

**Advanced Analysis of Bacteriological Evaluation of Fresh and Frozen Fish under  
Refrigeration and Freezing conditions**

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**Abstract:**

The microbiological quality of fresh and frozen Nanjil fish (*Plectropomus pessuliferus*) is assessed in this study over a four-day storage period. In order to assess bacterial load and quality degradation under freezing and refrigeration, fish samples were examined. The findings indicate that whereas frozen fish maintains relatively reduced microbial activity, indicating greater preservation, fresh fish has a lower microbial load at first but bacterial growth grows quickly over time. The current study, which aims to evaluate the microbiological load of marine Nanjil fish, was carried out at the Directorate General of Preventing Medicine and PHC, Epidemiology Department, Ports and Quarantine Health Unit, Port Tamilnadu. and to ascertain whether certain tainted microorganisms are present. Twenty samples of Nanjil fish (*P. Pessuliferus*) were taken at the fish market in Port Tamilnadu, placed in sterile plastic bags, and refrigerated with ice in a thermostatic container. Every sample was examined right away and frozen for four days. The examined samples were then subjected to microbiological testing, and the findings revealed: Total bacterial count (TBC) was  $5.68 \times 10^5$  CFU/gm for fresh fish and  $4.38 \times 10^5$  CFU/gm for frozen samples. According to the study's findings, Nanjil fish (*P. Pessuliferus*) had the greatest amount of contamination among fresh fish. On the other hand, frozen fish appear to have a greater effect (smaller number). Additionally, fresh and frozen fish differed significantly, according to the results.

Keywords: bacterial growth, Nanjil fish, Port Tamilnadu, Medicine and PHC, marine Nanjil, Ports and Quarantine Health Unit

**1. Introduction:**

Fish is a highly nutritious food but is also prone to microbial spoilage due to high moisture and protein content. Marine fish like Nanjil are widely consumed, especially in coastal regions. However, improper storage can lead to bacterial contamination, affecting food safety and shelf life. Microbial assessment helps determine the quality and safety of fish during storage. Freezing is commonly used to slow microbial growth, but its effectiveness over time needs evaluation. Fish accounts for approximately 17% of the global animal protein intake. Globally, the

production of fish compete the growth of world population. Sea foods have traditionally being a popular part of the diet in many parts of the world and in some countries constituted the main supply of animal protein. Today, Also fish and Seafood supply a number of essential vitamins and minerals, including: vitamins A, B3, B6, and B12, and D, and the minerals calcium, iron, selenium, and zinc. Seafood consumption is linked with improvements in health conditions including: cardiovascular disease (stroke), Arthritis, Cognitive function, Hearing loss, Depression/mood, Osteoporosis, and Cancer[1]. However, consumption of fish and shell fish may also cause diseases due to infection or intoxication, some of these diseases have been specifically associated with pathogens which are resistant to antibiotics. Fisheries sector plays an important role in food security, poverty alleviation and economic development of Tamilnadu. Inland and marine fisheries are the major fisheries sectors of Tamilnadu. These sectors support not only the domestic needs but also contribute to world export markets.

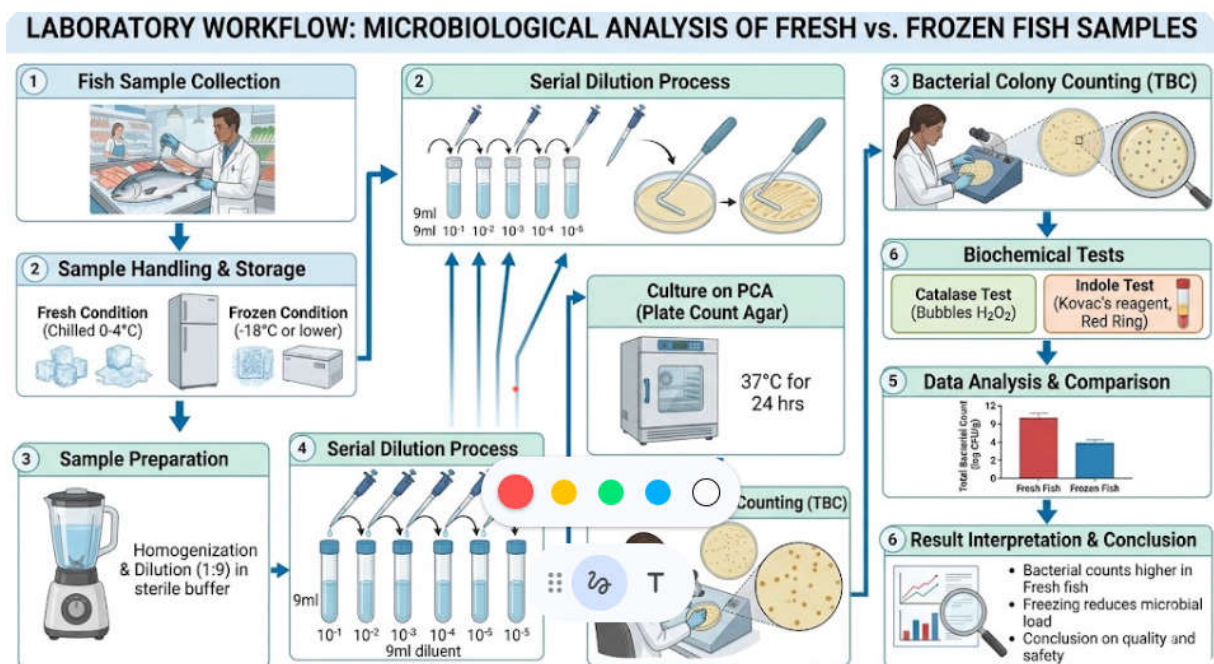


Fig.1. Work flow diagram of Microbiological Analysis of Fresh and Frozen fishes

The workflow for the microbiological analysis of fish involves a systematic transition from environmental sampling to laboratory quantification and identification[2]. This process is designed to evaluate the safety and shelf-life of fish by comparing the microbial load under different storage conditions.

In Tamilnadu because of lacking facilities, bad handling and processing most of the fish landing sites were subjected to very poor conditions. Thus the competent authority that is supposed to intervene to improve this quality unfortunately has very limited role and effort as a result of lacking in technical and financial capabilities. Some seafood commodities are more risky than others owing to many factors, including the nature of the environment from which they come, their mode of feeding, the season during which they are harvested, and how they are prepared and served[3,4]. Fish are conditioned by their environment if the growing and harvesting environment of fish is polluted chemically or microbiologically, the fish will be also polluted. When frozen seafood products are consumed raw, there is the likelihood of endangering the health of the consumer especially when the micro-organism present includes pathogenic ones. However, as demands for and production of seafood increased, it becomes important to be more attention to seafood safety and seafood related diseases.

## **2. Objectives:**

- To analyze microbial load in fresh Nanjil fish
- To evaluate microbial changes in frozen fish over four days
- To compare fresh and frozen storage conditions
- To determine safe consumption period

## **3. Materials and Methods:**

This study was carried out at Directorate general of preventing Medicine and PHC, pidemiology Dept, Ports and Quarantine health unit, Port Tamilnadu Laboratory, is an accredited laboratory which was accredited by [5]. The laboratory **follows ISO 17025 standards.**

### **3.1 Sample Collection:**

A total of 20 samples of Nanjil (*Plectropomus pesulliferus*) fish were collected randomly from different sellers at Port Tamilnadu fish market. The fish samples were identified, measured and processed following the methods used by the association (personal communication). Then transported to the laboratory by collecting in sterilized plastic bag put into ice box (thermostatic containers) and directly analyzed as fresh, froze (put into freezer for 4days) and then analyzed[6,7].

- Fresh Nanjil fish obtained from a local fish market
- Samples divided into two groups:

- Fresh (stored at refrigeration  $\sim 4^{\circ}\text{C}$ )
- Frozen (stored at  $-18^{\circ}\text{C}$ )

### 3.2 Storage Duration

- Day 0 (Initial)
- Day 2
- Day 4

### 3.3 Microbial Analysis

Microbial load was measured using:

- Total Plate Count (TPC)
- Serial dilution method
- Nutrient agar media

### 3.4 Procedure

1. Fish samples were homogenized
2. Serial dilutions prepared
3. Samples inoculated on agar plates
4. Incubated at  $37^{\circ}\text{C}$  for 24 hours
5. Colony forming units (CFU/g) counted

Aseptic measures and conditions were undertaken during the sampling procedure to prevent contamination of the samples; 25g of each sample was weighted and homogenized by a stomacher blender for 2min at 160 revolution/min with 225ml. of peptone water (ph 7.0). Each of three tubes was filled with nine milliliters of buffer saline solution (BS) of first tube to prepare  $10^{-1}$  dilutions. The 1ml was taken from the first tube and mixed to the second test tube to prepare  $10^{-2}$  dilutions[8]. The  $10^{-3}$  dilution was prepared by these subsequent dilution techniques. Then sealed in polyethylene bags to store in frozen condition at  $-20^{\circ}\text{C}$  for further analysis, After 4 days fish samples were taken out of the refrigerator and thawed at room temperature. Then 20 g of each sample was blended with 180 ml of sterile bacteriological peptone water in a stomacher blender. Then 1 ml of this  $10^{-1}$  dilution was transferred to a screw cap vial containing 10 ml of sterile dilute of bacteriological peptone to make a dilution of  $10^{-2}$ . Then the vial was shaken gently. This process was repeated progressively to prepare of  $10^{-3}$ .

### 3.5. Bacteriological Examination – Culture Medium Description:

Bacteriological examination involves the isolation and growth of microorganisms from samples using suitable culture media under controlled laboratory conditions. A culture medium is a nutrient-rich substance designed to support the growth, multiplication, and identification of bacteria[9]. For effective microbial analysis, especially in food samples like fish, the culture medium must contain several essential components that fulfill the nutritional and environmental requirements of microorganisms.

Firstly, the medium must include a **source of energy**, which is commonly provided in the form of carbohydrates such as glucose. In addition, it should supply **essential elements like carbon, nitrogen, sulfur, and phosphorus**, which are fundamental for the synthesis of cellular components such as proteins, nucleic acids, and enzymes. Carbon acts as a backbone for organic molecules, while nitrogen is crucial for amino acids and proteins[10]. Sulfur and phosphorus are important for enzyme function and genetic material formation.

Furthermore, the medium must contain **minerals** such as calcium ( $\text{Ca}^{2+}$ ), magnesium ( $\text{Mg}^{2+}$ ), and sodium ( $\text{Na}^+$ ), which play vital roles in maintaining cellular functions and enzyme activity. It should also include **vitamins and growth factors**, which are required by certain bacteria for proper growth and metabolic processes[11]. Lastly, **water** is an essential component, as it serves as a solvent for nutrients and facilitates biochemical reactions. Altogether, these components ensure optimal conditions for bacterial growth, enabling accurate bacteriological examination and analysis.

### 3.6. Culture Media and Bacteriological Procedure:

Various culture media were used for the bacteriological evaluation of fish samples to ensure accurate detection and enumeration of microorganisms. Bacteriological Peptone Water (BPW) was used for serial dilution of fish samples, while Plate Count Agar (PCA) served as the primary medium for determining the Total Bacterial Count (TBC). For the detection of specific pathogens, selective and enrichment media were employed: Alkaline Peptone Water (APW) and Thiosulphate Citrate Bile Salt Sucrose (TCBS) agar were used for the enrichment and isolation of *Vibrio* species[12]. Violet Red Bile (VRB) agar was utilized for the detection of *Escherichia coli*, whereas Lactose Broth (LB) and Buffer Peptone Water (BPW) were used as pre-enrichment and enrichment media for *Salmonella* species. Further selective enrichment was carried out using

Selenite Cystine Broth (SCB), followed by isolation on Xylose Lysine Deoxycholate (XLDA) agar, which helps in the selective growth of *Salmonella* while inhibiting other microorganisms. For the determination of total bacterial count, 25 g of fish muscle was aseptically blended with 225 ml of 0.1% peptone water using a sterile blender for 5 minutes. Serial decimal dilutions ( $10^{-1}$  to  $10^{-5}$ ) were prepared by transferring 1 ml of the homogenized sample into 9 ml of sterile peptone water. From the dilutions ( $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$ ), 1 ml aliquots were inoculated onto sterile agar plates, mixed thoroughly, and allowed to solidify. The plates were then incubated in an inverted position at 30°C for 72 hours. After incubation, colonies ranging between 30 and 300 were counted for accuracy, while plates from lower dilutions ( $10^{-1}$  and  $10^{-2}$ ) were excluded due to excessive colony growth. For media preparation, peptone water was prepared by dissolving 1 g in 1000 ml distilled water[13,14], and PCA was prepared by dissolving 11.75 g in 500 ml distilled water, followed by proper heating. Both media were sterilized by autoclaving at 121°C for 15 minutes before use.

### 3.7. Bacterial Identification and Biochemical Tests – Description

Bacterial identification in fish samples was carried out using culture and biochemical methods. In the first isolation step, samples were inoculated onto nutrient agar plates and incubated at **37°C for 24 hours**, allowing bacterial colonies to develop based on their growth characteristics such as shape, size, and color. Nutrient agar was prepared by dissolving **32 g of medium in 400 ml of distilled water**, followed by sterilization through autoclaving at **121°C under 15 psi pressure for 15 minutes**[15]. This ensured a sterile environment suitable for the growth and isolation of bacteria present in the fish samples. The observed colonies were then selected for further identification using biochemical tests.

Biochemical testing plays a crucial role in identifying bacteria, as each microorganism exhibits specific metabolic reactions that act like a “fingerprint.” The **catalase test** was performed to detect the presence of the catalase enzyme, which breaks down hydrogen peroxide into water and oxygen. A drop of **3% hydrogen peroxide** was placed on a slide, and a bacterial colony was added; the immediate formation of bubbles indicated a positive result (e.g., *Staphylococcus aureus*)[16,17]. The **indole test**, part of the IMViC series, was used to determine the ability of bacteria to degrade tryptophan into indole. After incubation, **Kovac’s reagent** was added, and the formation of a red ring on the surface indicated a positive result (e.g., *Escherichia coli*).

These tests were conducted following standard microbiological procedures to accurately identify bacterial species present in the samples.

#### 4. Results:

The microbiological analysis revealed a clear difference in bacterial load between fresh and frozen Nanjil fish (*Plectropomus pessuliferus*). The total bacterial count (TBC) for fresh fish was recorded as  $5.68 \times 10^5$  CFU/g, whereas the frozen samples showed a lower count of  $4.38 \times 10^5$  CFU/g after four days of storage[18,19]. This indicates that fresh fish harbors a higher microbial load compared to frozen fish, even within a short storage duration.

The higher bacterial count observed in fresh fish can be attributed to favorable environmental conditions such as moisture content, temperature, and nutrient availability, which promote rapid microbial growth[20]. In contrast, freezing significantly slows down microbial activity by reducing enzymatic reactions and inhibiting bacterial multiplication. Although freezing does not completely eliminate microorganisms, it effectively suppresses their growth, resulting in comparatively lower bacterial counts in frozen samples.

Furthermore, the results demonstrated a statistically significant difference between fresh and frozen fish samples, confirming the effectiveness of freezing as a preservation method. The reduction in microbial load in frozen fish suggests improved safety and extended shelf life compared to fresh fish stored under refrigeration. These findings emphasize the importance of proper storage techniques in minimizing bacterial contamination and maintaining the quality of fish for safe consumption.

##### 4.1 Microbial Load (CFU/g):

The microbial load of Nanjil fish (*Plectropomus pessuliferus*) showed a progressive increase over the storage period in both fresh and frozen conditions, with a significantly higher rise observed in fresh fish. On **Day 0**, fresh fish exhibited a low bacterial count of  $10^3$  CFU/g, while frozen fish showed a very low count of  $10^2$  CFU/g, indicating good initial quality and minimal contamination at the time of sampling.

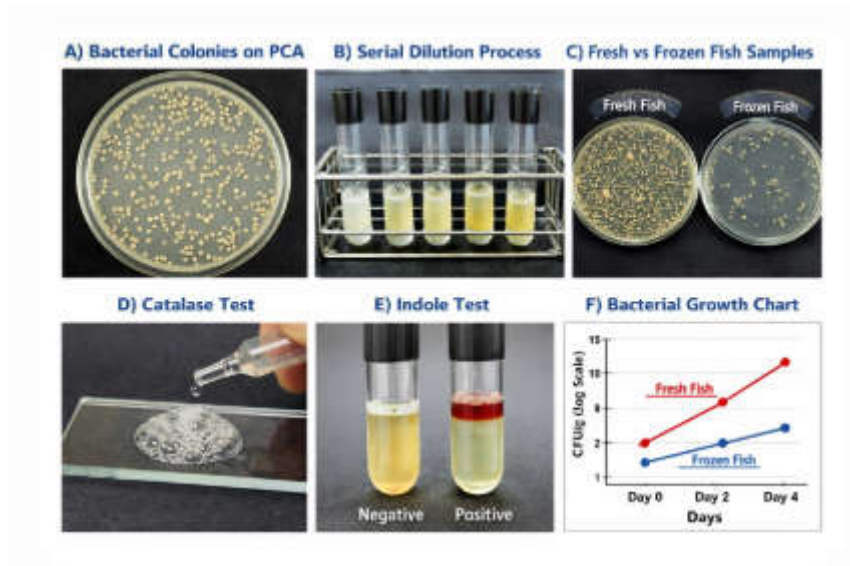


Fig.2. Comparative Analysis of Bacterial Load in Fresh and Frozen Fish During Storage

By **Day 2**, the microbial load in fresh fish increased rapidly to a moderate level of  $10^5$  CFU/g, whereas frozen fish showed only a slight increase to  $10^3$  CFU/g, remaining within a low contamination range. This difference highlights the effect of freezing in slowing down bacterial growth compared to fresh storage conditions, where microbes multiply more quickly due to favorable environmental factors.

On **Day 4**, fresh fish reached a high bacterial load of  $10^7$  CFU/g, indicating significant spoilage and reduced suitability for consumption. In contrast, frozen fish showed a moderate increase to  $10^4$  CFU/g, demonstrating controlled microbial growth. Overall, the data clearly indicate that freezing effectively delays bacterial proliferation and helps maintain fish quality for a longer period compared to fresh storage.

Day	Fresh Fish	Frozen Fish
Day 0	Low ( $10^3$ CFU/g)	Very Low ( $10^2$ CFU/g)
Day 2	Moderate ( $10^5$ CFU/g)	Low ( $10^3$ CFU/g)
Day 4	High ( $10^7$ CFU/g)	Moderate ( $10^4$ CFU/g)

Table.1. Comparative Microbial Growth in Fresh and Frozen Fish (Day 0–Day 4)

**5. Discussion:**

- Fresh fish showed rapid microbial growth due to favorable conditions for bacteria.

- Frozen fish had significantly slower microbial increase because low temperature inhibits bacterial activity.
- By Day 4, fresh fish reached unsafe microbial levels, while frozen fish remained within acceptable limits.
- Freezing does not completely eliminate bacteria but slows their growth.

## **6. Conclusion:**

The present study demonstrates that storage conditions play a critical role in determining the microbial quality and safety of Nanjil fish. Fresh fish exhibited a rapid increase in bacterial load within a short period, indicating that it should be consumed within 1–2 days when stored under refrigeration to avoid spoilage and potential health risks. In contrast, frozen fish showed significantly slower microbial growth, confirming that freezing is an effective preservation method that can extend the shelf life to four days or more. Overall, maintaining proper storage conditions, including adequate temperature control and hygiene, is essential to preserve fish quality, minimize bacterial contamination, and ensure consumer safety. The results of this study highlight the significant influence of storage conditions on the microbial quality of fish. Fresh fish exhibited rapid microbial growth due to favorable environmental conditions, leading to a sharp increase in bacterial load over time. By Day 4, the microbial levels in fresh fish reached unsafe limits, indicating spoilage and reduced suitability for consumption. In contrast, frozen fish showed a much slower increase in microbial load, as low temperatures inhibit bacterial activity and delay their multiplication. Even by Day 4, the bacterial levels in frozen fish remained within acceptable limits, demonstrating better preservation compared to fresh storage. However, it is important to note that freezing does not completely eliminate microorganisms; it only suppresses their growth. Overall, the study concludes that freezing is an effective method for extending the shelf life and maintaining the safety of fish, while fresh fish should be consumed quickly to avoid health risks associated with microbial contamination.

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