LWT - Food Science and Technology 234 (2025) 118564

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Preservation mechanism of perilla in chilled oysters based on diversity analysis of protease-producing microbiota and proteolytic enzymes during spoilage

Jie Yang[a,b,1], Xiaolong Zhu[a,1], Wenrui Su[c,d], Chen Huang[c,d], Xinyi Liu[c,d], Chao Su[c,d], Obafemi Ibitayo Obajemihi[d], Guangchao Liu[e,*]

[a] *College of Food Science and Engineering, Qingdao Agricultural University, Qingdao, 266109, China*
[b] *Qingdao Special Food Research Institute, Qingdao, 266109, China*
[c] *Royal Agricultural University Joint Institute for Advanced Agritechnology at QAU, Qingdao Agricultural University, Qingdao, 266109, China*
[d] *Royal Agricultural University, Cirencester, GL7 6JS, UK*
[e] *College of Life Sciences, Qingdao Agricultural University, Qingdao, 266109, China*

ARTICLE INFO

Keywords:
Oyster spoilage
Protease-producing bacteria
Metalloproteases and serine proteases
Perilla leaf extract (PLE)
Natural preservative

ABSTRACT

This study investigated the physicochemical characteristics and bacterial spoilage of refrigerated oysters from five Chinese provinces, alongside evaluating perilla leaf extract (PLE) as a natural preservative. A total of 62 strains of protease-producing bacteria, predominantly *Pseudoalteromonas*, *Shewanella*, and regionally *Vibrio*, were identified across samples, with serine proteases and metalloproteases as the dominant secreted enzymes, accelerating oyster spoilage. Metagenomic analysis revealed that PLE treatment reduced bacterial diversity, shifting dominant genera from spoilage-associated *Pseudoalteromonas* and *Shewanella* to *Pseudomonas* and *Marinomonas*. PLE effectively inhibited protein degradation, maintaining higher protein content compared to the control, while lower amino acid nitrogen levels on the seventh day of refrigeration. Furthermore, PLE suppressed the growth of 53 isolated protease-producing strains at 24 h and inhibited their extracellular protease activity, particularly metalloproteases and serine proteases. These findings demonstrate that protease-producing bacteria and their enzymes critically contribute to oyster spoilage, while PLE serves as a sustainable preservative by mitigating microbial deterioration and protease-driven quality loss. The results highlight PLE's dual antimicrobial and protease-inhibiting properties, offering an eco-friendly strategy to enhance seafood shelf life and meet consumer demand for natural preservation solutions.

1. Introduction

Oyster is widely recognized as an excellent source of high-quality protein, and their demand has increased in certain regions of the world (Ulagesan et al., 2022). China's annual oyster production exceeds 6,199,540 tons, with Fujian, Shandong, Guangdong, Liaoning, Jiangsu being the main producing areas (China Fishery Statistics Yearbook, 2023). Marine aquatic products are rich in nutrients, particularly high-quality protein, making them an important source of protein in human diets (Raja et al., 2024). Due to their high protein and moisture content, they are prone to discoloration, off-flavor development, and spoilage during processing, transportation, storage, and sales (Chand, 2024; Kontominas et al., 2021). This not only reduces their nutritional and edible value but may also lead to the formation of harmful substances, posing risks to health (Q. Yu et al., 2023). Compared with other commonly used preservation methods, e.g., chemical preservation, modified atmosphere preservation, and irradiation preservation, low-temperature preservation with the addition of medicinal and edible homologous substances is more acceptable to consumers. Medicinal and edible homologous plants are highly favored for their nutritional and medicinal values, and are popular in the "new Chinese health preservation" trend (Lu et al., 2022; H. Yu et al., 2017). Purple perilla (*Perilla frutescens*) is one of the first 60 medicinal and edible homologous plants identified by the Chinese National Health Commission, with various

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[*] Corresponding author. College of Life Sciences, Qingdao Agricultural University, Qingdao, Shandong Province, 266109, China.
E-mail address: gcliu@qau.edu.cn (G. Liu).
[1] Authors equally contributing.

https://doi.org/10.1016/j.lwt.2025.118564
Received 8 July 2025; Received in revised form 16 September 2025; Accepted 25 September 2025

health benefits, including antioxidant, anti-allergic, anti-inflammatory, cholesterol-lowering, and potential cancer risk reduction effects (D. K. D. K. Kim, Kang, & Rhee, 2023; Lee & Shin, 2024; Takemoto et al., 2024). As a resource of natural antimicrobial agents or antimicrobials, its entire plant contains active ingredients such as phenolic acids, volatile oils, flavonoids, and coumarin lactones, which can inhibit foodborne bacteria and fungi (Zhou et al., 2020; Zhu et al., 2024). Studies have shown that it has preservative effects on fruits, vegetables, livestock, poultry, and seafood (Li et al., 2018; Wang et al., 2024). For example, adding perilla extract to fish fillets has been proven to effectively inhibit lipid oxidation and microbial growth during refrigeration, significantly delaying spoilage while improving flavor and maintaining quality (Zhu et al., 2024).

The bacterial diversity and community composition in oysters have been studied through traditional culture-dependent methods and non-culture-dependent methods based on 16S rRNA sequencing (J. J. Kim & Kim, 2023; Y. M. Y. M. Kim, Choi, Cho, Rhee, & Kim, 2024). Different species and tissues of oysters, as well as their aquatic environment and storage conditions, all affect the richness and diversity of microorganisms in oysters (Scanes et al., 2021). It has been found that *Pseudoalteromonas* and *Vibrio* are dominant in spoiled Pacific oysters from South Australia and Sydney rock oysters from New South Wales, Australia (Madigan et al., 2014). In spoiled Pacific oysters from the Yellow Sea in China, *Pseudoalteromonas* and *Shewanella* are more abundant, while high-pressure treatment makes *Psychrobacter* dominant (Cao et al., 2018). In addition to the whole meat extracted from the half shell, the bacterial diversity and potential pathogens in oyster gills are also often studied because oyster gills are a filtering tissue with high bacterial counts and hygienic risk (Dor-Roterman et al., 2024; Sun et al., 2023; Vilke et al., 2023). The spoilage bacteria in the gills of oyster (*M. plicatula*) from Fujian Province, China stored at different temperatures were detected. During ambient and refrigerated storage, *Leuconostoc pseudomesenteroides* and *Lactococcus* spp. were found to be the most common major spoilage bacteria in the gills, respectively (H. Chen, Liu, Wang, Chen, & Chen, 2013).

Seafood spoilage involves microbial degradation of proteins by endogenous protease, as well as the release of amino acids and further essential degrading metabolites such as biogenic amines, ammonia, and volatile organic compounds (Laly et al., 2021; Zhuang et al., 2022; Zhuang et al., 2021). The protease-producing bacteria and secreted proteases are the causes of seafood spoilage and quality degradation. The enrichment of collagenolytic proteases secreted by bacteria is considered a virulence factor in marine organisms, and plays an important role in determining decaying jellyfish blooms. *Pseudoalteromonadaceae* dominates in the jellyfish bacterial community rich in protein/amino acid metabolism-related enzymes, producing extracellular enzymes related to proteolysis (Tinta et al., 2023). *Shewanella putrefaciens* exhibit high proteolytic activity towards fish protein and high amino acid deamination activity, cooperating with *Pseudomonas putida* to produce high levels of ammonia in grass carp meat (Zhuang et al., 2023). For refrigerated seafood, *Pseudomonas fluorescens*, as a specific spoilage microorganism, produces thermostable proteases that retain proteolytic activity after high-temperature treatment and maintain proteolytic activity during refrigerated storage (D. Wang et al., 2022; Zhang et al., 2021).

Previous studies have recognized the role of microorganisms in deterioration of seafood quality and examined the spoilage microbiota in different types of seafoods (Anagnostopoulos et al., 2022; Bai et al., 2025; Huang et al., 2022; Ren et al., 2025). Other studies have reported the significant role of protease-producing microorganism in seafood spoilage by inoculating them individually into seafood models (Tinta et al., 2023; Zhuang et al., 2023; D. Wang et al., 2022; Zhang et al., 2021). However, the analysis of the diversity of protease-producing bacterial microbiota was absent in these studies. Meanwhile, despite the demonstrated potential of these medicinal and edible homologous substances in addressing multiple challenges in the seafood industry through their antimicrobial, deodorizing, and anti-gout properties, systematic research on their inhibitory effects against protease-producing microorganisms and the secreted proteases in marine aquatic products remains limited. Therefore, a systematic evaluation of the diversity of protease-producing bacteria and their secreted proteases is essential not only to establish the relationship between protein degradation and seafood spoilage, but also to investigate the effects of purple perilla on these microorganisms and their proteolytic activities, which is crucial for elucidating the inhibitory mechanisms of medicinal and edible homologous substances on oyster spoilage and achieving precise preservation.

The purpose of this study included 1) to explore the characteristics of Pacific, Kumamoto, Fujian, and Hongkong oysters from five provinces of China in terms of bacterial spoilage during refrigerated storage; 2) to analyze the diversity of protease-producing bacteria obtained from the oyster samples and secreted proteases and their impact on oyster spoilage; 3) to evaluate the antimicrobial function of perilla extract and elucidate its key role and mechanism in inhibiting the spoilage of refrigerated oysters. This study holds significant scientific and practical value in understanding the microbial and enzymatic mechanisms driving the spoilage of marine aquatic products and promoting the comprehensive application of medicinal and edible homologous substances.

2. Materials and methods

2.1. Oyster samples

Pacific oysters (*M. gigas*) were harvested from the aquatic product market in Shandong Rushan, and Liaoning Dalian of China, respectively, Kumamoto oysters (*M. sikamea*), Fujian oysters (*M. angulata*), Hongkong oysters (*M. hongkongensis*) from commercial farms in Jiangsu Lianyungang, Fujian Zhangzhou, and Guangdong Zhanjiang, China, respectively. The oyster samples were purchased respectively in November and December 2023 and transported to the laboratory within 48 h after harvesting using blue ice packs. The oyster shell was cleaned with seawater pre-filtered through a 0.22 μm polypropylene microporous membrane, then placed on a sterile workbench for shell removal and meat extraction. The shelled oyster meat was washed again with filtered seawater, placed in sealed plastic bags and stored at 4 °C for subsequent experimental use.

2.2. Total colony count

The total colony count (TCC) was detected according to National Food Safety Standard GB 4789.2–2016. Firstly, 5 g of shelled oyster meat was homogenized with 45 mL physiological saline. Subsequently, the homogenized oyster sample was diluted serially 10 times with physiological saline. TCC was measured by pipetting and spreading 0.2 mL of each diluted sample onto a plate count agar (Hope Bio, China) and incubated at 28 °C for 3 days.

2.3. DNA extraction, metagenomic sequencing and analysis

Total genomic DNA was extracted from oyster samples using the EZNA® Soil DNA Kit (Omega Bio-tek, USA). Paired-end sequencing was performed on the Illumina Novaseq 6000 platform (Beijing Tsingke, China). Representative sequences for the non-redundant gene catalog were annotated based on the NCBI NR database, using blastp implemented in DIAMOND v0.9.19, with an e-value cutoff of $1e^{-5}$ for taxonomic annotations.

2.4. Screening of protease-producing bacteria

The screening plate consists of 5 g/L skimmed milk powder, 2 g/L yeast extract, 30 g/L synthetic sea salt, and 15 g/L agar. According to the procedure reported by Liu et al. (Liu et al., 2023), with slight modifications, the isolation of culturable protease-producing bacteria was carried out. In brief, 0.2 mL of the diluted oyster sample was spread onto the screening solid medium and incubated at 25 °C for 72 h. Colonies with visible hydrolysis zones were selected and sub-cultured at least three times to obtain purified strain.

2.5. 16S rRNA gene amplification and phylogeny

The bacterial 16S rRNA gene was amplified by colony PCR using universal primers 27F and 1492R according to the procedure reported by Yang et al. (Yang et al., 2023). Then the PCR products were sequenced by Tsingke Biotechnology Co., Ltd. (Qingdao, China). Isolates with more than two different bases in the 16S rRNA nucleotide sequence were considered as different strain. The phylogenetic tree was constructed using MEGA version 11, by the neighbor-joining method and the bootstrap model. The bacterial 16S rRNA sequences of the isolated strains have been uploaded to GenBank, and the accession numbers are presented in Supplementary Table S1.

2.6. Inhibition test on the proteases secreted by the isolated strains

The inhibitors used for protease inhibitor assay are 1.0 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1.0 mmol/L 1,10-phenanthroline (OP), 0.1 mmol/L pepstatin A (PA), and 0.1 mmol/L N-[N-(L-3-trans-carboxyirane-2-carbonyl)-L-leucyl]-agmatine (E64). The isolated culturable bacteria were cultured in liquid fermentation medium consisting of 5 g/L gelatin, 3 g/L casein, 2 g/L yeast extract, and 30 g/L synthetic sea salt, at 25 °C and 180 rpm for 4 days. The supernatant was collected by centrifuging at 12,000 rpm for 10 min at 4 °C, and then diluted with 50 mmol/L Tris-HCl (pH 8.0). The appropriately diluted supernatant was incubated with each inhibitor at 4 °C for 1 h, and then the residue proteolytic activity was detected by casein digestion according to a previously reported procedure (Yang et al., 2023). One unit of proteolytic activity is defined as the amount of enzyme that release 1 μg of tyrosine per minute per milliliter of reaction mixture. The inhibition ratio (%) is represented by the difference between the relative residue proteolytic activity of each sample with and without inhibitor.

2.7. Detection of protease-producing bacteria and their extracellular proteases' impact on oyster spoilage

Based on the procedure reported by Cui et al. (Cui et al., 2023) with slight modifications, the impact of bacteria on oyster spoilage was investigated. Six protease-producing bacterial strains belonging to genera *Pseudoalteromonas*, *Vibrio*, and *Shewanella* were selected. These bacteria were individually inoculated into fermentation medium and incubated at 28 °C for 48 h. By centrifuging at 10,000 rpm for 10 min at 4 °C, bacterial cells and crude enzyme solutions in the supernatant were obtained, respectively. The bacterial cells were washed twice with sterile saline and then resuspended in saline to obtain a bacterial suspension with a concentration of 10^6 CFU/mL. The treated oysters were soaked in 50 mL of the bacterial suspensions and stored in a sterile homogeneous bag at 4 °C, while the oysters soaked in saline as a blank control group. Texture characteristic testing on the oysters were performed on different dates. The crude enzyme solution in the supernatant and oyster homogenate were mixed at a volume ratio of 10:1, and incubated in a water bath shaker at 25 °C for 2 h. By centrifuging the mixture at 10,000 rpm for 30 min at 4 °C, the supernatant and precipitate was obtained, respectively. Then, the free amino acids in the supernatant were detected using the Folin phenol method (Yang et al., 2023), and the weight of the precipitate was measured.

2.8. Extraction of purple perilla and treatment of oysters

The leaves of purple perilla were crushed into powder using a high-speed grinder (DFT-100, Dade, China) with a motor power of 400W and a motor speed of 25000 r/min, followed by screening with a 100-mesh sieve. Ultrasonic-assisted extraction technology was employed for the extraction of perilla powder, utilizing an ultrasonic cleaner with liquid-to-material ratio of 20:1, ultrasonic power of 150 W, temperature of 80 °C, and extraction time of 60 min. After centrifugation at 8000 rpm for 10 min, the supernatant was collected and dried in a forced-air oven at 55 °C for 48 h, thereby preparing perilla extracts.

Perilla leaves extract (PLE) was dissolved with water to prepare an aqueous solution with a concentration of 50 mg/mL and filtered through a 0.22 μm polyethersulfone membrane syringe filter unit (Millex-GP, Merck, Germany). The oyster meat was soaked in 100 mL of PLE aqueous solution and stored at 4 °C. Oysters soaked in physiological saline served as the control group.

2.9. Detection of the impact of PLE on protease-producing bacteria and extracellular proteases

Based on the method described by Wang et al. (Wang et al., 2023) with slight modifications, we investigated the effect of PLE on oyster bacteria. Using an automatic microbial growth curve analyzer (Micro-Screen-HT, Jieling, China), we detected the inhibitory effect of 50 mg/mL of PLE on the growth of all these bacterial strains at a culture temperature of 37 °C, a rotation speed of 300 r/min, and a wavelength of 600 nm.

We selected six protease-producing bacterial strains belonging to genera *Pseudoalteromonas*, *Vibrio*, and *Shewanella*. These bacteria were inoculated into fermentation medium and incubated at 28 °C for 48 h. The crude enzyme in the supernatant was obtained by centrifuging at 10,000 rpm for 10 min at 4 °C. The appropriately diluted supernatant was incubated with 50 mg/mL of PLE at 4 °C for 1 h, then the residual proteolytic capability was detected by casein degradation according to a previously reported method (Yang et al., 2023).

2.10. Statistical analysis

The experiment was repeated in triplicate, and the values were presented in the form of mean ± SD. IBM SPSS Statistics version 27.0 (IBM Corporation, USA) was used to perform significance testing on the differences between groups. The significance level was set at a p-value <0.05.

3. Results and discussion

3.1. Analysis of bacterial taxonomic composition of oysters from China

Texture, color, and pH were key indicators in assessing the freshness of oyster. All these parameters of Pacific oysters (*M. gigas*) from Shandong Province and Liaoning Province, Kumamoto oyster (*M. sikamea*) from Jiangsu Province, Fujian oyster (*M. angulata*) from Fujian Province, Hongkong oyster (*M. hongkongensis*) from Guangdong Province after 7 days in refrigeration were presented in Supplementary Fig. S1 and Supplementary Table S2. Hardness and springiness decreased significantly (up to 44.66 % and 23.96 %, respectively). Color shifts ($\Delta E > 5$, notably in Shandong/Liaoning) and pH fluctuations further reflected spoilage trends. The average bacterial count of all fresh samples from five provinces was approximately 5.5 $\log_{10}$ CFU/g oysters. After 7 days of refrigerated storage, the bacterial counts of the oysters from Shandong, Jiangsu, Liaoning, Fujian, and Guangdong significantly increased, reaching 8.23 ± 0.04, 9.38 ± 0.06, 7.11 ± 0.05, 7.67 ± 0.01, 8.29 ± 0.07 $\log_{10}$ CFU/g, respectively (Fig. 1A), all of which were higher than the acceptable limit of 7 $\log_{10}$ CFU/g. Similar observations were made in the gills of the refrigerated oysters from British Columbia, New Brunswick, and Prince Edward Island, where the plate counts increased from an initial approximately 4.0 $\log_{10}$ CFU/g to 7.8, 7.9, and 8.8 $\log_{10}$ CFU/g oyster on day 8, respectively (Chen et al., 2019).

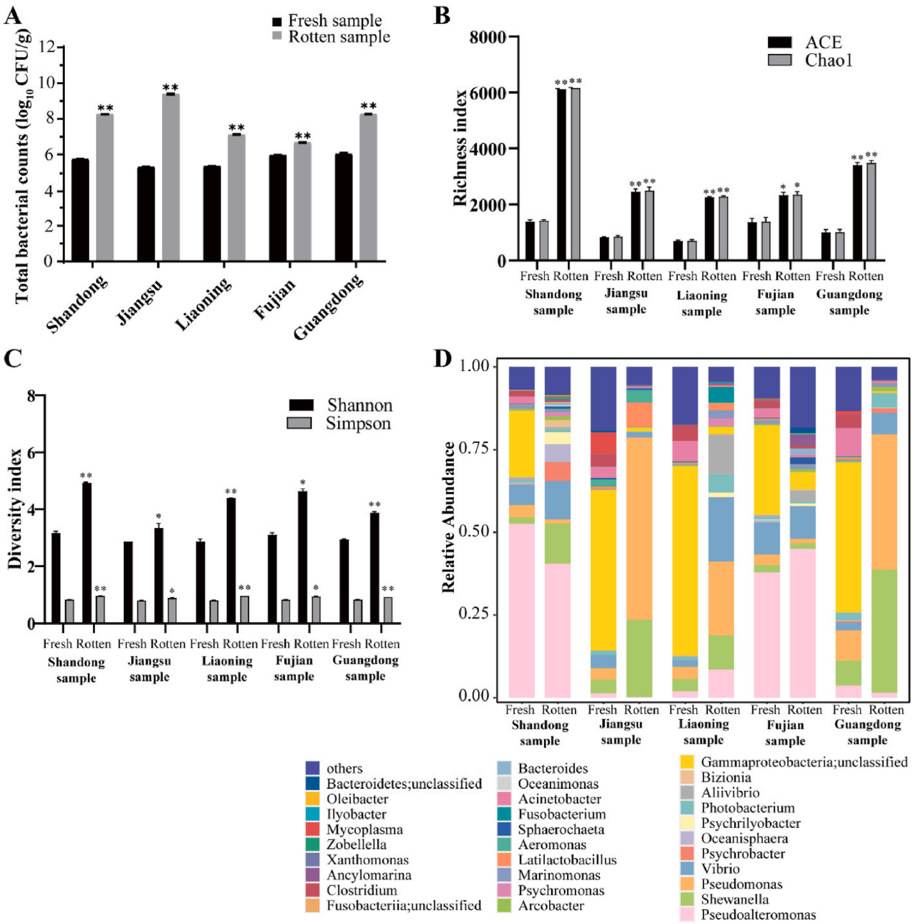

Fig. 1. Alpha diversity estimators and composition of bacterial communities of oysters from China at the fresh and rotten stages. (A) Average bacterial count, (B) richness index, (C) diversity index, and (D) composition at the genus level of bacterial communities of oyster samples. Oyster samples were collected from five provinces and were in the fresh and rotten stages, respectively. The black asterisks (*) and double asterisks (**) indicate significant differences between fresh oyster samples and their corresponding rotten samples, with p-values of $p < 0.05$ and $p < 0.01$, respectively.

Through metagenomic sequencing and taxonomic annotation, we conducted a study on the microbial community in oyster samples. The sequencing coverage exceeded 99.50 %, and the α diversity indices are detailed in Supplementary Table S3. The values of OTUs, Ace, and Chao express the richness of microbial community. The values of Shannon and Simpson (1-D) reveal the diversity of microbial community. The richness and diversity indices of all rotten oyster samples from Shandong, Jiangsu, Liaoning, Fujian, and Guangdong were higher than those of fresh samples (Fig. 1B and C), indicating that the richness and diversity of the microbial community in rotten oysters were significantly higher than those in fresh samples.

In the oyster samples, a total of 90 phyla, 141 classes, 244 orders, 499 families, 1566 genera, and 6205 species were identified. Fig. 1D and Supplementary Fig. S2 shows the top 30 genera in fresh and rotten oyster samples from five provinces of China. For fresh oyster samples, the genera *Pseudoalteromonas* (52.55 %), unclassified Gammaproteobacteria (20.00 %), and *Vibrio* (6.09 %) were definitively dominant in Shandong samples, accounting for 78.65 % of the total; the main genera were unclassified Gammaproteobacteria (48.47 %) and *Mycoplasma* (6.56 %) in Jiangsu samples; unclassified Gammaproteobacteria (57.35 %), *Thiothrix* (6.74 %), and *Acinetobacter* (6.21 %) were dominant in Liaoning samples; *Pseudoalteromonas* (37.86 %), unclassified *Gammaproteobacteria* (27.07 %), and *Vibrio* (9.72 %) were dominant in Fujian samples; unclassified Gammaproteobacteria (45.41 %), *Pseudomonas* (9.17 %), *Acinetobacter* (8.48 %), and *Shewanella* (7.63 %) were dominant in Guangdong samples. Oysters typically inhabit the nearshore intertidal zone and exhibit a filter-feeding nature. It has been reported that oyster can filter approximately 5–25 L of seawater per hour, during which it accumulates various substances from the environment (Li et al., 2024). Natural water bodies inherently contain a wide range of organic and inorganic compounds, complexes, suspended solids, and sediments (Chen et al., 2023). Human activities such as port transportation, discharge of urban domestic wastewater, and the use of antibiotics in aquaculture can also impact the water quality in which oysters live (Canty et al., 2020; Diner et al., 2023). The organic factors including total organic nitrogen (TON) and total organic carbon (TOC), the inorganic factors encompassing various heavy metals, the physical factors involving temperature, salinity, and pH, vary across different regional water bodies (Liu et al., 2024; Wang et al., 2021). Differences in organic, inorganic, and physical factors across regional water bodies lead to distinct microbial compositions in oysters from different origins. Similarly, research has found that the main bacteria in fresh Pacific oysters collected from an aquaculture farm in the Yellow Sea of China belong to the genera *Vibrio*, *Shewanella*, and *Pseudomonas*, with a total abundance of 45.8 % (Cao et al., 2018). In this study, these three genera were dominant in the fresh oyster samples from five provinces of China.

For the rotten samples, the main genera include *Pseudoalteromonas* (40.60 %), *Shewanella* (12.01 %), *Vibrio* (11.57 %), *Psychrobacter* (5.75 %) and *Oceanisphaera* (5.31 %) in Shandong samples; *Pseudomonas* (55.07 %), *Shewanella* (23.40 %) and *Latilactobacillus* (7.32 %) in Jiangsu samples; *Pseudomonas* (22.32 %), *Vibrio* (19.47 %), *Aliivibrio* (12.06 %), *Shewanella* (10.30 %), *Pseudoalteromonas* (8.51 %) and *Photobacterium* (5.51 %) in Liaoning samples; *Pseudoalteromonas* (45.02 %), *Vibrio* (9.71 %) and unclassified Gammaproteobacteria (5.35 %) in

Fujian samples; *Pseudomonas* (40.82 %), *Shewanella* (37.26 %) and *Vibrio* (6.48 %) in Guangdong samples. The genera *Pseudoalteromonas* and *Vibrio* have been identified as important genera in the spoilage process of Pacific oysters and Sydney rock oysters, indicating that there are common spoilage characteristics among oyster species (Bott et al., 2014).

3.2. Diversity of protease-producing bacteria of oysters from China

Proteases and protease-producing bacteria play crucial roles in nitrogen cycle of aquaculture systems (Wei et al., 2021). The extracellular proteases have been reported as one of the spoilage-related factors of seafoods, including salmon, red sea bream filets, sturgeon fillets, and crabs (Jahid et al., 2018; Myszka et al., 2024; Tan, Li, & Shang, 2023; D. Wang et al., 2022). To further understand the microbe-associated spoilage of oysters, we detected the protease-producing microorganisms in rotten oyster samples by using culture-dependent methods. In general, a large quantity of colonies with transparent zones formed on the protease-screening plates of the 10^{-1}-10^{-4} fresh oyster and the 10^{-1}-10^{-6} rotten oyster diluted samples after cultivation. The abundance of protease-producing bacteria in all fresh groups from five provinces was approximately 3.5 $\log_{10}$ CFU/g oysters. For rotten groups, the count of protease-producing bacteria of oysters from Shandong reached 8.25 ± 0.02 $\log_{10}$ CFU/g oysters, while those from Jiangsu,

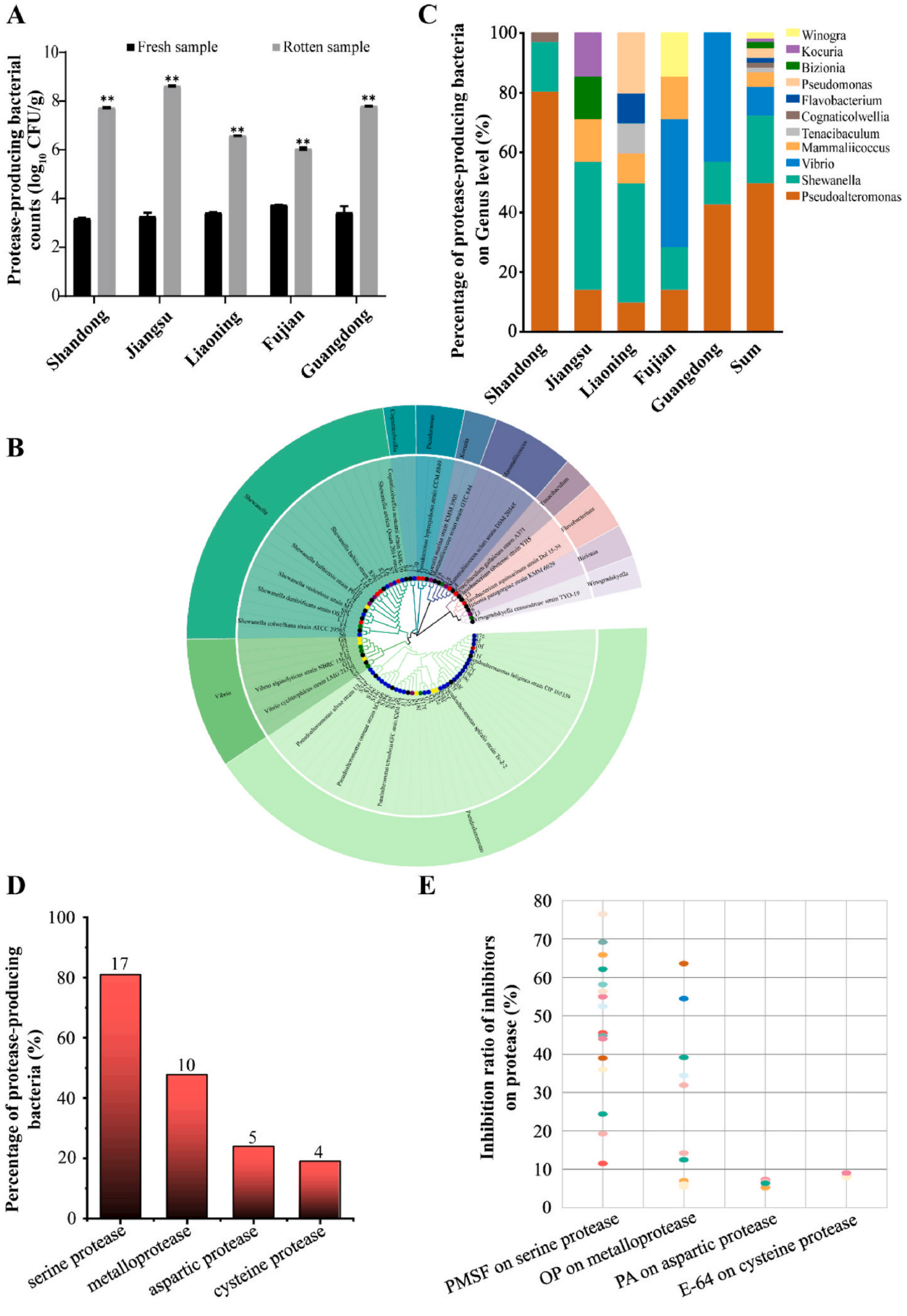

Fig. 2. Diversity analysis of culturable protease-producing bacteria isolated from rotten oyster samples and their secreted proteases. (A) Bacterial count, (B) phylogenetic relationships, (C) Genus-level diversity and distribution of protease-producing bacteria in oysters. Black ** indicates significant differences between fresh oyster samples and the corresponding rotten sample, $p < 0.0l$. (D) Percentage of serine protease, metalloprotease, aspartic protease, and cysteine protease in secreted proteases. The numbers on the bar graph indicate the quantity of each type of proteases secreted by bacterial strains. (E) The inhibition ratio of PMSF, OP, PA, and E64 on the secreted proteases. Different colored dots represent protease secreted by different bacterial strains.

Liaoning, Fujian, and Guangdong were 9.38 ± 0.04, 7.11 ± 0.03, 6.67 ± 0.02, and 8.26 ± 0.03 $\log_{10}$ CFU/g oysters, respectively, all of which were significantly higher than their corresponding fresh groups (Fig. 2A). These results suggest that protease-producing bacteria inhabiting oysters may play a key role in the spoilage process of oysters. It has been reported that extracellular proteases secreted by spoilage microorganisms degrade proteins during refrigeration, thereby disrupting the texture of seafood such as golden pomfret (X. Lou, Wen, et al., 2023; Lou, Hai, et al., 2023; X. W. Lou, Zhai, & Yang, 2021).

The culturable protease-producing bacterial strains were purified from rotten oyster samples, and their 16S rRNA genes were then amplified and sequenced. Ultimately, a total of 62 strains were identified, 31 strains from Shandong oyster, 7 from Jiangsu oysters, 10 from Liaoning oysters, 7 from Guangdong oysters, 7 from Fujian oysters (Supplementary Table S1). Fig. 2B shows the phylogenetic relationships of the protease-producing bacterial strains belonging to different genera. These 62 strains were classified into 11 genera (Fig. 2C). On the genus level of culturable protease-producing strains, strains belonging to *Pseudoalteromonas* and *Shewanella* dominated in all studied oyster samples, with total abundances of 96.77 %, 57.14 %, 50.00 %, 28.57 %, and 57.14 % in the rotten oysters from Shandong, Jiangsu, Liaoning, Fujian, and Guangdong, respectively. In addition, *Vibrio* was the dominant genus in oyster from Fujian and Guangdong. Strains belonging to genera such as *Mammaliieoccus*, *Tenacibaculum*, *Cognaticolwellia*, *Flavobacterium*, *Pseudomonas*, *Bizionia*, *Kocuria*, and *Winogra* were also identified as protease-producing bacteria in Chinese oysters.

3.3. Diversity analysis of extracellular proteases of bacteria isolated from oysters

Further inhibition test was conducted on the extracellular substances of bacterial strains derived from oysters to identify types of the extracellular proteases. The total 62 strains were respectively cultured in fermentation broth medium, and 21 strains belonging to the genera *Pseudoalteromonas*, *Cognaticolwellia*, *Shewanella*, *Mammaliicoccus*, *Flavobacterium*, *Pseudomonas*, and *Vibrio* produced sufficient extracellular proteases for subsequent detection. The effects of serine protease inhibitor (PMSF), metalloprotease inhibitor (OP), aspartic protease inhibitor (PA), and cysteine protease inhibitor (E64) on extracellular proteolytic activity were detected. The results are shown in Fig. 2D and. E and Supplementary Table S4. The extracellular proteolytic activity in 17 strains were inhibited by PMSF, with inhibition rates ranging from 11.54 % to 76.45 %, indicating that most of the oyster strains secrete serine proteases. Specifically, an inhibition ratio of up to 76.45 % was found in the *Vibrio* strain G8, implying that strain G8 mainly secretes serine protease (Supplementary Table S4). OP inhibited the proteolytic activity in 10 strains, indicating that most isolated strains secrete metalloproteases. Conversely, PA and E64 inhibited proteolytic activities in 5 and 4 strains, with inhibition rates below 10 %, suggesting that only a few strains secrete aspartic and cysteine proteases, and the secretion levels are relatively low.

3.4. Effects of protease-producing bacteria and their extracellular proteases on oyster spoilage

To investigate the impact of bacteria on oyster spoilage, we immersed oysters in bacterial suspensions belonging to genera *Pseudoalteromonas* (strains G3 and S3), *Vibrio* (strains G8 and F7), *Shewanella* (strains S7 and L6). The changes in texture properties of the oysters are presented in Supplementary Figure S3. As the refrigeration time increased, the textural parameters primarily represented by hardness and springiness exhibited a downward trend. As the main texture parameter, hardness was significantly lower than that of the control group on Day 8 (Fig. 3A). Additionally, protease secreted by these bacterial strains were collected and used to examine their effects on the oysters. After a 2-h treatment, the weight of the oysters significantly decreased (Fig. 3B), and the oysters released free amino acids (Fig. 3C). These results indicate that protease-producing bacteria and their extracellular proteases accelerate the corruption of oysters. It has been reported that *Pseudoalteromonas* and *Vibrio* are the main bacterial communities during the decomposition of jellyfish organic matter, while the proteins of jellyfish are mainly hydrolyzed by *Pseudoalteromonas*. Proteases is the main extracellular enzyme of *Pseudoalteromonas*, including microbial collagenase, serine protease, and prolyl oligopeptidase (Tinta et al., 2023). During the deterioration of grass carp meat, the synergistic action of *Shewanella putrefaciens* with *Pseudomonas putida* released more putrescine (Zhuang et al., 2023).

3.5. Effects of PLE on oyster spoilage: texture, oyster protein, and bacterial count

Perilla, as a medicinal and edible homologous herb, possesses a wide range of biological activities. In addition to its therapeutic applications for cough, depression, allergies, and other ailments (Wu et al., 2023), its antibacterial function has also been widely reported. The leaves were identified as the optimal part of purple perilla, surpassing stem and seed extracts, characterized by the highest concentrations of flavonoids and polyphenols, as well as significant antioxidant activity and effective antimicrobial activity against both G+ and G-bacterial strains (Supplementary Table S5, Supplementary Figure S4). The dry powder of PLE appears as a yellowish-brown, fine particulate solid (Supplementary Figure S5A). PLE powder is completely soluble in water, yielding a

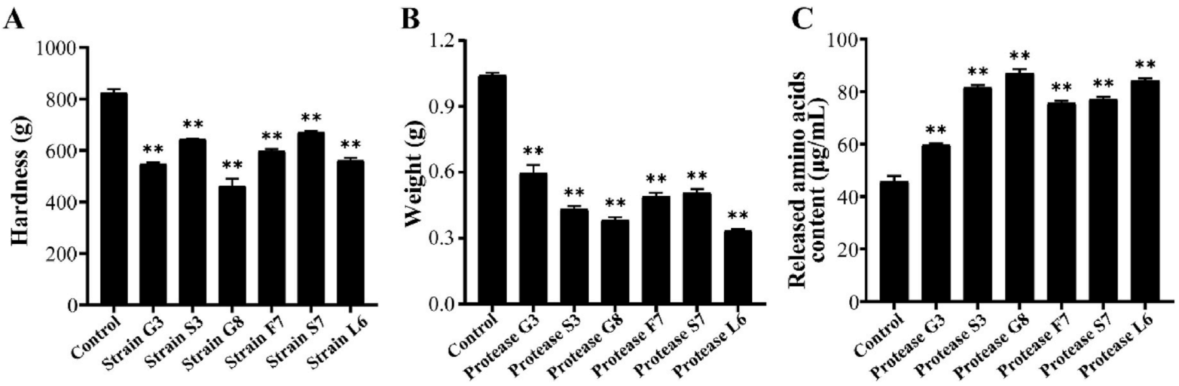

Fig. 3. The impact of protease-producing bacteria and their extracellular proteases on oyster spoilage. (A) Hardness of oysters socked in bacterial suspensions on Day 8. (B) Weight and (C) released amino acid content of oyster homogenate incubated in bacterial supernatant. Oysters or oyster homogenates soaked in physiological saline served as controls. The bacterial strains tested were *Pseudoalteromonas tetraodonis* G3, *Pseudoalteromonas tunicata* S3, *Vibrio alginolyticus* G8, *Vibrio atlanticus* F7, *Shewanella septentrionalis* S7, and *Shewanella septentrionalis* L6.

transparent solution retaining the unique flavor of perilla. The solutions show a clear concentration-dependent color gradient, which was light yellow at 20 mg/mL and light brown at 50 mg/mL (Supplementary Figure S5B). When oysters were treated with PLE solutions and subsequently stored, we observed that the distinctive aroma of perilla was present but mild. It did not overwhelm the natural fresh and marine notes of the oyster meat. Instead, it imparted a subtle, herbaceous nuance that we subjectively found to be complementary rather than disruptive. The impacts of PLE on the spoilage of oysters, including texture, protein, and bacterial count in oyster samples are represented in Fig. 4. The results suggest that PLE can significantly extend the shelf life of refrigerated oysters.

Hardness and elasticity are the most crucial texture parameters for refrigerated oysters, and they are also key criteria for consumers to evaluate food quality (Cheng et al., 2014). The texture properties of oysters, including hardness and elasticity, were measured, and the results under different treatments are presented in Supplementary Fig. S6A and B. For PLE-treated oysters, the hardness and elasticity values were 1088.70 g and 0.52, respectively, on Day 7; 805.08 g and 0.48 on Day 10; and 653.38 g and 0.42 on Day 13. Although both hardness and elasticity in the PLE group decreased with prolonged refrigeration time, they remained significantly higher compared to the corresponding control samples. These findings indicate that PLE effectively slowed the deterioration of texture properties in oysters during refrigeration. Hardness and elasticity are attributed to myofibrillar proteins and sarcoplasmic proteins (Feng et al., 2017; Paker & Matak, 2015). The loss of oyster hardness and elasticity is mainly due to the activity of autolytic enzymes and spoilage microorganisms that damage proteins (Lou, Hai, et al., 2023; X. Lou, Wen, et al., 2023).

Oysters are well-known sources of health-beneficial proteins (Venugopal & Gopakumar, 2017). However, due to their high protein content, numerous microorganisms, and active endogenous enzymes, oysters are prone to spoilage (Venugopal & Gopakumar, 2017). Supplementary Fig. S6C shows the measurement results of protein content in oysters during refrigeration at 4 °C. On the first day, there were no significant differences in protein content among the groups, ranging from 8.76–8.78 %. On the fourth and seventh days of refrigeration, the protein content in the PLE-treated group was 7.57 % and 6.17 %, respectively, which was significantly higher than that in control group (5.34 % and 4.45 %). Protein degradation in oysters directly leads to the breakdown of food texture, the production of undesirable flavors, and ultimately the formation of endogenous toxic and harmful substances (X. Liu et al., 2018). The significantly higher protein content observed in the PLE treatment group, compared to the control group, demonstrates that the addition of PLE effectively inhibits protein degradation in oysters, thereby positively influencing their quality and extending their shelf life.

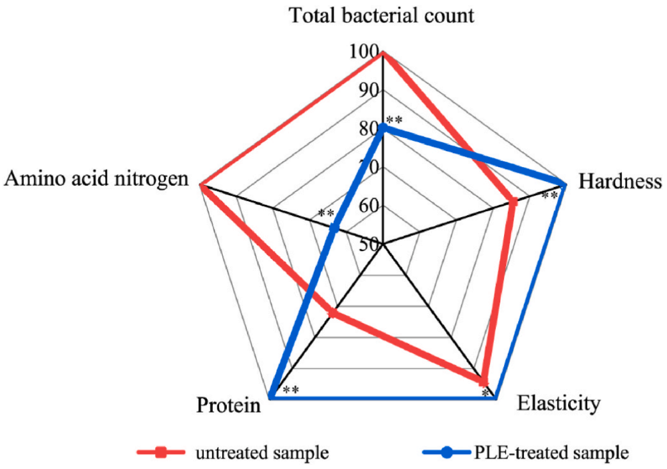

Fig. 4. The impact of PLE on the spoilage of oysters, average bacterial count, hardness, elasticity, protein, and amino acid nitrogen in oyster samples. Oysters soaked in physiological saline served as control group. Each graph is repeated at least three repeats. Black *, ** indicate significant differences between PLE-treated sample and their corresponding untreated sample at the same time point, with $p < 0.05$ and $p < 0.01$, respectively.

Amino acid nitrogen (AAN) is widely used as an indicator to assess the degree of fermentation in fermented products (Ruan et al., 2022; Zhao et al., 2013). A higher AAN value indicates a greater content of amino acids in the food, reflecting the extent of protein hydrolysis. As depicted in Supplementary Figure S6D, the AAN content of fresh oyster ranges from 0.223 ‰ to 0.226 ‰, with no significant differences observed among various groups. The AAN content in the perilla group on Days 4 and 7 was 0.231 ‰ and 0.234 ‰, respectively, which was notably lower than in control group (0.309 ‰ and 0.37 ‰). These results further demonstrate that PLE treatment significantly reduces the degradation of proteins in oysters.

The average bacterial count of all fresh samples was approximately 3.7 $\log_{10}$ CFU/g oysters. During refrigerated storage, the bacterial count of the untreated oysters significantly increased, reaching 5.45 $\log_{10}$ CFU/g after 4 days and 8.46 $\log_{10}$ CFU/g after 7 days, exceeding the acceptable limit of 7 $\log_{10}$ CFU/g (Supplementary Figure S6E). However, the bacterial count of oysters treated with PLE was significantly lower, reaching 4.16 $\log_{10}$ CFU/g after 4 days and 6.17 $\log_{10}$ CFU/g after 7 days. The results indicated that PLE can effectively inhibit the growth of microorganisms in oysters, delay spoilage, and extend the shelf life.

3.6. *Effect of PLE on bacterial taxonomic composition of oyster*

Through metagenomic sequencing combined with taxonomic annotation, we conducted a comprehensive analysis of the microbial community in oyster samples treated with or without PLE. The coverage rate of metagenomic sequencing exceeded 99.50 %. The systematically evaluated richness and diversity of bacterial community of oyster samples are presented in Supplementary Table S6. The richness and diversity indices of oyster samples treated with PLE were significantly lower than those of untreated samples (Fig. 5A), indicating that PLE treatment significantly reduced microbial richness and diversity of rotten oysters.

Microbial analysis of oyster samples revealed significant differences in community composition between untreated and PLE-treated groups. At the genus level, high-throughput sequencing identified 1488 genera in untreated oyster samples, compared to 550 genera in PLE-treated samples. The top 30 genera are presented in Fig. 5B and Supplementary Fig. S7. The microbial community in untreated oysters was dominated by *Pseudoalteromonas* (40.60 %), *Shewanella* (12.01 %), and *Vibrio* (11.57 %). In contrast, PLE treatment substantially altered the microbial profile, with *Pseudomonas* (39.52 %), *Vibrio* (12.58 %), and *Marinomonas* (10.29 %) emerging as the dominant genera. Notably, the relative abundance of spoilage-related *Pseudoalteromonas* and *Shewanella* decreased significantly to 9.90 % and 2.83 %, respectively, indicating a substantial shift in microbial community structure following PLE treatment. The results suggested the potential of PLE treatment in controlling spoilage microorganisms and modifying the microbial ecosystem in oyster samples.

3.7. *Effect of PLE on oyster protease-producing bacteria and extracellular proteolytic activity*

The effects of PLE on the growth of oyster-isolated protease-producing bacteria, which belongs to the genera *Pseudoalteromonas*, *Shewanella*, *Vibrio*, *Pseudomonas*, *Bizionia*, *Cognaticolwellia*, *Flavobacterium*, *Kocuria*, *Tenacibaculum*, and *Winogradskyella*, are shown in Table 1. At 12 and 24 h, the growth of 53 strains was inhibited by PLE; at 48 and 60 h, the growth of 42, and 35 strains, respectively, was inhibited. This indicates that PLE inhibits the growth of most protease-producing

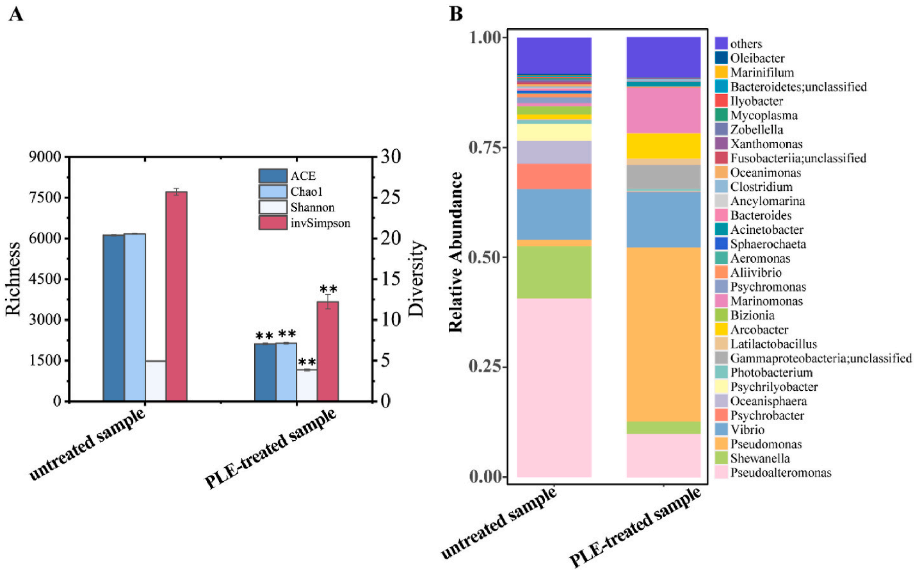

Fig. 5. Effects of PLE on Alpha diversity estimates and composition of bacterial communities in oyster samples. (A) Richness index and diversity index, (B) composition at the genus level of bacterial communities of oysters treated with or without PLE. Black ** indicate significant differences between PLE-treated sample and their corresponding untreated sample, with $p < 0.01$, respectively.

microorganisms within a certain period, thereby slowing down the decay rate of oysters.

To investigate the effect of PLE on extracellular protease activity, we selected six bacterial strains belonging to the genera *Pseudoalteromonas* (strain S14c), *Vibrio* (strains F7 and G8), *Pseudomonas* (strain L10), *Shewanella* (strain S7), *Flavobacterium* (strain L13), and conducted inhibition test to identify the types of extracellular proteases. The effects of metalloprotease inhibitor OP, serine protease inhibitor PMSF, aspartic protease inhibitor PA, and cysteine protease inhibitor E64 on extracellular protease activity were detected. The results are shown in Table 2. The extracellular protease activity of *Ps. ostreae* S14c and *V. atlanticus* F7 were inhibited by OP, with inhibition rates of 54.41 % and 63.55 %, respectively, indicating that they secrete metalloproteases. PMSF inhibited the protease activity of *V. alginolyticus* G8, *P. leptonychotis* L10, *S. septentrionalis* S7, *F. jumunjinense* L13, indicating that the isolated strains mainly secrete serine protease. Furtherly, we conducted inhibition experiments to determine the effect of PLE on extracellular proteases. PLE inhibited the extracellular protease activity of 6 strains, with inhibition rates ranging from 6.99 % to 34.22 % (Table 2). This indicates that PLE can inhibit the activity of extracellular proteases secreted by these strains to varying degrees, particularly metalloproteases and serine proteases.

Despite the promising antibacterial efficacy observed in this study, it is important to acknowledge its limitations. The current work primarily focused on the preservative potential of PLE, and a formal sensory evaluation with human subjects was not conducted. Although our preliminary observations suggested that the unique aroma of perilla added a complementary, herbaceous nuance to treated oysters instead of overwhelming their natural flavor, this remains subjective. The influence of the sensory attributes of PLE, particularly at higher concentrations, on consumer acceptability is yet to be quantitatively determined. Therefore, future research should include controlled sensory analysis panels. This is essential to comprehensively assess consumer perception and optimize the application of PLE as a natural preservative in seafood products, ensuring a balance between efficacy and sensory appeal.

It should also be noted that the drying process in the extraction of purple perilla was performed using a forced-air oven at 55 °C. The exposure to heat and oxygen during drying may potentially affect the stability of heat-sensitive compounds and consequently the overall bioactivity of PLE. This likely resulted in the need for higher concentrations in antimicrobial assays. Future studies will explore optimizing drying techniques, such as vacuum drying, freeze-drying, or lower-temperature processes, to better preserve the intrinsic bioactivity of the extract. This optimization may contribute to the use of lower concentrations of PLE to achieve the desired antimicrobial efficacy, thereby enhancing its practical application potential.

4. Conclusion

This study investigated the physicochemical characteristics and bacterial spoilage of Pacific, Kumamoto, Fujian, and Hongkong oysters from five Chinese provinces during refrigerated storage. Within 7 days, acceptability declined as bacterial counts exceeded 7 $\log_{10}$ CFU/g, and microbial richness and diversity increased significantly in spoiled oysters, dominated by *Pseudoalteromonas*, *Shewanella*, *Vibrio*, *Pseudomonas*, and *Aliivibrio*. Protease-producing bacteria were markedly more abundant in decayed oysters, with 62 strains isolated, among which *Pseudoalteromonas* and *Shewanella* were dominant overall, while *Vibrio* prevailed in Fujian and Guangdong samples. Inhibition tests revealed that most strains secreted serine proteases and metalloproteases, and experiments confirmed that these bacteria and their extracellular proteases accelerated spoilage. To mitigate decay, perilla leaf extract (PLE) was evaluated as a natural preservative. Optimized for high flavonoid and polyphenol content, antioxidant capacity, and antimicrobial activity, PLE extended oyster shelf life by preserving texture, suppressing microbial growth, reducing the abundance of spoilage-related genera, and inhibiting proteolytic activity, particularly metalloproteases and serine proteases. These results underscore the role of protease-producing bacteria in oyster spoilage and demonstrate the potential of PLE as an eco-friendly preservation method that aligns with consumer demand for sustainable food solutions.

CRediT authorship contribution statement

Jie Yang: Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Xiaolong Zhu:** Writing – original draft, Validation, Software, Methodology, Investigation, Data curation. **Wenrui Su:** Methodology. **Chen Huang:** Methodology. **Xinyi Liu:** Writing – review & editing, Supervision. **Chao Su:** Methodology. **Obafemi Ibitayo Obajemihi:** Writing – review &

Table 1
The effect of PLE on the growth of protease-producing strains isolated from oyster samples.

Genera	Strains	Gram	Growth			
			12 h	24 h	48 h	60 h
Pseudoalteromonas	*Ps. tunicate* S3	negative	-	-	-	-
	Ps. tunicata S1	negative	-	-	-	-
	Ps. tetraodonis S38c	negative	-	-	-	-
	Ps. tetraodonis S26f	negative	-	-	-	-
	Ps. tetraodonis S17f	negative	-	-	-	+
	Ps. tetraodonis J11	negative	-	-	+	+
	Ps. tetraodonis G7	negative	-	-	-	-
	Ps. tetraodonis G5	negative	-	-	-	-
	Ps. tetraodonis G3	negative	-	-	-	-
	Ps. tetraodonis F3	negative	-	-	-	-
	Ps. ostreae S25f	negative	-	-	-	-
	Ps. ostreae S1c	negative	-	-	-	-
	Ps. ostreae S16c	negative	-	-	-	-
	Ps. ostreae S14c	negative	-	-	-	-
	Ps. nigrifaciens S20f	negative	+	+	+	+
	Ps. nigrifaciens S8	negative	-	-	+	+
	Ps. nigrifaciens S6f	negative	-	-	+	+
	Ps. nigrifaciens S5f	negative	-	-	-	+
	Ps. nigrifaciens S37c	negative	-	-	-	+
	Ps. nigrifaciens S36c	negative	-	-	-	-
	Ps. nigrifaciens S27f	negative	+	+	+	+
	Ps. nigrifaciens S19f	negative	+	+	+	+
	Ps. nigrifaciens S11f	negative	-	-	-	-
	Ps. nigrifaciens S10f	negative	-	-	+	+
	Ps. nigrifaciens L3	negative	+	+	+	+
	Ps. elyakovii S22f	negative	-	-	-	-
	Ps. distincta S7f	negative	-	-	-	-
	Ps. distincta S32c	negative	-	-	-	+
	Ps. distincta S28f	negative	-	-	-	-
	Ps. distincta S18f	negative	-	-	+	+
	Ps. atlantica S13c	negative	-	-	+	+
Shewanella	*S. septentrionalis* S7	negative	-	-	-	-
	S. septentrionalis S39c	negative	-	-	+	+
	S. septentrionalis S12	negative	-	-	+	+
	S. septentrionalis L7	negative	-	-	-	-
	S. septentrionalis L6	negative	-	-	-	-
	S. septentrionalis L14	negative	-	-	-	-
	S. septentrionalis J4	negative	-	-	+	+
	S. septentrionalis J3	negative	-	-	-	+
	S. septentrionalis J10	negative	-	-	-	+
	S. septentrionalis G2	negative	-	-	+	+
	S. livingstonensis S8f	negative	-	-	-	+
	S. denitrificans L11	negative	-	-	-	-
	S. colwelliana S6	negative	-	-	-	-
	S. colwelliana F5	negative	-	-	+	+
Vibrio	*V. neocaledonicus* G11	negative	-	-	-	-
	V. atlanticus F7	negative	-	-	-	-
	V. alginolyticus G8	negative	-	-	-	-
	V. alginolyticus G1	negative	-	-	-	-
	V. alginolyticus F12	negative	-	-	-	-
Pseudomonas	*P. leptonychotis* L10	negative	-	-	-	-
	P. anguilliseptica L5	negative	-	-	-	-
Winogradskyella	*W. crassostreae* F13	negative	-	-	-	-
Tenacibaculum	*T. gallaicum* L4	negative	-	-	-	-
Kocuria	*K. tytonicola* J1	positive	+	+	+	+
Flavobacterium	*F. jumunjinense* L13	negative	-	-	-	-
Cognaticolwellia	*C. aestuarii* S4	negative	-	-	-	-
Bizionia	*B. paragorgiae* J6	negative	-	-	-	-

"–" indicates that microbial growth was inhibited by PLE at a specific time point.
"+" indicates that microbial growth was not inhibited at a specific time point.

Table 2
The effects of protease inhibitors and PLE on extracellular protease activity.

Strains	Inhibition ratio (%)				
	OP	PMSF	E64	PA	PLE
Pseudoalteromonas ostreae S14c	54.41	–	–	–	34.22 ± 0.11
Vibrio atlanticus F7	63.55	–	–	–	16.27 ± 0.09
Vibrio alginolyticus G8	–	76.45	–	–	19.67 ± 0.07
Pseudomonas leptonychotis L10	–	44.00	9.11	–	6.99 ± 0.01
Shewanella septentrionalis S7	–	58.14	–	–	25.40 ± 0.21
Flavobacterium jumunjinense L13	–	11.54	–	–	31.25 ± 0.16

editing. **Guangchao Liu:** Writing – original draft, Software, Resources, Data curation.

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.

Acknowledgments

This research was funded by the National Natural Science Foundation of China (32000004, 32102352), Enterprise entrusted project (20250049), the Innovation and Entrepreneurship Training Program for College Students in Shandong Province (S202410435011), and Qingdao Agricultural University Research Fund (1120088, QNYCX23043, QNDC20251184).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2025.118564.

Data availability

Data will be made available on request.

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