DOI: 10.26599/FSHW.2025.9250507

Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Effect of inoculation with *Weissella paramesenteroides* and *Lactiplantibacillus plantarum* on soy sauce fermentation

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ABSTRACT: This study aimed to investigate the effect of inoculation with *Weissella paramesenteroides* and *Lactiplantibacillus plantarum* on the quality of soy sauce. Three inoculation strategies were evaluated, the analysis results of flavor components and biogenic amine (BA) levels suggested that the optimal strategy for soy sauce fermentation was to inoculate *W. paramesenteroides* on day 1 (1.0×10^6 CFU/g), and *L. plantarum* on day 7 (1.0×10^6 CFU/g). This enhanced its organic acids (1.47-fold), volatiles (1.29-fold), and free amino acids (1.21-fold) flavors. It demonstrated a superior 2-Methoxy-4-vinylphenol production capacity and characteristic flavor components such as lactic acid, 1-octen-3-ol, benzeneacetaldehyde, and glutamic acid were increased by 1.38-, 1.45-, 4.10-, and 1.37-fold, respectively. Besides, the content of BA was reduced by 42.26%, and most of them were positively correlated with their precursors, suggesting that the addition of *W. paramesenteroides* and *L. plantarum* inhibited the growth of microorganisms involved in BA synthesis or degraded it. This work provides valuable information for the exploration and industrial application of functional microorganisms in soy sauce fermentation.

Keywords: Soy sauce; Inoculation; Functional microorganism; Flavor compounds; Biogenic amines;

1. Introduction

Soy sauce is a traditional condiment with a long history, which is very popular due to its unique flavor and occupies a major condiment market in East Asian countries[1]. Soy sauce fermentation is an open mixed microbial fermentation process, that uses soybeans and wheat as the main raw materials, resulting in the production of rich metabolites through the action of a variety of microorganisms and their enzyme systems[2]. So far, most of the production of soy sauce adopts the spontaneous fermentation method, bringing out the problems of a long fermentation period, unstable product quality, and low production efficiency[3]. With the improvement of living standards, people's demand for soy sauce quality and characteristics is gradually increasing. Recently, microbial inoculated fermentation to improve soy sauce flavor has attracted great attention.

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Received 10 September 2024
Received in revised from 23 October 2024
Accepted 2 January 2025

There are complex microbial communities involved in the fermentation of soy sauce, whose structural composition is critical for the formation of key flavor metabolites including volatile compounds, organic acids, amino acids, and sugars, which contribute to the final characteristic of soy sauce[4]. However, the metabolic activities of microbes during fermentation may develop potentially harmful substances like biogenic amines (BAs). BAs are primarily produced by microbial decarboxylation of the appropriate amino acids[5]. Excessive BAs not only significantly affect the flavor of food or even alter its composition, but also have serious toxic effects on the human body, causing damage to the nervous and cardiovascular system[6]. Currently, high levels of BAs have been detected in various types of soy sauce[7-8], which is of great concern. How to reduce the accumulation of BAs during soy sauce fermentation has become a hot research topic.

Several studies have been conducted using pure cultures of various microbial strains or mixed cultures of different strains to enhance soy sauce fermentation to improve the flavor and safety of soy sauce. For example, Wu et al. [9] reported that the co-culture of *Zygosaccharomyces rouxii and Lactobacillus fermentum* could increase the intensity of sauce-like, fruity, smoky, and caramel-like notes, as well as umami and kokumi taste in soy sauce. Zhang et al.[10] found that co-culturing *Zygosaccharomyces rouxii* QH-25 and *Candida versatilis* increased the volatile content of soy sauce by 1.61 times compared to the control. Guo et al.[11] revealed that the content of BAs was significantly decreased by co-culturing *Staphylococcus piscifermentans* with *Zygosaccharomyce srouxii* CICC1417 and *Torulopsis candida* CICC1019 during soy sauce fermentation. However, there are no studies that have comprehensively investigated the effects of inoculation with potentially functional microorganisms on soy sauce flavor and safety during the fermentation process.

In our previous study, two potential flavor-producing functional bacterial strains, *Weissella paramesenteroides*, and *Lactiplantibacillus plantarum*, were screened by metagenomic analysis and pure culture in a simulated soy sauce fermentation environment[12]. Additionally, the genera *Weissella* and *Lactobacillus* were found to be negatively correlated with the microorganisms responsible for BA production during soy sauce fermentation[13]. The purpose of this study is to verify and analyze the effects of *W. paramesenteroides* and *L. plantarum* on the quality of soy sauce during fermentation. Three different inoculated soy sauce fermentations by *W. paramesenteroides* and *L. plantarum* were investigated from the analysis of physicochemical properties, characteristic flavor compounds, and BAs. This study is of great value for expanding the soy sauce microbial database and promoting the industrial production of high-quality soy sauce in the future.

2. Materials and methods

2.1 Strains

Weissella paramesenteroides and *Lactiplantibacillus plantarum* used in this work were kept in our laboratory and isolated from the moromi of the soy sauce fermentation process and identified by matching to the BLAST (Basic Local Alignment Search Tool) base of the ITS sequencing results. In our previous study, *W. paramesenteroides* and *L. plantarum* showed good fermentation performance and metabolic capacity in pure cultures at 18% NaCl. They showed good salt tolerance and characteristic flavor-producing capabilities[12]. The two strains were individually inoculated and activated twice in MRS liquid medium (Solarbio Science & Technology Co., Ltd., Beijing, China) at 37 °C for 24-48 h. After centrifugation at 5600×g and 4 °C for 10 min, the bacterial cell pellets were collected. They were then twice washed in sterile water and resuspended at a concentration of 10^6 CFU/g.

2.2 Experimental procedure and sampling

The initial soy sauce moromi was a mixture of koji and brine (18%, w/v NaCl) in a ratio of 1:2.5, bottled into a 5-liter fermentation vat. The koji was produced according to our previous study[14]. Soaked soybeans were steamed in a pressure steam sterilizer (MVS-63, Alphavita Bio-scientific Co., Ltd. Dalian, China) at 121°C for 15 min and then mixed with roasted wheat at a ratio of 3:1 (w/w). After cooling, the material was inoculated with *A. oryzae* 3.042 seed koji at a ratio of 0.05% (w/w) and cultured for 36 hours at 30 °C. A suspension of *W. paramesenteroides* and *L. plantarum* was inoculated into the moromi at an initial concentration of 1×10^6 CFU/g, respectively. The preparation of soy sauce moromi was divided into the following four groups: (A) control group: uninoculated with *W. paramesenteroides* and *L. plantarum*; (B): inoculated with *W. paramesenteroides* on the day 1; (C): inoculated with *W. paramesenteroides* on the day 1 and *L. plantarum* on the day 7; (D): inoculated with *L. plantarum* on the day 7. The time to add *W.paramesenteroids* and *L.plantarum* depends on their appearance during the natural fermentation process of soy sauce[15]. All experiments were performed in triplicate. These samples were fermented at ambient temperature for 90 days, then sampled (collected on days 0, 3, 7, 15, 30, 45, 60, and 90), and stored at −80 °C (not exceeding three weeks), followed by physicochemical, flavor, and BA determinations.

2.3 Analysis of physicochemical properties

The pH of samples was detected by a pH meter (PHS-25; Shanghai Precision Scientific Instruments Company, Shanghai, China). The contents of total acid (TA), amino acid nitrogen (AN), and reducing sugar (RS) were determined according to our previous report[15].

2.4 Analysis of flavor metabolites

Based on a previous study conducted by Xiao et al. [16], the organic acids and amino acids in the samples were analyzed. The content of organic acids in soy sauce was determined by high-performance liquid chromatography (HPLC) (Model 1200, Agilent, USA). The temperature of the column (Aminex HPX87H, 300 mm × 7.8 mm, Bio-Rad) was kept at 35 °C, the mobile phase was 6 mmol/L H_2SO_4, the flow rate was maintained at 0.35 mL/min, and the detected wavelength of the UV detector was 210 nm. The amino acids of soy sauce were detected by an amino acid analyzer (AAA) (Sykam S-433D, Germany). The volatile metabolites of soy sauce were detected by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography-time-of-flight mass spectrometry (GC/Q-TOF MS) (Model 8890/7250, Agilent Technologies, USA). An 8 mL soy sauce sample was transferred to headspace vials (Supelco Company, USA), and 2-methyl-3-heptanone was added as an internal standard, and incubated at 50 °C with shaking for 30 min. Thereafter, the solid-phase microextraction (SPME) head was inserted into the headspace vials, and exposed to volatiles for 45 min before being placed into the injection port of a gas chromatograph (GC)[14]. The compounds were identified by matching them against the National Institute of Standards and Technology (NIST) 17.0 mass spectral database. The semi-quantification of each compound was achieved by comparing its peak area with that of the internal standard.

2.5 Biogenic amine analysis

Referring to our previous study[15], the amount of BA in the samples was evaluated by the HPLC system using a C18 column (5 μm, 4.6 mm × 250 mm; Nanologica, Sodertalje, Sweden) based on the Chinese national standard (GB 5009.208-2016).

2.6 Statistical analysis

All data were expressed as mean ± standard deviation (SD). SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) was employed to analyze the data, and GraphPad Prism 9.0 (Inc., La Jolla, CA, USA) was used to display the results. Principal component analysis (PCA) of flavor compounds was performed by SIMCA software (version 14.1, Umetrics AB, Umeå, Sweden). Spearman correlation analysis was performed by the data analysis platform genecloud tools (https://www.genescloud.cn/). The metabolite heatmap analysis was carried out by TBtools (Toolbox for Biologists; version 1.082, China).

3. Results and discussion

3.1 Effect of inoculation on physicochemical properties

The effects of inoculation on the physicochemical indexes of soy sauce during fermentation are displayed in Fig. 1 (a-d). As shown in Fig. 1a, the pH of moromi in the four groups gradually decreased with the fermentation proceeding. This may be related to bacterial fermentation products such as lactic acid and malic acid. In addition, the decrease in pH value may be due to the accumulation of free fatty acids, amino acids, and peptides produced by the hydrolysis of raw materials[17]. Among them, the pH value of the control group was higher than that of the inoculation groups, and the pH in group B was lowest, with only *W. paramesenteroides* added separately. Furthermore, the variations in total acid concentration are shown in Fig. 1b. This is consistent with the pH measurement results, with the final TA value reaching (2.04 ± 0.05) g/100 mL in group B. Indeed, in our previous study, we also found that culturing the bacteria under pure culture 18% NaCl conditions significantly increased lactic acid and oxalic acid levels (P < 0.05), with *W. paramesenteroides* producing the most organic acids[12]. *Weissella* is a group of lactic acid bacteria that are widely present in fermented foods, closely related to the food fermentation process and the formation of flavor substances in food. Among the *Weissella* strains, *W. paramesenteroides* is one of the most frequently isolated species from fermented foods. They not only contribute significantly to flavor production but also have potential probiotic properties. Fonseca et al.[18] observed that it has a positive effect in reducing the parasitic load of animals infected with Giardia.

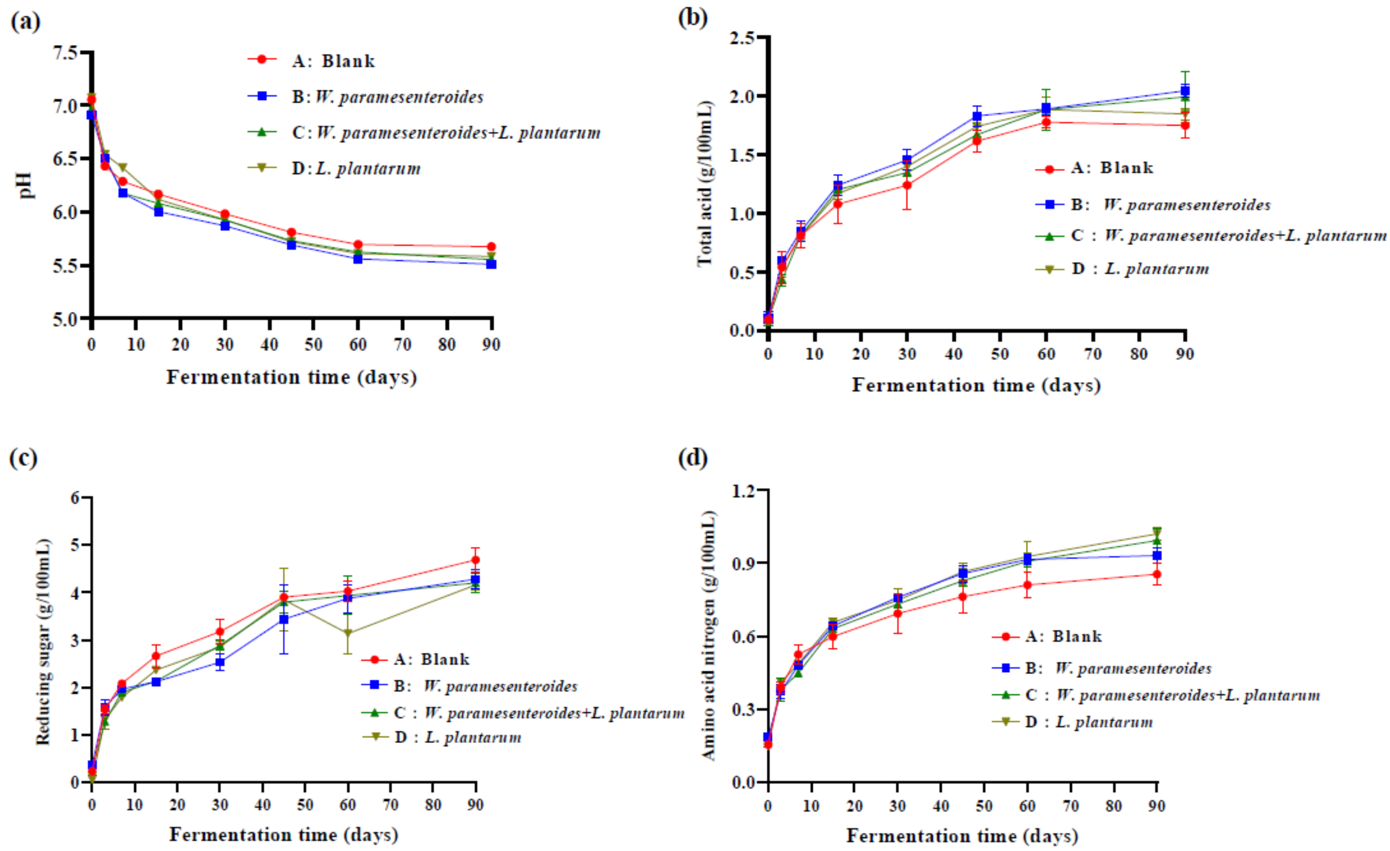


Fig.1. Effects of strains addition strategy on the soy sauce physicochemical indexes: (a) pH value; (b) Total acidity; (c) Reducing sugar content; (d) Amino nitrogen content.

The reducing sugar (RS) contents of the samples from the four groups at different fermentation times are shown in Fig. 1c. As the fermentation progresses, the RS content rises significantly, probably due to the high activity of amylase and glycosylase secreted by microorganisms, which hydrolyze the raw materials into glucose, maltose, and dextrin[19]. RS is the material basis of microbial metabolism in soy sauce fermentation. The addition of microorganisms accelerated the consumption of sugars, resulting in a greater reducing sugar in the control group compared to that in the inoculated group, which was consistent with previous reports[20]. At the end of fermentation, the highest reducing sugar content in the control group was (4.68 ± 0.24) g/100 mL, while the lowest reducing sugar content in group D was (4.15 ± 0.12) g/100 mL.

Amino acid nitrogen (AN) is a key indicator that reflects the quality of soy sauce and can determine the efficiency of protein utilization in the raw material[21]. As shown in Figure 1d, the AN content of the samples from the four groups increased continually during the fermentation process. After fifteen days of fermentation, the AN content of the blank group was significantly lower than that of the inoculated group ($P < 0.05$). This is consistent with our previous study, which found that *W. paramesenteroides* and *L. plantarum* have good amino acid production capacity[12]. At the end of fermentation, Group D had the highest AN content of (1.02 ± 0.03) g/100 mL. In contrast, several studies have found that there is no significant difference in AN concentration between the control group and the group inoculated with *Tetragenococcus halophilus* and *Zygosaccharomyces rouxii* or *Candida. versatilis* strains[22,17]. The same phenomenon was also observed in the study of fermented soy sauce inoculated with *Zygosaccharomyces rouxii*, *Kocuria kristinae*, and

Lactobacillus fermentum[9]. *W. paramesenteroides* and *L. plantarum* showed the potential to improve the soy sauce quality in inoculation fermentation applications.

3.2 Effect of inoculation on organic acids

Organic acids are important components in soy sauce, not only providing sourness but also serving as precursors for other ester aroma substances, greatly enriching the soy sauce flavor[23]. In addition, they play an important role in inhibiting the growth of spoilage or stray bacteria in soy sauce[24]. The concentrations of the seven main organic acids measured in this work are displayed in Fig. 2. Among them, lactic acid, oxalic acid, and citric acid were found to dominate throughout the fermentation process. Lactic acid is the main organic acid in traditional fermented soy sauce[25], which can promote saltiness and make the soy sauce taste smooth and long-lasting. After 90 days of fermentation, the content of lactic acid in groups A, B, C, and D was (7.63± 1.25), (8.30 ± 0.07), (10.59 ± 1.15), and (8.89 ± 0.56) g/L, respectively (Fig. 2a). These results demonstrated that the addition of lactic acid bacteria may increase the concentration of organic acids in soy sauce, which was in line with previous research[17]. Besides, citric acid is mainly derived from the tricarboxylic acid cycle, and trends to increase and decrease during fermentation (Fig. 2b), possibly due to its involvement in other reactions such as ester synthesis in the later stages of fermentation[4]. Similarly, its content was highest in group C and lowest in the control group on the 90th day of fermentation, as well as oxalic acid (Fig. 2c).

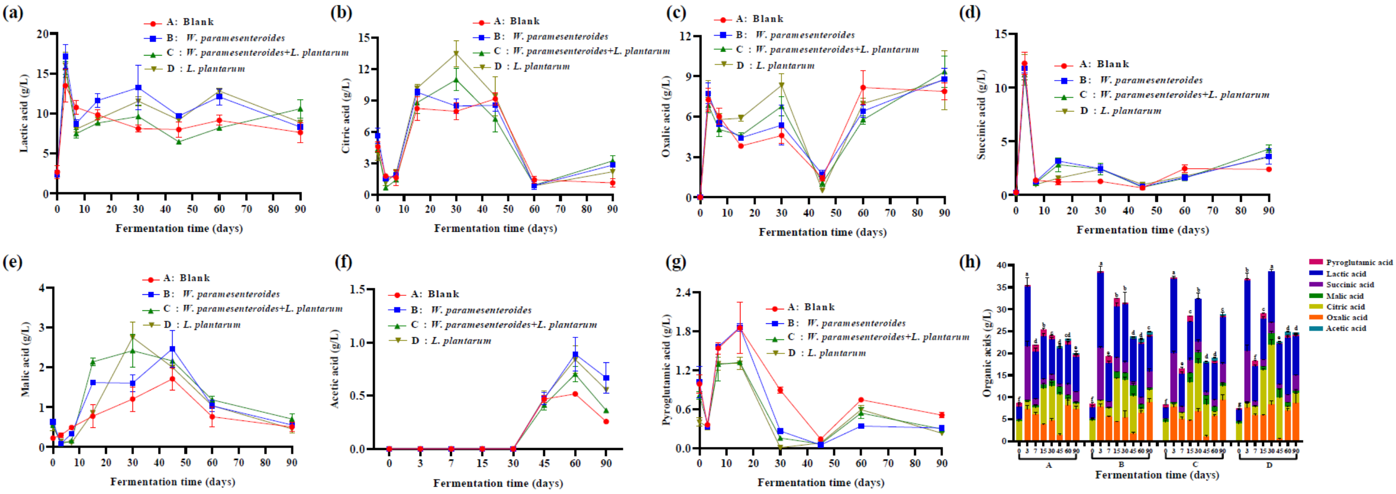
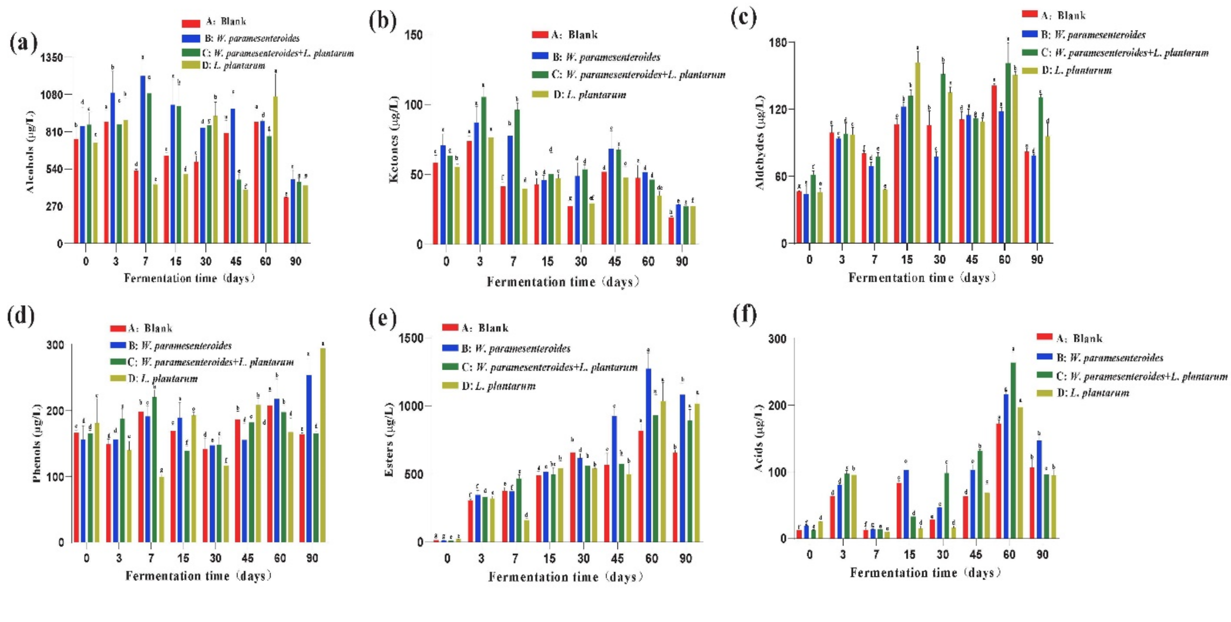


Fig. 2. Effects of strains addition strategy on organic acids in soy sauce: (a) Oxalic acid; (b) Citric acid; (c) Succinic acid; (d) Malic acid; (e) Lactic acid; (f) Acetic acid; (g) Pyroglutamic acid; (h) Total organic acids. Values at different time points for the same group marked by different letters are significantly different (P < 0.05).

The contents of the other four organic acids, succinic acid (Fig. 2d), malic acid (Fig. 2e), acetic acid (Fig. 2f), and pyroglutamic acid (Fig. 2g) fluctuated during the fermentation process. Except for pyroglutamic acid, the content of the other three organic acids in the inoculated group was gained at significantly higher levels (P < 0.05) than that in the control group. Pyroglutamic acid is mainly generated through non-enzymatic pyrolysis of glutamine[26]. It is worth noting that glutamic acid is a key amino acid for the umami flavor of soy sauce, primarily formed by hydrolysis of glutamine by glutaminase[27]. Typically, higher levels of pyroglutamic acid could indicate lower glutamic acid production. After 90 days of fermentation, the content of pyroglutamic acid in the control group was notably higher compared to the other three inoculation groups (P < 0.05). It suggested that the addition of *W. paramesenteroides* and *L. plantarum* may be beneficial to the production of glutamic acid during soy sauce fermentation. Notably, acetic acid, the precursor of ethyl acetate, which was identified as the characteristic flavor compound of soy sauce in previous research[15], was found to be detected only after 30 days of fermentation. In all soy sauce products, group B inoculated with *W. paramesenteroides* had the highest level of acetic acid, which was 2.64 times higher than that of the control group (P < 0.05). Liu et al.[17] observed that co-inoculation of soy sauce with *T. halophilus* and *Z. rouxii* resulted in a 2.29-fold increase in acetic acid production. As a volatile acid, acetic acid has a remarkable impact sourness of soy sauce and plays a role in the overall flavor harmonizing of soy sauce[28]. In general, at the end of fermentation, group C inoculated with *W. paramesenteroides* and *L. plantarum* depicted the highest total organic acid content (Fig. 2h), which was 1.47 times higher than that of the control group (P < 0.05). These results suggested that simultaneous inoculation of *W. paramesenteroides* and *L. plantarum* during soy sauce fermentation can synergistically promote the production of organic acids in soy sauce.

3.3 Effect of inoculation on volatile flavor compounds

Soy sauce's volatile flavor components have received widespread attention due to their influence on sensory quality and consumer acceptance. During the fermentation process of soy sauce, a total of 76 volatile components were identified, which can be categorized into seven groups, including esters (24), alcohols (9), aldehydes (7), phenols (9), acids (7), ketones (8) and others (12). The amounts of these compounds are detailed in Supplementary Table S1. The contents of alcohols, ketones, aldehydes, and phenols in the four groups of samples dynamically changed during the fermentation of soy sauce, with esters and acids initially tending to increase and then slightly decrease at the end of fermentation (Fig. 3a-f). The content of volatile compounds in the inoculation group was higher than that in the control group during the fermentation process. At the end of fermentation, group B had the highest levels of alcohols, ketones, esters, and acids, while groups C and D had the highest content of aldehydes and phenols, respectively. The total volatile compound content of groups B, C, and D was 1.50, 1.29, and 1.43 times substantially higher than that of the control group (P < 0.05), respectively (Table S1).



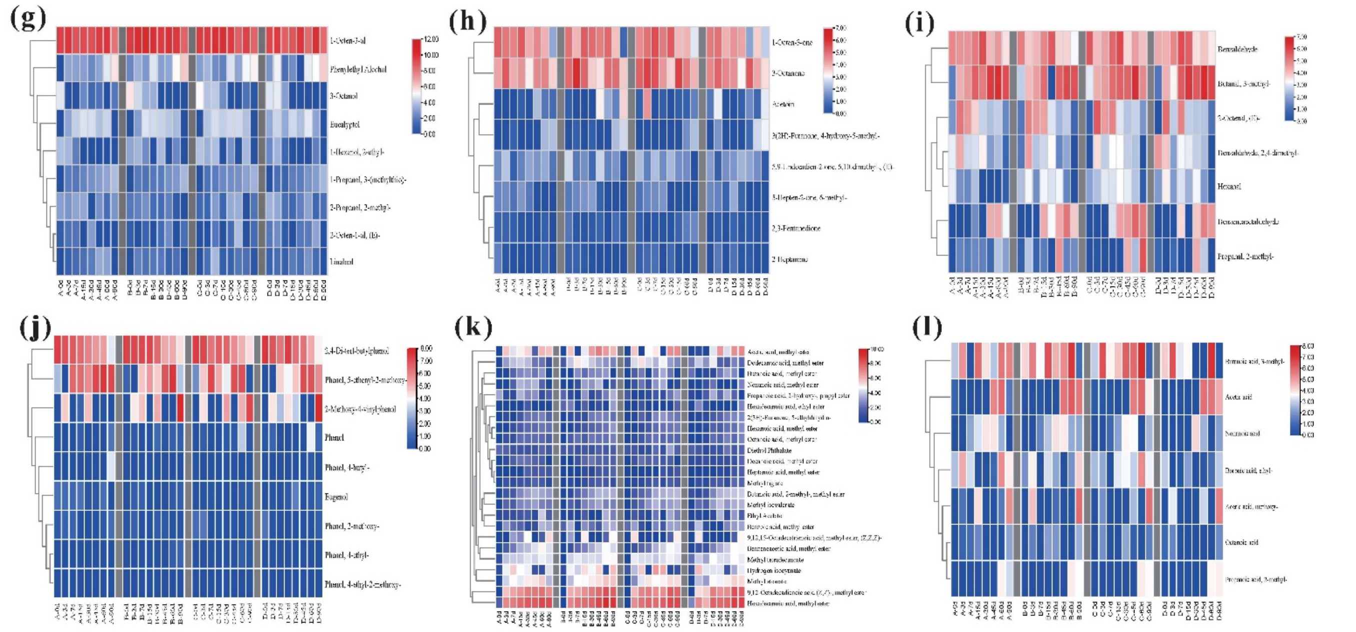


Fig. 3. Effect of strain addition strategy on volatile compounds in soy sauce and heat map analysis: (a), (g) Alcohols; (b), (h) Ketones; (c), (i) Aldehydes; (d), (j) Phenols; (e), (k) Esters; (f), (l) Acids. Values with the same color marked by different letters are significantly different (P < 0.05).

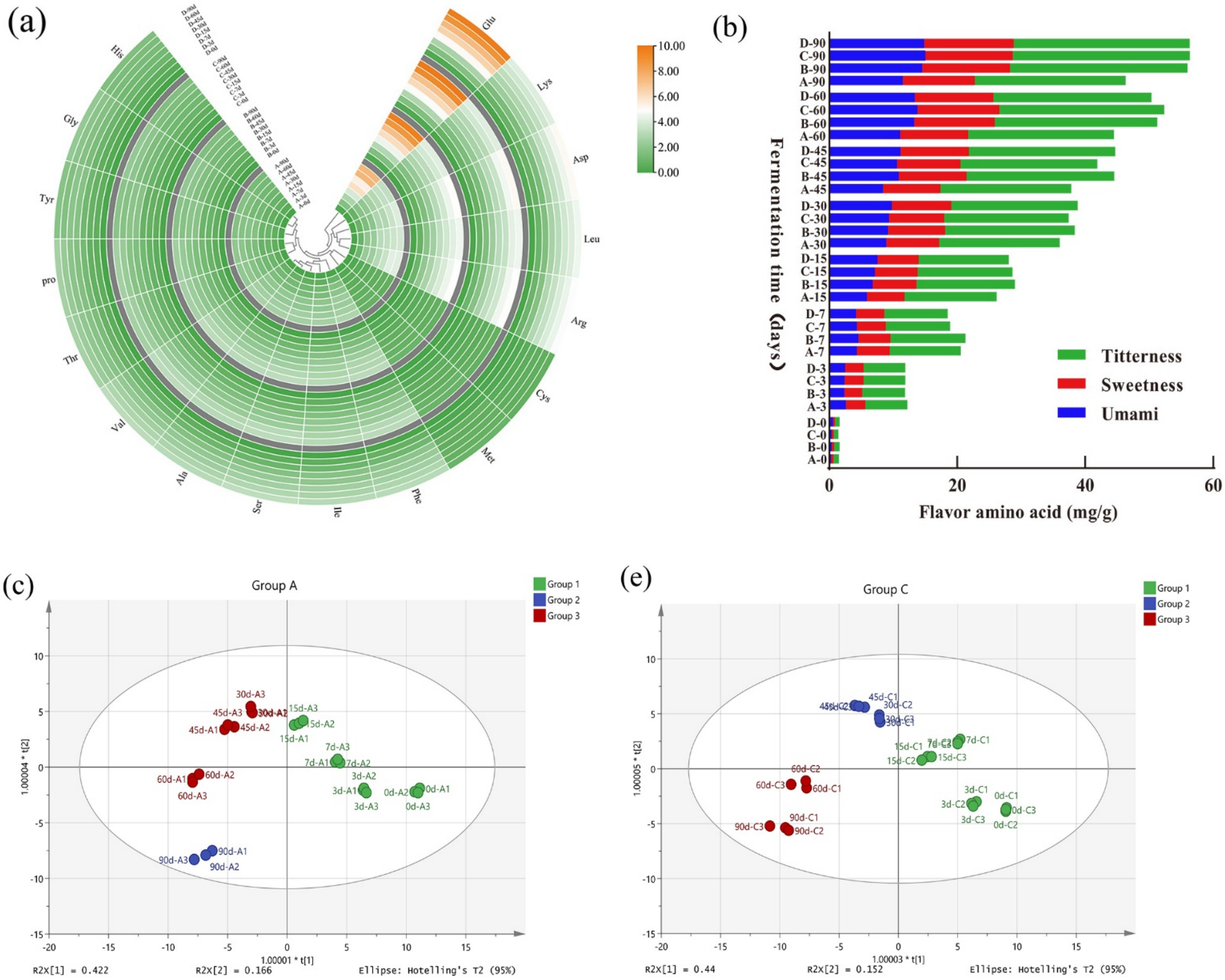
In order to better understand the impact of different inoculation strategies on soy sauce flavor, Fig. 3.g-1 shows cluster analysis of volatile compounds produced during each fermentation process. Among the alcohols, 1-octen-3-ol and phenylethyl alcohol were found to predominate during soy sauce fermentation (Fig. 3g). Generally, 1-octen-3-ol is an unsaturated alcohol that is a product of linoleic acid oxidation. Linoleic acid is a common edible flavoring with a strong mushroom flavor, which has also been reported in other fermented soy sauce flavor analyses[29-30]. In our previous study, it was identified as a characteristic volatile compound of Daqu and soy sauce by odor activity value (OAV) analysis [14]. At the end of fermentation, the concentrations of 1-octen-3-ol in groups A, B, C, and D were (269.28 ± 10.74), (385.81 ± 58.74), (392.89 ± 25.15) and (346.65 ± 46.78) μg/L, respectively. The highest content of 1-octen-3-ol was found in group C, which was 1.45 times higher than that of the control group (P < 0.05). This is consistent with our previous findings that it was found to be positively correlated with *W. paramesenteroides* and *L. plantarum*, suggesting that *W. paramesenteroides* and *L. plantarum* could synergistically facilitate its production[12]. Similarly, 3-octanone, another characteristic volatile compound of Daqu[14], was found to dominate among the ketone compounds during soy sauce fermentation (Fig. 3h). Consistently, at the end of fermentation, the content of 3-octanone in group C was 1.32 times richer than that in the control group (P < 0.05).

Additionally, aldehydes and esters are one of the key aromatic compounds in soy sauce because their high volatility and sensitivity to human olfactory receptors[31]. Among the aldehydes, benzaldehyde (almond aroma), butanal, 3-methyl- (chocolate and peach aroma), and benzeneacetaldehyde (honey-like aroma) exhibited higher concentrations during the soy sauce fermentation (Fig 3i), which was in line with previous research[32]. Benzeneacetaldehyde is one of the characteristic volatile compounds identified in soy sauce, and is also ethyl acetate[15]. Ethyl acetate mainly endows soy sauce with a fruity aroma and can be increased by the esterification of alcohols and acids[33]. Over time, at the end of fermentation, the levels of benzeneacetaldehyde and ethyl acetate were highest in groups C and B (Fig. 3i, k), which were 4.10 and 2.57 times higher than that in the control group (P < 0.01), respectively. In our previous study, six potential flavor-producing bacteria strains were cultured in a simulated soy sauce high-salt fermentation environment, and it was found that *W. paramesenteroides* produced the most of benzeneacetaldehyde[12]. These results indicated that *W. paramesenteroides* could produce benzeneacetaldehyde not only under pure culture conditions but also in complex colony environments, validating its potential flavor-producing function. Moreover, acetic acid, as one of the precursors of ethyl acetate, showed higher levels in group B at the late stage of soy sauce fermentation (Fig. 3l), which is in accordance with the changes in ethyl acetate content (Fig. 3k).

Furthermore, phenolic compounds also play an important role in the presentation of soy sauce flavor, with unique and active aroma characteristics, most of which have woodsy, roasted, smoky, and pungent aromas, etc., making the flavor of soy sauce more full-bodied[34]. Posteriorly, phenol, 5-ethenyl-2-methoxy- and 2,4-Di-tert-butylphenol showed a subsequent dominance during the soy sauce fermentation (Fig. 3j). Notably, 2-Methoxy-4-vinylphenol was significantly increased in the inoculated groups at the end of fermentation (Fig. 3j), which is a precursor for the production of 4-ethylguaiacol. 4-ethylguaiacol has been widely identified in soy sauce and is believed to be a characteristic flavor compound of soy sauce. It plays a critical role in the unique flavor of soy sauce by imparting a natural soy aroma as well as moderating the salty taste[35]. The content of 4-ethylguaiacol was detected at low levels in the samples of this study, as reported in previous research[17], which may be related to the fermentation environment. At the end of fermentation, 2-Methoxy-4-vinylphenol was not detected in the control group, with the highest level in group B being 225.53 μg/kg, indicating greater potential for conversion to 4-ethylguaiacol. In addition, 2-Methoxy-4-vinylphenol itself could increase the pungent or woody flavor of soy sauce, which contributed to the flavor of soy sauce, as determined in previous research[30]. Domínguez-Tornay et al.[36] reported that inoculation with *S. cerevisiae* and *L. plantarum* could effectively increase the production of 2-methoxy-4-vinylphenol in beer. The results further confirmed that inoculation with *W. paramesenteroides and L. plantarum* can increase the content of flavor compounds in soy sauce, with groups B and C showed higher levels of characteristic volatile compounds.

3.4 Effect of inoculation on free amino acids

Free amino acids (FAAs) markedly contributed to the formation of unique flavors and aromas in fermented foods, especially soybean-fermented foods containing protein as the main fermentation substrate[4]. FAAs level is one of the important indicators of soy sauce quality. A total of 17 amino acids were detected during the fermentation process, among which glutamic acid (Glu), aspartic acid (Asp), arginine (Arg), leucine (Leu), and lysine (Lys) were dominant (Fig. 4a), which is consistent with the composition of amino acid content in the Cantonese soy sauce that we previously identified[15]. The effects of different reinforcement methods on the content and proportion of FAAs are shown in Supplementary Table S2. As fermentation progresses, the content of each amino acid showed an upward trend. At the end of fermentation, the content of total amino acids in group C exhibited the highest level of 57.13 mg/mL, which was not remarkably different from groups B and D, with a 1.21-fold increase compared to the control group (P < 0.05). Notably, Glu was found to be significantly higher in the inoculated group compared to the control group, which echoed the changes in pyroglutamic acid content in each group (Fig. 2g). The amount of Glu in group C was 1.37 times higher than that in the control group (P < 0.05). Zhang et al.[10] found that inoculation of soy sauce moromi with *Z. rouxii* QH-25 increased the Glu contents by 10.76% compared with the control, while no significant difference was observed between after inoculation with *Z. rouxii* QH-1 or *C. versatilis*. These results suggested that co-inoculation of *W. paramesenteroides and L. plantarum* demonstrated a good ability to enhance free amino acid flavors during soy sauce fermentation.



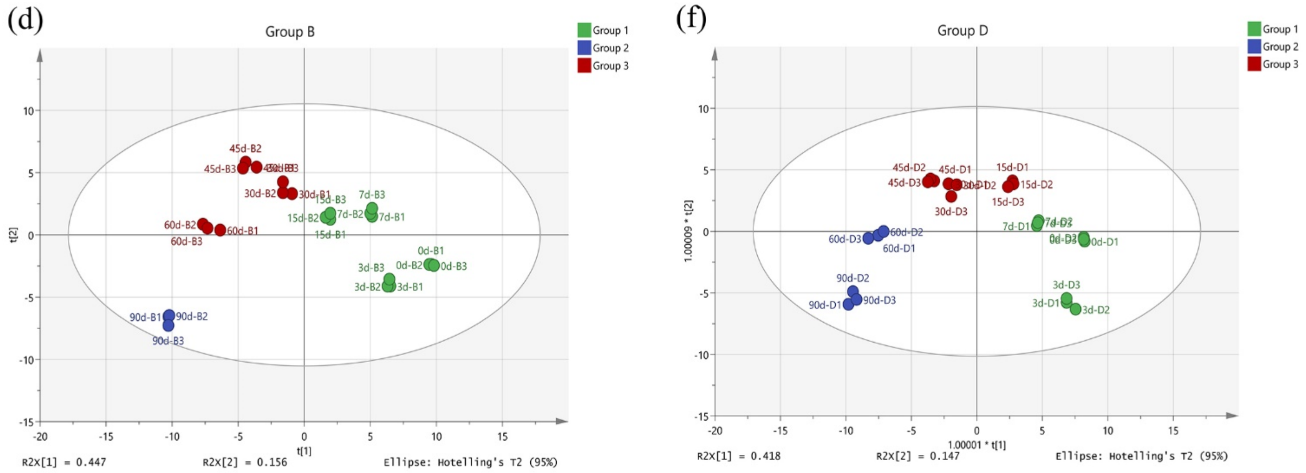
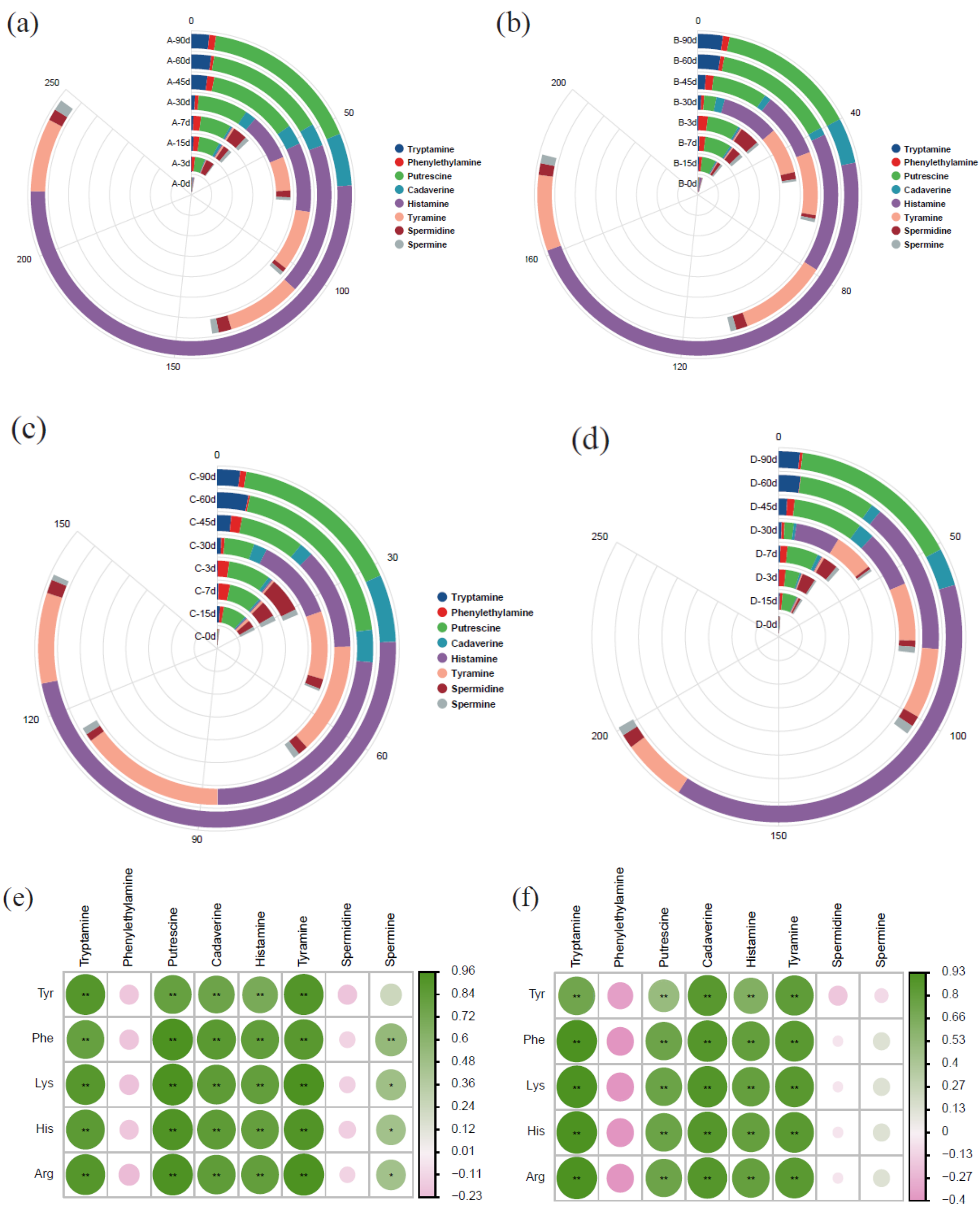


Fig.4. Effect of strain addition strategy on free amino acids in soy sauce ((a) Dynamic changes of free amino acids in soy sauce from the four groups; (b) Composition of different flavor amino acids in soy sauce from the four groups), and PCA analysis of flavor compounds during soy sauce fermentation ((c) Group A; (d) Group B; (e) Group C; (f) Group D).

In addition, according to the taste characteristics, amino acids can be mainly classified into three categories: umami, sweet, and bitter[37]. The percentage of bitter amino acids was found to be the highest in each group, which was in line with previous studies[38,39]. The total amino acid content of the three different categories at the end of fermentation was not significantly different in groups B, C, and D, but they were all higher than the control group (Fig. 4b). Furthermore, PCA analysis of the main flavor compounds of soy sauce, including organic acids, volatile compounds, and free amino acids, revealed that the development of flavor compounds in the four groups could be divided into three stages, with groups A and B equally divided into 0-15, 30-60, and 90 d (Fig. 4c, d), group C into 0-15, 30-45, and 60-90 d (Fig. 4e), and group D into 0-7, 15-45, and 60-90 d (Fig. 4f). From the viewpoint of these results, the addition of *W. paramesenteroides* and *L. plantarum* could enrich the flavor of soy sauce, and different fortification methods resulted in the different successional structure of soy sauce flavor during the fermentation process.

3.5 Effect of inoculation on biogenic amine

High levels of biogenic amines (BAs) in soy sauce are becoming increasingly concerning due to their potential toxic effects[8]. Certain studies have shown that the adding of starter culture to fermented food can reduce the content of BAs[11,40]. As shown in Figure 5, a total of eight types of BAs were detected in the four groups, among which histamine, tyramine, and putrescine dominated during the fermentation process, which is consistent with previous findings[41]. The total BA content of the four groups showed a comparable change during the first 15 days of fermentation and then presented an upward trend. After 90 days of fermentation, it was found that the BAs in the inoculated group were significantly lower than that in the control group ($P < 0.05$) (Table S3), the reduction in BAs level was most significant in group C. Likely, compared to the control group, the total of BAs decreased by 42.26%. In particular, the potentially more harmful histamine was reduced by 44.47% in group C. Guo et al.[11] found that co-inoculation of soy sauce mash with *S. piscifermentans* and salt-tolerant yeasts reduced the BAs production by 31.51% under laboratory-scale experiments.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C-60d
C-45d
C-30d
C-3d
C-7d
C-15d
C-0d
Tryptamine
Phenylethylamine
Putrescine
Cadaverine
Histamine
Tyramine
Spermidine
Spermine
(d)
0
50
100
150
200
250
D-90d
D-60d
D-45d
D-30d
D-7d
D-3d
D-15d
D-0d
(e)
Tryptamine
Phenylethylamine
Putrescine
Cadaverine
Histamine
Tyramine
Spermidine
Spermine
Tyr
Phe
Lys
His
Arg
0.96
0.84
0.72
0.6
0.48
0.36
0.24
0.12
0.01
−0.11
−0.23
(f)
Tryptamine
Phenylethylamine
Putrescine
Cadaverine
Histamine
Tyramine
Spermidine
Spermine
Tyr
Phe
Lys
His
Arg
0.93
0.8
0.66
0.53
0.4
0.27
0.13
0
−0.13
−0.27
−0.4

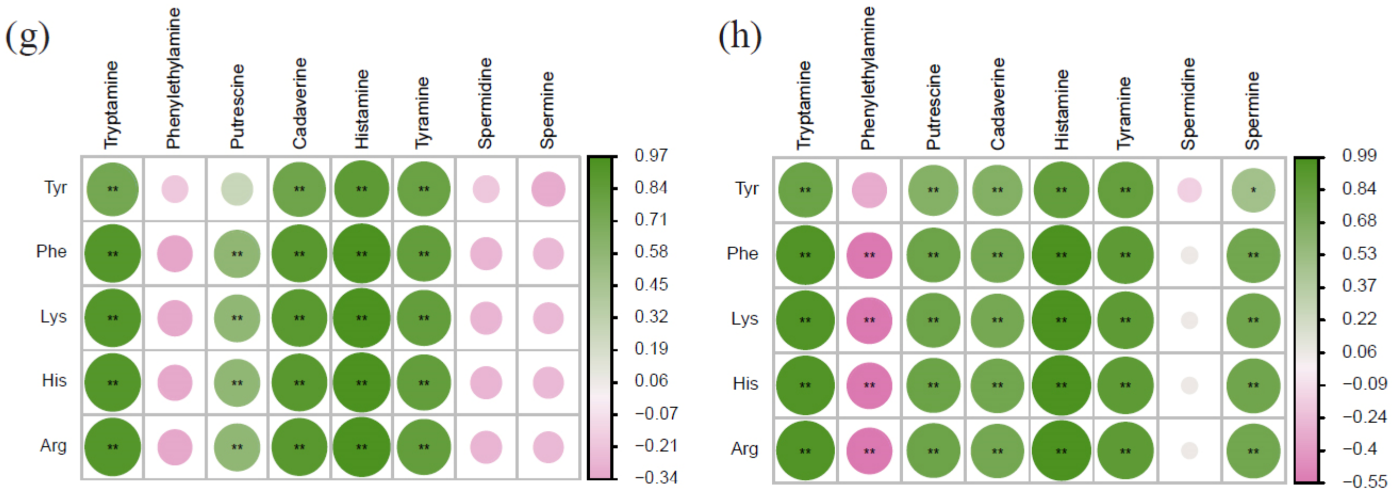

Fig.5. Effect of strain addition strategy on biogenic amines in soy sauce and correlation analysis of biogenic amines with precursor amino acids during soy sauce fermentation: (a), (e) Group A; (b), (f) Group B; (c), (g) Group C; (d), (h) Group D.

BAs are foremostly synthesized by corresponding amino acids under the action of microbial decarboxylases[42]. The main precursors of BAs detected in this paper include Tyr, Phe, Lys, His, and Arg, whose levels have been exhibited in Table S2 and were found to be higher in all inoculation groups than in the control group at the later stages of fermentation. Further, we explored the correlation between BAs and their precursors during the four fermentation processes. As shown in Figure 5b, most of the BAs in the four groups were positively correlated with their precursors, while spermidine and phenylethylamine were negatively associated with them. Based on these findings, we can infer that the reduction of BA by *W. paramesenteroides* and *L. plantarum* may mainly related to their inhibition of the growth of microorganisms involved in BA synthesis or their BA degradation capacity, rather than their suppression of the amount of precursor amino acids. Overall, the Group C inoculation method is most likely to become a good strategy for future application in factory fermentation to improve soy sauce flavor and reduce BA formation.

4. Conclusion

This study demonstrated that co-inoculation of *W. paramesenteroides* and *L. plantarum* could improve soy sauce's flavor and lower its BAs level. The changes in characteristic flavor compounds and BA content in the combination analysis revealed that the inoculation pattern of group C was more desirable to improve soy sauce quality. Compared with the control group, the content of organic acids, volatile substances, and free amino acids in Group C increased by 1.47 times, 1.29 times, and 1.21 times, respectively. The content of characteristic flavour compounds of soy sauce, such as lactic acid, 1-octen-3-ol, benzeneacetaldehyde, and glutamic acid, increased significantly. At the end of fermentation, the BA content of group C decreased by 42.26% compared to the control group. This study provides valuable insights into the validation and application of potential functional microorganisms in soy sauce fermentation.

Declaration of interests

The authors declared that there is no conflict of interest.

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