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Correlation between microbial community and flavour formation in dry-cured squid analysed by next-generation sequencing and molecular sensory analysis

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ABSTRACT

The sensory characteristics of dry-cured squid are closely related to its microbial community structure. To explore the relationship between microorganims and flavour formation, the microbial community and sensory characteristics of dry-cured squid at various processing stages were studied with next-generation sequencing and molecular sensory analysis. The most abundant genera in dry-cured squid were *Staphylococcus* and *Acinetobacter*, with relative abundance of 17.95% and 8.81%, respectively. Among the 44 volatile compounds identified, 11 had a relative odour activity value ≥ 1, including α-dicarbonyls, aldehydes, alcohols, sulphur compounds and trimethylamine. The concentrations of volatile and non-volatile compounds in squid samples increased greatly during the process. A significant correlation ($P < 0.05$) was observed between the main genera and sensory indicator compounds. *Staphylococcus*, as the dominant genus, was responsible for flavour formation in dry-cured squid. This study provides new evidence that microbial metabolism has an important effect on flavour development in dry-cured squid.

1. Introduction

Jumbo flying squid (*Dosidicus gigas*) is one of the fastest-growing squid species that are widely consumed. In 2018, it had a global production value of 0.9 million tonnes, accounting for 15% of total worldwide mollusc aquaculture production (FAO, 2020). Drying and curing are effective and common methods for its preservation, which can also improve the sensory qualities of squid products, including both odour and taste. Dry-cured squid is prepared with salt and either air-drying or oven-drying. The flavour development of dry-cured squid is a complicated biochemical course that relies on microbial metabolism and endogenous enzymes activity. However, the natural fermentation method under non-sterile fermentation conditions results in a variable microbial community and inconsistent quality and flavour of dry-cured squid.

Microbial metabolism occurring in the processing of dry-cured squid, including protein hydrolysis and lipolysis, determines the composition of sensory constituents and the unique flavour of squid products. Amino acids and oligopeptides are important taste-active compounds that can form through enzymatic hydrolysis. Taste-active peptides and their corresponding amino acids can produce unique flavour substances through the Maillard reaction, such as benzaldehyde and 4-ethylbenzaldehyde (Xu et al., 2021). Free fatty acids are vital aroma precursors that will contribute to the flavour of fermented fish products, and they are generated via lipolysis driven by endogenous enzymes, microbial activity and the exposure of air (Xu et al., 2018). Microbial metabolism is also involved in the oxidative biochemical degradation of fatty acids and the esterification of alcohols and acids (Wang et al., 2020). Besides, the formation of off-odour in spoiled dry-cured products is connected with the excessive protein degradation and lipid oxidation induced by the undesired growth of spoilage microorganisms (Zhou et al., 2022). Therefore, it is essential to reveal how the metabolic regulation of complex microbial communities affects the formation of flavour in dry-cured squid. Up to now, most of the current researches payed attention on the biochemical changes in squid fillets under non-enzymatic and enzymatic reactions, including the microstructure and content of the protein profile (Shui et al., 2021), volatile profile (Deng et al., 2015), Maillard browning (Geng et al., 2019), tenderisation in squid muscle (Xu et al., 2020), etc. Few studies about the complex microbial community in dry-cured squid have been reported. The correlation between microbial

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activity and sensory characteristics hasn't been investigated yet. The development mechanism of sensory qualities in dry-cured squid remains unkown.

For the study, next-generation sequencing (NGS) was applied to monitor the changes in the microbial communities of dry-cured squid during processing. Quantitative Insights Into Microbial Ecology 2 (QIIME2) is a microbiome analysis package that can provide sequence classification (Rai et al., 2021). The datasets obtained from QIIME2 are more accurate and the bacteria identified are relatively more abundant using QIIME2 than other bioinformatics tools, i.e. Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) and MOTHUR, mostly due to decreased abundance of unclassified sequences (Kaszubinski et al., 2020). QIIME2 has been applied extensively for bacterial diversity analysis. However, the application of QIIME2 to analyse the microbial community in fermented food has rarely been talked about. This work aims to describe the variations in the microbial community and sensory properties including volatile compounds, taste-active compounds and aroma characteristics of dry-cured squid during the process, and to investigate the relationship between volatile/non-volatile compounds and core functional microorganisms. Headspace solid-phase microextraction (HS-SPME) combined with gas chromatography–mass spectrometry (GC/MS), a processomics methodology that was known for its advances in monitoring metabolite fingerprinting of processed foods (Xia et al., 2022), was employed for volatile compounds profiling in various dry-cured squid samples. We hypothesise that the structure and abundance of microorganisms in dry-cured squid will transform during processing and further affect sensory qualities, thereby providing a new understanding of aroma formation mechanismin dry-cured squid.

2. Material and methods

2.1. Squid sample preparation

Frozen squid (*Dosidicus gigas*) were provided by Zhejiang Feirun Food Co., Ltd. The squid were packed in bubble chambers filled with ice and transported to the laboratory through a cold chain for sample preparation. After thawing at 4 °C, the squid were eviscerated, had their skin and tentacles removed, and then were washed with cold water. A total of 50 individual squid (600 ± 50 g in weight) were used for the processing. The squid flesh was placed neatly in a fermentation pot and mixed with 10% (w/w) salt under 10 ℃ for 6 h and then oven-dried at 45 °C for 24 h. Squid samples were gathered at various stages of processing for microbial and sensory analysis. To be specific, squid samples were collected at 3 h intervals during salting and 12 h intervals during drying. Six replicates of squid samples for each sampling point were sampled. All samples were stored at − 80 °C until analysis.

2.2. Microbial analysis

The cetyltrimethylammonium bromide (CTAB)/sodium dodecyl sulphate (SDS) method was used to extract the total genome DNA in fermented squid samples. The hypervariable V3–V4 region of 16S rRNA gene was amplified using the F341 and R806. After purification and library construction, sequencing was performed on Illumina NovaSeq 6000 platform (CA, USA) at Novogene Bioinformatics Technology Co., Ltd (China). Paired-end sequencing data were merged using 1.2.11 FLASH software (https://ccb.jhu.edu/software/FLASH/) and the Effective Tags were obtained by removing chimera sequences. Denoising was performed with the Divisive Amplicon Denoising Algorithm (DADA2) package to obtain initial Amplicon Sequence Variants (ASVs), and then ASVs with abundance of<5 were filtered out (Lima et al., 2021). QIIME2 software (https://qiime2.org/) was applied to perform species annotation and phylogenetic relationship construction. Alpha diversity and beta diversity were estimated based on normalised data.

2.3. Analysis of volatile compounds

For analysis, squid samples were smashed in a food crusher (HX-PB9322, AUX, Zhejiang, China) and then 2 g of the minced sample was put in a 20 mL headspace bottle. The internal standard used was 6 mg/L of 2,4,6-trimethylpyridine (TMP). A 75 μm Carboxen/poly-dimethylsiloxane fibre assembly (Supelco Analytical, Bellefonte, PA, USA), applied in HS-SPME–GC, was exposed in the headspace of the vial, which was sealed with a polytetrafluoroethylene silicone cap. The extraction was proceeded in a water bath at 60 °C for 50 min, and then the SPME fibre was quickly inserted into the GC inlet. Three biological replicates of each group were performed for the analysis of volatile compounds.

GC–MS analysis was carried out on a capillary GC column (60 m × 0.32 mm × 1 μm; DB-5MS, Agilent Technologies, Palo Alto, CA, USA) using an Agilent 7890A-5975C system (Palo Alto, CA, USA). The injection was set at 250 °C for 5 min in the splitless mode. The transfer line temperature was set at 250 °C. The helium carrier gas (>99.99% purity) was input at a constant flow rate of 1.2 mL/min. The column temperature was set at 30 °C for 2 min before being raised to 92 °C at 4 °C/min and maintained for 2 min, then increased to 120 °C at 5 °C/min, and further increased to 240 °C at 6 °C/min and held for 6 min (Zhao et al., 2022). The MS conditions were as follows: detector interface temperature, 280 °C; ion source temperature, 200 °C; mass range, 35–350 *m/z*; ionisation energy, 70 eV.

Volatile substances were temporarily identified by comparing their fragmentation pattern in mass spectra with those in the National Institute of Standards and Technology (NIST) 14.0 database (Gaithersburg, MD, USA) and confirmed by matching their Kovats retention indices (RI) with C5–C32 *n*-alkanes (ANPEL, Shanghai, China). For each compound, the relative concentration (μg/kg) and relative odour activity value (ROAV) were calculated according to the methods described in previous paper (Zhao et al., 2022).

2.4. Electronic nose analysis

The electronic nose applied to analyse the aroma characteristics in dry-cured squid samples was built in a laboratory in Zhejiang Gong-Shang University. This instrument has been successfully used to evaluate food sensory and quality (Chen et al., 2019). Fourteen gas sensor arrays were sensitive to various aroma substances (Table S1). After gas injection, the electrical signal was gathered through a signal acquisition system, managed with a signal processing system and then evaluated by an intelligent pattern recognition system. For the tests, the squid sample (10 g) was put into a 100 mL sealed vial for 0.5 h for incubation (25 ± 1 °C). After equilibrating for 120 s, the headspace vapor (5 mL) was injected into the system. The sample was detected for 90 s. The flow rate of pure air as the carrier gas was 100 mL/min. Three biological replicates for each group were used for the analysis.

2.5. Quantification of free amino acids (FAAs), 5′-nucleotides and organic acids

FAA analysis was performed according to Zhao et al. (2016). The squid samples (2 g) were homogenised using a food crusher (HX-PB9322, AUX, China) and then dissolved in sulphosalicylic acid. After being centrifuged (6300 × *g*, 10 min), the supernatants (100 μL) were applied to an automatic Sykam S-433D FAA analyser (Eresing, Germany) with a certain amount of FAAs as an external standard, including glutamic acid (Glu), aspartic acid (Asp), serine (Ser), threonine (Thr), glycine (Gly), alanine (Ala), lysine (Lys), proline (Pro), tyrosine (Tyr), methionine (Met), valine (Val), phenylalanine (Phe), leucine (Leu), isoleucine (Ile), histidine (His), cysteine (Cys) and arginine (Arg). The separation was conducted on an ion-exchange resin column (LCA K06/Na, 4.6 mm × 150 mm × 7 μm; Sykam Eresing, Germany).

5′-Nucleotides and organic acids from squid samples were both

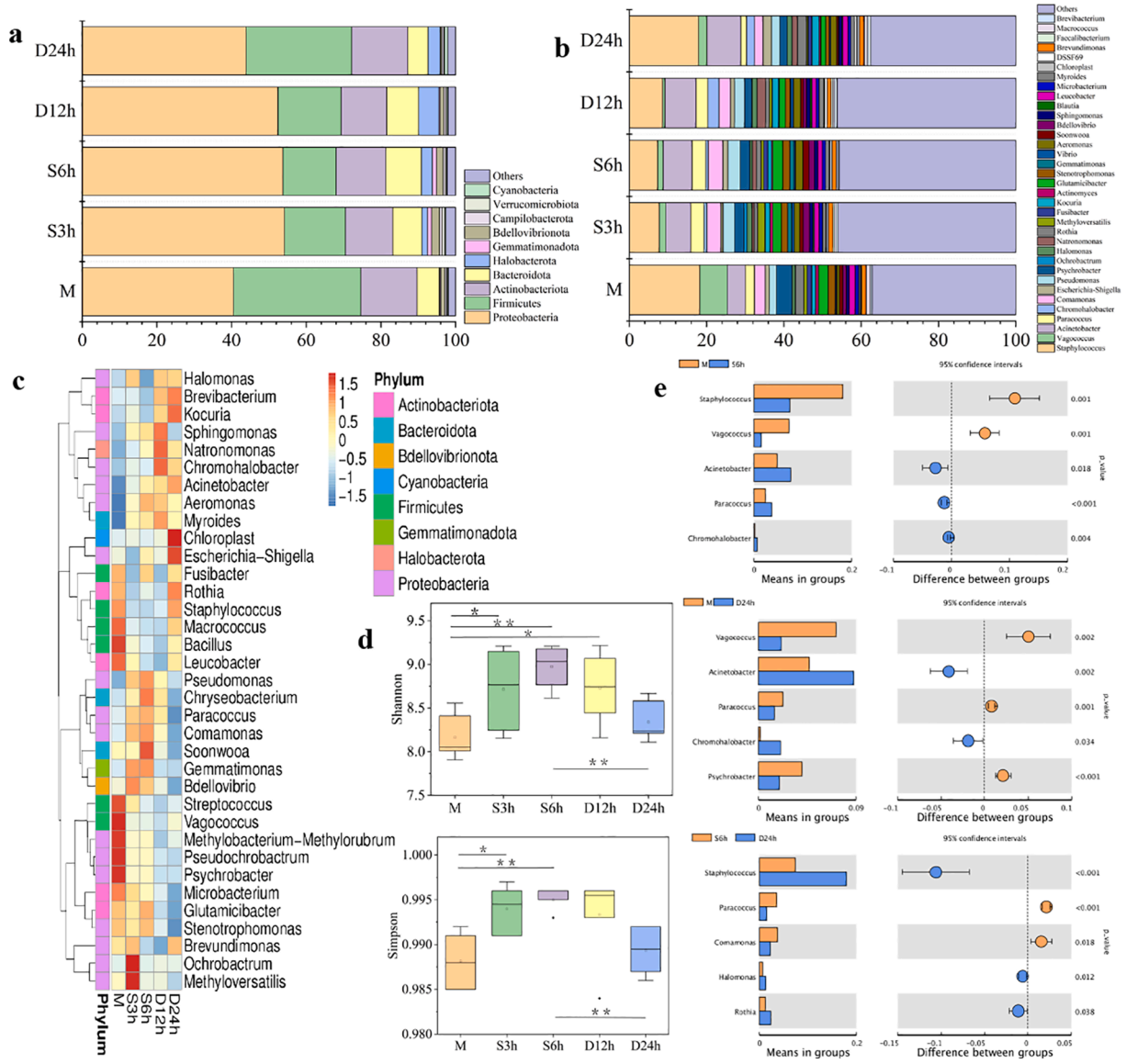

Fig. 1. Composition and diversity of microbiota in dry-cured squid samples. (a) Relative abundance of the bacterial community at the phylum level. (b) Relative abundance of the bacterial community at the genus level. (c) Top 35 most abundant genera. (d) Shannon and Simpson index of the bacterial community. (e) *t*-Test between different bacterial species groups. * and ** indicate significant ($p < 0.05$) and highly significant ($p < 0.01$) differences, respectively.

extracted with perchloric acid. The extract was centrifuged (6300 × *g*) at 4 °C for 10 min. The supernatants were neutralised with 10 M KOH. Then the solution was filtered through a 0.45 μm syringe filter and analysed by High Performance Liquid Chromatography (HPLC; Agilent 1260, Palo Alto, CA, USA). An aliquot of 10 μL of solution was injected into the system. For analysis of 5′-inosine monophosphate (5′-IMP), 5′-guanosine monophosphate (5′-GMP) and 5′-adenosine monophosphate (5′-AMP), an Agilent SB-C18 column (4.6 mm × 150 mm × 5 μm; Palo Alto, CA, USA) was applied for chromatographic separation. Phosphate buffer solution (pH 3.2) containing tetrabutylammonium hydrogen sulphate (1.4 mg/L) and methyl alcohol (4%, v/v) was used as the mobile phase, with a flow rate of 1.0 mL/min. The absorbance was measured at 254 nm. The column temperature was 25 °C. For analysis of organic acids, an AQ-C18 column (4.6 mm × 250 mm × 5 μm; Agilent, Palo Alto, CA, USA) was used. A kown mixture of organic acids were added as external standards. 10 mmol/L of dipotassium hydrogen phosphate (pH 2.55) was used as the mobile phase, with a flow rate of 0.5 mL/min. The absorbance was measured at 210 nm. The column temperature was 35 °C.

The taste activity value (TAV) of taste compound was calculated by calculating the ratio of the compound concentration to its threshold value in water. The threshold values were referenced from Wang et al. (2020).

2.6. Sensory evaluation

For sensory evaluation of squid samples, quantitative descriptive analysis was performed. The evaluation team contained 15 people, six males and nine females, aged from 24 to 35. The sensory attributes of squid samples were fully discussed. Seven descriptions of umami, sourness, sweetness, bitterness, saltiness, roast and fishy odour were finally determined. Monosodium glutamate (0.50 mg/mL), citric acid (0.50 mg/mL), sucrose (5.0 mg/mL), quinine sulphate (0.03 mg/mL) and sodium chloride (1.50 mg/mL), were applied as references of taste attributes. Raw squid samples (5 g) and commercial dry-cured squid samples (5 g) were used as the references of fishy and roast attributes, respectively. Before the evaluation, 10 g of squid samples were immersed in clean water for 10 min and steamed for 12 min. After cooled down naturally to room temperature, fifteen replicates at each sampling point were randomly presented to panelists in sealable plastic

cups coded with 3 digit numbers. The assessors were asked to open the cups and sniff the headspace above the samples to determine the intensities of roast and fishy odour, which defined as desirable roasted meaty flavour and undesirable amine smell, respectively. To evaluate the umami, sourness, saltiness, bitterness and sweetness of squid samples, pure water were provided to rinse the mouth between different samples. The intensity of every attribute was graded on a 11-point scale (0, none; 5, moderate; 10, extremely high intensity). Quantitative descriptive analysis was applied for the sensory evaluation of dry-cured squid samples.

2.7. Statistical analysis

SPSS 21.0 software (IBM, Armonk, USA) was applied for data statistical analysis. Z-score normalization was used for data pretreatment. The chemical data was subjected to one-way analysis of variance (ANOVA) using samples at various processing time as fixed factors. Duncan's multiple comparison was also performed for each group. The significance level in the analyses was considered at $P < 0.05$. The descriptive sensory data of squid samples for each group was expressed as the mean ± standard deviation (SD) of sensory scores obtained from 15 panelists. The electronic nose response data of squid samples were modeled with a principal component analysis (PCA) model using Origin 2019b (OrginLab, Northampton, MA, USA). Two principal components (PC1 and PC2) were used for samples discrimination. To elucidate the correlation between the microogranisms and sensory characteristics of dry-cured squid during processing, SiMCA 14.1 (Umetrics AB, Umea, Vasterbotten, Sweden) was used for bidirectional orthogonal partial least squares (O2PLS) analysis, in which microbiota data (X matrix) were mapped to contents of sensory compounds (Y matrix). A 7-fold cross-validation method was used to estimate the predictive ability of the correlation network model. The O2PLS qualtiy paratemers were validated by 200 random permutation tests.

3. Results and discussion

3.1. Microbial community of dry-cured squid samples

The bacterial composition dynamics of dry-cured squid during processing are shown in Fig. 1. The microbial strains can be assigned to 49 phyla, of which the percentage relative abundance is depicted in Fig. 1a. Seven bacterial phyla, Proteobacteria, Firmicutes, Actinobacteriota, Bacteroidota, Halobacterota, Gemmatimonadota and Bdellovibrionota, showed a relative abundance>1%. Proteobacteria, Firmicutes and Actinobacteriota were the most abundant phyla in dry-cured squid throughout the process, and their relative abundance was above 10%. At the beginning of the process, the relative abundance of Proteobacteria was 40.51%, increasing to a maximum of 54.15% after salting for 3 h then gradually decreasing to 43.91% after the drying stage. However, Firmicutes in dry-cured squid exhibited a different pattern of variation during processing, as its relative abundance decreased during the salting stage and then increased during 24 h of drying to a final value of 28.28%. The high proportion of Protobacteria and Firmicutes and their fluctuation during the various processing stages implies that they play a vital part in sensory development of dry-cured squid. Similar results have been reported in fermented seafood products (Zhang et al., 2021; Zhao et al., 2022).

Sequencing reads of 1050 genera at the genus level were classified during the various processing stages. From the results shown in Fig. 1b, *Staphylococcus* was the most dominant genera in the raw and dry-cured squid samples, with relative abundance of 18.21% and 17.95%, respectively. The trend of variations in the relative abundance of *Staphylococcus* and *Vagococcus* was consistent with that for Firmicutes, exhibiting an evident decrease in squid samples after salting and a subsequent increase after the processing stage. *Acinetobacter* became the second most dominant genus in the final product of dry-cured squid: its relative abundance increased from 4.68% to 8.81%.

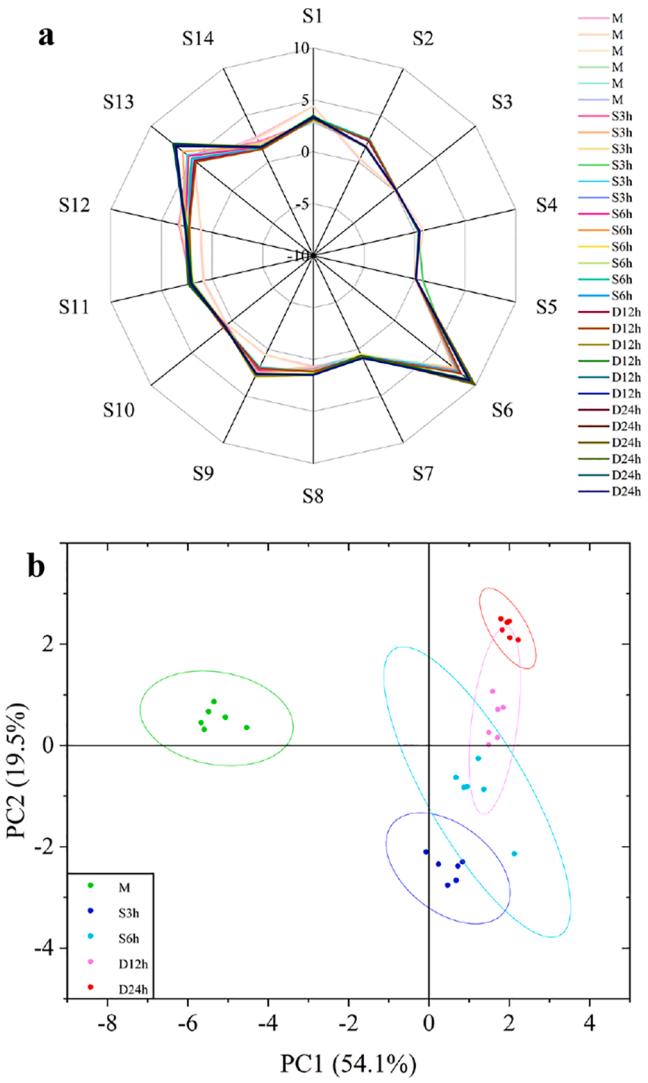

Fig. 2. Radar graph (a) and PCA scores diagram (b) of dry-cured squid samples associated with processing time by electronic nose analysis.

The differences among various processing stages are depicted in a heatmap (Fig. 1c). A significant difference in abundance of the top 35 genera was observed in squid samples throughout the process. The variation in the bacterial community structure of dry-cured squid can be ascribed to the complex and varying processing conditions, such as temperature and salt. It has been reported that halophilic and halotolerant bacteria are more able to exist in traditional fermented seafood products, which may play a vital part in the development of aroma, taste and colour, and shelf life in the ripening period (Lorentzen et al., 2015). In this study, the prevalence of *Staphylococcus* suggests its vital role in the flavour quality of dry-cured squid.

The estimates of bacterial diversity (Shannon and Simpson index) of dry-cured squid samples are shown in Fig. 1d. The Shannon and Simpson index of squid samples both increased after salting. Significant differences were observed between the M and S6h groups, as well as the S6h and D24h groups ($P < 0.05$). It has been suggested that both salting and drying have a serious effect on the bacterial diversity of dry-cured squid samples, although there was no apparent difference between M and D24h. *t*-Test analysis was used to identify the bacterial species with significant differences. From the results shown in Fig. 1e, it can be concluded that salting can suppress the growth of *Staphylococcus*, but drying is able to stimulate the development of this strain.

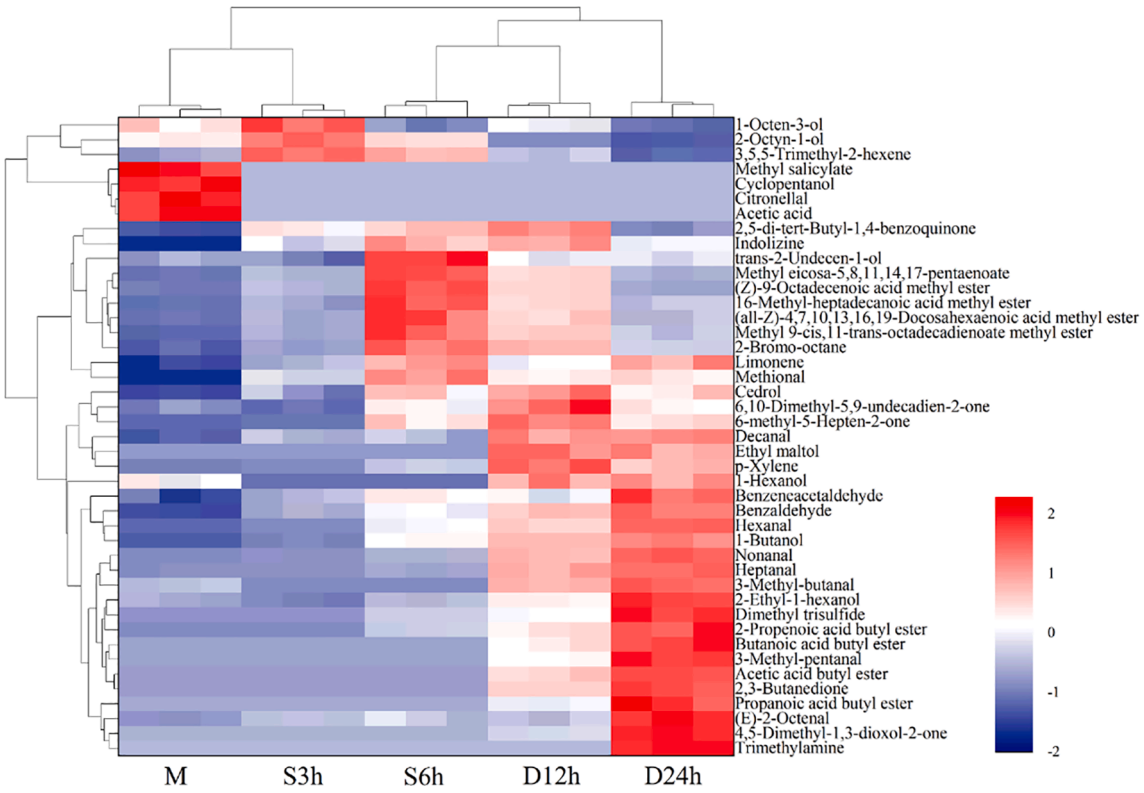

Fig. 3. Heatmap visualisation of the volatile compounds in various dry-cured squid samples during the process. Red squares indicate higher concentrations of the substances, while blue squares indicate lower concentrations.

3.2. Electronic nose analysis of dry-cured squid

In this work, an electronic nose was employed to characterise the complicated odour profile in dry-cured squid. The radar chart showed that the response levels of sensors S1, S2, S6, S8, S9, S11, S12 and S13 for the intensity of odours varied prominently among the squid samples from various processing stages, but no such discrepancies were observed by sensors S3 and S7 (Fig. 2a). The response signals obtained were statistically analysed by PCA (Fig. 2b). The figure shows that PC1 and PC2 accounted for 54.1% and 19.5%, respectively, which explained 73.6% of the difference, indicating that the principal components can reflect the overall odour of dry-cured squid throughout processing. The data show that the raw squid group was far away from the other groups and that D24h was completely separated from S3h and S6h, whereas the data points from S3h and S6h, S6h and D12h, D12h and D24h, respectively, were not well distinguished. The distances between the areas reflect the differences in volatile aroma components of the squid samples. According to the representative sensitive material types of gas sensors (Table S1), it was inferred that aldehydes, alcohols, ketones, amines and sulphur-containing compounds of dry-cured squid change as the processing progresses.

3.3. Volatile profile analysis by GC–MS analysis

Forty-four volatile substances were detected in dry-cured squid samples. Gas chromatograms of volatile compounds from different dry-cured squid samples are shown in Fig. S1. The relative concentrations of these compounds are demonstrated in Table S2. The variations of volatile compounds are demonstrated in a heat map (Fig. 3). It shows that the types and content of volatile compounds increased during processing. The relative concentrations of aroma compounds increased from 178.97 μg/kg (M) to 1278.82 μg/kg (D24h) after the entire fermentation process. A large amount of volatile compounds arise in the drying stage, which may be due to the metabolic reactions of flavour precursors such as fatty acids and amino acids during processing. Drying temperature is one of the vital factors impacting the sensory characteristics of dry-cured squid via the Maillard reaction and lipid oxidation (Deng et al., 2015).

The top five volatile compounds in dry-cured squid samples in terms of abundance were 2,3-butanedione (428.38 μg/kg), 1-butanol (220.35 μg/kg), hexanal (132.76 μg/kg), nonanal (87.60 μg/kg) and methional (65.39 μg/kg). The calculation of ROAV revealed that 11 compounds contributed significantly to the sensory perception of dry-cured squid (Table S3) (Gemert, 2011). 2,3-Butanedione, as the most abundant volatile compound with a low threshold, contributes a pleasant butter and popcorn odour to dry-cured squid. 2,3-Butanedione is the most well-known of the alpha-diketone substances that have been used as individual flavouring substances in the flavour industry, due to its natural presence in a widespread types of foods including fruits, vegetables, dairy products, poultry, beef and fish, and the offer of the desired taste sensation (Hallagan, 2017). Its addition to food has been permitted by the Food and Drug Administration (FDA) without causing a human health hazard. It has been suggested that 2,3-butanedione is formed through *C*2/C4 cleavage of the D-glucose moiety, which is a consequence of caramelisation and the Maillard reaction during the thermal processing and storage of foods (Zhang et al., 2019). The microbial community is also responsible for the formation of 2,3-butanedione in fermented food. Different species of LAB have been reported to produce abundant 2,3-butanedione and are considered as potential starters for the improvement of flavour (Zheng et al., 2020).

Studies have shown that aldehydes and alcohols make a prominent contribution to the flavour qualities of fermented seafood (Zhao et al., 2022), which are mainly originated from the breakdown of unsaturated fatty acids under the influence of microorganism activity or endogenous enzymes. There were 11 aldehydes and nine alcohols recognized in squid samples, of which the concentrations reached 425.28 and 259.51 μg/kg at the end of the process, respectively. The increase in the content of aldehyde and alcohols could be associated with fatty acids degradation and processing factors including salt, time and temperature (Det-

Table 1
Quantitative concentration of free amino acids, organic acids and 5′-nucleotides in dry-cured squid during the process.

	Threshold in water[a] (mg/100 g)	Concentration in dry-cured squid samples (mg/100 g)				
		M	S3h	S6h	D12h	D24h
Umami amino acids						
Aspartic acid	100	88.15 ± 0.45	14.27 ± 0.76	12.33 ± 0.82	22.87 ± 1.25	19.05 ± 0.66
Glutamic acid	30	20.23 ± 1.31^{c}	13.97 ± 0.94^{d}	12.15 ± 1.06^{d}	28.71 ± 0.85^{b}	43.66 ± 1.43^{a}
Sweet amino acids						
Serine	150	18.21 ± 2.31^{a}	12.04 ± 0.49^{b}	10.82 ± 0.51^{c}	21.35 ± 2.09^{a}	20.48 ± 1.87^{a}
Glycine	130	60.42 ± 3.89^{b}	52.41 ± 2.25^{c}	44.29 ± 1.90^{d}	70.32 ± 4.54^{a}	67.91 ± 2.96^{a}
Threonine	260	40.01 ± 1.25^{b}	26.42 ± 2.35^{c}	25.72 ± 1.66^{c}	62.51 ± 2.85^{a}	40.26 ± 0.98^{b}
Alanine	60	109.54 ± 5.78^{c}	77.65 ± 3.46^{d}	76.18 ± 2.71^{d}	129.45 ± 6.94^{b}	140.32 ± 10.28^{a}
Proline	300	120.42 ± 9.04^{b}	109.54 ± 8.22^{bc}	95.43 ± 7.35^{c}	109.55 ± 6.82^{bc}	140.17 ± 6.01^{a}
Lysine	50	23.75 ± 1.20^{b}	16.49 ± 0.78^{c}	16.89 ± 1.43^{c}	29.64 ± 2.41^{a}	30.44 ± 1.99=^{a}
Tyrosine	90	8.60 ± 0.51	6.41 ± 0.41	4.94 ± 0.27	14.27 ± 1.60	10.49 ± 0.52
Bitter amino acids						
Valine	40	14.23 ± 0.34^{ab}	11.24 ± 0.94^{c}	9.71 ± 0.73^{c}	13.78 ± 0.37^{b}	15.35 ± 0.62^{a}
Methionine	30	53.29 ± 2.04^{c}	39.85 ± 1.69^{d}	36.26 ± 2.05^{d}	80.17 ± 5.93^{a}	67.93 ± 4.27^{b}
Arginine	50	259.77 ± 13.53^{b}	150.35 ± 10.94^{d}	190.18 ± 12.53^{c}	339.57 ± 21.39^{a}	340.46 ± 19.07^{a}
Phenylalanine	90	10.06 ± 0.31^{c}	12.49 ± 0.44^{b}	7.24 ± 0.72^{d}	16.40 ± 0.82^{a}	11.83 ± 1.08^{bc}
Ileleucine	90	8.41 ± 0.47^{c}	7.23 ± 1.32 ^{cd}	6.30 ± 0.95^{d}	9.75 ± 0.58^{b}	11.46 ± 0.82^{a}
Leucine	190	18.46 ± 0.88^{c}	17.42 ± 1.04^{c}	17.08 ± 0.86^{c}	26.74 ± 2.06^{b}	34.15 ± 3.70^{a}
Histidine	20	100.43 ± 8.09^{b}	99.71 ± 6.43^{b}	97.54 ± 9.25^{b}	150.36 ± 12.64^{a}	139.58 ± 9.87^{a}
Organic acids						
Malic acid	50	516.67 ± 14.24^{d}	419.21 ± 22.59^{d}	590.61 ± 35.71^{c}	1333.55 ± 75.04^{a}	1123.12 ± 88.67^{b}
Lactic acid	126	437.65 ± 32.05^{c}	288.26 ± 13.97^{d}	459.28 ± 35.09^{c}	558.1 ± 22.80^{b}	880.89 ± 29.55^{a}
Acetic acid	12	2.42 ± 0.11^{d}	60.28 ± 4.58^{c}	190.52 ± 9.49^{a}	138.8 ± 7.06^{b}	138.04 ± 7.53^{b}
Fumaric acid	–	0.74 ± 0.06^{a}	0.19 ± 0.02^{d}	0.16 ± 0.01^{d}	0.63 ± 0.05^{b}	0.51 ± 0.03^{c}
5′-Nucleotides						
Hypoxanthine	120	97.62 ± 4.56^{b}	8.93 ± 0.03^{d}	80.85 ± 6.66^{c}	150.96 ± 9.51^{a}	145.41 ± 7.10^{a}
5′-Guanosine monophosphate	12.5	1.66 ± 0.12^{c}	3.42 ± 0.26^{b}	2.79 ± 0.32^{b}	3.36 ± 0.34^{b}	6.67 ± 0.53^{a}
5′-Adenosine monophosphate	50	3.08 ± 0.13	1.55 ± 0.12	10.86 ± 0.99	6.65 ± 0.45	2.37 ± 0.11
5′-Inosine monophosphate	25	11.41 ± 0.10	3.12 ± 0.22	0.93 ± 0.06	4.91 ± 0.33	15.50 ± 0.12

Notes: 1. Means with different superscripts in the same row are significantly different ($P < 0.05$, $n = 3$). 2. "-", not detected. 3. "a", referenced from Wang et al. (2020).

udom et al., 2021). 3-Methylbutanal (malty, caramel), heptanal (rancid, fat), methional (roast), hexanal (grass, fat), nonanal (fatty, green), 2-octenal (roast, fatty), 1-octen-3-ol (mushroom, fishy, fatty), decanal (soap, tallow) and were the dominant aldehydes. Nonanal can be considered as a potential marker for overall lipid oxidation in dry-cured squid products on account of the ever-increasing content with processing time. Methional, generated via the Maillard reaction and Strecker degradation and which showed high ROAV values in dry-cured squid samples, has been regarded as a vitally important contributor to the cooked-potato and meat like flavour in squid fillets dried by hot air (Deng et al., 2015).

Volatile sulphur compounds such as methional and dimethyl trisulphide are known to play an vital part in the formation of fish aroma (Song et al., 2021). They are potent odour-active compounds that can rapidly cause a strong odour even at extremely low concentrations due to their low odour thresholds. Dimethyl trisulphide contributes a strong odorant described as a fishy, fried garlic and onion-like aroma to dry-cured squid; its concentration increased rapidly during the drying stage. The formation pathways of volatile sulphur compounds such as methional and dimethyl trisulphide are based on the microbial or thermal degradation of sulphur-containing amino acids, including free, peptide and protein amino acids (Cardinal et al., 2020). Methanethiol is an important intermediate from Met metabolism, which is usually rapidly converted into dimethyl trisulphide through the Ehrlich pathway (Lu et al., 2018). The production of methional is an enzymatic reaction mediated by Met aminotransferase and α-keto acid decarboxylase activity with Met as a precursor (Che et al., 2020). In addition, the Maillard reaction between Met and ribose can also cause an increase in the amounts of methional and dimethyl trisulphide (Liu et al., 2020).

Trimethylamine (TMA) also plays a part in odour formation owing to its low threshold; it contributes to the development of the particularly intense smell of fermented fish (Shen et al., 2021). It has been reported that TMA is a major volatile compound in dried squid (Sukkhown et al., 2018). From the results, it can be seen that TMA showed a remarkable enhancement during the drying stage but was not detected in fresh and cured squid samples. The results are similar to those in previous reports on other dried seafood products (Hu et al., 2021). The increase in TMA during heating can be ascribed to the thermal decomposition of trimethylamine oxide (TMAO). Heating squid muscles can induce significant generation of TMA and dimethylamine (DMA) from TMAO, but the conversion differs relying on the heating temperature (Fu et al., 2007). This suggested that temperature significantly affects the formation of volatile compounds including α-dicarbonyls, carbonyl compounds, alcohols, sulphur compounds and TMA in dry-cured squid, indicating that the drying period is a vital stage for enhancing flavour quality. Besides, autochthonous microbiota succession is also involved in the formation of TMA in fermented fish products (Shen et al., 2021).

3.4. Analysis of non-volatile taste-active compounds

To investigate the key taste compounds contributing to taste development in dry-cured squid, the content of organic acids, amino acids and nucleotide acids was quantitatively determined (Table 1). The results demenstrate that the concentrations of FAAs undulated during processing, but ultimately enhanced after the final stage of drying. The taste-active FAAs accounted for 64.57% of total FAAs in dry-cured squid after processing, including Glu, Ala, Met, His and Arg. These five FAAs contributed significantly to taste, as all TAVs were ≥ 1. As is well known, Glu is one of the monosodium glutamate (MSG)-like components that work in synergy with 5′-nucleotides and contribute to the umami taste. Ala and Arg, which were the most abundant, are sweet-related amino acids responsible for the related desirable taste characteristics. His was found to be essential to the bitterness of dry-cured squid. It is worth mentioning that Met plays an vital role in the sulphurous taste of dry-cured squid. The increase in Met abundance was consistent with the production of volatile sulphur compounds including methional and dimethyl trisulphide, as Met is an important flavour precursor.

5′-Nucleotides are taste-active substances present in many kinds of

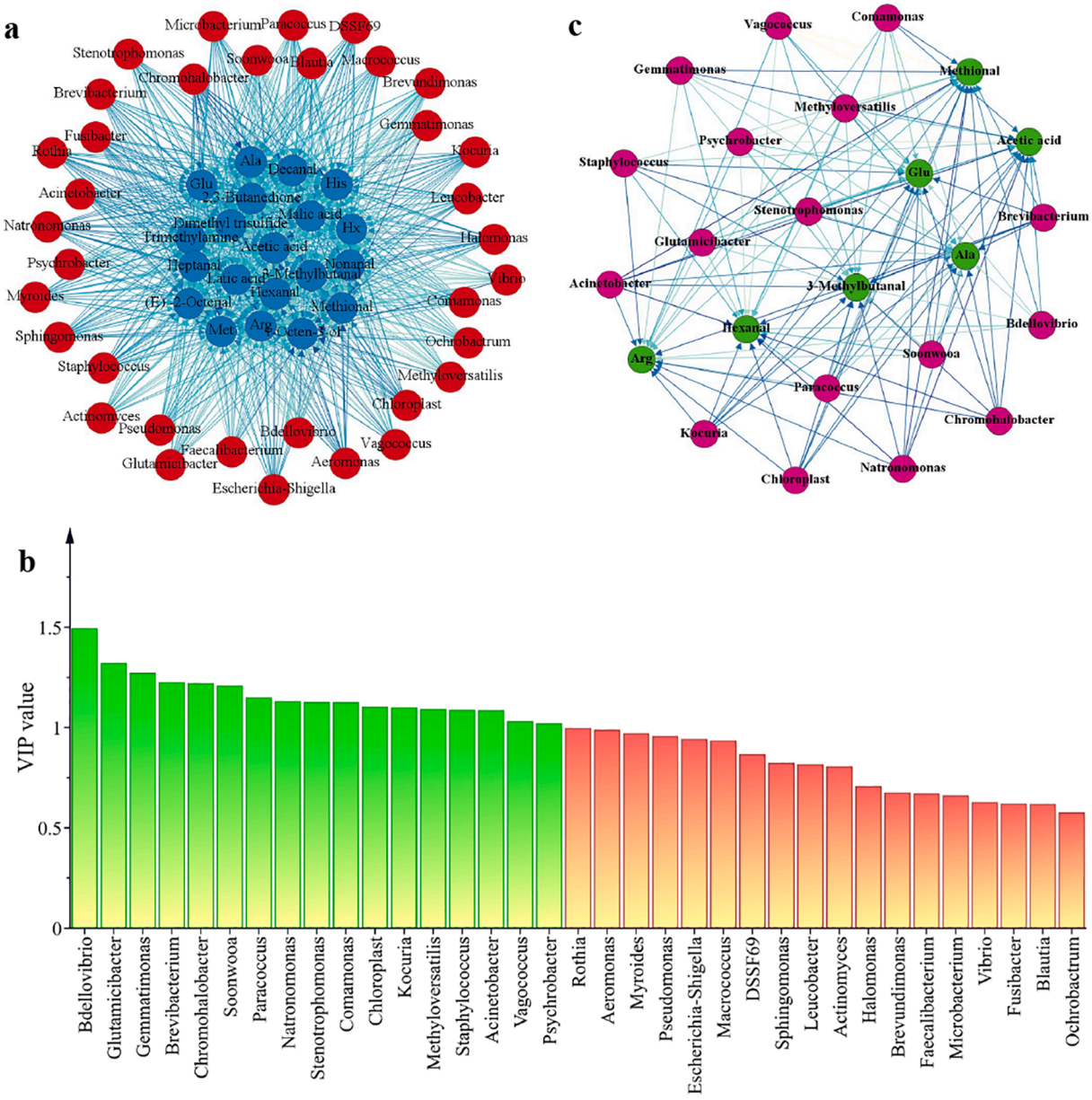

Fig. 4. Network model of the correlation between microorganisms and volatile substances by O2PLS modelling of dry-cured squid during the process. (a) Correlations between the top 35 genera and 17 sensory indicator substances. (b) Changes in VIP values of the top 35 microorganisms. (c) Correlations between the 17 core microorganisms (VIP > 1) and seven typical sensory indicators.

foods and are generated via the degradation of FAAs and adenosine triphosphate (ATP). From the results (Table 1), the total nucleotide content detected in dry-cured squid was 1.61 μg/kg, which was significantly higher than that of raw squid samples ($P < 0.05$). 5′-IMP, 5′-GMP and 5′-AMP are important umami-enhancing components that contribute to umami taste and act synergistically with MSG-like FAAs by combining with the same T1R1 + T1R3 receptor (Manninen et al., 2018). However, they may have barely any effect on the umami taste of dry-cured squid owing to their low abundance and TAV. Only the content of Hx in squid samples at the drying stage was higher than its threshold, indicating its contribution to a bitter flavour in dry-cured squid.

Malic acid, lactic acid and acetic acid were the predominant organic acids in dry-cured squid samples (1123.12, 880.89 and 138.04 mg/100 g, respectively) (Table 1). They were identified as taste-active compounds with a TAV ≥ 1, contributing a unique umami taste and sourness to dry-cured squid. Malic acid, with a strong ability to enhance sour and salty flavours (Pu et al., 2021), was the most abundant organic acid in dry-cured squid samples. Lactic acid was the second most dominant organic acid in dry-cured squid, conferring a unique sourness and thickness. The concentrations of organic acids increased significantly after processing ($P < 0.05$), which is associated with the synthesis and metabolism of fatty acids and amino acids and attributed to the increasing activity of enzymes from salt-tolerant microorganisms presented in dry-cured squid (De Vuyst & Leroy, 2020).

Overall, protein degadation was one of the main metabolic pathways to affect the taste characteristics of dry-cured squid. This result is consistent with a previous study (Liao et al., 2022). The variations in taste profile at different processing stages were correlated with the protease activity of exogenous and endogenous enzymes, under the influence of processing factors such as temperature, time and salt content (Ismail et al., 2020). The data show that the squid processed to the drying stage presented more umami taste, sweetness and bitterness than raw squid and cured squid samples, which implies that higher protease

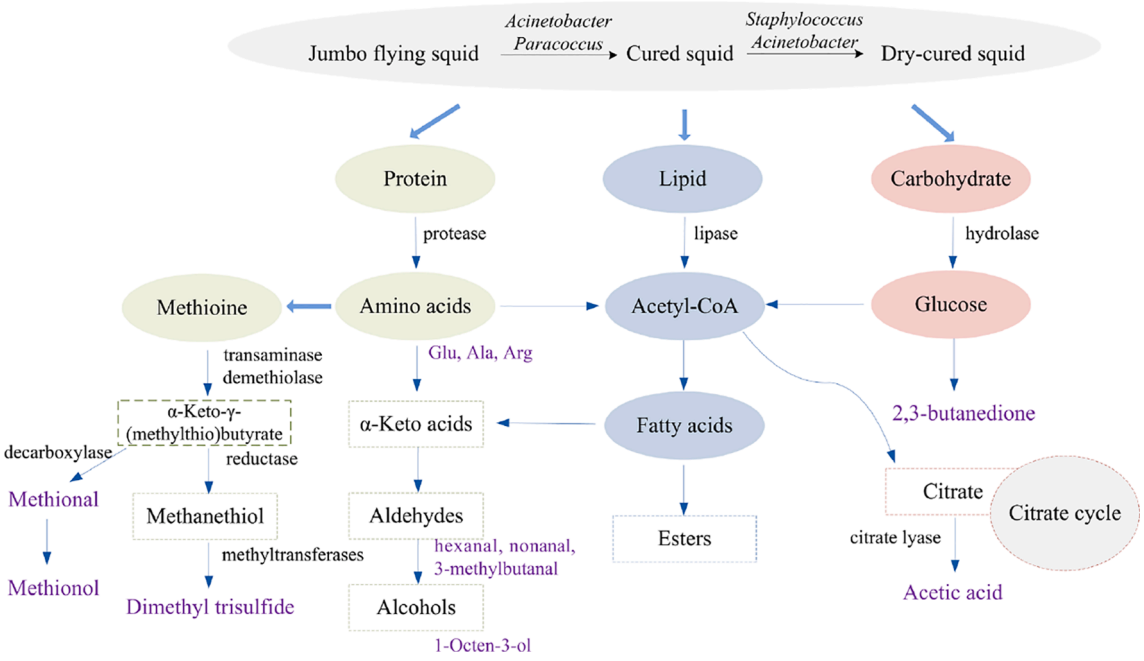

Fig. 5. Predicted metabolic network for the development of sensory characteristics in dry-cured squid during the process.

activity is participated in the taste-active compounds formation at high temperature.

3.5. Sensory evaluation of dry-cured squid samples

To measure the development of flavour throughout the process, organoleptic evaluation of dry-cured squid samples was conducted; the results are shown in Fig. S2. The raw squid samples showed the umami and fishy flavour. An intense saltiness rises in processed squid samples after the addition of salt. The umami taste, sourness, bitterness and sweetness of squid samples increased remarkably along with the processing time, fitting with the development of water-soluble taste components, such as FAAs (Glu, Ala, Met, Arg and His), organic acids (malic acid, acetic acid and lactic acid) and 5′-nucleotides (Hx), of which the concentrations were higher than their taste thresholds. In addition, the roast and fishy odour was also enhanced, which was consistent with the occurrence of a mass of flavour compounds (hexanal, nonanal, heptanal, 2-octenal, 1-octen-3-ol, methional and dimethyl trisulphide).

3.6. Analysis of relationship between bacterial community and sensory characteristics

The O2PLS method was applied to explore the relationship between microbial community and sensory characteristics during the process of dry-cured squid. The 17 sensory indicator compounds, including 11 principal volatile compounds (ROAV $\geq$ 1), five taste amino acids and one 5′-nucleotide (TAV > 1), were chosen to build a network model with the top 35 genera. The R^2_x, R^2_y and Q^2 values of the model were 0.57, 0.67 and 0.61, respectively. The 200 random permutation tests results showed that all calculated Q2 values were lower than the original points in the validation plot and the Q2 intercepted the vertical axis was below zero (Fig. S3). These findings indicated that the built O2PLS model is reliable for predicting the correlation between the abundance and structure of microorganisms and volatile/non-volatile compounds. Fig. 4a shows that all of the 35 genera were closely related to the 17 sensory indicator compounds. The Variable Importance in Projection (VIP) vector of the analysed genera was 0.58–1.50; 17 bacterial genera had a VIP > 1, including *Bdellovibrio*, *Glutamicibacter*, *Gemmatimonas*, etc. (Fig. 4b), which may imply that these genera have vital effects on the sensory qualities of dry-cured squid. Bidirectional O2PLS analysis was further conducted to investigate the correlation between dominant genera (VIP > 1) and sensory indicator compounds (Fig. 4c). It showed that the production of three aldehydes (hexanal, 3-methylbutanal and methional), three FAAs (Glu, Ala and Arg) and one organic acid (acetic acid) was remarkably correlated with the development of the dominant genera ($P < 0.05$).

The metabolism of food-inhabiting microbiota, such as lipolysis and proteolysis, determines the types and content of constituents, which play a vital role in the sensory characteristics of fermented food (Zhao et al., 2022; Zhou et al., 2022). As reported elsewhere, the formation of hexanal was positively correlated with oleic acid hydrolysis (Moretti et al., 2017). 3-Methylbutanal is generated by the Strecker reaction of α-keto acids through branched-chain amino acid aminotransferases. The generation of methional is also an enzymatic reaction mediated by microbial metabolism. The taste amino acids, including Glu, Ala and Arg, with a taste of umami, sweet and bitter, respectively, are generated by protease-mediated protein hydrolysis. The yield of acetic acid is promoted by citrate lyase producers through citrate metabolism (Ouattara et al., 2017). Microbial activity is involved in the formation of aroma and taste substances in dry-cured squid. It has been recorded that *Staphylococcus* spp., as the main genera in dry-cured squid, can degrade the branched-chain amino acids into methyl branched aldehydes (Hu et al., 2020). The network pathway for the formation of sensory compounds is demonstrated in Fig. 5. It shows that the sensory development of dry-cured squid was mainly via catabolism pathways of three major components: amino acids, fatty acids and glycogen.

4. Conclusions

The present study of dry-cured squid based on NGS and molecular sensory analysis provides a profound understanding of how the regulation of microbial metabolisms affects the formation of flavour in dry-cured squid. Microbial analysis demonstrated that *Staphylococcus* and *Acinetobacter* were the primary genera in various dry-cured squid samples. Eleven volatile compounds were identified to have a great contribution to the aroma characteristics of dry-cured squid. O2PLS analysis showed that hexanal, 3-methylbutanal and methional were highly correlated with 17 genera (VIP > 1, $P < 0.05$). Correlation analysis

revealed that the formation of taste-active compounds were closely related to the core microorganisms. *Staphylococcus* was involved in the regulation of microbial metabolism on the formation of flavour in dry-cured squid. The current study provides novel insights into the microorganisms composition and sensory characteristics of dry-cured squid. The results are beneficial to uncover the relationship between microbial community and flavour formation in dry-cured squid during processing. This study will assist future research on how microbial regulation affects sensory formation at the molecular level and provide guidance for industrial cultural starters.

CRediT authorship contribution statement

Dandan Zhao: Conceptualization, Methodology, Software, Writing – original draft, Data curation. **Jun Hu:** Visualization, Investigation. **Xuxia Zhou:** Supervision, Validation. **Wenxuan Chen:** Writing – review & editing.

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.

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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.2022.100376.

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