

Elucidating the interplay between lipases and lipid metabolites during the processing of dry-cured Wuchang fish (*Megalobrama amblycephala*)

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Abstract: This study systematically investigated lipid metabolic alterations during the dry-curing processing of Wuchang fish (*Megalobrama amblycephala*) using ultra-performance liquid chromatography-tandem mass spectrometry coupled with an in-house lipidomics database. A total of 1 370 lipid species were identified, classified into six major categories (fatty acids, glycerolipids (GL), glycerophospholipids (GP), prenol lipids, sphingolipids, and sterol lipids) and 41 subclasses. Orthogonal partial least squares-discriminant analysis revealed significant lipidomic divergence among three processing stages (fresh fish (CK), curing for 2 days (Y2), and drying for 2 days (G2)), with GL dominating the lipid profile and GP exhibiting marked depletion during salting. The lipid oxidation index elucidated competing effects of lipid hydrolysis (substrate depletion) and oxidation (metabolite generation), revealing that lipoxygenase-driven conversion of polyunsaturated fatty acids (PUFAs) to volatile aldehydes and alcohols predominated during salting, while reduced water activity in air-drying triggered PUFAs autooxidation and oxylipins accumulation. Post-processing nutritional enhancement was evident, with essential fatty acids (eicosapentaenoic acid, docosahexaenoic acid, linoleic acid, and oleic acid) significantly enriched in air-dried fish, constituting 18.869% of total free fatty acids, a substantial increase from 13.414% in fresh samples. This work provides critical insights into flavor formation mechanisms and quality control strategies for traditional dry-cured fish products.

Keywords: lipidomic; dry-cured Wuchang fish; ultra performance liquid chromatography; lipase

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1 Introduction

Wuchang fish (*Megalobrama amblycephala*) is rich in high-quality protein and polyunsaturated fatty acids (PUFAs), which are effective in preventing cardiovascular diseases^[1]. However, the lipids in fish are highly susceptible to oxidation during transportation, processing, and storage, leading to spoilage. Hubei Province, China, is one of the main production areas of Wuchang fish, where it is commonly processed into dried products. Dry-cured Wuchang fish, known for its balanced saltiness and unique flavor, is a traditional delicacy in China. The production process typically involves two main stages: salting and air-drying. During salting, salt penetrates the fish, acting as a preservative^[2] and enhancing flavor through enzymatic activity. The air-drying stage further reduces moisture content, improving storage stability. Nevertheless, the role of enzymes in flavor formation and lipid metabolism during the dry-curing process remains incompletely understood and requires further investigation.

Current research on cured fish products is limited, with most studies focusing on lipid changes in cured meats such as ham and marinated poultry. Previous studies suggest that lipid oxidation products (e.g., aldehydes, ketones, and alcohols) are key contributors to the characteristic flavors of cured meats, generated

through enzymatic degradation of proteins and lipids, fatty acids (FA) oxidation, autooxidation, and enzymatic oxidation^[3–4]. The quality of dry-cured Wuchang fish is closely related to the degree of lipid oxidation. Alfaia et al.^[5] found that moderate lipid oxidation enhances flavor, while excessive oxidation produces undesirable odors. Lipid hydrolysis during fermentation is primarily driven by endogenous lipases (acid lipase, neutral lipase, and phospholipase)^[6], which, along with microbial lipases, degrade active lipids into unsaturated fatty acids (UFAs). These are further oxidized by lipoxygenase (LOX) to generate volatile flavor compounds^[7–8]. Salt added during curing also significantly promotes lipid oxidation, as Na⁺ enhances LOX activity and accelerates lipid autooxidation, playing a crucial role in flavor development. However, the regulatory mechanisms of enzymes on lipid oxidation in dry-cured Wuchang fish, particularly the dual effects of NaCl on LOX activity and lipid autooxidation, remain poorly understood and warrant further exploration.

In this study, lipidomics combined with enzyme activity analysis were employed to investigate the dynamic changes in lipids during different processing stages (pre-processing, post-salting, and post-drying) of Wuchang fish. The aim is to elucidate the influence of enzymes on lipid oxidation during dry-curing and provide a

theoretical basis for optimizing the processing technology of dry-cured Wuchang fish.

2 Materials and methods

2.1 Sample preparation and salting

The Wuchang fish fillets used in the experiments were procured in June 2024 from Nanhu Comprehensive Fresh Market in Wuhan city, Hubei Province, China. The average length and weight of the fish were (22.59 ± 1.78) cm and (512.45 ± 2.68) g, respectively. After removing the scales and guts, the washed fish were evenly coated with 2% salt, and then placed in a freezer at 4 °C for 2 days. After the curing processing, the fish were rinsed with water, absorbed the surface water and dried in a desiccator (BYXX-50; Hangzhou Expro Mechanical Engineering Co., Ltd., Hangzhou, China) for 2 days $((15 \pm 2)^\circ\text{C})$. Three sampling points were set up during the dry-cured processing of Wuchang fish, which were fresh fish (CK), curing for 2 days (Y2), and drying for 2 days (G2). The samples were collected from the white muscle part of the fish back. This study followed strict hygiene procedures during processing. The experiments were executed with three parallel replicates to enhance the statistical validity of the data.

Oil sample preparation: referring to the method of Folch et al.^[9] with slight modifications. Weigh 3.00 g of sample into a centrifuge tube, add 25 mL of chloroform-methanol solution (2:1, V/V), homogenize in an ice-water bath for 40 s, transfer to a stoppered graduated cylinder and make up to 50 mL. Place at 4 °C for 1 h, then filter to remove proteins and connective tissue. Add 10 mL of physiological saline, centrifuge at 4 °C and 5 000 r/min for 10 min. Remove the upper clarified layer (water, methanol, and ionic impurities), transfer the remaining liquid to a centrifuge tube, evaporate the solvent using a nitrogen-blowing instrument, and store the residue at -80 °C for later use.

2.2 Volatile flavor compounds analysis

Volatile flavor compounds were extracted according to Liu et al.^[10], with a slightly modification. Gas chromatography-mass spectrometry (GC-MS) analysis was consisting of an Agilent 7890A gas chromatograph equipped with a 5975C mass spectrometer (Agilent Technologies, USA). Fish muscle (3.00 g) were placed into a headspace vial (20 mL) and equilibrated at 40 °C for 15 min. Solid phase microextraction fiber (50/30 µm DVB/CAR/PDMS extractor, Supelco, Bellefonte, PA, USA) was exposed to the vial headspace for 40 min at 40 °C. Then, the fiber desorbed at 250 °C for 5 min in the injection port of GC-MS. Volatile flavor compounds were separated on a DB-WAX column (60 m × 0.25 µm, 0.25 µm; Agilent Technologies, USA) with helium as the carrier gas at a flow rate of 1 mL/min. The temperature program was set as follows: the oven temperature was held at 40 °C, then increased to 100 °C by 2 °C/min, after that increased to 180 °C by 5 °C/min, finally rising to 250 °C by 8 °C/min held for 5 min. The mass scan was conducted in the range of 30–400 amu, and the temperature of ion source is 230 °C. 2-Octanol was used as internal standard to calculate the content of volatile flavor compounds.

2.3 Determination of lipid oxidation parameters

2.3.1 Thiobarbituric acid reactive substances (TBARS) value

The 2.00 g sample added with 10 mL of 10 g/100 mL trichloroacetic acid solution (containing 0.1 g/100 mL ethylene diamine tetraacetic

acid), homogenized for 30 s (1 000 r/min). Filtrate (3 mL) added 3 mL 0.02 mol/L thiobarbituric acid solution and water baths (95 °C) for 45 min. The absorbance was measured at a wavelength of 532 nm. 1,1,3,3-Tetraethoxypropane solution was used as the standard substance. The TBARS value was calculated using the method of Zhou et al.^[11].

2.3.2 Peroxide value (POV)

The 2.00 g oil sample was added 30 mL chloroform-glacial acetic acid (2:3, V/V) and 1 mL saturated potassium iodide solution, then shaken and placed in the dark for 3 min. Titration was performed with 0.01 mol/L sodium thiosulfate standard solution. When the solution appeared pale yellow, starch indicator (1 mL) was added and continued to drop until the blue solution disappeared as the titration endpoint. POV was calculated according to formula (1):

$$\text{POV}(\text{mmol/kg}) = \frac{(V - V_0) \times c}{2 \times m} \times 1000 \quad (1)$$

Where V is volume of sodium thiosulfate standard solution consumed (mL); V_0 is the volume of sodium thiosulfate standard solution consumed in the blank test (mL); c is the concentration of sodium thiosulfate standard solution (0.01 mol/L); m is weigh of sample (g); 1 000 was conversion factor.

2.4 Determination of the activities of phospholipase, acid lipase, and neutral lipase

Extraction of crude lipase: 5.00 g crushed samples were added into 25 mL phosphoric acid buffer solution (50 mmol/L, pH 7.5, and contained 0.05 mol/L ethylene glycol bis(2-aminoethylether)- N,N,N',N' -tetraacetic acid (EGTA)). Then homogenized at 15 000 r/min for 10 s with 3 times, and with ice water bath 30 min. The supernatant was collected after centrifugation for 15 min at 10 000 r/min.

The 0.1 mL crude lipase liquid was mixed with 2.8 mL of pH 5.0 buffer solution containing 0.1 mol/L disodium hydrogen phosphate, 0.05 mol/L citric acid, 0.8 mg/mL bovine serum albumin, and 5 mg/10 mL Triton X-100, the 4-methyl umbelliferyl oleate (0.1 mL, 1 mmol/L) as the reaction substrate at 37 °C for 30 min. Then 0.5 mL HCl (1 mol/L) was used to quench the enzyme reaction of acid lipase and phospholipase. And the activity of neutral lipase was stopped by ice bath. The fluorescence value of the reaction solution was measured by enzyme-labeled apparatus at excitation wavelength of 328 nm and emission wavelength of 470 nm (Spark®; Tecan Laboratory Equipment Co., Ltd., Switzerland). An enzyme activity unit is defined as: under the condition of 37 °C, 1 g enzyme protein produces 1 nmol of 4-methylumbellyl ketone within 1 h, and the result was expressed as U/(h·g)^[12].

2.5 Determination of moisture content

Accurately weigh 2 g of the comminuted sample and dry it in an oven at 105 °C to constant weight. Moisture content was calculated according to formula (2):

$$\text{Moisture content (\%)} = \frac{2 - m}{2} \times 100 \quad (2)$$

Where m is the mass after drying to a constant weight (g).

2.6 Determination of LOX activity

The 5.00 g sample was mixed with 25 mL phosphoric acid buffer solution (pH 7.0, containing 5 mmol/L EGTA) in an ice water for

4×10 s at a speed of 20 000 r/min. The homogenized sample was stored for 30 min, and centrifuged for 15 min at $12\,000 \times g$. The supernatant was the crude lipase solution. Then 200 μ L 0.5 g/100 mL sodium linoleic acid was used as the reaction substrate, then 2.8 mL of 50 mmol/L citric acid buffer solution (pH 5.5) was added, and the absorption value at 234 nm was determined after being mixed evenly at room temperature. When the absorption value was stable, 0.1 mL crude enzyme liquid was added, and the change of absorption value in 1 min was recorded. One unit (U) of LOX activity is defined as increased absorbance at 234 nm per gram of protein per minute, and the result was expressed as U/min-g sample.

2.7 Lipid profiles analysis

2.7.1 Sample extraction

Take out the 20 mg oil sample from the -80°C refrigerator and thaw it on ice. Add 50 μ L of pure methanol, vortex well and centrifuge (12 000 r/min, 4°C) for 30 s. Homogenize (30 Hz) the sample for 20 s with a steel ball and centrifuge (3 000 r/min, 4°C) for 30 s. Subsequently, collect the upper-layer supernatant and add 1 mL of the extraction solvent (MTBE:MeOH = 3:1, V/V) containing internal standard mixture (10 μ g/mL PC(12:0/0:0), 10 μ g/mL Cer(d18:1/4:0), and 10 μ g/mL PC(13:0/13:0)). After whirling the mixture for 15 min, 200 μ L of water was added. Vortex for 1 min and centrifuge at 12 000 r/min for 10 min. The 200 μ L of the upper organic layer was collected and evaporated using a vacuum concentrator. The dry extract was reconstituted using 200 μ L mobile phase B prior to ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis.

2.7.2 UPLC conditions

The sample extracts were analyzed using an UPLC-electrospray ionization (ESI)-MS/MS system (UPLC, ExionLC AD, AB Sciex Pte. Ltd., Singapore; MS, QTRAP[®] System, AB Sciex Pte. Ltd., Singapore). The analytical conditions were as follows, UPLC: Thermo Accucore[™] C₃₀ column (2.1 mm $\times$ 100 mm, 2.6 μ m); solvent system: mobile phase A was acetonitrile-water (60:40, V/V, 0.1% formic acid, 10 mmol/L ammonium formate), mobile phase B was acetonitrile-isopropanol (10:90, V/V, 0.1% formic acid, 10 mmol/L ammonium formate); gradient program: the volume ratio of mobile phases A and B were 80:20 at 0 min, 70:30 at 2.0 min, 40:60 at 4 min, 15:85 at 9 min, 10:90 at 14 min, 5:95 at 15.5 min, 5:95 at 17.3 min, 80:20 at 20 min; flow rate was 0.35 mL/min; temperature was 45°C ; injection volume was 2 μ L. The effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (QTRAP)-MS/MS.

2.7.3 ESI-QTRAP-MS/MS conditions

Linear ion trap (LIT) and triple quadrupole (QQQ) scans were acquired on a QTRAP[®] LC-MS/MS system, equipped with an ESI turbo ion-spray interface, operating in positive and negative ion modes and controlled by Analyst 1.6.3 software (Sciex, USA). The ESI source operation parameters were as follows: ion source, turbo spray; source temperature 500°C ; ion spray voltage 5 500 V (positive), $-4\,500$ V (negative); ion source gas 1, gas 2 and curtain gas were set at 45, 55, and 35 psi, respectively; the collision gas was medium. Instrument tuning and mass calibration were performed with 10 and 100 μ mol/L polypropylene glycol solutions in QQQ and LIT modes, respectively. QQQ scans were acquired as multiple

reaction monitoring (MRM) experiments with collision gas (nitrogen) set to 5 psi. Declustering potential (DP) and collision energy (CE) for individual MRM transitions was done with further DP and CE optimization. After acquiring lipid MS data from various samples, integrate the peak areas of all compounds and correct the MS peaks of the same lipid across different samples^[13]. The relative contents of lipid molecules were calculated based on peak areas.

2.7.4 Calculation of lipid oxidation index (LOI)

The LOI was calculated according to formula (3):

$$\text{LOI} = \frac{A_i}{B_i} \quad (3)$$

Where A_i represents the combined relative content of oxidized lipids; B_i represents the combined relative content of non-oxidized lipids.

2.8 Statistical analysis

The data were analyzed through one-way analysis of variance (ANOVA) via SPSS 25.0 (SPSS Inc., Chicago, IL, USA), and a level of $P < 0.05$ was considered statistically significant. Metabolic pathways of metabolites screened from correlative network were further retrieved using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Orthogonal partial least squares-discriminant analysis (OPLS-DA) and visualization were performed using R language.

3 Results and discussion

3.1 Variations in volatile flavor compounds in dry-cured Wuchang fish processing

During the processing of dried Wuchang fish, variations in volatile flavor compounds are presented in Table 1. A total of 30 volatile flavor compounds were identified by GC-MS, including 13 alcohols, 3 aldehydes, 2 ketones, 2 terpenes, 6 alkanes, 2 esters, 1 carboxylic acid, and 1 thiazole. It is evident that the content of volatile flavor compounds increased significantly after processing, and the odor of the fish meat became richer after dry-salting and air-drying, with the most noticeable increase in the content of alcohols and aldehydes, forming the unique odor of dry-cured Wuchang fish. The GC-MS detection results clearly show that the processing methods of curing and drying can significantly promote the formation of volatile flavor substances, which is consistent with the research results of Wu et al.^[14]. From CK to Y2 stage, enzymatic hydrolysis (neutral lipase, acid lipase, and phospholipase) and LOX-catalyzed oxidation drove significant odorant changes. During the curing process, alcohols and aldehydes that represent the flavor of dry-cured Wuchang fish began to form, and in aquatic products, they are both regarded as the main components of fishy odor, originating from the oxidation of lipids. 1-Octene-3-ol is a product generated by the oxidation of arachidonic acid under the action of LOX, with a mushroom and grassy odor^[15]. Nonyl aldehyde is an oxidation product of linolenic acid (LNA)^[16]. In the stage from Y2 to G2, 3-methyl-1-butanol began to form, possibly from the decomposition of secondary hydroperoxides of FAs produced during the curing stage or from the Strecker degradation of amino acids, while ketones (lipid oxidation/Maillard products) increased, reducing fishy odor^[17–18].

Table 1 Volatile flavor compounds in the processing of dry-cured Wuchang fish.

No.	Volatile flavor compounds	Retention time (s)	Contents (ng/g)		
			CK	Y2	G2
Alcohol					
1	Ethyl alcohol	7.127 0	nd	1.07 ± 0.12	174.96 ± 32.17
2	Propyl alcohol	9.220 7	nd	nd	4.05 ± 1.44
3	<i>n</i> -Butanol	11.824 1	nd	nd	1.95 ± 0.42
4	1-Pentene-3-ol	12.189 1	nd	4.03 ± 0.47	2.11 ± 0.64
5	3-Methyl-1-butanol	13.545 3	nd	nd	28.14 ± 3.93
6	1-Amyl alcohol	14.845 3	nd	5.48 ± 0.93	2.33 ± 0.27
7	1-Hexanol	18.393 6	nd	20.27 ± 5.59	10.77 ± 1.64
8	3-Octanol	19.916 3	nd	0.46 ± 0.23	0.91 ± 0.08
9	1-Octene-3-ol	22.242 5	0.95 ± 0.18	19.75 ± 3.09	6.84 ± 1.07
10	1-Heptanol	22.537 3	nd	0.71 ± 0.08	0.99 ± 0.01
11	2-Ethylhexanol	24.037 0	1.07 ± 0.09	12.93 ± 0.58	1.29 ± 0.08
12	2,3-Butanediol	26.356 9	nd	1.06 ± 0.38	20.59 ± 4.75
13	Octa-ethylene glycol	45.889 4	0.40 ± 0.03	0.57 ± 0.09	0.61 ± 0.06
Aldehyde					
14	Isovaleric aldehyde	6.858 4	nd	nd	50.92 ± 11.30
15	Caproaldehyde	10.240 0	nd	4.88 ± 1.04	2.07 ± 0.42
16	Nonyl aldehyde	20.063 6	nd	nd	5.00 ± 0.95
Ketone					
17	3-Hydroxy-2-butanone	16.181 8	0.62 ± 0.12	nd	23.44 ± 2.14
18	Cyclohexanone	16.629 8	nd	1.18 ± 0.08	nd
Terpene					
19	Bornan-2-one	25.773 9	nd	0.60 ± 0.04	nd
20	Linalool	26.619 8	nd	0.72 ± 0.05	nd
Alkanes					
21	Trichloromethane	8.752 4	0.84 ± 0.02	0.52 ± 0.32	nd
22	Bromodichloromethane	12.023 9	0.90 ± 0.17	nd	nd
23	Dichloromethane	6.968 7	nd	0.54 ± 0.08	nd
24	<i>m</i> -Xylene	11.975 8	nd	2.75 ± 0.17	nd
25	<i>p</i> -Xylene	13.033 0	nd	1.82 ± 0.12	nd
26	1,2-Xylene	12.037 8	nd	2.06 ± 0.77	nd
Esters					
27	Ethyl acetate	6.369 4	nd	nd	12.44 ± 5.93
28	Ethyl lactate	18.035 3	nd	nd	2.81 ± 0.35
Others					
29	Acetic acid	22.716 8	nd	nd	1.45 ± 0.07
30	4-Methylthiazole	31.109 2	nd	1.69 ± 0.40	nd

Note: nd, not detected.

3.2 Changes of lipid oxidation of dry-cured Wuchang fish during processing

As depicted in Figures 1A and B, the variations in the TBARS value and POV in the fish meat throughout the entire dry-curing process are illustrated. During the entire preparation phase of dry-cured Wuchang fish, the TBARS value increased from 0.05 (CK group) to 1.63 mg/kg (G2 group), and the POV increased from 0.47 (CK group) to 4.18 mmol/kg (G2 group). The degree of lipid oxidation in the fish meat exhibited a significant upward trend ($P < 0.05$). In previous studies, lipid oxidation, due to its distinct reactive characteristics, is categorized into three types: autooxidation, enzymatic oxidation, and photooxidation. Given that the entire preparation phase was conducted under light-free conditions, the changes in TBARS value and POV were primarily attributed to enzymatic catalysis and autooxidation^[19]. It is worth noting that FAs serve as substrates for lipid oxidation reactions, and the rate of lipid hydrolysis is likewise a crucial factor influencing lipid oxidation.

During the curing process of fish, the FAs produced are mainly attributed to the endogenous lipases in the fish meat, which play a crucial role in the hydrolysis of lipid molecules^[20–22]. Figure 1C displays the activities of phospholipase, acid lipase, and neutral lipase, as well as the moisture content in the fish meat during the drying and curing stages. Among these three types of lipases, the activity of neutral lipase is the highest, indicating that neutral lipase plays a primary role in the processing^[23]. The activities of phospholipase, acid lipase, and neutral lipase in fresh fish meat are 55.82, 39.82, and 80.02 U/(h·g), respectively, and all of them significantly decreased during the processing ($P < 0.05$). Meanwhile, the moisture content in the fish meat showed a significant decline during the processing, which may be an important reason for the decrease in lipase activity. However, the activity of LOX shown in Figure 1D presented a different result. As the moisture content decreases, the concentration of NaCl in the fish meat also increases, forming an environment with high salt concentration and low water activity. Throughout the preparation process of air-dried

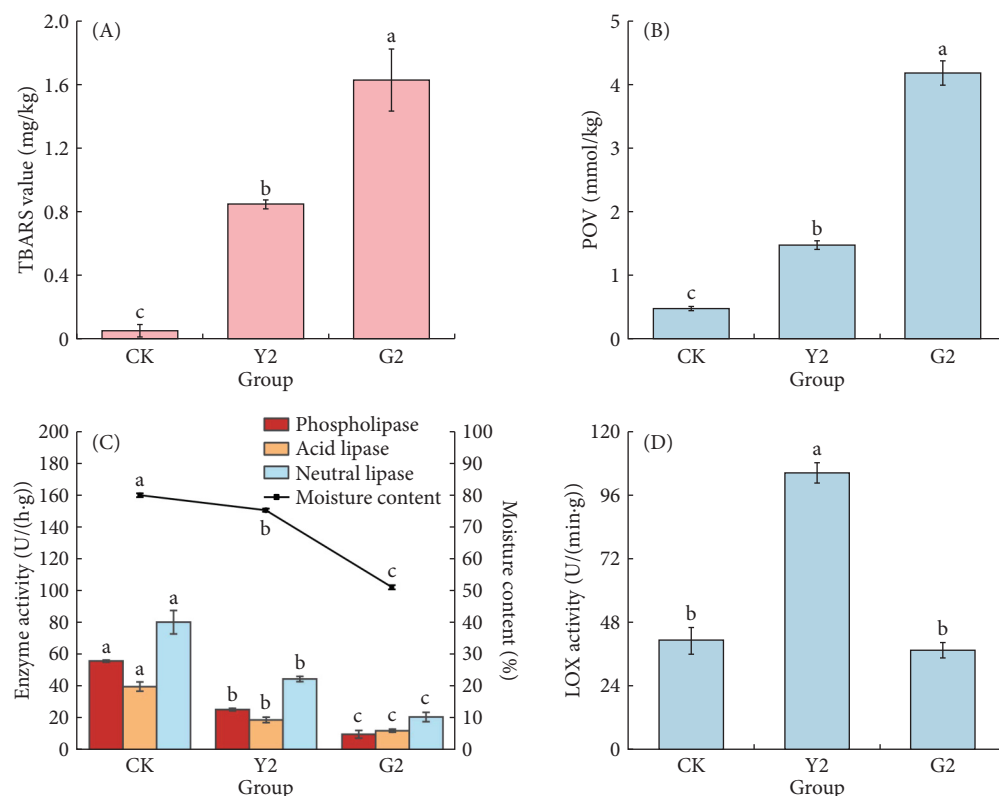


Figure 1 Changes in (A) TBARS value, (B) POV, (C) enzyme (phospholipase, acid lipase, neutral lipase) activities, moisture content, and (D) LOX activity during the processing of dried Wuchang fish. Different letters indicate significant differences among samples ($P < 0.05$).

Wuchang fish, the LOX activity first increased and then decreased, with the highest LOX activity measured in the Y2 group sample. Similar results have been found in the studies of Cao et al.^[30], which may be related to the activating effect of salt. During the curing process from CK to Y2, salt permeates into the fish meat, causing the Na^+ concentration to rise and begin to activate LOX. From Y2 to G2 in the drying process, the moisture content in the fish meat gradually decreases, and the activating effect of Na^+ also gradually increases. However, with the reduction of moisture content, the water activity in the fish meat also significantly decreases, and the inhibitory effect on LOX gradually increases^[24]. When the drying reaches a certain time, the inhibitory effect of water activity on LOX in the fish meat is greater than the promoting effect of Na^+ on LOX, and the LOX activity gradually decreases. Therefore, autoxidation plays a larger role in the lipid oxidation of fish meat in the later stages of air-drying. It is worth noting that the oxidation reaction catalyzed by LOX occupies a dominant position in the entire processing process and is the main driving factor of total lipid oxidation. During the curing process of fish meat, LOX catalyzes the oxidation of PUFAs^[25], generating hydroperoxides and a series of oxidation products, as well as aldehydes and other special flavor substances, which lead to an increase in POV.

3.3 Changes of lipid of dry-cured Wuchang fish during processing

3.3.1 Lipid classification of dry-cured Wuchang fish during processing

Lipid remodeling during dry-curing of Wuchang fish was revealed by UPLC-MS/MS-based lipidomics, lipidomic profiling of Wuchang fish during dry-curing processing was performed using UPLC-MS/MS coupled with a customized MetWare database.

Quality control (QC) samples demonstrated high reproducibility, enabling the identification of 1 370 lipid species across six major categories: FA (69), glycerolipids (GL, 458), glycerophospholipids (GP, 727), prenol lipids (PR, 1), sphingolipids (SP, 85), and sterol lipids (ST, 30), which were further divided into 41 subclasses. Ionization mode analysis showed 805 lipid species detected in positive mode (spanning all classes) compared to 565 in negative mode (predominantly 35 FA, 525 GP, and 5 ST), as illustrated in Figure 2B. Sankey diagram visualization (Figure 2A) revealed dynamic compositional shifts: GL dominated as the primary lipid class throughout processing, while GP exhibited significant depletion during curing stages. These systematic changes in lipid profiles suggest active metabolic reprogramming involving lipid hydrolysis and structural modification during salting and air-drying processes.

3.3.2 Differential lipids of dry-cured Wuchang fish during processing

OPLS-DA of lipid content measurements across three processing stages (Figures 2C–E) revealed clear separation of samples along the X axis, indicating significant intergroup differences. After 200 permutation tests, as shown in Figure 3, the validation parameters demonstrated no overfitting in pairwise comparisons: CK vs. Y2 ($Q^2 = 0.973$, $R^2_Y = 0.986$, $R^2_X = 0.8$), Y2-G2 ($Q^2 = 0.988$, $R^2_Y = 0.998$, $R^2_X = 0.816$), and CK-G2 ($Q^2 = 0.979$, $R^2_Y = 0.99$, $R^2_X = 0.83$). These results confirm that the OPLS-DA models accurately reflect the true biological variation among sample groups.

Heatmaps of the top 20 differential lipids (variable importance in projection (VIP) > 1) identified by OPLS-DA (Figures 2F–H) demonstrated that GP, GL, and FA dominated the VIP rankings across all pairwise comparisons (CK vs. Y2, Y2 vs. G2, and CK vs. G2). In the CK-Y2 stage, 11 GP species (including lysophosphatidylethanolamine (LPE) (20:2/0:0), LPE(18:3/0:0),

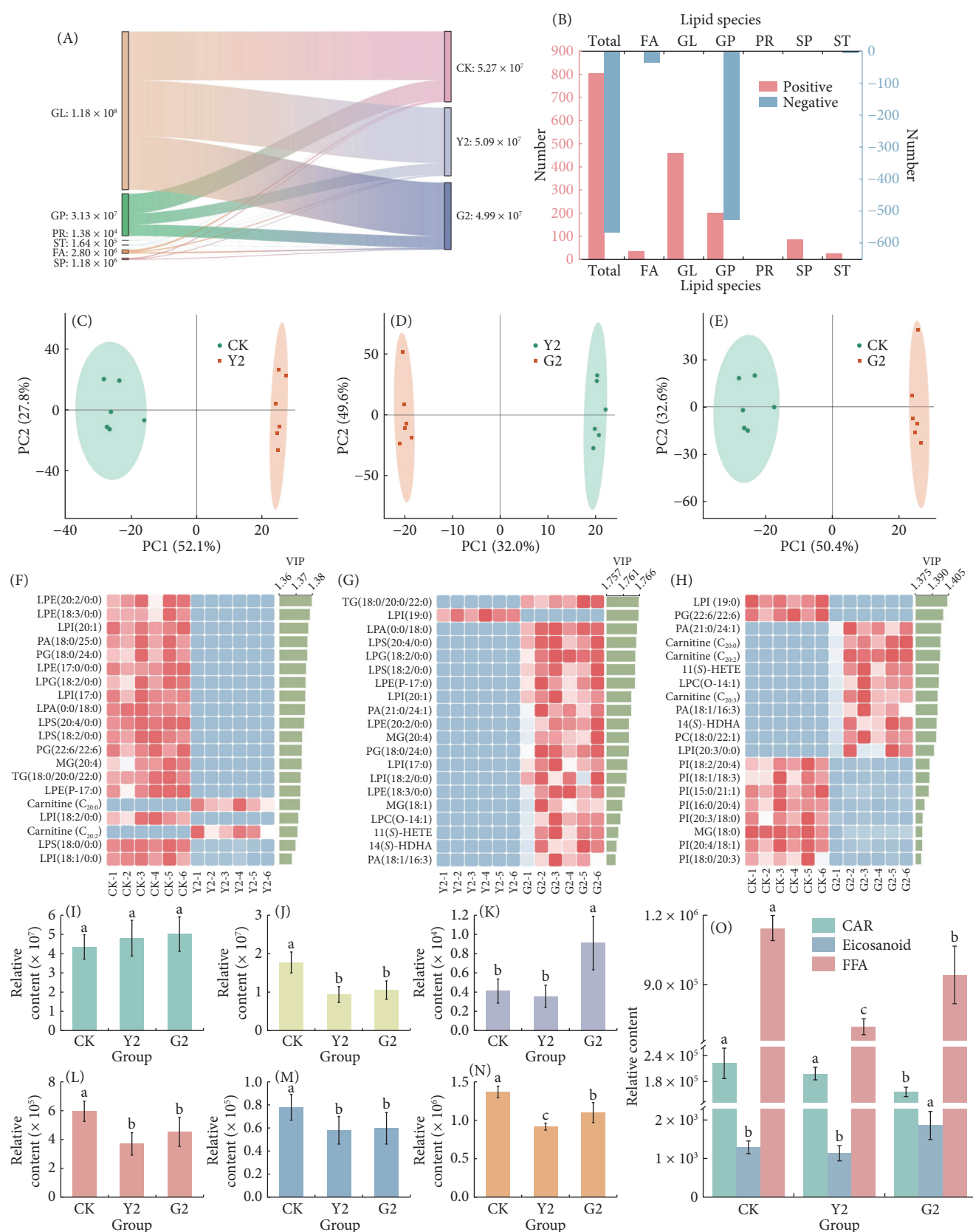


Figure 2 (A) Sankey diagram illustrating the relative abundance of different lipid classes during drying, with widths representing relative lipid content; (B) number of lipid species identified in positive and negative ionization modes; OPLS-DA of lipids between (C) CK and Y2 groups, (D) Y2 and G2 groups, and (E) CK and G2 groups; heatmap of the top 20 lipids ranked by VIP values for (F) CK vs. Y2, (G) Y2 vs. G2, and (H) CK vs. G2; changes in (I) GL, (J) GP, (K) PR, (L) SP, (M) ST, (N) FA, (O) CAR, eicosanoid, and FFA content. Different letters indicate significant differences among samples ($P < 0.05$). 11(S)-HETE, 11(S)-hydroxyeicosatetraenoic acid; 14(S)-HDHA, 14(S)-hydroxy docosahexaenoic acid; LPC, lysophosphatidylcholine; PI, phosphatidylinositol.

lysophosphatidylinositol (LPI) (20:1), phosphatidylglycerol (PG) (18:0/24:0), lysophosphatidylglycerol (LPG) (18:2/0:0), LPI(17:0), lysophosphatidic acid (LPA) (0:0/18:0), lipopolysaccharide (LPS) (20:4/0:0), LPS(18:2/0:0), LPE(P-17:0), and LPI(18:2/0:0)) and two GL species (monoglyceride (MG) (20:4) and triglycerides (TG) (18:0/20:0/22:0)) exhibited significant increases, followed by marked decreases during Y2-G2. This pattern aligns with the activation of phospholipases during salting, which hydrolyze GP into free fatty acids (FFAs) and other small molecules, while neutral and acid lipases degrade GL, releasing additional FA. Notably, in the Y2-G2 phase, most top-ranked GP, GL, and FA species showed upward trends. Although overall lipase activities (acid lipase, neutral lipase, and phospholipase) declined during air-drying, residual enzymatic activity in the initial drying phase likely facilitated partial hydrolysis of TG and GP, generating diacylglycerols (DAG), monoacylglycerols (MAG), and FA. Concurrently, reduced water activity suppressed microbial growth and enzyme activity but promoted lipid aggregation, leading to localized accumulation of GL and GP.

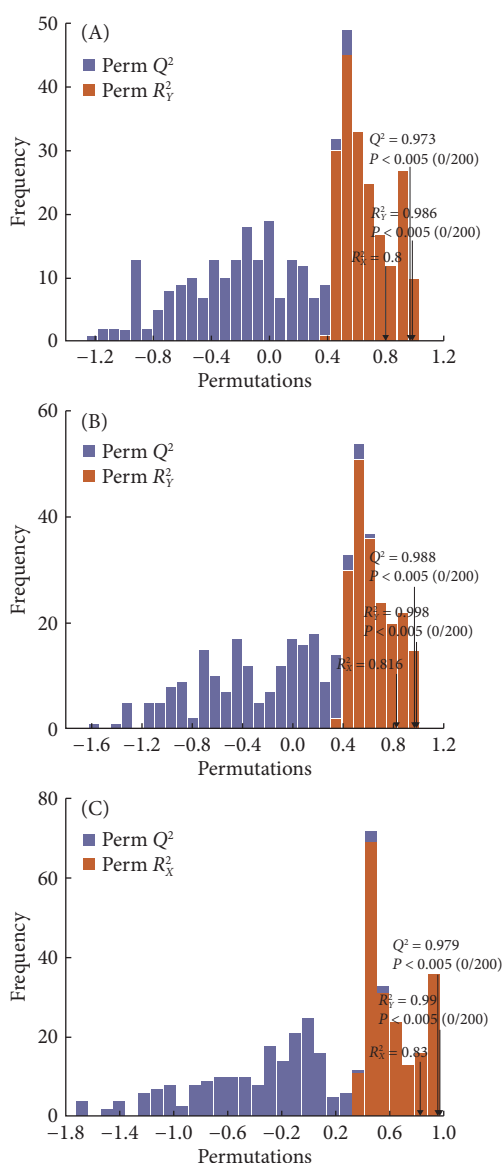


Figure 3 Plot of 200 permutation tests for (A) CK vs. Y2, (B) Y2 vs. G2, and (C) CK vs. G2.

As illustrated in Figures 2I–N, the total GL content exhibited a slight but non-significant upward trend throughout processing, while GP decreased significantly during salting ($P < 0.05$). The inverse trends of GP depletion and GL accumulation suggest enhanced GP-to-GL conversion during salting^[26]. Given the critical role of GPs in maintaining biomembrane integrity^[27], their decline may reflect cellular membrane disruption, facilitating catalyst access to lipid substrates and accelerating oxidative reactions. PR increased markedly during air-drying ($P < 0.05$), whereas SP and ST mirrored GP dynamics, declining sharply in salting ($P < 0.05$) and rising slightly in drying. Contrary to expectations, total FA content decreased during salting, likely attributable to LOX activity. High LOX activity during salting catalyzed PUFAs oxidation, driving lipid degradation and generating volatile flavor compounds. Figure 2O delineates FA subclass dynamics: carnitines (CAR) declined persistently; eicosanoids decreased marginally in salting but rose significantly during drying ($P < 0.05$); FFAs dropped in salting then rebounded in drying ($P < 0.05$). During air-drying, diminished LOX activity reduced lipid peroxidation, while reduced moisture content promoted FA accumulation through water activity-mediated lipid aggregation.

3.3.3 FFAs dynamics of dry-cured Wuchang fish during processing

During salting, microbial activity facilitates oxidative cleavage of UFAs at double bonds, generating volatile flavor compounds such as aldehydes, ketones, alkanes, and alcohols that contribute to the characteristic fish flavor^[28]. As shown in Figure 4A, analysis of 28 FFAs revealed a pronounced decrease in most FFAs during salting, followed by significant post-drying increases in key species, including C_{18:2} (linoleic acid (LA)), C_{18:1} (oleic acid (OA)), a cholesterol-regulating monounsaturated fatty acid^[29], C_{20:5} (eicosapentaenoic acid (EPA)), C_{22:6} (docosahexaenoic acid (DHA)), and C_{20:4} (arachidonic acid (AA)). As illustrated in Figures 4C–E, essential fatty acids (EFAs), including LA, LNA, AA, EPA, and DHA, accounted for a higher proportion of total FFAs in dried fish. The proportion of EFAs in total FFAs in air-dried fish muscle was 18.869%, far higher than 13.414% in fresh fish. Despite their relatively low absolute concentrations, these EFAs, including EPA, DHA (ω -3 PUFAs with hypolipidemic, anti-inflammatory, and neuroprotective properties)^[30–32], LA (an ω -6 PUFA critical for membrane integrity and immune modulation)^[33–34] hold significant nutritional value due to their inability to be endogenously synthesized in humans.

3.3.4 Analysis of potential lipid metabolic pathways of dry-cured Wuchang fish during processing

KEGG enrichment analysis of lipids during salting (CK-Y2) and air-drying (Y2-G2) stages identified 11 and 12 significantly enriched pathways, respectively (Figures 5A and B). Both stages shared dominant pathways including GP metabolism and GL metabolism, consistent with the results reported by Liu et al.^[30]. GP are one of the main components of cell membranes and involved in various cellular functions, including cell signaling, membrane structure maintenance, and energy metabolism^[35]. The degradation of GP is catalyzed by phospholipases, which hydrolyze GP into lysophospholipids, FA, glycerol, DAG, and phosphatidic acid (PA). Concurrently, GL metabolism involves lipase-catalyzed sequential hydrolysis of TG to DAG and MAG, with neutral lipases degrading lipid droplets into FA and glycerol, a critical mechanism for lipid mobilization. Similarly, neutral lipases play a crucial role in

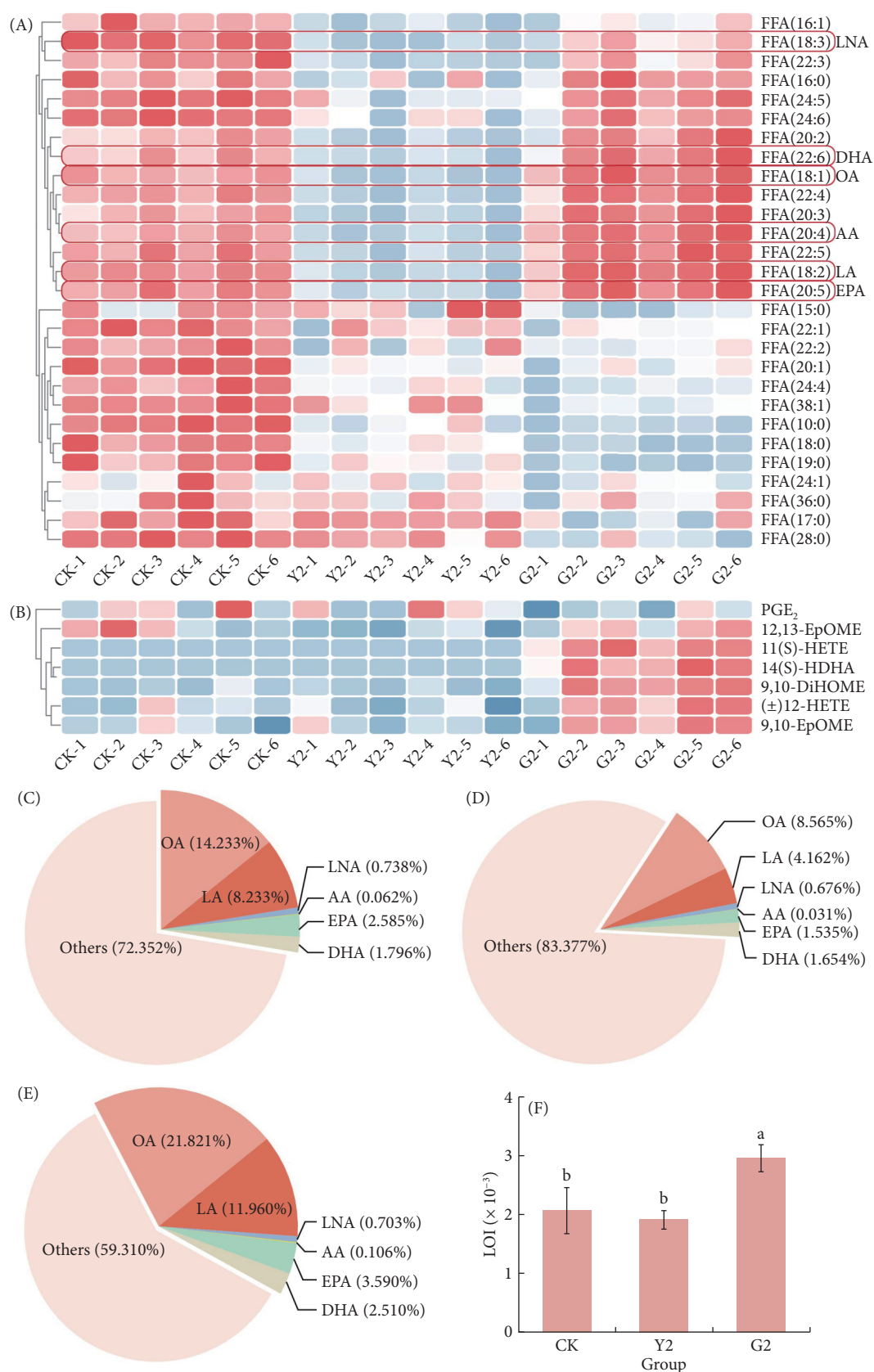


Figure 4 Heatmap of (A) FFAs and (B) oxylipins in fish during the processing of dried Wuchang fish. Ratio of EFAs to total FA content in the (C) CK, (D) Y2, and (E) G2 groups. (F) Changes in LOI. Different letters indicate significant differences among samples ($P < 0.05$). PGE₂, prostaglandin E₂; EpOME, *cis*-epoxyoctadecenoic acid; 9,10-DiHOME, 9,10-dihydroxy-12-octadecensäure.

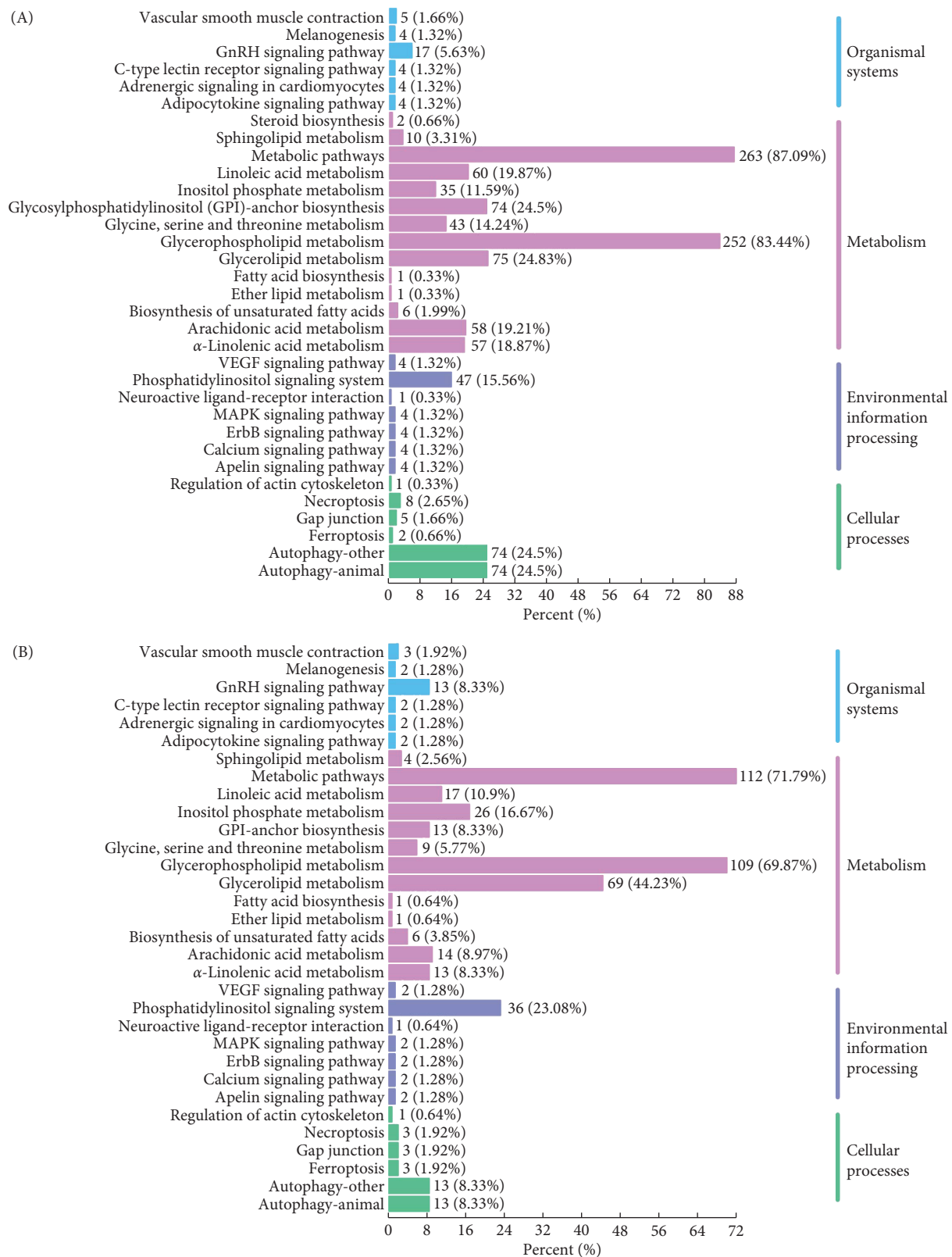


Figure 5 KEGG classification of differential lipids in the (A) CK-Y2 stage and (B) Y2-G2 stage. GnRH, gonadotropin-releasing hormone; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; ErbB, erythroblastic oncogene B.

processing by hydrolyzing GL within cytoplasmic lipid droplets, breaking them down into FA and glycerol^[36].

Oxylipins, oxidative metabolites derived from PUFAs through autoxidation or enzymatic actions (cyclooxygenase, LOX, and

cytochrome P450), were analyzed using the LOI. As shown in Figure 4F, LOI decreased insignificantly during the CK-Y2 stage (salting, $P > 0.05$) but increased significantly in the Y2-G2 stage (air-drying, $P < 0.05$), which appears contradictory to prior

observations. This paradox stems from the opposing effects of GL/GP hydrolysis (reducing LOI by substrate depletion) and PUFAs oxidation (increasing LOI via oxylipin formation). Analysis of PUFAs subclasses (Figure 4A), including $C_{18:3}$, $C_{22:3}$, $C_{24:5}$, $C_{24:6}$, $C_{20:2}$, $C_{22:6}$, $C_{22:4}$, $C_{20:3}$, $C_{20:4}$, $C_{22:5}$, $C_{18:2}$, and $C_{20:5}$, revealed an initial decline during CK-Y2 followed by a rebound in Y2-G2. During CK-Y2, volatile flavor compounds were extensively generated (Figure 4B), yet oxylipins content remained stable ($P > 0.05$) despite high activities of neutral lipase, acid lipase, phospholipase, and LOX. This indicates concurrent lipid hydrolysis and oxidation during salting, with LOX-driven oxidation dominating to convert PUFAs into volatile aldehydes and alcohols rather than accumulating oxylipins. In Y2-G2, enzyme activity decreased due to reduced water activity, slowing hydrolysis and oxidation. However, residual PUFAs underwent autooxidation, leading to oxylipins accumulation. Thus, LOI elevation in Y2-G2 reflects autooxidation-driven oxylipins synthesis despite reduced enzymatic activity. Notably, PUFAs-related pathways (LA, AA, and α -LNA metabolism) were prominent across both stages. LA undergoes desaturation/elongation to form EPA and DHA, while AA is converted via cyclooxygenases and LOX into bioactive mediators regulating inflammation and vascular function^[37]. During air-drying, the emergence of biosynthesis of UFAs highlights oxidative degradation of LA, EPA, and DHA into volatile aldehydes, ketones, and alcohols, pivotal for flavor development. These interconnected pathways (spanning membrane degradation, enzymatic hydrolysis, and oxidative modifications) collectively underpin lipid remodeling and flavor biogenesis in dry-cured fish, with reduced LOX activity and water activity-driven lipid aggregation further modulating FA dynamics during processing.

4 Conclusions

This study elucidated the intrinsic relationship between lipid remodeling and lipase activities during the processing of dry-cured Wuchang fish through lipidomics analysis. Oxidative indices (TBARS value and POV) increased significantly ($P < 0.05$) throughout processing. Lipidomic profiling identified 1 370 lipid species categorized into six major classes (FA, GL, GP, PR, SP, and ST) and 41 subclasses, whose dynamic changes strongly correlated with phospholipase, neutral lipase, and LOX activities. The LOI revealed that LOX-driven conversion of PUFAs to flavor-active aldehydes and alcohols dominated during salting, while reduced water activity in air-drying promoted PUFAs autooxidation and oxylipins accumulation. Notably, EFAs constituted 18.869% of total FFAs in air-dried fish, a significant increase from 13.414% in fresh samples. Collectively, these findings provide novel insights into the lipid-lipase interplay governing quality evolution in traditional dry-cured fish products.

Conflict of interests

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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Authors contribution

Chuanfeng Hu: experiments, data curation, writing—original draft, methodology. Xuan Liu: investigation, formal analysis, writing—review & editing. Tianyu Xiao: validation, visualization, writing—review & editing. Yi Deng: writing—original draft, experiments. Dongyin Liu: writing—original draft, formal analysis. Yingqing Bai: writing—review & editing. Yu Qiao: writing—review & editing, project administration, funding acquisition, research methodology.

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