Proteomics and metagenomics reveal the relationship between microbial metabolism and protein hydrolysis in dried fermented grass carp using a lactic acid bacteria starter culture

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A microbial starter culture is expected to improve the quality of traditional fermented fish products. \textit{Lactobacillus plantarum} was selected for grass carp fermentation due to its high proteolytic activity. To investigate its effects on protein hydrolysis of dried fermented fish, the protein profile and microbial community were analysed by using proteomics and metabolomics. The myofibrillar protein and collagen profiles showed remarkable variation after processing, changes that were related to the development of flavour and texture in fish samples. The starter culture had a marked effect on the microbial composition. \textit{Macrocarcus} and \textit{Staphylococcus} were the dominant genera, with a relative abundance of 24.79% and 12.53%, respectively. There were significant correlations (P < 0.05) between the dominant genera and the major peptidase genes and quality-related proteins. These findings suggest that microbial activity is involved in proteolysis and affects the flavour and texture of dried fermented fish.

1. Introduction

The grass carp (\textit{Ctenopharyngodon idella}) is one of the most widely distributed freshwater species in the world (Yu et al., 2020). As a traditional fermented fish product, dry-cured grass carp is popular due to its high nutritional value and unique flavour. It is prepared from raw grass carp by mixing with salt (generally >10%, w/w) and spices and then air-dried. However, spontaneous fermentation may cause negative influences on the safety and quality of fish products due to the complex microbial communities and instability (Zhao, Hu, & Chen, 2022a). Besides, high salt intake in the diet has been linked to a risk of cardiovascular and cerebrovascular diseases and has drawn much attention from researchers (Y. Yang et al., 2021b). Lactic acid bacteria (LAB), which are found in various naturally fermented fish, have been applied as starter cultures in fermented fish products to the shorten fermentation time, to control undesirable fermentation and to improve sensory characteristics and nutritional quality (Y. Zhou et al., 2021). Microbial starter cultures with proteolytic activity can attenuate undesirable sensory characteristics that arise from the use of NaCl substitutes (Martini et al., 2021).

During microbial fermentation, protein hydrolysis occurs due to the combined action of endogenous acidic proteases in muscles, mainly sarcoplasmic cathepsins, and microbial proteolytic activity, resulting in the release of large amounts of low-molecular-weight peptides and free amino acids (FAAs) originating from myofibrillar proteins (Martini et al., 2021). The breakdown of muscle proteins has a pronounced effect on the development of meat texture and sensory characteristics (Liao et al., 2022). The accumulation of small peptides and FAA has been related to organoleptic qualities. Kokumi-active \(\gamma\)-glutamyl peptides enhance the umami taste of monosodium glutamate (MSG) (J. Yang et al., 2021a), while peptides containing leucine (Leu) at the C-terminus and glycine (Gly) at the N-terminus are considered bitter (Saha and Hayashi, 2001). Aromatic amino acids, including tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Trp) – each containing an aromatic ring in the side chain – are important precursors of aromatic compounds (Fernandez-Espla and Rul, 1999; Ma et al., 2021). Production of branched aldehydes, such as 3-methylbutanal and 2-methylbutanal, is related to proteolysis and amino acid degradation. These aldehydes, which contribute a nutty and cheesy flavour, are mainly formed from Strecker degradation of Leu via the Ehrlich reaction.
(López-Pedruso et al., 2019). Textural changes in muscles are mostly attributed to changes in muscle proteins. Cytoskeletal proteins, which are mainly myofibrillar and include actin, myosin, titin, troponin, nepilin, desmin, vinculin, tubulin, dynein, spectrin, clathrin, keratin and vimentin, as well as collagen, play a vital role in meat texture formation (López-Pedruso et al., 2021; Lyu and Ertbjerg, 2022; Petrova et al., 2015; Yu et al., 2020).

Studies have reported that autochthonous microorganisms or industrial starter cultures are beneficial to develop the unique colour, flavour and texture of fermented food (Hu et al., 2022; Wen et al., 2021; Xiao et al., 2020). The inoculation of Lactobacillus plantarum could increase the levels of sweet-tasting amino acids and improve the overall acceptance of mackerel seasonings (Y. Zhou et al., 2021). The aroma profile of fermented fish products using L. plantarum as starters was improved by effectively diminishing the aliphatic aldehydes (Gao et al., 2020). Som-fug, a Thai fermented fish mince, inoculated with L. plantarum exhibited higher hardness and adhesiveness than fish mince without inoculum (Riebroy et al., 2008). However, little is known about the mechanism of protein hydrolysis of fermented fish during microbial fermentation. Hence, research is needed to determine the key protein(s) that specific microbial enzymes or genes affect during complex microbial fermentation. Therefore, it is essential to investigate the variation in protein composition under microbial fermentation and to identify key genes and enzymes that contribute to proteolysis. Combining metagenomics and proteomics is particularly relevant to uncover the regulatory mechanism of microbial fermentation on sensory characteristic and texture quality in fermented fish products. In this study, we applied L. plantarum, a bacterium we isolated from commercially available traditional dry-cured fish, as a starter culture for fermentation due to its high proteolytic activity. In our first attempt, L. plantarum showed the ability to produce more FAAs and oligopeptides in fish samples compared with that in traditional dry-cured fish. We hypothesised that microbial activity in grass carp fermented using a L. plantarum starter culture has a marked influence on the fish muscle protein profile. To test this hypothesis, we characterised the variations in the protein profile and composition of microorganisms and microbial peptidase in fermented fish samples and examined correlations between microbial metabolism and proteolysis.

2. Materials and methods

2.1. Preparation of LAB suspension and fish samples

L. plantarum, which we isolated from commercially available traditional dry-cured fish, was maintained in 20% sterile glycerine and stored at -80°C in Zhejiang Academy of Agricultural Sciences. The strain was cultured in de Man, Rogosa and Sharpe (MRS) medium under anaerobic condition at 37°C for 18 h. Bacteria were harvested by centrifugation (6000 × g, 10 min, 4°C), washed with 0.85% (w/w) NaCl and then resuspended in 0.85% (w/w) NaCl to a final concentration of approximately 1 × 10^8 colony-forming units (CFU)/mL.

Live grass carp were purchased from a farmer’s market in Shiqiao Road in Hangzhou. The fish were slaughtered by percussion and then gutted, and the scales, gills and skins were removed. After being washed with cold water, the fish were placed in a clean sampling box equipped with ice packs and delivered to the laboratory. A total of 60 individuals (1800 ± 50 g in weight, 1–1.5 years old) were used for fermentation. The flesh was cut into small pieces (4 cm × 4 cm × 1.5 cm) before fermentation. Next, 3% (w/w) NaCl was spread evenly over the fish fillets, following the inoculation of starter culture (10 mL/100 g) in a sterile environment. The fish fillets were then placed in aseptic plastic bags. After being hot-sealed, the bags containing the fish sample were placed in an incubator. After incubation at 37°C for 18 h, the pieces were dried for 24 h at 30°C in an air oven. Dorsal muscles of fish samples were collected at various fermentation stages for analysis. Specifically, raw fish samples (M), fish samples after fermentation (F) and fish samples after fermentation and drying (D) were taken. The control with natural fermentation was also collected for FAAs analysis. There were six biological replicates per group; all samples were stored at -80°C until use.

2.2. Proteomics

2.2.1. Protein extraction

Protein extraction was performed by using the method of Gu et al. (2022), with some modifications. In brief, fish samples were homogenised by using a HK-PB9322 cooking machine (AUX, Ningbo, China) at 4°C. Twenty milligrams of each sample was ground in liquid nitrogen and lysed with lysis buffer (100 mM NH4HCO3, 8 M urea, pH 8), followed by 5 min of ultrasonication on ice. The lysate was centrifuged at 12000 g for 15 min at 4°C. The supernatant was reduced with 10 mM DL-dithiothreitol (DTT) for 1 h at 56°C, and subsequently alkylated with adequate iodoacetamide (IAM) for 1 h at room temperature in the dark. The samples were mixed with four volumes of precooled acetone by vortexing and then incubated at -20°C for 2 h. The precipitate was collected by centrifuging at 12000 g for 15 min at 4°C. After washing twice with cold acetone, the pellet was dissolved in dissolution buffer containing 100 mM triethylammonium bicarbonate (TEAB, pH 8.5) and 8 M urea. The concentrations of extracted proteins were determined by using the Bradford protein assay.

2.2.2. Trypsin digestion

One hundred 20 µg of extracted protein was adjusted to 100 µL with lysis buffer containing 100 mM TEAB (pH 8.5) and 8 M urea. Proteins were digested at 37°C for 16 h with Sequencing Grade Modified Trypsin (Promega) in 100 mM TEAB, using an enzyme-to-protein ratio of 1:50. The digested sample was acidified with formic acid (FA). After centrifugation at 12000g for 5 min at room temperature, the supernatant was desalted by using a C18 cartridge (Sep-Pak, Waters Associates, Milford, MA, USA). Finally, the digested samples were lyophilised and stored at -20°C until liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

2.2.3. LC-MS/MS analysis

LC-MS/MS experiments were performed on a Q Exactive HF–X mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled with an EvoSip One UHPLC system (Gainesville, FL, USA). The lyophilised protein samples were redisolved in 10 µL of 1% LFA in water. After centrifugation at 14000g for 20 min at 4°C, 1 µL of supernatant was separated in a homemade analytical column (15 cm × 150 µm, 1.9 µm) at a flow rate of 200 nL/min, where a binary mobile phase was used: 1 mL/LFA in water (A) and 1 mL/LFA in acetonitrile/water (8:2, v/v) (B). Nanospray Flex (Thermo Fisher Scientific) was used as the electrospray ion source for electrospray ionisation (ESI) coupled to MS, with a spray voltage of 2.1 kV and a capillary temperature 320°C. The MS datasets were collected in a data-dependent acquisition mode using the following procedure: scan range, 350–1500 m/z; mass resolution, 60000 (m/z = 200); automatic gain control (AGC) target, 3e6; and maximum injection time, 20 ms. The MS/MS scans of the 40 most intense ions were performed with the higher-energy C-trap dissociation (HCD) method, with a resolution of 15000 (m/z = 200) and a normalised collision energy of 27%. The AGC target, maximum injection time, intensity threshold and dynamic exclusion were set to 1e5, 45 ms, 2.2e4 and 20 s, respectively.

2.2.4. Database searching, quantification and bioinformatic analysis

The resulting spectra were searched against the grass carp database (972222-uniprot-Grass-carp-2743745.fasta, 31869 sequences) downloaded from uniprot (https://www.UniProt.org/) by using Proteome Discoverer 2.2 (Thermo Fisher Scientific). Mass tolerance for precursor ion and product ion were set to 10 ppm and 0.02 Da, respectively. The fixed modification was carboxymethyl. Methionine oxidation was set as dynamic modification. Acetylation was specified as N-terminal modification. A maximum of 2 missed cleavage sites were allowed. The
false discovery rate (FDR) of both peptide and protein identification were set to be no more than 1%. The protein quantification results were analysed statistically with Student’s t-test. Significance of the differential proteins between various groups was estimated. There were three comparisons: F versus M, D versus F and D versus F.

Gene Ontology (GO) analysis was conducted by using the inter-proscan program against non-redundant protein databases (including Pfam, PRINTS, ProDom, SMART, ProSite and PANTHER). Differentially abundant proteins (DAPs) were used for cluster heat map analysis and GO enrichment analysis.

2.3. Metagenomic analysis

2.3.1. Genomic DNA extraction, library preparation and sequencing

Microbial DNA of each fish sample was extracted by using the E.Z.N.A. A. Stool DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer’s protocol. DNA concentration and purity were assessed by spectrophotometry with a Nanodrop 2000 (Thermo Fisher Scientific). DNA purity was estimated by fluorometric quantification using a Qubit 3.0 system (Life Invitrogen, Waltham, MA, USA) (Fu et al., 2021). DNA integrity was verified with 1% agarose gel electrophoresis observed under ultraviolet light. DNA of fish samples meeting the criteria for sequencing was stored at -20°C pending further analysis. Sequencing libraries were constructed and sequenced at Shanghai Biozeron Biological Technology Co. Ltd. In brief, 1 μg of DNA per sample was sheared by using a Covaris S220 Focused Ultrasonicator (Woburn, MA, USA). Sequencing libraries were constructed with fragment lengths of approximately 450 bp. All samples were sequenced on the Illumina Hiseq X instrument (Illumina, San Diego, CA, USA) with paired-end 150 bp sequencing. Raw sequence reads underwent quality trimming using Trimmomatic v0.36 (parameters: illuminaclip-adapter, 2:30:10; slidingwindowe-4, 15; minlen, 75) to remove adaptor contaminants and low-quality reads (Bolger et al., 2014). Reads that passed quality control were then mapped against the human genome with Burrows–Wheeler Alignment v0.7.12-r1039 (parameters: -M -k 32 -t 16) to remove human genome sequence contamination.

2.3.2. Metagenomic de novo assembly and gene prediction and annotation

Clean sequence reads were assembled by using Megahit v1.1.1-2-g02102e1 (parameters: --min-contig-len, 500). The open reading frames (ORFs) of assembled contigs were predicted with Prodigal v2.6.3 (parameters: -p meta), and all ORFs were aligned to a set of unique genes after clustering with CD-HIT v4.8.1 (parameters: -n, 9; -c, 0.95; -G, 0; -M, 0; -d, 0; -aS, 0.9; -r, 0; -T, 40) to remove redundancy. The longest sequence of each cluster was considered the representative sequence of each gene in the unique gene set. Salmon v1.1.0 was applied to calculate the gene abundance. Taxonomic assignment of predicted genes was conducted with BLASTP alignment against the integrated NR database using DIAMOND v0.9.22.123 (parameters: blastp –evalue 0.00001). The amino acid sequences of inferred proteins translated from predicted genes were functionally annotated by comparing them against the peptidase database (MEROPS v12.4).

2.4. Quantification of FAs

FAs in fish samples were separated and quantified by ion exchange chromatography according to Zhao et al. (2016). Minced fish samples (2 g) were fully mixed with 100 mL sulphasalicylic acid and then centrifuged at 6300 g for 10 min at 4°C. A 100-μL aliquot of the supernatant was applied to an S-433D automatic analyser (Sykam, Eising, Germany) equipped with an ion-exchange resin column (150 mm × 4.6 mm × 7 μm; LCA K06/Na, Sykam). A known mixture of FAs was added as an external standard, including aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), Gly, alanine (Ala), cysteine (Cys), valine (Val), methionine (Met), isoleucine (Ile), Leu, Tyr, Phe, histidine (His), lysine (Lys), arginine (Arg) and proline (Pro).

2.5. Texture profile analysis (TPA)

TPA of fish samples was performed by using a TA-XT Plus Texture Analyser with a P/36R probe (Stable Micro System, Godalming, UK). There were six replicates of each fish sample (1 cm × 3 cm × 3 cm) at each sampling time. The measured parameters include chewiness (g), hardness (g), adhesiveness, springiness and gumminess (g). The TPA parameters were as follows: time interval between the first and the second compressions, 5.0 s; working distance, 50%; strain; crosshead speed, 1.0 mm/s; trigger force, 5 g (Jia et al., 2022).

2.6. Sensory evaluation analysis

Quantitative descriptive analysis was conducted for sensory evaluation analysis. The evaluation team consisted of 15 people (7 males and 8 females, aged 25–30 years). The flavour and taste qualities of grass carp samples were fully discussed. Seven descriptions of fishy smell, roast, saltiness, bitterness, sweetness, sourness and umami were finally determined. Before the evaluation, grass carp samples were immersed in pure water for 10 min, steamed for 15 min, and then cooled down naturally to room temperature. Fifteen replicates for each sample were provided to assessors in sealed plastic cups. The odour intensities of headspace above the samples in the cups were determined. Pure water was used to rinse the mouth after testing each samples. A 11-point scale (10, extremely high intensity, 5, moderate; 0, none) was applied for the tests.

2.7. Data analysis

SPSS Statistics v21.0 (IBM, Armonk, NY, USA) was used for statistical analysis. TPA and FAA data were subjected to one-way analysis of variance (ANOVA) using samples as the fixed factor, followed by Duncan’s multiple range test. To determine differences in protein profiles, principal component analysis (PCA) was performed and heat maps were generated using R v3.6.3 (R Foundation for Statistical Computing, Vienna, Austria). For metagenomic analysis, comparative analysis between samples was conducted with R software using Bray–Curtis dissimilarity. A classified statistical heat map of peptidase genes was generated by using R software. To investigate the correlation between microbial activity and the protein profile, the Mantel test was conducted using Quantitative Insights Into Microbial Ecology (QIIME) v1.9.1 software (http://qiime.org/), and Pearson’s correlation heat map was constructed by using R software.

2.8. Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the iProX partner repository with the dataset identifier PXD037634. The metagenomics data are available in National Center for Biotechnology Information (NCBI) under the project accession SRR404784.

3. Results and discussion

3.1. The microbial community of dried fermented fish

We conducted metagenomic analysis to determine the microbial composition of different fish samples during fermentation and thus to understand the correlation between microbiota and the protein profile. Based on principal coordinates analysis (PCoA) (Fig. 1a), fish samples had a different microbial composition depending on the processing stage. The metagenomic DNA sequences were classified taxonomically at both the phylum and genus levels. As shown in Fig. 1b, the microbial flora was assigned to 58 phyla among the fish samples. In fresh grass carp, Proteobacteria was the most abundant (71.32%), followed by Chordata (10.01%), Firmicutes (7.69%), Streptophyta (6.46%),
Bacteroidetes (2.11%) and Chlamydiae (1.04%). The relative abundance of Firmicutes in fish samples kept increasing during fermentation, which showed a marked increase to 36.12% after 18 h of fermentation and then increased progressively to 55.14% after drying, making it the dominant phylum in dried fermented fish. The relative abundance of Proteobacteria decreased constantly during the entire process, accounting for 42.34% of classified sequences in fish samples at the end of drying. It has been reported that Proteobacteria and Firmicutes are the main bacterial species in dried fermented fish products, suggesting their vital role in the development of muscle quality (Q. Zhang et al., 2021).

At the genus level, there were 1299 genera identified among the fish samples, and 34 of them had a relative abundance > 1% (Fig. 1c). *Burkholderia* (23.95%), *Achromobacter* (20.09%) and *Serratia* (18.55%) were the most abundant genera in raw fish samples, the levels of which decreased remarkably during fermentation, with < 2% abundance by the end of processing. After inoculation and primary fermentation, the proportion of *Lactobacillus* increased, but it only reached a fraction of microbial flora. *Macrococcus*, *Staphyloccocus*, *Morganella* and *Citrobacter* became the dominant genera in the final fermented fish products. The results indicated that both primary fermentation and drying have a significant impact on the microbial community structure in fish samples. In a previous study of dry-cured fish, *Cobetia*, *Staphylococcus* and *Ralstonia* became the most abundant genera after processing (Zhao, Hu, & Chen, 2022a). Therefore, it is deduced that inoculation with *L. plantarum* dramatically altered the microbial composition. The relative abundance of *Macrococcus* and *Citrobacter* increased drastically in fish samples after fermentation, with the initial relative abundance of 0.61% and 0.30% increasing to 17.23% and 13.06%, respectively. These changes might be because the development and metabolism of *L. plantarum* provide a more suitable environment for the growth of these microbes. We speculate that acid-tolerant bacteria have a competitive advantage in the microbial community of fermented fish at a low pH due to acid production. During the drying process, the proportion of *Macrococcus*, *Staphyloccocus* and *Morganella* increased substantially, with a final relative abundance of 24.79%, 12.53% and 8.87%, respectively; the relative abundance of *Citrobacter* decreased slightly to 8.22%.

Macroccus and *Staphyloccocus* are closely related: they generally share the same niches in the food matrix and their growth is associated with the development of texture, colour, aroma and flavour of fermented foods (Mazhar et al., 2020; Ramos et al., 2021; Xiao et al., 2020).

### 3.2. The secreted peptidase profile of microbial taxa

Proteases secreted by microorganisms are responsible for protein hydrolysis and accelerating maturation of various types of fermented food (Ramos et al., 2021). Peptidases are a type of protease that can catalyse the cleavage of the peptide bonds between amino acid residues of proteins (Nguyen et al., 2019). A total of 78207 genes encoding the peptidases and peptidase inhibitors were identified in the fish samples according to the Merops database, and variations in the top 50 were examined (Fig. 2, heatmap with data presented in transcripts per million [TPM], and Table S1). The abundance of peptidase genes in microbes changed during processing. It can be observed that most of these peptidase genes had an upward trend in relative expression during processing. MER0427791 was the most abundant peptidase gene in raw fish samples and showed a remarkable decrease in TPM during fermentation. Among the top 50 genes, 40 peptidase genes were annotated to *Macrococcus*, with elevated TPM in processed fish samples. As reported previously, methionyl aminopeptidase has the potential to hydrolyse proteins and thus produce FAAs (Flores et al., 2000). PepS aminopeptidase is considered to be involved in flavour development of dairy products through hydrolysing bitter peptides and liberating aromatic amino acids (Fernandez-Espla and Rul, 1999). Hence, *Macrococcus* may play an important role in protein proteolysis.
Fig. 2. Variations in the top 50 genes encoding peptidases and peptidase inhibitors of microorganisms in different fish samples.
3.3. Qualitative and quantitative analysis of dried fermented fish proteins

A total of 633 proteins were identified in the M, F and D grass carp samples by using the label-free proteomic approach. The molecular weight of all identified proteins is shown in Fig. 3a. Proteins >100 kDa were the most abundant in grass carp samples, accounting for around 20% of identified proteins, followed by 15% and 16% for 10–20 kDa and 20–30 kDa proteins, respectively. The PCA score plot showed that 71.39% of the variability was explained by the two principal components, which accounted for 59.18% and 12.21% of the total variance, respectively (Fig. 3b). There was pronounced separation between D samples and other (M and F) samples, while there was some overlap between F and M samples. These results indicate greater variation in the identified proteins occurred during drying. Protein degradation in fermented fish samples was induced by the activity of microbial enzymes and endogenous proteases and affected by the processing conditions (pH, temperature, etc.). It was suggested that the alteration of protein profile in grass carp samples during the first 18 h of fermentation was a slow, gradual process. Therefore, no significant differences in protein abundance between F and M were observed. However, the microbial community structure and peptidase profile of grass carp samples was seriously influenced by the introduction of LAB starters, resulting in the obvious change of microbial composition. These could explain why there is no significant differences in protein abundance but significant differences showed in microbial composition between F and M.

There were three comparisons of grass carp protein profiles during fermentation. Fig. 3c shows DAPs in a heat map, and Table S2 presents critical information about the proteins. There were variations in 199 DAPs, including 165 proteins with a fold change (FC) ≥ 1.5 or ≤ 0.67 and P ≤ 0.05, and 34 unique proteins. Of those DAPs, 27 were upregulated (FC ≥ 1.5) and seven were downregulated (FC ≤ 0.67) in fish samples after 18 h of fermentation, suggesting that inoculation with L. plantarum induced mild proteolysis. It is evident that more low-molecular-weight proteins were generated in fish samples during this period. After drying, there were up to 129 DAPs, including 25 upregulated proteins and 104 downregulated proteins. This finding indicates marked protein degradation occurred in fish muscles during 24 h of low-temperature drying. There were 46 upregulated proteins and 91 downregulated proteins in the final fermented fish samples compared with the raw fish samples.

The normalised abundance of the top 30 DAPs in the various fish samples is shown in Fig. 3d. In the raw fish samples, fast skeletal myosin 10C-type S1 (B0FYX6), 10C light meromyosin (A6NA51), myosin-binding protein C (A0A3N0Y2F0), parvalbumin (A0A3N0Y405) and troponin I (A0A3N0ZAY0) were most abundant. Processing, especially drying, had a pronounced influence on the abundance of most of the highly abundant proteins. The expression of the top 3 most abundant proteins in the raw fish samples declined significantly after processing. It has been reported that protein degradation plays an important part in flavour development and texture changes in fermented food (Xiao et al., 2020; C.-Y. Zhou, Wu, et al., 2019b). In this study, DAPs related to flavour and texture development in fish samples were screened and the
variations in their relative abundance are shown in a heat map (Fig. 4). The myofibrillar structure was weakened during processing, denoted by the significantly downregulated myofibrillar proteins. In the F versus M comparison, fast skeletal myosin 30C-type S1 (B0FYX8), the most abundant DAP in the raw fish samples, decreased significantly during fermentation, suggesting breakdown of muscle structure after inoculation with the starter culture. In the D versus F comparison, there were 17 cytoskeletal DAPs (8 upregulated and 9 downregulated), indicating that drying had a marked effect on the myofibrillar protein composition. Compared with raw fish samples, the expression of fast skeletal myosin 10C-type S1 (B0FYX6), myosin heavy chain (A0A3NOXHY0, A8R0Q7 and B1PF34), troponin I (A0A3NO2ZAY0), troponin T (A0A3NOXN9 and A0A3NOZAD6), myosin-7 (A0A3NOXFJ8 and A0A3NOYFS2), tubulin alpha chain (A0A0H3U093) and keratin (A0A3NOYWN9) declined significantly in dried fermented fish. Besides, heat shock proteins are closely associated with meat tenderness and can protect structural proteins from being degraded (Jia et al., 2022). Degradation of myofibrillar proteins could be modulated by heat shock proteins and cytochrome c limiting the size of the aggregated proteins (Ding et al., 2021). In this study, heat shock protein 90α (A0A3NOYWG3 and A0A2P1K680) and cytochrome c (A0A3NOY5G0) were downregulated during drying (Table S2), data demonstrating that proteolysis occurred in fish muscles.

As an extracellular matrix structural constituent, collagens are responsible for the textural quality of fish muscles (Yu et al., 2020). It has been reported that increased expression of meat collagens is correlated with greater shear force, hardness and chewiness (Jia et al., 2022; Song et al., 2022). In this work, collagen α chain proteins (A0A3NOZ806, A0A3NOXFT6, A0A3NOY9S3, A0A3NOYC21 and A0A7D4XU00) in dried fermented fish samples were upregulated sharply after processing. The variations in collagen content may be associated with altered water distribution and mobility within muscle (Baldi et al., 2019).

Proteolysis in fermented food also plays a crucial role in the production of active peptides. In addition to contributing to taste and flavour, LAB can augment the release of bioactive peptides (Gallego et al., 2018). Troponin T (A0A3NOXN9 and A0A3NOZAD6), a myofibrillar protein involved in muscle contraction and reported to be a good source of novel angiotensin-converting enzyme (ACE) inhibitory peptides (Katayama et al., 2008), exhibited intense breakdown in the drying, implying an increasing abundance of active peptides.

3.4. GO analysis of DAPs

GO annotation was applied to understand the functional characteristics of proteins in dried fermented fish samples. Fig. 5 shows the variations in the relative abundance of the protein, while blue indicates a lower relative abundance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
functional annotation classification of DAPs in the M versus F, F versus D and M versus D comparisons (P < 0.05). The proteins were categorised into three groups based on their functional activities, namely biological process, cellular component and molecular function. For the M versus F comparison, the 24 DAPs were associated with oxygen binding (GO:0019825), organic cyclic compound binding (GO:0097159) and heterocyclic compound binding (GO:1901363) (Fig. 5a). For the F versus D comparison, the 174 DAPs were mainly distributed in cellular component (75.3%), followed by biological process (21.3%) and molecular function (3.4%), and most of them (87.9%) were downregulated (Fig. 5b). In the D versus M comparison, most of the 687 DAPs belonged to the cellular component (55.8%), molecular function (27.9%) and biological process (16.3%), and 73.7% of the DAPs were downregulated (Fig. 5c). The top 5 GO terms in the D versus M comparisons were related to cellular component, including intracellular organelle (GO:0043232), cytoplasmic part (GO:0044444), intracellular non-membrane-bound organelle (GO:0043232), macromolecular complex (GO:0032991) and cell part (GO:0044464), suggesting the breakdown of cellular structure and the occurrence of protein hydrolysates in dried fermented fish samples. It was worth mentioning that the expression of ribosomal proteins, which help maintain transfer RNA (tRNA) stability in muscles (B. Zhang et al., 2020), was also markedly decreased after drying. It has been suggested that ribosomal proteins can serve as indicators of textural properties in meat (Song et al., 2022). The GO enrichment results provide further perspectives in how the quality of fish changes during fermentation and drying.

3.5. The texture and sensory analysis of dried fermented fish

To observe the changes in texture profile of fish samples during processing, TPA was determined (Table 1). The results showed that the hardness of fish samples was obviously decreased after 18 h of
Table 1
Changes in the texture profile of dried fermented fish samples.

| Parameters | M     | F     | D     | Control |
|------------|-------|-------|-------|---------|
| Hardness (g) | 261.12 ± 33.97 | 206.63 ± 13.10 | 6441.59 ± 314.90 |       |
| Adhesiveness | 4.80 ± 0.04 | 7.42 ± 0.41 | 77.0 ± 8.47 |       |
| Springness | 0.44 ± 0.02a | 0.61 ± 0.01a | 0.59 ± 0.08a |       |
| Gumminess (g) | 86.64 ± 5.70 | 104.5 ± 8.77 | 3091.69 ± 147.91 |       |
| Chewiness (g) | 127.49 ± 11.76 | 2325 ± 43.52 | 6494.22 ± 462.74 |       |

Notes: All of the values are presented as means ± SD. Letters a-b refer to significance (P < 0.05) between different fish samples.

3.6. The relationship between microbial metabolism and protein degradation

To find out the correlation between microbial metabolism and protein degradation, we firstly conducted redundancy analysis (RDA) between dominant genera or peptidase genes and FAAs to investigate the influence of microbial activity on FAA composition. Fig. 6A shows correlations between the dominant genera and FAAs. Macrococcus, Staphylococcus, Klebsiella, Vibrio, Morganella, Kurthia, Metalysinibacillus and Bacillus were correlated positively with 15 FAAs, namely Asp, Gly, Glu, Ala, Ser, Thr, Val, Tyr, Met, Pro, Lys, Ile, Phe, Leu and His. Meanwhile, Burkholderia, Achromobacter, Serratia, Cucumis, Acinetobacter and Soonwooa were correlated positively with Arg. Fig. 6B shows the relationship between the top 30 peptide genes and FAAs. Most of the peptide genes (23/30) were correlated positively with the above-mentioned 15 FAAs. MER0700839, MER0199362, MER0208403 and MER0207602 were closely linked to Arg. These results suggest that the development of microorganisms, as well as elevated expression of peptide genes, had a pronounced effect on FAA production. Moreover, Macrococcus and Staphylococcus were correlated more closely with FAAs than other genera, indicating their high proteolytic activity and great contribution to FAA production.

Next, we calculated Pearson’s correlation coefficients to evaluate the relationship between the abundance of microorganisms or expression of peptidase genes and FAAs to investigate the relationship between microbial metabolism and the protein profile. As shown in Fig. 7a, the development of Kurthia, followed by Staphylococcus, Vibrio, Metalysinibacillus, Bacillus, Klebsiella, Morganella and Macrococcus, was highly correlated with variations in abundance of myofibrillar proteins and collagens in fermented fish samples, suggesting that these strains contribute greatly to protein hydrolysis. Kurthia was closely related with 25 DAPs, including 14 upregulated and 11 downregulated proteins. Kurthia species are capable of converting metmyoglobin to oxymyoglobin, which is more desirable for preserving and improving the colour characteristics of meat products (Arthura et al., 1993). This indicates that Kurthia is involved in the colour formation of fish samples by degrading muscle proteins. Macrococcus and Staphylococcus, as the most abundant strains in dried fermented fish, were highly correlated with 12 and 22 DAPs, respectively, suggesting their important role in proteolysis. There was also a close correlation between the major peptidase genes and principal DAPs (Fig. 7b). A0A120N0J19 (myosin heavy chain) and A0A7D74XU0 (collagen type I alpha 2) were correlated significantly with most peptide genes (P < 0.001). This was consistent with the strong correlation between Macrococcus and the two proteins (P < 0.001, Fig. 7a) and could be explained by the fact that most of the detected peptide genes were annotated to Macrococcus (as described above). Previous studies have demonstrated that Macrococcus species play an important role in ripening and organoleptic quality development in fermented foods.
Fig. 6. Redundancy analysis (RDA) of dominant genera and free amino acids (FAAs) (A), and RDA analysis of main peptidase genes and FAAs (B).
(Liang et al., 2019; Mazhar et al., 2020), due to their abundant proteolytic and lipolytic enzymes, while the main enzyme in *Macrococcus* has cell-envelope proteinase activity, with limited peptidolytic activity (Mazhar et al., 2020). In this study, the high correlation between peptidase genes and FAA production and the protein profile suggest that *Macrococcus* played a major role in the development of sensory and texture qualities due to proteolysis.

4. Conclusions

We have demonstrated that microbial metabolism is involved in protein hydrolysis of fish muscle during microbial fermentation. The LAB starter culture had a pronounced influence on the microbial community structure of fermented fish, although *Macrococcus* and *Staphylococcus* became the dominant microorganisms in the final products. The protein profile changed markedly during processing. There were 199

Fig. 7. Heat map of Pearson’s correlation coefficients showing the relationships between the dominant genera (a) or the major peptidase genes (b) and the principal differentially abundant proteins. Yellow indicates a positive correlation, blue indicates a negative correlation and green indicates no correlation. *P* < 0.05, **P** < 0.01, ***P** < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
DAPs, including myofibrillar proteins and collagens related to taste and texture quality. RDA demonstrated that dominant genera and peptidase genes were highly correlated with FAA production. There were significant correlations between the dominant strains as well as the major peptidase genes and 38 DAPs (including cytoskeletal proteins and collagens). Based on peptidase profile analysis, most of the top peptidase genes were annotated to *Macrococcus*, indicating its vital role in proteolysis. These results are useful to uncover the relationship between microbial metabolism and protein hydrolysis. This study provides guidance for applying a microbial starter culture to improve the sensory qualities of fermented fish.

CRediT authorship contribution statement

Dandan Zhao: Conceptualization, Data curation, Writing – original draft. Yunqiong Cheng: Methodology, Software. Jun Hu: Visualization, Investigation. Xuxia Zhou: Validation. Chaogeng Xiao: Supervision. 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfbs.2022.11.016.

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