

Research Article

Attachment of *Salmonella Typhimurium* to Fish Surfaces from a Suspension in Seawater and the Effect of Subsequent Washing

Sahna Don¹, Manjusha Lekshmi¹, Binya Bhusan Nayak¹, Sanath H Kumar^{1*}

¹QC Laboratory, Post-Harvest Technology, ICAR-Central Institute of Fisheries Education, Versova, Mumbai, 400061, India

*Correspondence author: Sanath Kumar H, Principal Scientist, ICAR-Central Institute of Fisheries Education (CIFE), Seven Bungalows, Andheri West, Mumbai, Maharashtra, India; Email: sanathkumar@cife.edu.in

Citation: Don S, et al. Attachment of *Salmonella Typhimurium* to Fish Surfaces from a Suspension in Seawater and the Effect of Subsequent Washing. J Clin Immunol Microbiol. 2024;5(1):1-7.

<https://doi.org/10.46889/JCIM.2024/5104>

Received Date: 04-03-2024

Accepted Date: 25-03-2024

Published Date: 02-04-2024



Copyright: © 2024 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CCBY) license

(<https://creativecommons.org/licenses/by/4.0/>).

Abstract

Background: *Salmonella* contamination of seafood is an important consumer health concern. Anthropogenic contamination of coastal-marine water is an important source of seafood contamination with *Salmonella*. In this study, we aimed to investigate the extent of *Salmonella* attachment when fish is exposed to contaminated seawater and also, the effect of washing on the removal of *Salmonella* attached to fish surface.

Methods and Findings: Bombay duck (*Harpodon nehereus*) fish was dipped in seawater artificially contaminated with 5 log CFU/ ml *Salmonella Typhimurium* for 30, 45 and 60 seconds and the number of bacteria present on the fish surface was quantified by surface plating. Separately, the effect of rinsing *Salmonella*-contaminated fish for 30, 45 and 60 seconds on the removal of *Salmonella* from the fish surface was investigated. The results showed attachment of bacteria in significant numbers, in the range of 3.6 - 4.16 log CFU/g, within 60 seconds of exposure to *Salmonella*-contaminated seawater. However, no significant reduction in the *Salmonella* counts was observed after 30 to 60 seconds of rinsing of fish in freshwater.

Conclusion: The results suggest that *Salmonella Typhimurium* can readily attach to the fish surface from a seawater medium and resist detachment by routine washing procedures.

Keywords: *Salmonella* Contamination; Fish; Seafood; *Salmonella Typhimurium*

Introduction

Food-borne infections due to Non-Typhoidal *Salmonella* (NTS) are estimated to be 3.4 million globally, with considerable morbidities and mortalities [1]. Seafood is often reported to be a vehicle of non-typhoidal *Salmonella* and studies have reported the isolation of diverse serovars from fish and shellfish all over the world [2-6]. *Salmonella* is not an indigenous microorganism of

fish. Therefore, its presence in seafood is attributed to faecal contamination of coastal-marine waters by humans, as well as contamination during transportation and processing attributable to unhygienic handling [7,8]. In highly populated countries like India with inadequate sanitation infrastructure, there is a high chance of seafood contamination due to the discharge of untreated or partially treated sewage into the coastal environment. Therefore, seafood caught from contaminated coastal and estuarine waters is more likely to harbour *Salmonella* in them [9].

There are many reports of detection of *Salmonella* in water sources, including surface waters, natural resources and drinking water. This is particularly concerning as water plays a pivotal role in contaminating harvested fish, with both seawater and potable water from public supply being utilized for fish washing practices in India. Regrettably, there seems to be a lack of attention given to the sanitary conditions of coastal waters, which are frequently employed for primary washing of fish after catch. In retail markets, washing of seafood is often by potable water; however, the risk remains, as the water quality might not always be monitored. Another notable concern is the use of non-potable water for the preparation of ice, which can serve as a

potential source of contamination of *Salmonella* in seafood. This apprehension is well founded with multiple reports of NTS being detected in finfish and shellfish samples from retail markets of India [5,10-15]. The bacterium reaches the water sources by direct faecal contamination, agricultural runoff, sewage overflows etc [10]. There are few reports of food-borne *Salmonella* outbreaks in which contaminated water was found to be the source of bacterial introduction into the products. *Salmonella* serotypes obtained from the products were detected in the irrigation water and water used for washing the food products [11]. The attachment dynamics of *Salmonella* in seafood are rarely investigated. With this background, the experiments were designed to simulate the effect of washing practices prevailing in landing centres and the retail markets of India on the contamination of fish with *S. Typhimurium*.

Material and Methods

Collection and Preparation of Fish Sample

Fresh Bombay duck fish (*Harpadon nehereus*) was collected from a landing centre in Mumbai, India. The samples collected were aseptically transported to the laboratory in sterile sample bags in chilled condition. Fish was cut into steaks of 10 g each and stored in sterile bags on ice until use. Prior to contamination experiments, the fish samples were dipped briefly in water maintained at 90°C for few seconds to eliminate the surface microflora.

Preparation of Bacterial Culture and Artificial Inoculation of Seawater

S. Typhimurium ATCC 14028 was used as the reference culture in all the experiments. The stock bacterial culture was initially grown in 10 ml Luria Bertani (LB) broth (Hi-Media, Mumbai, India) at 37°C for 12 h in a shaking incubator. One-millilitre of the broth culture was transferred to 100 mL LB broth and incubated at 37°C with shaking for 12 h. The bacterial cells were harvested from the culture by centrifuging at 12000×g for 10 min and the pellet was washed twice with 20 ml Phosphate Buffered Saline (PBS) and centrifuged at 12000 rpm for 10 min. Using previously determined reference curve of counts versus absorbance at OD540, the bacterial suspension was adjusted to an absorbance value corresponding to ~8 log CFU/ml. From this bacterial suspension, 1ml each was added in into 1 L sterile seawater, maintained at ambient temperature. The resultant count in seawater was approximately 5 log CFU/ml as determined by standard plate count method.

Contamination Experiment

An experiment was designed to study the effect of dipping/ washing of seafood in *Salmonella*-contaminated seawater on attachment by the bacterium. In this experiment, fish sample (10 g) was dipped and swirled gently in the contaminated seawater (500 ml) maintained in 500 ml beakers (Borosil, India), for three different durations viz., 30 sec, 45 sec and 1 min. The experiment was performed in triplicate. To quantify the number of bacteria attached to fish surface, the sample (10 g) was homogenized in 90 ml PBS, 10-fold serially diluted in the same buffer and multiple dilutions were spread plated on Xylose Lysine Deoxycholate (XLD) agar. *Salmonella* appears as red colony with black centre on XLD. Ten representative colonies from each sample were subjected to PCR confirmation using a *Salmonella*-specific PCR targeting the *invA* gene [16].

Effect of Washing on The Removal Bacteria from Fish Surface

In the second experiment, the effect of washing *Salmonella*-contaminated fish on the level of bacterium was tested. For this, fish samples (n=3) of 10 g each were dipped in seawater (500 ml) contaminated with 5 log CFU/ml *S. Typhimurium* for 30 sec. Immediately after this, fish was washed by swirling in boiled sterile potable water, maintained at ambient temperature (500 ml) for 30, 45 and 60 seconds. *Salmonella* counts on the fish sample before and after washing with fresh water were enumerated on XLD agar and representative isolates were PCR confirmed.

In both the experiments, the counts from duplicate plates were averaged and mean plate counts from triplicate samples were determined. Statistical analysis was carried out by performing analysis of variance using the software SPSS 16 (IBM SPSS Statistics for Windows, version 24.0, IBM Corp., Armonk, NY). A P-value of <0.05 was considered statistically significant.

Results and Discussion

Non-typhoidal *Salmonella* in seafood is derived from human and animal sources. Considering the diverse reservoirs of this bacterium, seafood contamination with *Salmonella* offers a formidable challenge [17,18]. Scientific evidences suggest that *Salmonella Typhimurium* is able to survive in seawater for 12-54 days, although longer survival of 32 to 74 weeks has also been reported [19,20]. *Salmonella* can survive in fresh water for 54 days and in sediment for 119 days [21]. Studies have shown that

Salmonella can survive in seawater longer than *E. coli*, although contrasting results indicating better survival of *E. coli* than *Salmonella* exist [22,23]. In tropical seawater and freshwater, *Salmonella* could survive for up to 16 weeks and 24 weeks, respectively at ambient temperature [24]. *Salmonella* can remain viable in frozen seafood for considerable period of time. Our recent study using *S. Typhimurium* showed that in fish (*Harpodon nehereus*) and shrimp (*Parapenaeopsis styliifera*) inoculated with 8 log CFU/g and stored in frozen conditions, the bacterium was viable for up to 90 days [25]. At a lower inoculation level of 4 log CFU/g, *Salmonella* was viable for up to 60 days in frozen fish, while in frozen shrimp, the bacterium could survive for only 15 days [25]. The ability of *Salmonella* to survive for longer periods of time in seawater offers greater chances of contamination of fish and shellfish. It has come to attention that fishermen frequently extract water from the vicinity of coastal areas to cleanse their catch aboard their vessels. Additionally, the practice of dipping the catch in coastal waters at the landing centres has been noted. These actions carry the potential to escalate the risk of *Salmonella* contamination significantly. However, studies on the contamination dynamics of seafood such as the attachment rate, effects of washing at various stages of handling fish from harvest to consumer on *Salmonella* attached to fish surface are lacking. To understand the extent of *Salmonella* attachment to fish surface, fish samples were dipped in seawater artificially contaminated with 5 log CFU/g for 30-60 seconds. Fish exposed to *Salmonella Typhimurium* for 30 seconds had a count of 3.30 log CFU/g, while 45 and 60 second exposure resulted in a count of 4 log CFU/g (Fig. 1). These results indicate that *Salmonella Typhimurium* can attach quickly to fish surface within 60 seconds and depending on the numbers in the surrounding medium, the number of bacteria attaching to fish surface can be as high as 80%.

In the second experiment, the effect of washing for 30-60 seconds on the level of *Salmonella* on fish surface was investigated. The results indicated that washing in freshwater for up to 60 seconds did not result in significant detachment of *Salmonella* from fish surface. The count of *S. Typhimurium* on fish immediately after dipping in contaminated seawater were in the range of 3.5- 4 log CFU/g. After 30-60 seconds of washing in fresh water, the counts on counts on fish remained in the range of 3.03-3.42 log CFU/g (Fig. 2). No significant difference ($P>0.05$) could be observed between 30, 45 and 60 seconds of washing. Representative colonies from XLD plates amplified the *invA* gene by PCR (Fig. 3). This observation suggests that simple washing procedures in the form of rinsing fish in fresh water do not ensure removal of *S. Salmonella Typhimurium* from fish surface.

Even though there are studies investigating the bacterial attachment on food matrices such as meat, poultry and dried fruits, similar data on seafood is by far, lacking. In a study, *Salmonella* cells showed a very rapid attachment to poultry skin cells within a few seconds of exposure to *Salmonella* cell suspension [26]. These observations are in agreement with the results of our study, where high numbers of *Salmonella* were detected after dipping in artificially contaminated seawater. *Salmonella* attachment to raw food surfaces such as the meat primarily depends on the concentration of cell suspension rather than the presence of *flagella* and *fimbriae* [26]. The bacterial population will be higher on the surface then, but with a continuous immersion, the bacterial internalization to the underlying skin and entrapment in muscles occurs [27,28]. The meat tissues take up water and the bacterial cells get entrapped again. Immediate attachment of *Salmonella* cells to vegetable surfaces exposed to bacterial suspensions have been demonstrated in various studies. A study conducted on apple disks to analyses the bacterial attachment pattern, observed that the attachment of *Salmonella* to apple disks was dependent on the concentration of bacteria rather than the exposure period [29].

A clear explanation of the bacterial attachment to biotic surfaces is yet to be defined. It is considered to be dependent on the combination of multiple factors, including the bacterial cell surface and physicochemical properties of the substratum, such as hydrophobicity and charge [30]. Initial attachment to plant surfaces is dependent on the cell surface structures and the ability to form biofilms. Subsequent survival is influenced by regulation of genes related to stress regulation, virulence and metabolism [30]. Components of the cell envelope, specifically capsules, fimbriae and outer membrane polymers, cellulose, fimbriae and O antigen play important roles in the attachment of *S. enterica* onto plant surfaces [31]. The contribution of the surface components of *Salmonella* to fish surface attachment and resistance to subsequent washing is an interesting area of investigation. Bacterial cells attached to the product surface become more resistant to cell injury and become more viable at different stress conditions such as low temperature and hyperosmosis [30]. A combination of ultrasound and organic acid was found to be effective in reducing *Salmonella* and *E. coli* load in green pepper and melon [32]. Disinfectant Chlorine dioxide at a concentration of 5- 20 ppm reduced the *Salmonella* load in water samples by 5 log almost immediately. But a comparatively longer time immersion was required for *Salmonella* reduction from tomato samples [33]. Polyethylene glycol film was found to prevent the adhesion of *S. Typhimurium* and *E. coli* on tomato surfaces by 90% [34]. Similar studies are lacking in the case of fish and shellfish. There is a need to develop effective packaging and antimicrobial treatments for the control of *Salmonella* and other pathogenic bacteria in

seafood. The strict implementation of sanitary protocols, including chlorination of the water used has the potential to substantially mitigate the associated risk.

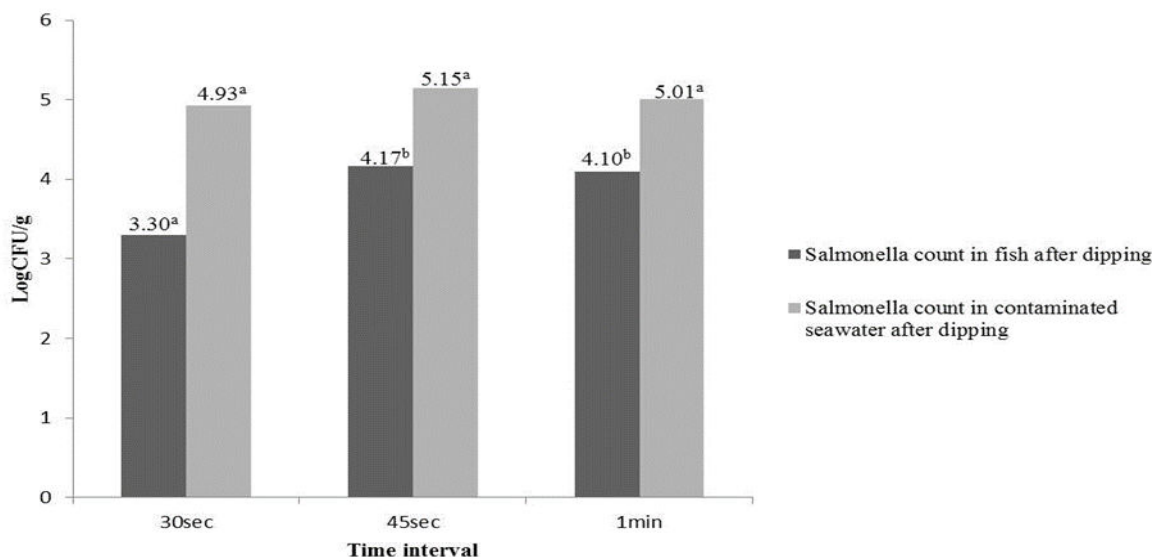


Figure 1: Attachment of *Salmonella Typhimurium* to fish surface after 30, 45 and 60 seconds of dipping in artificially contaminated seawater. Different superscripts on the graph indicate significant ($P < 0.05$) difference in counts at different time intervals. Initial count of *S. Typhimurium* in seawater = 5.08 ± 0.07 log CFU/ml.

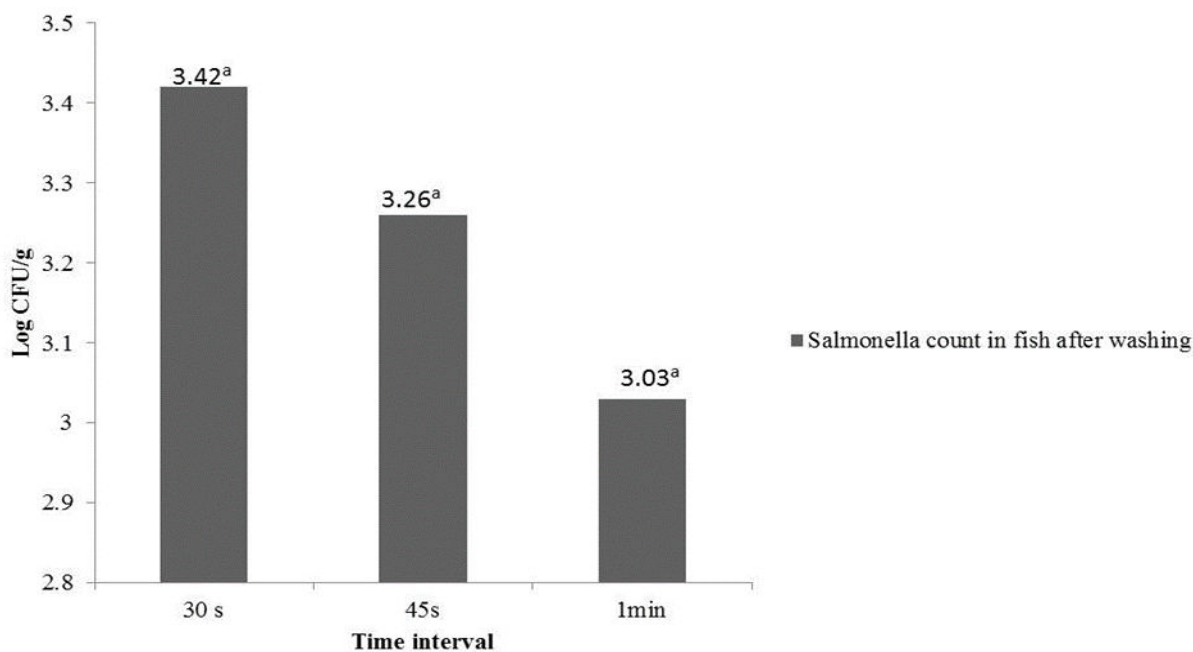


Figure 2: Effect of washing for 30 sec, 45 sec and 60 sec on levels of *Salmonella Typhimurium* in artificially contaminated fish. Identical superscripts on bars indicate no significant difference in *Salmonella* counts after washing. Initial count of *S. Typhimurium* in seawater = 4.89 ± 0.06 log CFU/ml. Counts on fish immediately after dipping in *Salmonella* contaminated seawater = 3.64 ± 0.18 log CFU/g.

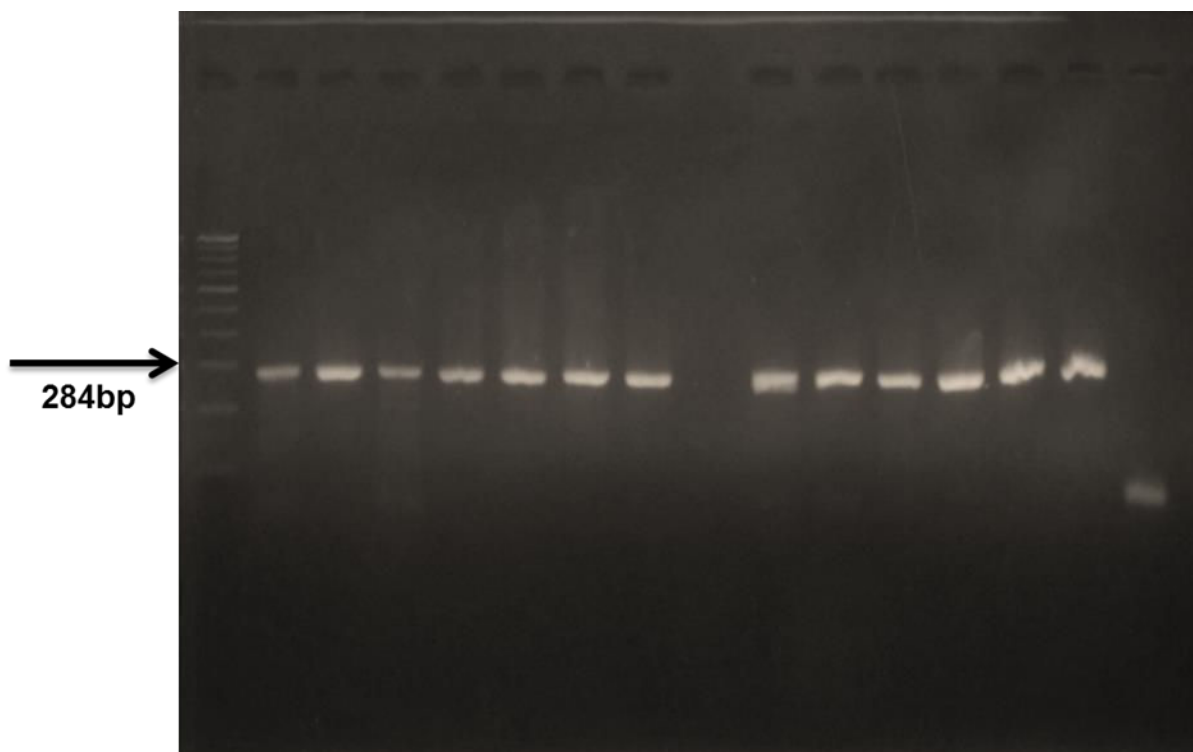


Figure 3: Confirmation of *S. Typhimurium* re-isolated from artificially contaminated fish using of *invA*-specific primers. Lane M: 100 bp DNA Molecular weight marker (StepUp™, GeNei, India); lanes 1- 12: Colonies from XLD plates; lane P: Positive control (*S. Typhimurium* ATCC 14028); lane N: Negative control.

Conclusion

In conclusion, *Salmonella* is a recurrently reported contaminant in seafood products. Washing the seafood with sterile waters does not guarantee the removal of *Salmonella* from the seafood matrix. This calls for effective and stringent sanitary practices to be followed by food handlers in order to minimize the contamination of seafood samples with this bacterium.

Conflict of Interests

The authors have no conflict of interest to declare.

Acknowledgements

Authors thank Director, ICAR- Central Institute of Fisheries Education, Mumbai for help and support in carrying out this research work.

References

- Haselbeck AH, Panzner U, Im J, Baker S, Meyer CG, Marks F. Current perspectives on invasive nontyphoidal *Salmonella* disease. *Curr Opin Infect Dis*. 2017;30(5):498-503.
- Barrett KA, Nakao JH, Taylor EV, Eggers C, Gould LH. Fish-associated foodborne disease outbreaks: United States, 1998-2015. *Foodborne Pathog Dis*. 2017;14(9):537-43.
- Iwamoto M, Ayers T, Mahon BE, Swerdlow DL. Epidemiology of seafood-associated infections in the United States. *Clin Microbiol Rev*. 2010;23(2):399-411.
- Martinez-Urtaza J, Liebana E. Investigation of clonal distribution and persistence of *Salmonella* Senftenberg in the marine environment and identification of potential sources of contamination. *FEMS Microbiol Ecol*. 2005;52(2):255-63.
- Prabhakar P, Lekshmi M, Ammini P, Nayak BB, Kumar S. *Salmonella* contamination of seafood in landing centers and retail markets of Mumbai, India. *J AOAC Int*. 2020;103(5):1361-5.
- Kumar HS, Sunil R, Venugopal MN, Karunasagar I, Karunasagar I. Detection of *Salmonella* spp. in tropical seafood by polymerase chain reaction. *Int J Food Microbiol*. 2003;88(1):91-5.
- Amagliani G, Brandi G, Schiavano GF. Incidence and role of *Salmonella* in seafood safety. *Food Res Int*. 2012;45(2):780-8.

8. Carrasco E, Morales-Rueda A, García-Gimeno RM. Cross-contamination and recontamination by *Salmonella* in foods: A review. *Food Res Int*. 2012;45(2):545-56.
9. Novoslavskij A, Terentjeva M, Eizenberga I, Valciņa O, Bartkevičs V, Bērziņš A. Major foodborne pathogens in fish and fish products: a review. *Ann Microbiol*. 2016;66(1):1-15.
10. Jung AV, Le Cann P, Roig B, Thomas O, Baurès E, Thomas MF. Microbial contamination detection in water resources: interest of current optical methods, trends and needs in the context of climate change. *Int J Environ Res Public Health*. 2014;11(4):4292-310.
11. Levantesi C, Bonadonna L, Briancesco R, Grohmann E, Toze S, Tandoi V. *Salmonella* in surface and drinking water: occurrence and water-mediated transmission. *Food Res Int*. 2012;45(2):587-602.
12. Kumar R, Surendran PK, Thampuran N. Distribution and genotypic characterization of *Salmonella* serovars isolated from tropical seafood of Cochin, India. *J Appl Microbiol*. 2009;106(2):515-24.
13. Deekshit VK, Kumar BK, Rai P, Srikumar S, Karunasagar I, Karunasagar I. Detection of class 1 integrons in *Salmonella* Weltevreden and silent antibiotic resistance genes in some seafood-associated nontyphoidal isolates of *Salmonella* in south-west coast of India. *J Appl Microbiol*. 2012;112(6):1113-22.
14. Adesiji YO, Deekshit VK, Karunasagar I. Antimicrobial-resistant genes associated with *Salmonella* spp. isolated from human, poultry and seafood sources. *Food Sci Nutr*. 2014;2(4):436-42.
15. Shabarinath S, Kumar HS, Khushiramani R, Karunasagar I, Karunasagar I. Detection and characterization of *Salmonella* associated with tropical seafood. *Int J Food Microbiol*. 2007;114(2):227-33.
16. Rahn K, De Grandis SA, Clarke RC, McEwen SA, Galan JE, Ginocchio C, et al. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol Cell Probes*. 1992;6(4):271-9.
17. Kumar S, Lekshmi M, Parvathi A, Nayak BB, Varela MF. Antibiotic resistance in seafood-borne pathogens. *Foodborne Pathogens and Antibiotic Resistance*. 2016;397-415.
18. Stephen J, Mukherjee S, Lekshmi M, Kumar S, Varela M. Antibiotic resistance in fishborne pathogens of public health significance: An emerging food safety issue. *Microbiology*. 2021.
19. Smith JJ, Howington JP, McFeters GA. Survival, physiological response and recovery of enteric bacteria exposed to a polar marine environment. *AEM*. 1994;60(8):2977-84.
20. Davidson MC, Berardi T, Aguilar B, Byrne BA, Shapiro K. Effects of transparent exopolymer particles and suspended particles on the survival of *Salmonella enterica* serovar *Typhimurium* in seawater. *FEMS Microbiol Ecol*. 2015;91(3):005.
21. Moore BC, Martinez E, Gay JM, Rice DH. Survival of *Salmonella enterica* in freshwater and sediments and transmission by the aquatic midge *Chironomus tentans* (Chironomidae: Diptera). *AEM*. 2003;69(8):4556-60.
22. Evison LM. Comparative studies on the survival of indicator organisms and pathogens in fresh and sea water. *Water Sci Technol*. 1988;20(11-12):309-15.
23. Chandran A, Hatha AM. Relative survival of *Escherichia coli* and *Salmonella typhimurium* in a tropical estuary. *Water Res*. 2005;39(7):1397-403.
24. Sugumar G, Mariappan S. Survival of *Salmonella* spp. in freshwater and seawater microcosms under starvation. *Asian Fisheries Science*. 2003;247-55.
25. Don S, Ammini P, Nayak BB, Kumar SH. Survival behaviour of *Salmonella enterica* in fish and shrimp at different conditions of storage. *LWT*. 2020;132:109795.
26. Thomas CJ, McMeekin TA. Effect of water uptake by poultry tissues on contamination by bacteria during immersion in bacterial suspensions. *J Food Prot*. 1984;47(5):398-402.
27. Benedict RC, Schultz FJ, Jones SB. Attachment and removal of *Salmonella* spp. on meat and poultry tissues. *J Food Saf*. 1990;11(2):135-48.
28. Lillard HS. Factors affecting the persistence of *Salmonella* during the processing of poultry. *J Food Prot*. 1989;52(11):829-32.
29. Liao CH, Sapers GM. Attachment and growth of *Salmonella Chester* on apple fruits and *in-vivo* response of attached bacteria to sanitizer treatments. *J Food Prot*. 2000;63(7):876-83.
30. Giaouris E, Nesse LL. Attachment of *Salmonella* spp. to food contact and product surfaces and biofilm formation on them as stress adaptation and survival strategies. *Salmonella: Prevalence, Risk Factors and Treatment Options* 2015;111-36.
31. Barak JD, Jahn CE, Gibson DL, Charkowski AO. The role of cellulose and O-antigen capsule in the colonization of plants by *Salmonella enterica*. *MPMI*. 2007;20(9):1083-91.

32. de São José JFB, de Medeiros HS, Bernardes PC, De Andrade NJ. Removal of *Salmonella enterica* Enteritidis and *Escherichia coli* from green peppers and melons by ultrasound and organic acids. *Int J Food Microbiol.* 2014;190:9-13.
33. Pao S, Kelsey DF, Khalid MF, Ettinger, MR. Using aqueous chlorine dioxide to prevent contamination of tomatoes with *Salmonella enterica* and *Erwinia carotovora* during fruit washing. *J Food Prot.* 2007;70(3):629-34.
34. Zhang M, Yang F, Pasupuleti S, Oh JK, Kohli N, Lee IS, et al. Preventing adhesion of *Escherichia coli* O157: H7 and *Salmonella Typhimurium* LT2 on tomato surfaces via ultrathin polyethylene glycol film. *Int J Food Microbiol.* 2014;185:73-81.

Journal of Clinical Immunology & Microbiology



Publish your work in this journal

Journal of Clinical Immunology & Microbiology is an international, peer-reviewed, open access journal publishing original research, reports, editorials, reviews and commentaries. All aspects of immunology or microbiology research, health maintenance, preventative measures and disease treatment interventions are addressed within the journal. Immunologist or Microbiologist and other researchers are invited to submit their work in the journal. The manuscript submission system is online and journal follows a fair peer-review practices.

Submit your manuscript here: <https://athenaeumpub.com/submit-manuscript/>