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NMR-based metabolic analysis of *Bacillus velezensis* DZ11 applied to low-salt fermented coarse fish involved in the formation of flavor precursors

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Abstract

Microbial fermentation can be responsible for flavor production and increasing the nutritional quality of fermented fish foods. The study aimed to isolate non-halophilic bacteria possessing proteolysis and lipolysis properties as starter cultures for low-salt content fermentation of coarse fish. The strains with high enzyme activities were screened and multiple growth characteristics were analyzed. The content of amino acid nitrogen and trichloroacetic acid (TCA)-soluble peptides in fermented fish was also investigated under different fermentation conditions. Nuclear magnetic resonance (NMR) metabolomics analysis was utilized to monitor the metabolite profile of fermented fish paste. The isolate was identified as *Bacillus velezensis* DZ11. The strain DZ11 exhibited obvious hydrolysis properties of lipids and proteins. During the fermentation of fish paste, a total of 40 differential metabolites were identified, of which multiple metabolites changed significantly, including nutrient-related substances and flavor precursors. Salt concentration and strain DZ11 had important effects on the differential metabolites. *B. velezensis* DZ11 under longer fermentation time promoted the formation of flavor precursors and potentially increased the nutritional substances of low-salt fermented fish.

Keywords: *Bacillus velezensis*; NMR; metabolite profiles; fermentation; low-salt.

Practical Application: This work demonstrates the feasibility of *Bacillus velezensis* DZ for low-salt fermented fish paste.

1 Introduction

A variety of fermented aquatic foods, including fish sauce, fish paste, shrimp paste and sour fish, are widely consumed in many countries (Isola et al., 2022; Kesika et al., 2022). These traditional fermented aquatic foods are generally produced through spontaneous fermentation and by selecting low-value fish and shrimp in addition to 20%-30% salt for 1-3 years (Han et al., 2022; Wang et al., 2019; Wang et al., 2022). As spontaneous fermentation is usually implemented by endogenous enzymes and microorganisms that are primarily derived from raw ingredients or the surrounding environment, high salt contents affect the function of endogenous enzymes and the metabolic activity of other microorganisms, leading to a prolonged fermentation time. Thus, a highly salted environment in traditional aquatic foods fermentation process raises concerns, such as adverse health effects, difficulty in controlling the fermentation process and a strong odor that limits consumer acceptability. Therefore, there is an increasing demand for low-salt fermented aquatic foods with improved flavor.

Various methods have been exploited to improve the quality of fermented aquatic products quality and accelerate the fermentation process by lowering the salt content (Liu et al., 2017), changing the temperature (Gao et al., 2020a), adding protease (Chancharoonpong & Hsieh, 2022) and functional starters (Auttanak et al., 2022; Chuprom et al., 2016). However, reducing salt only by physical approaches may adversely affect flavor and taste (Singracha et al., 2017). Therefore, the application of non-halophilic microorganisms for developing low-salted fermented aquatic food with good flavor and nutrition is considered one option in line with the modern healthy consumption concept.

It has been documented that lactic acid bacteria (Zhou et al., 2021), *Bacillus piscicola* (Tepkasikul et al., 2022), *Tetragenococcus halophilus* and *Virgibacillus* (Lapsongphon et al., 2013; Montriwong et al., 2015; Udomsil et al., 2017) were used to low-salt fermented fish products under laboratory conditions. Different from the commercial starter cultures, the starter cultures selected from autochthonous microorganisms should be better adapted to the fermentation environment (Santa et al., 2014). Microorganisms from naturally fermented foods are also attractive sources of enzymes owing to their non-toxic nature and specific metabolic capabilities. Therefore, it is necessary to isolate the predominant microorganisms from traditional fermented fish foods to accelerate the fermentation process as well as produce low-salt fermented fish products with a characteristic flavor. This study aimed to isolate strains with proteases and lipase-producing ability from traditional fish sauce and investigate the effects of the isolated strains on low-salt fermentation of coarse fish.

Metabolomics studies provide the "snapshot" of metabolic profiles in fermented foods and may be helpful for systematically understanding fermentation mechanisms and flavor compound formation. NMR-based metabolomics method is non-invasive to samples, non-biased to compounds and allows for real-time and dynamic monitoring (Mielko et al., 2021). In this study,

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NMR-based metabolomics combined with multivariate data analysis was applied to investigate metabolite alterations during the processing of fermented fish paste. The changes in the main metabolites were measured to characterize the differences in the properties of fermented fish paste in relation to fermentation factors, including microbial starter additions and salinity. These results make it possible to guide the efficient processing to produce fermented fish products with high quality standards. This study would be beneficial for producing low-salt fermented aquatic foods and the development of a rapid fermentation process.

2 Materials and methods

2.1 Chemicals and reagents

The 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid, sodium salt (TSP-d_4) was purchased from Tokyo Chemical Industry Co., Ltd., Japan. Deuterium water (D_2O: 99.8% purity) was purchased from Shanghai Macklin Biochemical Company, China. All other reagents were of analytical grade.

2.2 Isolation and preparation of bacterial strains

Traditional fish sauce samples fermented for 12 months were collected from a fish sauce-processing factory located in Shantou, Guangdong Province, China. Protease-producing and lipase-producing bacteria were isolated from the traditional high-salt fish sauce. Bacteria were isolated using a Gibbons culture medium containing 5% NaCl and a spread plate technique (Yeannes et al., 2011). Bacterial colonies with different morphological characteristics were selected and purified.

To test the presence of extracellular proteases, the colonies were grown on skim milk agar plates containing 5% NaCl at pH 7.0 and incubated under the aerobic condition at 30 °C for 48 h. Protease activity can produce large clear zones around the colonies where the precipitated skim milk is solubilized (Lloyd-Jones et al., 2010). The most active protease-producing bacteria were selected according to the zone of clearing and preservation diameter.

To determine the lipase-producing ability of the selected isolates, the preliminary screening for lipase-producing strains was carried out in petri dishes using agar spot assay. In brief, the strains were spotted on a lipase screening medium containing 1% Tween 80 and incubated at 37 °C for 48 h (Ilesanmi et al., 2020). A sedimentation circle surrounded lipase-producing strains, and the most zone-forming bacterial strain was selected for further analysis.

2.3 Molecular identification of the isolated strain

The isolated bacterial strain was identified using 16S rDNA gene sequence analysis by a commercial service (GENEWIZ, Suzhou, China). Afterwards, the sequences were identified by BLAST in the NCBI nucleotide database for strain identification, and several known sequences of type strains were downloaded, and multiple sequence comparisons were performed using MEGA 7 software to establish a phylogenetic tree.

2.4 Growth characteristics of the isolated strain

The isolate was added to Gibbons liquid medium according to the additional amount of 2% and incubated for 48 h. The growth of the isolate at different salinity (0%-25% NaCl, w/v), temperature (20-40 °C) and pH (5.0-9.0) was performed by measuring the OD value of 600 nm at 24 h and 48 h.

2.5 Laboratory fish paste production

Freshwater small miscellaneous fish (*Hemiculter leucisculus*) was obtained from the Dongfeng Market in Zhenjiang, Jiangsu Province, China. To study the effect of the strain on low-salt fermentation of fish paste, the small miscellaneous fish was gilled and offal sterilized at 115 °C for 15 min, then distilled water was added to homogenize. Fish pulp was mixed with a reduced-salt content (9%) and 5% (v/w) isolated strain and fermented in 500 mL jars. The isolate was subcultured twice continuously in Gibbons liquid medium, and the final cell density was adjusted to 7-8 log CFU/mL. The fermentation jars were stirred every five days, and the fermentation samples were collected on day 20 and 40 for analysis. Additionally, as uninoculated controls, the fresh fish was gilled, offal and homogenized without sterilization, then mixed with a reduced-salt (9%) and high-salt (25%) content, respectively. All the samples were incubated in an incubator at 25 °C for 40 days of fermentation. Thus, four different groups were formulated: starter culture groups for 20 days (DZ11-20) and 40 days (DZ11-40), low-salt control group (LS-40) and high salt control group (HS-40).

2.6 Determination of Amino Acid Nitrogen (AAN) and TCA-soluble peptide

The AAN of all samples were determined using the formoltitration method (Ruan et al., 2022) according to the recommended detection method under Chinese national standards (GB5009.235-2016 - Chinese Standard, 2016). The TCA-soluble peptide content was measured following the method described earlier (Sriket et al., 2012).

2.7 Sensory evaluation

Sensory evaluation of the samples was carried out according to the method of Zhou et al. (2021). The 10 evaluation participants (five women and five men, aged 25-35 years old) were trained prior to sensory assessment. Sensory (bitter, umami, fishy, sour, ammonia, rancid, salty and fermented smell) evaluation scores were made using a 10-point scale.

2.8 NMR sample preparation and spectral acquisition

Metabolite extraction was performed according to the previous method (Markkinen et al., 2022) with some modifications. The fish paste samples were centrifuged at 10000 rpm for 10 minutes at 4 °C. The 450 μL of the supernatant, 70 μL sodium phosphate buffer and 70 μL solvent of TSP-d4 (5 mM TSP-d4 in D_2O) were mixed. After mixing, the sample was transferred into a 5 mm NMR tube for NMR-based metabolomics analysis. At 0 ppm, TSP-d4 was used as the chemical shift reference. D_2O offered a lock signal. ^1H, ^{13}C, and 2D NMR spectroscopy were performed

with a Bruker 400 Ultrashield TM spectrometer system (USA, 400 MHz magnetic field) using the standard PRESAT pulse sequence.

2.9 NMR data processing and analyses

Acquired spectra have employed a series of procedures, including phase, baseline corrections and calibrating under manual correction using MestReNova (version 14.0) software. Peaks within NMR spectra were assigned with reference to known chemical shifts and peak multiplicities. The processed spectra region was divided into 0.01 ppm size bins within a range of 0.5 and 9.0 ppm, and the water region of δ 4.4-5.0 was removed. All of the bucketed regions were normalized to the total integrated area of the spectra.

The metabolites were identified and quantified based on chemical shift and the Biological Magnetic Resonance Data Bank in addition to other literature references (Lee et al., 2022). Data sets were conducted in a multivariate statistical analysis using MetaboAnalyst 5.0 software (Cui et al., 2022). Principal component analysis (PCA) and heatmap correlation were applied to the NMR datasets. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using the *Bacillus subtilis* pathway library as a reference.

2.10 Statistical analysis

Except for NMR analysis, data were statistically analyzed independently, at least in triplicates. All data were shown as mean ± standard deviation. IBM SPSS (version 26) and Origin software were used for statistical data analysis.

3 Results and discussion

3.1 Screening and identification of proteolytic and lipolytic isolates

This study isolated Bacillus velezensis DZ11 exhibiting the highest protease (Figure 1A) and lipase-producing activities (Figure 1B). The strain DZ11 was gram-positive, rod-shaped bacteria (Figure 1C). The colony of the stain showed morphological characterization of light yellow, dry surface and uneven edges.

A phylogenetic analysis based on 16S rRNA gene sequences showed that the strain DZ11 formed a tight phyletic lineage with the strain of Bacillus velezensis FZB42 and was most closely related to the strain, displaying a 99.79% sequence identity. These results clearly suggested that the strain DZ11 is a member of B. velezensis. The taxonomic position of the strain DZ11 biotype is presented in Figure 1D.

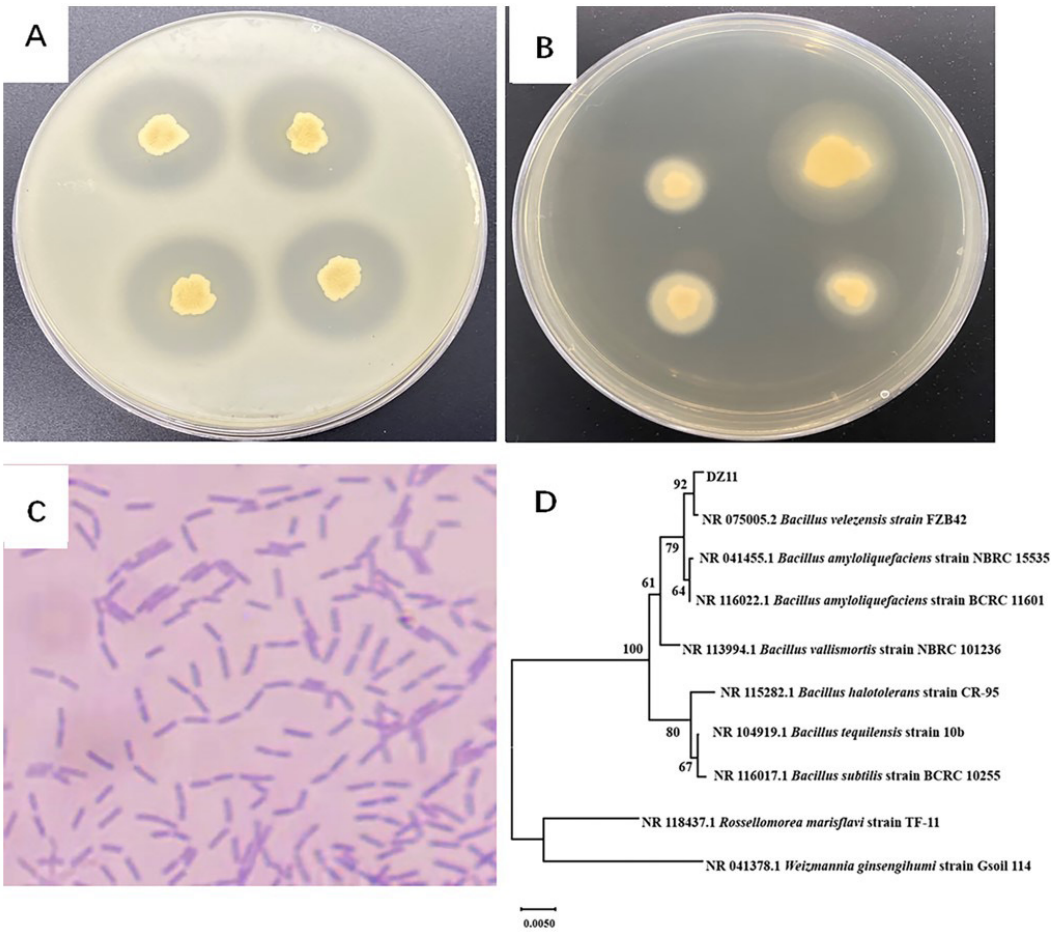

Figure 1. The appearance of clear zones surrounding the colony on agar plates containing skim milk with protease-producing activities of *Bacillus velezensis* DZ11 (A); containing tween 80 with lipase-producing activities of *B. velezensis* DZ11 (B); Gram stain analysis under the light microscope of *B. velezensis* DZ11 (C); phylogenetic tree showing the relationships between *B. velezensis* DZ11 and its related species (D).

The protease and lipase activities of DZ11 were observed by agar diffusion assays, indicating that the strain DZ11 could break down proteins and lipids. Proteolysis and lipolysis were the main biochemical reactions during fish sauce fermentation (Mohamed et al., 2012). Protease and lipase activities produced by some microorganisms contributed to producing the good flavor of fermented foods (Kanjan et al., 2021). Therefore, as a starter culture, *B. velezensis* DZ11 is a good candidate for further investigation in the production of flavor-determining components of fermented fish paste.

In addition, strain DZ11 was able to grow well between 20 °C and 30 °C. When the temperature was above 30 °C, the growth ability of the strain decreased significantly. Meanwhile, the strain DZ11 was not sensitive to the change in pH and could grow well at a pH of 5.0-8.0. Nevertheless, the strain DZ11 could not tolerate higher concentrations of sodium chloride and grew in a suitable range of salt concentrations from 0 to 10%. When the salinity was greater than 10%, DZ11 showed very little growth. The growth curve of DZ11 was measured under optimal growth conditions, showing that the phase of rapid increases of the strain was from 8 h to 24 h (Figure 2A). According to the growth curve and growth characteristics, the incubation activity of the strain DZ11 was correlated to low acidic conditions and pH-independent, thereby indicating that DZ11 may be conducive to low-salt fermentation of fish paste.

3.2 Changes in AAN and TCA-soluble peptide contents

The correlations of salt and the strain DZ11 on AAN and TCA-soluble peptide contents of fermented fish samples are shown in Figure 2B. The free amino acid in food is commonly measured in the form of AAN, and ANN is a reflection of the taste properties of fermented foods. The concentration of AAN in DZ11-groups increased significantly and can reach 0.79 g/100 mg after 40 days of fermentation. ANN in the uninoculated control groups was significantly lower than that of the inoculated group after day 40. The change in ANN content reflects the number of primary amino groups in fermented fish products and is closely related to the degradation of polypeptides and proteins (Zhu et al., 2021).

As presented in Figure 2B, the concentration of TCA-soluble peptides in the inoculated groups first increased and then decreased as fermentation continued. A significant difference in the content of TCA-soluble peptides between the two inoculated samples and the uninoculated controls was also observed. After 40 days of fermentation, only lower content of TCA-soluble peptides of uninoculated control samples was detected. This observation could be the combined effects of microorganisms and the salt concentration on proteolytic enzyme activity. TCA-soluble peptides can usually reflect the degradation of protein caused by endogenous enzymes or microbial proteases (Jia et al., 2019). The results indicated that the TCA-soluble peptides in the starting inoculated samples were produced from protein hydrolysis and could be catalyzed into free amino acids by the strain DZ11 proteinases.

3.3 sensory evaluation analysis

The sensory evaluation profiles based on Quantitative Descriptive Analysis (QDA) are shown in Figure 2C. The bitter, fermented smell and sour ammonia scores in the DZ11-20 group were slightly higher than those in other groups. The umami score in the DZ11-40 group increased significantly. In addition, salt and fish were the main tastes in the HS-40 group.

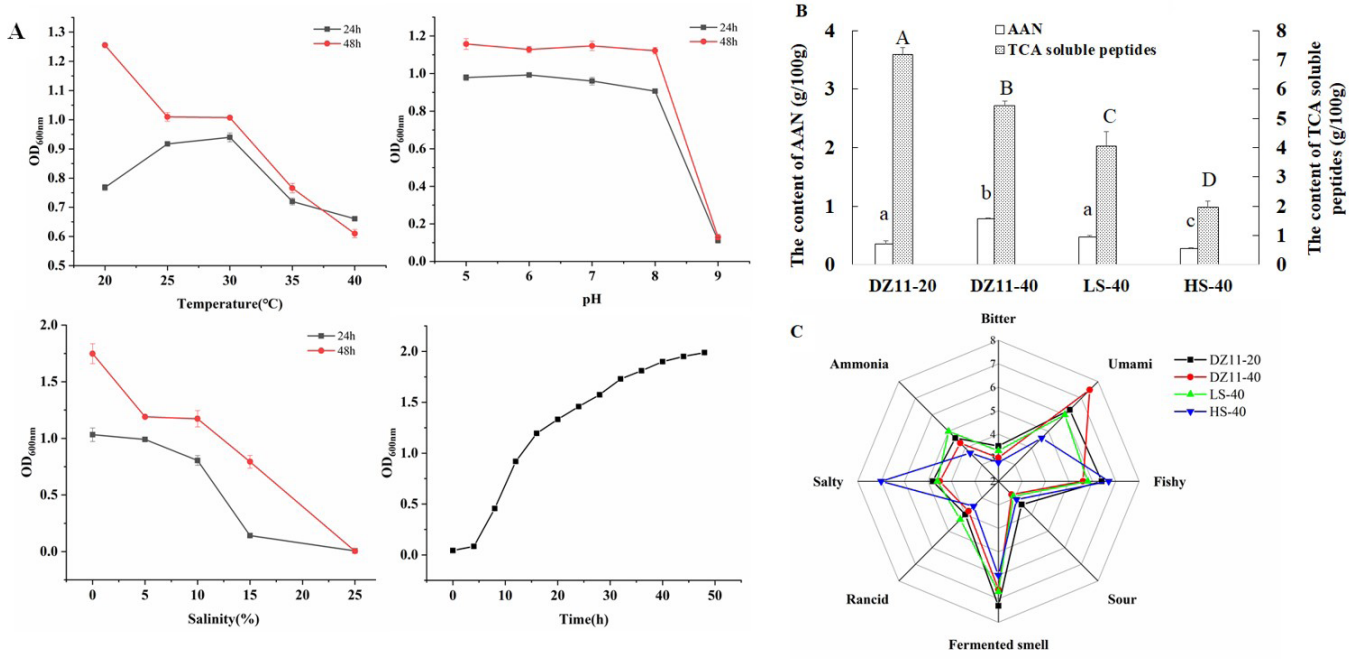

Figure 2. Effects of temperature, pH, and salinity on the growth curve of DZ11 (A). AAN and trichloroacetic acid (TCA) soluble peptides of fermented fish samples (B), different lowercase letters (a-c) and capital letters (A-D) indicate significantly different ($P \leq 0.05$). Sensory profiles of fermented fish samples based on QDA testing (C).

3.4 Metabolic profiles of four samples with different fermentation conditions

A total of 40 metabolites were identified from the four fermented fish samples based on the ^{1}H NMR spectroscopy analysis (Figure 3). Generally, the metabolites of samples were composed of amino acids, organic acids, sugars, nucleotides and some other primary or secondary metabolites. These metabolites included 22 amino acids and their derivatives, 8 organic acids, 1 amine (histamine), 3 nucleotides and their derivatives (uracil, IMP, inosine), glucose and other compounds (UDPG, glycerol phosphocholine, phosphocholine, glycerol 3-phosphorate, choline).

3.5 Metabolism changes of fermented fish with different salt concentrations

PCA was applied to examine the two groups' separation: the high-salt control (HS-40) and low-salt group (LS-40). To visualize the changes in metabolites due to the different treatments of salt, the heatmap was plotted in a red-blue color scale using the transformed NMR data (Figure 4). The heatmap presented different levels of metabolites. The deeper blue and darker red color on the heatmap illustrated the lower and higher concentration of metabolites, respectively.

Traditional fermented fish foods are produced by long-term fermentation and high salt solution without using starter cultures. Therefore, the salt concentration is one of the critical factors affecting fermented food quality through metabolites changes (Auttanak et al., 2022). As shown in Figure 4A, there was a clear separation between the high-salt and low-salt control groups. The result indicated that the effect of salt was detectable in the metabolome of fermented fish samples. From the hierarchical clustering heatmap (Figure 4B), there was a higher amount of only 11 metabolites in the high-salt group (HS-40), identified as formate, choline, phosphocholine, threonine, creatine, lactate, hypotaurine, UDPG, tyrosine, inosine and tryptophan, while most metabolites were lower. More particularly, a total of 10 major compounds were not detected in HS-40, consisting of 6 amino acids, 2 organic acids, histamine and uracil, indicating that the fermented fish with high salt concentrations could not produce these compounds or the levels were too low to be detected.

Some halophilic bacteria such as halophilic lactic acid bacteria, *Tetragenococcus*, *Virgibacillus*, *Bacillus*, *Pediococcus* and *Staphylococcus* were isolated from high-salt environments as the dominant microbe (Kanjan et al., 2021; Ma et al., 2022; Udomsil et al., 2017). In this study, the production of lactate, formate and UDPG in response to high salinity also indicated the existence of a small number of halophilic bacteria. Choline, phosphocholine, hypotaurine and creatine could maintain cell structural integrity as osmoprotectant molecules. This result aligned with the report on salting processed seafood (Chen et al., 2016). However, high-salt environments can inhibit the growth and reproduction of most non-halophilic microorganisms. Additionally, a high osmotic pressure exerted from a high salt condition can result in limited microbial enzyme activity (Auttanak et al., 2022). Meanwhile, high-salt environments can inhibit the activity of endogenous enzymes of fish. Accordingly, the content of most metabolites in the high-salt sample was significantly lower.

The heatmap (Figure 4B) also showed that more diverse and higher flavor precursors (amino acids, organic and glucose) were produced in low-salt fermented fish paste. However, fish fermentation under low-salt concentration could increase the possibility of the production of histamine. The formation of biogenic amines or undesirable tastes and flavor compounds caused by spoilage microorganisms might take place at a low salt concentration (Klomklao et al., 2006). Therefore, further studies related to flavor, inhibition of biogenic amines production and safety of low-salt fermented fish are surely necessary.

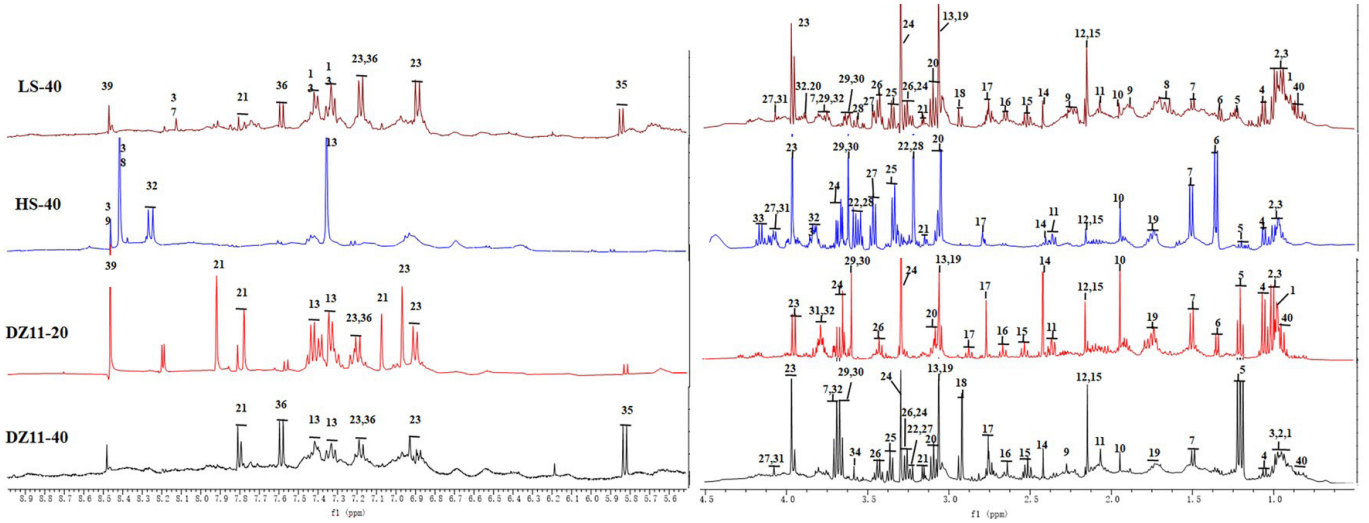

Figure 3. Representative 1H NMR spectra (δ0.5-9.0 ppm) obtained from the different groups. Keys: 1, Isovalerate; 2, Isoleucine; 3, Leucine; 4, Valine; 5, 3-Hydroxybutyrate; 6, Lactate; 7, Alanine; 8, Arginine; 9, GABA; 10, Acetate; 11, Glutamate; 12, Methionine; 13, Phenylalanine; 14, Succinate; 15, Glutamine; 16, citrate; 17, Aspartate; 18, lysine; 19, Ornithine; 20, Creatine; 21, Histidine; 22, Choline; 23, Tyrosine; 24, Glucose; 25, Hypotaurine; 26, Taurine; 27, Phosphocholine; 28, Threonine; 29, Glycerol -3-phosphate; 30, Glycerophosphocholine; 31, Creatinine; 32, Inosine; 33, UDPG; 34, Glycine; 35, Uracil; 36, Tryptophan; 37, Histamine; 38, Formate; 39, IMP; 40, 2-Methybutyrate.

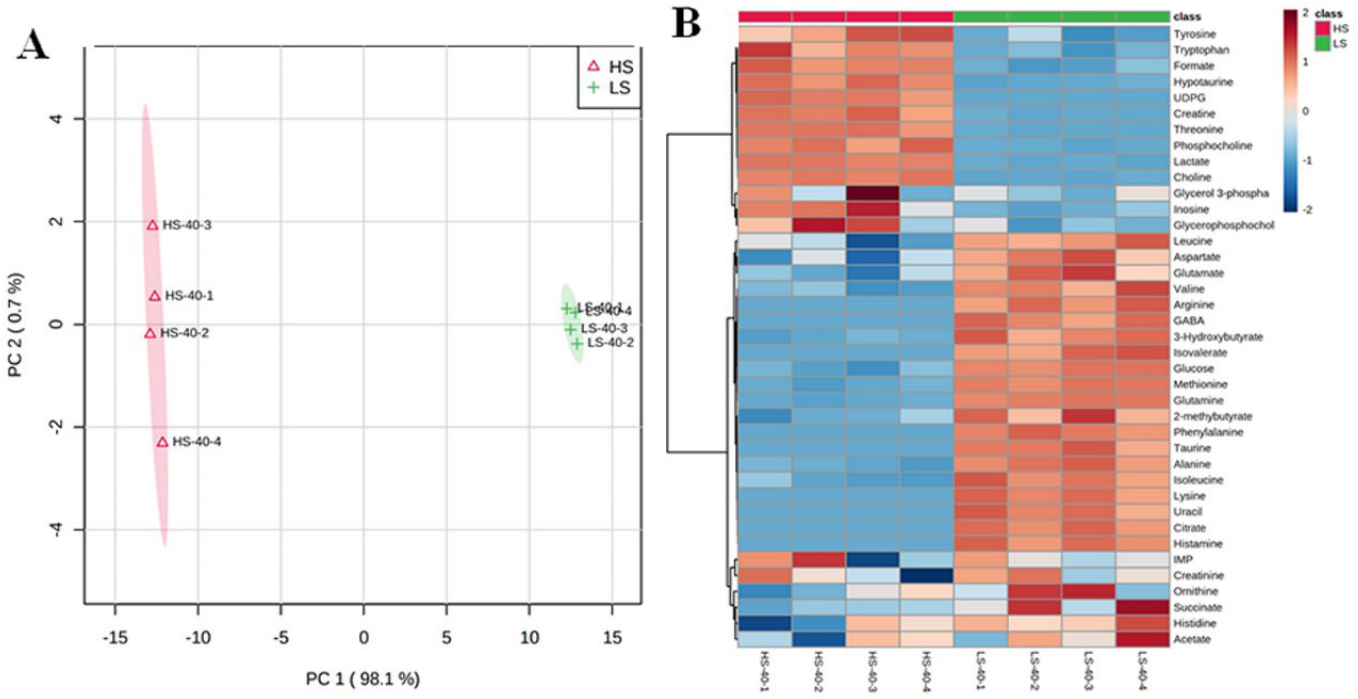

Figure 4. PCA comparison results and hierarchical clustering heatmap analysis of metabolites variations of groups. PCA score plot (A) and heatmap (B) of the two groups (HS-40 and LS-40).

3.6 Metabolic analysis of the stain DZ11 at different fermentation stages

Fermentation time is essential for producing fermented foods with optimal taste and functional properties. Multivariate statistical analysis was further performed for metabolic differences of samples inoculated with DZ11 at different fermentation stages. For the comparisons of the metabolite concentrations, the samples were subject to fold-changes (FC) analysis. As shown in Figure 5A, it can be observed that there were 9 metabolites that were significantly down-regulated in the sample after 40 days of fermentation (FC: 2.13-4.95, $P < 0.05$). On the other hand, 9 metabolites were significantly up-regulated as compared to those in the sample after 20 days of fermentation (FC: 1.34-5.1, $P < 0.05$).

Among these metabolites, the content of taste-active amino acids increased, of which bitter (valine, leucine, histidine and isoleucine), umami (glutamate and glycine) and sour amino acids (glutamate and histidine) were more abundant, indicating that the strain DZ11 initiated proteolysis and was responsible for modifying amino acid patterns at the early stage of fermentation (DZ11-20). The relative content of succinate and acetate increased in the early fermented sample and reduced in the late fermented sample. Succinate and acetate serve the sour taste. Succinate was also reported to impart a strong umami taste to seafood (Wang et al., 2019). Acetate might impart a strong and pungent aroma (Xiao et al., 2022). The two organic acids were derived from carbohydrate fermentation and converted to flavor compounds, such as esters, ketones, alcohols and aldehydes. Therefore, fermented fish paste for 20 days with DZ11 had more abundance of taste-active compounds, which was consistent with the sensory evaluation results.

Apart from the metabolites mentioned above, glycerol 3-phosphate also increased at the beginning of the fermentation and then decreased. Glycerol 3-phosphate might be produced as a result of lipid degradation by DZ11 lipases. Furthermore, the degradation of amino acids could produce higher glycerol-3-phosphate through pyruvate or oxaloacetate (Kryachko et al., 2020) in the early fermented sample (Figure 5D). After 40 days of fermentation, the significantly increased content of lysine suggested that a large amount of pyruvate was used as a precursor for lysine synthesis and the flux toward the TCA cycle, which resulted in the increase in citrate during fermentation (Gao et al., 2020b).

In addition, BCFAs, such as 2-methybutyrate, isovalerate, and 3-hydroxybutyrate, were distinctly increased. The significant increase in their content further verified that BCAA was effectively degraded into BCFAs by the strain DZ11 (Figure 5D). The production of BCFAs is meaningful because BCFAs contribute to important flavor precursors of fermented fish and bioactive activities.

Throughout the fermentation process, the strain DZ11 was metabolically active in producing more flavor precursor and taste compounds during the early fermentation period. As fermentation progressed, nutritional and bioactive compounds were formed. Therefore, the effect of strain DZ11 on low-salt fermented fish paste was greatly influenced by fermentation time. Increasing fermentation time is required to activate multiple metabolic pathways.

A

Metabolite class	Metabolite	Fold increase DZ40/DZ20	Fold decrease DZ20/DZ40
Amino acids	Glutamate		2.13
	Glycine		2.43
	Histidine		4.95
	Lysine	5.1	
	Leucine		2.18
	Glutamine	1.68	
	Valine		2.07
	GABA	1.54	
	Methionine	2.09	
	Taurine		2.05
Organic acids	3-Hydroxybutyrate	1.49	
	2-methybutyrate	1.34	
	Succinate		2.17
	Acetate		1.50
	Citrate	2.3	
Other	Glycerol 3-phosphorate		2.19
	Creatinine	2.54	
	Uracil	2.51	

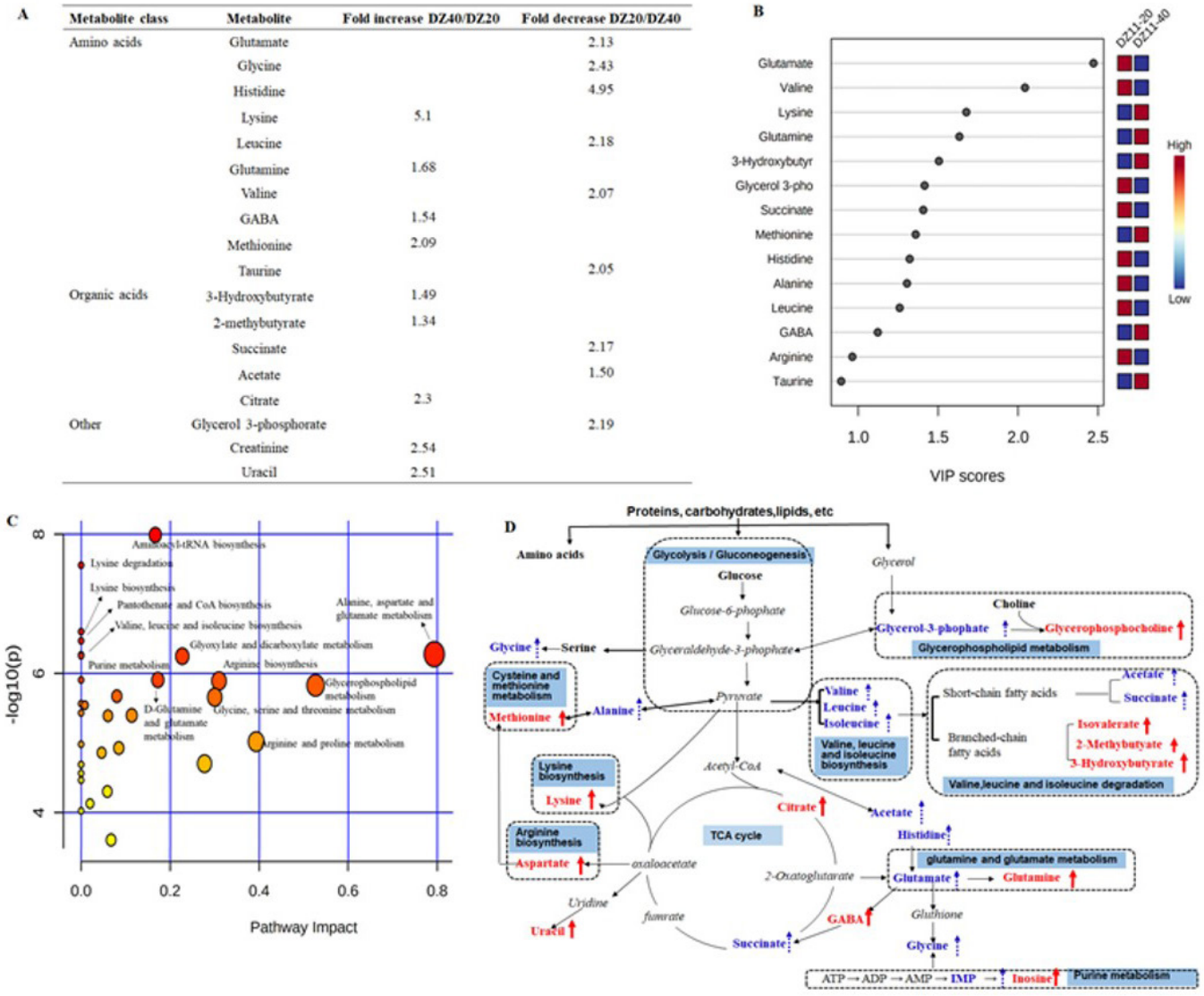

Figure 5. Fold change of metabolites (A); screened metabolites with VIP score > 1 (B); overview of the pathway analysis of DZ11 (C); integration map of altered metabolic pathways involved in DZ11 during the fermentation of low-salt fish paste (D). Note: The blue dotted arrows represent metabolites with higher relative content in the DZ11-20 group, and red arrows represent metabolites with higher relative content in the DZ11-40 group; metabolites in italic were not detected, while metabolites in bold black did not change significantly. Main strain-specific pathways were shadowed by blue.

3.7 Analysis of metabolic pathways related to the strain DZ11

The vital metabolites that contributed to the discrimination between the group DZ-20 and group DZ-40 were identified, and VIP scores were further calculated (Figure 5B). The results revealed that 12 metabolites (glutamate, valine, lysine, glutamine, 3-hydroxybutyrate, glycerol 3-phosphate, succinate, methionine, alanine, histidine, leucine, GABA) with VIP value > 1 were possible biomarkers for the characterization of the strain DZ11. Based on these selected candidates and the pathway database of the KEGG, pathway analysis was carried out to identify the related pathway that was caused by DZ11 treatment. In total, 38 pathways were predicted, and 11 pathways were regarded as significant metabolic pathways related to the strain DZ11 (Figure 5C). As shown in Figure 5C, the selected pathways were lysine degradation; lysine biosynthesis; pantothenate and CoA biosynthesis; alanine, aspartate and glutamate metabolism; valine, leucine and isoleucine degradation; valine, leucine and isoleucine biosynthesis; glyoxylate and dicarboxylate metabolism; D-Glutamine and D-glutamate metabolism; glycerophospholipid metabolism; purine metabolism; glycine, serine and threonine metabolism.

The assumptive metabolic pathways in DZ11 adapted from the KEGG database are shown in Figure 5D. Early in fermentation, energy sources, nucleosides and free amino acids were produced as a result of the degradation of nutrients, such as protein, carbohydrates and lipids in fish. During the fermentation of fish paste, DZ11 utilized these small metabolites and increased the metabolic flux of amino acids, forming succinate, acetate and other taste compounds. Amino acid metabolism was found to

be the most influenced pathway. Most amino acids appeared an increase, and the contents of glutamate, isoleucine, leucine, valine, isoleucine, histidine and glycine decreased at the late fermentation stage. Particularly, as the important precursors of flavor compounds, branched-chain amino acids (valine, leucine, and isoleucine) could potentially enrich the flavor of products. Via the branched-chain amino acid biosynthesis and degradation pathways, there was an increase in the BCFA content, which was essential for the peculiar odor precursors and nutrition of the fermented fish paste. Generally, GABA levels in natural organisms were known to be too low to extract and were mainly increased by microbial fermentation (Woraratphoka et al., 2022). GABA was produced from glutamate by glutamate decarboxylase activity of some microbial strains (Gao et al., 2020b; Jung et al., 2018; Xiao et al., 2022). The elevated level of GABA might explain the reduced level of glutamate observed in the sample inoculated with DZ11 after 40 days of fermentation.

4 Conclusion

In this study, *B. velezensis* DZ11 exhibited obvious proteolysis and lipolysis characteristics. Salt contents and application of starter cultures with DZ11 could affect the metabolic profile of fermented fish paste. The addition of DZ11 positively affected the flavor of fermented fish paste by increasing the metabolism of free amino acids and the synthesis of some flavor precursors, which was closely associated with 12 potential metabolic pathways. Therefore, the low-salt fermentation incubated with DZ11 was feasible for producing high content of flavor precursor and nutrient compounds of fermented fish paste. This study provided a new way to develop low-salt fermented fish foods with good flavor and nutrition.

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