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Survivability of Pathogens under Different Growth Conditions in Seawater

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Abstract

There is need to assess the effects of variability in temperature as well as salinity on the fate of pathogens in marine foods and environments due to the links between climate change and disease epidemiology. Here, the survivability of four pathogenic bacteria (*Escherichia coli* O157:H7, *E. coli* ST2747, *Salmonella* sp., and *Salmonella enterica* subsp. *Enterica* Typhimurium) under various temperature regimes (4, 10, 25 and 50ºC in natural and artificial seawaters for 10 days) and salt concentrations (0.5×, 1× and 2× for 14 days) was evaluated. Antibiotic susceptibility profiling of the bacteria was also carried out using disk diffusion method. In general, temperature affected survival of the pathogens in both natural and artificial seawaters. Growth was mostly lowest at 4ºC, but not significantly different from that at 10ºC, for all organisms. However, significantly higher bacterial growth was observed, particularly in the first 5 days, at 25 and 50ºC, for most of the organisms. Survival of all organisms in natural seawater was higher at 50 than at 25ºC in the later stage of incubation, indicating greater bacterial persistence at higher temperature. In comparison, persistence of all organisms, except *E. coli* ST2747, was lowest in the 0.5× than in both 1× and 2× salt concentrations, though not significant (P>0.05). All organisms demonstrated high sensitivity to ofloxacin but showed varied multiple resistance to cefuroxime, ampicillin, nitrofurantoin and amoxicillin/clavulanate. These findings imply that in the future, if negative impacts of climate change are not mitigated, pathogenic organisms which pose serious health risks persist longer in seawater environments.

Keyword: Antibiotic susceptibility, Climate change, *E. coli* O157:H7, Public health, *Salmonella enterica*, Seawater

1. Introduction

There is an increasing need to understand the influence of certain environmental growth conditions, such as changes in temperature and seawater salt concentration, on the persistence

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of pathogenic organisms in seawater as increasing evidence shows a link between climatic change and increased disease epidemics and pathogen virulence (Harvell *et al*., 2002). Runoff from agricultural wastewaters and manure has been observed to lead to transfer of enteric pathogenic bacteria, primarily *Escherichia coli*, *Salmonella* spp. and *Shigella* spp. into the open sea. This transfer could be direct, such as from runoff or flow water, or indirectly through animals and contaminated freshwater which flows into the sea (Williams *et al*., 2007). These discharges into aquatic ecosystems are on the increase (Obi *et al*., 2016), and depending on the quality and quantity of these discharges, they are capable of producing physicochemical and ecological disturbances in the receiving waters (Morifiigo *et al*., 1990; Obi *et al*., 2016).

However, problems that arise from these are the potential public health hazards that are associated with the recreational use of the receiving waters (Borrego *et al*., 1983) and the consumption of foods from such waters, especially filter-feeding molluscs which are often eaten raw (Iwamoto *et al*., 2010). Seafood are high in nutritional contents, but they are prone to bacterial contamination and could pose serious health risk to consumers, especially when eaten raw or undercooked (Amagliani *et al*., 2012). Pathogenic *E. coli* and *Salmonella* sp. have been of public health concern to seafood producers and consumers, and a major cause of import bans and rejections in international markets (WHO, 2001; Norhana *et al*., 2010).

Escherichia coli, a facultative anaerobic organism belonging to the family Enterobacteriaceae, is a natural inhabitant of the intestinal tracts of humans and warm-blooded animals. However, there are certain strains of *E. coli* that have been found to be pathogenic (Chandra *et al*., 2013). Among these is the enterohaemorrhagic *E. coli*, which has *E. coli* O157:H7 as the most common member. In addition to being haemorrhagic, *E. coli* O157:H7 has been known to be verocytotoxin-producing, or Shiga-toxin-producing (Pennington, 2010). This organism has been implicated in several cases of haemorrhagic colitis, haemolytic uremic syndrome and bloody diarrhoea worldwide (Effler *et al.*, 2001; Olsen *et al*., 2002; Rangel *et al*., 2005; Rybarczyk *et al*., 2017). Studies conducted in eight countries over a period of 24 years revealed that the major source of transmission of *E. coli* was via faecal–oral route, with over 40% of the total of outbreaks being traced to contaminated foods. Other sources include dairy products, animal contact, water, and the environment (Snedeker *et al.*, 2009). The presence of *E. coli* and *Salmonella* sp. has also been demonstrated in ready-to-eat seafood, which require minimal heating and seafood that are eaten raw (Amagliani *et al*., 2012; Boss *et al*., 2016); and have been isolated from sea and brackish water of both tropical and temperate regions (Kirs *et al.*, 2010). There are reports which have suggested that *Salmonella* sp. survive longer than *E. coli*

in sea and freshwater environments (Huss, 1997; Sugumar and Mariappan, 2003). This is possibly because, compared to other Gram-negative bacteria, *Salmonella* sp. are relatively resistant to many environmental factors (WHO, 1988). For instance, they are capable of growth at 5–47^{o}C, with an optimum growth temperature of 37^{o}C (Olgunoğlu, 2012). They have also been observed to grow between 2–54^{o}C, although growth below 7^{o}C and above 48^{o}C is only observed under laboratory conditions and in mutant strains respectively (Bremer *et al*., 2003).

Antibiotic use and misuse over the last few decades has led to the increase in the number of antibiotic-resistant microorganisms in the environment. Due to the characteristic quick distribution of antibiotic resistance among pathogens, many previously potent antibiotics have been rendered useless, and are now unable to treat bacterial infections they once were effective against (Sengupta *et al*., 2013). In light of this growing challenge against antimicrobial resistance, the problem has come to be recognized as one of the most pressing public health concerns in this century (WHO, 2014; Woolhouse *et al.*, 2016). Antibiotic resistance in *E. coli* and *Salmonella* is of important public health concern because the organisms are very common in humans, animals, food and environmental samples.

Microbial pathogens in seawater are exposed to some biotic and abiotic factors which may cause stress including dilution, temperature change, pH, salinity, sedimentation, nutrient deficiencies, predation, and sunlight amongst others (Rhodes and Kator, 1990; Sinton *et al*., 2002; Chandran and Hatha, 2005). The effects may be acerbated by the influence of climate change which has varying impacts on marine life (Eissa and Zaki, 2011). For instance, warming of seawater has been associated with intensified metabolic rates of organisms and stratification of the water column. Barange and Perry (2009) projected that the combined effects of temperature and salinity changes which arise from climate warming will reduce the density of the ocean and increase vertical stratification. This will further lead to additional stress for marine organisms and their associated microflora. The pattern of salinity in the ocean has been changing over a course of decades and this change affects both water cycle and marine salinity; with fluctuations in salinity accompanied by ionic disruptions in microbial cells (Durack *et al*., 2012). However, there is limited information on the factors influencing survival of *E. coli* O157:H7 and *Salmonella* sp. under marine growth conditions.

Therefore, this study was designed to assess the effects of variability in temperature and salinity on the, survival of *E. coli* and *Salmonella* species (*E. coli* O157:H7, *E. coli* ST2747, *Salmonella*

sp., and *Salmonella enterica* subsp. *Enterica* Typhimurium). Also the pattern of susceptibility of these organisms to antibiotics was assessed.

2. Materials and Methods

Materials

The culture media used in this study – nutrient agar, nutrient broth, sorbitol MacConkey agar (SMAC), MacConkey agar with salt (MCA), Salmonella-Shigella agar (SSA) as well as the cefixime-potassium tellurite (CT) were purchased from Oxoid, Basingstoke, UK. Mueller-Hinton agar (MHA) and the multi-disc antibiotics (CM-12-8NR100) were obtained from Rapid Lab., UK. The other chemicals used are of analytical grade and obtained from Sigma-Aldrich, UK. All culture media were prepared following the manufacturers' instructions with little modification. Instead of normal deionized water, saline water (9% NaCl w/v) was used to prepare the agar media. Based on preliminary study, greater cell recovery and growth was recorded on the culture agar media prepared in this manner. Sterilization was done by autoclaving at 121°C for 15 minutes. The nutrient agar was used to enumerate total viable bacteria, and MacConkey agar with salt to enumerate the total coliform bacteria in the samples.

Bacterial strains and cultural conditions

Escherichia coli O157:H7 was isolated on sorbitol MacConkey agar supplemented with the antibiotics cefixime and potassium tellurite (CT-SMAC) while *Salmonella* species were enumerated on Salmonella-Shigella agar. The nutrient broth was used to cultivate the pure isolates to obtain a large inoculum growing at exponential phase for the survival studies. *E. coli* strain ST2747 and *Salmonella enterica* subsp. *Enterica* Typhimurium were previously isolated from environmental water in Ilorin and identified using 16S rRNA gene analysis. The isolates of *E. coli* O157:H7 and *Salmonella* sp. were further verified by culturing on EMB, then Gram stained, and tested for indole, methyl-red, Voges-Proskauer, and citrate. They were further tested using the O157 latex agglutination test and H7 antisera.

Animal and water sample collection

Samples of squids (*Sepiella ornate*), shrimps (*Penaus notialis*), and seawater were collected around the Oniru Beach, Lagos, Nigeria at two different sampling times between April and June, 2016. Sampling was carried out in very early hours of the morning. The animals (about 60 shrimps and 4 squids) were obtained from the fishermen who harvested them in the open

sea and collected into clean sealable sterile plastic bags. Seawater samples were collected in the open sea about 400 m offshore into 5 L sterile polypropylene screw-cap bottles 20 cm below the surface. All samples were placed in an ice chest (4^{o}C) and transported to the laboratory in Ilorin and analyses started on the same day of collection and not more than 8 hours after collection. (FDA, 1992). Temperature (25–27°C) and pH (7.7–8.3) of seawater samples were determined on site while salinity (24–33%) was measured using the $AgNO_3$ titration method of Harvey (1955) in the laboratory.

Sample preparation and bacteriological analysis

The squid was dissected with sterile scalpels under aseptic conditions in a UV-sterilised cabinet to separate the parts (skin, tissue, gut, tentacles and buccal cavity). A gram of each of the parts of the squid was weighed and macerated with ethanol-sterilised mortar and pestle to get a paste which was introduced into test tubes containing 10 ml sterile saline water (9% NaCl w/v). Shrimps were blended using mortar and pestle and 1 g of the mashed shrimp was weighed and introduced into a test tube containing 10 ml sterile saline water. The contents of the test tubes were vortexed for 30 seconds to dislodge the microbial cells from the animal parts. Subsequently, appropriate ten-fold serial dilutions were made and 1 ml aliquots of the least two dilutions were inoculated on nutrient agar plates using pour plate technique. Similarly, the seawater was first serially diluted in saline water before 1 ml aliquots of the appropriate dilutions were inoculated onto the agar plates (AOAC, 1995a, b; Environment Agency, 2009). Incubation of all agar plates was done at 37^{o}C for 24-48 hours, except for CT-SMAC agar, which was at 45^{o}C. The whole procedure was carried out carefully under aseptic conditions to prevent cross contamination with the materials used in sample processing.

Growth, harvest and quantification of purified cell pellets

Distinct colonies of *E. coli* O157:H7 and *Salmonella* sp. recovered from the seafood as well as the previously isolated *E. coli* strain ST2747 and *Salmonella enterica* subsp. *Enterica* Typhimurium were retrieved from their appropriate selective media into nutrient broth placed in 250-ml conical flasks. The conical flasks containing the individual isolate were incubated on a rotary shaker at 100 rpm at 37^{o}C for 72 hours to allow the growth of cell till exponential phase under aerobic condition. Harvest of cell pellets was done by centrifugation at 3500 × *g* for 10 minutes. Centrifugation was repeated twice until a clear supernatant was obtained over the cell pellets at the bottom of the tubes. The supernatant broth was then carefully decanted and discarded. The washed cell pellets were re-suspended in sterile phosphate buffer solution

(PBS: 8g NaCl, 0.2g KCl, 1.44g Na_2HP0_4, 0.24g KH_2PO_4 per litre of distilled water; pH 7.2) and the process repeated 3–4 times. The PBS was used to wash the remnant of the broth from the cells. The pure cell pellets were finally re-suspended in PBS and stored in the refrigerator until usage, but not more than 2 days. The purified cell suspension was quantified using MacFarland's standard (adding 1% $BaCl_2$ with 1% H_2SO_4) and taking the absorbance at a wavelength of 600nm using a spectrophotometer (Genesys 20 model). Where necessary, the suspensions of the individual isolates were appropriately diluted with PBS to give approximately 9.0×10^8 cfu/ml.

Pathogen survival studies under different temperature and salt concentration regimes

There were two sets of experiments carried out under the survival studies: (i) Comparative evaluation of effect of temperature at 4, 10, 25, and 50°C on the survival of test isolates in natural and artificial seawater after 0, 1, 3, 5, 7 and 10 days of inoculation; and (ii) Comparative evaluation of the effect of salt concentration at 0.5×, 1×, and 2× of normal concentration (see Table 1 for salt composition) on the survival of test isolates after 0, 1, 3, 5, 7, 10 and 14 days of inoculation. The formulations of the artificial seawater were modified from Subow (1931) (Table 1):

Table 1: Modified artificial seawater concentrations.

Salt composition	**Artificial seawater concentration (g/l)**		
	0.5×	1×	2×
NaCl	13.20	26.52	53.00
$MgCl_2$	1.22	2.45	4.88
$MgSO_4$	1.65	3.31	6.60
$CaCl_2$	0.57	1.14	2.28
KCl	0.36	0.73	1.45
$NaHCO_3$	0.10	0.20	0.40
NaBr	0.04	0.08	0.17
pH	7.50	8.15	9.20

The collected natural seawater and compounded artificial seawater were first autoclaved for three consecutive times to allow all vegetative and spore-forming cells to be completely killed. The test organisms used for the survival studies were isolates of *E. coli* 0157:H7 and *Salmonella* sp. recovered from the gut of the squid as well as the typed isolates of *Salmonella enterica* subspecies *enterica* serovar Typhimurium 08-1736 and *Escherichia coli* ST2747

(previously recovered from freshwater and identified using PCR 16S rRNA gene sequencing). The typed isolates were used as standard control organisms and assayed in the same manner as the test organisms in natural and artificial seawaters.

Although the carbon, nitrogen and phosphorus contents of the natural seawater were not determined, in order to provide sufficient nutrient sources in the artificial seawater for bacterial growth, minimal basal salts (MBS: 0.3g NaCl, 0.6g $(NH_4)_2SO_4$, 0.6g KNO_3, 1.5g MgSO4.7H2O, 0.75g Na_2HP0_4, 0.25g KH_2PO_4 and 1.0g glucose per litre of distilled water) solution was prepared and added to both natural and artificial seawater setups.

To determine the effect of temperature, the experiment was carried out in 250-ml conical flasks with a working volume of 100 ml into which 1 ml of the bacterial suspension was added to deliver washed cell concentration of approximately 1×10^4 cfu/ml. There were a total of 32 setups comprising 8 flasks each for a test organism; with 4 of these flasks containing natural seawater and the other 4 flasks artificial seawater. Eight conical flasks containing either natural or artificial seawater with a particular test isolate were incubated for 10 days at 4, 10, 25 and 50°C, respectively. The flasks for the 4°C were placed in the freezer compartment while the ones for the 10°C were placed in the fridge compartment of two separate refrigerators and the their temperatures appropriately adjusted. The flasks for the 25°C were placed on the work bench at room temperature, and finally the ones for the 50°C were placed in a water bath maintained at that temperature. At the defined intervals for a period of 10 days, 1 ml was pipetted from each of the flasks and appropriate 10-fold serial dilutions made. Aliquots (1 ml) were then removed from the least two dilutions and plated aseptically on the appropriate selective media and incubated at 37ºC for *Salmonella* isolates and at 45ºC for *E. coli* isolates. *E. coli* O157:H7 was inoculated onto CT-SMAC, *E. coli* ST2747 onto MCA, and both *Salmonella* strains on SSA. Cell enumerations were done at 24 and 48 hours. The lower limit of detection was set at 0.95 $\log_{10}$ CFU/ml.

To assay for the effect of salt concentration, 12 conical flasks, with 100 ml working volume, were used and the experiment carried out at room temperature of 25ºC. The 0.5× setups represented diluted seawater with half of salt contents of the normal (i.e. 1×) and 2× setups represented highly concentrated seawater with twice the salt contents of the normal (i.e. 1×). A set of 4 flasks contained a specific salt concentration with different test isolates added to each of them. The survival measurements for 14 days were carried out as previously described above.

Antibiotic susceptibility assay

To standardize the inoculum used, the test isolates were first individually cultured in nutrient broth for 72 hours at 37ºC to produce cells growing at exponential phase. The cell pellets were harvested and purified as previously described above. The turbidity of the final bacterial suspension was adjusted to 0.5 MacFarland standard. The antibiotic susceptibility assay was carried out according to Kirby Bauer disk diffusion technique (Bauer *et al*., 1966), where 1 ml of the cell suspension (*ca*. 1.2×10^6 CFU/ml) was evenly spread on Mueller-Hinton agar plates using sterile swabs and incubated aerobically for 6 hours; then the multi-disc rings of antibiotics were carefully placed on the plates and incubated at 37˚C for a further 24 hours. The diameters of inhibition zones around the antibiotic discs were measured and the average values of three readings reported (to the nearest mm). The results were interpreted using the Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2016). The following antibiotics were used: ceftazidime (30 µg), gentamycin (10 µg), nitrofurantoin (30 µg), augmentin (30 µg), amoxicillin (30 µg), ciprofloxacin (30 µg), ofloxacin (30 µg), and cefuroxime (30 µg).

Statistical analysis

The $\log_{10}$ transformed data of the survival studies showed an initial steady growth phase of appropriately 3–5 d in many of the treatments, and followed by a period of linear decline or logarithmic death. The linear polynomial equation was fitted only to the portion of data representing the decline phase and used to calculate the decline rate (*D*-values: days to achieve a $\log_{10}$ reduction in cell number) of the test organisms under the various temperature and salinity regimes. The differences in the *D*-values were evaluated by ANOVA ($P < 0.05$), and the mean differences evaluated using post-hoc Tukey's test. Differences between the natural and artificial seawaters with respective to temperature and organism type were analysed by two-way ANOVA. Statistical analyses were performed with SigmaStat v.3 (IBM SPSS Inc. Chicago, IL, USA).

3. Result and Discussion

Occurrence of heterotrophic and coliform bacteria, E. coli O157:H7 and Salmonella sp. in seafood and seawater

Table 2 highlights the heterotrophic bacteria populations in different parts of squids, shrimps and seawater. The mean viable bacteria numbers ranged from 7.00–68.00 $\times 10^5$ cfu/g in the squid samples. These values were higher than those recovered from shrimps (1.97–2.45 $\times 10^5$ cfu/g) and in seawater (6.10–11.80 $\times 10^5$ cfu/ml). Similarly, total coliform number was

generally higher in the squid parts (2.80–12.00 × 10^4 cfu/g) than in shrimps (3.50–4.70 × 10^3 cfu/g) or seawater (0–1.10 × 10^4 cfu/ml). Recovered *E. coli* O157:H7 numbers ranged from 1.00–6.90 × 10^2 cfu/g while *Salmonella* number ranged from 0–2.60 × 10^2 cfu/g from the squid samples; lower numbers of the pathogens (0–3.50 × 10^1 and 4.70–6.00 × 10^1 cfu/g, respectively) were recovered in shrimps. *E. coli* O157:H7 number ranged from 0–1.10 × 10^1 cfu/ml, while no *Salmonella* was recovered in the seawater. Various authors have also detected these organisms in seafood and seawater (del Refugio Casteneda Chavez *et al*., 2005; Gopal *et al*., 2005; Koonse *et al.*, 2005; Kumar *et al*., 2008; Norhana *et al*., 2010). In a water surveillance carried for over a year, del Refugio Casteneda Chavez et al. (2005) reported very high occurrences of *E. coli* (88.6–97.1%) and *Salmonella* sp. (86.7–100%) in La Mancha and Alvarado lagoons on the Atlantic coast of Veracruz, Mexico. The detection of these pathogens in shrimps and squids further affirms the general recommendation that seafood should not be eaten raw. In the light of concerns raised by the presence of these pathogens in the tested seafood, increased inspection and regular monitoring of seafood processors and processing factories should be carried out by appropriate regulatory bodies. In addition, handlers of these foods are encouraged to develop and execute hazard analysis critical control point (HACCP) systems.

Table 2: Bacterial populations in seafoods and seawater.

Bacterial group (cell number)ǂ	Squid 1				Squid 2					Shrimp		Seawater	
	(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)	(e)	1	2	1	2
Heterotrophic bacteria (× 10^5 cfu/g; cfu/ml)	34.0	7.0	38.0	66.0	12.0	4.60	68.0	19.0	19.0	1.97	2.45	11.8	6.10
Total coliforms (× 10^3 cfu/g; cfu/ml)	120.0	10.0	65.0	1.0	40.0	30.0	40.0	5.30	28.0	3.50	4.70	2.0	11.0
E. coli O157:H7 (× 10^1 cfu/g; cfu/ml)	6.8	0.0	3.5	0.0	4.8	7.0	11.4	5.9	5.7	0.0	3.5	0.0	1.1
Salmonella/Shigella sp. (× 10^1 cfu/g; cfu/ml)	5.0	0.0	26.0	0.0	6.0	5.2	18.0	0.0	24.0	6.0	4.70	0.0	0.0

ǂ Values for (a) buccal, (b) skin, (c) gut, (d) viscera and (e) tentacle are in cfu/g and values for seawater in cfu/ml.

Effect of temperature on survival of pathogens

Temperature has been reported by several authors to be an important factor for the survival of *E. coli* and *Salmonella* sp. in environmental samples with the general observation that higher temperatures result in increased die-off of the organisms (Arrus *et al*., 2006; Garcia *et al*., 2010; Blaustein *et al*., 2013). The survival of the four test organisms in natural and artificial seawaters

at various temperatures was monitored over 10 days and the results presented in Figure 1. In general, temperature affected the survival of the pathogens in both natural and artificial seawaters. Growth was mostly lowest at the refrigerator (4^{o}C) condition, and was not significantly different from that at 10^{o}C, for all organisms (Figure 1). However, significantly higher bacterial growth was observed, particularly in the first 5 days, at 25 and 50^{o}C for most of the test isolates, as compared to growth at lower temperatures. Interestingly, in the natural seawater, while the initial increases in growth of all organisms between days 1 and 5 were greater at 25^{o}C, there were no appreciable differences in growth of most organisms at 10 and 25^{o}C in the later stage (days 5 to 10). Noteworthy is the observation that growth of all organisms in natural seawater was higher at 50 than at 25^{o}C in the later stage of incubation (Figure 1). For instance, growth of *Salmonella* sp. and *Salmonella enterica* in natural seawater, though negligible at day 1, increased appreciably to peak at day 3 and only declined slightly to persist at the relatively highest cell numbers between days 7 and 10 days of incubation. Comparatively, the pathogens apparently persisted better at 50^{o}C in the natural than in the artificial seawater at the end of the incubation, with the exception of *E. coli* O157:H7. Khalil (2016) reported the growth and survival of *E. coli* O157 strains in damaged leafy green vegetables in Egypt. The pathogen populations increased with increasing temperature from 8 to 22^{o}C under storage conditions.

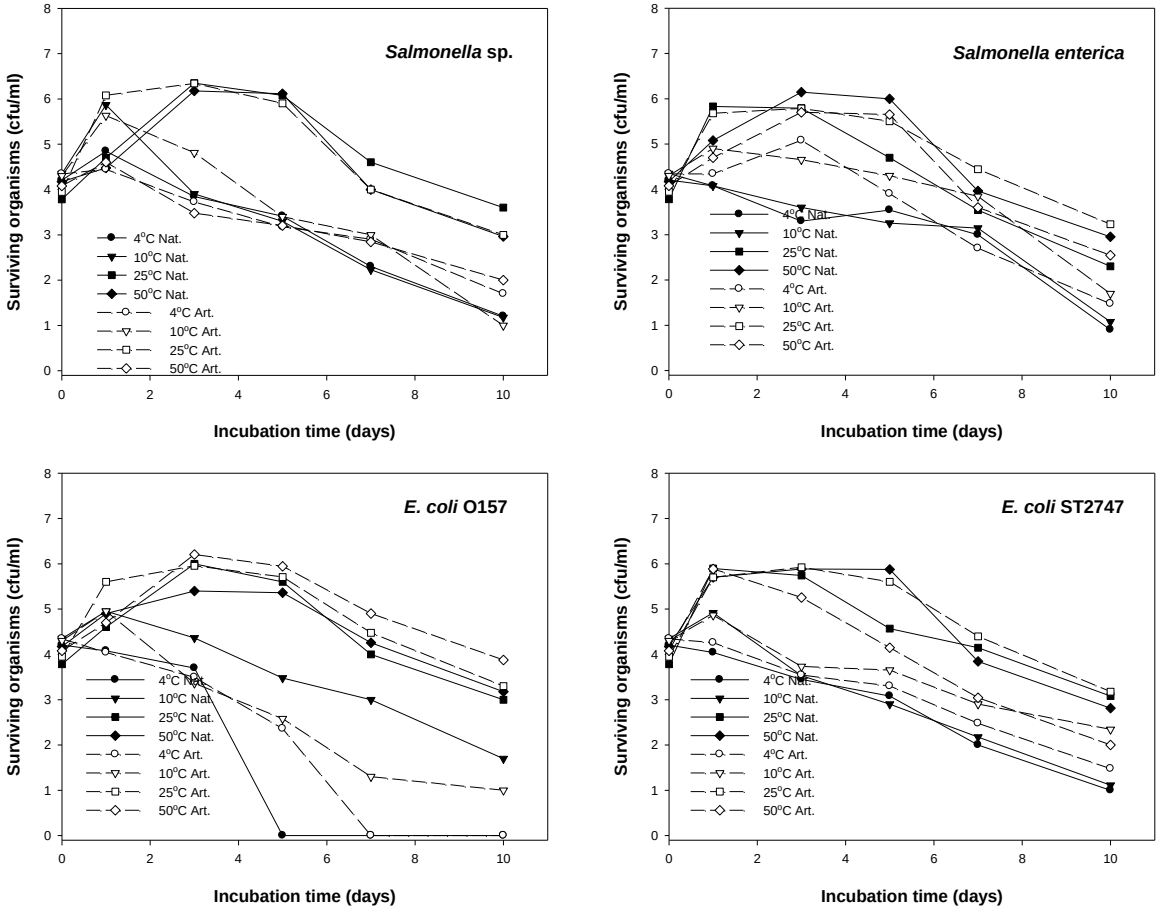

Figure 1: Effect of temperature on survival of the test isolates in natural and artificial seawaters.

The lag observed in the growth of the organisms at 4 and 10ºC can be attributed to the slowdown of the organisms' metabolism, which consequently extends the detrimental action of seawater on the long run (Jones, 1971). Therefore, even though the bacteria are not actively growing under these low temperature regimes, they are still metabolically active and would persist for longer time than actively growing cells which are utilizing available nutrients at a much higher rate. This means an increase in temperature might lead to greater stress and energy consumption for the organisms than a decrease in temperature (Semenov *et al*., 2009). For example, in low-temperature environments, the metabolism and substrate utilization rate of *E. coli* are markedly reduced (Blaustein *et al*., 2013). Furthermore, when temperature reaches around 5ºC, *E. coli* and *Salmonella* can enter a viable but non-culturable (VBNC) state, which allows continuous survival without the ability to divide (Gupte *et al*., 2003; Na *et al*., 2006). This is corroborated by various studies which have shown that enteric bacteria such as *E. coli* and *Salmonella* are more stable, and thus survive longer, at low temperature than higher ones in both fresh and marine waters (Carlucci and Pramer, 1960; Vasconcelos and Swartz, 1976; Lessard and Sieburth, 1983; Popovic *et al*., 2010). Furthermore, enteric pathogens, with few

exceptions, typically do not replicate in aqueous environment. This is because, on entry, they are gradually inactivated, with high inactivation rates usually correlating with increasing temperature (Schijven and de Roda Husman, 2005). Depending on the growth pattern of a bacterium, there may be an initial rapid growth in water with high temperature, as reported for *E. coli* by Freeman *et al*. (2009). Like all other physicochemical factors, however, temperature does not act alone and its effects are usually dependent upon other growth conditions.

Effect of salt concentration on survival of pathogens

Results of the effect of salt concentration (0.5×, 1× and 2×) on *Salmonella* sp., *Salmonella enterica*, *E. coli* O157:H7, and *E. coli* ST2747 is presented in Figure 2. Growth patterns of *Salmonella* sp. and *Salmonella enterica* followed similar trends, though the initial rate of decline after the rapid increase to peak at day 3 was more pronounced in *Salmonella* sp. Likewise, growth patterns of *E. coli* O15:H7 and *E. coli* ST2747 were similar at all the three salt concentrations; cell numbers gradually increased to peaks on day 5 and declined steadily thereafter. In comparison, generally, persistence of all organisms, except *E. coli* ST2747 was slightly lowest in the 0.5× than in both 1× and 2× salt concentrations. However, this was not significant. While growth of both *Salmonella* sp. and *S. enterica* were usually higher at the early stage, there seems not to be appreciable difference in the cell numbers of the Salmonellae as compared with those of *E. coli* species investigated in this study after 7 days of incubation, at the different salt concentrations.

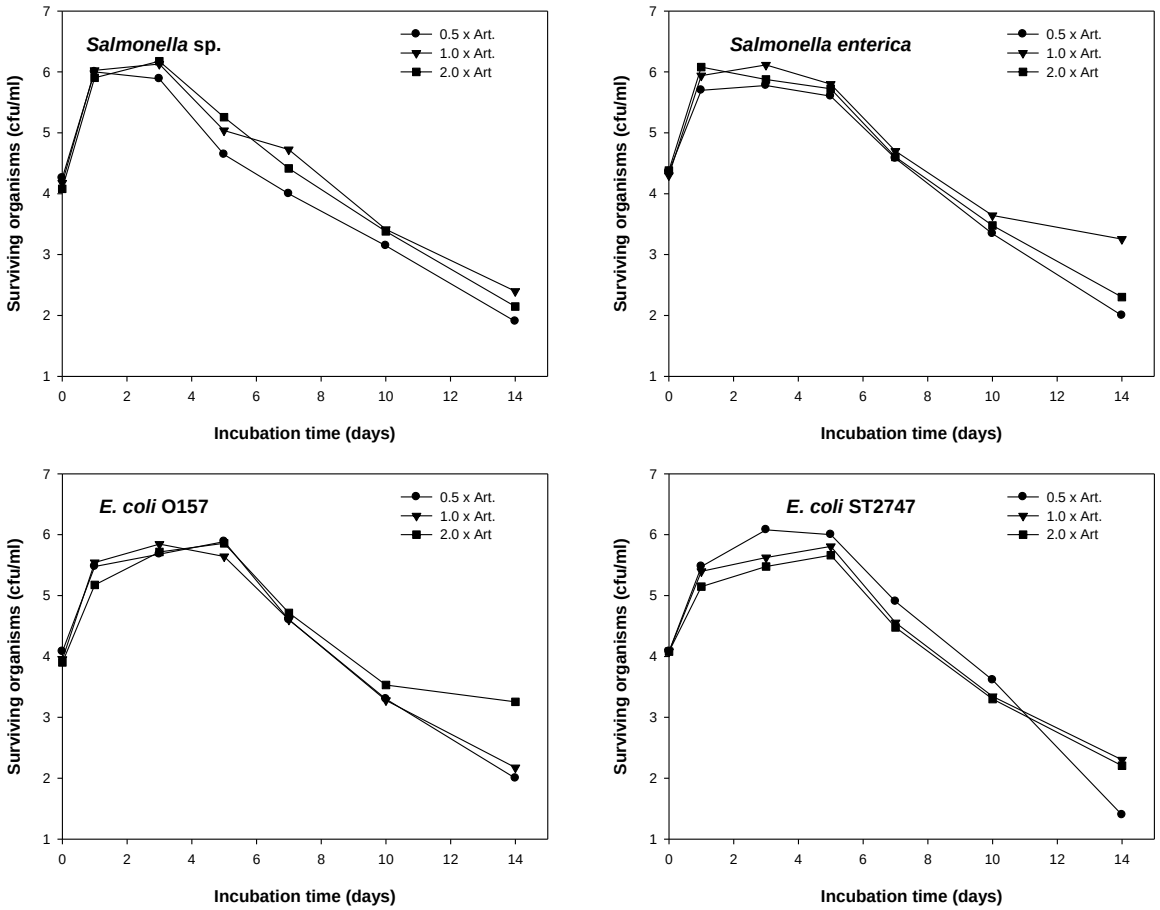

Figure 2: Effect of seawater salt concentrations on survival of the test isolates.

These observations are in tandem with the work of Kaspar and Tamplin (1993), who described the effects of salinity on the survival of vibrios and found that although high salinity may have effects on the survival of bacteria, they are not as striking as the effects of temperature. This observation may be because the rapid deaths of enteric bacteria in seawater are not primarily a result of its high salinity, and the normal salt concentrations of marine waters are not detrimental to these organisms (ZoBell and Feltham, 1933; Nusbaum and Garver, 1955; Chandran and Hatha, 2005). However, the low rate of persistence observed at low salt concentration with some of the test strains contradicts the study of Anderson *et al*. (1979) who evaluated the survival of *E. coli* in seawater at different salt concentrations and found that decreasing salinity was accompanied by increasing survival of the isolate.

Antibiotics susceptibility profiles of test isolates

Results of the antibiotic susceptibility profiles of the test isolates are presented in Table 3. *Salmonella* sp. was highly sensitive to ciprofloxacin (23 mm), cefuroxime (18 mm), ceftazidime (16 mm), gentamycin (14.6 mm) and ofloxacin (11 mm) but exhibited resistance

to amoxycillin/clavulanate, nitrofurantoin, and ampicillin. *Salmonella enterica* was sensitive to a different set of antibiotics: ciprofloxacin (16.3 mm), ofloxacin (12 mm), ampicillin (8.6 mm), ceftazidime (8 mm), and nitrofurantoin (6.6 mm) but exhibited resistance to cefuroxime, gentamycin, and amoxycillin/clavulanate. *E. coli* O157:H7 was sensitive to ciprofloxacin (17.3 mm), ofloxacin (17 mm), gentamycin (14.3 mm), and amoxycillin/clavulanate (11.3 mm) but exhibited resistance to cefuroxime, ceftazidime, nitrofurantoin, and ampicillin. *E. coli* ST2747 exhibited varied sensitivity to all the antibiotics excluding cefuroxime and ampicillin. All test isolates demonstrated sensitivity to ciprofloxacin and ofloxacin, while only one organism each was sensitive to either cefuroxime or ampicillin (Table 3).

Table 3: The antibiotic susceptibility profiles of test isolates.

Isolates	Zone of inhibition (mm)$^{\Phi}$							
	CAZ	CRX	GEN	CIP	OFL	AUG	NIT	AMP
Salmonella sp.	16.3±0.9	18.0±0.0	**14.6±0.4**	23.0±0.8	11.0±0.8	0.0±0.0	0.0±0.0	0.0±0.0
Salmonella enterica	8.0±0.8	0.0±0.0	0.0±0.0	16.3±1.6	12.0±1.6	0.0±0.0	6.6±0.9	8.6±0.4
AST Breakpoints (R,I,S)	≤17, 18-20, ≥21	≤14, 15-22, ≥23	≤12, 13-14, ≥15	≤20, 21-30, ≥31	NA	≤13, 14-17, ≥18	≤14, 15-16 ,≥17	≤13, 14-16, ≥17
E. coli O157:H7	0.0±0.0	0.0±0.0	*14.3±0.9*	17.3±0.4	**17.0±1.4**	11.3±4.7	0.0±0.0	0.0±0.0
E. coli ST2747	9.0±0.0	0.0±0.0	4.6±0.4	20.0±0.0	**20.0±0.0**	11.0±0.0	7.0±0.0	0.0±0.0
AST Breakpoints (R,I,S)	≤17, 18-20, ≥21	≤14, 15-22, ≥23	≤12, 13-14, ≥15	≤15, 16-20, ≥21	≤12, 13-15, ≥16	≤13, 14-17, ≥18	≤14, 15-16, ≥17	≤13, 14-16, ≥17

KEY: CAZ = Ceftazidime, CRX = Cefuroxime, CIP = Ciprofloxacin, AUG = Amoxycillin/Clavulanate, AMP = Ampicillin, GEN = Gentamycin, OFL = Ofloxacin, NIT = Nitrofurantoin; NA: Not applicable; Φ: values in bold mean organisms are sensitive to the antibiotics.

Antibiotic resistance has become a big problem worldwide, with resistant bacteria emerging from different sectors such as animal husbandry, agriculture, veterinary and human medicines. Such bacteria are transmitted into aquatic environments and their products (Amagliani *et al*., 2012) where horizontal transfer can occur between them, seafood and humans through a variety of means (Newell *et al.*, 2010). The antibiotic resistance pattern observed in organisms isolated in this study is of huge public health concern. The resistance against ampicillin which was demonstrated by most of the pathogens has been documented by some authors. For instance, Kumaran *et al*. (2010) reported that 56.25% *E. coli* stains from seafood in India were resistant to ampicillin and more than 35% resistant to ciprofloxacin. In a related study, Boinapally and Jiang (2007) described the isolation of a *Salmonella* strain resistant to ampicillin, ceftriaxone, gentamicin, streptomycin and trimethoprim from shrimps. Similarly, Yan *et al*. (2010) described *Salmonella* serovars from seafood resistant to various categories of antibiotics such as beta-lactams, aminoglycosides, nitrofurans, sulfonamides, quinolones and fluoroquinolones,

According to Ryu *et al*. (2012), seafood may act as the reservoir for multi-resistant bacteria and facilitate the dissemination of the resistance genes.

In Turkey, Matyar *et al*. (2008) isolated *E. coli* strains resistant to heavy metal (cadmium) and antibiotics (cefazolin, nitrofurantoin, cefuroxime and ampicillin) from shrimp samples originally from Iskenderun Bay. The authors recognized the presence of these bacteria in foods as a potential risk for public health. In Brazil, there have been reports on the presence of non-resistant and antibiotic-resistant *E. coli* in farming area and in fresh shrimp Likewise, Duran and Marshall (2005) isolated antibiotic-resistant bacteria, including *E. coli*, from ready-to-eat shrimp and concluded that this product may be considered as an international vehicle of antibiotic-resistant human pathogens.

4. Conclusion

The findings in this study demonstrates that *E. coli* and *Salmonella* can thrive and survive under artificial and slightly modified seawater environments. The survival of enteric bacteria depend on many physicochemical factors, among which temperature and salt concentrations were selected for the current study. Due to the variation and fluctuations observed in natural marine environment, it is perhaps impossible to give an accurate estimate of survival rates without taking all biotic and abiotic factors into consideration. However, studies on the effects of temperature and salinity on the survivability of these organisms will go a long way in further explaining their persistence in seawater and subsequently seafood harvested from such waters. Further work may involve the study of the optimum conditions of temperature and salinity for the longest survival period of the organisms. Simultaneous variation and shuffling of culture conditions (temperature and salinity) will reveal the ideal conditions that positively or negatively affect the survivability of the test strains.

The findings on the survivability of the pathogenic organisms under various temperature and salt concentration portend a serious public health risk, particularly as there is potential increase in global temperature and rise in seawater level as well as increased pollution of marine environments with organic and inorganic materials. This implies that in the future, if these negative impacts of climate change are not mitigated, pathogenic organisms will survive more and persist longer in seawater and foods.

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