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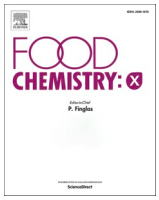

Effects of enhanced fermentation with high-yielding strains of Tetramethylpyrazine on flavor quality of Douchiba

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ABSTRACT

Douchiba (DCB) is a nutritious food rich in various functional components such as Tetramethylpyrazine (TTMP), and the strain fermentation is crucial for enhancing its quality. This work utilized *Bacillus subtilis* S2–2 and *Hyphopichia burtonii* S6-J1 with high TTMP production for fermentation of soybeans to optimize the pre-fermentation process and to evaluate the flavor quality of mature DCB. The concentration of TTMP in DCB fermented by mixed microbial (MG) was 2.95 times higher than that of of the control. Furthermore, the concentrations of taste substances, organic acids, free amino acids, and free fatty acids in MG were significantly increased. 87 flavor compounds were detected by gas chromatography-ion mobility spectrometry. The content of aldehydes, alcohols, esters, acids, and pyrazines flavor compounds was higher in MG, with esters and alcohols being notably higher than in other groups. Additionally, the highest comprehensive score of flavor quality was obtained in MG by principal component analysis.

1. Introduction

Douchi (DC) is one of the four major representatives of traditional fermented soybean products in China. Its derivative, Douchiba (DCB), is a unique traditional fermented condiment in Guizhou, China. DCB is produced with local high-quality soybeans as raw materials from winter solstice to the Spring Festival every year through a series of processes including soaking, steaming, microbial cultivation, salt sprinkling, fermentation, mashing, drying, molding, aging, etc. In order to prepare DCB with a unique flavor, a delicious taste, and a black appearance, its production cycle is usually as long as 1 to 1.5 years (Wang, Wen, et al., 2023). At present, the production of DCB is mainly performed in the traditional way of small farmers' workshops and still has some problems such as a long production cycle and an unstable flavor quality. Therefore, in order to further improve the flavor quality of DCB and realize industrial production, it is necessary to explore the dominant fermentation microorganisms in DCB and screen the strains with excellent production performances for enhanced fermentation.

The traditional open fermentation makes the microbial community of DC complex and leads to the diversity and uniqueness of the flavors of DC. In *bacterial*-type DC, *Bacillus* spp. and *Staphylococcus sciuri* are significantly correlated with the abundances of volatile acids, alcohols, esters, pyrazines, and other compounds (Qin et al., 2006). The network graph analysis results of Yongchuan DC proved the positive correlations between *Staphylococcus*, *Escherichia_Shigella*, and 1-octene-3-ol, ethyl isobutyrate (Lan et al., 2023). The mixed microbial fermentation with *bacillus subtilis* and *pediococcus pentosaceus* can optimize the DC fermentation process and improve the nutritional properties and flavor quality of DC (Wang, 2022). Although natural fermentation can significantly improve the flavor diversity and uniqueness of DC, the stability of flavor and quality is reduced due to the differences in the microbiota and their metabolites during the fermentation process. However, the optimization of DC fermentation process by microbial inoculation can ensure the stability of its flavor and quality. Therefore, it is necessary to optimize the DC fermentation process by inoculating microorganisms.

The strong flavor is a key quality of traditional fermented DCB in China and also determines consumer acceptability. The volatile compounds of traditional fermented DCB include seven major groups of compounds, among which acids and heterocyclic compounds (mainly pyrazines) respectively rank first and second (Qin & Ding, 2007).

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Pyrazines present the flavors of DC and nuts with a low odor threshold but high odor intensity and are the main components determining the characteristic flavor of DCB, especially tetramethylpyrazine (TTMP) (Wang, Wen, et al., 2023). TTMP can dilate blood vessels and inhibit platelet adhesion and aggregation as well as thrombosis, displaying physiological and pharmacological effects, such as treating hyperlipidemia and high cholesterol, preventing cardiovascular diseases, and inhibiting tumor cell proliferation (Li, Sng, et al., 2023). Therefore, increasing the concentration of TTMP in DCB may contribute to the flavor and quality of DCB. At present, increasing TTMP concentration in DCB by enhanced fermentation was seldom reported.

In order to increase the content of TTMP in DCB and improve the flavor and quality of DCB, in this study, single and mixed microbial fermentation methods of DCB and the optimization of fermentation conditions were investigated with soybeans as the raw material and *Bacillus subtilis* S2–2 and *Hyphopichia burtonii* S6-J1 strains screened by our laboratory for high TTMP production from DCB. Based on the comparison results of two fermentation methods, the changes in basic physicochemical indexes, active ingredients, and flavor of DCB were investigated. This study provides the theoretical basis for the quality improvement and industrial production of DCB.

2. Materials and methods

2.1. Materials and reagents

Soybeans were purchased in a local supermarket. *Bacillus subtilis* S2–2 and *Hyphopichia burtonii* S6-J1 were screened and preserved in our laboratory. Acetoin standard, TTMP standard, neutral protease kit, and the agar media and liquid media of LB and YPD were purchased from Beijing Solarbio Technology Co., Ltd. The rest of reagents were analytically pure and purchased from Tianjin Fuyu Chemical Co., Ltd.

2.2. Sample preparation

The selected soybeans were soaked for 18 h and steamed (121 °C) for 30 min. Then sterilized soybeans were cooled to room temperature in an ultra-clean bench for subsequent experiments.

Cooled soybeans were fermented according to the optimized conditions to obtain the single microbial fermentation group (SG): soybean (50 g), the inoculation volume of *Bacillus subtilis* S2–2 (2 %), fermentation temperature (45 °C), and fermentation time (4 d). The single microbial blank control group (SB) was obtained by placing cooled soybeans in an open environment for 30 min, stirring every 15 min, and pre-fermentation under the same conditions.

Cooled soybeans were fermented according to the optimized conditions to obtain the the mixed microbial fermentation group (MG): soybean (60 g), inoculation volume (6 %), fermentation temperature (30 °C), fermentation time (4 d), and *Bacillus subtilis* S2–2: *Hyphopichia burtonii* S6-J1 (7:1). The mixed microbial blank control group (MB) was obtained by placing cooled soybeans in an open environment for 30 min, stirring every 15 min, and pre-fermentation under the same optimized mixed microbial fermentation conditions.

The above pre-fermented DCB was ground and mashed. After 4 % salt was added and mixed, the mixture was placed in cleaned and sterilized earthenware pots for 15 d natural fermentation. Then, the fermented products were taken out from pots for molding. Finally, the characteristic fermentation components were determined.

2.3. Analysis methods

2.3.1. Determination of basic indexes

The moisture, pH, and NaCl content were determined according to the method of Guo et al. (2024). Nitrite was determined according to the method of Wang, Sui, et al., 2023. Protease activity was determined with a neutral protease kit.

2.3.2. Determination of TTMP and acetoin

TTMP was determined with the method of Li, Liu, et al., 2023. The conditions of high performance liquid chromatography (HPLC) were set as follows: the mobile phase (methanol: ultrapure water (0.05 % trifluoroacetic acid) = 7: 3 (*V*/V)), column temperature at 30 °C, detection wavelength at 278 nm, the flow rate of 1 mL/min, and the injection volume of 5 μL.

Acetoin was determined with the method of Li, Liu, et al., 2023. The column was an Aminex HPX-87H (Bio-Rad). The injection volume was 20 μL. The mobile phase was 5 mM H_2SO_4. The flow rate was 0.6 mL/min. The column temperature was 60 °C and a refractive index detector was used.

2.3.3. Determination of flavor substances

Amino acid nitrogen, reducing sugars, and organic acids were determined according to the method of Guo et al. (2024). Total acids were determined according to the method of Lin et al. (2024). Soluble proteins/peptides were determined according to the method of Tan (2021).

2.3.4. Determination of the free amino acids and free fatty acids

Free amino acids and free fatty acids were determined according to the method of Zhang, Han, et al. (2024) with slight modifications.

The contents of free amino acids in DCB were examined with high-performance liquid chromatography (Agilent 1100 HPLC, Agilent Technologies, Santa Clara, CA, USA). HPLC conditions were as follows. The mobile phase A consisted of water (0.1 % formic acid), and the mobile phase B was acetonitrile (2.5 mmol/L ammonium formate, 0.1 % formic acid). The elution program was as follows: 0–0.5 min, A: B = 96:4; 0.5–2.5 min, A: B = 90:10; 2.5–5 min, A: B = 72:28; 5–7 min, A: B = 5:95; 7–9 min, A: B = 96:4.

The contents of free fatty acids in DCB were analyzed based on a gas chromatography (Agilent 7890 A, Agilent Technologies, USA) with a DB-225 capillary column (20.0 m × 0.10 mm × 0.10 μm, Agilent, USA). The injection volume was 1 μL and the split ratio was 10:1. The carrier gas was high purity helium, and the flow rate was 1.0 mL/min. The initial temperature of the column oven was 50 °C for 0.5 min, then increased to 194 °C at 35 °C/min for 3.5 min, finally increased to 240 °C at 9 °C/min for 1.0 min. The peak area of the targeting data was calculated with MassHunter quantitative software and the concentration was calculated with the standard curve method.

2.3.5. Determination of volatile flavor compounds

Volatile flavor compounds were determined according to the previous study with some slight modifications (Lu et al., 2024).

Volatile compounds in DCB were analyzed based on a GC–IMS (FlavourSpec®, G.A.S., Dortmund, Germany). Briefly, 1 g of DCB sample was placed into a 20 mL headspace glass vial. After heating at 80 °C for 20 min, the sample (200 μL) was added into injector at 85 °C. Volatile compounds were isolated by an MXT-WAX (30 m, 0.53 mmID, df1.0 μm, Restek, USA) at 60 °C. The carrier (nitrogen, 99.9 %) was programmed as follows: 2 mL/min for 0–2 min, 10 mL/min for 2–5 min, increased from 10 mL/min to 100 mL/min within 5–25 min, and 100 mL/min for 25–30 min. The flow rate of drift gas and temperature in the drift tube were 150 mL/min and 45 °C, respectively. The flow rate of drift gas and temperature in the drift tube were 150 mL/min and 45 °C, respectively.

The retention index (RI) of volatile compounds was calculated with n-ketones C4-C9 as external references. The identification of volatile compounds was performed via the comparison of RI and drift time based on the GC-IMS library.

2.4. Data processing

Origin 2021 and SIMCA 14.1 were used for plotting and SPSS 20.0 was used for significance tests. Experimental data were expressed as mean ± standard deviation and $P < 0.05$ indicated a significant

Table 1
Content of basic physicochemical indexes in different samples.

sample	Hydration (%)	Neutral protease activity (U/g)	pH	NaCl (%)	nitrite (mg/kg)
SG	25.98 ± 1.48^{b}	1.07 ± 0.06^{a}	6.60 ± 0.04^{c}	1.47 ± 0.12^{c}	0.45 ± 0.08^{b}
SB	21.33 ± 1.27^{c}	0.32 ± 0.02^{b}	7.11 ± 0.04^{ab}	1.67 ± 0.28^{ab}	0.64 ± 0.05^{a}
MG	33.52 ± 2.93^{a}	1.08 ± 0.05^{a}	7.42 ± 0.03^{a}	1.64 ± 0.09^{ab}	0.48 ± 0.08^{b}
MB	21.42 ± 1.23^{c}	0.44 ± 0.04^{b}	7.46 ± 0.02^{a}	1.92 ± 0.07^{a}	0.75 ± 0.10^{a}

Note: SG was single microbial fermentation and SB was its control; MG stands for mixed microbial fermentation and MB was its control; All data are the mean ± standard deviation of three replicates. Means followed by different letters within the same column are significantly different ($P < 0.05$) from each other.

difference.

3. Results and discussion

3.1. Optimization of pre-fermentation

In the single microbial fermentation experiments of soybeans, fermentation time, the amount of soybeans, fermentation temperature, and inoculation volume had significant influences on TTMP yield (Supplementary Materials Table A.3). Fermentation time had the most significant influence on TTMP yield of single microbial fermentation, followed by the amount of soybeans. The optimal combination of the above factors for single microbial fermentation was determined as follows: soybean (50 g), inoculation volume (2 %) fermentation temperature (45 °C), and fermentation time (4 d) (Supplementary Materials Table A.1). In the mixed microbial fermentation of soybeans, fermentation temperature, fermentation time, amount of soybeans, inoculation volume, and the ratio of bacterial strain to yeast strain had significant influences on TTMP yield (Supplementary Materials Table A.4). Fermentation temperature had the most significant influence on TTMP yield of mixed microbial fermentation, followed by fermentation time. The optimal combination of the above factors for mixed microbial fermentation were determined as follows, soybean (60 g), inoculation volume (6 %), fermentation temperature (30 °C), fermentation time (4 d), and strain ratio (7:1) (Supplementary Materials Table A.2). By verifying the results of the above orthogonal experiments, the concentration of TTMP in single microbial fermentation reached 305.11 μg/g, which was 10.9 % higher than that obtained before optimization (275.23 μg/g). The concentration of TTMP in mixed microbial fermentation was 340.11 μg/g, which was 21.4 % higher than that obtained before optimization (280.17 μg/g). The improvements showed that the optimization of the pre-fermentation process was obvious, so the optimized conditions were adopted in subsequent experiments.

3.2. Basic physicochemical indexes

After 15 days of later fermentation of DCB, the physicochemical indexes were showed in Table 1. The moisture concentration in the enhanced fermentation group was significantly higher than that in the control group because the more intense microbial metabolism and interactions in the open natural fermentation consumed more moisture (Zhao et al., 2023). Protease activity ranged from 0.32 U/g to 1.08 U/g. Protease activity in the samples of the enhanced fermentation group was the highest, thus facilitating the synthesis of ammonium, the precursor of TTMP (Wang, Qiu, et al., 2023). The determined pH ranged from 6.60 to 7.46, which was in line with the normal pH range of foods acceptable to the human body. The microorganisms in the enhanced fermentation group could utilize sugars to produce organic acids (Chiara et al., 2024), which lowered the pH value compared to that in the control group. NaCl concentration ranged from 1.47 % to 1.92 % and nitrite concentration ranged from 0.45 mg/kg to 0.75 mg/kg and was in line with national standards (≤ 20 mg/kg) (Song et al., 2024).

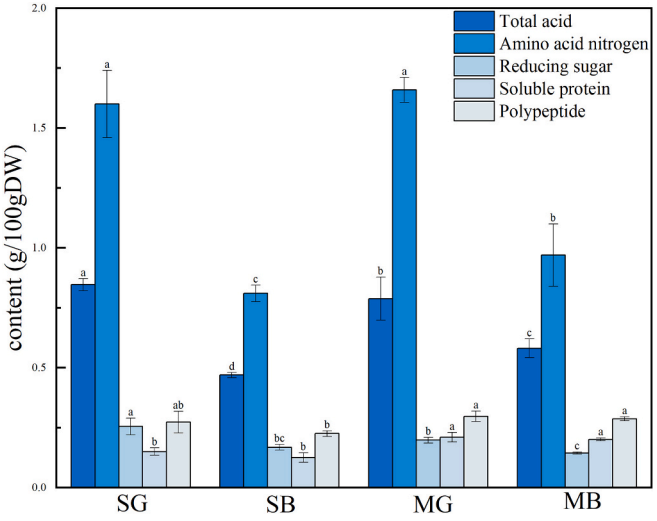

Fig. 1. Contents of taste substances in different samples (g/100gDW). SG was single microbial fermentation and SB was its control; MG stands for mixed microbial fermentation and MB was its control; The above values are on a dry basis, where the reducing sugar content is in mg/mL. All data are the mean ± standard deviation of three replicates. Means followed by different letters within the same column are significantly different ($P < 0.05$) from each other.

3.3. TTMP and acetoin

TTMP and acetoin concentrations in the enhanced fermentation group were significantly higher than those in the control group. TTMP concentration in MG was the highest (312.73 μg/g), which was 2.95 times of that in the control group (106.11 μg/g). Compared with TTMP content in fermented soybean (58.41 μg/g) with *B. subtilis* E20 by Wang, Li, & Xie, 2023, TTMP content in MG was increased by 5.35 times. However, the content of acetoin in SG was higher than that in MG because the acidic environment was conducive to the accumulation of acetoin, as confirmed by the increased amount of biomass and the activated acetolactate synthase (ALS, also called “pH 6 enzyme” responsible for acetolactate formation in the acetoin pathway) in acidic conditions (Zhu & Xu, 2010). In summary, the synthesis and accumulation of TTMP and related substances in DCB could be promoted by inoculating high-yielding strains of TTMP and optimizing the pre-fermentation process conditions. The results provided the basis for increasing TTMP production in DCB.

3.4. Taste substances

Fig. 1 shows the concentrations of taste substances in different DCB samples. Total acids affected the microbial growth and fermentation of DCB as well as the formation of taste. With the addition of strains, the enhanced fermentation group contained abundant microorganisms. Microorganisms grew and multiplied rapidly and produced a large number of secondary metabolites, such as acetic acid and lactic acid (Liao et al., 2023), which increased the total acid concentration in the enhanced fermentation group. The concentration of reducing sugars was higher in the enhanced fermentation group, indicating that the enzymatic activity in the enhanced fermentation group was high and led to the high conversion rate of reducing sugars. The difference in the concentration of reducing sugars in DCB was related to the amount of amylase produced by microorganisms as well as the activity of amylase (Guo et al., 2024). The content of amino acid nitrogen indicates the degree of hydrolysis of soy proteins and the level of free amino acids

Table 2
Contents of organic acids in different samples (mg/g).

Organic acid	SG	SB	MG	MB
Oxalic acid	2.00 ± 0.13^{b}	0.49 ± 0.11^{d}	2.25 ± 0.14^{a}	0.75 ± 0.10^{c}
Tartaric Acid	1.00 ± 0.10^{a}	/	0.25 ± 0.11^{b}	0.07 ± 0.01^{c}
Malic acid	3.25 ± 0.15^{d}	4.50 ± 0.13^{c}	5.03 ± 0.25^{b}	5.50 ± 0.20^{a}
Lactic acid	3.50 ± 0.50^{d}	5.00 ± 0.30^{c}	10.25 ± 0.57^{b}	12.75 ± 0.77^{a}
Acetic Acid	3.75 ± 0.10^{d}	0.50 ± 0.12^{c}	2.00 ± 0.80^{b}	0.49 ± 0.12^{a}
Citric acid	0.16 ± 0.02^{c}	0.35 ± 0.09^{b}	0.86 ± 0.10^{a}	0.20 ± 0.08^{c}
Succinic acid	27.43 ± 0.86^{c}	29.60 ± 0.50^{b}	40.26 ± 1.55^{a}	27.35 ± 1.21^{c}
Total content	41.09 ± 0.83^{c}	40.44 ± 0.97^{c}	60.91 ± 2.30^{a}	47.11 ± 1.35^{b}

Note: SG was single microbial fermentation and SB was its control; MG stands for mixed microbial fermentation and MB was its control; All data are the mean ± standard deviation of three replicates. Means followed by different letters within the same column are significantly different ($P < 0.05$) from each other.

during the fermentation process (Liao et al., 2023). In addition, the content of amino acid nitrogen is also related to umami, which is important for improving the flavor quality of DCB. According to the experimental results, it was found that the content of amino acid nitrogen in the enhanced fermentation group was significantly higher than that in the control group, suggesting that the enhanced fermentation group might have the better flavor quality. The concentrations of both soluble proteins and peptides were higher in MG because several microbial species with the synergistic effect produced abundant enzymes that acted on hydrophobic groups and amino acids, improved the solubility and promoted the degradation of high molecular weight proteins into low molecular weight proteins and peptides (Tan, 2021). In conclusion, enhanced fermentation by inoculation with *Bacillus subtilis* S2–2 and *Hyphopichia burtonii* S6-J1 significantly increased the contents of taste substances in DCB.

3.5. Organic acids

Table 2 shows the presence of seven major organic acids in DCB samples, including oxalic acid, tartaric acid, malic acid, lactic acid, acetic acid, citric acid, and succinic acid. These organic acids were likely generated via microbial metabolic activity, protein hydrolysis, and fermentation, or other processes (Cui et al., 2020). Organic acids in fermented soybean products regulate the flavor and increase appetite (Jia et al., 2019). The concentrations of organic acids in MG sample were significantly higher and the dominant organic acid was succinic acid. The metabolic pathways and enzymatic catalysis were related to the succinic acid production in various microorganisms such as *bacillus* and *yeasts* (Chen & Nielsen, 2016). The inoculation of advantageous species in the fermentation of DCB accelerated the accumulation of organic acids. In the decomposition process, through the oxidative tricarboxylic acid pathway pyruvic acid was converted into acetyl coenzyme A and reacted with oxalylacetic acid to produce citric acid, which was then converted into butanedioic acid (Kiira et al., 2016). Therefore, the low concentration of citric acid in the samples of DCB might be ascribed to the conversion of most citric acid.

3.6. Free amino acids and free fatty acids

Free amino acids are crucial for the taste and flavor characteristics of fermented soy products. Free amino acids are the primary nitrogen source for microbial growth and metabolism and largely determine the flavor properties of DC (Le et al., 2020). The concentrations of free amino acids in samples were determined (Table 3). In total, 20 free amino acids were detected, including 7 essential amino acids. The total concentrations of free amino acids were the highest in SG, followed by MG. Among all DCB samples, glutamine exhibited the highest content, followed closely by threonine and alanine, and while cystine had the lowest content, aligning with the previous research results (Zhang, Han, et al. (2024)).

Table 3
Composition and content of free amino acids in different samples (g/100 g).

Name	SG	SB	MG	MB
Alanine	1.82 ± 0.20^{b}	2.02 ± 0.08^{a}	1.71 ± 0.09^{c}	1.67 ± 0.07^{c}
Glycine	0.93 ± 0.06^{b}	0.82 ± 0.15^{b}	0.80 ± 0.11^{b}	1.21 ± 0.13^{a}
Serine	1.37 ± 0.19^{a}	1.14 ± 0.05^{bc}	1.06 ± 0.08^{c}	1.32 ± 0.09^{ab}
Threonine*	2.07 ± 0.60^{ab}	1.89 ± 0.38^{c}	2.21 ± 0.19^{a}	2.10 ± 0.14^{b}
Sweet	6.19 ± 0.27^{a}	5.87 ± 0.18^{b}	5.78 ± 0.21^{b}	6.30 ± 0.19^{a}
Arginine	0.41 ± 0.06^{b}	0.47 ± 0.08^{a}	0.46 ± 0.08^{ab}	0.33 ± 0.06^{c}
Histidine	0.31 ± 0.09^{a}	0.29 ± 0.07^{ab}	0.28 ± 0.01^{ab}	0.25 ± 0.02^{b}
Isoleucine*	0.68 ± 0.11^{a}	0.48 ± 0.08^{b}	0.60 ± 0.12^{ab}	0.58 ± 0.11^{ab}
Leucine*	0.88 ± 0.20^{a}	0.63 ± 0.15^{b}	0.76 ± 0.15^{ab}	0.72 ± 0.19^{ab}
Methionine	0.25 ± 0.09^{a}	0.25 ± 0.04^{a}	0.25 ± 0.08^{a}	0.24 ± 0.04^{a}
Phenylalanine*	0.34 ± 0.04^{a}	0.35 ± 0.08^{a}	0.35 ± 0.03^{a}	0.34 ± 0.04^{a}
Tyrosine	0.31 ± 0.09^{b}	0.35 ± 0.12^{a}	0.29 ± 0.08^{b}	0.25 ± 0.11^{c}
Valine*	0.66 ± 0.18^{a}	0.53 ± 0.12^{b}	0.61 ± 0.21^{ab}	0.58 ± 0.11^{ab}
Bitter	3.84 ± 0.11^{a}	3.35 ± 0.13^{b}	3.60 ± 0.10^{ab}	3.29 ± 0.14^{b}
Aspartic acid	0.11 ± 0.03^{a}	0.06 ± 0.01^{b}	0.04 ± 0.01^{c}	0.07 ± 0.02^{b}
Glutamic acid	0.45 ± 0.04^{a}	0.28 ± 0.08^{c}	0.26 ± 0.09^{c}	0.38 ± 0.10^{b}
umami	0.56 ± 0.09^{a}	0.34 ± 0.07^{c}	0.30 ± 0.05^{c}	0.45 ± 0.10^{b}
Asparagine	0.27 ± 0.02^{a}	0.21 ± 0.04^{b}	0.22 ± 0.02^{b}	0.20 ± 0.01^{b}
Glutamine	2.83 ± 0.07^{a}	2.42 ± 0.36^{b}	2.80 ± 0.12^{a}	2.45 ± 0.10^{b}
Cystine	0.04 ± 0.01^{a}	0.03 ± 0.01^{b}	0.03 ± 0.01^{b}	0.03 ± 0.01^{b}
Lysine *	1.43 ± 0.16^{a}	1.29 ± 0.33^{ab}	1.33 ± 0.21^{ab}	0.98 ± 0.18^{b}
Tryptophan *	0.85 ± 0.16^{a}	0.91 ± 0.11^{a}	0.66 ± 0.12^{b}	0.81 ± 0.21^{a}
Proline	0.52 ± 0.14^{b}	0.53 ± 0.09^{b}	0.61 ± 0.16^{a}	0.50 ± 0.12^{b}
Odourless	5.94 ± 0.11^{a}	5.39 ± 0.17^{ab}	5.65 ± 0.14^{ab}	4.97 ± 0.21^{b}
Total Amino Acids	16.53 ± 1.23^{a}	14.96 ± 0.98^{c}	15.31 ± 1.66^{b}	15.01 ± 1.78^{c}
Essential Amino Acids	6.91 ± 0.21^{a}	6.08 ± 0.16^{c}	6.52 ± 0.19^{ab}	6.11 ± 0.17^{c}

Note: * denotes essential amino acids; SG was single microbial fermentation and SB was its control; MG stands for mixed microbial fermentation and MB was its control; All data are the mean ± standard deviation of three replicates. Means followed by different letters within the same column are significantly different ($P < 0.05$) from each other.

By classifying the detected free amino acids into four groups according to their flavor-presenting characteristics, it was found that sweet and bitter amino acids were the major flavor-presenting amino acids in DCB. It was observed that this result was similar to that of Wang, Wen, et al., 2023. However, in the actual consumption over a long period of time, DCB showed a different flavor profile with a strong umami flavor and a slight bitterness. This may be due to the fact that the final flavor profile of DCB depended on the equilibrium and interactions among different flavor components. However, the exact mechanism remains to be explored. In conclusion, DCB was rich in free amino acids, and the interactions or synergistic effects among its components jointly

Table 4
Fatty acids composition and content of different samples (g/100 g).

Name	SG	MG	SB	MB
C4:0	0.55 ± 0.04^{b}	0.86 ± 0.02^{a}	0.81 ± 0.05^{a}	0.46 ± 0.08^{c}
C6:0	0.73 ± 0.11^{b}	0.91 ± 0.19^{a}	0.29 ± 0.08^{c}	0.22 ± 0.07^{d}
C8:0	0.81 ± 0.12^{a}	0.64 ± 0.13^{c}	0.56 ± 0.11^{d}	0.74 ± 0.14^{b}
C10:0	0.46 ± 0.12^{b}	0.55 ± 0.10^{a}	0.56 ± 0.12^{a}	0.23 ± 0.06^{c}
C11:0	0.37 ± 0.09^{d}	0.47 ± 0.12^{b}	0.45 ± 0.11^{c}	0.70 ± 0.15^{a}
C12:0	0.83 ± 0.23^{a}	0.73 ± 0.18^{b}	0.86 ± 0.19^{a}	0.36 ± 0.12^{c}
C13:0	0.74 ± 0.23^{a}	0.63 ± 0.18^{b}	0.41 ± 0.15^{c}	0.15 ± 0.09^{d}
C14:0	0.30 ± 0.09^{c}	0.72 ± 0.22^{a}	0.17 ± 0.10^{d}	0.35 ± 0.09^{b}
C14:1	0.72 ± 0.12^{b}	0.95 ± 0.23^{a}	0.57 ± 0.16^{c}	0.12 ± 0.06^{d}
C15:0	0.98 ± 0.23^{a}	0.95 ± 0.18^{a}	0.45 ± 0.19^{c}	0.71 ± 0.22^{b}
C15:1	0.66 ± 0.14^{ab}	0.61 ± 0.19^{b}	0.42 ± 0.12^{c}	0.73 ± 0.24^{a}
C16:0	0.60 ± 0.12^{b}	0.89 ± 0.15^{a}	0.95 ± 0.16^{a}	0.54 ± 0.11^{b}
C16:1	0.44 ± 0.11^{c}	0.65 ± 0.12^{b}	0.14 ± 0.05^{d}	0.90 ± 0.12^{a}
C17:0	0.57 ± 0.12^{c}	0.84 ± 0.11^{a}	0.31 ± 0.08^{d}	0.64 ± 0.13^{b}
C17:1	0.67 ± 0.11^{b}	0.65 ± 0.14^{b}	0.26 ± 0.09^{c}	0.89 ± 0.12^{a}
C18:0	0.35 ± 0.09^{c}	0.55 ± 0.11^{b}	0.74 ± 0.17^{a}	0.75 ± 0.14^{a}
C18:1n9c	0.62 ± 0.12^{b}	0.95 ± 0.16^{a}	0.57 ± 0.11^{c}	0.41 ± 0.09^{d}
C18:1n9t	0.95 ± 0.23^{a}	0.84 ± 0.21^{b}	0.77 ± 0.17^{c}	0.57 ± 0.18^{d}
C18:2n6c	0.74 ± 0.18^{b}	0.85 ± 0.26^{a}	0.45 ± 0.09^{c}	0.44 ± 0.11^{c}
C18:2n6t	0.55 ± 0.10^{b}	0.74 ± 0.18^{a}	0.45 ± 0.09^{c}	0.71 ± 0.19^{a}
C18:3n6	0.54 ± 0.12^{b}	0.54 ± 0.08^{b}	0.49 ± 0.10^{c}	0.83 ± 0.21^{a}
C18:3n3	0.97 ± 0.19^{a}	0.55 ± 0.12^{b}	0.14 ± 0.08^{c}	0.97 ± 0.11^{a}
C20:0	0.76 ± 0.20^{a}	0.72 ± 0.19^{a}	0.66 ± 0.12^{a}	0.83 ± 0.21^{a}
C20:1	0.54 ± 0.18^{c}	0.74 ± 0.16^{b}	0.83 ± 0.11^{a}	0.50 ± 0.10^{d}
C20:2	0.85 ± 0.23^{a}	0.84 ± 0.21^{a}	0.64 ± 0.17^{b}	0.46 ± 0.12^{c}
C20:3n6	0.81 ± 0.11^{a}	0.84 ± 0.14^{a}	0.35 ± 0.09^{b}	0.26 ± 0.07^{c}
C21:0	0.86 ± 0.18^{a}	0.16 ± 0.11^{c}	0.43 ± 0.14^{b}	0.79 ± 0.21^{a}
C20:3n3	0.85 ± 0.29^{a}	0.93 ± 0.27^{a}	0.53 ± 0.18^{c}	0.72 ± 0.16^{b}
C20:4n6	0.93 ± 0.20^{a}	0.73 ± 0.12^{b}	0.87 ± 0.19^{ab}	0.38 ± 0.08^{c}
C20:5n3	0.77 ± 0.13^{a}	0.44 ± 0.19^{b}	0.13 ± 0.08^{c}	0.77 ± 0.10^{a}
C22:0	0.77 ± 0.22^{a}	0.55 ± 0.14^{b}	0.18 ± 0.09^{c}	0.18 ± 0.10^{c}
C22:1n9	0.79 ± 0.21^{b}	0.61 ± 0.09^{c}	0.10 ± 0.02^{d}	0.88 ± 0.19^{a}
C22:2	0.84 ± 0.23^{a}	0.94 ± 0.22^{a}	0.59 ± 0.19^{b}	0.85 ± 0.14^{a}
C23:0	0.64 ± 0.18^{a}	0.38 ± 0.11^{b}	0.30 ± 0.09^{b}	0.28 ± 0.07^{b}
C24:0	0.21 ± 0.09^{d}	0.74 ± 0.14^{a}	0.57 ± 0.19^{b}	0.32 ± 0.10^{c}
C22:6n3	0.76 ± 0.19^{a}	0.53 ± 0.08^{c}	0.43 ± 0.11^{d}	0.63 ± 0.15^{b}
C24:1	0.84 ± 0.22^{a}	0.85 ± 0.19^{a}	0.60 ± 0.15^{b}	0.22 ± 0.09^{c}
Saturated fatty acids (SFA)	10.52 ± 0.21^{b}	11.29 ± 0.18^{b}	8.71 ± 0.15^{c}	15.75 ± 0.22^{a}
Monounsaturated fatty acids (MUFA)	5.37 ± 0.11^{ab}	5.99 ± 0.17^{a}	3.66 ± 0.11^{c}	5.00 ± 0.18^{b}
Polyunsaturated fatty acids (PUFA)	9.45 ± 0.21^{a}	8.80 ± 0.19^{b}	5.66 ± 0.20^{c}	7.23 ± 0.14^{bc}
Total Fatty Acids	25.33 ± 0.18^{a}	26.07 ± 0.19^{a}	18.03 ± 0.82^{c}	20.47 ± 0.83^{b}

Note: SG was single microbial fermentation and SB was its control; MG stands for mixed microbial fermentation and MB was its control; All data are the mean ± standard deviation of three replicates. Means followed by different letters within the same column are significantly different ($P < 0.05$) from each other.

determined the unique flavor of DCB.

Free fatty acids are both nutrients and precursors for the formation of the flavor of DCB and take part in the formation of DCB aroma substances through esterification reactions in the later fermentation stage (Xie et al., 2017). The concentrations of free fatty acids in DCB are shown in Table 4. In total, 37 free fatty acids were detected. The total content of free fatty acids showed the significant difference among samples. The total content of free fatty acids in SG and MG was significantly higher than that in the control group, and the total content in MG was the highest (26.07 g/100 g), which was 1.45 times higher than that in the control group. The composition of free fatty acids in DCB is mainly pentadecanoic acid, α-linolenic acid, oleic acid, etc. It is observed that this result is similar to the research findings of Xie et al. (2017).

According to the number and position of double bonds in the carbon chain, fatty acids can be classified as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). Among them, saturated fatty acids had the highest content and the concentration of saturated fatty acids in MB was significantly higher than that in other groups, suggesting that microorganisms had an effect on the concentration of saturated fatty acids, but this effect remained to be further explored. Unsaturated fatty acids played an important role in the formation of flavor substances in DCB and the increase in their concentration could enhance the flavor. Monounsaturated fatty acids are mainly oleic acid, which has the effect of reducing cholesterol and blood sugar (Sergio et al., 2007). The content of oleic acid in MG was the highest (0.95 g/100 g), suggesting the potential in the quality improvement of DCB. Linoleic acid, linolenic acid, and α-linolenic acid are currently polyunsaturated fatty acids with high nutritional values. The linoleic acid content was the highest in MG sample, followed by SG sample, indicating that enhanced fermentation by inoculating *Bacillus subtilis* S2–2 and *Hyphopichia burtonii* S6-J1 strains could effectively promote the generation of these polyunsaturated fatty acids with high nutritional values. The result is significant for the subsequent directional regulation of the production of DCB.

3.7. Volatile compound analysis

3.7.1. Volatile compounds of DCB

The volatile compounds in different samples were analyzed. A total of 87 volatile compounds were detected, including 21 aldehydes, 17 esters, 15 ketones, 15 alcohols, 6 pyrazines, 4 acids, and 9 other compounds (Table 5). The content of aldehydes was the highest, followed by ketones and esters.

Aldehydes have distinctive sweet, floral, and fruity aromas, which can enhance the flavor quality of DCB (Li, Peng, et al., 2023). In DCB samples, the highest content of aldehydes was observed and could be attributed to the high contents of unsaturated fatty acids and proteins in DCB. Aldehydes mainly originated from protein hydrolysis and oxidation of unsaturated fatty acids during the fermentation process (Wang et al., 2020). The contents of aldehydes in SG and MG were much higher than that in the control group, and a variety of aldehydes were present in

Table 5
Composition and relative content of volatile flavors in different samples based on GC-IMS (%).

Nane	GAS	MW	RI	RT (*sec*)	Dt (a.u)	SG	MG	SB	MB
Butanoic acid	C107926	88.1	1628	1678.927	1.1615	0.66 ± 0.16^{a}	0.64 ± 0.10^{a}	0.52 ± 0.08^{a}	0.50 ± 0.05^{a}
2-Methylpropanoic acid	C79312	88.1	1589.6	1495.102	1.1507	0.70 ± 0.04^{a}	0.68 ± 0.02^{a}	0.63 ± 0.10^{a}	0.49 ± 0.11^{b}
acetic acid-M	C64197	60.1	1490.5	1108.668	1.0551	1.09 ± 0.06^{a}	0.78 ± 0.12^{b}	0.75 ± 0.06^{b}	0.65 ± 0.04^{c}
acetic acid-D	C64197	60.1	1490.1	1107.326	1.1599	0.91 ± 0.11^{a}	0.25 ± 0.03^{b}	0.22 ± 0.04^{b}	0.08 ± 0.01^{c}
Acids (4)						3.37 ± 0.17^{a}	2.35 ± 0.15^{b}	2.11 ± 0.17^{ab}	1.73 ± 0.02^{c}
Benzaldehyde-M	C100527	106.1	1534	1264.315	1.1557	0.39 ± 0.11^{b}	0.42 ± 0.02^{b}	0.27 ± 0.07^{c}	0.64 ± 0.09^{a}
Benzaldehyde-D	C100527	106.1	1533.3	1261.631	1.474	0.05 ± 0.01^{c}	0.10 ± 0.03^{b}	0.05 ± 0.00^{c}	0.20 ± 0.02^{a}
citronellal	C106230	154.3	1485.6	1092.566	1.3585	0.34 ± 0.07^{a}	0.13 ± 0.02^{b}	0.08 ± 0.01^{b}	0.06 ± 0.01^{b}
Methional	C3268493	104.2	1470.2	1042.92	1.0934	0.21 ± 0.10^{a}	0.08 ± 0.00^{b}	0.08 ± 0.01^{b}	0.08 ± 0.01^{b}
(E)-2-hexenal	C6728263	98.1	1259.8	558.126	1.1692	0.54 ± 0.11^{a}	0.49 ± 0.09^{a}	0.27 ± 0.08^{b}	0.08 ± 0.01^{c}
3-Methyl-2-butenal-M	C107868	84.1	1211.3	488.823	1.0929	1.14 ± 0.09^{a}	0.63 ± 0.07^{ab}	0.47 ± 0.06^{b}	0.25 ± 0.07^{c}
3-Methyl-2-butenal-D	C107868	84.1	1211.7	489.386	1.3613	1.27 ± 0.13^{a}	0.69 ± 0.09^{b}	0.26 ± 0.08^{c}	0.17 ± 0.05^{c}
heptanal-M	C111717	114.2	1185.6	456.206	1.3348	0.07 ± 0.01^{c}	0.23 ± 0.09^{a}	0.14 ± 0.08^{b}	0.17 ± 0.04^{b}
heptanal-D	C111717	114.2	1187	457.833	1.6892	0.32 ± 0.12^{a}	0.15 ± 0.07^{b}	0.20 ± 0.08^{b}	0.12 ± 0.04^{b}
2-Methyl-2-pentenal	C623369	98.1	1161.9	429.529	1.5081	0.81 ± 0.13^{a}	0.35 ± 0.10^{b}	0.18 ± 0.03^{c}	0.32 ± 0.09^{b}
2-methyl-(E)-2-butenal-M	C497030	84.1	1112.4	378.778	1.0939	1.26 ± 0.15^{a}	0.97 ± 0.12^{b}	0.92 ± 0.13^{b}	0.99 ± 0.11^{b}
2-methyl-(E)-2-butenal-D	C497030	84.1	1112	378.452	1.3501	2.58 ± 0.21^{b}	3.19 ± 0.23^{a}	1.07 ± 0.18^{d}	2.01 ± 0.20^{c}
Hexanal-M	C66251	100.2	1098.2	365.439	1.2627	0.94 ± 0.19^{a}	0.74 ± 0.13^{c}	0.85 ± 0.11^{b}	0.64 ± 0.15^{d}
Hexanal-D	C66251	100.2	1098.6	365.764	1.5641	1.68 ± 0.12^{a}	1.51 ± 0.14^{a}	0.96 ± 0.11^{b}	1.07 ± 0.15^{b}
butanal	C123728	72.1	850.2	231.207	1.1142	5.96 ± 0.66^{c}	7.36 ± 0.56^{b}	8.12 ± 0.96^{a}	7.42 ± 0.43^{b}
Propanal-M	C123386	58.1	822.3	221.013	1.0651	1.51 ± 0.29^{a}	1.56 ± 0.18^{a}	1.54 ± 0.12^{a}	1.41 ± 0.21^{a}
Propanal-D	C123386	58.1	822.3	221.013	1.1461	4.97 ± 0.67^{b}	5.24 ± 0.78^{ab}	5.54 ± 0.39^{a}	5.48 ± 0.56^{a}
2-Methylpropanal	C78842	72.1	798.7	212.752	1.0869	1.46 ± 0.13^{a}	1.49 ± 0.14^{a}	1.35 ± 0.17^{a}	1.42 ± 0.12^{a}
2-methylbutanal	C96173	86.1	851.4	231.665	1.1568	3.47 ± 0.32^{a}	2.99 ± 0.21^{b}	2.98 ± 0.20^{b}	2.65 ± 0.22^{c}
(E)-2-octenal	C2548870	126.2	1444.5	965.14	1.3356	0.41 ± 0.12^{a}	0.28 ± 0.08^{b}	0.06 ± 0.01^{c}	0.06 ± 0.00^{c}
octanal	C124130	128.2	1263.5	563.833	1.4195	0.42 ± 0.11^{b}	0.39 ± 0.13^{b}	0.16 ± 0.07^{c}	1.00 ± 0.12^{a}
Aldehydes (21)						29.79 ± 1.00^{a}	29.00 ± 0.85^{ab}	25.54 ± 0.98^{c}	26.45 ± 0.48^{b}
Linalool-M	C78706	154.3	1482.8	1083.174	1.223	0.86 ± 0.09^{b}	1.50 ± 0.11^{a}	0.70 ± 0.10^{bc}	0.40 ± 0.04^{c}
Linalool-D	C78706	154.3	1485.2	1091.225	1.686	0.04 ± 0.01^{a}	0.03 ± 0.00^{a}	0.04 ± 0.00^{a}	0.03 ± 0.00^{a}
3-Methyl-1-butanol-M	C123513	88.1	1218	497.838	1.2481	0.76 ± 0.14^{a}	0.54 ± 0.10^{b}	0.49 ± 0.09^{bc}	0.39 ± 0.10^{c}
3-Methyl-1-butanol-D	C123513	88.1	1217.6	497.275	1.4902	0.42 ± 0.10^{a}	0.32 ± 0.08^{c}	0.35 ± 0.05^{b}	0.20 ± 0.04^{d}
butan-1-ol-M	C71363	74.1	1154.4	421.396	1.1821	0.84 ± 0.13^{a}	0.74 ± 0.14^{a}	0.69 ± 0.09^{b}	0.72 ± 0.13^{a}
butan-1-ol-D	C71363	74.1	1153.4	420.42	1.3785	1.24 ± 0.15^{a}	1.16 ± 0.11^{a}	0.83 ± 0.09^{b}	0.88 ± 0.10^{b}
2-Methyl-1-propanol-M	C78831	74.1	1105.5	372.271	1.1737	0.97 ± 0.18^{a}	0.80 ± 0.15^{bc}	0.72 ± 0.13^{c}	0.84 ± 0.14^{b}
2-Methyl-1-propanol-D	C78831	74.1	1103.8	370.644	1.3655	0.45 ± 0.12^{c}	0.84 ± 0.19^{a}	0.72 ± 0.14^{ab}	0.62 ± 0.14^{b}
1-Propanol-M	C71238	60.1	1051.2	329.888	1.1099	0.61 ± 0.11^{b}	0.70 ± 0.09^{a}	0.54 ± 0.12^{c}	0.60 ± 0.13^{b}
1-Propanol-D	C71238	60.1	1050.7	329.562	1.2513	0.57 ± 0.11^{b}	0.67 ± 0.13^{a}	0.64 ± 0.10^{ab}	0.57 ± 0.09^{b}
ethanol	C64175	46.1	942.6	268.469	1.1461	2.26 ± 0.22^{a}	2.36 ± 0.19^{a}	2.08 ± 0.12^{a}	1.62 ± 0.12^{b}
2-methylbutan-1-ol	C137326	88.1	747.5	195.825	1.2291	1.15 ± 0.14^{a}	1.17 ± 0.09^{a}	1.14 ± 0.11^{a}	0.89 ± 0.09^{b}
2-Propanol	C67630	60.1	942.8	268.569	1.2324	2.03 ± 0.16^{b}	2.42 ± 0.22^{a}	2.33 ± 0.19^{a}	2.30 ± 0.21^{a}
(*Z*)-Hex-3-enol	C928961	100.2	1367.8	765.667	1.2308	0.78 ± 0.15^{ab}	1.02 ± 0.10^{a}	0.58 ± 0.13^{b}	0.42 ± 0.10^{c}
3-Methyl-3-buten-1-ol	C763326	86.1	1212	489.756	1.4439	0.09 ± 0.02^{b}	0.75 ± 0.12^{a}	0.13 ± 0.05^{b}	0.15 ± 0.07^{b}
Alcohols (15)						13.46 ± 1.38^{b}	14.90 ± 1.18^{a}	11.98 ± 1.08^{b}	10.36 ± 1.29^{c}
pyrazine,2,3,5,6-tetramethyl	C1124114	136.2	1462.5	1018.768	1.2097	0.26 ± 0.09^{a}	0.28 ± 0.10^{a}	0.21 ± 0.08^{bc}	0.18 ± 0.02^{c}
Trimethylpyrazine-M	C14667551	122.2	1407.5	863.121	1.169	1.07 ± 0.11^{b}	1.28 ± 0.13^{a}	1.11 ± 0.09^{b}	1.18 ± 0.09^{b}
Trimethylpyrazine-D	C14667551	122.2	1408	864.463	1.6236	0.32 ± 0.06^{ab}	0.42 ± 0.07^{a}	0.25 ± 0.05^{b}	0.22 ± 0.03^{b}
2,5-dimethylpyrazine-M	C123320	108.1	1328.2	679.36	1.1159	2.29 ± 0.13^{c}	2.55 ± 0.11^{a}	2.49 ± 0.12^{ab}	2.45 ± 0.11^{ab}
2,5-dimethylpyrazine-D	C123320	108.1	1327.8	678.638	1.5016	1.59 ± 0.13^{a}	1.52 ± 0.14^{a}	1.03 ± 0.09^{b}	1.49 ± 0.10^{a}
3-isobutyl-2-methoxypyrazine	C24683009	166.2	1534.2	1265.104	1.2911	0.73 ± 0.17^{b}	0.62 ± 0.12^{b}	0.30 ± 0.07^{c}	1.13 ± 0.13^{a}
Pyrazines (6)						6.26 ± 0.26^{ab}	6.67 ± 0.30^{a}	5.37 ± 0.29^{b}	6.66 ± 0.38^{a}
Acetoine-M	C513860	88.1	1294.8	614.353	1.0617	1.46 ± 0.13^{a}	0.27 ± 0.01^{b}	0.19 ± 0.02^{b}	0.11 ± 0.02^{b}
Acetoine-D	C513860	88.1	1295.3	615.076	1.3326	3.43 ± 0.14^{a}	1.16 ± 0.00^{b}	0.08 ± 0.00^{c}	0.07 ± 0.00^{c}
3-Octanone-M	C106683	128.2	1263.8	564.324	1.3095	0.87 ± 0.09^{a}	0.20 ± 0.08^{b}	0.14 ± 0.04^{bc}	0.05 ± 0.00^{c}
3-Octanone-D	C106683	128.2	1262.3	562.071	1.7191	0.85 ± 0.11^{a}	0.12 ± 0.08^{b}	0.06 ± 0.01^{bc}	0.03 ± 0.00^{c}
heptan-2-one-M	C110430	114.2	1190.9	462.388	1.2642	0.99 ± 0.14^{a}	0.82 ± 0.10^{b}	0.48 ± 0.08^{c}	0.82 ± 0.09^{b}
heptan-2-one-D	C110430	114.2	1193	464.99	1.6347	0.77 ± 0.11^{b}	1.07 ± 0.11^{a}	0.15 ± 0.06^{c}	1.10 ± 0.11^{a}
Cyclopentanone-M	C120923	84.1	1141.4	407.732	1.1147	2.27 ± 0.11^{b}	1.77 ± 0.09^{d}	2.46 ± 0.13^{a}	2.07 ± 0.16^{c}
Cyclopentanone-D	C120923	84.1	1140.1	406.431	1.3263	4.59 ± 0.17^{a}	3.01 ± 0.14^{b}	4.62 ± 0.16^{a}	2.72 ± 0.11^{b}
1-Penten-3-one	C1629589	84.1	1043.2	324.178	1.088	0.44 ± 0.11^{c}	0.46 ± 0.09^{c}	0.63 ± 0.13^{b}	0.81 ± 0.16^{a}
4-methyl-2-pentanone-M	C108101	100.2	1040.4	322.259	1.1753	0.34 ± 0.09^{b}	0.43 ± 0.12^{a}	0.42 ± 0.10^{a}	0.44 ± 0.12^{a}
4-Methyl-2-pentanone-D	C108101	100.2	1033	317.163	1.4793	2.28 ± 0.22^{d}	2.61 ± 0.18^{c}	3.07 ± 0.21^{b}	3.94 ± 0.22^{a}
2,3-butanedione	C431038	86.1	1000.5	295.537	1.1694	1.31 ± 0.19^{a}	1.04 ± 0.09^{b}	1.22 ± 0.12^{ab}	0.94 ± 0.11^{c}
Pentan-2-one	C107879	86.1	996.6	293.076	1.3702	1.03 ± 0.10^{c}	2.17 ± 0.16^{b}	1.16 ± 0.13^{c}	2.65 ± 0.20^{a}
Butan-2-one	C78933	72.1	910.1	254.76	1.2473	5.79 ± 0.34^{c}	6.30 ± 0.68^{b}	8.08 ± 1.01^{a}	8.28 ± 0.34^{a}
Cyclohexanone	C108941	98.1	1296	616.383	1.1725	0.26 ± 0.09^{c}	3.22 ± 0.19^{b}	4.33 ± 0.24^{a}	4.67 ± 0.31^{a}
Ketones (15)						26.67 ± 3.15^{b}	24.64 ± 3.75^{c}	27.10 ± 0.79^{b}	28.71 ± 0.27^{a}
Ethyl lactate-M	C97643	118.1	1366.4	762.424	1.1525	0.96 ± 0.08^{a}	0.11 ± 0.02^{b}	0.04 ± 0.01^{c}	0.03 ± 0.00^{c}
Ethyl lactate-D	C97643	118.1	1366.7	763.146	1.5454	0.57 ± 0.10^{a}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}	0.03 ± 0.00^{b}
isobutyl butyrate	C539902	144.2	1163.7	431.481	1.3394	1.58 ± 0.14^{a}	1.15 ± 0.11^{b}	0.79 ± 0.13^{b}	0.94 ± 0.11^{b}
butyl acetate-M	C123864	116.2	1086	355.679	1.2381	0.43 ± 0.11^{b}	0.77 ± 0.13^{a}	0.48 ± 0.09^{b}	0.23 ± 0.08^{c}
butyl acetate-D	C123864	116.2	1086	355.679	1.6186	0.76 ± 0.13^{a}	0.44 ± 0.09^{b}	0.40 ± 0.10^{b}	0.09 ± 0.01^{c}
Ethyl 3-methylbutanoate-M	C108645	130.2	1062.6	338.111	1.2489	1.02 ± 0.13^{a}	0.85 ± 0.10^{a}	0.41 ± 0.09^{b}	0.39 ± 0.06^{b}

(*continued on next page*)

Table 5 (*continued*)

Nane	GAS	MW	RI	RT (*sec*)	Dt (a.u)	SG	MG	SB	MB
Ethyl 3-methylbutanoate-D	C108645	130.2	1063.5	338.762	1.6523	0.35 ± 0.09^{b}	0.69 ± 0.11^{a}	0.12 ± 0.03^{c}	0.18 ± 0.08^{c}
2-Methyl butanoic acid ethyl ester-M	C7452791	130.2	1077.9	349.498	1.2335	0.82 ± 0.08^{c}	1.31 ± 0.11a	0.95 ± 0.09^{ab}	1.01 ± 0.10^{ab}
2-Methyl butanoic acid ethyl ester-D	C7452791	130.2	1079.6	350.799	1.6531	0.21 ± 0.07^{b}	0.61 ± 0.12^{a}	0.20 ± 0.05^{b}	0.08 ± 0.01^{c}
Ethyl butanoate-M	C105544	116.2	1050.3	329.236	1.2061	0.23 ± 0.06^{a}	0.25 ± 0.10^{a}	0.15 ± 0.07^{b}	0.22 ± 0.09^{a}
Ethyl butanoate-D	C105544	116.2	1049.6	328.746	1.5611	0.80 ± 0.13^{b}	2.25 ± 0.21^{a}	0.08 ± 0.01^{c}	0.03 ± 0.00^{c}
Ethyl acetate-M	C141786	88.1	896.4	249.135	1.0978	0.85 ± 0.12^{c}	1.18 ± 0.12^{a}	0.90 ± 0.12^{b}	0.77 ± 0.09^{d}
Ethyl acetate-D	C141786	88.1	895	248.608	1.3375	5.68 ± 0.77^{a}	5.71 ± 1.08^{a}	5.06 ± 0.45^{b}	2.75 ± 0.23^{c}
isobutyl acetate	C110190	116.2	1027.1	313.102	1.2324	1.58 ± 0.15^{b}	1.90 ± 0.18^{a}	1.41 ± 0.23^{b}	1.47 ± 0.18^{b}
3-methylbutyl propanoate	C105680	144.2	1193.8	465.928	1.3608	0.17 ± 0.08^{c}	0.74 ± 0.15^{b}	1.04 ± 0.13^{a}	0.66 ± 0.15^{b}
Isobutyl 3-methylbutanoate	C589593	158.2	1186.9	457.668	1.3894	0.81 ± 0.13^{a}	0.83 ± 0.19^{a}	0.14 ± 0.08^{c}	0.59 ± 0.15^{b}
Ethyl isobutyrate	C97621	116.2	976.9	283.805	1.5609	0.49 ± 0.13^{b}	1.50 ± 0.23^{a}	0.52 ± 0.14^{b}	0.23 ± 0.12^{b}
Esters (17)						17.31 ± 0.46^{b}	20.30 ± 0.56^{a}	12.72 ± 0.48^{c}	9.68 ± 0.17^{d}
Ethanol 2-butoxy	C111762	118.2	1444.1	963.755	1.2047	0.29 ± 0.13^{a}	0.03 ± 0.00^{b}	0.04 ± 0.00^{b}	0.04 ± 0.00^{b}
2,6-Dimethylpyridine-M	C108485	107.2	1252.3	546.858	1.0947	1.39 ± 0.12^{a}	0.89 ± 0.10^{b}	0.94 ± 0.13^{b}	0.58 ± 0.08^{b}
2,6-Dimethylpyridine-D	C108485	107.2	1253.4	548.548	1.4472	0.88 ± 0.10^{a}	0.57 ± 0.09^{b}	0.26 ± 0.08^{bc}	0.12 ± 0.07^{c}
2-ethoxyethanol	C110805	90.1	1273.5	579.537	1.0938	0.46 ± 0.08^{b}	0.35 ± 0.09^{c}	1.03 ± 0.11^{a}	1.04 ± 0.10^{a}
2-pentylfuran	C3777693	138.2	1239.7	528.264	1.2552	1.65 ± 0.13^{a}	1.20 ± 0.11^{b}	1.11 ± 0.10^{b}	0.74 ± 0.12^{c}
butylcyclohexane	C1678939	140.3	1078.7	350.148	1.2627	0.76 ± 0.10^{a}	0.79 ± 0.11^{a}	0.47 ± 0.08^{b}	0.28 ± 0.02^{c}
(Z)-beta-ocimene	C3338554	136.2	1254	549.335	1.2201	0.24 ± 0.09^{c}	1.04 ± 0.11^{b}	2.55 ± 0.21^{a}	2.45 ± 0.26^{a}
2,5-dimethylfuran	C625865	96.1	946.9	270.359	1.3762	1.69 ± 0.23^{c}	2.43 ± 0.25^{b}	2.36 ± 0.17^{b}	4.12 ± 0.31^{a}
beta-myrcene	C123353	136.2	1153.2	420.181	1.6262	0.07 ± 0.01^{c}	0.45 ± 0.10^{b}	0.05 ± 0.00^{c}	1.46 ± 0.13^{a}
Others (9)						7.42 ± 1.43^{b}	7.74 ± 0.42^{b}	8.81 ± 0.32^{b}	10.83 ± 0.10^{a}

Note: SG was single microbial fermentation and SB was its control; MG stands for mixed microbial fermentation and MB was its control; All data are the mean ± standard deviation of three replicates. Means followed by different letters within the same column are significantly different ($P < 0.05$) from each other; D for dimer, M for monomer.

all four groups of samples, mainly including butanal, propanal, 2-methylbutyraldehyde, and hexanal. Among them, hexanal is a typical substance with a beany flavor in fermented soybean products and has a low threshold value, so it has a great impact on the overall flavor of DCB (Wang et al., 2021). 2-methylbutyraldehyde is an aldehyde compound with a high concentration and a coffee and nutty flavor in DCB and mainly generated by the Strecker reaction of amino acids or degraded by microorganisms (Han et al., 2013). The concentration of 2-methylbutyraldehyde was the highest in SG samples because the higher fermentation temperature of SG samples during the pre-fermentation stage promoted the Strecker degradation of amino acids and thus increased the concentration of 2-methylbutyraldehyde.

Ketone compounds can be produced through amino acid degradation or the Maillard reaction, and they contribute to the reduction of fishy odors in DCB (Li et al., 2021). Ketone compounds including acetoin, 2-pentanone and 2-heptanone were detected in DCB samples. Acetoin is an important flavor substance and also a precursor of TTMP. Its relative concentration in SG was higher than that in MG, because the competition relationship between *yeasts* and *Bacillus subtilis* for the nitrogen source in MG samples resulted in the reduction in the production of acetoin compared to that in SG samples (Fan et al., 2021). 2-pentanone presents an apple flavor. 2-heptanone is one of the products of linoleic acid degradation and endows DCB with a cheesy flavor.

Esters are generated by esterification between alcohols and fatty acids during fermentation and have a low flavor threshold and a fruity flavor (Hu et al., 2022). The concentration of esters was the highest in MG, followed by SG, and the lowest in the control group. Most of these esters were ethyl esters, which contributed prominently to the flavor of DC (Han et al., 2013). The relative concentration of ethyl acetate in the enhanced fermentation group was higher and ethyl acetate was significantly positively correlated with *Bacillus* spp. (Zhao et al., 2022). The contents of ethyl isobutyrate and ethyl butanoate in MG were significantly higher than those in other groups and they both had fruity flavors and lower thresholds, which made the taste of MG samples better. The result provided the basis for the flavor improvement in the subsequent enhanced fermentation of DCB.

Alcohols endow DCB with a typical sauce flavor of black soybeans (Nie et al., 2022), and originate from the microbial fermentation and metabolism of amino acids and sugar compounds (Liao et al., 2023). Fifteen alcohols were detected in DCB samples. The relative concentrations of most of alcohols were the highest in MG because alcohols were the metabolites mainly generated by *yeasts* through glycolytic metabolism, decarboxylation reactions, or amino acid deamidation (Chen et al., 2023). The content of ethanol was the highest, but its odor threshold was relatively high, so it was not considered as an important contributor to the overall flavor of DC (Hu et al., 2022). Linalool had a lower odor threshold and presented floral and orange odors, which made the flavor of DCB even more intense. Moreover, the content of linalool in MG was significantly higher than that in other groups, thus endowing MG with the better flavor profile.

The species of acids and pyrazines detected in DCB samples were less, but they also contributed to the flavor of DCB. Acids originate from various pathways such as *bacterial* metabolism and amino acid degradation (Tan, 2021). Butyric acid and isobutyric acid with an irritating odor were detected in all four samples. The concentrations of butyric acid and isobutyric acid in MG were lower than those in SG, indicating that MG might yield the better flavor. Pyrazines with a cocoa or nutty aroma originate from non-enzymatic browning and microbial action and are essential to the flavor of DCB (Wang, Wen, et al., 2023). Six pyrazines, including TTMP and 2,3,5-trimethylpyrazine, were detected in the samples. Among six pyrazines, TTMP had the higher concentration. The concentration of TTMP showed no significant difference between SG and MG samples, but the concentration of TTMP in SG and MG samples were higher than that in the control group, suggesting that enhanced fermentation could accumulate TTMP. In addition, 2-n-pentylfuran has a soybean and grassy flavor and laurin has a floral and sweet aroma (Lin et al., 2022).

SG and MG increased the contents of aldehydes, alcohols, esters, acids, pyrazines, and other volatile compounds in DCB. Among them, esters and alcohols in MG were significantly increased. These volatile compounds jointly endowed DCB with a unique taste and flavor. The results suggested a way to improve the flavor quality of DCB.

3.7.2. *Clustered heat map of volatile compounds of DCB*

In order to explore the similarity and differences among samples, the detected volatile compounds were analyzed with PCA and hierarchical clustered heat maps. The four samples were well differentiated from each other (Fig. 2A). The closer distance between two samples indicated the more similarity in the flavor substance composition between them. The similarity between SB and MB samples was higher, but the

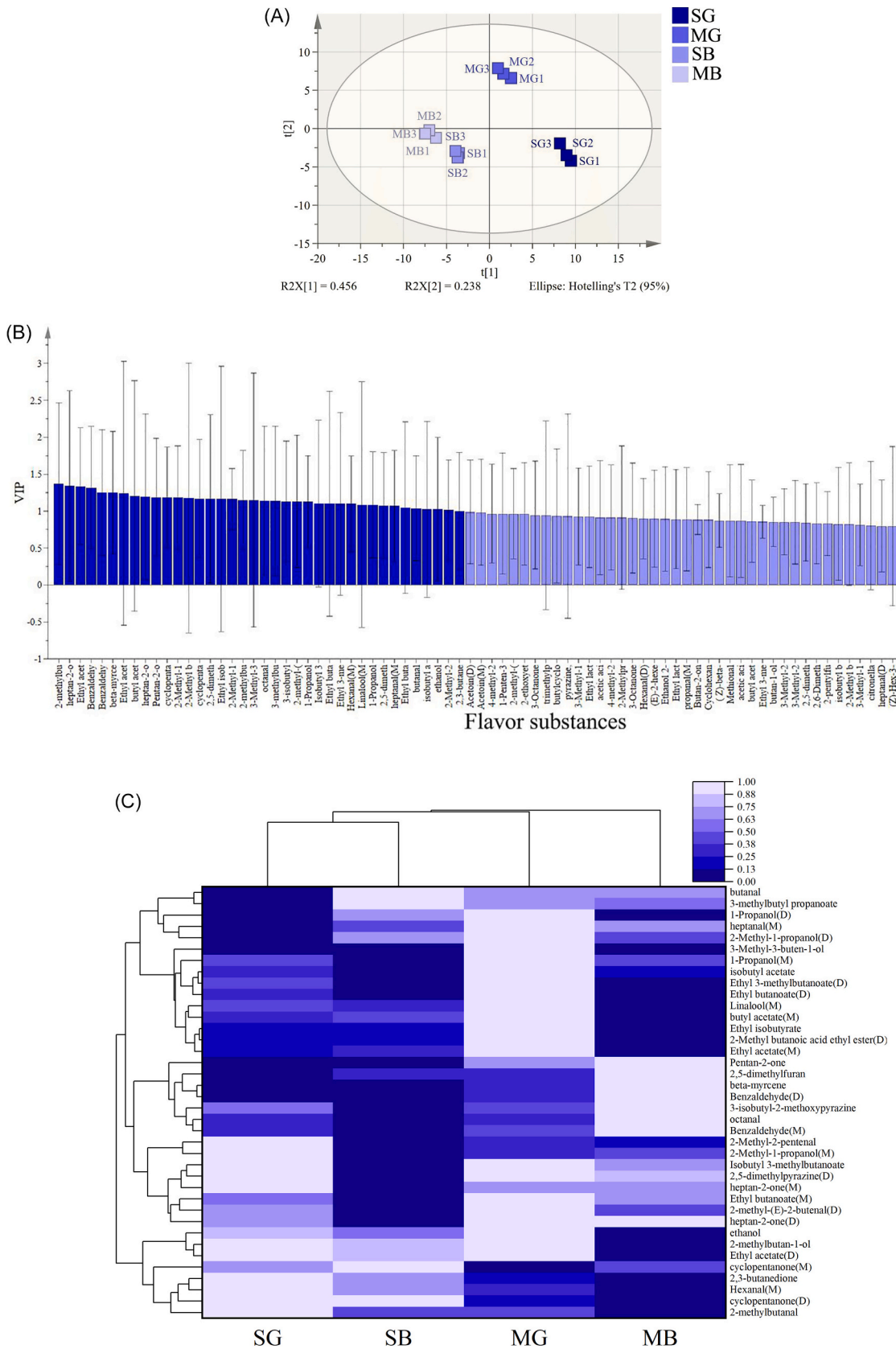

Fig. 2. Flavor substances of (A) PCA analysis, (B) VIP and (C) clustering heat map in different samples. SG was single microbial fermentation and SB was its control; MG stands for mixed microbial fermentation and MB was its control.

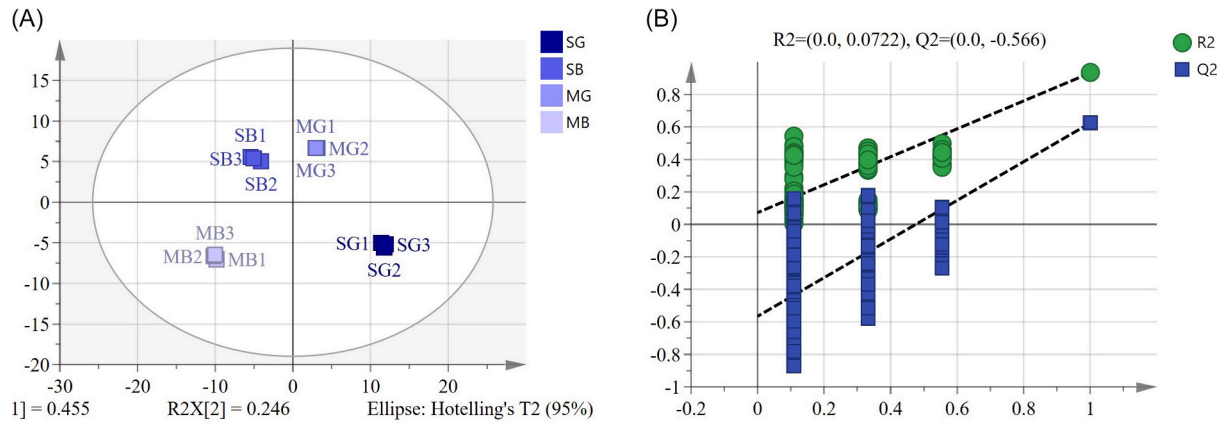

Fig. 3. All indicators of (A) PCA analysis and (B) plot of permutation test in different samples. SG was single microbial fermentation and SB was its control; MG stands for mixed microbial fermentation and MB was its control.

differences among other samples were significant. Variable importance in projection (VIP) was further used to screen out the key markers that had important influences on the flavor aroma profile of DCB. With VIP > 1 as the screening criterion, a total of 38 flavor substances were obtained (Fig. 2B). In order to further visualize the key volatile flavor compounds in different DCB samples, the relative content data of 38 flavor compounds with VIP > 1 were clustered in a heat map (Fig. 2C). Some differences were found among the four samples. Among the 38 selected flavor compounds, 20 flavor compounds contributed to MG and esters were the most abundant. Eleven flavor compounds contributed to SG and ketones were the most abundant. In contrast, fewer flavor compounds contributed to their control groups, including 8 flavor compounds in MB and 4 flavor compounds in SB.

3.8. Principal component analysis

The principal component analysis was performed with the data of basic physicochemical indexes, including the concentrations of TTMP, acetoin, taste substances, organic acids, free amino acids, free fatty acids, and volatile compounds in different samples (Fig. 3A). The R2X [1] = 0.455 and the R2X [2] = 0.246, indicating that the model samples had good reliability, and that the samples could be clearly distinguished from each other. The cross-validation analysis was performed with 200 permutation tests to verify the reliability of the model (Fig. 3B). The intercepts of R2 and Q2 were respectively 0.0722 and − 0.566, indicating that there was no overfitting problem in the model and the OPLS-DA model was reliable (Zhang, Huang, et al., 2024). Therefore, the original model could interpret the differences between the samples. On the basis of principal component analysis, the components with eigenvalues greater than 1 were selected as principal components and 3 principal component factors were extracted based on the cumulative contribution rate. The contribution rates of PC1, PC2, and PC3 variance were 40.68 %, 33.11 %, and 26.21 %, respectively. The cumulative contribution rate of the three principal components was as high as 100 %, indicating that 100 % of the eigenvalues of variables could be interpreted with the three principal components. According to the composite scoring model, the fermentation samples of the top three categories of comprehensive quality were MG (0.54), SG (0.45), and MB (−0.36). The higher composite scores indicated that the samples had the better comprehensive quality.

In conclusion, Through mixed fermentation with *Bacillus subtilis* S2–2 and *Hyphopichia burtonii* S6-J1, the flavor and quality of DCB were improved, and it had better comprehensive quality. The data provided the theoretical basis for the subsequent directional regulation of DCB production.

4. Conclusion

In the study, *Bacillus subtilis* S2–2 and *Hyphopichia burtonii* S6-J1 were used to optimize the pre-fermentation process of DCB and the optimized conditions were adopted to prepare DCB by single or mixed microbial enhanced fermentation. The quality characteristics of DCB samples were analyzed. Optimization results of the pre-fermentation process of DCB revealed that the TTMP concentration in single microbial fermentation was 10.9 % higher than that obtained before optimization. The TTMP concentration in mixed microbial fermentation was 21.4 % higher than that obtained before optimization. The concentrations of taste substances, organic acids, free amino acids, fatty acids, and volatile flavor compounds in enhanced fermentation samples, especially MG, were higher than those in the control group. MG also reduced the irritating odor caused by butyric acid and improved the comprehensive taste of DCB. Based on PCA results, the mixed microbial fermentation group was clearly differentiated from other groups and had the highest comprehensive score. In conclusion, TTMP concentration in DCB could be increased to improve the quality of DCB by inoculating *Bacillus subtilis* S2–2 and *Hyphopichia burtonii* S6-J1. Therefore, the study provided the theoretical basis and technical support for obtaining high-quality DCB starter and promoting the industrial production of DCB.

CRediT authorship contribution statement

Panpan Yang: Writing – review & editing, Writing – original draft, Software, Formal analysis, Data curation. **Qin Wang:** Methodology, Formal analysis, Data curation. **Yurou Yang:** Methodology, Data curation. **Anyan Wen:** Writing – review & editing, Resources, Funding acquisition. **Haiying Zeng:** Methodology, Investigation, Funding acquisition. **Na Liu:** Resources, Project administration. **Likang Qin:** Resources, Project administration, Methodology, Conceptualization.

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

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.102037.

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