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Identification and analysis of primary bacteria during fermentation of stinky Mandarin fish (*Siniperca chuatsi*)

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Abstract

The present work aimed to investigate the changes in physicochemical properties, volatile flavour compounds, and bacterial diversity of stinky Mandarin fish during fermentation, and identify the primary bacteria influencing the fermentation. After fermentation, the moisture, crude protein, and crude fat contents of fish decreased by 7.41, 34.15, and 5.95%, respectively. Proteobacteria was the most dominant phylum throughout fermentation, accounting for > 50% during days 1 - 2 of fermentation. At the genus level, the bacterial composition exhibited more diversity during days 1 - 2 of fermentation. However, from day 3 onward, *Psychrilyobacter*, *Vibrio*, and *Psychrobacter* gradually emerged as the dominant genera. Additionally, 22 key flavour compounds were identified by calculating their odour activity values. Redundancy analysis revealed that the most abundant flavour species emerged during days 6 - 7 of fermentation. *Psychrilyobacter*, *Psychrobacter*, *Fusobacterium*, and *Morganella* exhibited a positive correlation with these species.

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Introduction

The fermentation industry has experienced significant growth, resulting in increased popularity among consumers of fermented foods. This includes various products, such as fermented dairy products, soy-based ferments, and wines. The unique flavours of these foods are due to small molecular volatile compounds that impart a distinctive taste to the fermented products (Dimidi *et al*., 2019). Stinky Mandarin fish is a typical fermented product with a long history (Li *et al*., 2013b). The traditional preparation of stinky Mandarin fish depends on the intuition and empirical expertise of seasoned artisans, resulting in difficulties in achieving consistent quality across batches due to the intricacies of the fermentation. The processing of traditional fermented fish can be divided into dry-cured, water-cured, and mixed fermentation methods (Wu *et al*., 2017). The dry curing method typically occurs at room temperature; however, its drawbacks included insufficient brine penetration, leading to uneven fermentation, elevated salt concentrations, and a more pronounced odour of fermented fish. However, the wet curing method involves complete immersion of the whole fish in brine, fully submerged under substantial weights at a low temperature. Low temperatures can inhibit the rapid microorganism propagation while simultaneously ensuring greater stability in low-temperature fermentation, preventing the volatilisation of some flavour substances.

Mandarin fish is characterised by its rapid growth, tender flesh, and high nutritional value (Yang *et al*., 2019). Fermented stinky Mandarin fish emits a mildly rotten odour. Under the influence of intrinsic or environmental microorganisms, the rich proteins and lipids in fish are metabolically degraded into small molecular volatile flavour compounds (VFCs). The VFCs are implicated in the special flavours of fermented stinky Mandarin fish, and the headspace

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solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) technique has effectively facilitated the qualitative and quantitative analyses of these VFCs in the samples (Yin *et al*., 2019). The VFCs of stinky Mandarin fish are diverse and categorised into alcohols, acids, esters, aldehydes, ketones, aromatics, and nitrogenous and sulphurous compounds. A previous study reported that the VFCs in stinky Mandarin fish included trimethylamine (TMA), indole, sulphurous compounds, 1-octen-3-ol, acetic acid, esters, phenols, and aldehydes (Li *et al*., 2013a). Additionally, TMA, indole, 1-octen-3-ol, and dimethyl trisulphide have also been identified in stinky Mandarin fish (Ke *et al*., 2020). Indole, TMA, and sulphurous compounds are the primary sources of the "special odour" in stinky Mandarin fish, and these compounds are closely associated with microorganisms.

The study of flavour must include the examination of microorganisms due to their impact on flavour development. Recently, high-throughput sequencing technology utilising 16s rRNA gene amplicons has been widely employed to analyse microbial communities across various ecosystems, including industrial wastewater, soil, and raw milk (Wei *et al*., 2022; Belleggia *et al*., 2022). The significant contributions of *Vagococcus*, *Peptostreptococcus*, *Acinetobacter*, *Psychrobacter*, and *Enterococcus* to the flavour of stinky Mandarin fish have been identified during fermentation (Yang *et al*., 2022). Stinky Mandarin fish exhibited a significant concentration of lactic acid bacteria, with *Lactobacillus sakei* accounting for 63% of the total bacterial population. Other bacteria identified included *Lactococcus garvieae*, *Lactococcus lactis*, *Lactococcus raffinolactis*, *Vagococcus* sp., *Enterococcus hermanniensis*, *Macrococcus caseolyticus*, and *Streptococcus parauberis* (Dai *et al*., 2013). A previous study reported that *Pseudomonas* and *Carnobacterium* were more prevalent in Mandarin fish before fermentation, while *Psychrobacter* and *Vagococcus* were overwhelmingly dominant in Mandarin fish after fermentation, accounting for 59.96 and 26.97% of the total population, respectively (Wu *et al*., 2021).

The present work measured the physical and chemical properties, and the flavour and bacteria involved in stinky Mandarin fish fermentation, and aimed to identify the key flavours in stinky Mandarin fish, and the relationship between bacteria and these essential flavours during fermentation.

Materials and methods

Stinky Mandarin fish fermentation and sampling

Mandarin fish samples were procured from Anhui Huiziyihao Investment Co. in Huangshan, Anhui, China. The wet curing method was used to ferment the stinky Mandarin fish. Briefly, fresh raw fish were thawed, gutted, scaled, cleaned, and arranged in a fermentation tank in a single layer, mixed thoroughly with approximately 6% salt and 0.02% spices (pepper and ginger). The fish-to-brine ratio was 1.25:1 (with fish measured in kg and brine in L). The top layer was covered with stones, and fermented at 10 ± 2°C for 1 w. The fermentation broth was used for bacteriological analysis, and three fish were extracted daily from them as parallel experimental groups, and frozen at -18°C for subsequent analysis.

Determination of physical and chemical properties

The moisture, fat, and crude protein contents were assessed according to Shen *et al*. (2020). Briefly, fish samples were thawed in water and subsequently cut into 1 × 1 × 1 cm cubes. The texture was analysed using a P/36R cylindrical probe with a pre-test speed of 2 mm/s, a test speed of 1 mm/s, and a post-test speed of 1 mm/s at 50% compression. Each sample was measured thrice with two parallel sets, and the average value was calculated.

The quantification of thiobarbituric acid reactive substance (TBARS) was performed according to Xu *et al*. (2022). Briefly, 5 g of the sample were homogenised with 50 mL of trichloroacetic acid solution (7.5%, w/v) for 1 min. After shaking at 50°C for 30 min, the filtrate was collected by filtration through neutral filter paper. Subsequently, 5 mL of the filtrate was mixed with 5 mL of 2-thiobarbituric acid (0.02 mol/L), and reacted at 90°C for 30 min. The absorbance of the reaction solution was measured at 532 nm. The TBARS value was expressed as mg MDA (malondialdehyde)/kg of sample using 1,1,3,3-tetraethoxypropane as the standard curve.

The total volatile base-N (TVB-N) was measured for each sample using the Kjeldahl method adapted from Yang *et al*. (2020), and expressed as mg/100 g. Briefly, the fish was cut, and 10 g of each sample was combined with 100 mL of distilled water, and sonicated for 15 min after 10 mL was extracted and incorporated into a 1% (w/v) MgO suspension. The absorbent solution was 2% H_3BO_3, while the

mixed indicator was 1 g/L methyl red anhydrous ethanol solution and 1 g/L bromocresol green anhydrous ethanol solution in a 1:5 ratio. The mixed indicator was combined with the absorbent solution in a 1:100 ratio, and titrated with 0.01 M HCl for quantification.

High-throughput sequencing of 16s rRNA

Genomic DNA extraction and polymerase chain reaction (PCR) amplification

This experiment was performed according to Quast *et al*. (2013). The 16S amplicon sequencing and analysis were performed by OE Biotech Co., Ltd. (Shanghai, China). Genomic DNA was extracted using a DNA extraction kit, and subsequently subjected to agarose gel electrophoresis to assess purity and concentration. The sample was subsequently placed in a centrifuge tube, and diluted to 1 ng/μL with sterile water when required. PCR was performed using Takara Ex Taq high-purity enzyme to ensure amplification efficiency and accuracy. Bacterial diversity was identified in the 16S V3-4 region (using primers 343F and 798R), with the front-end primer TACGGRAGGCAGCAG and the back-end primer AGGGTATCTAATCCT.

PCR product mix and purification

The amplicon quality was assessed using gel electrophoresis, purified with AMPure XP beads, and subsequently amplified using PCR. After purification with AMPure XP beads, the final amplicon was quantified using the Qubit dsDNA assay kit. Equal amounts of purified amplicon were pooled for subsequent sequencing.

Bioinformatic analysis

The raw sequencing data were in FASTQ format. Paired-end reads were subsequently preprocessed using Cutadapt software to identify and remove the adapter. After trimming, paired-end reads underwent filtering for low-quality sequences, denoising, merging, and detecting and removing chimera reads using DADA2 with the default parameters of QIIME2 (2020.11). The software produced the representative reads and the ASV abundance table. The representative read of each ASV was selected using the QIIME2 package. All representative reads were annotated and blasted against Silva database Version 138 (or unite) (16s/18s/ITS rDNA) using q2-feature-classifier with the default parameters.

Volatile flavour compounds

The VFCs were identified using HS-SPME-GC-MS, following a modified method described by Ke *et al.* (2020). Briefly, the fish samples were chopped, and 5 g of each sample was combined with 10 μL of 0.01% 2,4,6-trimethylpyridine into a 20 mL headspace vial. The SPME (divinylbenzene/ carbon molecular sieve/ polydimethylsiloxane (DVB/CAR/PDMS) coated extraction head; Supelco, USA) needle was subsequently inserted into the headspace vial without touching the fish or the vial wall. Extraction was performed thrice for each sample at 70°C for 40 min in a water bath.

Gas chromatography-mass spectrometry analysis of VFCs was performed using QP-2010 gas chromatography-mass spectrometry (Shimadzu, Japan) and a DB-WAX capillary chromatography column (30 × 0.20 mm, 0.25 μm; Agilent, USA).

The gas chromatography conditions were as follows: the initial temperature was established at 30°C, and maintained for 1 min; the temperature was increased to 92°C at 4°C/min, and maintained for 2 min; subsequently, the temperature was increased to 200°C at 5°C/min, and further to 240°C at 6°C/min, and maintained for 6 min. Helium was used as the carrier gas at a 1 mL/min flow rate, and there was no split injection.

The operating parameters of the MS were as follows: ionisation mode, electron energy of 70 eV, interface temperature of 250°C, ion source temperature of 250°C, mass spectrometry scan range of 29 - 450 *m/z*, and data acquisition in full scan mode.

Statistical analysis

All experimental data were obtained in triplicate, and expressed as mean ± SD. The physical and chemical properties of the fish were analysed and tabulated using Excel 2016. Origin 2021 was used to perform a fish flavour heatmap and cluster analysis. GraphPad Prism software (version 9) was used to plot line graphs. Statistical Package for the Social Sciences software (version 26.0) was used to analyse the data statistically. Canoco5 was used to perform redundancy analysis.

Results and discussion

Moisture, crude protein, and crude fat contents in stinky Mandarin fish during fermentation

Table 1 shows the moisture, crude protein, and fat contents during stinky Mandarin fish fermentation. Moisture was the most critical part of the fish body mass, accounting for approximately 70 - 80%. The moisture content of the fish increased with prolonged fermentation time, starting at 79.57% at the beginning of fermentation, and decreasing by 8.48% at the end of fermentation. The precipitation of water was due to the presence of salt. The decrease in moisture content in fish flesh could have been due to natural evaporation of water and salt-induced osmosis, with the stinky Mandarin fish demonstrating a 32.44% reduction in moisture during fermentation (Yang *et al*., 2017). Additionally, the fat and protein contents decreased during fermentation, which could have been due to fat and protein breakdown facilitated by specific lipases and proteases. The fat content decreased from 4.05 to 2.65 g/100 g, representing a decrease of 34.15%, while the protein content decreased by 5.95%. Decreased level of protein decomposition maintained more protein. It retained the textural features of fish hardness and elasticity, while fat decomposition may have generated free fatty acids and other small-molecule compounds that enhanced the flavour profile of stinky Mandarin fish.

Table 1. Moisture, fat, and protein contents during 7-day natural fermentation of stinky Mandarin fish.

Day	Moisture (%)	Protein (g/100 g)	Fat (g/100 g)
1	79.57 ± 0.08^{a}	18.45 ± 0.21^{a}	4.05 ± 0.07^{a}
2	79.56 ± 0.10^{a}	18.20 ± 0.14^{ab}	3.40 ± 0.28^{b}
3	78.87 ± 0.07^{b}	18.10 ± 0.14^{ab}	3.30 ± 0.14^{b}
4	78.63 ± 0.02^{c}	17.90 ± 0.14^{bc}	3.15 ± 0.07^{bc}
5	78.15 ± 0.04^{d}	17.65 ± 0.21^{cd}	3.15 ± 0.07^{bc}
6	77.89 ± 0.01^{e}	17.65 ± 0.07^{cd}	2.85 ± 0.21^{cd}
7	73.68 ± 0.04^{f}	17.35 ± 0.07^{d}	2.65 ± 0.07^{d}

Different lowercase superscripts in similar columns indicate significant difference ($p < 0.05$).

Textural properties, TBARS, and TVB-N of stinky Mandarin fish during fermentation

Figure 1a depicts the textural properties of stinky Mandarin fish during fermentation. The presence of brine during fermentation reduced the water content in the fish, resulting in a gradual increase in its hardness. The hardness values increased from 1,466.433 to 2,311.092 g, and the chewiness values increased from 1,108.928 to 1,765.101 g. The decrease in hardness and chewiness of fish may have resulted from partial protein degradation as fermentation duration increased (Xiao *et al*., 2022). Apart from that, the springiness, adhesiveness, and resilience all improved following fermentation.

TBARS indicates the change in malondialdehyde and the degree of lipid oxidation in fish and meat products (Fan *et al*., 2021). Figure 2b depicts the variation of TBARS and TVB-N. The TBARS value increased from 0.7 to 1.8 mg/kg, representing a 61.11% increase, which occurred gradually during fermentation. However, it remained within the permissible range (TBA content in fresh meat products was not permitted to exceed 5 mg/kg).

TVB-N is frequently employed as a standard for assessing fish freshness (Zhang *et al*., 2022). It indicates the extent of fish spoilage; a higher value signifies a greater degree of spoilage (Xu *et al*., 2022). The present work observed an increase in TVB-N from 24.35 to 68.10 mg/100 g during fermentation, indicating enhanced protein decomposition and oxidation. A previous study reported that the decomposition produces compounds that contribute to the characteristic flavours of fermented stinky Mandarin fish (Yang *et al*., 2020).

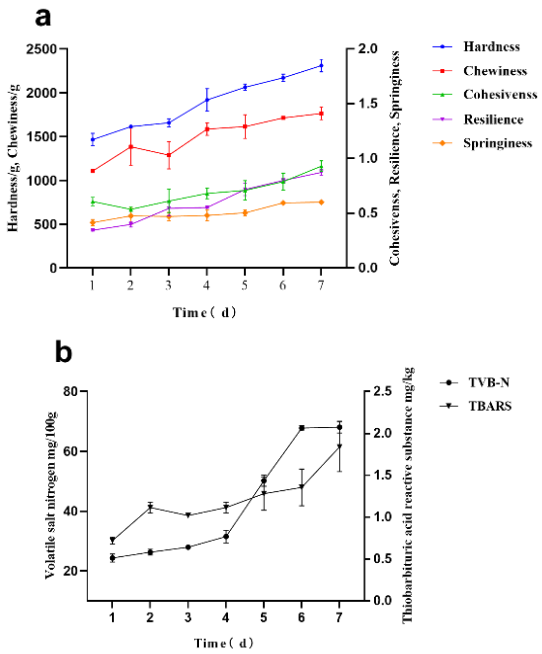
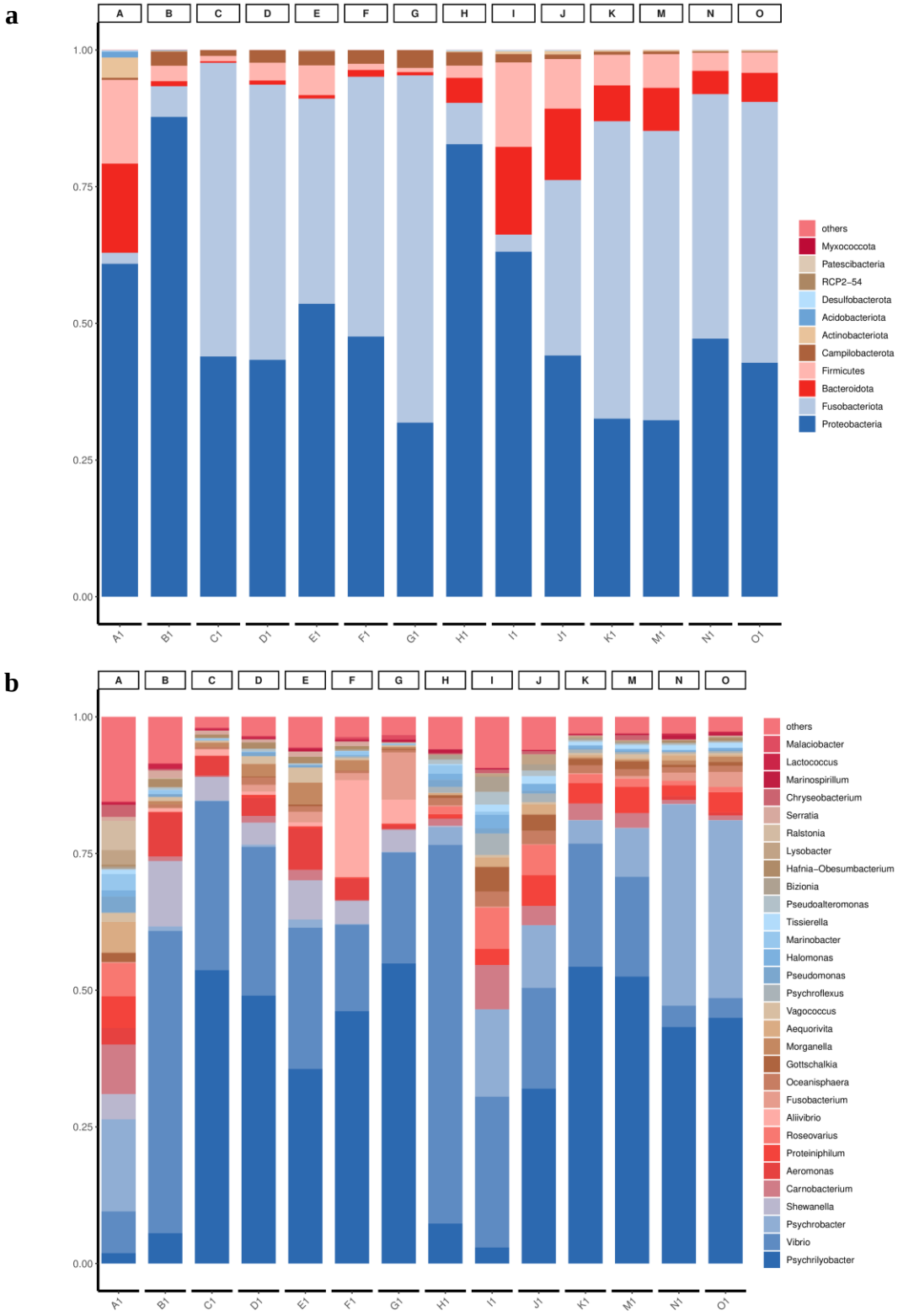


Figure 1. Physical and chemical properties of stinky Mandarin fish during 7-day natural fermentation. **(a)** Textural properties, and **(b)** TBARS and TVB-N.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Actinobacteriota
Campilobacterota
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Vagococcus
Aequorivita
Morganella
Gottschalkia
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Carnobacterium
Shewanella
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Vibrio
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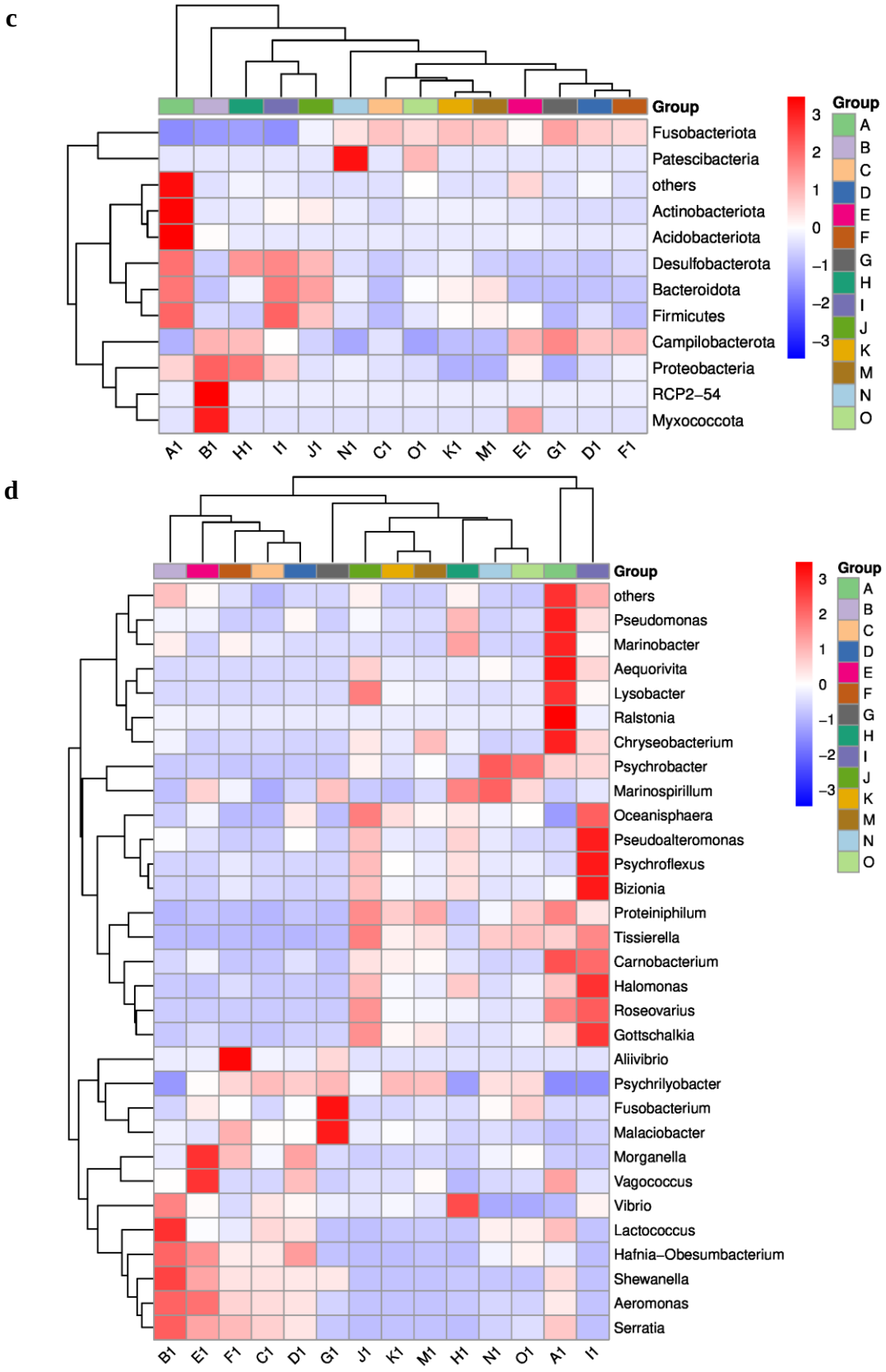

Figure 2. Changes in bacterial community structure of stinky Mandarin fish and fermentation broth during fermentation. **(a)** Histogram of changes in bacterial community structure at phylum level. **(b)** Histogram of changes in bacterial community structure at genus level. **(c)** Heatmap of bacterial species abundance at phylum level. **(d)** Heatmap of bacterial species abundance at genus level; A1 - G1 are fish samples, and H1 - O1 are fermentation broth samples.

Bacterial succession during fermentation

Bcaterial communities involved in the fermentation of stinky Mandarin fish were classified using 16s rRNA high-throughput sequencing at the phylum and genus levels. Figure 2a illustrates the bacterial species at the phylum level in the stinky Mandarin fish and the fermentation broth, revealing 11 bacterial phyla. The dominant phylum in the stinky Mandarin fish and fermentation broth during days 1 - 2 of fermentation was Proteobacteria, which exhibited an increase, while Fusobacteria demonstrated a decrease. Subsequently, both phyla emerged as the dominant phyla in the middle and late stages of fermentation. Figure 2b demonstrates the bacteria at the genus level in stinky Mandarin fish and fermentation broth, revealing 30 bacterial genera. The genus *Psychrilyobacter* was less prevalent, while other bacterial genera were more widely distributed on days 1 - 2. However, the genus *Vibrio* decreased after day 3, and the genus *Psychrilyobacter* increased to become the dominant genus in the middle and late stages of fermentation. *Psychrilyobacter*, *Psychrobacter*, and *Vibrio* were the dominant genera during fermentation. *Psychrobacter* was common in low-temperature fermented foods, and found in refrigerated meat, seafood products, and milk. *Psychrobacter* is a mildly deleterious bacterium that could use carbohydrates in fermented foods and decomposing fats to generate various acids, aldehydes, ketones, and sulphurous compounds. However, it does not produce TMA (Broekaert *et al*., 2013). Figures 2c - 2d depict the heatmap of bacterial community species abundance, revealing that *Actinobacteria* and *Acidobacteria* exhibited higher species abundance at the onset of fermentation, which subsequently decreased with fermentation. The heatmap of species richness indicates a disparity in species abundance between stinky Mandarin fish and fermentation broth.

Figure 3 displays the combination of species evolutionary tree and abundance. The bacterial phyla that exhibited high abundance were Actinobacteriota, Bacteroidota, Campylobacterota, Firmicutes, Fusobacteriota, and Proteobacteria. The species heatmap indicates that the abundance of Bacteroidota in the fermentation broth was higher, while the abundance of Proteobacteria in the fermentation broth was lower than that found in the stinky Mandarin fish. Additionally, the bacterial diversity in stinky Mandarin fish and fermentation broth samples was also found to be similar.

Volatile flavour compounds during stinky Mandarin fish fermentation

Moderate fermentation facilitated the development of characteristic aromas and good organoleptic properties of stinky Mandarin fish (Yang *et al*., 2021). We detected 80 VFCs during fermentation, and classified them into nine groups: 19 alcohols, four acids, six esters, 13 aldehydes, eight ketones, 17 hydrocarbons, nine aromatic compounds, two nitrogenous compounds, and two sulphurous compounds. Figure 4a presents the proportion of each compound at various fermentation stages. Alcohols and aromatic compounds accounted for the largest proportion during fermentation. As the fermentation progressed, the proportion of aldehydes and hydrocarbons decreased, whereas esters, sulphurous compounds, and nitrogenous compounds increased. Figure 4b indicates the composition of each class of compounds at different stages of fermentation. The compounds consistently predominant during fermentation were alcohols and aromatic compounds, followed by hydrocarbons. The concentrations of acids, esters, ketones, nitrogenous, and sulphurous compounds generally increased during fermentation.

Odour activity value (OAV) is commonly used to estimate the contribution of odour compounds, with compounds exhibiting OAV ≥ 1 regarded as the primary contributor to the flavour of the sample. A higher OAV indicates a more significant flavour contribution (Tan *et al*., 2022). Table 2 illustrates the OAV values of VFCs derived from literature and books highlighting 22 compounds with OAV ≥ 1. During the daily fermentation process, the compounds with an OAV ≥ 1 included 1-octen-3-ol, acetic acid, hexanal, octanal, nonanal, and indole; 18 compounds exhibited an OAV ≥ 1 on the final day of fermentation. Several essential flavour substances listed in the table, including 1-octen-3-ol, TMA, indole, and sulphurous compounds were reported in other studies. These substances were converted into a thermogram (Figure 4c). The colour gradients and dimensions of the circles indicate the magnitude of the OVA value; a larger circle with a redder colour indicates a more significant contribution of the substance to the flavour of stinky Mandarin fish. The graph indicates a substantial increase in flavour value

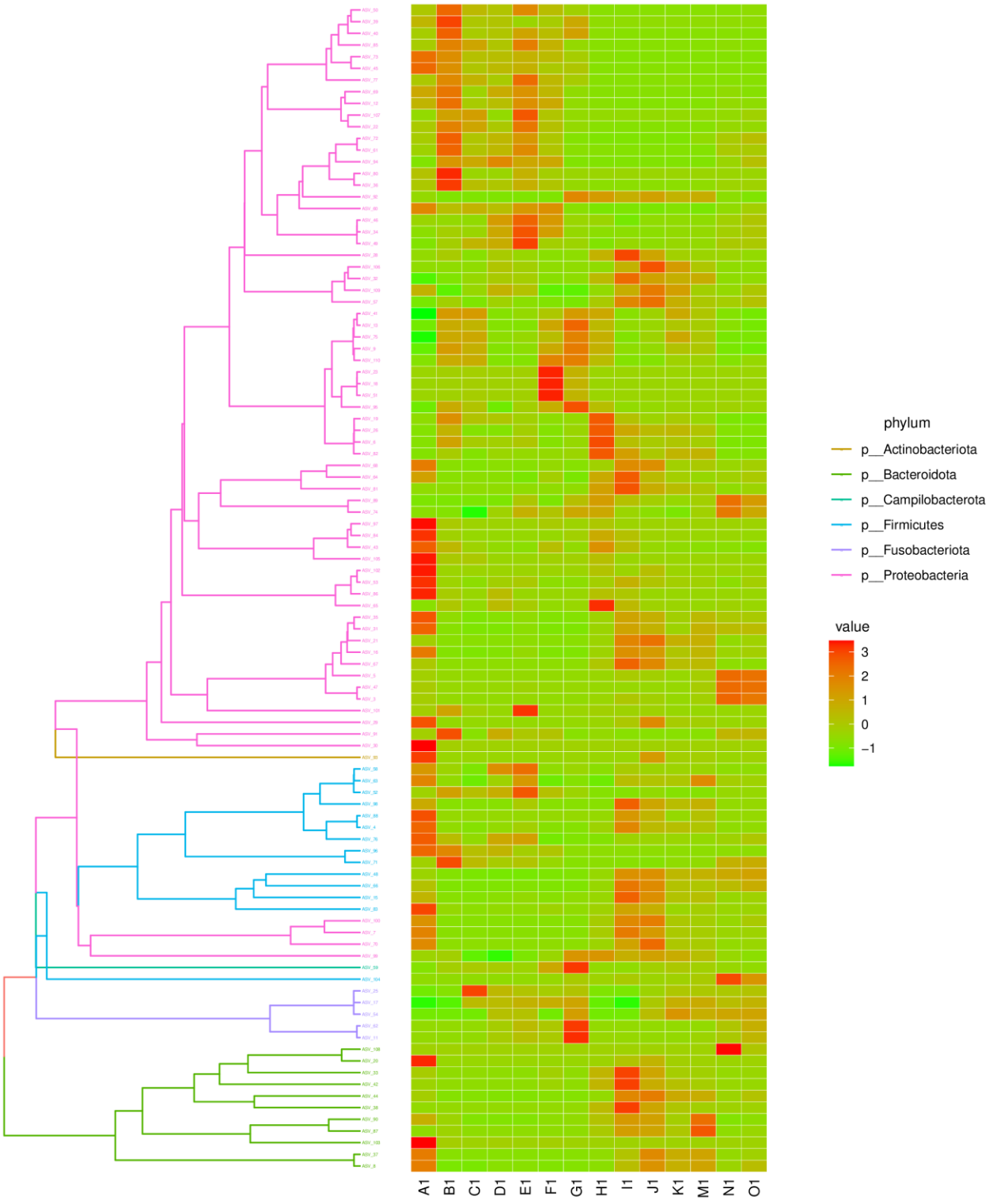

Figure 3. Phylogenetic evolutionary tree and species abundance combination map. Evolutionary tree plot on the left, with phylum as gate information; abundance plot on the right, corresponding to the abundance of ASV in each sample on the left.

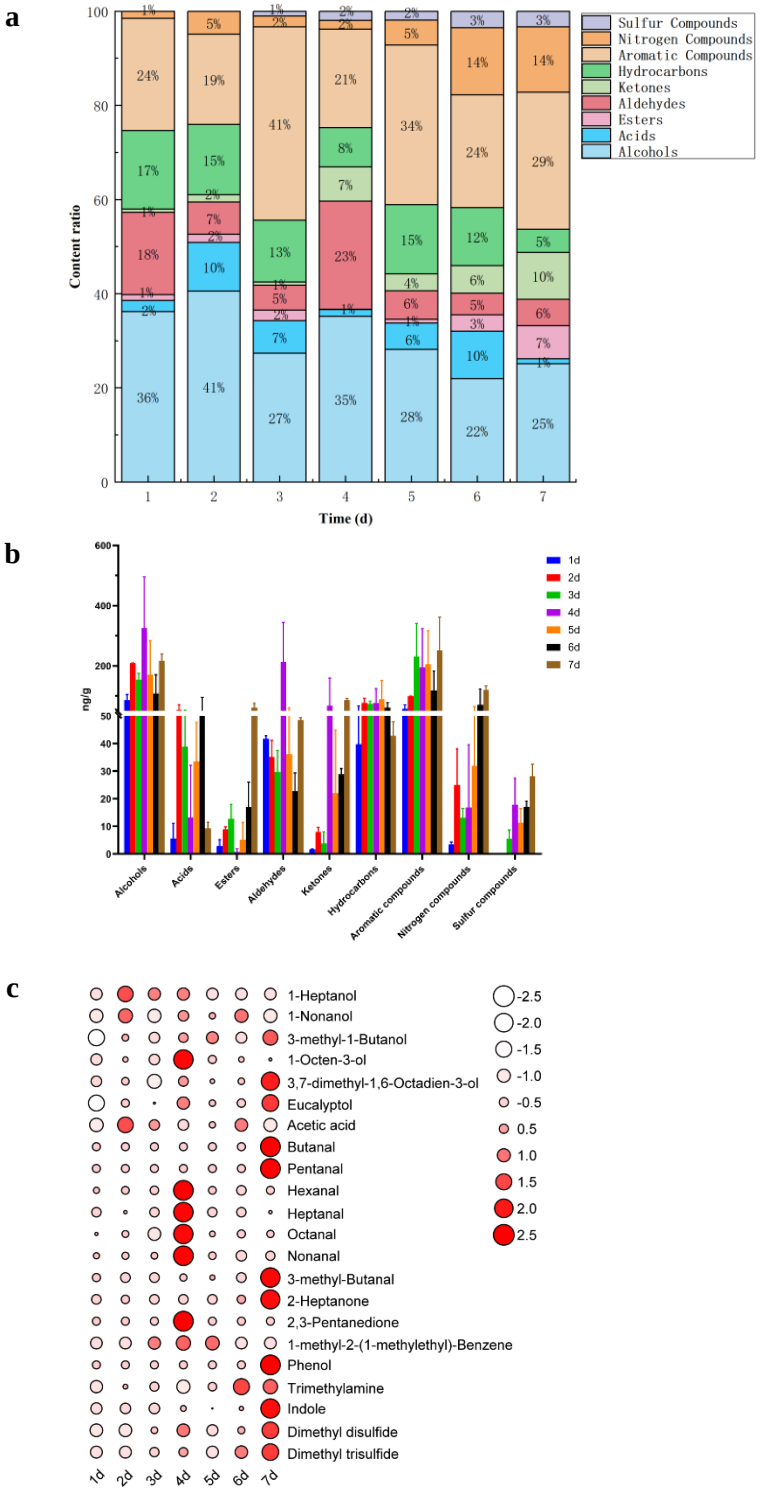

Figure 4. Volatile flavour compounds during fermentation of stinky Mandarin fish. **(a)** Histogram of cluster analysis of different types of volatile flavour compounds. **(b)** Contents of different types of volatile flavour compounds. **(c)** Heatmap of volatile flavour compounds (OAV ≥ 1). The darker the colour of the circle, the greater the contribution of the compound to the flavour.

Table 2. Odour activity values (OAV ≥ 1) of volatile flavour compounds of stinky Mandarin fish during 7-day natural fermentation.

Compound	Threshold (μg/L)	OAV						
		1 d	2 d	3 d	4 d	5 d	6 d	7 d
1-Nonanol	0.9	1.006	3.078	0.955	2.363	1.767	2.866	—
1-Heptanol	4.8	—	1.397	1.056	1.057	0.290	0.000	0.320
3-methyl-1-Butanol	4	0.404	2.836	1.363	3.257	3.712	1.319	4.430
1-Octen-3-ol	1.5	6.133	15.704	8.613	49.330	12.430	15.505	11.545
Linalool	2.5	0.738	1.750	0.000	3.721	2.213	1.918	6.660
Eucalyptol	0.26	—	14.603	19.247	31.444	16.351	14.130	41.423
Acetic acid	0.006	59917.573	8891.326	6228.108	2011.410	3438.772	7228.305	908.370
Butanal	1	—	—	—	—	—	—	3.594
Pentanal	0.15	—	—	—	—	—	—	11.263
Hexanal	4.5	3.609	4.447	3.622	33.402	4.415	2.382	4.121
Heptanal	2.9	0.500	1.073	0.602	6.485	0.837	0.604	1.079
Octanal	0.6	5.421	3.069	3.270	14.222	3.449	2.598	2.940
Nonanal	1.1	6.183	5.976	6.179	22.747	5.734	3.681	4.604
3-methyl-1-Butanal	0.2	7.684	9.383	—	3.927	11.490	—	44.414
2,3-Pentanedione	30	—	—	—	2.112	—	—	0.804
2-Heptanone	1	—	1.357	—	—	—	9.872	27.637
1-methyl-2-(1-methylethyl)-Benzene	8.4	0.361	0.986	1.171	1.395	1.333	0.473	—
Phenol	31	—	—	0.978	0.406	—	0.962	5.907
Trimethylamine	1.4	1.634	15.515	7.202	0.984	8.135	33.637	29.721
Indole	0.031	437.388	105.912	96.670	499.280	661.786	762.787	2529.845
Dimethyl disulphide	0.16	—	—	22.489	62.400	—	41.010	84.345
Dimethyl trisulphide	1.5	—	—	1.287	5.277	7.583	7.012	9.823

"—" indicates that the compound was not detected at this stage of the fermentation of stinky Mandarin fish.

on day 7, primarily due to nitrogenous and sulphurous compounds, and some aldehydes and alcohols. Considerable contributors included linalool, eucalyptol, indole, 3-methyl-butanal, hexanal, nonanal, 2-heptanone, disulphide dimethyl, TMA, and dimethyl trisulphide.

Alcohols significantly contributed to stinky Mandarin fish fermentation, exhibiting the most remarkable diversity among the detected compounds, with higher levels observed during fermentation. The alcohols identified in the present work with an OAV ≥ 1 included 1-heptanol, 1-nonanol, 3-methyl-1-dutanol, 1-octen-3-ol, 3,7-dimethyl-1,6-octadien-3-ol, and eucalyptol. 1-Octen-3-ol has a mushroom-like, vegetal, and oily aroma, and is a common flavouring agent in fermented fish products (Olivares *et al*., 2009). Heptanol has a grape-like aroma; nonanol has a citrusy odour; eucalyptol has a refreshing herbal, camphor-like odour; linalool is associated with the spices introduced during fermentation, exhibiting a woody odour, generally not a metabolite of microbial metabolism.

Aldehydes typically have a pleasant taste with a low threshold, and a significant impact on the overall flavour of the fermented product (Li *et al*., 2022); they are primarily generated through protein hydrolysis and the oxidation of unsaturated fatty acids during fermentation. Hexanal and nonanal contribute floral, fatty, and grassy flavour to stinky Mandarin fish, while benzaldehyde has almond and nutty flavour. 2-Heptylone has a fruity odour, and facilitates fruit flavour formation in stinky Mandarin fish, while 2,3-pentanedione facilitates creamy, caramel odours, and nutty flavour formation in stinky Mandarin fish.

Esters are typically produced through the esterification of alcohols and acids, imparting a fruity and sweet taste to the fermented product that can obscure the undesirable sourness of fermentation (Wen *et al*., 2022). The ester content in fermented Mandarin fish was minimal. More aromatic compounds were identified during fermentation; although the number of species was not dominant, their concentration increased during fermentation. 1-methyl-2-(1-methylethyl)-benzene and phenol were the two aromatic compounds with elevated OAV. Phenolic compounds may produce pungent, sour, and roasted flavours (Al-Dalali *et al*., 2020), and these two compounds contributed to the flavour profile of the stinky Mandarin fish.

Nitrogenous compounds were identified throughout fermentation, while sulphurous compounds were identified on day 3. The nitrogenous compounds were primarily TMA and indole, with TMA as the characteristic ichthyosis compound, and an indicator for assessing fish freshness (Heising *et al*., 2014). TMA was identified on day 1 of fermentation, and its concentration increased approximately 15 times after fermentation completion, reaching 41.61 μg/kg. The indole concentration increased with fermentation, significantly enhancing the fish flavour. Indole is generated through the microbial metabolism of tryptophan; low indole concentration emits an aroma, while high indole concentration emits an odour. The threshold of indole was considerably low, significantly influencing the flavour of the stinky Mandarin fish. However, the indole concentration decreased due to evaporation during steaming (Yang *et al*., 2020).

Sulphurous compounds may be generated from methionine, which undergoes a transamination reaction facilitated by microbial enzymes to yield methionol, and followed by decarboxylation, deamination, or desulphuration (Wang *et al*., 2021). Herein, the sulphurous compounds were detected from day 3, and gradually increased with fermentation, primarily dimethyl disulphide and dimethyl trisulphide compounds. TMA, indole, and sulphurous compounds have lower thresholds and exert a more significant impact on flavour. They were also the primary contributors to the flavour in the final fermentation samples.

Redundancy analysis

Redundancy analysis was performed to investigate bacterial community succession and product characteristics, including physicochemical properties (moisture, fat, protein, TBARS, and TVB-N) and VFCs [OAV ≥ 1]). Figure 5 reveals that fermentation time can be divided into pre-fermentation, middle and late fermentation, and final fermentation. Day 1 was for the pre-fermentation, days 2 - 5 were for the late fermentation, and days 6 - 7 were for the final fermentation. No significant correlation existed between VFCs and day 1 of fermentation. Accumulation commenced on day 2, and there was an increase in predominant VFCs on days 6 and 7, consistent with those depicted on the last day of the heatmap. Water, fat, and protein were

positively correlated with day 1, and decreased with the increase in fermentation time, indicating a decrease in their contents due to fermentation and decomposition. Conversely, malondialdehyde and volatile salt and nitrogenous substances emerged from the decomposition of fermented fat and protein, leading to the accumulation of TBARS and TVB-N contents in the middle and late stages of fermentation, which were positively correlated with the final fermentation and the predominant VFCs. The concentration of VFCs in stinky Mandarin fish contributed to the distinctive flavour of the fish. These conditions cannot be separated from the role of microorganisms, with the dominant flavour substances (sulphide, indole, linalool, 2-heptanone, and eucalyptol) during the final fermentation period exhibiting a positive correlation with *Psychrilyobacter*, *Fusobacterium*, *Psychrobacter*, and *Morganella*. These genera emerged as dominant during the critical stinky Mandarin fish fermentation period. They were positively correlated with TBARS and TVB-N, while water, fat, and protein demonstrated a negative correlation with these genera. Redundancy analysis revealed that food metabolism in fish and bacterial interaction contributed to the production of VFCs, with the presence of bacterial genera being influenced by specific VFCs.

Conclusion

The present work aimed to deepen the study of bacteria involved in stinky Mandarin fish fermentation. It demonstrated that the dominant genera involved in the fermentation process of stinky Mandarin fish were *Psychrilyobacter*, *Fusobacterium*, *Psychrobacter*, and *Morganella*, which significantly contributed to the formation of the flavour compounds. Linalool, eucalyptol, 3-methyl-1-butanol, 1-octen-3-ol, acetic acid, sulphurous compounds, indole, 2-heptanone, butanal, and hexanal were significant VFCs detected in stinky Mandarin fish. *Psychrobacter* was the dominant genus in the fermentation process, and it could promote the VFCs in stinky Mandarin fish. Bacteria facilitated stinky Mandarin fish fermentation and unique odour formation. Initially, fermentation technology was utilised for food preservation; however, it is currently used to prepare stinky Mandarin fish, giving it a special quality and flavour. Numerous bacteria were involved in stinky Mandarin fish fermentation, and the fermentation system may be associated with the interaction among them. Furthermore, bacterial and endogenous enzymes promoted the decomposition of fats and proteins, contributing to the distinctive flavour of stinky Mandarin fish. The formation of flavour during Mandarin fish fermentation is associated with the decomposition of large molecules, including proteins and lipids. The decomposition of large molecules may be linked to the presence of bacteria and the enzymes secreted by them. However, their specific mechanisms and pathways of action require further studies.

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