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Virulence and multidrug resistant *Salmonella enterica* isolated from *Cardisoma armatum* and *Mercenaria mercenaria* sold in Port Harcourt, Nigeria

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ABSTRACT

Cardisoma armatum (crab) and *Mercenaria mercenaria* (clam) are highly esteemed sources of protein globally, cherished for their nutritional value. However, the consumption of contaminated seafood can pose severe health risks. *Salmonella enterica* is a significant concern due to its propensity to cause foodborne illnesses. This study aimed to determine virulence and multidrug resistant *Salmonella enterica* isolated from *Cardisoma armatum* and *Mercenaria mercenaria* sold in Port Harcourt, Rivers State, Nigeria. Eight seafood samples were collected from two different markets, Creek Road Market and Mile One Market, and subjected to standard bacteriological procedures such as culture, isolation and identification. A total of 18 *Salmonella enterica* were isolated and identified. The results revealed significantly elevated levels of *Salmonella enterica* in the seafood samples, with notable variations ($p \leq 0.05$) across different seafood types. In raw and parboiled crab samples; Total Heterotrophic Bacteria (THB) counts ranged from 5.40±0.28 to 2.15±1.63 ($x10^6$ CFU/g); 1.6±0.09 to 1.69±0.62 ($x10^6$ CFU/g), Faecal Coliform counts ranged from 6.1±0.86 to 6.4±0.91 ($x10^4$ CFU/g); 2.58±1.30 to 3.72±2.38 ($x10^4$ CFU/g), and Total *Salmonella* counts ranged from 3.00±1.69 and 5.00±0.14 ($x10^2$ CFU/g) 2.52±2.09 and 2.10±1.56 ($x10^2$ CFU/g. In raw and parboiled clam samples in both markets showed Total Heterotrophic Bacteria (THB), counts ranging from 1.98±0.37 to 3.79±2.98 ($x10^6$ CFU/g); 1.38±0.43 to 2.21±0.33 ($x10^6$CFU/g), Faecal Coliform Count of 3.50±2.12 to 2.10±0.57 ($x10^4$ CFU/g) 0.00±0.00 and 3.75±1.77 ($x10^4$ CFU/g); and Total *Salmonella* counts between 1.25±0.35 and 3.00±2.83 ($x10^2$ CFU/g); 1.13±1.59 to 0.50±0.71 ($x10^2$ CFU/g) with significant differences ($p \leq 0.05$). All *Salmonella enterica* isolates were 100% positive for Motility, Starch, Capsule, Protease, and Haemolysin, and 83.3% produced biofilm. *Salmonella enterica* was more prevalent in raw crab (26.7%) and clam (33.33%) samples when compared to parboiled samples. *Salmonella enterica* were highly sensitive to Ofloxacin, Gentamicin, Streptomycin, and Ciprofloxacin (88.9%, 88.9%, 94.4%, 88.9%), while they were highly resistant to Pefloxacin and Ampicillin (44.4% and 33.35%), respectively. All *Salmonella enterica* isolates had a MAR index greater than 0.2. These findings highlight multidrug-resistant *Salmonella enterica* in *Cardisoma armatum* (crab) and *Mercenaria mercenaria* (clam) with high prevalence from these markets, emphasizing the need for improved food safety measures and monitoring due to the potential risk of seafood-borne illnesses and antibiotic abuse discouraged.

Keywords: *Cardisoma armatum*, *Mercenaria mercenaria*, multidrug resistance, *Salmonella enterica*, virulence.

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INTRODUCTION

Cardisoma armatum (crab) is found in the world's oceans, in fresh water and on land, particularly in tropical regions and feeds from the surrounding water, accumulating bacteria (Richard and Neil, 2001).

Mercenaria mercenaria (clam) are known as filter feeders because of their mode of feeding. The cilia in the clams' gills can trap the tiny food particles from the water and move them down to their mouth, where they can be consumed. The water is pushed out through the other syphon (Chen et al., 2008).

When bacteria pick up several genetic elements from diverse sources through distinct conjugation, transduction, or transformation pathways, they become resistant to multiple drugs (Hakanen et al., 2017). Ayukekbong et al. (2017) reported that in addition to acquired resistance, bacteria possess chromosomally encoded intrinsic resistance mechanisms. Examples of these mechanisms include the marRAB locus of *Salmonella enterica*, which confers intrinsic resistance to a variety of antibiotics, including tetracyclines, chloramphenicol, cephalosporins, penicillins, nalidixic acid, fluoroquinolones, rifampin, and organic solvents.

Salmonella pathogenicity islands (SPIs) are chromosomal regions in bacterial cells that encode gene clusters that encode different virulence characteristics, such as invasion and toxin genes (Foley et al., 2013). Different functions are played by the SPIs in the pathogenesis and pathogenicity of *Salmonella*. Virulence is the measure of the pathogenicity of an organism. The degree of virulence is related directly to the ability of the organism to cause disease despite host resistance mechanisms; it is affected by numerous variables such as the number of infecting bacteria, route of entry into the body, specific and nonspecific host defence mechanisms, and virulence factors of the bacterium (Ryan et al., 2017).

Secreted proteins play a crucial role in the pathogenesis of infectious diseases caused by *Salmonella enterica*. A remarkably large number of fimbrial and non-fimbrial adhesins are present in Salmonella and mediate biofilm formation and contact with host cells. Secreted proteins are also involved in host-cell invasion and intracellular proliferation, two hallmarks of Salmonella pathogenesis (Ryan et al., 2017). The key virulence traits and factors of *Salmonella enterica*, such as invasion or intracellular replication inside the host's cells, have been approached by various methods, such as screening for attenuated mutants, and this has resulted in the identification of many single genes that contribute to the virulence traits at the molecular and cellular levels (Gerlach and Hensel, 2007). Many virulence factors have been demonstrated to play diverse roles in the pathogenesis of *Salmonella* infections. These factors included flagella, capsule, plasmids, adhesion systems, and type 3 secretion systems (T3SS) encoded on the *Salmonella* pathogenicity island (SPI)-1 and SPI-2 and other SPIs (Daigle, 2008; Sabbagh et al., 2010). Hence, this research focuses on determining the virulence and multidrug resistant *Salmonella enterica* from *Cardisoma armatum* and *Mercenaria mercenaria* sold in Port Harcourt, Nigeria.

MATERIALS AND METHODS

Description of the study area

Two markets in Rivers State, Creek Road and Mile 1 Market, in Port Harcourt Local Government Area (PHALGA), were used for the study due to the high patronage of vendors of the shelled seafood every day, coupled with the easy accessibility of the market.

Sample Collection

A total of 8 samples of raw and parboiled *Cardisoma armatum* and *Mercenaria mercenaria* were purchased from the 2 markets in 2023, placed in sterile polythene bags, then put in ice chests, and aseptically transported to the Department of Microbiology Laboratory, Rivers State University, Port Harcourt, Nigeria, for bacteriological analysis (Figure 1 and 2).

Microbiological analyses

Bacterial enumeration

The enumeration of the aerobic heterotrophic plate count and *Salmonella* count was carried out using Nutrient agar, *Salmonella-Shigella* agar and Eosin Methylene Blue Agar for faecal coliform count. The stock analytical unit was done by weighing 10 grams of the edible part of the raw and parboiled seafood samples and homogenizing them in 90 ml of sterile normal saline for enumeration, isolation and identification. Ten-fold serial dilution was performed by pipetting 1 ml of the samples into 9 ml of sterile normal saline. About 0.1 ml of the appropriate dilutions (10^1 and 10^3) were inoculated in duplicates onto already prepared sterile plates of Nutrient, *Salmonella-Shigella* and Eosin Methylene Blue Agar, respectively using the spread plate technique and incubated at 37^oC and 44.5^oC for 24 hours after which the colonies with a metallic green sheen were counted and recorded.

Isolation and identification of Salmonella enterica

Salmonella enterica was isolated by picking representative or discreet colonies based on their size, margin, surface, elevation, texture, transparency and coloration (blackish) on *Salmonella-Shigella* agar and sub-cultured onto nutrient agar plates before incubating at 37^0C for 24 hours to obtain pure cultures. The pure

Figure 1. *Cardisoma armatum* (Crab).

Figure 2. *Mercenaria mercenaria* (Clam).

cultures were stored in 10% (v/v) glycerol suspension at -4^{0}C to prevent damage to the pure cultures during drying for further analysis**.** Identification of the organism was further conducted through biochemical test procedures such as citrate utilization test, methyl red, Catalase, Coagulase, Indole test, Starch hydrolysis, Voges Proskauer test, sugar fermentation test and specifically hydrogen sulphide production and triple sugar iron agar to confirm *Salmonella enterica* (Cheesbrough, 2005).

Test for virulence factors

The virulence property of the bacterial isolates was assayed to determine the bacterial capacity to cause disease (Chakraborty and Nishith, 2008). The virulence factors assayed included haemolytic activity, DNase, motility test, capsule, biofilm formation, protease and coagulase activities.

Antibiotic susceptibility profiling

The Kirby Bauer disk diffusion method was used on sterile Mueller-Hinton agar to assess the antimicrobial susceptibility profiles of the isolates to conventional antibiotics. Standardization of the *Salmonella* isolates was conducted by adjusting to 0.5 McFarland turbidity standards (x10^8 cells). The swab was deeped into the cell suspension and streaked over the surface of the agar plates, rotating the agar plate 60^0 to ensure appropriate distribution of the inoculum. The plates were air-dried for 3–5 min. Standard antibiotics disk impregnated with Gentamicin (10 µg), Ceftriaxone Sulbactam (45 µg), Nalidixic acid (30 µg), Cefexime (5 µg), Ampiclox (10 µg), Cefuroxime (300 µg), Nitrofurantoin (300 µg), Levofloxacin (5 µg), Impenem/Cilastatin (10 µg), Augmentin (30 µg), Ofloxacin (5 µg) and Cefotaxime (25 µg), were aseptically placed on the surface of the inoculated agar plate with sterile forceps. The disk was pressed down to make full contact with the surface of the agar. The plates were then incubated for 24 hours at 33 to 35^{0}C in an inverted position. The zones of inhibition were measured in millimetres (mm) and interpreted following the clinical and laboratory standards institute guidelines (CLSI, 2017).

Determination of Multiple Antibiotic Resistance (MAR) index

According to Krumperman (1985), multiple antibiotic resistance refers to bacterial isolates' resistance to three or more different antibiotic classes. Multiple antibiotic resistance (MAR) index was determined by using the formula MAR = a/b, where ‘’a’’ represents the number of antibiotics to which the test isolates depicted resistance and ‘’b’’ refers to the total number of antibiotics to which the test isolate has been tested for susceptibility.

Data analyses

Analysis of variance (ANOVA) was used to assess the significance of the collected data, and the Duncan multiple range test was utilized to differentiate the means where differences occurred Using the Statistical Package for Social Science (SPSS) version 25 (Bewick et al., 2004).

RESULTS

The results of the Total Heterotrophic Bacteria (THB),

Faecal Coliform Count (FCC) and *Salmonella* count (TSC) for raw and Parboiled crabs and clams in Creek Road and Mile 1 Market are shown in Table 1-4. Generally, the study recorded a significantly high population of *Salmonella* in the seafood samples with significant differences ($p \leq 0.05$) across the various seafood locations. In Creek Road and Mile 1 markets, the total heterotrophic bacteria count ($x10^6$ CFU/g) for raw crab ranged between 5.40±0.28 and 2.15±1.63, faecal coliform count ($x10^4$ CFU/g) ranged between 6.1±0.86 and 6.4±0.91 and total *Salmonella* count ($x10^2$ CFU/g) ranged from 3.00±1.69-5.00±0.14. The total heterotrophic bacteria count for parboiled crab ($x10^6$CFU/g) ranged between 1.6±0.09 and 1.69±0.62, faecal coliform count ($x10^4$ CFU/g) ranged between 2.58±1.30 and 3.72±2.38, the total Salmonella count ($x10^2$ CFU/g) ranged between 2.52±2.09 and 2.10±1.56.

The total heterotrophic bacteria count for raw clam ($x10^6$ CFU/g) ranged between 1.98±0.37 and 3.79±2.98, faecal coliform count ($x10^4$ CFU/g) ranged between 3.50±2.12 and 2.10±0.57, and total *Salmonella* count ($x10^2$ CFU/g) was between 1.25±0.35 and 3.00±2.83. The total heterotrophic bacteria count for parboiled clam ($x10^6$ CFU/g) was between 1.38±0.43 and 2.21±0.33, faecal coliform count ($x10^4$ CFU/g) was between 0.00±0.00 and 3.75±1.77, and total *Salmonella* count ($x10^2$ CFU/g) is between 1.13±1.59 and 0.50±0.71 with significant difference ($p \leq 0.05$) in the total heterotrophic bacteria count, faecal coliform count and total *Salmonella* count.

Eighteen *Salmonella enterica* were isolated from the *Cardisoma armatum* and *Mercenaria mercenaria* from the markets sampled. The prevalence of *Salmonella enterica* in raw and parboiled seafood samples from Creek Road and Mile 1 markets is shown in Figure 3. Specifically, the prevalence of *Salmonella enterica* in raw crabs and clams samples were 26.7% and 33.33% respectively, and were higher than the parboiled samples. *Salmonella enterica* had the highest prevalence from the Creek Road market in the raw clams and crabs samples than the parboiled samples.

The virulence potentials showed that all the isolates of *Salmonella enterica* were 100% positive for motility, starch, capsule, protease and haemolysin, and 83.3% produced biofilm, as shown in Table 5.

The results of the susceptibility pattern of *Salmonella enterica* in raw and parboiled samples of crabs and clams are shown in Table 6. The result indicated a higher number of the *Salmonella enterica* isolates were susceptible to Ofloxacin, Gentamicin, Streptomycin and Ciprofloxacin (88.9%, 88.9%, 94.4% and 88.9%) respectively and was highly resistant to Pefloxacin and Ampicillin (44.4% and 33.35%). Generally, *Salmonella enterica* isolates were resistant to Gentamicin (5.6%), Augmentin (22.2%), Ofloxacin (5.6%), Streptomycin (5.6%), Trimethoprim (11.1%), Chloramphenicol (27.8%), Ciprofloxacin (5.6%) and Spectinomycin (22.2%) (Table 5). All *Salmonella enterica* had MAR indices greater than 0.2, which indicates a high-risk source of contamination (Table 7).

Table 1. Bacterial count of raw *Cardisoma armatum* (Crab).

Locations	THB $x10^6$ CFU/g	FCC $x10^4$ CFU/g	TSC $x10^2$ CFU/g
Creek Road	5.40±0.28[a]	6.1±0.86[a]	3.00±1.69[a]
Mile 1	2.15±1.63[a]	6.4±0.91[a]	5.0±0.14[a]
P-value	0.081	0.872	0.406

*Means with same alphabet shows that there is no significant difference ($p \geq 0.05$).

Table 2. Bacterial count of parboiled *Cardisoma armatum* (Crab).

Locations	THB $x10^6$ CFU/g	FCC $x10^4$ CFU/g	TSC $x10^2$ CFU/g
Creek Road	1.6±0.09[a]	2.58±1.30[a]	2.52±2.09[a]
Mile 1	1.69±0.62[a]	3.72±2.38[b]	2.10±1.56[a]
P-value	0.577	0.044	0.313

*Means with same alphabet shows that there is no significant difference ($p \geq 0.05$).

Table 3. Bacterial count of raw *Mercenaria mercenaria* (Clam).

Locations	THB $x10^6$ CFU/g	FCC $x10^4$ CFU/g	TSC $x10^2$ CFU/g
Creek Road	1.98±0.37[a]	3.50±2.12[a]	1.25±0.35[a]
Mile 1	3.79±2.98[a]	2.10±0.57[a]	3.00±2.83[a]
P-value	0.376	0.911	0.502

*Means with same alphabet shows that there is no significant difference ($p \geq 0.05$).

Table 4. Bacterial count of parboiled *Mercenaria mercenaria* (Clam).

Locations	THB x10^6 CFU/g	FCC x10^4 CFU/g	TSC x10^2 CFU/g
Creek road	1.38±0.43^a	0.00±0.00^a	1.13±1.59^a
Mile 1	2.21±0.33^a	3.75±1.77^a	0.50±0.71^a
P-value	0.375	0.910	0.501

*Means with same alphabet shows that there is no significant difference (p≥0.05).

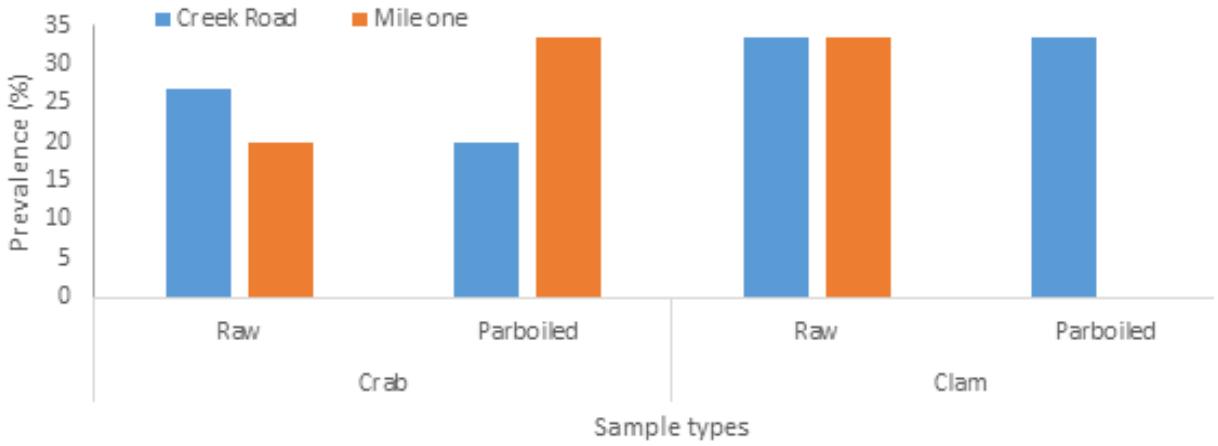

Figure 3. Prevalence of *Salmonella enterica* from the seafood samples.

Table 5. Virulence potentials of *Salmonella enterica* in seafoods.

Organism (n)	Capsule n(%)	Haemolysin n(%)	Motility n(%)	Biofilm n(%)	Protease n(%)	Starch n(%)
	+ve	+ve	+ve	+ve	+ve	+ve
Salmonella enterica (18)	18(100)	18(100)	18(100)	15(83.3	18(100)	18(100)

Key: Positive (+ve).

Table 6. Susceptibility pattern of *Salmonella enterica.*

Antibiotics	Conc. (µg)	*Salmonella enterica* (n=18)		
		Resistant n(%)	Intermediate n(%)	Susceptible n(%)
Gentamicin	10	1(5.6)	1(5.6)	16(88.9)
Augmentin	30	4(22.2)	1(5.6)	13(72.2)
Pefloxacin	10	8(44.4)	6(33.3)	4(22.2)
Ofloxacin	5	1(5.6)	1(5.6)	16(88.9)
Streptomycin	5	1(5.6)	0(0.0)	17(94.4)
Trimethoprim	30	2(11.1)	1(5.6)	15(83.3)
Chloramphenicol	30	7(27.8)	8(44.5)	5(27.8)
Spectinomicin	30	4(22.2)	5(27.8)	8(50)
Ciproflox	10	1(5.6)	1(5.6)	15(88.9)
Ampicilin	30	6(33.3)	2(11.1)	10(55.6)

Table 7. Multiple Antibiotic Resistance Index.

Organism (n)	MAR Index					
	0.0	0.1	0.2	0.3	0.5	0.6
Salmonella enterica (18)	7(38.8)	6(33.3)	1(5.5)	1(5.5)	2(11.1)	1(5.5)

DISCUSSION

Seafood is a popular source of minerals and vitamins and is consumed by a large number of people, neglecting its health problems. The presence of *Salmonella* in seafood samples indicates poor hygiene and poor sanitary conditions, and the quality of seafood depends on the quality of water from which the seafood is harvested and the sanitary condition of the landing centres (Dons et al., 2011).

In the crabs and clams samples, *Salmonella* counts were higher in raw samples obtained from Creek Road and Mile 1 markets than in the parboiled samples. Kumar et al. (2009) had similar counts from fresh seafood in India. Sampson et al. (2020) and Barika et al. (2023) also recorded similar results in their works on cockles, prawns and whelks from Creek Road market. The high *Salmonella* load in the sample from the Creek Road and Mile 1 market is attributable to poor environmental conditions, ingestion of microorganisms that reside in their natural habitat by the seafood during feeding, poor handling by market vendors and sources of water used for washing and storage of samples before purchase (Takeda, 2011). The parboiled samples from the various markets had lower counts because parboiling could eliminate some microorganisms from the samples (Odu et al., 2017). The contamination of the seafood by *Salmonella enterica* may be a result of the source of water used for preparation, the distance between areas of catch and polluted areas where anthropogenic activities (human and animal faeces) are predominant (Takeda, 2011). The presence of *Salmonella* in seafood samples indicates poor hygiene, and the quality of seafood depends on the quality of water from which the seafood is harvested and the sanitary condition of the landing centres (Dons et al., 2011). The counts from the raw and parboiled crabs and clam samples revealed that for the total heterotrophic bacterial count, faecal coliform count and the total *Salmonella* count, Creek Road market had the highest count. The high bacterial counts in the prawn sample observed in the Creek Road market could also be attributed to the poor environmental condition such as disposal of faeces into open waters of the harvest area.

The presence of *Salmonella* in seafood samples indicates sanitary issues (Dons et al., 2011). The prevalence of *Salmonella enterica* from the seafood from the different markets was high. *Salmonella enterica* in the raw crabs and clam samples respectively were more predominant than in the parboiled samples from the Creek Road market which could be due to the continuous dumping of human wastes and faeces into these water bodies. The seafood accumulates the organism during filter feeding and the nature and activities within the respective markets. Consequently, the Creek Road market is always crowded with a poor understanding of the implications of contamination (Robertson, 2007). The high prevalence may also be due to poor sanitary conditions, hand hygiene and cross-contamination (Bose et al., 2014). However, the level of *Salmonella* in the seafood could also be as a result of the nutritional composition of the different seafood (Ajao et al., 2009). The high prevalence of *Salmonella enterica* in seafood could also be attributed to the fact that *Salmonella enterica* is halo-tolerant, and they maintain a low level of ionic concentrations to synthesize compatible solute to balance the osmotic level inside the cytoplasm with the outer medium (Okonko et al., 2009). The results are in line with reports of other studies in Nigeria by various authors (Al-Hindi et al., 2011).

Salmonella enterica were positive for capsule, protease, starch, motility, haemolysin and produced biofilm**.** The presence of capsule and the ability to produce biofilm is a virulent factor because it can aid the organism to survive for a long period in a seemingly hostile environment, such as even a food processing facility, partially due to its ability to endure various stress, such as sanitizers, pH and temperature and its ability to form biofilm leading to persistence in food (Håstein et al., 2006)**.** *Salmonella* has flagella that provides it with the ability to move in a directed manner, which is a crucial virulence factor for its pathogenesis. Motility allows *Salmonella* to swim through mucus layers, penetrate the intestinal epithelium, and reach the underlying tissues. These help the bacterium initiate infection and evade host defenses (Kutsukake, 2014). *Salmonella* produces proteases, enzymes that can degrade host proteins. These proteases can play a role in Salmonella pathogenicity by facilitating the bacterium's entry into host cells, evasion of the host immune system, and damage to host tissues (Behnsen et al., 2015). Starch hydrolysis is the ability of *Salmonella* to break down starch into simpler sugars using amylase enzymes. This capability may help *Salmonella* to utilize starch as a nutrient source in various environments, such as the gastrointestinal tract, where starch can be a component of the diet or a carbohydrate source present in host tissues (Skinner and Guilfoyle 2014).

The results of the antibiotic-resistant pattern of *Salmonella enterica* in raw and parboiled samples of crabs and clams reveal important insights into the antibiotic abuse and potential risks associated with seafood sold in Rivers State. This study demonstrates the susceptibility profiles of *Salmonella enterica* isolates and their Multiple Antibiotic Resistance (MAR) Index as a result of acquired resistance. The implications of these findings are significant for public health and food safety.

A substantial portion of *Salmonella enterica* was found to be susceptible to certain antibiotics, specifically Ofloxacin, Gentamicin, Streptomycin, and Ciprofloxacin. This susceptibility to these antibiotics indicates potential treatment options in cases of *Salmonella* infections resulting from the consumption of contaminated seafood. This information could benefit healthcare professionals

when treating affected individuals (Smith and Johnson, 2023). Conversely, the results show that *Salmonella enterica* isolates exhibited a high level of resistance to Pefloxacin and Ampicillin. This high level of resistance to these antibiotics is concerning and highlights the limited effectiveness of these drugs in treating Salmonella infections associated with seafood consumption (Johnson and Brown, 2022). Furthermore, the MAR Index of all *Salmonella enterica* exceeded 0.2, indicating a high-risk source of contamination. The MAR Index suggests that these isolates have developed resistance to multiple antibiotics, which may complicate the treatment of infections and increase the risk of the spread of antimicrobial resistance (Garcia and Martinez, 2019).

CONCLUSION AND RECOMMENDATION

The study provided valuable insights into the potential risks associated with the consumption of *Cardisoma armatum* and *Mercenaria mercenaria* in this region. The findings of this research indicate that *Salmonella* is a prevalent pathogen in these seafood, and it possesses various virulence factors that can pose a significant threat to public health. Furthermore, antibiotic resistance among the isolated *Salmonella* strains is a matter of concern, as it limits the effectiveness of treatment options in cases of infections. Additionally, the identification of multiple antibiotic-resistant *Salmonella* strains highlights the importance of prudent antibiotic use and stewardship in both human and veterinary medicine to prevent the further development and spread of antibiotic resistance. The presence of *Salmonella* strains emphasizes the need for stringent food safety measures in the seafood supply chain in Rivers State. Adequate measures such as proper handling, storage and cooking of seafood must be promoted to reduce the risk of *Salmonella* contamination.

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