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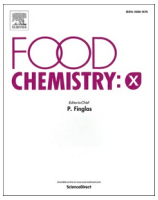

Characterization of key aroma compounds in gray sufu fermented using Leuconostoc mesenteroides subsp. Mesenteroides F24 as a starter culture

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ABSTRACT

Gray sufu is a traditional fermented bean product with strong flavor in China, but traditional fermentation methods often lead to its off-flavor. This study was performed to investigate the flavor quality characteristics of gray sufu fermented using *L. mesenteroides* F24. Results showed 220 volatile compounds in gray sufu, among which alcohols and esters were the main volatiles. Inoculation with *L. mesenteroides* F24 considerably affected the contents of flavor substances in gray sufu and substantially increased the main flavor compounds. In addition, 29 kinds of key volatile compounds were identified by analyzing the ROAVs. Four unique key flavor substances were found in gray sufu inoculated with *L. mesenteroides* F24. This study is the first report on the feasibility of *L. mesenteroides* F24 as a promising starter culture to improve the flavor quality of gray sufu. The results provide a theoretical basis for improving the processing and quality control of gray sufu.

1. Introduction

Gray sufu is a very distinct traditional fermented soybean product in China due to its unique flavor and creamy taste. This food is a popular side dish served with breakfast rice or steamed bread (Han, Rombouts, & Nout, 2001). Gray sufu is traditionally produced from salted pehtzes after their infusion with brine and tofu yellow serofluids, which are residues from making tofu (Hesseltine and Wang, 1986). These brines comprise sulfurous compounds, esters, alcohol, aldehydes, polyphenols, and aromatic compounds, which exhibit strong flavor properties (Sun et al., 2020). Moreover, given its diverse biological active substances (e.g., isoflavandiol, amino acids, and vitamin B_{12}), the soybean and its fermentation process also provide gray sufu with various nutrients, which render it with oxidation resistance and capability to lower blood pressure and cholesterol and reduce bone loss (Gu et al., 2018).

Aroma is one of the most important quality attributes that contribute to the quality of gray sufu. The acceptance of gray sufu by the consumers is also dependent on the aroma. Hence, studying aroma characteristics is critical to improving the flavor and quality of this food (Wang et al., 2020). Its off flavor is the most important quality issues with gray sufu; it results from various factors, such as raw materials, fermentation time, fermentation temperature, and air quality, during fermentation. Therefore, investigating the favorable characteristics of gray sufu helps in improving its product quality, production processes, and food safety. Microbial regulation-based modern fermentation technology can control precisely the fermentation of gray sufu, and the results are critical to enhancing product quality and safety compared with traditional spontaneous fermentation. However, gray sufu is currently produced through an open fermentation process, which introduces numerous uncertainties, especially unpredictable microorganisms. The lack of high-quality starters is a significant bottleneck restricting the industrialization of gray sufu production. Thus, enterprises are actively exploring for a suitable starter to improve the flavor of gray sufu. Although previous studies have shown that microorganisms play a crucial role in the formation of the flavor of fermented soybean paste, rice wine and other fermented foods, their findings were based on unproven correlation analysis (An et al., 2020; Yang et al., 2021; Qian et al., 2023), and no verification experiment using a single strain as a starter has been performed.

Leuconostoc is found in various fermented foods, such as kimchi (Seo,

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Park, Kim, Lee, Na, & Son, 2018), pickled chili peppers (Ye et al., 2022), fermented coffee (Thayanna et al., 2022), and suancai (He et al., 2020). He et al. found significant positive correlation between *Leuconostoc* and several volatile compounds (VOCs), such as 1-hexanol, *cis*-hept-4-enol, 1-octanol, linalool, phenylethyl alcohol, 3-phenylpropanol, and 2,5-dimethyl-benzaldehyde. In our previous studies, *Leuconostoc* was the dominant bacterium during the fermentation of gray sufu (Sun et al., 2020). Therefore, we speculated that *Leuconostoc* may contribute significantly to its flavor. To confirm our hypothesis, we isolated a new strain of *Leuconostoc* from gray sufu and identified it as *Leuconostoc mesenteroides* subsp. *mesenteroides* F24 (*L. mesenteroides* F24). To our best knowledge, *Leuconostoc* has not been investigated as an adjunctive starter in the processing of gray sufu.

With the advancement of flavoromics, more interactions between flavors and VOCs of foods have been decoded. The most commonly used method for separating and quantifying VOCs from foodstuffs is gas chromatography-mass spectrometry (GC–MS) (Liao et al., 2020). Wang et al. (2020) detected 55 VOCs in unfermented stinky tofu brine using solid-phase microextraction (SPME-)-GC–MS. Meanwhile, An et al. identified the key flavor compounds in traditional fermented soybean paste of Northeast China using SPME-GC–MS. The results showed the feasibility of SPME-GC–MS to detect VOCs of gray sufu. Therefore, the main objectives of the current study were as follows: to identify the aroma compounds in gray sufu fermented by *L. mesenteroides* F24 using GC–MS; to assess the aroma contributions of these compounds to gray sufu fermented using *L. mesenteroides* F24 through quantification of aroma-active compounds and calculation of relative odor activity values (ROAVs); to determine the key aroma compounds in gray sufu fermented by *L. mesenteroides* F24 via aroma recombination and omission tests. The findings can potentially help elucidate the aroma-active compounds in gray sufu and provide a solid basis for *L. mesenteroides* F24 as an excellent adjunctive starter for gray sufu.

2. Materials and methods

2.1. Preparation and collection of gray sufu

The preparation of gray sufu was mainly divided into early and end fermentation. During the early fermentation, fresh soybean curds sprayed with a certain proportional mixed suspension of *Mucor* sp. and *Rhizopus microsporus* at the ratio of 1:1 (v/v) were placed on sterile wooden trays in a fermentation chamber with a controlled temperature of 28 °C and relative humidity (80%). After 7 days of fermentation, the curds were immersed in salt water (20%) to produce fresh sufu pehtzes. At the end of fermentation, the pehtzes were placed in a 350 mL glass jar containing a mixture of brine and tofu yellow serofluids, which are the soy whey residues obtained from making tofu. The adjunctive starter (*L. mesenteroides* F24) was added to the mixture of brine and tofu yellow serofluids at an initial cell density of approximately 10^6 CFU/mL. Two different batches were prepared using (1) spontaneous fermentation (group S) and (2) inoculation with *L. mesenteroides* F24 (group L). Then, the jars were sealed and kept at 28 °C for 28 days to complete the fermentation. Fresh pehtzes (0 days) and gray sufu were taken at random at days 7, 14, 21, 28, and 28 of fermentation. The mixture of brine and tofu yellow serofluids and pehtzes came from Changchun Zhu Laoliu Food Co., Ltd. Then, the samples at each time point were ground separately. All samples were stored at − 80 °C, and sample analyses were completed within two weeks.

2.2. Extraction of volatile compounds

VOCs from the samples were extracted using the method described by An et al. (2020), with several modifications. The frozen samples were ground, weighed, mixed with 10 μL of the internal standards (i.e., 2-octanol [40 μg/mL] and carbetamide [100 μg/mL]) per tube, and transferred to 10 mL gas-tight glass vessels. A carboxen/divinylbenzene/polydimethylsiloxane solid-phase microextraction (CAR/PDMS SPEM) tip (Agilent Technologies, USA) was inserted into each tube and incubated at 40 °C for 30 min. The fiber was then withdrawn and injected into the gas chromatograph.

2.3. GC × GC-TOF-MS analysis

An Agilent 7890A gas chromatograph equipped with a LECO Pegasus 4D mass spectrometer was used in this experiment. The VOCs were separated using a DB-WAX (30 m × 0.25 mm × 0.25 μm) and a DB-17MS (2 m × 100 μm × 0.10 μm) with an oven temperature programmer held at 40 °C for 3 min, ramped at 5 °C/min to 250 °C, and held for 5 min. The carrier gas was helium (>99.99%) with a constant flow rate of 1.0 mL/min. The mass spectrometer conditions were an electron ionization mode and an electronic energy of 70 eV. The interface temperature was set to 270 °C, the ion source temperature was 250 °C, and the mass scan range was 33–500 AMU.

2.4. Identification and quantification of aroma compounds

Each VOC was identified by comparing its mass spectrum from database libraries (NIST). Retention indices (RIs) and retention times were assessed with the data available in the published literature and online library. The VOCs were semi-quantitatively identified based on their peak areas relative to those of the internal standard. All samples were analyzed in triplicates.

2.5. Characterization of key aroma compounds

The ROAVs explain the contribution of each VOC to the aroma profile. Components with ROAV $\geq$ 1 were generally accepted to contribute to the overall flavor of the sample, whereas components with 0.1 $\leq$ ROAV < 1 had an important modifying contribution (Zhang et al., 2020). The ROAV was calculated according to Equation 1 and 2.

$$ROAV = \frac{OAV}{OAV_{max}} \times 100 \quad (1)$$

$$OAV = \frac{C}{T} \quad (2)$$

where OAV is the odor activity value (OAV) of an arbitrary VOC; OAV_{max} is the maximum OAV of all compounds in the sample; C and T are the detection and threshold concentrations of an aroma compound, respectively. Among them, the thresholds were taken from https://pubchem.ncbi.nlm.nih.gov/compound/.

2.6. Quantitative descriptive sensory analysis

Quantitative descriptive sensory analysis was conducted as described by Chen et al. (2021), with minor modifications. Ten panelists (five men and five women; average age: 27 years old) who have been familiar with the fermentation and flavor of gray sufu for a long time have been trained regularly to evaluate the sensory quality of gray sufu. Eight relevant flavor attributes, namely, fishy, fruity, gasoline, pungent, rancid, butter, sweet, and herbal, were selected after the panel discussion. Definitions and references for these terms were based on the ROAV analysis. The odor intensities were defined as 0 = no smell, 1 = very weak, 2 = weak, 3 = medium, 4 = strong, and 5 = very strong. The average of the final scores of the sensory group indicated the flavor profile of gray sufu. Each experiment was repeated thrice at room temperature.

2.7. Aroma recombination

Deodorized matrixes were prepared as described by Chen et al. (2021), with some modifications. VOCs with ROAVs $\geq$ 1 and the same

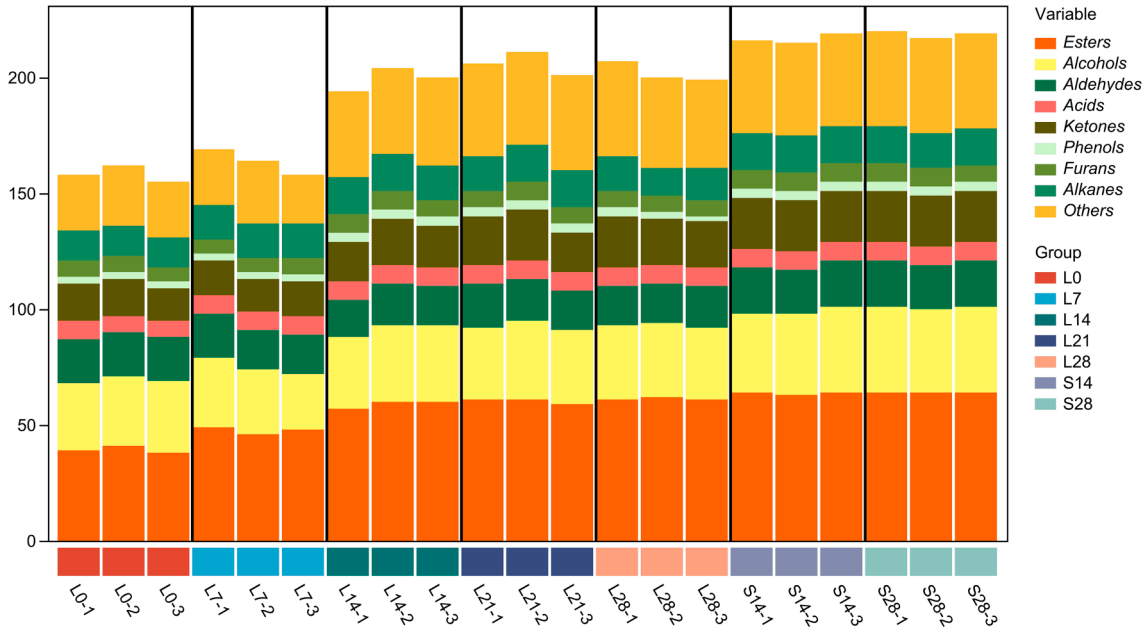


Fig. 1. Relative abundance of each volatile compound in gray sufu samples.

amount of high-content VOCs were added to the deodorization matrix for recombinant models 1 and 2, respectively. The models were equilibrated at room temperature for 10 min. Then, the intensities of the eight different odor types produced by the chemicals were evaluated. Odor intensity was evaluated as described in Section 2.6. The recombinant samples were encoded and placed at room temperature, and the evaluators were instructed to sniff the samples for 90 s for evaluation. Each sample was assessed thrice at 10-min interval. The results were plotted in the form of a spider web diagram.

2.8. Omission experiments

A triangle test was used to select the VOCs that contributed greatly to the flavor of gray sufu (Tan et al., 2021). We omitted one or more VOCs from the recombinant model, including two complete recombinant models and one missing model. The omission model and complete recombination models were evaluated by ten panelists to select a model that differed considerably from the other two models.

2.9. Statistical analysis

All results from independent triplicates were expressed as mean ± standard deviation. Heatmaps were drawn using pheatmap (v3.3.2) in R. Data were analyzed using Graphpad Prism 8. For comparison between three or more samples, one-way analysis of variance followed by Tukey's multiple comparison tests were used. In addition, $P < 0.05$, $P < 0.01$, and $P < 0.001$ were used to denote significance, high significance, and very high significance, respectively.

3. Results and discussion

3.1. GC × GC-TOF-MS analysis of VOCs in gray sufu fermented by L. Mesenteroides F24

The concentrations of VOCs in gray sufu with spontaneous fermentation and inoculated with *L. mesenteroides* F24 were measured by headspace SPME (HS-SPME) and GC × GC-TOF-MS (Supplementary Table S1). A total of 220 VOCs were detected in gray sufu in spontaneous fermentation (group S) and inoculation with *L. mesenteroides* F24 (group L) at different fermentation periods. These VOCs can be divided into nine categories based on their chemical structures (Fig. 1). The structures and numbers of the chemicals detected in gray sufu on the 28th day of fermentation in group S were as follows: esters, 64; alcohols, 37; ketones, 22; aldehydes, 20; acids, 8; phenols, 4; furans, 8; alkanes, 16; and others, 41. Meanwhile, the following compounds were detected on the 28th day after the addition of *L. mesenteroides* F24: esters, 61; alcohols, 32; ketones, 20; aldehydes, 17; acids, 8; phenols, 3; furans, 7; alkanes, 14; and other compounds, 39. As shown in Fig. 1, ester and alcohol compounds were the dominant VOCs, and they accounted for 29.09% and 16.82% of the VOCs in group S and 30.34% and 15.92% of those in group L, respectively. Esters and alcohols are the most abundant volatiles in sufu products according to Chen et al. (2022).

Among all flavor chemicals, esters account for>60% of the total mass of flavor substances. Given that most esters have low odor thresholds, they may contribute remarkably to odor (Xu et al., 2022). Esters are formed by lower saturated fatty acids and saturated fatty alcohols, which can mainly endow fruit, flower, sweet, and milk flavors to fermented food (Sidira, Kandylis, Kanellaki, & Kourkoutas, 2016; Xu et al., 2022). On the 28th day of fermentation, esters exhibited the highest types and contents (Figs. 1 and 2). The contents of ester compounds (e.g., ethyl acetate [fruity], ethyl 4-methylpentanoate, methyl acetate [fruity], ethyl butyrate [fruity], methyl 4-methylpentanoate, and propyl butyrate [cream] [Table S1]) in group L after 28 days of fermentation were significantly higher than those in group S. These results indicate that inoculation with *L. mesenteroides* F24 enhanced ester synthesis and allowed the gradual accumulation of esters during fermentation, which provided gray sufu with a fruitier and creamier aroma (Zhong et al., 2021).

Alcohols, including ethanol, isopropyl alcohol, 1-propanol, 1-octanol, 1-heptanol, 1-hexanol, 1-pentanol, and 3-pentanol, were the second most widely detected compounds (Fig. 2). In this study, these alcohols showed higher contents during the fermentation of gray sufu. Yang et al. (2020) also observed that these alcohols are the main VOCs in raw soymilk. Ethanol was the most abundant alcohol compound produced during the fermentation of gray sufu. During fermentation, the change in volatiles and the accumulation of odor were mainly caused by three biochemical pathways, namely, ethanol fermentation metabolism, amino acid catabolism, and fatty acid catabolism. The biosynthesis of volatiles derived from ethanol fermentation was responsible for the "fermented" off-flavors (Xuan et al., 2022). By contrast, the introduction

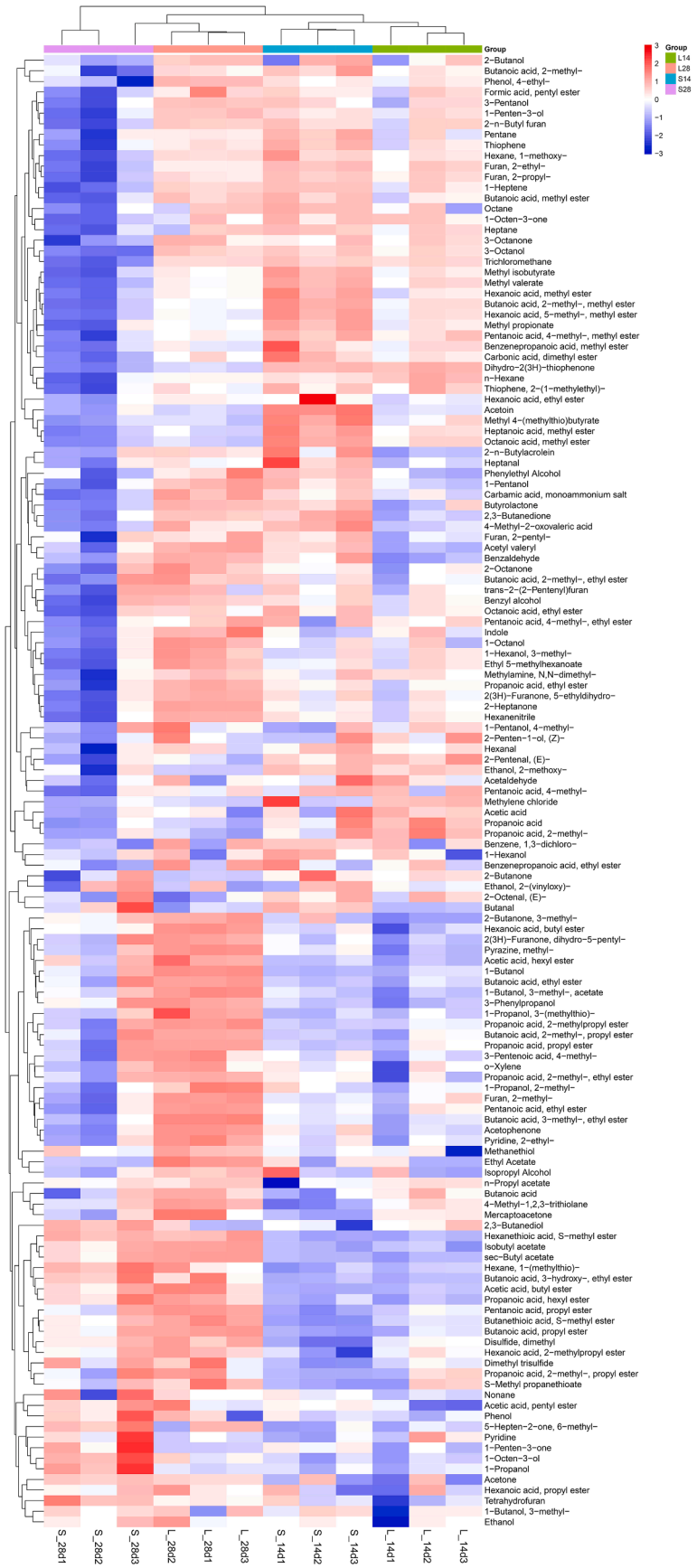


Fig. 2. Heatmap and cluster analysis of volatile flavor compounds of gray sufu. Blue and red areas represent the minimum and maximum contents, respectively.

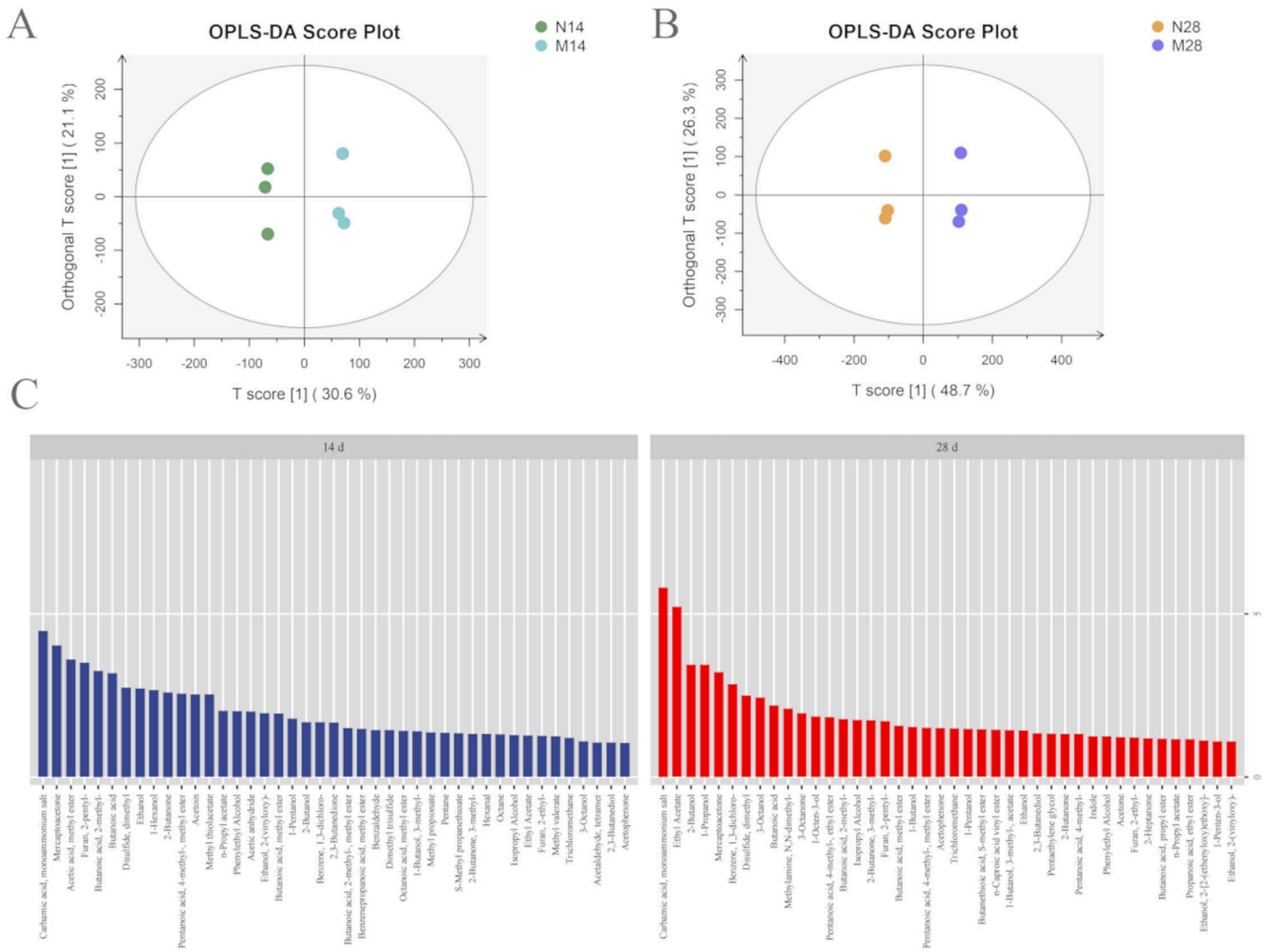


Fig. 3. Orthogonal partial least square discriminant analysis of groups L (inoculation with *L. mesenteroides* F24) and S (spontaneous fermentation) on (A) day 14 and (B) day 28 of fermentation; (C) the VIP value of the differential metabolites.

of *L. mesenteroides* F24 imparted a strong green and buttery aroma and significantly increased the contents of 1-octanol (green), 1-heptanol (herbal), 1-hexanol (fruity), and 1-penten-3-ol (fruity).

1-Octen-3-one (herbal), 2-octanone (cheese), 3-octanone (herbal), acetone (sweet), and acetophenone (floral) were the main ketone compounds detected during the fermentation of gray sufu. Ketones are mainly produced following the Maillard reaction and thermal modification of amino acids in food and oxidation of free fatty acids (Sevindik et al., 2022). Moreover, aldehyde contents, such as those of benzaldehyde (bitter almond), hexanal (leafy), nonanal (lime), and octanal (fruity), increased during the fermentation of group L. The formation of aldehydes may be related to the oxidative degradation of unsaturated fatty acids and the Strecker degradation of amino acids. In addition, microbial aminotransferase can convert free amino acids to α-ketoacids, which are further degraded to the corresponding aldehydes by various decarboxylases during fermentation (Ye et al., 2022). Therefore, such process may be the action mechanism of *L. mesenteroides* F24 that led to the accumulation of aldehyde compounds.

The volatile acids found in all samples were mainly acetic acid (sour), butanoic acid (penetrating and obnoxious odor), 2-methylbutanoic acid, propanoic acid (rancid odor), and isobutyric acid. Acids were the microbial metabolites produced during fermentation; they were responsible for the fruity, fatty, and sour aromas and contributed significantly to the sensory properties of gray sufu. During fermentation, the acid content gradually increased, possibly due to the oxidation of alcohols and hydrolysis of esters (Zhang et al., 2021). The contents of butanoic acid and 2-methylbutanoic acid in group L were higher than those in group S, possibly because branched-chain amino acids can be converted into organic acids through the first step of transamination after oxidation (Sevindik et al., 2022). Five phenolic substances, namely, phenol (medicinal), guaiacol (smoky), *N*-methyltyramine, 4-ethylphenol (woody), and 4-ethyl-2-methoxyphenyl (spicy), were also identified in this study. These compounds imparted a smoky, herbal, and spicy aroma to gray sufu (Sun et al., 2021).

Eight furan compounds were detected in gray sufu. Furans are mainly derived from the intermediate or final products of Maillard reactions between sugars and amino acids, primarily in fruity and smoky aroma (Sun et al., 2021). Hydrocarbons, which are possibly derived from the oxidation and degradation of fatty acids, are often aromatic and sweet, but higher thresholds, especially alkanes, have minimal contribution to the overall flavor (Ye et al., 2022). In addition, sulfur compounds, such as dimethyl disulfide, dimethyl sulfide, and dimethyl trisulfide, had been reported to act as aromatic components and precursors in reactions to produce more complex aromatic compounds. The contents of dimethyl disulfide, dimethyl sulfide, and dimethyl trisulfide were significantly higher in group L than in group S, and these compounds contributed to the production of more flavor substances during fermentation. Pyrazine is an important compound responsible for food flavor, and its flavor threshold is low. This compound contributed greatly to the formation of gray sufu flavor and mainly projected meaty,

Table 1
Aroma profile, odor characteristic, and the aromatic compounds in the gray sufu by HS–SPME–GC–MS.

No	[a]Compound CID	Aroma compound	[b]Odor characteristic	[c]Odor Threshold (mg/kg)
1	31,276	3-Methylbutyl Acetate	fruity, banana, sweet, fragrant	0.017
2	8103	1-Hexanol	fruity, ethereal, green, flower, sweet	0.2
3	6569	2-Butanone	fruity, ethereal, ether, camphor	50
4	5,364,752	Trans-2-Pentenal	pungent green, fruity	–
5	7710	5-Pentyloxolan-2-One	a coconut-like	–
6	19,707	2,3-Hexanedione	fruity, creamy, oily, sweet, fatty, caramel, butter	–
7	70	4-Methyl-2-Oxovaleric Acid	fruity	–
8	177	Acetaldehyde	pungent, fruity, suffocating, fresh, green	0.21
9	31,272	Butyl Acetate	fruity	0.31
10	8908	Hexyl Acetate	sweet, fruity	–
11	6584	Methyl Acetate	fruity	180
12	12,348	Pentyl Acetate	banana-like	0.26
13	7643	Methyl 3-Phenylpropionate	fruity, wine, floral sweet	–
14	11,552	3-Methylbutanal	apple-like	0.06
15	24,020	Ethyl 2-Methylbutyrate	fruity	–
16	62,572	Ethyl 3-Hydroxybutyrate	fruity, grape, green	–
17	7945	Ethyl 3-Methylbutanoate	fruity, vinous, apple-like	–
18	7762	Ethyl Butanoate	pineapple	–
19	12,180	Methyl Butyrate	apple-like	–
20	7770	Propyl Butyrate	pineapple, apricot	–
21	8857	Ethyl Acetate	fruity, ethereal, green, anise, weedy, balsam, sweet	0.1
22	12,529	Pentyl Formate	fruity	–
23	19,602	2-Pentylfuran	fruity	–
24	7826	Methyl Heptanoate	fruity, orris	–
25	7775	Isobutyl Hexanoate	fruity, cocoa odor	–
26	12,294	Butyl Hexanoate	pineapple	–
27	7824	Methyl Hexanoate	pineapple, ethereal	–
28	12,293	Propyl Hexanoate	ethereal, pineapple-blackberry	–
29	8038	Isobutyl Acetate	fruity, floral	0.037
30	6544	Isophorone	fruity, cooling, green, leather, cedarwood, sweet	5.4
31	65,425	Methyl 4-(Methylthio) Butyrate	fruity, cabbage, pineapple, sulfury, cheese	–
32	7909	Methyl Isobutyl Ketone	fruity, ethereal	0.88
33	11,160	Methyl Isovalerate	fruity	50
34	7997	*n*-Propyl Acetate	mild, fruity odor	0.18
35	16,106,703	Pentanal	fruity, malt, bready, nutty, pungent	0.07
36	527	Propanal	suffocating, fruity	1
37	7342	Ethyl Isobutyrate	fruity odor	–
38	12,571	Propyl Isobutyrate	pineapple odour	–
39	10,895	Isobutyl Propionate	pineapple	–
40	7749	Ethyl Propionate	fruity, rum-like, ethereal odour	–
41	12,217	Pentyl Propionate	apple-like odor.	–
42	7803	Propyl Propionate	fruity (apple, banana, pineapple) odour	–
43	7758	*sec*-Butyl Acetate	fruity odor	–
44	8129	1-Heptanol	herbal, violet, green, leafy, coconut, sweet	0.2
45	61,346	1-Octen-3-one	herbal, earthy, metal, musty, mushroom, dirty	–
46	8051	2-Heptanone	herbal, fruity, soap, coconut, sweet, spicy	0.07
47	11,527	3-Octanol	herbal, mushroom, minty, nut, citrus, woody	–
48	246,728	3-Octanone	herbal, lavender, mushroom, fresh, herb, sweet	6
49	18,827	1-Octen-3-ol	raw, fishy, oily, earthy, fungal, chicken	–
50	1146	Trimethylamine	fishy, amine odor	0.6
51	261	Butanal	pungent, aldehyde	0.009
52	19,310	Dimethyl Trisulfide	cabbage, fish, sulfurous, cooked, sulfur, meaty	0.003
53	8125	1-Octene	gasoline	0.005
54	7500	Ethylbenzene	sweet, gasoline-like odor	0.09
55	8058	*n*-Hexane	gasoline-like odor	0.0064
56	8141	Nonane	gasoline, alkane	650
57	356	Octane	gasoline-like	150
58	12,020	1-Penten-3-ol	butter, pungent, tropical, horseradish, green	3
59	6590	Isobutyric Acid	sharp, butter	1
60	650	2,3-Butanedione	butter, caramel, oily, creamy, sweet, pungent	0.0086
61	179	Acetoin	butter	–
62	6560	2-Methyl-1-Propanol	solvent, ether, wine, bitter	40
63	31,265	Ethyl Caproate	wine-like odour	–
64	7799	Ethyl Octanoate	wine, brandy, fruity floral odour	–
65	8091	Methyl Octanoate	wine, fruity, orange	–
66	6568	2-Butanol	wine, sweet, oily, apricot	3.2
67	5,281,168	2-Hexenal	leafy, apple, cheesy, vegetable, fat, banana	–
68	5,283,324	Trans-2-Octenal	green-leafy odor	0.061
69	6184	Hexanal	leafy, fruity, sweaty, grass, fatty, aldehydic	–
70	996	Phenol	medicinal, acid, ink, creosote	5.5
72	8093	2-Octanone	fatty, green, cheese, floral, bitter, fruity	248
73	454	Octanal	fat, green, aldehydic, lemon, citrus, fatty	–
74	5,283,321	2,4-Heptadienal	fatty, green aroma	0.03

(*continued on next page*)

Table 1 (*continued*)

No	[a]Compound CID	Aroma compound	[b]Odor characteristic	[c]Odor Threshold (mg/kg)
75	9862	6-Methyl-5-Hepten-2-one	strong fatty, green citrus-like odour	–
76	12,756	Gamma-Caprolactone	herbaceous, sweet odour	–
77	11,124	Methyl Propionate	sweet, fruity, rum	50
78	6344	Methylene Chloride	sweet, pleasant odor, chloroform	214
79	7237	o-Xylene	sweet	1.8
80	7654	Phenethyl Acetate	sweet, rose, honey	–
81	180	Acetone	sweet, fruity, etherous	20
82	241	Benzene	bitter almond, fruit, vanilla	4.68
83	13,357	Methyl 2-Methylbutyrate	sweet, fruity, apple	–
84	10,885	Isobutyl Butyrate	a sweet, fruity, apple, pineapple	–
85	7302	Butyrolactone	a faint, sweet, caramel	20
86	7918	Acetic Anhydride	pungent, vinegar	1.44
87	6561	Isobutyraldehyde	pungent	0.3
88	8030	Thiophene	pungent	–
89	7150	Methyl Benzoate	a pungent, heavy, floral	–
90	261	Butyraldehyde	pungent, aldehyde	–
91	8005	1-Chlorobutane	pungent	1.64
92	62,444	Methyl Thiobutyrate	putrid, rancid, sour, pungent cabbage odor	–
93	1032	Propanoic Acid	pungent disagreeable, rancid	0.066
94	1049	Pyridine	putrid, rancid, sickening, fishy, sour, amine	0.95
95	8130	Heptanal	citrus, wine-lee, rancid, ozone, fatty, herbal	–
96	263	1-Butanol	oil, vanilla, fruit, fusel, sweet, balsam	0.5
97	31,260	Isoamyl Alcohol	oil, alcoholic, burnt, whiskey, malt, banana	0.1
98	62,465	4-Ethyl-2-Methoxyphenol	sweet, spicy, medicinal	–
99	957	1-Octanol	burnt, orange, rose, waxy, chemical, metal	24
100	6276	1-Pentanol	balsamic, vanilla, balsam, sweet, fusel, oil	0.4
101	31,234	3-Phenylpropanol	balsamic	–
102	1031	1-Propanol	alcoholic, fermented, alcohol, musty, fusel, pungent	7
103	522,834	2-Acetyl-1-Pyrroline	roast, nut, roasted, ham, sweet, nutty	–
104	11,251	3-Methyl-2-Butanone	acetone	2.5
105	5,364,920	Trans-2-Penten-1-ol	green	–
106	5,364,919	Cis-2-Penten-1-ol	green	–
107	5,284,503	3-Hexen-1-ol	grassy, green	–
108	11,747	2,3-Pentanedione	creamy, nutty, pungent, cream, sweet, butter	0.005
109	61,021	Metaldehyde	menthol	–
110	176	Acetic Acid	sour, vinegar, pungent	1.2

nutty, and roasted potato aromas. These compounds accumulated in the gray sufu in group L, which resulted in a more abundant flavor. Pyridine was generated later in fermentation. Pyridine forms when aldehydes and ketones are produced during lipid oxidation. Thus, the formation of pyridine is another consequence of the lipid oxidation pathway. Pyridine has a low threshold and various flavors. High concentrations of pyridine result in unpleasant pungent tastes, such as fishy, and its low concentrations lead to pleasant aroma characteristics, such as those of toast, corn, and cookies (Li et al., 2023).

Table 1 (*continued*)

No	[a]Compound CID	Aroma compound	[b]Odor characteristic	[c]Odor Threshold (mg/kg)
111	12,587	4-Methylpentanoic Acid	sour, penetrating	–
112	7410	Acetophenone	acacia, flower, must, pungent, hawthorn, almond	65
113	6054	Phenylethyl Alcohol	rose	7.02
114	240	Benzaldehyde	bitter almond, fruit, vanilla	0.05
115	244	Benzyl Alcohol	faint	5.5
116	7284	2-Methylbutyraldehyde	unpleasant	0.04
117	7523	2-Ethylpyridine	unpleasant	–
118	1068	Dimethyl Sulfide	wild radish, cabbage	0.0098
119	517,232	Carbamic Acid	ammonia	46.8
120	12,021	Dimethyl Carbonate	pleasant	–
121	6212	Trichloromethane	pleasant, etheric, nonirritating	192
122	7865	Methyl Formate	pleasant	2000
123	8452	Cyclopentanone	minty	–
124	264	Butanoic Acid	penetrating, obnoxious	1
125	13,852	Dihydro-2(3H)-Thiophenone	burnt, garlic	–
126	12,232	Dimethyl Disulfide	garlic, sulfurous, diffuse, intense onion	0.03
127	78,925	2-(Methylthio)Ethanol	meat	–
128	8019	2-Methoxyethanol	ether	0.22
129	8028	Tetrahydrofuran	ether	31
130	14,505	2-Acetylfuran	coffee	–
131	18,554	2-Ethylfuran	smoky, burnt	–
132	10,797	Methylfuran	spicy, smoky	–
133	8900	Heptane	ethereal, alkane, sweet	230
134	8027	Pyrrole	ethereal, nutty, warm, sweet	–
135	798	Indole	feces	0.5
136	878	Methanethiol	rotten cabbage	–
137	31,289	Nonanal	lime, grapefruit, fat, rose, green, fresh	0.0035
138	31,242	P-Ethylphenol	woody-phenolic, sweet	–
139	11,529	Butyl Propionate	earthy, sweet	–
140	88,454	Hexyl Propionate	earthy, acrid	–
141	31,252	2,5-Dimethyl Pyrazine	earthy, potato	1
142	521,869	S-Methyl Propanethioate	fruity, milk	–
143	7976	Methylpyrazine	nutty, cocoa	0.25
144	9256	Thiazole	nutty, pyridine, meaty	–
145	8200	Tetraethylene Glycol	mild	–

[a,b]Aromatic compounds CID and aroma descriptor were derived from the web pages: (https://pubchem.ncbi.nlm.nih.gov/compound/).
[c]Odor thresholds were taken from the web pages: (https://pubchem.ncbi.nlm.nih.gov/compound/) and (Lu et al., 2022).

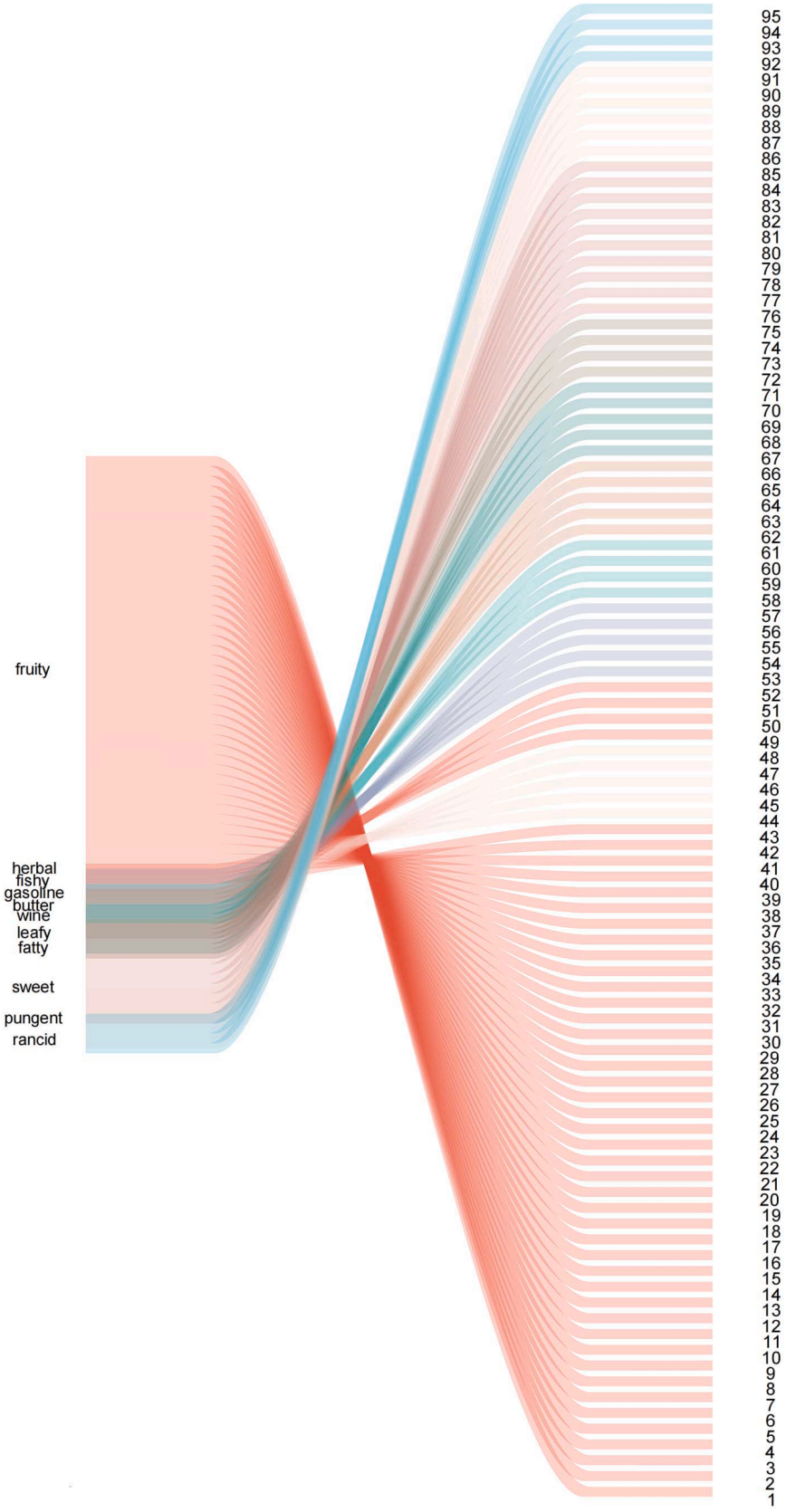


Fig. 4. Tracing diagram of flavor compound origin. Note: the numbered compounds in the figure are shown in Table 1.

3.2. Screening differential flavor metabolites in gray sufu fermented by F24

To further clarify the effect of adding *L. mesenteroides* F24 on the flavor of gray sufu, we analyzed the flavor compounds in groups S and L using the OPLS-DA model. As shown in Fig. 3A and 3B, group S was significantly distinct from group L when the fermentation was performed for 14 and 28 days. This result indicated that *L. mesenteroides* F24 had a significant effect on the flavor profile during spontaneous fermentation. In the current model, the values of R^2X, R^2Y, and Q^2Y were 0.517, 0.998, and 0.721 for 14 days, respectively, and the corresponding values for 28 days were 0.75, 0.999, and 0.936. These findings showed that the model fitted well, and the predictability was acceptable (Fig. 3A, B). However, given that a large amount of data in OPLS-DA were not useful for classification, they greatly interfered with the analysis, thereby reducing the discrimination capability of the model (Kang et al., 2022). Therefore, the use of variable importance in the projection (VIP) is an effective method for selecting key flavor substances. Variables with VIP values > 1 are generally perceived to have significant differences between different categories and play an important role in classification. Thus, differential flavor metabolites were screened using VIP values > 1. As shown in Fig. 3C, the VIP values of 43 and 42 VOCs were > 1 on the 14th and 28th days of fermentation, respectively. On the 14th day, compared with those in group S, nine flavor compounds in group L were upregulated, and the other 34 were downregulated. The key VOCs of group L included mercaptoacetone, butanoic acid, dimethyl disulfide, methyl thiolacetate, *n*-propyl acetate, 2-butanol, dimethyl trisulfide, and 3-methyl-2-butanone (VIP > 1), which may be responsible for the fruity, penetrating, obnoxious, sulfurous, cooked-meat, wine, sweet, oily, apricot, and acetone-like odors. These aromas enhanced the characteristic flavor of gray sufu. Most of the nine compounds were sulfur-containing compounds, which were produced by amino acid degradation or lipid metabolism (Elena et al., 2022). This result indicated that *L. mesenteroides* F24 had significant effects on amino acid degradation and lipid metabolism. Although the addition of *L. mesenteroides* F24 reduced the content of some alcohols, esters, and aldehydes in the middle stage of fermentation, these compounds were significantly higher than those in group S on day 28 of fermentation, and they enhanced the fruity aroma of gray sufu. Thus, a correlation existed between *L. mesenteroides* F24 and the formation of volatile flavor substances, such as alcohols, aldehydes, and esters, was indicated. This finding was consistent with the report of He et al. (2020) and Lopez et al. (2022) studied the bread fermented by *L. mesenteroides*. The results showed that *L. mesenteroides* fermented significant branched-chain amino acids and metabolites, among which the BCAA metabolite is also related to flavor compounds (e.g., 3-methylbutyraldehyde) (Lopez et al., 2022). This phenomenon may also be the main reason why *L. mesenteroides* promoted the accumulation of flavor in gray sufu.

3.3. Analysis of flavor compounds combined with flavoromics

A total of 220 VOCs were detected in all samples (Fig. 1). These VOCs were further analyzed, and the aroma activities of 142 compounds were determined from PubChem database. Table 1 shows the aroma characteristics and odor of gray sufu. A total of 142 compounds formed the final flavor profile of gray sufu, with 95 compounds exhibiting important aroma attributes for all samples (Fig. 4): nos. 1–43, important fruity aroma; nos. 44–48, herbal aroma; nos. 49–52, fishy odor; nos. 53–57, gasoline odor; nos. 58–61, butter aroma; nos. 62–66, wine aroma; nos. 61–71, leafy aroma; nos. 72–75, fatty aroma; nos. 76–85, sweet aroma; nos. 86–91, pungent odor; and nos. 92–95, rancid odor. Moreover, the compounds with fruity aromas were mostly esters and alcohols. Esters are mostly short-chain fatty acid esters, such as ethyl acetate and ethyl butyrate. The esters accumulated in the fermentation group inoculated with *L. mesenteroides* F24, which indicates that the introduction of *L. mesenteroides* F24 improved the flavor quality of gray sufu. Short-chain fatty acid esters are a group of low-molecular-weight chemicals that are mainly produced by the esterification of alcohols with short-chain fatty acids. In fermented foods, these esters are mainly synthesized by enzymes from the carboxylic ester hydrolase superfamily (EC 3.1.1.-) in microorganisms, thus forming a unique flavor that affects the sensory quality and flavor characteristics (Xu et al., 2021). In addition, the unique flavor of gray sufu has been attributed to its unpleasant odor, such as gasoline, pungent, and rancid smell, due to the presence of nitrogen and sulfur-containing compounds. Nitrogen- and sulfur-containing compounds mainly include protein and amino acids in the substrate, which are converted into derivatives, such as ammonia, pyrazine, pyrrole, thiazole, trimethylamine, and amine compounds by microbial metabolism during fermentation (Lu, Liu, & Xu, 2022). Most of these flavor compounds also accumulated in large amounts on gray sufu during cofermentation, possibly due to the transformation of protein and amino acids in the substrate promoted by *L. mesenteroides* F24. These flavor compounds usually produce fishy and pungent odors. The above results indicated the overall flavor profile and change in the gray sufu inoculated with *L. mesenteroides* F24.

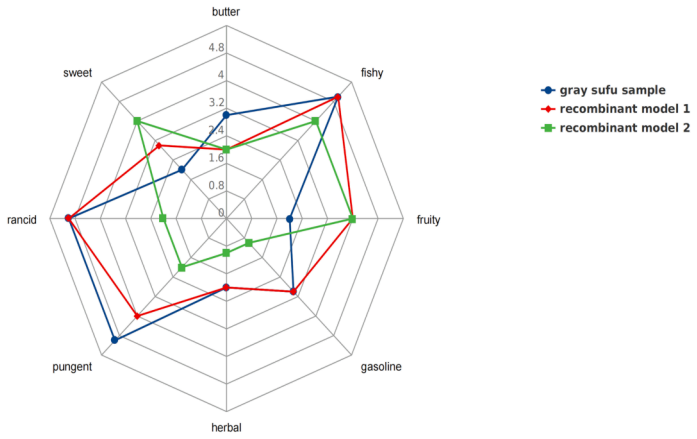
3.4. Identification of key flavor compounds in gray sufu fermented by L. Mesenteroides F24

Because the samples under study may contain dozens or even hundreds of volatile chemicals, absolute quantification is incredibly time-consuming and expensive. In the present study, our only goal is to screen a few compounds with the highest OAV. The relative odor activity value (ROAV) is a technique that allows for the analysis of the relative percent (C%) of these compounds (Cheng et al., 2023). This technique has also been widely used to identify the primary flavoring ingredients in food, such as fragrant rapeseed oil and camellia seed oil (Fang et al., 2022; Liang et al., 2023). A total of 43 main aroma components (ROAV > 0.1) were identified in groups L and S at different fermentation stages, and 29 key aroma components (ROAV > 1) were detected (Table S2). Although significant differences were found in the key aroma components among the samples, 18 key aroma components, such as dimethyl trisulfide, *n*-hexane, 2-methylbutyraldehyde, ethyl acetate, and pentanal, were identified in all samples, and they provided fishy, gasoline-like, unpleasant, fruity, amine-like, buttery, pungent, balsamic, creamy, bitter almond-like, garlic-like, feces-like, and lime-like odors. These aromas together constitute the main flavor characteristics of gray sufu. In addition, at the end of fermentation, compared with group S, group L contained four unique key flavor substances, namely, ethyl acetate, 2,3-pentanedione, isobutyl acetate, and 1-heptanol, which endowed gray sufu with fruity, creamy, floral, and a unique herbal aroma. Fermentation with *L. mesenteroides* F24 might have upregulated the key genes and proteins, such as alcohol dehydrogenase family, alcohol O-acetyltransferase, acetyl-CoA C-acetyltransferase, and acyl-CoA thioester hydrolase. However, this process might have also downregulated aldehyde dehydrogenase family to participate in the glycolysis/gluconeogenesis pathway, starch and sucrose metabolism pathway, amino sugar and nucleotide sugar metabolism pathway, tricarboxylic acid cycle, and pyruvic acid metabolism pathway, thus promoting the formation of ethyl acetate, alcohol and other esters (Liu, Qin, Hu, & Miao, 2023). In the future, through the complementary analysis of transcriptome and protein group, the formation mechanism of key flavors in gray sufu fermented by *L. mesenteroides* F24 will be explored.

3.5. Aroma recombination and omission tests

Based on the results of GC–MS detection, flavoromics, and ROVA analysis, the gray sufu flavor was more diversified in group L than in group S. Therefore, the gray sufu samples fermented for 28 days in group L were selected for the aroma omission and recombination experiments to verify the results of the ROAV analysis and GC–MS. Recombinant

A



B

No.	Odorants omitted from the complete recombination 1	n	Significance
1	All compounds	10	***
1-1	Dimethyl Trisulfide	9	***
1-2	Dimethyl Disulfide	9	***
1-3	n-Hexane	7	**
1-4	Ethyl Acetate	5	*
1-5	2,3-Butanedione	6	*
1-6	Benzaldehyde	6	*
1-7	2,3-Pentanedione	4	*
1-8	Isobutyl Acetate	5	*
1-9	1-Heptanol	4	*

Fig. 5. (A) Aroma profiles of the original sample and recombinant model. (B) Omission experiments using complete recombination 1; n: number of panelists who perceived correctly the differences in the triangle test; significance: *** ($P < 0.001$), very highly significant); ** ($P < 0.01$, highly significant); * ($P < 0.05$, significant).

experimental models were prepared using an odorless matrix, aromatic compound standards with ROAV > 1 and the same amount of high-content VOCs, and approximately 60% ultrapure water (Chen et al., 2021). As shown in Fig. 5A, subtle differences were observed in the sweet, buttery, and pungent flavors between recombinant model 1 and the original sample, but without statistical difference ($P > 0.05$). This phenomenon may be related to masking, inhibition, or synergy between compounds with ROAV < 1 and ROAV ≥ 1 (Zhang, Gao, Xia, & Jiang, 2022). However, a significant difference ($P < 0.05$) was found in the flavor between the recombinant model 2 and the original samples, indicating that the results of GC–MS detection and ROAV analysis were reliable.

Therefore, recombination model 1 was selected for the omission experiment. The experiment was omitted to study the effect of specific compounds or a group of compounds on the overall odor of gray sufu. As shown in Fig. 5B, 10 omission models were prepared, in which a single compound or all compound groups were missed. Three models showed extremely significant differences ($P < 0.001$), one missing model exhibited highly significant differences ($P < 0.01$), and six missing models indicated significant differences ($P < 0.05$). When all compounds were omitted, a very significant difference was observed ($P < 0.001$), indicating the successful establishment of an odorless matrix. Extremely significant differences ($P < 0.001$) were also detected when dimethyl trisulfide and dimethyl disulfide were removed separately. These compounds are associated with flavors such as fishy and radish (Table 1), which may be the main source of odor in gray sufu. In addition, the absence of *n*-hexane, ethyl acetate, 2,3-butanedione, benzaldehyde, 2,3-pentanedione, isobutyl acetate, and 1-heptanol contributed to the flavor to a certain extent. Thus, the key flavor substances of gray sufu were determined.

4. Conclusion

This study explored for the first time the feasibility and advantages of *L. mesenteroides* F24 for improving the flavor of gray sufu, a traditional fermented soybean product in China. The results revealed the important characteristics of VOCs in gray sufu, with alcohols and esters as the main VOCs. The results of multivariate statistical analysis showed that the content levels of VOCs in the gray sufu inoculated with *L. mesenteroides* F24 were significantly different from those in the naturally fermented one. The key odors in gray sufu were identified by the flavor database method and ROAV analysis. *L. mesenteroides* F24 significantly improved the aroma quality of gray sufu. The main flavor substances of gray sufu are dimethyl trisulfide, dimethyl disulfide, *n*-hexane, ethyl acetate, 2,3-butanedione, benzaldehyde, 2,3-pentanedione, isobutyl acetate and 1-heptanol. This study is the first comprehensive report on the use of *L. mesenteroides* F24 in assisting the fermentation of gray sufu. The findings may help in understanding the aroma quality of gray sufu and provide a theoretical basis for the processing and quality control of gray sufu products. Thus, *L. mesenteroides* F24 is a promising adjunct strain to promote the modern industrial production of traditional gray sufu.

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

No data was used for the research described in the article.

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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.2023.100881.

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