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Research Note

Identification and Characterization of *Aeromonas* spp. as a Specific Spoilage Organism in Chilled Storage of Rohu fish (*Labeo rohita*): Implications for Preservation Strategies

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Abstract

Rohu (*Labeo rohita*) is regarded as the most preferred carp in India. However, maintaining its freshness during transport is challenging. Chilling is commonly used for short-term preservation, but spoilage bacteria can remain active in ice, compromising fish quality. This study aims to identify specific spoilage bacteria (SSB) in rohu during 14-day period of chilled storage. The 16S rRNA sequencing of isolates from chilled rohu identified *Aeromonas jandaei* as the dominant bacterial species. Further, *A. jandaei* spiked rohu samples confirmed it as the SSB. The study findings highlight *A. jandaei* as SSB in chilled rohu, which can help processors to control its growth through better control of temperature, packaging, or use of preservatives.

Keywords: *Aeromonas jandaei*, rohu, chilling, TMA, microorganisms, SSB

Introduction

Fish is a highly nutritious source of dietary protein. It is also one of the most perishable commodities that require quick storage, careful handling, and efficient distribution. The US FDA and EU recommend maintaining temperatures between 0–4°C to inhibit microbial growth and prevent spoilage (FDA, 2017). Thus, using ice or chilled storage is the most practical and cost-effective method for preserving raw fish. Low temperatures significantly suppress the growth of bacteria, mold, and other microorganisms that cause spoilage (Erkmen, 2016). Previous studies have demonstrated that chilled storage alone or coupled with specific additives can extend the shelf life of fish and fishery products (Banerjee & Maheswarappa, 2019). Generally, initial spoilage begins enzymatically, followed by bacterial growth. Hence, identifying specific spoilage bacteria (SSB) is key to maintaining quality and shelf life (Snyder, Martin & Weidmann, 2024). Specifically, psychrotrophic bacteria, which thrive at low temperatures, are the main spoilage agents in refrigerated meat (Taormina, 2021). In this investigation, the identification of SSB may facilitate the management of potential threats from the development of microorganisms responsible for spoilage during chilled storage. Food processors can control the development of SSBs during chilling by adjusting the temperature, using modified packaging, or adding preservatives. Thus, it is crucial to identify and understand the development pattern of specific microorganisms to implement effective control measures that ensure the safety and quality of food products. Rohu, a key aquaculture species in India, is widely transported under iced conditions. This study focuses on identifying SSB in chilled rohu, facilitating shelf-life extension and quality control during low-temperature storage.

Material and Methods

The study consisted of two parts. The first part aimed to identify SSB by examining the progression of spoilage bacteria during 14 days of storage in ice. Representative colonies from different storage stages

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were analyzed using both biochemical and molecular methods. Bacteria that consistently increased in number and became dominant by the end of the experiment were identified as SSB. In the second phase, the identified SSB was inoculated into fish during ice storage. A faster rate of spoilage and higher bacterial growth, compared to control, confirmed the spoilage potential of the identified SSB (Fig. 1).

Live rohu weighing between 1000 and 1050 g were procured from a local fish market in Nalasopara, Mumbai, India. The fish were stunned, washed with ice-cold water, and packed in thermally insulated (expanded polystyrene) storage containers with alternating layers of fish and flake ice in a 1:1 ratio. The ice was replaced daily, and the samples were stored for up to 14 days. Samples were collected on days 1, 3, 5, 7, 9, 11, and 14 for sensory, chemical, and microbiological analyses. Following the identification of SSB in Phase I, a new set of fish was used in Phase II, stored under similar conditions and inoculated with the identified SSB at 10^3 cfu/g for the inoculation study (Gkana, Chorianopoulos, Grounta, Koutsoumanis, & Nychas, 2017). Samples were analyzed using the same methods as in phase I.

Microorganisms were isolated from samples collected on different days, as described earlier (Joshi, Nayak, Balange, Kumar, & Manjusha, 2025). Briefly, tissue samples from stored fish were crushed in physiological saline solution, serially diluted and spread on agar plates. The inoculated plates were incubated at 37°C for 36-48 h to allow microbial growth. Total plate count (TPC) was determined using the spread plate technique on nutrient agar, according to previous reports (Emire & Gebremariam, 2009). Selected colonies were further evaluated using specific biochemical tests for classification. In addition, the spoilage potential of the suspected SSB was also evaluated by performing proteolytic and lipolytic tests.

The biochemical characteristics, including pH, Trimethylamine (TMA) (Huss et al.,1995; Malle & Tao, 1987), Total Volatile Basic Nitrogen (TVB-N) (Beatty & Gibbons, 1937), Peroxide value (PV) (Andina, Riyanto, & Rohman, 2017) Free fatty acids (FFA) (Rathod & Pagarkar, 2013) and Thiobarbituric acid (TBA) (Tarladgis, Watts, Younathan, & Dugan, 1960) were assessed to measure the spoilage process over time. Additionally, sensory analysis was done based on the hedonic scale sensory scorecard (Rathod & Pagarkar, 2013).

DNA extraction and PCR amplifications were performed as described earlier (Joshi et al., 2025). Briefly, DNA was extracted using the lysate method using TE buffer, with minor modifications (Muhammed, Bindu, Jini, Prashanth, & Bhaskar, 2015). The DNA fragments were amplified using the 16S rRNA of selected isolates. This study used a universal primer set for amplification, as described previously (DeLong, 1992). The primers used were 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-TACGGTTACCTTGTTACGACTT-3'.

PCR product purification was performed using the GeneJet Gel Extraction Kit (Thermo Fisher Scientific, India) following the manufacturer's instructions. DNA (90/ µL) was purified using a column-based extraction method involving binding buffer and

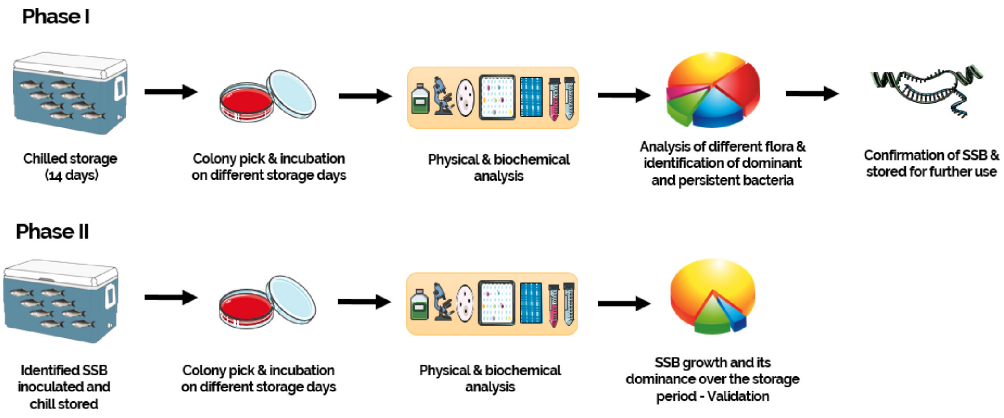

Fig. 1. Experimental workflow illustrating the identification and confirmation of specific spoilage bacteria in chilled rohu.

centrifugation (SLM-MCF-14K, GeNei, India). The eluted DNA was sequenced by Eurofins, Bangalore. The resulting sequences were converted to FASTA format and analyzed using BLAST, with the highest match used to identify the bacterial species.

Results and Discussion

The total plate count (TPC) in chill-stored rohu rose from 3×10^3 cfu/g on day 1 to 8.7×10^8 cfu/g by day 14 (Table 1). The bacterial load increased gradually until day 7, followed by a sharp rise, indicating the onset of spoilage. Biochemical analysis identified five genera: *Aeromonas*, *Pseudomonas*, *Yersinia*, *Shigella*, and *Bacillus*. Further, BLAST analysis of 16S rRNA sequences confirmed the bacterial species. *Bacillus*, *Yersinia*, and *Pseudomonas* spp. made up 66% of the flora on day 1 but decreased to 7% by day 9. *Aeromonas* spp. increased from 33% to 100% by day 11, becoming the dominant species. Further, to qualify as an SSB in protein-rich foods like fish, bacteria must exhibit proteolytic or lipolytic activity. *Aeromonas* isolates, identified as *A. jandaei*, showed both gelatinase and lipase activity, confirming them as the SSB in ice-stored rohu.

Table 1. Changes in bacteria in ice-stored rohu

Sampling Day	TPC count (cfu/g)	% contribution	Biochemically identified genus	Molecularly identified spp. (% Seq. match)	Accession No. (Gen Bank)
1	3×10^3	33.33	*Aeromonas* spp.	ND	-
		40.00	*Shigella* spp.	ND	-
		26.66	*Bacillus* spp.	ND	-
3	6×10^3	33.33	*Aeromonas* spp.	*Aeromonas jandaei* (92.46%)	OR678534.1
		11.66	*Shigella* spp.	*Shigella sonnei* (100%)	CP053751.1
		25.00	*Bacillus* spp.	*Bacillus firmicutes* (92.46%)	KY849501.1
		08.33	*Yersinia* spp.	*Yersinia ruckeri* (92.77%)	MN505172.1
		21.66	*Pseudomonas* spp.	*Pseudomonas* spp. (98.34%)	MN945258.1
5	1.40×10^5	50.00	*Aeromonas* spp.	*Aeromonas jandaei (97.45%)*	OR678534.1
		09.28	*Shigella* spp.	*Shigella sonnei* (96.60%)	CP053751.1
		18.57	*Bacillus* spp.	*Bacillus firmicutes* (78.81%)	KY849501.1
		05.71	*Yersinia* spp.	*Yersinia ruckeri* (90.06%)	MN505172.1
		16.42	*Pseudomonas* spp.	*Pseudomonas* spp. (98.34%)	MT576536.1
7	2.30×10^5	69.56	*Aeromonas* spp.	*Aeromonas jandaei* (97.45%)	OR678534.1
		04.78	*Shigella* spp.	*Shigella sonnei* (94.69%)	CP053751.1
		12.17	*Bacillus* spp.	*Bacillus firmicutes* (87.10%)	KY849501.1
		03.04	*Yersinia* spp.	*Yersinia ruckeri* (89.17%)	MN505172.1
		10.43	*Pseudomonas* spp.	*Pseudomonas* spp. (100%)	MT576540.1
9	4.6×10^8	93.87	*Aeromonas* spp.	*Aeromonas jandaei* (94.89%)	OR678534.1
		01.14	*Shigella* spp.	*Shigella sonnei* (94.44%)	CP053751.1
		02.14	*Bacillus* spp.	*Bacillus firmicutes* (85.48%)	KY849501.1
		00.59	*Yersinia* spp.	*Yersinia ruckeri* (89.17%)	MN505172.1
		02.24	*Pseudomonas* spp.	*Pseudomonas* spp *(96.81%)*	MT576536.1
11	6.4×10^8	100.00	*Aeromonas* spp.	*Aeromonas jandaei (97.45%)*	OR678534.1
14	8.7×10^8	100.00	*Aeromonas* spp.	*Aeromonas jandaei (97.45%)*	OR678534.1

ND: Not detected

Sensory quality declined after day 5, with early spoilage signs appearing by day 7. By day 9, observations such as muddy odour, dark gills, and soft flesh indicated that the fish had reached rejection limits. By day 14, strong decay odour, yellow belly, and severe texture loss confirmed advanced spoilage, making the fish unfit for consumption. Besides, rohu's pH dropped from 6.78 to 6.19 over 14 days. Spoilage indicators (TMA, TVBN, PV, FFA, TBA) rose notably from day 3, marking the onset of spoilage. By day 9, these values exceeded freshness limits, indicating significant quality loss linked to increased bacterial load (Fig. 2)

Inoculation studies are essential for understanding spoilage under realistic storage conditions. Rohu was chilled and spiked with *A. jandaei* at 10^3 cfu/g and during storage, bacterial growth increased from 3×10^3 to 1.28×10^9 cfu/g by day 14. These results confirmed the sustained dominance of *A. jandaei* throughout the study, effectively suppressing the growth of other microbes, as shown in Table 2.

In this study, the pH decreased from 6.22 ± 0.04 to 6.02 ± 0.13 by the 14th day of storage, which is close to pH 6, potentially promoting protein denaturation. The study revealed that all measured spoilage indicators like TMA, TVBN, PV, TBARS, and FFA exceeded acceptable freshness limits more rapidly in the inoculated group compared to the control.

Chilling is a viable method for preserving fish, but bacterial contamination remains a significant concern during chilled storage. To ensure proper preservation over extended periods, ice must be regularly replenished as it melts. However, the growth of psychrotrophic bacteria poses a significant challenge during storage (Yeasmin, Reza, Shikha, Khan, & Kamal, 2010). They produce extracellular enzymes that degrade proteins and lipids (Brasca et al., 2017; Wei, Wang, Sun, & Pu, 2019), ultimately reducing food quality. Previous reports suggest that identifying the main SSB is essential for developing effective control strategies to combat psychrotrophic spoilage in chilled products. This study identified the spoilage microbes in *L. rohita* during chilled storage, with a rapid increase in bacterial load observed from day 3 (Duarte, Silva, Pinto, Barroso, & Gil, 2020). The investigations revealed that the chill-stored rohu harbored *Bacillus, Aeromonas, Shigella, Pseudomonas,* and *Yersinia* spp., with *Aeromonas* spp., dominating after day 5. Sequence analysis confirmed *A. jandaei* dominance in the late spoilage stages, coinciding with rapid changes in spoilage indicators, suggesting its role as the SSB. Also, its protein and lipid degradation ability supported its role as SSB. This may be attributed to its ability to secrete bacteriocin-like substances, cold-active enzymes, and biofilm formation, which enhance colonization and suppress competitors, especially at 0–4°C (Ghanbari & Jami, 2013). In support, 36 *Aeromonas* species have been

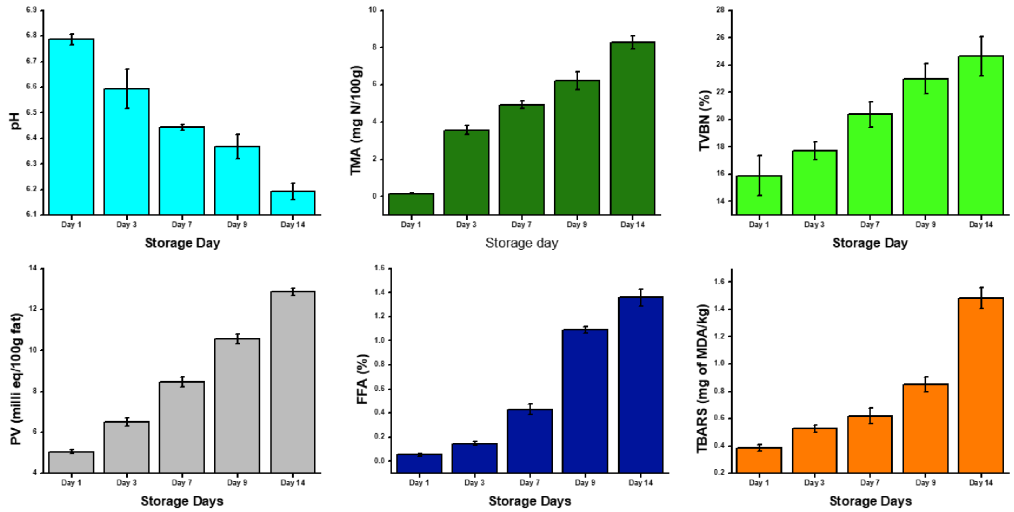

Fig. 2. Measurement of markers of decomposition during chilled storage. Data are presented as mean ± SEM (n = 3). TMA, Trimethylamine; TVBN, Total Volatile Basic Nitrogen; PV, Peroxide value; FFA, Free fatty acids; TBARS, Thiobarbituric Acid Reactive Substances.

Table 2. Total count of *A. jandaei* during chilled storage following inoculation of *A. jandaei* 10^3 cfu/g

Storage Day	Species	Count (cfu/g)	~% to Total Count (TC)
Day 1	*Aeromonas jandaei*	1×10^3	100
	Other spp.	0	0
Day 3	*Aeromonas jandaei*	2×10^3	66
	Other spp.	1×10^3	33
Day 5	*Aeromonas jandaei*	9×10^4	75
	Other spp.	3×10^4	25
Day 7	*Aeromonas jandaei*	2.1×10^5	77
	Other spp.	6×10^4	23
Day 9	*Aeromonas jandaei*	6.8×10^8	94
	Other spp.	4×10^7	6
Day 11	*Aeromonas jandaei*	1.03×10^9	100
	Other spp.	0	0
Day 14	*Aeromonas jandaei*	1.28×10^9	100
	Other spp.	0	0

TC: Total count on respective day; Other species include *Bacillus* spp., *Yersinia* spp., and *Pseudomonas* spp., but their precise count is not mentioned as it was determined in the phase I of the study.

linked to fish spoilage (Fernandez-Bravo & Figueras, 2020), with *A. jandaei* recognized as one of the major SSB in chilled storage across multiple fish species. Collectively, identifying *A. jandaei* as the SSB highlights the need to control spoilage bacteria. However, fish quality largely depends on prompt post-harvest handling. Delayed chilling accelerates microbial growth and tissue decay. Therefore, strict hygiene and temperature control during handling and transport are essential to minimize spoilage and extend shelf life (Getu, Misganaw, & Bazezew, 2015; Sone, Skara, & Olsen, 2019). Identification of SSB helps to customize the packaging that minimizes the SSB growth. Specifically, nanoencapsulation enables controlled release of natural antimicrobials via nanocarriers, enhancing their stability (Chadha, 2021).

Together, this study identifies *A. jandaei* as the SSB in *L. rohita* during chilled storage, highlighting its ability to thrive at 0°C and dominate the microbial community. Its proteolytic and lipolytic activities contribute to spoilage by breaking down fish muscle components. Inoculation studies confirmed its spoilage potential. However, the study has certain limitations, including reliance on a single fish species, analysis of a limited number of isolates, and the use of non-selective media. Future research should include multiple species and employ advanced methods. However, understanding *A. jandaei* as SSB offers a basis for targeted spoilage control. Integrating eco-friendly strategies, such as antimicrobial-loaded biodegradable films, can help reduce spoilage and extend the shelf life of fish products, particularly rohu, a widely consumed fish in the Indian subcontinent.

Ethical approval

The experiment and subsequent handling and sampling of the experimental fish were carried out as per the Guidelines of ICAR-Central Institute Fisheries Education.

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