Fishery Technology 54 (2017) : 202 - 208

Effect of Washing on the Adhesion of *Yersinia enterocolitica* on Fish and Shrimp Muscle Surface

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Abstract

Water is used traditionally with or without a sanitizing agent to wash fresh seafood and to reduce pathogens attached to the surface. Ability of *Yersinia enterocolitica* to get attached to fish and shrimp muscle and detached during water washing was studied. Dressed tissues were dipped in suspension containing *Yersinia enterocolitica* 10^8-10^9 cells per ml and washed sequentially. Bacterial counts and tetrazolium salt reduction were estimated. The study revealed that first wash was more effective compared to two subsequent washes. The bacterial reduced from 5.145 log CFU g^{-1} to 4.445 log CFU g^{-1} in fish and 5.05 to 3.7 log CFU g^{-1} in shrimp after 3 washings. The cells which remained bound after washing were more active than those which got washed and were measured by per cell activity of the cells monitored by rate of formazan formed. The washing was found to be more effective in removing bacteria in shrimp tissue than in fish tissue provided initial quality of water was good.

Keywords: Tissue attachment, washing effects, respiration indicator, *Yersinia enterocolitica*

Introduction

Yersinia enterocolitica is a common food-borne pathogen causing yersiniosis in humans in Europe (Bottone, 1997). This zoonotic agent is responsible for gastrointestinal disease, reactive arthritis and erythema nodosum (Fedriksson-Ahomaa, 2006). Yersiniosis can be acquired through consumption of contaminated food and water (Kapperud, 1991; Drummond et al., 2012). The foodstuff that generally gets contaminated with *Y. enterocolitica* includes packaged or unpackaged meat products, vacuumed packed red meat, milk, eggs, meat, various poultry meat, vegetables, edible shellfish, including untreated fresh water (Andersten et al., 1991; Hanna et al., 1985; Ostroff et al., 1994; Belgian, 2004; Siddique et al., 2009; Atobla, 2012; Mahdavi et al., 2012). Chilled food may also pose risk of infection due to survival nature of *Y. enterocolitica* at low temperatures of 29.7°F (Fratamico et al., 2005; FDA, 2011). The transfer of pathogenic strains of *Y. enterocolitica* to humans occurs primarily through food consumption if hygienic protocols are not followed during food processing and/or storage (Atobla, 2012).

The mode of transmission of *Y. enterocolitica* is through faecal-oral route via contaminated food (Bari et al., 2011; Arora et al., 2012) similar to other enteric pathogens. Contamination in fish may occur through washing water and ice, sea water used for processing, contact surfaces and processing line, and from hands of workers (Guðbjörnsdóttir et al., 2005; Jannat et al., 2007; Shikongo-Nambabi et al., 2012).

The usual practice at the landing centers is to wash fish with near-shore water, before off loading to landing centers. Fish is also periodically washed at landing centers, market, pre-processing areas and also at industries. Washing is done to decrease the microbial population on the surface of meat (Crouse et al., 1988; Kotula et al., 1974). To ascertain washing efficacy, basic information on microbial attachment is essential (Chung et al., 1989). Hence, this study was carried out to investigated the efficiency of washing step to reduce *Y. enterocolitica* population in the seafood which is now known to present may be due to unhygenic handling practices.

Materials and Methods

Tissues of Indian mackerel (*Rastrelliger kanagurta*) and Kiddi Shrimp (*Parapeneopsis stylifera*) were used

Received 10 March 2017; Revised 05 September 2017; Accepted 05 September 2017

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as experimental samples for this study. The fish and shellfish were procured from Versova retail market near the landing centre, Mumbai, India, under aseptic conditions in sterile sampling bags (Nasco whirl Pak; HiMedia, India) in ice box for analysis. Samples were dressed hygienically and washed thoroughly with sterile water (three times) and washed with 70% ethanol to to reduce the surface bacterial population. They were further washed two times with distilled water to remove alcohol.

Y. enterocolitica (ATCC 23715) was used for this study. Tryptic soy agar (TSA) and tryptic soy broth (TSB) were used for culture and enumeration unless specified otherwise. Sterile physiological saline was used as diluent. All the media used were procured from HiMedia, Mumbai, India.

Organisms were grown in TSB at room temperature and incubated for 18-24 h. The cells were harvested by centrifugation at 6000 g for 15 min at 4°C. The pellets were washed once in sterile saline and resuspended in normal saline. Suspension had approximately 10^8-10^9 cells ml^{-1} as estimated by spread plate counting method.

Hygienically dressed fish and shrimp muscles were dissected aseptically and immersed in the suspension described above and were allowed to drain for 15 min in a sterile beaker. Nine volumes of sterile water was added, shaken for 30 seconds and drained. This was considered as first wash and subsequently two more washes were given. Samples were picked for bacteriological analysis after each washing.

Total plate count was determined by spread plate technique on TSA plates. Ten-fold dilutions were made from the samples and 0.1 ml portions were spread plated onto TSA in duplicates. The plates were incubated at room temperature for 24 - 48 h and the counts of colonies were recorded. Further five colonies from each plate were selected and confirmed biochemically as per BAM (2007).

Formazan assay was performed as described by Nayak et al. (2005) for determination of bacterial respiration. 2, 3, 5-Triphenyltetrazolium Chloride (TTC) (HiMedia, Mumbai, India) was used as redox indicator. The colourless compound (TTC) reduced to red colour (triphenyl formazan) is indicator of bacterial respiration (Emswiler et al., 1976). TTC (1%) stock solution was added to 10 ml of samples to give the final concentration of 0.1% without any enriching substrate. After overnight incubation in dark at room temperature, the reactions were stopped by addition of paraformaldehyde solution to a final concentration of 2%. Ethanol was added to extract the formazan. The samples were centrifuged at 10 000 g for 10 min and filtered through polycarbonate filter (GTTP, Millipore). The absorbance of the extracts was measured at 488 nm in 1 cm path length cuvettes using spectrophotometer (ThermoSpectronic, England). Calculations of the TTC reduction rate were based on a specific absorbance of 1.42×10^4 M^{-1} cm^{-1} for TTC formazan. The calculation was based on the following expression:

$$A = 3\ Hs/17.2V\ X\ absorbance$$

Where, 3 converts the activity to hour units, H is the homogenate volume, s is the slope of the calibration curve in millicoulombs per absorbance unit (Packard & Healy, 1968), 17.2 is the electrochemical equivalent in milli coulombs of 1 µl O_2 and V is the volume of filterate.

Results and Discussions

The investigation on effect of washing on removal of *Y. enterocolitica* revealed a reduction in attached bacterial number after washing the fish muscle with sterile water as shown in Table 1. The attached population of *Y. enterocolitica* on fish muscle after three washes decreased from 5.145 to 4.45 log CFU g^{-1}. The first wash was found to be more effective than two subsequent washes. In the subsequent two washes, the average change in the counts of organism was 0.186 log CFU g^{-1} and 0.105 log CFU g^{-1} respectively. The absorbances of formazan due to the reduction of TTC by bacteria in fish muscles were also measured. The absorbance of each washing showed similar pattern of decrease as the bacterial count measured by TPC (Fig. 1).

The effect of three washes on detachment of *Y. enterocolitica* from shrimp muscle is shown in Table 2. As the shrimp muscles are very different from fish muscles, bacteria showed different kind of ability to adhere on the shrimp muscle. After the first wash there was nearly one log reduction of attached bacterial population in shrimp which was much higher compared to the first wash in fish tissue. In further washing, the removal was not significant (p-value >0.05) where only a reduction of 0.26 and 0.08 log CFU g^{-1} were observed for second and third washings respectively. The absorbance of extracted formazan showed similar pattern of reading (Fig. 2).

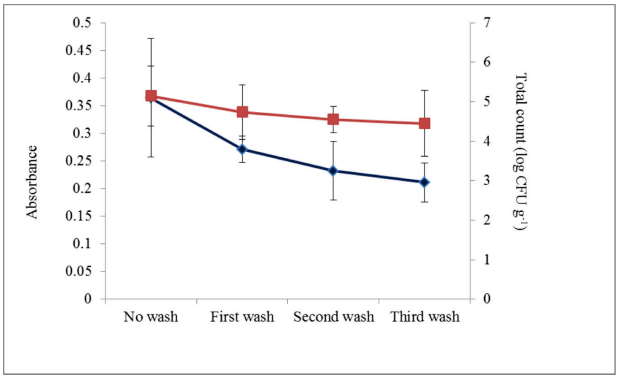

Fig. 1. Effect of washing on detachment of *Y. enterocolitica* to fish muscles

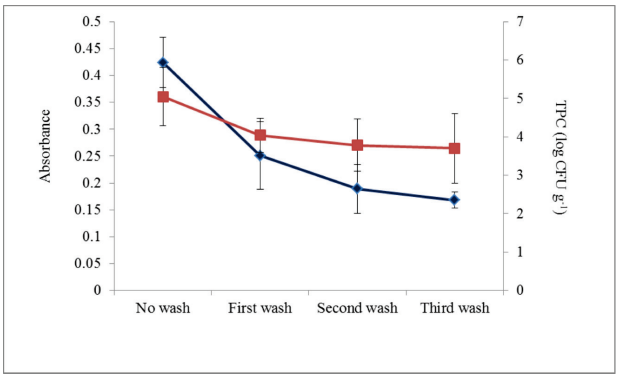

Fig. 2. Effect of washing on detachment of *Y. enterocolitica* to shrimp muscles

Bacteria can reach a newly exposed muscle surface from surrounding environment, contaminated surfaces and through cross contamination from other carcasses (Piette & Barriga, 1994). Bacteria are known to attach to meat tissues (Özdemir, 2006) which are difficult to remove. The use of chemicals to remove bacteria on raw meat is not acceptable by importing countries because of possible risk of residues in the meat and ithe ssue of disposal waste. Generally physical methods are highly encouraged. The common decontamination protocols include washing with water in the form of a spray or by complete immersion (Corry et al., 2007). One of the early studies on bacterial attachment to meat was done by Notermans & Kampelmacher (1974), who used bacterial suspensions to immerse broiler meat and then drained. The bacteria transferred from the suspensions to the meat surface during immersion were considered to be attached. The same immersion method, with minor modifications, was used in most subsequent studies of bacterial attachment to meat (Notermans & Kampelmacher, 1975; Firstenberg-Eden et al., 1978; McMeekin & Thomas, 1978; Firstenberg-Eden et al., 1979; Butler et al., 1980; Notermans et al., 1980; Thomas & McMeekin, 1981; Farber & Idziak, 1984; Lillard, 1985; Lillard, 1988; Dickson & Crouse, 1989). In this study also immersion method was used. Bacterial attachment to meat surface is a rapid process that occurs in the first minute of contact between bacterial suspension and meat tissue (Bouttier et al., 1997). It has been reported that a large numbers of bacteria adhered to the meat or became entrapped in channels and crevices at the meat surface (Firstenberg-Eden, 1981). It was therefore expected that bacteria have been attached to the fish and shrimp tissues used in this study which could be found after washing. Washing and rinsing of fruits and vegetables prolonged the shelf life by reducing the number of microorganisms on their surfaces as described by Ukuku et al. (2005). However, only a portion of pathogenic microorganisms may be removed with this simple treatment. Similarly, our study indicated that initial (first) washing was effective in reducing the number of *Y. enterocolitica* by 0.41 log CFU g^{-1} and 0.6 and 0.7 log CFU g^{-1} in subsequent washing

Bacterial or microbial respiration indicator (TTC) provides clue for count of bacteria and their activities. The absorbance of the formazan formed by bacterial respiration was measured. The average absorbance followed exactly the similar pattern as the total plate count. The absorbance was converted to molar concentration of formazan from the specific absorbance value which gave a means of comparing the activities of bacteria before washing and after each subsequent washing. The activities of cells derived as described above is presented in Table 3.

Table 1. Total plate count and respiration activities of *Y. enterocolitica* attached to fish muscles

	No wash[#]	First wash[#]	Second wash[#]	Third wash[#]
Absorbance	0.364 ± 0.107	0.271 ± 0.024	0.232 ± 0.053	0.211 ± 0.035
TPC (log CFU g^{-1})	5.145 ± 0.761	4.736 ± 0.693	4.55 ± 0.33	4.445 ± 0.835

[#] represents Mean ± SE; n=3

The activity of organism attached to fish muscle was in the range of 15.28 - 44.57 pmol $cell^{-1}$ h^{-1} with lowest activity recorded for the cells before wash and subsequently for the cells after wash. Cells attached to shrimp muscle showed the activity of 196.2 pmol $cell^{-1}$ h^{-1} after third wash as compared to 22 pmol $cell^{-1}$ h^{-1} activity of all the cells before washing.

Carpenter et al. (2011) studied the attachment efficacy of bacteria of public health significance bacteria on water wash. He rinsed cooked products with cold water for 5 min and found that there was 0.26 log cfu cm^{-2} of *L. moncytogenes* reduction in turkey roll and water wash reduced the attachment by 0.48 log cfu cm^{-2} in different products. Water wash could not lower the counts of *Salmonella* recovered from both chicken skin and pork belly. Distilled water reduced counts by 1.0 log cfu cm^{-2} of *L. monocytogenes* from inoculated frankfurters (Sofos & Georgnaras, 2010). The reduction in bacterial population achieved with rinsing procedure is expected to be one log cycle, which is clearly insufficient to bring a meaningful improvement in the bacterial quality of meat (Lillard, 1988). Post-harvest washing of fresh produce can be an important measure to reduce pathogen contamination. However, not all washing methods and washing solutions are effective (Olaimat & Holley, 2012).

Fish muscles are arranged in layer where gaps develop after death of fish. In the course of handling, pathogens enter the gaps and either attach or harbor themselves in cervices from where it is difficult to remove. The proportion of the cells removed by different washing steps has been represented as percentage of cells present in the previous wash. This was done to understand the efficiency of each wash, even though each wash was given exactly in the same manner. The values are presented in Table 4. This result indicates that washing was more effective in case of shrimp compared to fish muscles as muscle structures are different.

The first wash was very effective compared to other two washes which removed 61.15 and 90.32% of *Y. enterocolitica* cells from fish and shrimp respectively, while removal in the third washing was 21 and 16% respectively. The result indicates that as the number of washing increases the efficiency in removal is reduced.

In general, microbial reduction rate is affected by mechanical force of washing (Richard & Cooper, 1995). Spray treatment tested with trout reduced the bacterial level by one log cfu and led to the extension of shelf life. They recommended that chlorine dips cannot be used with fresh fish because simple washing and evisceration was more effective than chlorine dip. In this study, the bacterial densities were monitored by total plate count and bacterial reduction of tetrazolium salt. TTC is colourless, but is reduced to the bright triphenyl red formazan (TF) (Zhivich et al., 1991). The formazan formation occurs only in living cells with active mitochondria and is directly proportional to the number of viable cells (Matalon & Sandine, 1986). *In-vitro* studies indicate that the sensitivity of the

Table 2. Total plate count and respiration activities of *Y. enterocolitica* attached to shrimp muscles

	No wash[#]	First wash[#]	Second wash[#]	Third wash[#]
Absorbance	0.424	0.251	0.189	0.168
TPC (log CFU g^{-1})	5.05 ± 0.76	4.04 ± 0.45	3.78 ± 0.68	3.7 ± 0.91

[#] represents Mean ± SE; n=3

Table 3. Activity of cells of *Y. enterocolitica* attached to fish and shrimp muscles

	pmol $cell^{-1}$ h^{-1}			
	No wash	First wash	Second wash	Third wash
Fish	15.28	29.2	38.26	44.57
Shrimp	22.12	133.76	177.16	196.2

Table 4. Percent reduction of *Y. enterocolitica* during washing from initial count

	Change in number of cells (%)			
	Fish		Shrimp	
	In respect to original wash	In respect to previous wash	In respect to original wash	In respect to previous wash
First wash	61.15	61.15	90.32	90.32
Second wash	74.54	34.25	94.63	44.95
Third wash	80.04	21.69	95.52	16.67

TTC reduction method may exceed that of the classical culture techniques, as it allows for the detection of bacteria on the surface of the implant even if the number of the bacteria colonizing the biomaterial is below the detection threshold of culture techniques (Jaloza et al., 2009).

TTC metabolizes to form formazan in bacteria adhering to the surface (Reœliñski et al., 2010). TTC has been used to investigate microbial activity in stone (Bartosch et al., 2003) and on the surfaces of synthetic hernia implants (Reœliñski et al., 2010). Its applications also include the detection of hydrocarbon-oxidizing bacteria in oil-contaminated water and soil specimens (Olaga et al., 2008). Since the per cell activity rates seems to be higher as the washes progresses, it appears that the cells that remain bound are active one.

The study clearly indicates the efficiency of first washing to remove the *Y. enterocolitica* cells from both fish and shrimp tissues. The reduction in bacterial count was more in case of bacteria attached to shrimp muscles compared to fish muscles which may be due the fact that fish muscles and shrimp muscle are very different from each other. Probably the main reason is structural differences among fish and shrimp tissues. Respiration activity of cells also clearly indicates that the increment in activity per cell got increased as the washes progress and it seems that the cells that remain bound are active ones. The study insists on the usage of potable water for washing of seafoods which may help to reduce the contamination of various pathogens of human health significance.

Acknowledgements

Authors thank Dr. W. S. Lakra, Director, ICAR-Central Institute of Fisheries Education, Mumbai (India) for extending facilities for the work carried out. The financial assistance provided by Indian Council of Agricultural Research (ICAR), New Delhi (India) to the first author as fellowship is also gratefully acknowledged.

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