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Research article

# Characteristics of halophilic lactic acid bacteria isolated from fermented snakehead fish (*Channa striata* Bloch)

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## Abstract

**Importance of the work**: This work lays the groundwork for further exploration into the use of halophilic lactic acid bacteria (LAB) in developing high-quality fermented fish products.

**Objectives**: To isolate and characterize halophilic LAB isolated from fermented snakehead fish (*Channa striata* Bloch), a traditional Vietnamese 'mam' product.

**Materials and Methods**: Fermented snakehead samples were collected from local markets and transported to the laboratory under sterile conditions. The fish samples were homogenized in saline solution and serially diluted. Halophilic LAB were isolated by plating onto Man Rogosa Sharpe agar supplemented with 5% NaCl, followed by anaerobic incubation at 30°C for 48 hr. Strains were evaluated for their growth ability in de Man, Rogosa, Sharpe medium containing varying concentrations of NaCl (5–15%), at varying growth temperatures (30–45°C), as well as for their levels of proteolytic activity.

**Results**: In total, 18 LAB strains were isolated from 4 fermented snakehead samples, with identification performed using 16S rRNA gene sequencing. The strain with superior properties, identified as *Tetragenococcus halophilus*, had signifcant proteolytic activity, crucial for enhancing the flavor and nutritional profile of fermented fish. The isolated strains demonstrated robust growth in high-salinity conditions, specifically at 10% NaCl, with optimal growth observed at 30°C and 35°C. Additionally, most strains had high levels of protein hydrolysis.

**Main finding**: The halophilic LAB *Tetragenococcus halophilus* FF18.2 was successfully isolated and screened. Due to its ability to thrive in high-salt environments and degrade proteins, the addition of this bacterial strain as a starter culture may contribute to the development of high-quality, safe and potentially more nutritious fermented fish products.

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## Introduction

Fermented fish, transliterated as 'mam' from Vietnamese, is a traditional condiment that is important in the culinary heritage of Southeast Asia, particularly in Vietnam (Ruddle and Naomichi, 2010). This distinctive product is created through the fermentation of various types of fish, often combined with salt and sometimes additional ingredients such as rice, spices or herbs (Ruddle and Naomichi, 2010). Commonly used fish include anchovies, mackerel and catfish, each contributing unique flavors and characteristics to the final product (LeGrand et al., 2020). Typically, the process involves mixing fresh fish with a large amount of salt and compacting the mixture to create an anaerobic environment that is conducive to the growth of specific microorganisms (Ruddle and Naomichi, 2010).

Traditional fermentation processes involve complex microbial communities that contribute to the unique flavors and textures of fermented fish products. Various types of bacteria have been identified in fermented fish products, including *Bacillus, Micrococcus, Staphylococcus, Streptococcus, Pediococcus* and halophilic lactic acid bacteria (LAB), particularly *Tetragenococcus* (Narzary et al., 2021). Halophilic LAB enhance traditional fermented fish products by thriving in a high salinity environment and improving flavor, nutrition and food safety (Nguyen et al., 2024). Halophilic LAB are known for their ability to withstand elevated salt concentrations, making them ideal candidates for fermenting foods that require a high salt content such as 'mam', where these bacteria ferment sugars and other substrates present in the fish and added ingredients, resulting in the production of lactic acid, volatile compounds and amino acids (Zhang et al., 2020). Halophilic LAB are not only crucial for acidifying and preserving fermented fish products but also for the production of enzymes, such as proteases, which break down proteins into smaller peptides and amino acids (Zhang et al., 2020). This enzymatic activity not only enhances the digestibility of the fermented product but also contributes to the development of complex flavors that are highly prized in culinary applications (Xu et al., 2020). Therefore, characterizing halophilic LAB allows for deeper understanding of this microbial diversity and its implications for fermentation dynamics.

Traditional fermented fish products are integral to many cultures and cuisines. In these products, halophilic LAB play a critical role in the fermentation process, contributing to the preservation of fish products through acidification and the inhibition of spoilage organisms and pathogens (Cai et al., 2024). By isolating and characterizing halophilic LAB, researchers can help preserve these traditional practices while also fostering innovation. In addition, the halophilic LAB strains are recognized for their high potential in biotechnological applications beyond traditional fermentation. For example, these strains can be utilized in the development of functional foods, probiotics and novel fermentation processes (Khushboo et al., 2023, Nguyen et al., 2024). Therefore, the search for strains with outstanding characteristics can lead to innovative applications in the food industry.

Despite the importance of halophilic LAB in fermentation, there is limited understanding of their diversity and functional capabilities, particularly in the context of fermented fish. Therefore, the current study aimed to isolate and select halophilic LAB from mam samples, focusing on their proteolytic activity. Identifying strains with high protein hydrolysis could uncover new opportunities for improving the quality and nutritional value of fermented fish products. Thus, molecular identification and characterization of these bacteria were applied to contribute to the broader knowledge of the microbial communities involved in traditional fermentation processes and their potential applications in the food industry.

## Materials and Methods

### *Sample collection and isolation*

Samples of fermented snakehead fish (*Channa striata* Bloch) were collected from local markets in Chau Doc town and Phu Tan district (An Giang province, Vietnam) known for traditional fermentation practices. The isolation process was conducted as described by Cui et al. (2012). Specifically, 25 g of each sample were diluted tenfold in 0.85% NaCl, supplemented with 0.1% (volume per volume, v/v) Tween 80. Then 0.1 mL of the sample was spread onto de Man, Rogosa, Sharpe (MRS) agar (Merck; Germany) containing 5% NaCl and 0.5% $CaCO_3$ to encourage the growth of halophilic LAB. The plates were incubated under anaerobic conditions at 30°C for 48 hr. Distinct colonies forming $CaCO_3$-solution zones were selected and re-cultured to obtain pure isolates. Then, the isolates were characterized based on cell morphology, Gram staining and catalase assay.

### *Evaluation of growth properties*

The isolated strains were cultured for growth in liquid MRS medium for 48 hr. The optimal growth temperature was assessed using a 5% (v/v) inoculum from the proliferated culture that was introduced into 200 mL of MRS medium containing 10% NaCl to mimic the conditions of high-salinity environments typical of traditional fermented fish products.

Next, the cultures were incubated at 30°C, 35°C, 40°C or 45°C for 48 hr based on the conditions commonly used in food fermentation. Salt tolerance was evaluated using a 5% (v/v) inoculum from the proliferated culture that was added to 200 mL of MRS medium containing NaCl at concentrations of 5%, 10% or 15%. Subsequently, these cultures were incubated at 35°C for 48 hr. The bacterial density was determined by spreading samples onto MRS agar. The percentage change in bacterial density was calculated using the formula: Percentage change = 100 × (Final density − Initial density) / Initial density.

### *Proteolytic activity*

The proteolytic activity of the halophilic LAB was assessed using fish broth containing 10% NaCl. The fish broth was prepared by boiling fish and distilled water in a 1:2 ratio for 20 min, followed by filtration through cheesecloth to collect the filtrate (Udomsil et al., 2010). Subsequently, 10% NaCl was added to the filtered fish broth, the pH was adjusted to 7.0 and the mixture was autoclaved at 121°C for 15 min. A 2% inoculum was added to 100 mL of the prepared fish broth and incubated at 30°C for 7 d under anaerobic conditions in an anaerobic chamber (Shel Lab; Sheldon Manufacturing Inc.; USA). The bacterial density was determined using the spread plate method on MRS agar containing 5% NaCl and 0.5% $CaCO_3$, incubated at 30°C. Upon completion of the incubation period, the cell cultures were centrifuged at 10,000×*g* at 4°C for 10 min and the supernatants were collected. The remaining protein content in the supernatant was determined using bovine serum albumin as the standard, following the method outlined by Bradford (1976). The proteolytic activity was expressed as the percentage change in the protein content, with the protein levels of non-inoculated fish broth incubated under the same conditions (30°C for 7 d under anaerobic conditions) serving as the reference point, being assigned a value of 100%.

### *Identification of halophilic LAB*

Each isolate was identified based on 16S rRNA gene sequence analysis. Genomic DNA was extracted and subjected to polymerase chain reaction (PCR) using the primers 5' AGAGTTTGATCMTGGCTCAG 3' and 5' TACGGYTACCTTGTTACGACTT 3' (Nga, 2018). The PCR reaction was performed on a Mastercycler Eppendorf PCR machine (Eppendorf, Germany), with a total reaction volume of 25 µL, comprising 0.5 µL of template DNA, 12.5 µL of PCR master mix, 1.25 µL of each primer (10 pmol/µL) and 9.5 µL of distilled water. The thermal cycling conditions were an initial denaturation at 96°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min each cycle, annealing at 40°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 10 min. The PCR products were purified using a PureLink™ DNA Purification Kit (Invitrogen, Thermo Fisher Scientific Inc., USA) and subsequently sequenced. The obtained sequencing results were compared for genetic similarity using the Nucleotide BLAST tool available in the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch). A phylogenetic tree was constructed using the maximum parsimony method with the MEGA version 4.0 software (Udomsil et al., 2010).

### *Statistical analysis*

All experiments were conducted using three biological replicates, and the results were presented as mean ± SD values. One-way analysis of variance (ANOVA) was performed using the Statgraphics 18 software (Statgraphics Technologies Inc., USA) to evaluate the significance of differences between groups. Subsequently, Duncan's test was applied to identify statistically significant differences ($p < 0.05$).

## Results

### *Isolation and characterization*

Among the four fermented fish samples, the study identified 18 strains exhibiting the characteristic properties of LAB, specifically being Gram-positive and catalase-negative (Table 1). These findings revealed a diverse array of halophilic LAB present in the fermented fish. Notably, four groups of bacteria were identified, with cocci being the predominant morphology observed in the fermented fish products. Furthermore, only two strains of the class Bacilli were detected.

**Table 1** Shape class of halophilic lactic acid bacterial strains isolated from fermented fish.

| Shape class | Cell arrangement | Number of strains | Identification |
|---|---|---|---|
| Bacilli | Isolated | 2 | FF8, FF14 |
| Cocci | Clustered | 4 | FF18.1; FF18.2; FF3.1; FF4.2 |
| Cocci | Isolated | 7 | FF5; FF7; FF9; FF10; FF11; FF15.2; FF16.1 |
| Cocci | No arrangement | 5 | FF1.1; FF2.1; FF6; FF12; FF13 |

Growth of isolates under different temperature conditions

The growth of the isolated strains was assessed over the range 30–45°C. As shown in Table 2, most of the isolated strains had good growth at 30°C. Compared to the initial density, the strains with the highest growth rates were FF9 (512% increase), FF3.1 (475% increase) and FF6 (254% increase). Similarly, the majority of strains were able to survive and grow at 35°C, although the growth rates were lower than those observed at 30°C. However, strains, such as FF10, FF14, FF15.2 and FF2.1 decreased in cell density slightly compared to the initial measurements, with reductions of 0.009%, 0.135%, 0.264% and 0.109%, respectively. At 40°C, the bacterial viability decreased significantly, with some strains exhibiting reduced cell density and others being completely eliminated (FF11, FF12, FF15.2, FF2.1 and FF8). By 45°C, most isolated strains were unable to survive, with only a few strains (FF14, FF3.1, FF4.2, FF5, FF6 and FF9) maintaining viability, albeit with a substantial reduction in cell density.

### *Growth of isolates at various salt concentrations*

Evaluation of salt tolerance in isolated strains is essential for identifying strains with potential applications in industrial and scientific research. In this study, the salt content of the fermented fish samples was in the range 10–12%. In addition, the salt tolerance of the isolated strains was examined at concentrations both lower and higher than that of the samples, specifically at 5%, 10% and 15%.

The changes in bacterial cell density under varying salt concentrations are detailed in Table 3, indicating that the isolated strains had good growth at a salt concentration of 5%. Compared to the initial density, the strains with the highest growth rates were FF4.2 (245%), FF12 (230%) and FF3.1 (190%), while the strain with the lowest growth rate was FF13 (1%). Similarly, most strains were able to survive and grow at a salt concentration of 10%, although the growth rates were lower than those observed at 5%. However, compared to the initial measurements, some strains experienced a decrease in cell density such as FF13 (7%), FF14 (3%) and FF15.2 (9%). At a salt concentration of 15%, most of the isolated strains maintained viability, but there was a significant reduction in cell density. Notably, strains FF18.1 (0.6%) and FF18.2 (0.8%) continued to survive and grow.

### *Proteolytic activity*

Research into the protein hydrolysis capabilities of LAB is essential for understanding their metabolic mechanisms and for utilizing these microorganisms in the production of new fermented food products with high nutritional value and unique flavors. Consequently, this study evaluated the protein hydrolysis potential of halophilic LAB. Based on the data shown in Table 4, after 7 d of incubation, the pH of the culture medium decreased slightly from an initial value of 6.95 to a range of 5.74–6.71. This reduction in pH may be attributed to the acid production resulting from the metabolic activity of the halophilic LAB.

**Table 2** Growth of halophilic lactic acid bacterial strains under different temperature conditions.

| Isolate | Change in cell density* (%) | | | |
|---|---|---|---|---|
| | 30°C | 35°C | 40°C | 45°C |
| FF1.1 | $40.115 \pm 6.498^{ijk}$ | $0.073 \pm 0.031^{gh}$ | $-0.270 \pm 0.054^{f}$ | x |
| FF10 | $158.389 \pm 17.994^{f}$ | $-0.009 \pm 0.046^{hi}$ | $-0.991 \pm 0.184^{h}$ | x |
| FF11 | $52.778 \pm 16.839^{hij}$ | $0.351 \pm 0.043^{e}$ | x | x |
| FF12 | $70.691 \pm 26.821^{hi}$ | $0.086 \pm 0.073^{fgh}$ | x | x |
| FF13 | $195.455 \pm 9.201^{e}$ | $0.520 \pm 0.005^{b}$ | $-0.895 \pm 0.218^{h}$ | x |
| FF14 | $28.369 \pm 4.958^{jklm}$ | $-0.135 \pm 0.129^{j}$ | $-0.536 \pm 0.011^{g}$ | $-1.784 \pm 0.187^{f}$ |
| FF15.2 | $37.77 \pm 6.063^{ijkl}$ | $-0.264 \pm 0.015^{k}$ | x | x |
| FF16.1 | $85.783 \pm 2.9567^{h}$ | $0.271 \pm 0.041^{cde}$ | $0.159 \pm 0.062^{cde}$ | x |
| FF18.1 | $232.863 \pm 19.032^{cd}$ | $0.296 \pm 0.016^{cd}$ | $0.109 \pm 0.020^{de}$ | x |
| FF18.2 | $206.898 \pm 6.232^{de}$ | $0.197 \pm 0.009^{def}$ | $0.036 \pm 0.012^{e}$ | x |
| FF2.1 | $4.447 \pm 1.202^{klm}$ | $-0.109 \pm 0.153^{ij}$ | x | x |
| FF3.1 | $475.000 \pm 33.301^{b}$ | $0.639 \pm 0.057^{a}$ | $0.288 \pm 0.022^{bc}$ | $-0.184 \pm 0.076^{a}$ |
| FF4.2 | $119.867 \pm 11.082^{g}$ | $0.166 \pm 0.025^{efg}$ | $-0.261 \pm 0.035^{f}$ | $-1.014 \pm 0.097^{d}$ |
| FF5 | $215.400 \pm 19.821^{de}$ | $0.217 \pm 0.066^{de}$ | $0.137 \pm 0.027^{cde}$ | $-0.726 \pm 0.027^{c}$ |
| FF6 | $253.765 \pm 16.258^{c}$ | $0.487 \pm 0.009^{b}$ | $0.229 \pm 0.009^{bcd}$ | $-1.297 \pm 0.189^{e}$ |
| FF7 | $0.479 \pm 5.348^{m}$ | $0.001 \pm 0.120^{hi}$ | $0.377 \pm 0.022^{ab}$ | x |
| FF8 | $2.528 \pm 9.278^{lm}$ | $0.024 \pm 0.026^{h}$ | x | x |
| FF9 | $511.949 \pm 41.178^{a}$ | $0.657 \pm 0.012^{a}$ | $0.464 \pm 0.007^{a}$ | $-0.410 \pm 0.021^{b}$ |

x = no bacterial growth.

* Percentage change in bacterial density, calculated as 100 × (Final density - Initial density) / Initial density.

Mean±SD in the same column with different lowercase superscript letters are significantly different ($p < 0.05$).

**Table 3** Growth of halophilic lactic acid bacterial strains at various salt concentrations.

| Isolate | Change in cell density* (%) | | |
|---|---|---|---|
| | 5% NaCl | 10% NaCl | 15% NaCl |
| FF1.1 | 74.283 ± 6.266$^{hi}$ | 13.460 ± 6.958$^{de}$ | -61.996 ± 1.715$^{d}$ |
| FF10 | 139.068 ± 4.966$^{d}$ | 31,606 ± 1.129$^{c}$ | -74.715 ± 0.903$^{g}$ |
| FF11 | 100.375 ± 16.818$^{f}$ | 320.006 ± 1.045$^{a}$ | -38.525 ± 4.908$^{a}$ |
| FF12 | 229.977 ± 20.712$^{b}$ | 101.140 ± 3.612$^{b}$ | -63.082 ± 4.477$^{de}$ |
| FF13 | 1.449 ± 0.941$^{m}$ | -7.154 ± 10.722$^{ij}$ | -84.999 ± 0.187$^{h}$ |
| FF14 | 54.981 ± 2.122$^{jk}$ | -3.558 ± 4.005$^{hij}$ | -60.317 ± 2.524$^{d}$ |
| FF15.2 | 58.674 ± 3.308$^{jk}$ | -9.440 ± 3.081$^{j}$ | -87.008 ± 0.089$^{hi}$ |
| FF16.1 | 69.884 ± 2.818$^{hij}$ | 10.303 ± 4.231$^{ef}$ | -92.446 ± 2.479$^{i}$ |
| FF18.1 | 50,196 ± 1.773$^{k}$ | 8.160 ± 0.652$^{efg}$ | 0.638 ± 2.972$^{a}$ |
| FF18.2 | 81.452 ± 8.659$^{gh}$ | 11.561 ±2.664$^{e}$ | 0.822 ± 8.718$^{a}$ |
| FF2.1 | 120.918 ± 4.792$^{e}$ | 33.963 ± 5.638$^{c}$ | -53.936 ± 2.298$^{c}$ |
| FF3.1 | 189.590 ± 9.713$^{c}$ | 21.252 ± 5.474$^{d}$ | -63.962 ± 1.443$^{de}$ |
| FF4.2 | 245.538 ± 5.596$^{a}$ | 7.652 ± 6.460$^{efg}$ | -90.872 ± 2.487$^{i}$ |
| FF5 | 84.510 ± 4.173$^{gh}$ | 0.056 ± 5.799$^{ghi}$ | -84.482 ± 1.407$^{h}$ |
| FF6 | 58.747 ± 0.717$^{jk}$ | 1.389 ± 1.203$^{fghi}$ | -88.800 ± 1.356$^{hi}$ |
| FF7 | 66.253 ± 9.663$^{ij}$ | 21.401 ± 4.598$^{d}$ | -68.035 ± 1.536$^{ef}$ |
| FF8 | 30.698 ± 5.876$^{l}$ | 2.330 ± 3.994$^{fgh}$ | -71.673 ± 1.826$^{fg}$ |
| FF9 | 93.600 ± 2.705$^{fg}$ | 7.510 ± 3.754$^{efg}$ | -71.121 ± 0.266$^{fg}$ |

* Percentage change in bacterial density, calculated as 100 × (Final density - Initial density) / Initial density.
Mean±SD in the same column with different lowercase superscript letters are significantly different ($p < 0.05$).

**Table 4** Changes in bacterial growth, protein content and pH of fish broth containing 10% NaCl inoculated with halophilic lactic acid bacterial strains.

| Isolate | Δ Cell count* (log CFU/mL) | Δ Protein content** (%) | pH |
|---|---|---|---|
| FF1.1 | 0.11 | 20.16 ± 0.71$^{j}$ | 6.54 |
| FF10 | 1.19 | -35.95 ± 1.50$^{d}$ | 5.88 |
| FF11 | 0.20 | -30.67 ± 0.55$^{e}$ | 6.71 |
| FF12 | 0.13 | -35.53 ± 1.54$^{d}$ | 5.74 |
| FF13 | 0.16 | -17.58 ± 0.76$^{h}$ | 6.51 |
| FF14 | 0.12 | 37.57 ± 1.15$^{k}$ | 6.42 |
| FF15.2 | 0.17 | -39.64 ± 1.15$^{c}$ | 6.53 |
| FF16.1 | 0.18 | -13.57 ± 1.15$^{i}$ | 6.61 |
| FF18.1 | 0.33 | -41.68 ± 0.65$^{b}$ | 5.88 |
| FF18.2 | 0.30 | -44.75 ± 1.05$^{a}$ | 5.89 |
| FF2.1 | 0.21 | -38.43 ± 1.01$^{c}$ | 6.08 |
| FF3.1 | 0.25 | -21.92 ± 1.53$^{g}$ | 6.44 |
| FF4.2 | 0.13 | -31.86 ± 1.53$^{e}$ | 6.57 |
| FF5 | 0.27 | -21.58 ± 1.05$^{g}$ | 6.65 |
| FF6 | 0.10 | -27.80 ± 1.26$^{f}$ | 6.54 |
| FF7 | 0.18 | -31.08 ± 1.00$^{e}$ | 6.62 |
| FF8 | 0.27 | -31.47 ± 0.53$^{e}$ | 6.32 |
| FF9 | 0.23 | -35.09 ± 0.50$^{d}$ | 6.14 |

CFU = colony forming unit.
*difference in bacterial count relative to the initial count.
**difference in protein content relative to non-inoculated fish broth incubated under the same conditions, expressed as a percentage.
Mean±SD in the same column with different lowercase superscript letters are significantly different ($p < 0.05$).

Monitoring the protein hydrolysis capacity revealed that the protein concentration in the fish broth decreased compared to the initial concentration, demonstrating the ability of halophilic LAB to hydrolyze fish proteins. A greater reduction in protein levels indicates a higher amount of protein utilized, supporting the growth of these halophilic LAB. Based on the results in Table 4, certain strains, such as FF13 and FF16.1, had proteolytic activity of approximately 17% and 13%, respectively. In contrast, other strains, including FF18.1 and FF18.2, had significantly higher proteolytic activity levels, reaching nearly 44%. The proteases produced by the halophilic bacteria can hydrolyze proteins in high-salinity environments, suggesting that the isolated strains had stable protease production capabilities in saline conditions. A notable finding was that while the majority of isolated strains reduced the protein content in the fish broth, two strains (FF1.1 and FF14) increased the protein concentration, suggesting that the proteins from these microorganisms may be secreted during the cultivation process.

### *Identification of halophilic lactic acid bacteria*

The study identified the bacterial strain FF18.2 (Fig. 1), which had high salt tolerance and significant protein hydrolysis capabilities, based on molecular biology techniques.

The sequences of the 16S rRNA gene were compared to entries in global gene bank databases using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), revealing a 99% similarity with *Tetragenococcus halophilus* FF18.2 (The nucleotide sequence data were deposited in the GenBank database under accession number PQ628373.1). The construction of a phylogenetic tree revealed that the isolated strain FF18.2 had a high degree of similarity to the *T. halophilus* (Fig. 2).

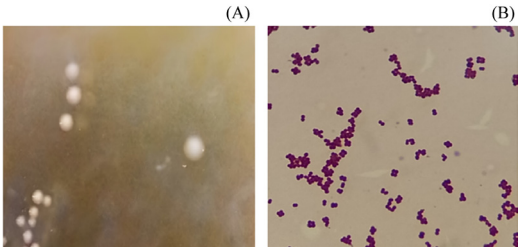


**Fig. 1** Bacterial strain FF18.2: (A) colony characteristics; (B) morphology.

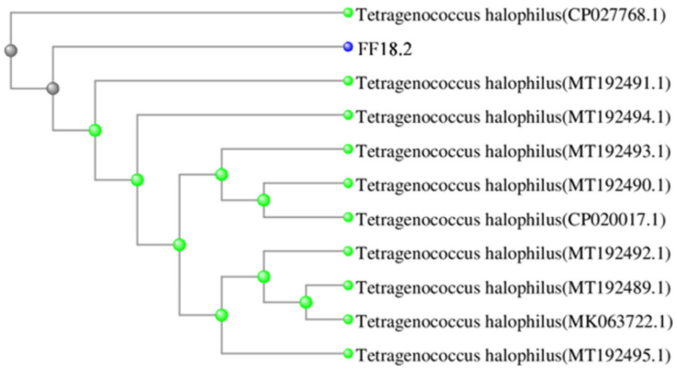


**Fig. 2** Phylogenetic tree of bacterial strain FF18.2 constructed by comparing 16S rRNA gene sequences

## Discussion

The isolation and selection of halophilic LAB that produced protease from fermented fish represents a major advancement in understanding the microbial dynamics involved in traditional fermentation processes. The current study successfully identified several LAB strains with notable proteolytic activity, which is crucial for enhancing the flavor, nutritional value and safety of fermented fish products.

The current results showed the abundance of halophilic LAB in fermented fish products. Among the isolates, cocci morphology was found to be more prevalent than bacilli. This finding was consistent with the report by Karyantina et al. (2020), which indicated that most isolates from traditional fermented fish in Central Java, Indonesia, were cocci. Metagenomic analyses of traditional Indonesian fermented fish, transliterated as 'budu', from Indonesian, identified LAB from the family *Enterococcaceae* and the genus *Vagococcus*, as well as pathogenic bacteria such as *Staphylococcus cohnii*, *Peptostreptococcus russelli*, *Clostridium disporicum* and *S. baltica* (Marlida et al., 2024). The literature reviewed primarily consisted of culture-dependent analyses that relied on microbiological viable counts, allowing for the identification of the main microbial populations involved in the fish fermentation process, including the LAB *Staphylococcus* spp., *Bacillus* spp. and yeasts (Belleggia and Osimani, 2023). Thus, the microbial community in fermented fish is highly diverse. However, in the current study, reliance on a single sampling point may not have fully captured the diversity needed for a comprehensive analysis. This limitation could affect the generalizability of the findings; therefore, future studies should consider multiple sampling points and times to obtain a more diverse and representative dataset.

Based on molecular techniques, particularly 16S rRNA gene sequencing, the strain exhibiting the highest proteolytic capability was identified as *T. halophilus* FF18.2. This strain's high similarity (99%) to known sequences in databases reinforced its relevance in fermented fish applications. Several studies have reported the presence of *T. halophilus* in various salt-fermented products. For example, this bacterium has been isolated from shoyu mash (Hanagata et al., 2003), Indonesian soy mash, transliterated as 'kecap' from Indonesian (Roling and van Verseveld, 1996) and Thai fish sauce transliterated as 'nam pla' from Thai (Thongsanit et al., 2002). *T. halophilus*, isolated from fermented fish products, is known not only for its protease production, which plays an important role in the formation of volatile compounds (Udomsil et al., 2010), but also for its aspartate decarboxylase activity, which converts aspartate to alanine. This conversion is associated with taste alteration and helps prevent the accumulation of biogenic amines (Wakinaka et al., 2019). The ability of this bacterium to thrive in high-salinity conditions is essential for its role in the fermentation of fish products, as it contributes greatly to both flavor development and preservation. Genome-resolved metaproteomic analysis of the microbiota and metabolic pathways revealed the taste-related protein composition in traditional fermented fish products. This composition is associated with genera, such as *Halanaerobium*, *Psychrobacter*, *Photobacterium* and *Tetragenococcus* and is linked to amino acid metabolism, including the metabolism of alanine, aspartate, glutamate and histidine, as well as lysine degradation and arginine biosynthesis (Wang et al., 2022).

The proteolytic activity observed among the isolated strains is particularly noteworthy. LAB and their proteolytic systems play a crucial role in determining the organoleptic properties of fermented products (Kieliszek et al., 2021). Proteases produced by LAB contribute substantially to the proteolysis of fish proteins, resulting in the formation of bioactive peptides and amino acids that enhance taste and nutritional profiles (Ajayeoba and Ijabadeniyi, 2023). The current study demonstrated that certain strains had high levels of protein hydrolysis. Consequently, *T. halophilus* may play a major role in protein hydrolysis during the snakehead fish fermentation process, which typically contains 12–15% NaCl. These isolates are capable of hydrolyzing proteins into oligopeptides and amino acids, which are crucial

for developing complex flavors and aromas in fermented fish products (Wang et al., 2021). Furthermore, the proteolytic enzymes produced by LAB have been utilized to generate potent bioactive peptides through protein hydrolysis (Phupaboon et al., 2023). These bioactive peptides produced by LAB have been shown to possess potential health benefits (Guo et al., 2023), highlighting their potential application in the food industry, particularly in the development of fermented fish products with enhanced sensory qualities.

The evaluation of growth characteristics under varying salt concentrations and temperatures provided insights into the adaptability of the isolated strains. Most isolates had robust growth at lower salt concentrations (5%) and at the salt concentration present in the samples (10%). However, at higher salt concentrations, viability decreased significantly. According to Nguyen et al. (2024), low-salt bacteria can survive in salt concentrations in the NaCl range 1–3%, while moderate halophiles can thrive in NaCl concentrations of 3–15%. Bacteria that can survive in NaCl concentrations of 15–30% are classified as extreme halophiles. Thus, most of the isolated strains in the current study fall within the category of moderate halophiles. Evaluating the salt tolerance of isolated LAB is essential for selecting suitable strains for applications in the food industry and for gaining a deeper understanding of the mechanisms by which these bacteria adapt to harsh environmental conditions (Nguyen et al., 2024).

The current results indicated that the majority of the isolated strains had optimal growth at 30°C and 35°C. These temperatures align with the typical conditions for many mesophilic LAB (Chen et al., 2015), suggesting that these strains are well-adapted to moderate thermal environments, which are common in traditional fermentation processes. As the temperature increased to 40°C, there was a notable decline in bacterial growth, which could be attributed to the stress experienced by the strains at elevated temperatures that could have affected metabolic processes and enzyme activity (Adamberg et al., 2003). At 40°C, some strains exhibited reduced viability, indicating that while they could tolerate moderate heat, their growth rates were negatively impacted beyond optimal levels. At 45°C, most strains were unable to survive, highlighting the thermal limits of these halophilic LAB. This finding underscored the importance of temperature control during fermentation processes, as exceeding optimal temperatures can lead to decreased microbial viability and, consequently, a reduction in the quality of the final product.

The findings from the current study have important implications for the production of fermented fish. Producers can enhance the flavor complexity and nutritional value of their products by utilizing halophilic LAB with strong proteolytic activity (Cai et al., 2024). Additionally, these LAB can potentially improve the safety and shelf life of fermented fish by outcompeting spoilage organisms and pathogenic bacteria through acid production and other antimicrobial mechanisms (Cai et al., 2024). However, notably, lipase activity also contributes to the development of flavor and aroma in fermented fish products (Ye et al., 2024). Therefore, further studies are warranted to assess the lipase-producing ability of these strains and their influence on the sensory quality of fish sauce. Additionally, studies focused on the sensory evaluation of fermented fish products made with these LAB could provide valuable insights into consumer preferences and market potential. More recent surveys have indicated that consumers are increasingly seeking products that offer unique flavors and health benefits, particularly those associated with probiotics and bioactive compounds (Ali and Ali, 2020; Siddiqui et al., 2023). Exploring the genetic and metabolic pathways of these bacteria may also reveal novel applications in food biotechnology.

## Conclusion

This study successfully isolated and characterized halophilic LAB from fermented fish, highlighting their great potential for application in food biotechnology. The isolated strains demonstrated robust growth in high-salinity environments, particularly at 10% NaCl, with optimal growth being achieved at 30°C and 35°C. Notably, the majority of the strains demonstrated strong proteolytic activity, with the strain having the highest proteolytic capability identified as *T. halophilus* FF18.2. This bacterium is essential for enhancing the flavor and nutritional value of fermented fish products. The practical applications of these LAB strains may lead to the development of high-quality, safe and potentially more nutritious fermented fish products. This research should be useful in industrial applications, paving the way for further investigations into LAB and high-salt fermentation environments.

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## Conflict of Interest

The authors declare that there are no conflicts of interest.

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