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Research Article

EVALUATION OF MICROBIOLOGICAL TRAITS, ANTIBIOTIC RESIDUE AND FORMALDEHYDE RESIDUE BY TRACKING SEASONAL VARIATIONS IN A SELECTED FISH SPECIES COLLECTED FROM DIFFERENT FISH MARKETS IN PUNJAB, INDIA

Pargi Narendrakumar Arjunsinh[1], Surjya Narayan Datta[1*], Anuj Tyagi[2]

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ABSTRACT: The study aimed to assess microbiological parameters, antibiotic concentrations, and formaldehyde residues in four fish species - *Wallago attu*, *Labeo rohita*, *Pangasianodon hypophthalmus*, and *Rastrelliger kanagurta* - sourced from both inland and marine sectors, encompassing culture and capture fisheries. These fish were collected from the Amritsar, Jalandhar, Ludhiana, Mohali, and Bathinda fish markets in Punjab, India, over four distinct seasons (post-monsoon, winter, pre-monsoon, and monsoon) from September 2021 to August 2022. The study focused on evaluating Total Plate Count (TPC), *Escherichia coli*, and *Staphylococcus aureus*. Additionally, it investigated antibiotic residues and formaldehyde presence to ensure consumer safety. TPC levels in various fish species ranged from 1.19-3.22×10^4 cfu/g across different seasons and markets. Elevated levels of *E. coli* (2.46 - 4.46×10^2 cfu/g) and *S. aureus* (2.33 - 4.73×10^3 cfu/g), exceeding permissible limits, presented a notable public health concern. The monsoon season recorded the highest microbiological counts in all fish species sampled. Bathinda's fish market had higher average microbiological values compared to the other markets, potentially due to factors like ice quality, hygiene standards during packaging, and storage and transportation processes. In terms of antibiotic residues, the concentrations of chloramphenicol (0.001 - 0.019 µg/kg), nitrofurantoin (0.001 - 0.026 µg/kg), and oxytetracycline (0.001 - 0.028 µg/kg) in the selected species were found to be below permissible limits, ensuring the fish's safety for consumption. No formaldehyde residues were detected in any of the fish species across all seasons and markets, further confirming their safety. Addressing bacterial contamination through scientific interventions remains essential for public health and environmental sustainability.

Keywords: Fish markets, Commercial fish, Antibiotic residue, Formaldehyde residue, Consumer safety.

INTRODUCTION

Aquatic ecosystems reflect potential fish diseases through the prevalence of general contamination. Bacterial presence within fish typically manifests in three primary regions: the gills, intestines, and outer slime layer. The vulnerability of fish to bacterial contamination arises at various stages of handling, processing, and transportation [1]. The degradation process in harvested fish initiates promptly following capture. Bacteria responsible for spoilage rapidly infiltrate the muscle tissue of fish. To mitigate the susceptibility to bacterial growth and ensuing spoilage, meticulous attention is required during handling procedures. Particularly, vigilance is necessary in subtropical regions due to the elevated ambient temperatures that can precipitate the swift proliferation of bacteria within fish if the cold chain is not followed. Implementing and maintaining cold chain conditions play a pivotal role in retarding enzymatic and biochemical activities, along with microbial growth inhibition. This, in turn, extends the shelf life of fish [2]

Aquaculture operations have commonly integrated antibiotics for both preventive and therapeutic purposes. A notable challenge encountered by the shrimp and

[1]*Department of Fisheries Resource Management,* [2]*Department of Aquatic Environment, College of Fisheries, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004 , Punjab, India.*
**Corresponding author. e-mail: surjya30740@gmail.com*

fish industries revolves around the outbreak of bacterial-induced diseases. These infectious ailments perpetually pose a substantial risk, leading to considerable stock losses and concerns regarding animal welfare [3]. To address these concerns, antibiotics have become an indispensable tool in managing infectious diseases within the realm of aquaculture. Their utilization extends across various aquatic species, including fish and shrimp, in India and globally.

However, the implementation of antibiotics demands careful consideration due to their potential repercussions. While antibiotics are instrumental in curing infections, their residues could adversely influence the quality of the final product if not administered judiciously [4]. If antibiotics are used indiscriminately, the resulting residues might infiltrate edible tissues, thereby posing hazards to public health. Consequences may encompass allergies, toxic manifestations, disruptions in intestinal microbial balance, and the emergence of drug-resistant strains. Consumption of antibiotic residues by individuals can also disrupt gut ecology, promoting the development of antibiotic-resistant microorganisms. Furthermore, antibiotic residues can contribute to the onset of ailments like "grey syndrome," a condition characterized by cyanosis and cardiovascular collapse, particularly in neonates [5]. Beyond health concerns, the presence of antimicrobial residues can diminish the market value and export potential of aquaculture products. Given these potential hazards linked with the occurrence of these compounds within edible tissues, diligent attention to this form of contamination is imperative [6].

A well-balanced diet should incorporate fish and seafood, which are excellent sources of protein. However, these aquatic foods are inherently susceptible to microbial spoilage and post-harvest metabolic changes due to their composition of fats, free amino acids, and high water content. Because of their perishable nature, fish and seafood can only be maintained in a fresh state for a limited duration when stored on ice. Unfortunately, fishermen and fish dealers frequently resort to the imprudent use of formaldehyde as a preservative agent to extend the shelf life of fish and shellfish. Formaldehyde, the simplest aldehyde compound, is notorious for its reactivity. It exists in two forms: gaseous formaldehyde and liquid formalin [7]. Characterized by its colourless appearance, pungent odor, eye irritant properties, toxicity, and flammability, formaldehyde is often produced through the oxidation of methanol. It finds common use as a disinfectant and a preservative agent for organic materials [8].

Formaldehyde has been classified as a Group 1 human carcinogen by the International Agency for Research on Cancer (IARC) [9]. In addition to being naturally produced through enzymatic processes in fish and seafood, formaldehyde can also result from biochemical reactions such as lipid oxidation triggered by microbial activity. These reactions may cause physical damage to the fish or produce undesirable compounds like biogenic amines [10]. To extend the shelf life of fish during transportation, some fish traders use formalin by injecting or spraying it onto the fish before it reaches domestic distribution channels. Formalin remains widely used in the fish trade due to its low cost, availability, and ease of application [11].

This study aimed to assess the microbiological quality, antibiotic concentration, and formaldehyde residues in four fish species - *Wallago attu*, *Labeo rohita*, *Pangasianodon hypophthalmus*, and *Rastrelliger kanagurta* - sourced from the fish markets of Amritsar, Jalandhar, Ludhiana, Mohali, and Bathinda in Punjab, India. The study was performed from September 2021 to August 2022 covering different seasons.

MATERIAL AND METHODS

Selection of fish markets, fish species, and sample collection

Fish markets in five districts of Punjab, India, namely Amritsar (31°37' 50.969"N, 74°52' 30.93"E), Jalandhar (31°20' 2.605"N, 75°34' 13.205"E), Ludhiana (30°55' 17.501"N, 75°54' 5.642"E), Mohali (30°43' 49.67"N, 76°42' 2.46"E), and Bathinda (30°12' 29.399"N, 74°57' 56.785"E) were selected. The data was collected over a one-year period, from September 2021 to August 2022. Sampling was carried out at regular intervals to cover different seasons: post-monsoon (October to December), winter (January to March), pre-monsoon (April to June), and monsoon (July to September). The selection process took into consideration the marketing of fish obtained from both culture and capture fisheries in Punjab, as well as fish imported from other states. *L. rohita* (culture fishery), *P. hypophthalmus* (culture fishery), *W. attu* (capture fishery), and *R. kanagurta* (capture fishery) muscle samples were collected in fresh condition from markets in sterile containers and transported in an insulated box to the laboratory. Microbiological parameters were analyzed immediately after the collection of samples. Samples for analysis of antibiotics and formaldehyde residuals were preserved at - 20^{0}C till further analysis.

Analysis of samples for microbial load

This present study encompassed an evaluation of key microbiological parameters, including Total plate count, and enumeration of *Escherichia coli* and *Staphylococcus aureus* following the standard methods of APHA [12].

Total plate count (TPC)

Tryptone soya broth (soybean casein digest medium) (Merck, Molecular grade) and agar powder (Merck, Bacteriological grade) were used for the enumeration of total plate count. 1ml each of the required dilutions was transferred in separated serial petri dishes in duplicate. 18-20 ml of sterile tryptone soya broth and agar powder was spread on Petri dishes, mixed well, and allowed to solidify. Incubation of petri dishes was done at 37 ± 1ºC for 48 hours. After 48 hours the colonies were counted.

Calculation

TPC (CFU/g) = Average count x Reciprocal of the dilution x Correction factor

Enumeration of *Escherichia coli*

MacConkey's Agar (Merck, Bacteriological Grade) was used for the enumeration of *E. coli.* 1 ml of each of the required dilutions was transferred in separated serial petri dishes in duplicates. 18-20 ml of sterile MacConkey Agar was spread on Petri dishes, mixed well, and allowed to solidify. Incubation of Petri dishes was done at 37 ± 1ºC for 48 hours. After 48 hours the colonies were counted.

Calculation

E. coli (CFU/g) = Average count × Reciprocal of the dilution × Correction factor.

Enumeration of *Staphylococcus aureus*

Baird-Parker agar medium (Merck, Bacteriological Grade), egg yolk emulsion, and potassium tellurite solution (Merck, Molecular grade) were used for enumeration of *S. aureus*. 1 ml each from the required dilutions were transferred in separated serial petri dishes in duplicates. 18-20 ml of sterile Baird-Parker agar medium, egg yolk emulsion, and potassium tellurite solution were spread on Petri dishes, mixed well, and allowed to solidify. Incubation of petri dishes was done at 37 ± 1ºC for 48 hours. After 48 hours the colonies were counted.

Calculation

S. aureus (CFU/g) = Average count × Reciprocal of the dilution × Correction factor.

Assessment of antibiotic residues

In the context of Indian aquaculture practices, antibiotics such as Oxytetracycline, Nitrofurantoin, and Chloramphenicol are commonly employed. Consequently, the presence of residue from these antibiotics within fish and fisheries products cannot be discounted. Thus, residues of Chloramphenicol, Nitrofurantoin, and Oxytetracycline in the edible muscle of four designated fish species *W. attu*, *L. rohita*, *P. hypophthalmus*, and *R. kanagurta* across Amritsar, Jalandhar, Ludhiana, Mohali and Bathinda fish markets during various seasons were assessed. Antibiotic residue in fish flesh was assessed by commercially available enzyme-linked immune sorbent assay (ELISA) kits, *i.e.* EuroProxima Oxytetracycline (code no. 5091OTC), Ridascreen Chloramphenicol (code no. R1511) and Ridascreen Nitrofuranamoz (code no. R3722)[13].

Estimation of Chloramphenicol and Nitrofurantoin

A volume of 50 µl from each standard or prepared sample was added to separate duplicate wells. Then, 50 µl of the conjugate was introduced to each well and incubated for 30 minutes at room temperature. The wells were subsequently washed with 250 µl of washing buffer, and the liquid was discarded. Next, 100 µl of substrate/chromogen solution was added to each well, followed by a 15-minute incubation in a dark place at room temperature. Finally, 100 µl of stop solution was applied to each well, and the absorbance was measured at 450 nm.

Calculation

Chloramphenicol concentration (µg/l) =
concentration read from the standard curve × 0.25

Nitrofurantoin concentration (µg/l) = concentration read from the standard curve × 2

Estimation of oxytetracycline

Each well was filled with 300 µl of rinsing solution. Then, 100 µl of sample dilution buffer was added in duplicate to wells H1, and H2 (blank), and 50 µl of sample dilution buffer was pipetted in duplicate into wells A1 and A2. Fifty microliters of each standard solution were also pipetted in duplicate into the respective wells. The remaining wells received 50 µl of each sample solution in duplicate. Additionally, 50 µl of conjugate was pipetted into all wells except for H1 and H2. The microtiter plate was then sealed and incubated for one hour in the dark at room temperature. After incubation, the solution was discarded, and the plate was washed three times with rinsing buffer. Subsequently, 100 µl of substrate solution was added to each well, and the plate was incubated for approximately 30 minutes in the dark at room temperature. Finally, 100 µl of stop solution was added to each well, and absorbance values were immediately measured at 450 nm.

Calculation:

Oxytetracycline concentration (µg/l) = concentration read from the standard curve × 20.

Determination of formalin in selected fish

To detect formalin residues, the 'HiRapid Formalin Test Kit,' developed by ICAR-Central Institute of Fisheries Technology, Kochi, and commercialized by HiMedia Laboratories Pvt. Ltd., was used. A reagent strip (F-1) was removed from the bottle and swabbed on the fish surface or cut portions of the fish 3 to 4 times across different areas. One drop of reagent F-2 was then added to the swabbed strip, and the result was observed after 1.5 to 2 minutes. The resulting colour was compared with the reference colour chart provided in the kit [14]. Statistical analysis was performed using SPSS version 20 software.

RESULTS AND DISCUSSION

Total plate count (TPC) in selected fish

The Total plate count (TPC) for *Wallago attu* ranged from 2.23 to 3.40 × 10 cfu/g across different seasons and markets (Table 1). The lowest TPC in the Amritsar market was 2.23 × 10^4 cfu/g during the post-monsoon season, with the highest reaching 2.96 × 10^4 cfu/g during the monsoon. In Jalandhar, the TPC ranged from 2.60 × 10^4 cfu/g post-monsoon to 3.26 × 10^4 cfu/g in the monsoon. Similarly, in Ludhiana, the lowest value was 2.30 × 10^4 cfu/g post-monsoon, while the highest was 2.76 × 10^4 cfu/g during the monsoon. The Mohali market recorded a TPC range of 2.36 × 10^4 cfu/g post-monsoon to 2.93 × 10^4 cfu/g during the monsoon. In Bathinda, TPC ranged from 2.76 × 10^4 cfu/g post-monsoon to 3.40 × 10^4 cfu/g during the monsoon. Statistical analysis showed no significant seasonal fluctuations in TPC ($p > 0.05$).

For *L. rohita*, the TPC ranged between 1.56 and 3.50 × 10^4 cfu/g across seasons and markets (Table 1). In the Amritsar market, the lowest TPC was 1.56 × 10^4 cfu/g during both the post-monsoon and winter seasons, while the highest was 2.23 × 10^4 cfu/g during the monsoon. Statistical analysis found no significant seasonal differences ($p>0.05$). In Jalandhar, TPC ranged from 2.83 × 10^4 cfu/g post-monsoon to 3.23 × 10^4 cfu/g during the monsoon. In Ludhiana, the TPC was 1.73 × 10^4 cfu/g in both the post-monsoon and winter seasons, with the highest value at 2.36 × 10^4 cfu/g in the monsoon. In Mohali, TPC ranged from 2.73 × 10^4 cfu/g post-monsoon to 3.13 × 10^4 cfu/g during the monsoon. Similarly, Bathinda recorded a TPC range of 3.03 × 10^4 cfu/g post-monsoon to 3.50 × 10^4 cfu/g during the monsoon. Seasonal variation in TPC was not statistically significant ($p>0.05$). The TPC for *P. hypophthalmus* varied between 1.92 × 10^4 and 3.60 × 10^4 cfu/g across different seasons and markets (Table 1). In Amritsar, the lowest TPC was 2.43 × 10^4 cfu/g during the post-monsoon season, while the highest was 3.00 × 10^4 cfu/g in the pre-monsoon. Statistical analysis revealed no significant seasonal differences in TPC ($p>0.05$). In Jalandhar, the TPC ranged from 3.00 × 10^4 cfu/g post-monsoon to 3.26 × 10^4 cfu/g during the pre-monsoon and monsoon seasons. Ludhiana showed a TPC range of 1.92 × 10^4 cfu/g post-monsoon to 3.16 × 10^4 cfu/g in the pre-monsoon. In Mohali, the lowest TPC was 2.73 × 10^4 cfu/g post-monsoon, while the highest was 3.20 × 10^4 cfu/g during the pre-monsoon. In Bathinda, the TPC ranged from 2.96 × 10^4 cfu/g post-monsoon to 3.60 × 10^4 cfu/g during the monsoon season. Seasonal variability in TPC was not statistically significant ($p>0.05$).

The Total plate count (TPC) for *R. Kanagurta* ranged from 0.87 × 10^4 to 1.86 × 10^4 cfu/g across different seasons and markets (Table 1). In the Amritsar fish market, TPC values ranged from a low of 0.87 × 10^4 cfu/g in the post-monsoon season to a high of 1.30 × 10^4 cfu/g during the monsoon. No significant seasonal variations in TPC were observed ($p>0.05$). In Jalandhar, TPC values ranged from 1.30 × 10^4 cfu/g post-monsoon to 1.53 × 10^4 cfu/g in the monsoon. Ludhiana saw a TPC range from 1.13 × 10^4 cfu/g post-monsoon to 1.53 × 10^4 cfu/g during the monsoon. In Mohali, TPC values varied from 1.23 × 10^4 cfu/g post-monsoon to 1.43 × 10^4 cfu/g during the monsoon. In Bathinda, TPC ranged from 1.43 × 10^4 cfu/g post-monsoon to 1.86 × 10^4 cfu/g during the monsoon season. No statistically significant differences in TPC were found across the seasons ($p>0.05$). Roopma *et al.* [15]

Table 1. Total Plate Count (Mean ± SE) (×10^{4}cfu/g) in selected fish species marketed in different fish markets during the study period.

Species name	Season	Amritsar	Jalandhar	Ludhiana	Mohali	Bathinda
W. attu	Post-monsoon	2.23±0.26	2.60±0.17	2.30±0.23	2.36±0.29	2.76±0.26
	Winter	2.26±0.20	2.76±0.20	2.36±0.20	2.56±0.20	2.93±0.24
	Pre-monsoon	2.40±0.23	2.70±0.20	2.56±0.26	2.66±0.26	2.86±0.26
	Monsoon	2.96±0.48	3.26±0.23	2.76±0.20	2.93±0.23	3.40±0.23
L. rohita	Post-monsoon	1.56±0.20	2.83±0.17	1.73±0.20	2.73±0.23	3.03±0.17
	Winter	2.03±0.20	3.03±0.17	1.73±0.17	3.03±0.17	3.13±0.17
	Pre-monsoon	2.03±0.23	3.06±0.14	2.23±0.20	2.96±0.29	3.26±0.23
	Monsoon	2.23±0.20	3.23±0.23	2.36±0.17	3.13±0.29	3.50±0.23
P. hypophthalmus	Post-monsoon	2.43±0.23	3.00±0.20	1.92±0.83	2.73±0.29	2.96±0.29
	Winter	2.70±0.17	3.23±0.23	2.70±0.20	2.90±0.17	3.33±0.23
	Pre-monsoon	2.80±0.17	3.26±0.20	3.16±0.26	2.90±0.17	3.23±0.27
	Monsoon	3.00±0.17	3.26±0.29	3.06±0.26	3.20±0.20	3.60±0.26
R. kanagurta	Post-monsoon	0.87±0.38	1.30±0.17	1.13±0.14	1.23±0.20	1.43±0.20
	Winter	1.20±0.20	1.43±0.20	1.23±0.14	1.33±0.20	1.63±0.26
	Pre-monsoon	1.13±0.14	1.43±0.20	1.33±0.14	1.33±0.20	1.63±0.20
	Monsoon	1.30±0.20	1.53±0.20	1.53±0.14	1.43±0.20	1.86±0.23

The values did not differ significantly species wise within the seasons from different sites.

Table 2. *E. coli* count (Mean ± SE) (×10^{2}cfu/g) in selected fish species marketed in different fish markets during the study period.

Species name	Season	Amritsar	Jalandhar	Ludhiana	Mohali	Bathinda
W. attu	Post-monsoon	2.33±0.88	3.33±0.88	2.00±0.57	3.33±0.88	4.33±1.20
	Winter	2.67±0.88	4.00±0.57	3.00±0.57	3.67±0.88	4.67±0.88
	Pre-monsoon	3.00±0.57	3.67±0.88	3.33±1.20	4.00±0.57	5.00±0.57
	Monsoon	2.67±0.88	4.33±1.20	3.67±0.88	4.67±0.88	5.33±1.20
L. rohita	Post-monsoon	1.33±0.33	3.00±1.15	2.83±0.31	2.00±0.57	3.67±0.88
	Winter	2.33±0.88	3.33±1.20	2.90±0.26	1.67±0.66	4.67±0.88
	Pre-monsoon	3.00±1.15	4.67±0.88	3.33±0.26	2.67±0.88	5.67±0.88
	Monsoon	3.33±0.88	4.33±1.20	3.57±0.43	3.67±0.88	5.00±0.57
P. hypophthalmus	Post-monsoon	3.00±0.57	4.33±1.20	3.67±0.88	4.67±0.88	5.33±1.20
	Winter	2.67±1.20	4.67±0.88	3.67±0.88	4.00±0.57	5.67±0.88
	Pre-monsoon	2.00±0.57	5.00±0.57	4.00±1.15	3.67±0.88	4.67±0.88
	Monsoon	2.33±0.88	5.33±1.20	4.67±0.88	4.33±1.20	5.67±0.88
R. kanagurta	Post-monsoon	2.00±0.57	2.00±0.57	2.33±0.88	3.00±1.15	3.00±1.15
	Winter	2.67±1.20	3.00±0.57	3.00±0.57	3.33±0.88	3.33±1.20
	Pre-monsoon	2.00±0.57	3.00±0.57	2.67±0.88	3.00±0.57	3.00±0.57
	Monsoon	2.33±0.88	3.67±0.88	3.00±0.57	3.67±0.88	3.67±0.88

The values did not differ significantly species wise within the seasons from different sites.

highlighted the relationship between fish meat quality and microbial contamination, noting that TPC levels in *Wallago attu* rose from 2.18 ± 0.02 log cfu/g at the beginning of storage to 6.87 ± 0.1 log cfu/g by the 30th day at -12 ± 2°C, surpassing the permissible limit of 6 log cfu/g by the 20th day. Similarly, Dhanapal *et al.* [16] observed that TPC in *L. rohita* increased from 6 × 10^2 cfu/g on day 1 to 7.4 × 10^4 cfu/g by the 18th day under ice storage. Patil *et al.* [17] also reported that TPC in *P. hypophthalmus* started at 4.82 × 10^2 cfu/g on day 0 and rose to 1.36 × 10^4 cfu/g by day 16 during storage at 4 ± 2°C. The monsoon season showed elevated TPC levels across all fish species, likely due to increased bacterial presence, which then decreased post-monsoon and remained lower. Bathinda's fish market displayed higher TPC levels, likely due to suboptimal hygiene practices compared to other markets. However, the TPC levels observed throughout this study remained within the acceptable limit of 7 × 10^4 log cfu/g, as reported by Sathishkumar *et al.* [18].

Escherichia coli count in selected fish

The study examined *E. coli* counts in four fish species across different seasons and markets. For *W. attu*, the counts ranged from 2.00 to 5.33 × 10^2 cfu/g, with generally lower values recorded in the post-monsoon season and higher values during the monsoon (Table 2). However, these variations were not statistically significant (p>0.05), indicating minimal seasonal impact. In *L. rohita*, *E. coli* counts ranged from 1.33 to 5.67 × 10^2 cfu/g, with lower counts in post-monsoon and winter seasons and higher counts during the monsoon. Seasonal differences were also statistically insignificant (p>0.05). *P. hypophthalmus* had *E. coli* counts from 2.00 to 5.67 × 10^2 cfu/g, with peaks during the monsoon or winter, but these variations were not statistically significant (p>0.05). For *R. kanagurta*, counts ranged from 2.00 to 3.67 × 10^2 cfu/g, showing lower levels in post-monsoon and pre-monsoon, with higher levels in winter and monsoon. Across all species, seasonal variations in *E. coli* counts were not statistically significant, suggesting that factors other than seasonality, such as water quality and market hygiene practices, may influence contamination levels. Despite the lack of significant seasonal impact, the higher counts observed during the monsoon may indicate increased bacterial loads in the water. Improved hygiene and handling practices are crucial for ensuring fish safety. Seasonal variations did not result in statistically significant differences in *E. coli* counts among fish from the markets in Amritsar, Jalandhar, Ludhiana, Mohali, and Bathinda (p>0.05).

Vishwanath and Lilabati [19] found no *E. coli* in *W. attu* and noted that ice storage for 3-6 days can effectively eliminate *E. coli*. While small amounts of *E. coli* are typically not problematic, improper handling can significantly increase levels, posing a risk of foodborne illness. Bordoloi and Muzaddadi [20] reported *E. coli* counts in *L. rohita* from Agartala, Tripura, with imported fish showing values from 7.2 × 10^3 to 2.4 × 10^5 cfu/g, and locally produced fish ranging from 2.9 × 10^3 to 5.7 × 10^4 cfu/g, both surpassing the allowable limit of 100 cfu/g. Arefin *et al.* [1] examined *E. coli* in *P. hypophthalmus* from Dhaka markets, noting counts of 5 × 10^3 cfu/g and 9.9 × 10^4 cfu/g before washing, and 3.5 × 10^2 cfu/g and 6.1 × 10^3 cfu/g after washing. For *R. kanagurta*, *E. coli* levels ranged from 2.00 to 3.67 × 10^2 cfu/g, which is above the acceptable limit of 20 cfu/g for *E. coli*, as established by ICMSF and FSSAI [23, 24].

Staphylococcus aureus count in selected fish

The quantification of *S. aureus* in *W. attu* showed variability based on season and market (Table 3). In fish markets in Amritsar, Jalandhar, Ludhiana, Mohali, and Bathinda, the lowest counts were observed in the post-monsoon season, while the highest were recorded during the monsoon. However, these differences were not statistically significant (p>0.05).

The study also evaluated *S. aureus* counts in other fish species. For *L. rohita*, counts ranged from 2.77 to 4.20 × 10^3 cfu/g, with lower counts post-monsoon and higher counts during the monsoon, across all markets. Seasonal differences in *S. aureus* levels were not statistically significant (p>0.05). In *P. hypophthalmus*, counts ranged from 3.83 to 5.06 × 10^3 cfu/g, showing a similar trend with lower counts post-monsoon and higher counts during the monsoon, but with no significant seasonal impact (p>0.05). For *R. kanagurta*, counts varied by season and market, with the lowest being 2.03 × 10^3 cfu/g post-monsoon in the Amritsar market and the highest being 2.33×10^3 cfu/g during the monsoon. Variations between seasons were present but not statistically significant (p>0.05). Overall, while *S. aureus* levels varied with the seasons and markets, these variations did not reach statistical significance. This suggests that other factors, such as handling and storage practices, may have a greater influence on bacterial contamination. Further research is needed to explore the impact of these factors on food safety and public health, focusing on contamination sources and preventive measures.

Vishwanath and Lilabati [19] reported *S. aureus* levels of 2.47-3.07 log cfu/g in *W. attu* muscle, which aligns with our findings of 2.46 - 3.66 × 10^3 cfu/g in various fish markets across Punjab. Bordoloi and Muzaddadi [20] observed *S. aureus* counts ranging from 2.2 × 10^4 to 8.9 × 10^5 cfu/g in *L. rohita* from different regions, and 2.6 × 10^4 to 7.9 × 10^5 cfu/g locally in Tripura, with no significant differences between local and out-of-state fish. Our study also reported high *S. aureus* counts in *L. rohita* ranging from 2.77 to 4.20 × 10^3 cfu/g across Punjab. Hasan *et al.* [21] found *S. aureus* counts averaging 3.66 × 10^3, 2.29 × 10^3, and 2.25 × 10^3 cfu/g in *P. hypophthalmus* from three local markets, which is slightly lower than our

Table 3. *S. aureus* count (Mean ± SE) (×10³ cfu/g) in selected fish species marketed in different fish markets during the study period.

Species name	Season	Amritsar	Jalandhar	Ludhiana	Mohali	Bathinda
W. attu	Post-monsoon	2.46±0.23	2.73±0.29	2.46±0.20	2.60±0.28	2.90±0.26
	Winter	2.56±0.20	2.93±0.34	2.66±0.31	2.76±0.29	3.06±0.31
	Pre-monsoon	2.73±0.29	3.13±0.29	2.73±0.29	2.90±0.32	3.36±0.31
	Monsoon	3.00±0.28	3.40±0.28	3.10±0.23	3.23±0.34	3.66±0.31
L. rohita	Post-monsoon	3.10±0.28	2.93±0.34	3.26±0.26	3.36±0.26	2.83±0.31
	Winter	3.26±0.31	3.73±0.29	3.53±0.20	3.43±0.29	2.90±0.26
	Pre-monsoon	3.56±0.26	3.86±0.24	3.83±0.29	3.66±0.26	3.33±0.26
	Monsoon	3.70±0.26	2.77±1.23	4.20±0.28	3.86±0.31	3.56±0.43
P. hypophthalmus	Post-monsoon	3.83±0.23	4.26±0.35	3.96±0.29	4.23±0.29	4.33±0.34
	Winter	3.90±0.32	4.56±0.26	4.13±0.34	4.30±0.36	4.36±0.29
	Pre-monsoon	4.16±0.35	4.56±0.39	4.30±0.23	4.73±0.34	4.60±0.26
	Monsoon	4.30±0.26	5.00±0.37	4.43±0.29	4.86±0.35	5.06±0.46
R. kanagurta	Post-monsoon	2.03±0.26	2.43±0.34	2.20±0.26	2.33±0.26	2.66±0.31
	Winter	2.16±0.29	2.66±0.26	2.40±0.23	2.40±0.23	2.83±0.29
	Pre-monsoon	2.23±0.31	2.80±0.23	2.46±0.31	2.63±0.31	3.20±0.26
	Monsoon	2.33±0.31	2.00±0.90	2.70±0.23	2.73±0.37	3.33±0.29

The values did not differ significantly species wise within the seasons from different sites.

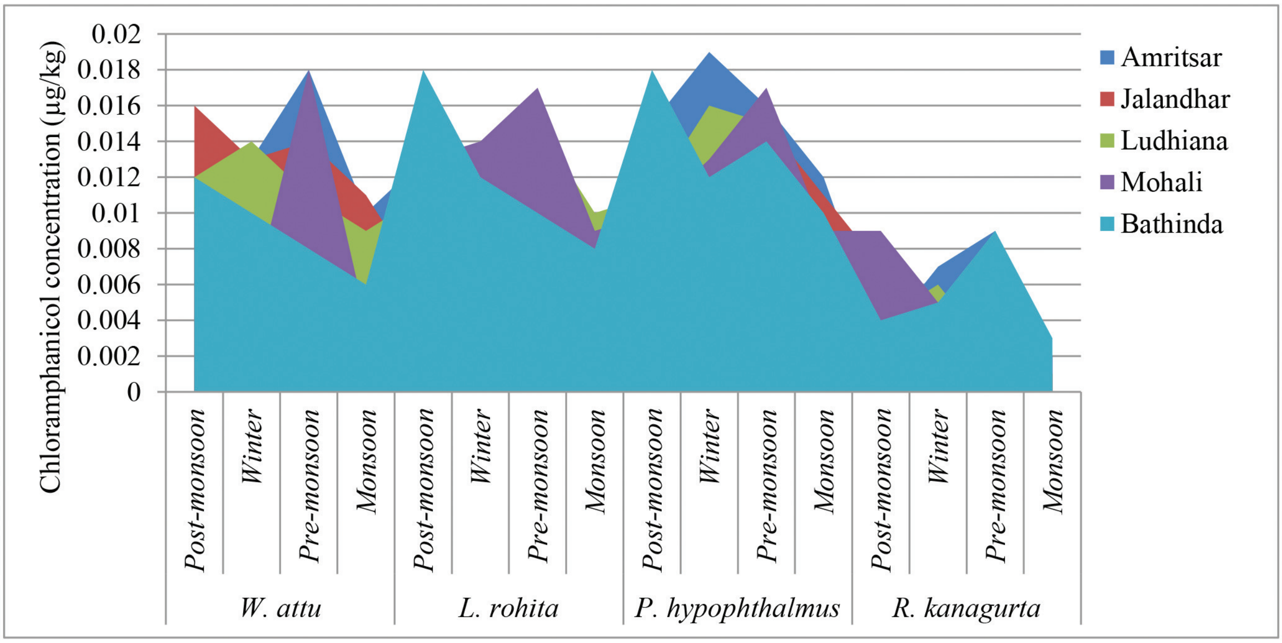

Fig. 1. Chloramphenicol concentration (µg/kg) of selected fish marketed in different fish markets during the study period.

range of 3.83 to 5.06 × 10³ cfu/g. Murthy *et al.* [22] reported all *R. kanagurta* samples having *S. aureus* levels below 100 cfu/g, suggesting safety, in contrast to our findings of 2.00 to 3.33 × 10³ cfu/g, which exceed this limit. Our results indicate a notably high presence of *S. aureus* in *R. kanagurta*, with counts ranging from 2.00 to 3.33 × 10³ cfu/g, surpassing the maximum permissible limit of 100 cfu/g. This highlights the need for rigorous safety measures. According to ICMSF and FSSAI [23, 24], acceptable limits are <5.0 × 10⁵ cfu/g for total plate count, 20.0 cfu/g for *E. coli*, and 100 cfu/g for *S. aureus*. The *E. coli* counts in *R. kanagurta* ranged from 2.00 to 3.67 × 10² cfu/g, which is above the acceptable limit.

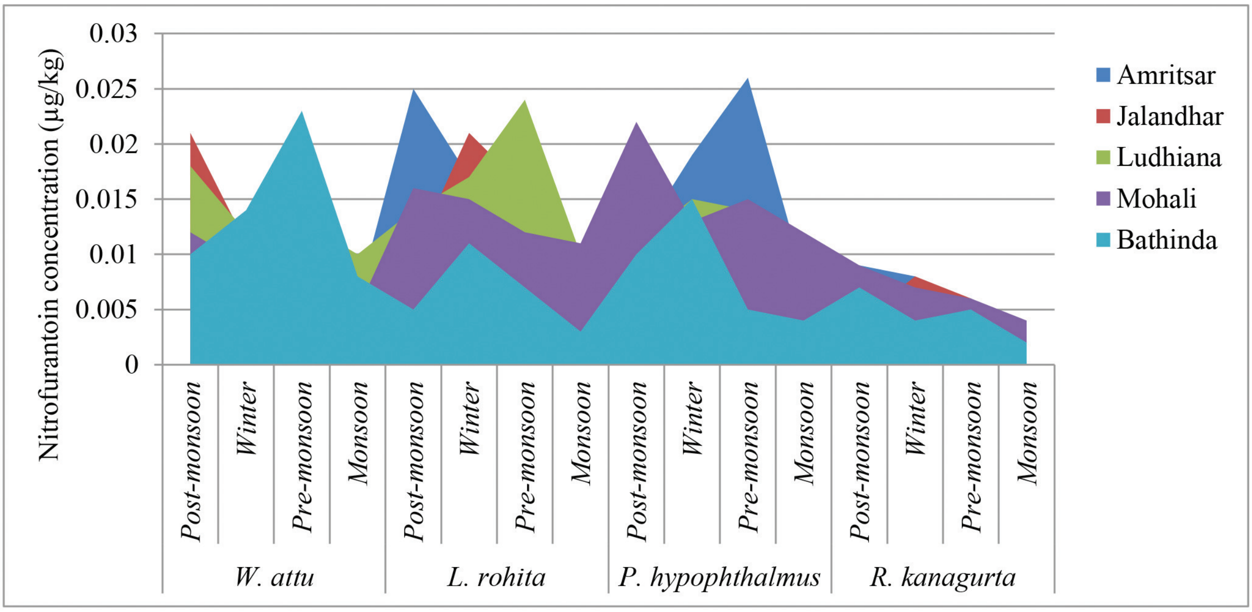

Fig. 2. Nitrofurantoin concentration (µg/kg) of selected fish marketed in different fish markets during the study period.

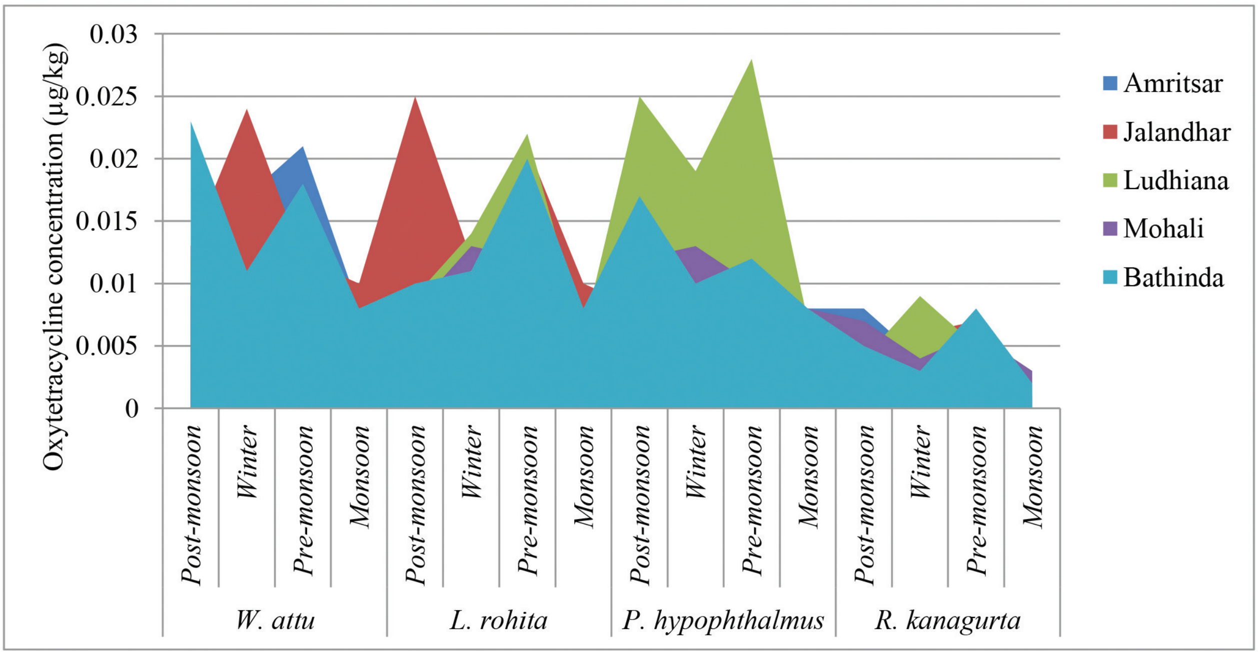

Fig. 3. Oxytetracycline concentration (µg/kg) of selected fish marketed in different fish markets during the study period.

Antibiotic residues in fish

Chloramphenicol concentration in selected fish

Chloramphenicol levels were assessed in four fish species - *W. attu*, *L. rohita, P. hypophthalmus,* and *R. kanagurta* - across different markets and seasons (Fig. 1). For *W. attu*, concentrations varied between 0.004 and 0.018 µg/kg, with Amritsar showing 0.010-0.018 µg/kg, Jalandhar 0.011-0.016 µg/kg, Ludhiana 0.009-0.014 µg/kg, Mohali 0.004-0.018 µg/kg, and Bathinda 0.006-0.012 µg/kg. In *L. rohita*, levels ranged from 0.005 to 0.018 µg/kg, with Amritsar recording 0.006-0.013 µg/kg, Jalandhar 0.005-0.013 µg/kg, Ludhiana 0.010-0.015 µg/kg, Mohali 0.009-0.017 µg/kg, and Bathinda 0.008-0.018 µg/kg. *P. hypothalamus* had concentrations from 0.008 to 0.019 µg/kg, with Amritsar ranging from 0.012-0.019 µg/kg, Jalandhar 0.011-0.015 µg/kg, Ludhiana 0.008-

0.016 μg/kg, Mohali 0.009-0.017 μg/kg, and Bathinda 0.010-0.018 μg/kg. *R. kanagurta* exhibited lower levels, between 0.001 and 0.009 μg/kg, with Amritsar showing 0.002-0.009 μg/kg, Jalandhar 0.001-0.007 μg/kg, Ludhiana 0.001-0.006 μg/kg, Mohali 0.002-0.009 μg/kg, and Bathinda 0.003-0.009 μg/kg. Overall, higher concentrations of chloramphenicol were observed during the pre-monsoon and post-monsoon seasons across all markets and fish species, with Amritsar and Mohali showing consistently higher levels.

According to the Food Safety and Standards Authority of India (FSSAI) [24], the maximum permissible residual level of chloramphenicol in fish is <0.3 μg/kg. The chloramphenicol concentrations found in *W. attu* (0.004 to 0.018 μg/kg), *L. rohita* (0.005 to 0.018 μg/kg), *P. hypophthalmus* (0.008 to 0.019 μg/kg), and *R. kanagurta* (0.001 to 0.009 μg/kg) were all below this limit. *R. kanagurta* was sourced from marine waters, while *W. attu* and *L. rohita* were from inland fisheries, and *P. hypophthalmus* was imported from other states. This suggests that antibiotic residues in the water sources of these fish species were within acceptable limits.

Nitrofurantoin concentration in selected fish

Nitrofurantoin levels were measured in *W. attu, L. rohita, P. hypophthalmus,* and *R. kanagurta* across different markets and seasons (Fig. 2). In *W. attu*, concentrations ranged from 0.005 to 0.023 μg/kg. Specifically, in Amritsar, levels varied from 0.006 μg/kg during the monsoon to 0.014 μg/kg in the pre-monsoon. In Jalandhar, concentrations ranged from 0.008 μg/kg in the monsoon to 0.021 μg/kg in the post-monsoon. In Ludhiana, values ranged from 0.010 μg/kg in the monsoon to 0.018 μg/kg in the post-monsoon. Mohali showed concentrations from 0.005 μg/kg in the monsoon to 0.012 μg/kg in the post-monsoon, and Bathinda had levels from 0.008 μg/kg in the monsoon to 0.023 μg/kg in the pre-monsoon.

For *L. rohita,* nitrofurantoin concentrations ranged from 0.003 to 0.025 μg/kg. In Amritsar, levels ranged from 0.008 μg/kg in the monsoon to 0.025 μg/kg in the post-monsoon. In Jalandhar, concentrations ranged from 0.004 μg/kg in the monsoon to 0.021 μg/kg in winter. Ludhiana showed values from 0.010 μg/kg in the monsoon to 0.024 μg/kg in the pre-monsoon. In Mohali, levels ranged from 0.011 μg/kg in the monsoon to 0.016 μg/kg in the post-monsoon, while in Bathinda, concentrations ranged from 0.003 μg/kg in the monsoon to 0.011 μg/kg in winter. Nitrofurantoin levels in P. hypophthalmus ranged from 0.004 to 0.026 μg/kg. In Amritsar, levels varied from 0.008 μg/kg in the monsoon to 0.026 μg/kg in the pre-monsoon. In Jalandhar, concentrations ranged from 0.006 μg/kg in the monsoon to 0.015 μg/kg in winter. Ludhiana showed values from 0.009 μg/kg in the monsoon to 0.015 μg/kg in winter. In Mohali, concentrations ranged from 0.012 μg/kg in the monsoon to 0.022 μg/kg in the post-monsoon, while Bathinda had levels from 0.004 μg/kg in the monsoon to 0.015 μg/kg in winter. In *R. kanagurta*, nitrofurantoin concentrations ranged from 0.001 to 0.009 μg/kg. In Amritsar, the lowest level was 0.001 μg/kg in the monsoon, and the highest was 0.009 μg/kg in the post-monsoon. Jalandhar showed levels from 0.002 μg/kg in the monsoon to 0.008 μg/kg in winter. Ludhiana had concentrations ranging from 0.001 μg/kg in the monsoon to 0.009 μg/kg in the post-monsoon. In Mohali, levels ranged from 0.004 μg/kg in the monsoon to 0.009 μg/kg in the post-monsoon, and in Bathinda, they ranged from 0.002 μg/kg in the monsoon to 0.007 μg/kg in the post-monsoon.

These concentrations were consistently below the maximum permissible residual level for nitrofurantoin in fish set by the Food Safety and Standards Authority of India (FSSAI), which is <1.0 μg/kg [24]. This suggests effective environmental monitoring of water sources for these fish species. A study by Khan and Lively [25] noted that, in 2018, 70% of imported shrimp samples tested in the USA had nitrofurantoin residues exceeding the detection limit, ranging from 0.4 to 4.4 ppb. This underscores the need for stringent monitoring to prevent exposure to harmful antibiotic residues in fish and fishery products.

Oxytetracycline concentration in selected fish

Oxytetracycline levels in *W. attu* ranged from 0.007 to 0.024 μg/kg across different seasons and markets (Fig. 3). In Amritsar, concentrations varied from 0.008 μg/kg in the monsoon to 0.021 μg/kg in the pre-monsoon. Jalandhar had levels between 0.010 μg/kg in the monsoon and 0.024 μg/kg in winter. In Ludhiana, the concentration ranged from 0.007 μg/kg in the monsoon to 0.012 μg/kg in the post-monsoon. For Mohali, oxytetracycline levels varied from 0.008 μg/kg in the monsoon to 0.011 μg/kg in the pre-monsoon. Bathinda showed concentrations from 0.008 μg/kg in the monsoon to 0.023 μg/kg in the post-monsoon. In *L. rohita*, oxytetracycline concentrations ranged from 0.005 to 0.025 μg/kg. In Amritsar, the levels ranged from 0.008 μg/kg in the monsoon to 0.014 μg/kg in the pre-monsoon. Jalandhar had levels between 0.010 μg/kg in the monsoon and 0.025 μg/kg in the post-monsoon. In Ludhiana, concentrations ranged from

0.006 µg/kg in the monsoon to 0.022 µg/kg in the pre-monsoon. Mohali showed levels from 0.005 µg/kg in the monsoon to 0.013 µg/kg in winter. Bathinda had concentrations from 0.008 µg/kg in the monsoon to 0.020 µg/kg in the pre-monsoon.

For *P. hypophthalmus*, oxytetracycline levels ranged from 0.007 to 0.028 µg/kg. In Amritsar, concentrations ranged from 0.008 µg/kg in the monsoon to 0.015 µg/kg in the post-monsoon. Jalandhar had levels from 0.007 µg/kg in the monsoon to 0.011 µg/kg in winter. Ludhiana's concentrations ranged from 0.007 µg/kg in the monsoon to 0.028 µg/kg in the pre-monsoon. Mohali showed levels from 0.008 µg/kg in the monsoon to 0.013 µg/kg in winter, and Bathinda had concentrations from 0.008 µg/kg in the monsoon to 0.017 µg/kg in the post-monsoon. The concentration of oxytetracycline in *R. kanagurta* ranged from 0.001 to 0.009 µg/kg. In Amritsar, the levels ranged from 0.001 µg/kg in the monsoon to 0.009 µg/kg in the post-monsoon. Jalandhar showed concentrations from 0.002 µg/kg in the monsoon to 0.008 µg/kg in winter. Ludhiana had levels between 0.001 µg/kg in the monsoon and 0.009 µg/kg in the post-monsoon. In Mohali, concentrations varied from 0.004 µg/kg in the monsoon to 0.009 µg/kg in the post-monsoon, and Bathinda had levels from 0.002 µg/kg in the monsoon to 0.007 µg/kg in the post-monsoon. All measured concentrations of oxytetracycline in *W. attu*, *L. rohita, P. hypophthalmus,* and *R. kanagurta* were well below the maximum permissible limit of 100 µg/kg (0.1 ppm) as set by FSSAI [24], indicating that the levels of this antibiotic residue are within acceptable safety limits.

Formaldehyde concentration in selected fish

The study assessed formaldehyde residue in four fish species (*W. attu*, *L. rohita, P. hypophthalmus*, and *R. kanagurta*) from five different fish markets in Punjab. The results showed that no formaldehyde residue was detected in any of the fish species across various seasons and markets, indicating that these fish samples were free from formaldehyde and met safety standards.

In a related study, Yeasmin *et al.* [26] investigated formalin presence in fish from different markets in Mymensingh, Bangladesh. They detected formalin in imported *L. rohita* from several markets, including BAU Market, Kewatkhali Market, Mecchua Bazar, Natun Bazar, and Sankipara Bazar. However, no formalin was found in locally sourced *L. rohita* from the Mymensingh district. This highlights the need for greater consumer awareness about the risks of formalin use in fish preservation, stricter enforcement of food safety regulations, and the promotion of safer methods for preserving fish quality without using harmful chemicals.

CONCLUSION

The study revealed that TPC in fish species across seasons and markets remains within acceptable limits. However, *E. coli* and *S. aureus* levels exceeded permissible limits in all tested species. These high counts were likely due to poor water quality, and hygiene during packaging, storage, and transportation. *R. kanagurta* from marine capture fisheries had the lowest microbial counts, while *P. hypophthalmus* from inland culture fisheries exhibited the highest. The monsoon season recorded the highest microbial loads, attributed to increased bacterial presence in water. Fish markets were generally fair in organization but lacked advanced facilities, affecting fish quality. Concentrations of chloramphenicol, nitrofurantoin, and oxytetracycline remained below permissible limits, indicating safety. Formaldehyde was absent in all species tested across seasons, confirming the fish was safe for human consumption.

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