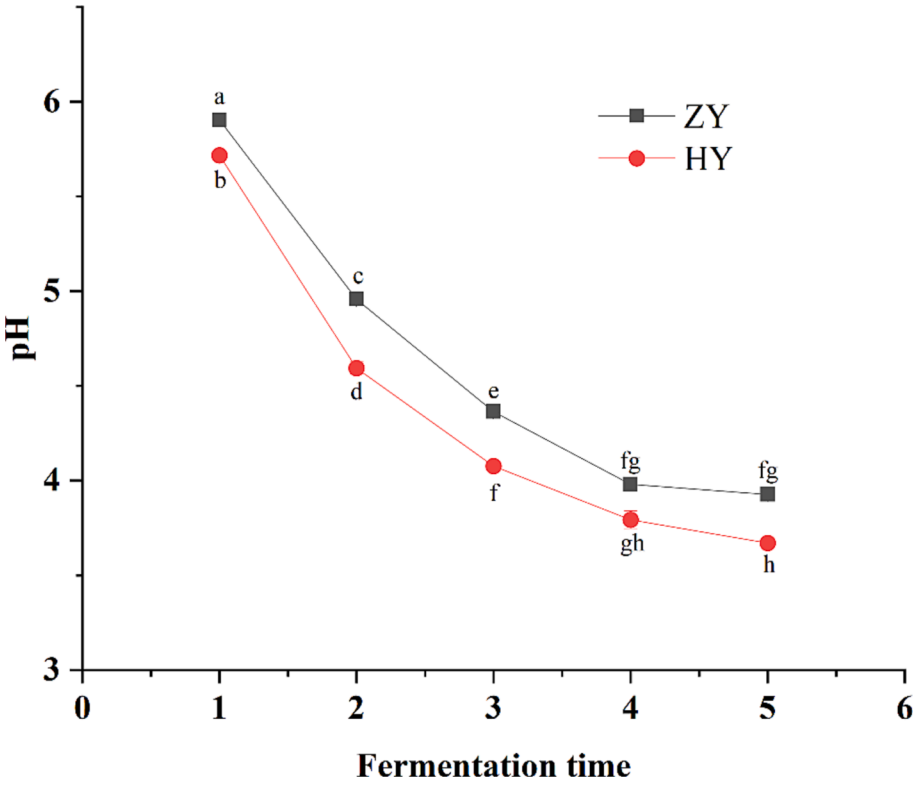


Fig. 4. Changes in pH during the fermentation time of YJS. The different letters in the line chart were shown as a significant difference between samples ($p < 0.05$).

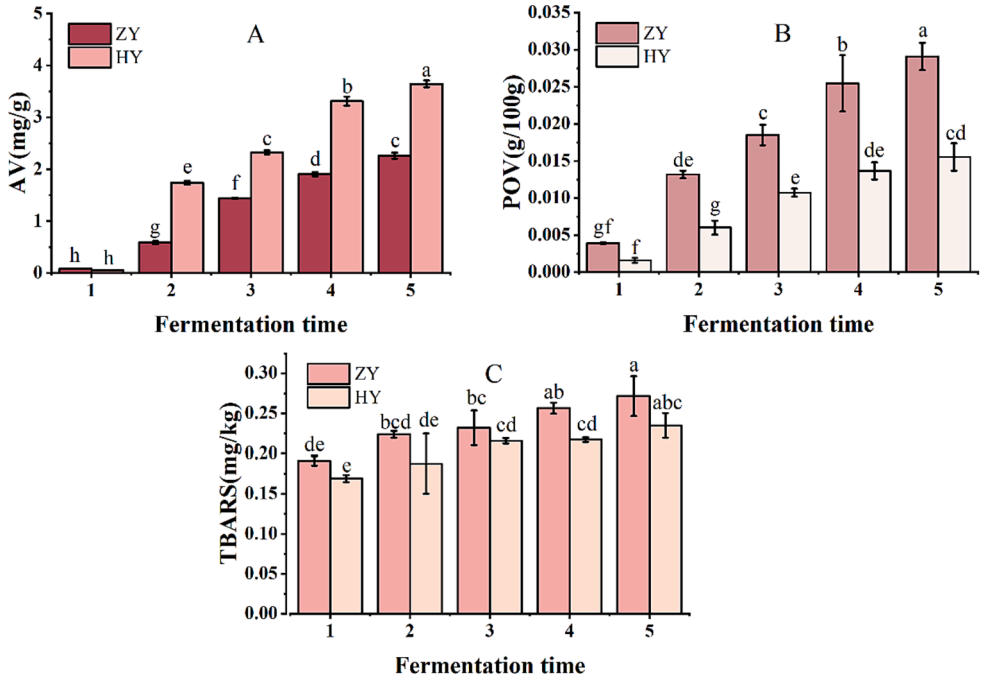


Fig. 5. (A) Change of AV during the fermentation time of YJS, Fig. 2 (B) Change of POV during the fermentation time of YJS, Fig. 2 (C) Change of TBARS during the fermentation time of YJS. The different letters in the bar chart were shown as a significant difference between samples ($p < 0.05$).

The TBARS value suggests the number of fat secondary oxidation products (final products) and is a broad measure of the fat oxidation degree (Beatriz, Juan, Samuel, & Josep, 2014). TBARS value exhibited a gradual increase during the fermentation of YJS (Fig. 5C), as observed in the increasing secondary products with the deepening of oxidation. After 16 days of fermentation, TBARS value reached 0.27 mg/kg in the ZY group and 0.23 mg/kg in the HY group, which were lower than those of the ZY group. This may be caused by the degradation of aldehydes or the reaction with other compounds to produce some flavor compounds during the fermentation of YJS, leading to lower TBARS value in the inoculated fermented YJS (Huang et al., 2014). This result is similar to that of Bozkurt and Erkmen (2002) in bacon. Therefore, inoculated fermentation can effectively slow down the increase of TBARS value in YJS.

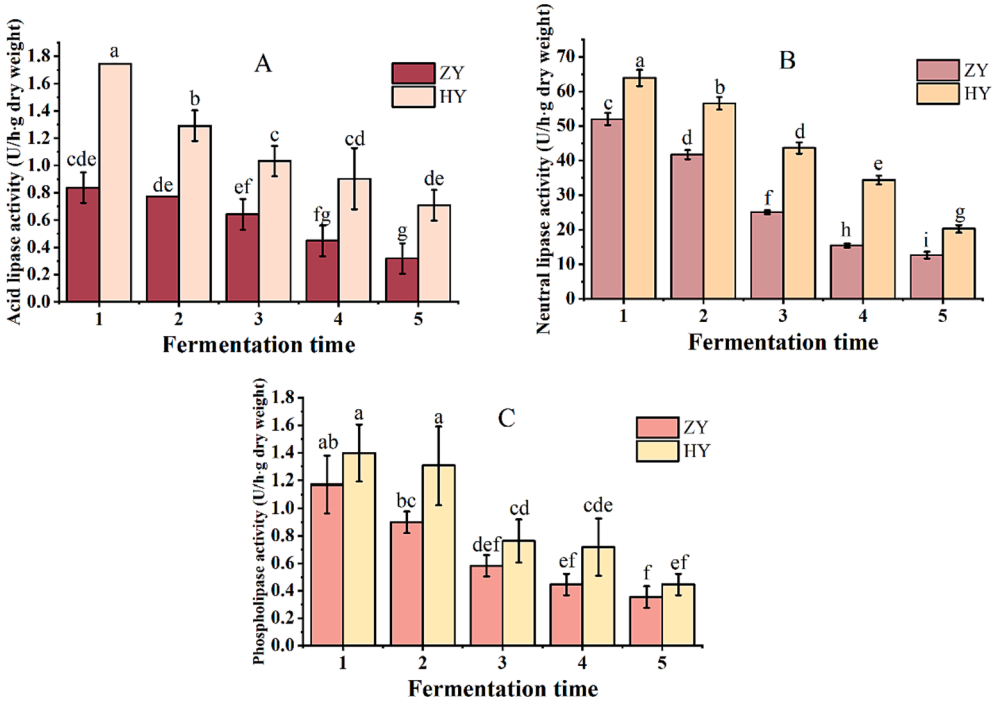


Fig. 6. (A) Change of acid lipase during the fermentation time of YJS, Fig. 3 (B) Change of neutral lipase during the fermentation time of YJS, Fig. 3 (C) Change of phospholipase during the fermentation time of YJS. The different letters in the bar chart were shown as a significant difference between samples ($p < 0.05$).

3.3. Measurement of lipase activity

3.3.1. Acid lipase

Through the fermentation of YJS, the acid lipase activity kept showing a decreasing trend (Fig. 6A), while that of the HY group was significantly higher than that of the ZY group in all cases ($p < 0.05$). During the late stage of fermentation, the acid lipase activity in both groups of YJS slowly decreased, indicating that acid lipase mainly acted on lipid hydrolysis in the pre-fermentation stage (Xu et al., 2018). Yu et al. (2021) showed that the optimum pH of acid lipase was about 5.5, and the pH of both YJS groups decreased with the fermentation process, suggesting that the pH change caused by microorganisms during fermentation influences the activity of acid lipase. Although the pH in the HY group was lower than that in the ZY group, the acid lipase activity was higher in the HY group, which may be resulted from the microorganisms contributing to some lipase activity (Xu et al., 2018). Therefore, the inoculated fermentation could slow down the reduction of acid lipase activity in YJS.

3.3.2. Neutral lipase

The neutral lipase activity in both groups of YJS decreased with the increasing fermentation time (Fig. 6B), but that was much higher than the acid lipase activity and phospholipase activity, suggesting that the neutral lipase exerted a vital role in the hydrolysis of lipids during the fermentation of YJS. This result is similar to that given by Xu et al. (2018). After 16 days of fermentation, the neutral lipase activity was 12.65 U/h·g in the ZY group and 20.25 U/h·g in the HY group, which was significantly higher than that of the ZY group ($p < 0.05$). The optimum pH of neutral lipase is 7.0–7.5, and the pH is below 7.0 through the fermentation process of YJS. Therefore, the neutral lipase activity lowers with the decreasing pH. The catalytic action of proteases or peroxidation during lipid oxidation may also lead to a reduction in enzyme activity.

3.3.3. Phospholipase

During the fermentation process, the phospholipase activity of YJS showed the same decreasing trend in both groups (Fig. 6C), which was in consistence with the results of their study (Jin, Zhang, Yu, Lei, & Wang, 2011; Zhou & Zhao, 2007). It is indicated that phospholipases have high activity in the neutral to alkaline pH range (Kenneally, Leuschner, & Arendt, 1998). However, the fermentation system was acidic all the time during the fermentation of YJS, which may be one of the reasons for the reduction of YJS phospholipase activity. The slightly higher phospholipase activity in the HY group may be associated with the lipase secreted by the microorganism compared to the ZY group (Xu et al., 2018). This result indicates that inoculated fermentation can delay the reduction of phospholipase activity.

In conclusion, the inoculated fermentation could slow down the reduction of lipase activity in YJS and enhance the degree of lipid hydrolysis. This result conforms to the analysis in the above AV.

3.4. Lipoxygenase activity measurement

Lipoxygenase catalyzes the oxidation of fatty acids and the further breakdown of hydroperoxides into small ketones as well as aldehydes that affect the flavour of the product (Buchhaupt, Guder, Etschmann, & Schrader, 2012). As shown in Fig. 7, the lipoxygenase activity was characterized with a decreasing trend through the fermentation process of YJS, which may be associated with the inactivation of hydroperoxides (Fu, Xu, & Wang, 2009). After 16 days of fermentation, lipoxygenase was 5.77 U/min·g in the ZY group and 3.39 U/min·g in the HY group, which was significantly lower than that in the ZY group ($p < 0.05$). Xu et al. (2018) considered that lower pH can also reduce the activity of lipoxygenase and that microorganisms are less able to break down lipoxygenase. Thus, inoculated fermentation reduces the activity of lipoxygenase, inhibits the oxidation of unsaturated fatty acids, slows down the deterioration process and enhances the safety of food products.

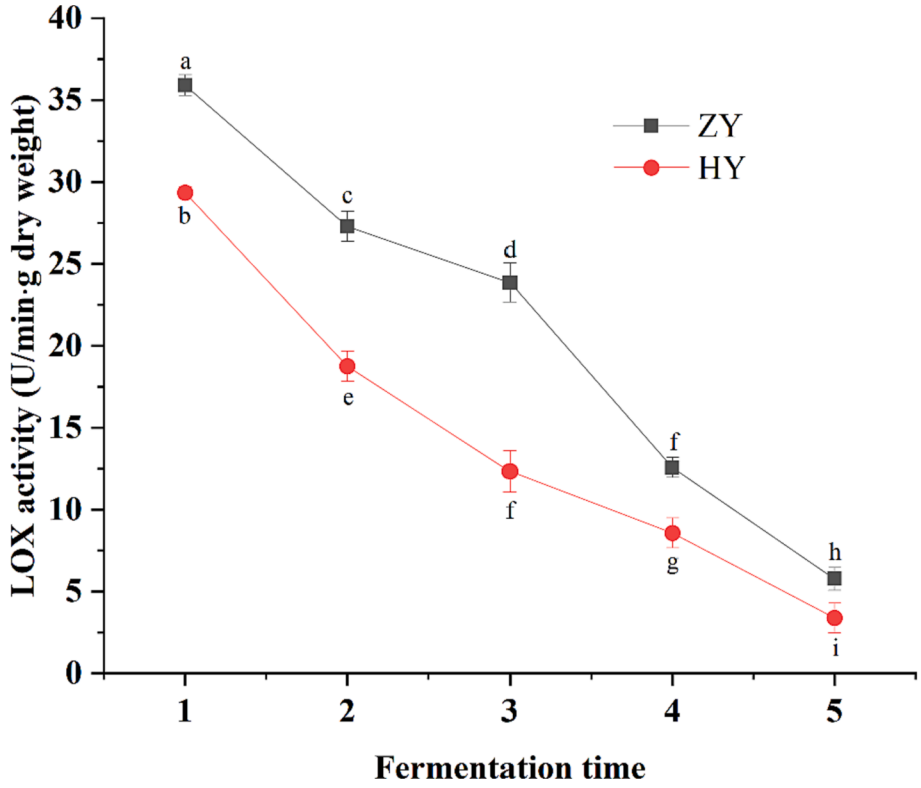


Fig. 7. Changes in lipoxygenase activity during fermentation of YJS. The different letters in the line chart were shown as a significant difference between samples ($p < 0.05$).

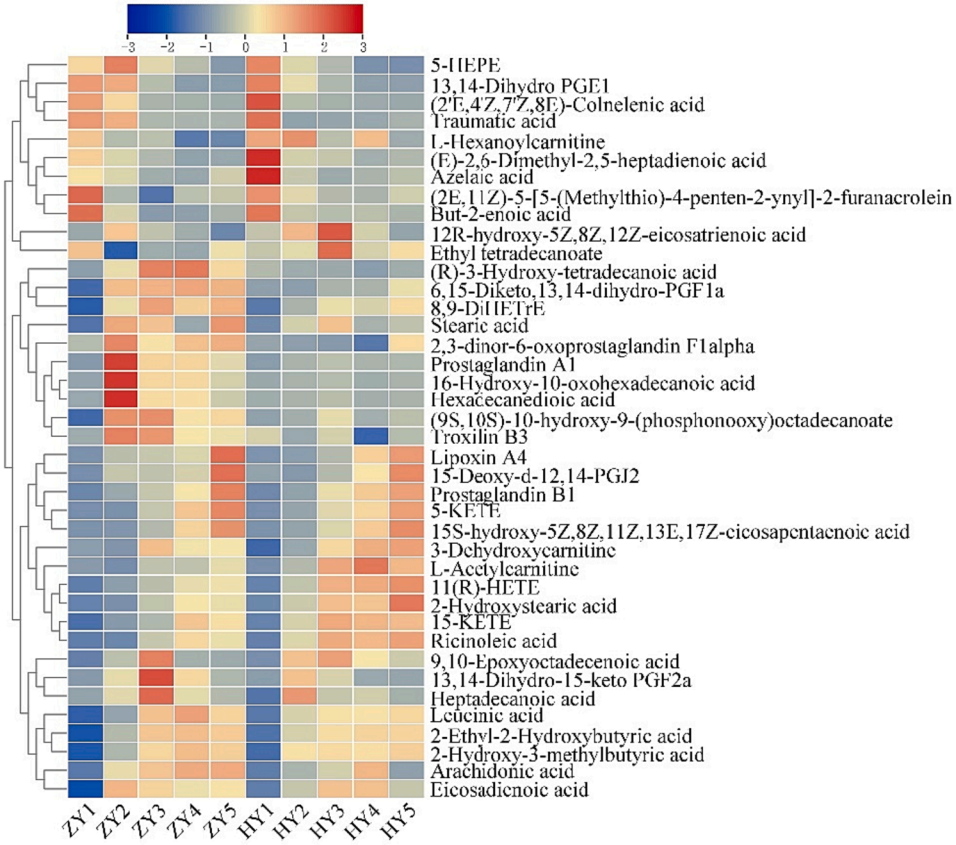


Fig. 8. Thermogram visualisation of differential fatty acid metabolites in the natural and inoculated fermentation groups for different periods of fermentation of YJS.

3.5. Fatty acid metabolite heat map visualization

Totally 40 differential fatty acid metabolites with VIP > 1 and $p < 0.05$ were chosen to make a heat map to visualise the trends in the concentration of differential fatty acid metabolites at different stages of fermentation. Each column in Fig. 8 represents a YJS sample at different fermentation times. Each row denotes a metabolite. The colour of the heat map refers to the relative expression of the metabolite in that group of samples, with the colour from blue to white and then to red, suggesting a gradual increase in metabolite abundance. As presented in Fig. 8, inoculated fermentation can influence the accumulation of metabolic substances. As fermentation progressed, 3-Dehydroxycarnitine, L-Acetylcarnitine, 11(R)-HETE, 2-Hydroxystearic acid, 15-KETE and Ricinoleic acid gradually accumulated and were more highly expressed in the inoculated fermentation group. Studies have indicated that L-Acetylcarnitine has neuroprotective, neurotrophic and analgesic activity (Formaggio, Rimondini, Delprete et al., 2022). By contrast, (R)-3-Hydroxy-tetradecanoic acid, 6,15-Diketo,13,14-dihydro-PGF1a, 8,9-DiHETrE, Stearic acid, 2,3-dinor-6-oxoprostaglandin F1alpha, Prostaglandin A1, 16-Hydroxy-10-oxohexadecanoic acid, Hexadecanedioic acid, (9S,10S)-10-hydroxy-9-(phosphonooxy)octadecanoate, and Troxilin B3 accumulated gradually in the natural fermentation group. 5-HEPE, 13,14-Dihydro PGE1, (2′E,4′Z,7′Z,8E)-Colnelenic acid, Traumatic acid, L-Hexanoylcarnitine, (E)-2,6-Dimethyl-2,5-heptadienoic acid, and Azelaic acid were highly expressed in the early stages of fermentation and gradually lowered as fermentation progressed, while inoculated fermentation increased the expression of these substances in YJS. Therefore, it is known that 5-HEPE and Azelaic acid function in reducing obesity and insulin resistance (Chae, Wu, Shin, & Lee, 2023; Zong, Wang, Liu, Fan, & Yang, 2023), and its increased expression contributes to the obesity-reducing effects of YJS. As *Lactiplantibacillus* is active in the fatty acid metabolic pathway in fermented foods (Zhang, Shang, Liu, Hu, & Yi, 2023), fermenting YJS by inoculation with *Lactiplantibacillus* can produce rich fatty acid metabolites. In this study, inoculated fermentation increased the accumulation of fatty acid metabolites, including L-Acetylcarnitine, 5-HEPE and Azelaic acid in YJS as well as improved the quality of YJS.

3.6. PCA analysis

Differential analysis of fatty acid metabolites in positive and negative ion modes was made. As displayed in Fig. 9A, the cumulative variance of the first principal component (PC1) and the second principal component (PC2) accounted for 31.2 % and 24.5 % of the total variance, respectively. PC1 (31.2 %) and PC2 (24.5 %) explained 55.7 % of the variance in the data. Fig. 9B presents totally 46.5 % of the variance in the data. In the positive and negative ion modes, the YJS from the two groups fermented for 0 days could be obviously separated from the YJS from the other fermentation time periods, suggesting that the microorganisms could use the carbon source material in the YJS for rapid fermentation and active metabolic activity in the first 4 days (Zhao, Su, Mu, Jiang, & Mu, 2020). This causes a greater variation of fatty acid metabolites in the YJS. As fermentation progressed, the metabolic profiles were more similar and less clearly separated in the PCA plots, probably because fewer carbohydrates became available in the YJS and the fermentation capacity of the *Lactiplantibacillus* diminished, leading to less significant differences in metabolite changes.

3.7. Screening for differential fatty acid metabolites

Totally 109 fatty acid metabolites were detected in the positive and negative ion models. To visualise the differential fatty acid metabolites

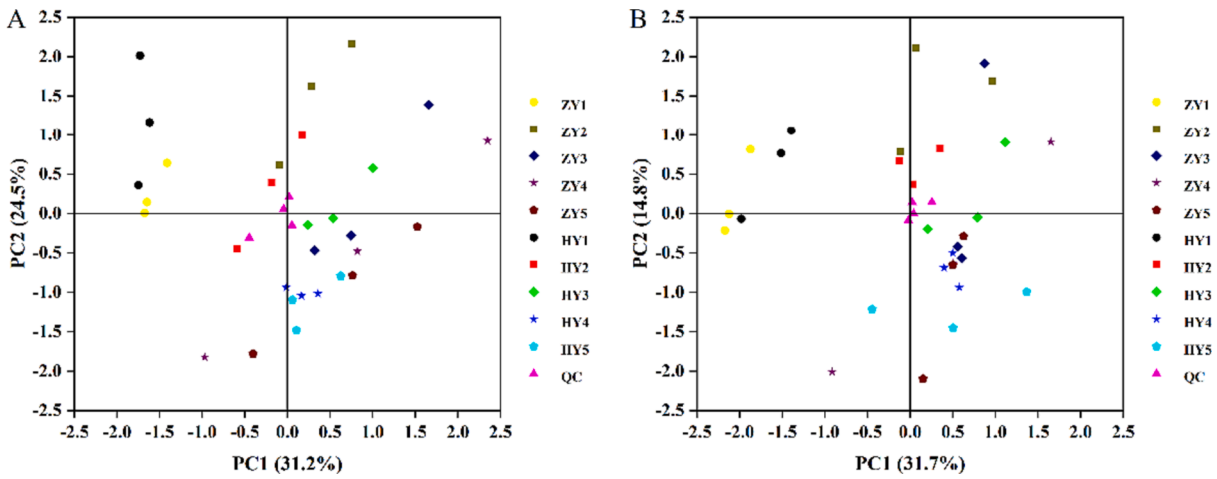


Fig. 9. PCA scoring of fatty acid metabolites during YJS fermentation in positive ion (A) and negative ion (B) mode.

between the inoculated and control groups, the differential fatty acid metabolites between the groups were screened based on the VIP values presented in the OPLS-DA model and the P-values in the *t*-test (VIP > 1, $p < 0.05$). After screening, 40 differentially metabolised fatty acids were obtained. The resulting differentials were visualised and plotted as volcano plots, where red dots, grey dots and green dots indicate significant up-regulation, no significant difference, and significant down-regulation, respectively. As can be seen from graph, 1, 5, 3, 1 and 0 peaks were significantly up-regulated, and 0, 6, 1, 1 and 0 peaks were significantly down-regulated on days 0, 4, 8, 12 and 16 of fermentation, respectively (Fig. 10). On day 4 of fermentation, both the up-regulated and down-regulated substances increased, indicating that on day 4, the microorganisms could use the carbohydrates and other nutrients in YJS for rapid fermentation, enabling metabolites to accumulate (Zhao et al., 2020). During the later stages of fermentation, metabolite up- and down-regulated substances are reduced, implying that fatty acid metabolites may be converted to flavour substances including aldehydes, ketones and esters at this time (Zang et al., 2020). As a result, inoculated

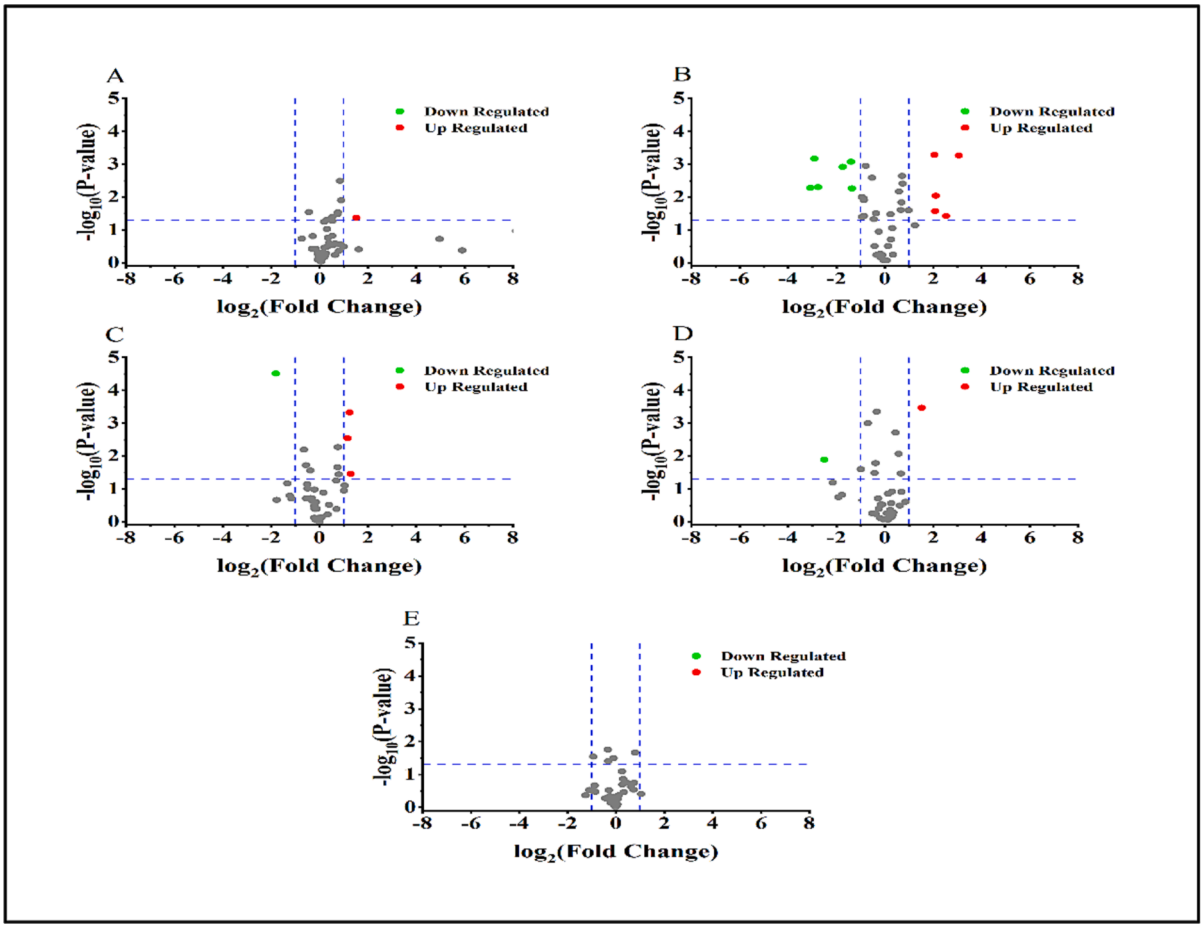


Fig. 10. Volcano diagram for different stages of fermentation of YJS (Fig. 10A shows 0 days, Fig. 10B shows 4 days, Fig. 10C shows 8 days, Fig. 10D shows 12 days and Fig. 10E shows 16 days of fermentation).

fermentation causes the accumulation of fatty acid metabolites in YJS.

3.8. Analysis of differential fatty acid metabolic pathways

In this study, the identified differential fatty acid metabolites were enriched in the KEGG database for pathway analysis using the Metabolomics Pathway Analysis online software. Three significantly different metabolic pathways, namely arachidonic acid metabolism, unsaturated fatty acid biosynthesis and linoleic acid metabolism were identified. Loach and pepper were used as the main raw materials to make YJS, where the fat and sugar substances (mainly glucose) are the main substrates for the metabolic production of fatty acids. Thus, fat and glucose are taken as the starting point for metabolic pathways. As displayed in Fig. 11, there are pyruvate produced from glucose by the glycolytic pathway and acyl-CoA produced from fat by β-oxidation produce acetyl-CoA that is a direct raw material for the synthesis of fatty acids. Additionally, acetyl-CoA generates stearic acid via fatty acid metabolism, while that produces hexadecanedioic acid via fatty acid elongation and unsaturated fatty acid synthesis in the cutin, suberine and wax biosynthesis pathway. The TCA cycle is a key intermediate in the synthesis of differential fatty acids. Fat degradation into the TCA cycle results in the metabolism of Traumatic acid and (2′E,4′Z,7′Z,8E)-Colnelenic acid via α-linolenic acid. In the meanwhile, fat degradation into the TCA cycle can be metabolized by linoleic acid to produce 9,10-Epoxyoctadecenoic acid and Arachidonic acid. Thus, arachidonic acid can be synthesized via the arachidonic acid pathway to 5-KETE, Lipoxin A4 and 8,9-DiHETrE. The presence of arachidonic acid enhances the blood pressure-lowering and lipid-lowering properties of YJS. Based on a heat map of the differential fatty acid metabolites of YJS, inoculation fermentation increased the expression of Traumatic acid and (2′E,4′Z,7′Z,8E)-Colnelenic acid, which could thus enhance the anti-inflammatory, anti-thrombotic and anti-cancer effects in YJS.

3.9. Analysis of the association between microorganisms and fatty acid metabolites

The formation of fatty acid metabolites during the fermentation of YJS is closely associated with the growth and reproduction of microorganisms. To explore the correlation between fatty acid metabolites and microorganisms, the results of pyrosequencing species annotation were associated with the fatty acid metabolites of YJS (Fig. 12). *uncultured_bacterium_o_Chloroplast* (B1) was positively correlated with six differential fatty acid metabolites, including 5-HEPE, Azelaic acid and L-Hexanoylcarnitine to a great extent. It is indicated that the presence of *uncultured_bacterium_o_Chloroplast* (B1) promotes the synthesis of these metabolites. Thus, in the pre-fermentation period, when *uncultured_bacterium_o_Chloroplast* (B1) was the dominant bacterium in both groups, the expression of these substances was higher in the inoculated fermentation group than that in the natural fermentation group. During the late stage of YJS fermentation, *Lactiplantibacillus* (B2) was the dominant bacterium in both groups of YJS. However, the relative abundance of *Lactiplantibacillus* (B2) was higher in the inoculated fermentation group than that in the natural fermentation group, and thus over 10 differential fatty acid metabolites that were significantly positively correlated with *Lactiplantibacillus* (B2), including 15- KETE, Lipoxin A4, L-Acetylcarnitine, 3-Dehydroxycarnitine, and Ricinoleic acid were all expressed in higher amounts in the inoculated fermentation group than that in the natural fermentation group. Although the relative abundance of *Pantoea* (B7), *Leuconostoc* (B9) and *uncultured_bacterium_c_Subgroup_6* (B10) was lower, they were also significantly and positively correlated with over 10 differentially metabolized fatty acids, indicating that these three bacteria are directly or indirectly involved in fatty acid metabolism and may be the main functional bacteria in fatty acid metabolism. *Lactiplantibacillus* (B2) is widely used as a probiotic in fermented foods and has the property of improving the quality of fermented foods (Liao, Shen, Manickam et al., 2023; Zhang, Huang, Ma, Tang, Li, & Zhang, 2023). Studies have indicated that *Pantoea* (B7) facilitates the formation of aromatic substances in fermented products (Zhao et al., 2020). *Fructobacillus_tropaeoli* (B4), as the dominant bacterium in the late stage of fermentation of naturally fermented YJS, had significantly positive relationships with eight differential fatty acids, including 15-KETE, L-Acetylcarnitine, and 11(R)-HETE, while the expression of all these substances during fermentation was lower in the natural fermentation group than that in the inoculated fermentation group. It is possible that the main source of these substances was less influenced by *Fructobacillus_tropaeoli* (B4).

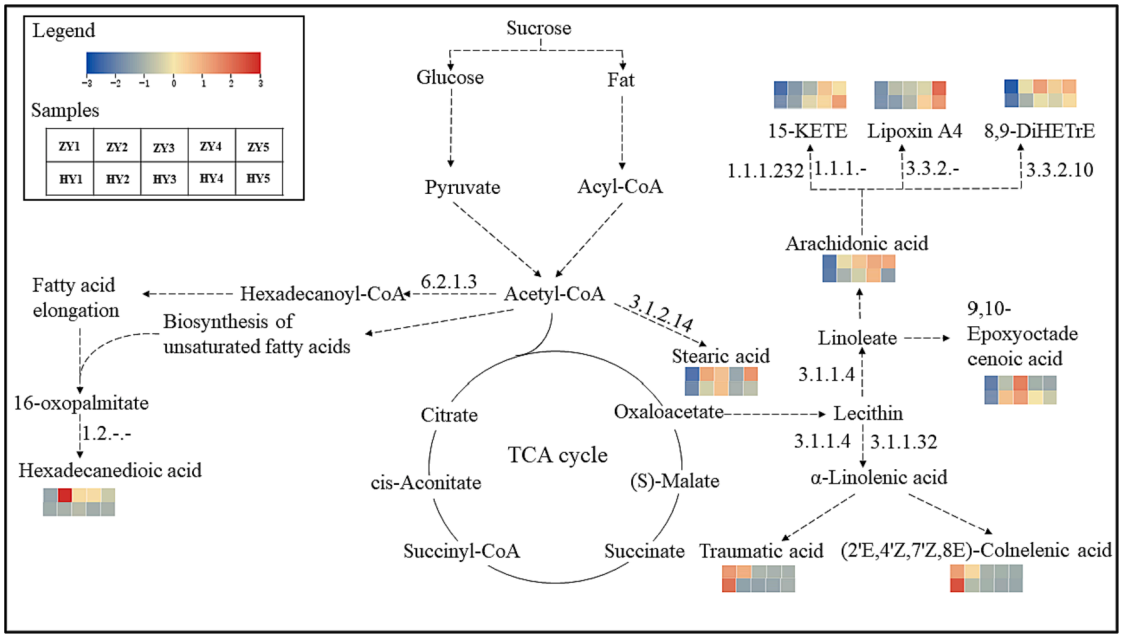
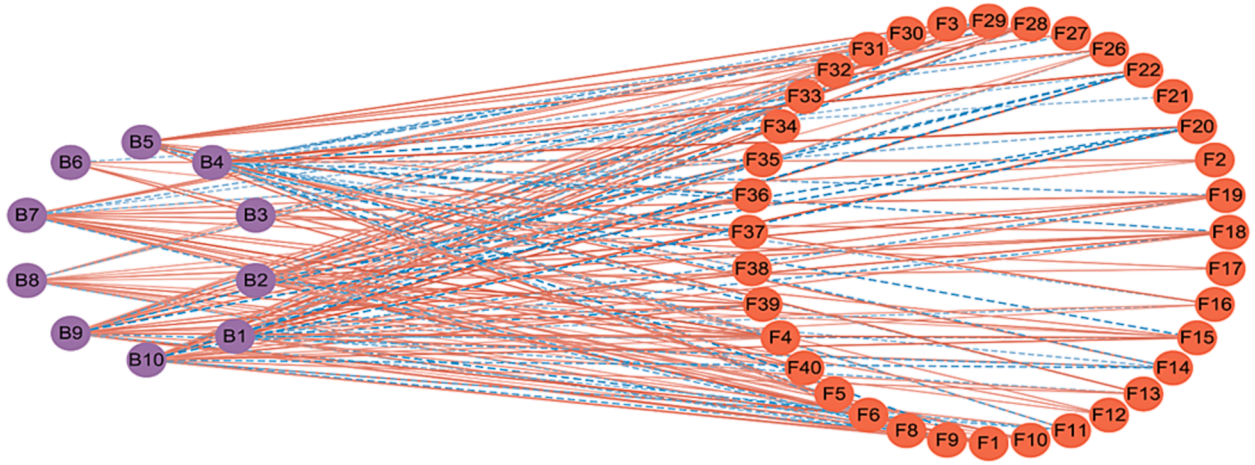


Fig. 11. Predicted major metabolic pathways of differential fatty acid metabolites during fermentation of YJS (markers in the figure indicate the expression levels of differential fatty acid metabolites during fermentation of YJS).



B1	uncultured_bacterium_o_Chloroplast	F8	L-Hexanoylcarnitine	F25	Stearic acid
B2	Lactiplantibacillus	F9	L-Acetylcarnitine	F26	Ricinoleic acid
B3	Weissella	F10	(9S,10S)-10-hydroxy-9-(phosphonooxy)octadecanoate	F27	9,10-Epoxyoctadecenoic acid
B4	Fructobacillus_tropaeoli	F11	5-HEPE	F28	2-Ethyl-2-Hydroxybutyric acid
B5	uncultured_bacterium_f_Mitochondria	F12	Troxilin B3	F29	Leucinic acid
B6	Pectobacterium	F13	11(R)-HETE	F30	Hexadecanedioic acid
B7	Pantoea	F14	(2'E,4'Z,7'Z,8E)-Colnelenic acid	F31	2-Hydroxystearic acid
B8	uncultured_bacterium_f_Enterobacteriaceae	F15	15-Deoxy-d-12,14-PGJ2	F32	Ethyl tetradecanoate
B9	Leuconostoc	F16	3-Dehydroxycarnitine	F33	But-2-enoic acid
B10	uncultured_bacterium_c_Subgroup_6	F17	16-Hydroxy-10-oxohexadecanoic acid	F34	5-KETE
F1	(2E,11Z)-5-[5-(Methylthio)-4-penten-2-ynyl]-2-furanacrolein	F18	Arachidonic acid	F35	Prostaglandin B1
F2	2,3-dinor-6-oxoprostaglandin F1alpha	F19	8,9-DiHETrE	F36	15S-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid
F3	15-KETE	F20	(E)-2,6-Dimethyl-2,5-heptadienoic acid	F37	Traumatic acid
F4	6,15-Diketo,13,14-dihydro-PGF1a	F21	12R-hydroxy-5Z,8Z,12Z-eicosatrienoic acid	F38	Azelaic acid
F5	Prostaglandin A1	F22	13,14-Dihydro PGE1	F39	2-Hydroxy-3-methylbutyric acid
F6	Lipoxin A4	F23	Eicosadienoic acid	F40	(R)-3-Hydroxy-tetradecanoic acid
F7	13,14-Dihydro-15-keto PGF2a	F24	Heptadecanoic acid		

Fig. 12. Network diagram of correlations between dominant species of YJS and differential fatty acid metabolites (red solid lines indicate positive correlations, blue dashed lines indicate negative correlations). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Weissella (B3) was the third dominant bacterium in the inoculated fermentation group. The inoculation effect of *Weissella* (B3) is shown in its ability to rapidly lower the pH in the YJS system in the pre-fermentation phase, providing an acidic environment for other bacteria and facilitating the correlation of other bacteria in the YJS with the differential metabolites (Jiang, Liu, et al., 2022). Meanwhile, *uncultured_bacterium_f_Mitochondria* (B5) was significantly negatively correlated with the synthesis of 17 differential fatty acid metabolites and significantly positively associated with the synthesis of five differential fatty acids. Different from that, *uncultured_bacterium_f_Enterobacteriaceae* (B8) was significantly negatively related to the synthesis of two differential fatty acids and significantly positively correlated with the synthesis of nine differential fatty acids. Therefore, the formation of fatty differential metabolites in YJS is closely associated with seven bacterial species, namely *uncultured_bacterium_o_Chloroplast* (B1), *Lactiplantibacillus* (B2), *uncultured_bacterium_f_Mitochondria* (B5), *Pantoea* (B7), *uncultured_bacterium_f_Enterobacteriaceae* (B8), *Leuconostoc* (B9), and *uncultured_bacterium_Subgroup_6* (B10).

4. Conclusion

To conclude, this study demonstrated that during the fermentation of YJS, the dominant classes are *Bacilli* and *Oxyphotobacteria*. After 12 days of fermentation, *Lactiplantibacillus* (40.51 %) and *Weissella* (20.43 %) were the dominant species in the HY group. Inoculated fermentation lowers the growth of *Fructobacillus_tropaeoli*. During the fermentation process, there are interactions between bacteria, while the fermentation of the inoculated fermentation shortens the fermentation cycle of YJS. Inoculation fermentation increased the degradation of fat in YJS and delayed the process of fat oxidation in YJS. The accumulation of fatty acid metabolites was increased in the inoculated fermentation group, when the involved main metabolic pathways included arachidonic acid metabolism, unsaturated fatty acid biosynthesis and linoleic acid metabolism. Correlation analysis demonstrated that seven bacterial species in YJS were strongly correlated with 40 differential fatty acid metabolites. Moreover, the findings of the present study provide more knowledge of the main bacterial species and fatty acid metabolites of YJS. However, the theoretical mechanisms concerning the coordination role of autochthonous starter cultures and fatty acid metabolites on the flavour in YJS need to be further investigated.

CRediT authorship contribution statement

Hongyan Chen: Writing – original draft, Visualization, Validation. **Lu Liu:** Supervision. **Lu Jiang:** Supervision. **Wenkang Hu:** Software. **Qin Cen:** Methodology. **Rui Zhang:** Investigation. **Fuyi Hui:** Formal analysis. **Jiamin Li:** Data curation. **Xuefeng Zeng:** Resources, Project administration, Funding acquisition.

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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