Ethiopian Journal of Environmental Studies & Management 19(4): 465 – 477, 2026
ISSN:1998-0507 doi: https://ejesm.org/doi/v19i4.6
Submitted: May 30, 2026 Accepted: July 3, 2026

ACID, BILE SALT, THERMOTOLERANCE AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF INFANT FOOD BORNE ENTEROBACTERIACEAE ISOLATES

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Abstract
The likelihood of contamination and spread of pathogenic bacteria increases when infant foods are consumed without adequate heating, which helps to effectively control or eliminate potentially harmful bacteria that might cause food-borne illnesses. Food-borne pathogens encounter multiple obstacles such as acidic conditions in the stomach and bile in the small intestine, which serves as a protective mechanism against infections in humans. The aim of this study was to determine the acid, bile salt, thermotolerance and antibiotic susceptibility pattern of Enterobacteriaceae isolates of infant food origin. Thirty Enterobacteriaceae isolates of infant food origin were used in this study. The isolates were screened for acid, bile salt and thermotolerance. Antibiotic sensitivity test was done using the Kirby-Bauer disc diffusion method and multiple antibiotic resistance index was evaluated. Thereafter, molecular techniques were used to carry out the plasmid profiling of the isolates with the highest MAR index. Results of the acid tolerance assay revealed that Raoultella ornithinolytica and Cronobacter sakazakii recorded highest viabilities of 89.40% and 84.41% at pH 4.5 and 3.0 respectively, while all isolates showed bile salt tolerance ranging from 84% to 99.32%. Thermotolerance studies showed that Proteus mirabilis (6.64 min) and Klebsiella spp. (5.70 min) had the highest D-values at 45 ℃ and 60 ℃ respectively. All isolates recorded MAR index (0.24 - 0.60) higher than the permissible limit of 0.20. The presence of multiple antibiotic-resistant bacteria isolates with a high level of tolerance to heat, acid and bile salt, in food products intended for infant consumption is of significant health concern.

Keywords: *Acid and bile salt tolerance, Antibiotic susceptibility profile, Cronobacter sakazakii, Infant food, Thermotolerance, Raoultella ornithinolytica*

Introduction

Enterobacteriaceae, a group of notable Gram-negative bacteria are opportunistic pathogens that can cause human diseases under favorable conditions (Wiles *et al*., 2008). These microorganisms have been categorized by Chap *et al*. (2009) on their ability to cause aliments in neonates. They include; *Salmonella* spp. and *Enterobacter sakazakii* (*Cronobacter* spp.) with strong proof of causing disease, making up Category A, while, bacterial species included in Category B were; *Citrobacter koseri, Citrobacter fruendii, Enterobacter*

cloacae, Pantoea agglomerans, Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, and *Acinetobacter* spp. These prokaryotes have been reported to show possible causality of infectious diseases (Dougnon *et al*.,2017). Infants may come into contact with harmful amounts of food-borne microorganisms when they start eating solid meals The frequent occurrence of infant foods being contaminated is unquestionably connected to the absence of fundamental hygiene practices. (Beuchat *et al*., 2013).

The likelihood of contamination and spread of pathogenic bacteria increases when weaning foods are consumed without adequate heating. A critical step, which involves subjecting the food to further heat in order to effectively control or eliminate potentially harmful bacteria that might cause food-borne illnesses is often overlooked (Ijabadeniyi and Pillay, 2017). Food-borne pathogens encounter multiple obstacles once they enter the host, such as acidic conditions in the stomach and bile in the small intestine, which serves as a protective mechanism against infections in humans. The ability of a pathogen to tolerate bile salts is crucial for its survival in the small intestine (Nwagu *et al*., 2020). Some members of the Enterobacteriaceae have been reported to have a high level of tolerance to heat, and as common inhabitants of the gastrointestinal tracts of warm-blooded animals, they may have the ability to withstand certain acidic conditions and bile concentrations (Fakruddin *et al.,* 2014; Bai *et al*.,2019).

The emergence of antibiotic resistance has undergone fast evolution in recent decades, making it one of the most notable public health challenges of the 21st century. Bacterial pathogens have developed specialized drug defense methods as a result of the extensive use of antibiotics, particularly broad-spectrum ones (Larsson and Flach, 2022). The mechanisms of antibiotic resistance are subsequently spread in the environment *via* horizontal gene transfer (HGT) between bacteria as well as lysogenic bacteriophages (Wojcicki, 2019). Multiple investigations have shown the discovery of Enterobacteriaceae that produce β-lactamase enzymes, extended-spectrum beta-lactamases (ESBLs) and carbapenemases that are resistant to numerous classes of antibiotics (Bahr *et al*., 2018; Gashaw *et al*., 2018). The aim of this study was to determine the acid, bile salt, heat tolerance and antibiotic susceptibility pattern of Enterobacteriaceae isolates of infant food origin.

Methodology

Collection of Bacterial Isolates

Thirty bacterial isolates belonging to the Enterobacteriaceae family were cultured from locally processed soyabean infant food supplements. The isolates were identified based on biochemical characteristics and molecular characterized. They consist of species of the genera *Enterobacter, Proteus, Salmonella, Klebsiella, Raoutella* and *Cronobacter*.

Determination of Acid Tolerance of the Bacterial Isolates

Two (2) ml portion of bacterial cultures previously cultivated overnight in tryptic soy broth (TSB) was respectively standardized using a 0.5 McFarland turbidity standard to achieve a cell density of approximately 1.5×10^8 cfu/ml. Subsequently, the test organism was placed in 10ml each of newly prepared TSB with an adjusted pH of 3.0 and 4.5 and incubated at a temperature of 37 ℃.

Samples were obtained at various time intervals (ranging from 0 to 5 hours) and placed on TSA plates. The plates were thereafter placed in an incubator set at a temperature of 37 ^{o}C for a duration of 24 hours. Afterwards, the number of viable cells was counted. The relative survival of each organism was determined using the following calculation:

Viability % = $\frac{Nt}{No}$ x 100---------- eqn (1)

Nt represents the logarithm of colony forming units (cfu) at intervals of 1, 3, and 5 hours.

No represents the logarithm of the cfu at 0 hours or the initial logarithm of the cfu count, as described by Nwagu *et al*. (2020).

Determination of Bile Salt Tolerance of Bacterial Isolates

A 2ml portion of bacterial cultures that were cultivated overnight in TSB (Tryptic Soy Broth) was calibrated using a 0.5 McFarland turbidity standard to achieve a cell density of approximately 1.5 x 10^8 colony-forming units per milliliter (cfu/ml). Subsequently, the test organism was placed in 10ml of newly made TSB (tryptic soy broth) that was supplemented with a control s 0.3% weight/volume of bile salts. The incubation took place at a temperature of 37ºC for a duration of 0 to 5 hours. Following each hour, the number of viable cells was measured and the results were reported as a percentage of the total number of cells using the formula:

Viability % = $\frac{Nt}{No}$ x 100---------- eqn (2)

Nt represents the logarithm of the colony-forming units (cfu) at intervals of 1, 3, and 5 hours. No represents the logarithm of the cfu at 0 hours or the initial logarithm of the cfu count, as described by Fakruddin *et al*. (2014) and Nwagu *et al*. (2020).

Determination of Thermotolerance of Bacterial Isolates

A 2ml portion of each bacterial culture that was cultivated overnight in TSB (Tryptic Soy Broth) was adjusted to a cell density of approximately 1.5 x 10^8 cfu/ml using a 0.5 McFarland turbidity standard. Subsequently, the thermotolerance of each individual sample was assessed by placing it in 20 ml of tryptic soy broth (TSB) that had been equilibrated to the desired temperature, ranging from 45 to 60°C, in a water bath. At regular time intervals ranging from 0 to 10 minutes, small volumes of 0.1ml were placed on the surface of Tryptic Soy Agar (TSA) plates that contained 1% sodium pyruvate. The plates were then placed in an incubator at a temperature of 37°C for a duration of 48 hours. The thermotolerance parameters (D and Z values) were determined using the application of basic regression analysis, as described by Iversen *et al*. (2004).

Determination of Antibiotic Susceptibility Profile of Bacterial Isolates

The Kirby-Bauer disc diffusion test was conducted by cultivating colonies of the test organism in Mueller-Hinton broth at a temperature of 37°C for a duration of 18-24 hours. The cultivated colonies were then collected and suspended in a solution of 0.85% normal saline. The concentration of the bacterial suspension was modified to match a 0.5 McFarland turbidity standard, which is equivalent to 1.5 × 10^8 colony forming units per milliliter (cfu/ml). Next, the sample was applied to Mueller-Hinton agar plates using a sterile swab stick. Subsequently, discs containing antibiotics were firmly positioned on the surface of the dry plates that had been inoculated. This was done using sterile forceps. The plates were then incubated at a temperature of 37°C for a period of 18-

24 hours. The diameter of the zone of inhibition around each disc was measured and interpreted according to the suggested standard set by the Clinical Laboratory Standard Institute (CLSI). The study utilized various pharmacological classes and specific antibiotics, including β-lactams such as Amoxacillin (AM) and Augumentin (AU), Quinolones such as Ciprofloxacin (CIP), sparfloxacin (SP), and pefloxacin (PEF), Aminoglycosides such as gentamycin (GEN), a macrolide called Erythromycin (ERY), fluoroquinolone named Ofloxacin (OFX), and Cloramphenicol (CMP). Also, a sulphonamide known as Trimethoprim/sulfamethoxazole (TMZ/SMZ) was used. The investigations were conducted in three distinct and separate biological duplicates. The evaluation of the multiple antibiotic resistance (MAR) index was conducted in the following manner:

MAR index = $\frac{Y}{nx}$ ---------- eqn (3)

Where Y= number of resistance score
n= number of isolates used
x= number of antibiotics tested
(CLSI, 2017).

Determination of the Plasmid Profile of the Antibiotic-Resistant Bacterial Isolates

The QIAGEN Plasmid Purification mini kit was used to isolate the plasmids. The alkaline lysis approach was employed for plasmid isolation. The integrity of the isolated plasmid was assessed by running it on a 1% Agarose gel to confirm amplification. The 1XTAE buffer was made and then utilized to prepare a 5% agarose gel. The suspension was heated in a microwave for a duration of 5 minutes. The liquefied agarose was cooled to a temperature of 60°C and then dyed with 3µl of ethidium bromide solution with a concentration of 0.5 g/ml. A comb was placed into the grooves of the casting tray, and the liquefied agarose was poured into the tray. The gel was left undisturbed for a duration of 20 minutes to undergo solidification and create the wells. The 1XTAE buffer was added to the gel tank until the gel was just covered. Two microliters (2 µl) of 10X blue gel loading dye, which enhances visibility and density of the samples for simple loading into the wells and monitoring the gel's progress, were added to 10µl of each PCR product. These samples were then placed into the wells after loading the 100-3000bp DNA ladder into well 1. The gel underwent electrophoresis at a voltage of 120V for a duration of 45 minutes. It was then observed using UV trans-illumination and captured in a photograph. The sizes of the PCR products were determined by comparing their mobility to that of a molecular weight ladder that was run alongside the experimental samples in the gel (Daniel *et al*., 2016).

Results

The acid tolerance of Enterobacteriaceae at pH 3.0 is depicted in Figure 1. The findings indicated that *C. sakazakii* exhibited the highest level of acid tolerance at pH 3.0, with viability percentages of 98.00%, 90.65%, and 84.41% after 1, 3, and 5 hours of incubation, respectively. The acid tolerance investigations conducted on the isolates at pH 4.5 revealed that *Salmonella enterica* exhibited the maximum viability of 98.96% after 1 hour of incubation. On the other hand, *R. ornithinolytica* had viabilities of 96.76% and 89.40% after 3 and 5 hours of incubation, respectively (Fig. 2).

Figure 3 displays the findings of the bile salt tolerance test. The findings

indicated that all isolates screened in this investigation exhibited viabilities ranging from 93.00% to 99.46% after 1-3 hours of incubation. After incubating for 5 hours, the viabilities varied from 84.00% (*R. ornithinolytica*) to 98.04% (*K. pneumoniae*).

The thermotolerance results of the Enterobacteriaceae isolates are displayed in Figure 4. At a temperature of 45°C, the D-value of *R. ornithinolytica* was found to be the lowest at 2.17 minutes, but *Proteus mirabilis* had the greatest D-value of 6.64 minutes. At a temperature of 60°C, *R. ornithinolytica* exhibited the shortest D-value of 1.87 minutes, whilst *Klebsiella* spp. demonstrated the longest D-value of 5.70 minutes. Additionally, *C. sakazakii* exhibited significant thermotolerance at temperatures of 45°C (with a survival time of 6.27 minutes) and 60°C (with a survival time of 4.38 minutes). The antibiotic susceptibility test findings of the isolates are displayed in Table 1. The findings indicated that majority (96.67%, 86.67%, and 70%) of the isolates exhibited susceptibility to ofloxacin, streptomycin and gentamycin respectively. However, 56.67% were resistant to quinolones (ciprofloxacin and sparfloxacin), 43.33% were resistant to Chloramphenicol, while 36.67% of the isolates exhibited resistance to β-lactam antibiotics, specifically Amoxicillin and Augmentin, as well as sulphonamide used.

Figure 5 displays the multiple antibiotic resistance (MAR) index. The Multiple Antibiotic Resistance (MAR) index of the isolates varied between 0.24 (*K. pneumonia*) and 0.60 (*R. ornithinolytica*). Plate 1 depicts the plasmid profile of the isolates with the highest MAR index.

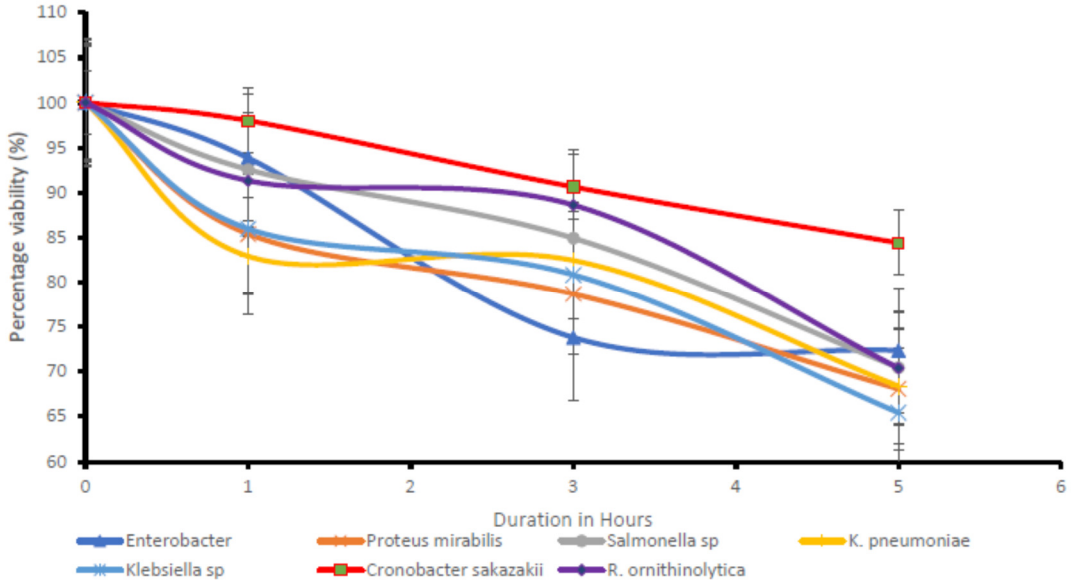

Fig. 1: Acid tolerance of Enterobacteriaceae isolates at pH 3.0

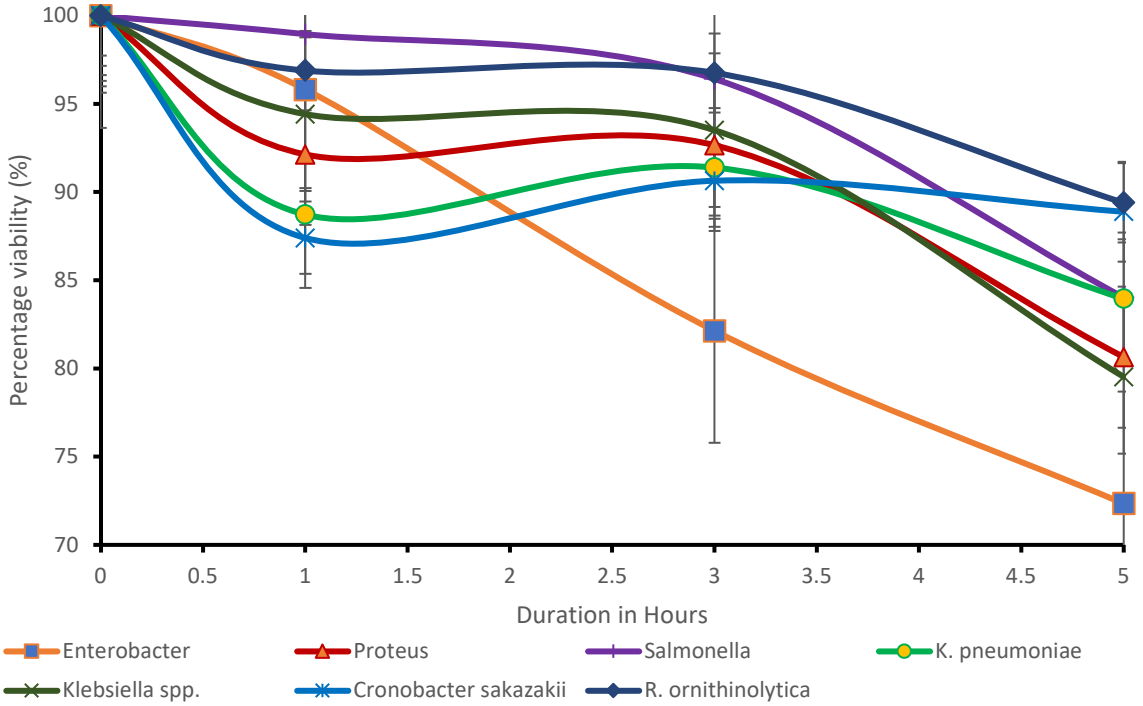

Fig. 2: Acid tolerance of Enterobacteriaceae isolates at pH 4.5

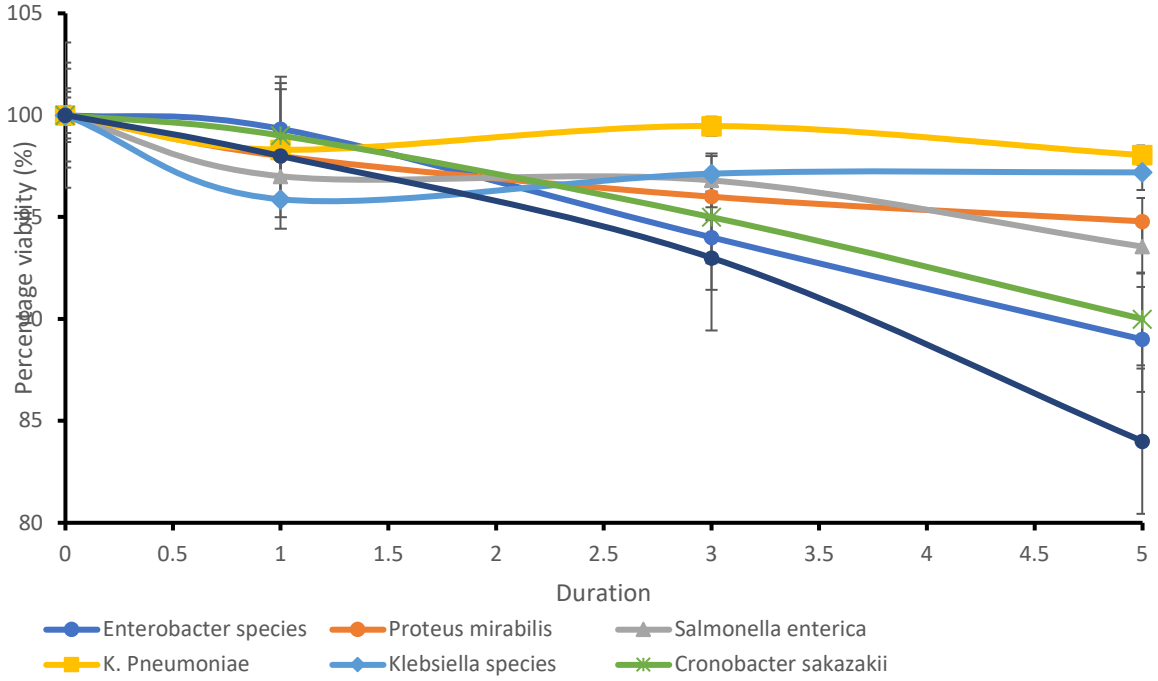

Fig. 3: Bile salt (0.3%) tolerance of Enterobacteriaceae isolates

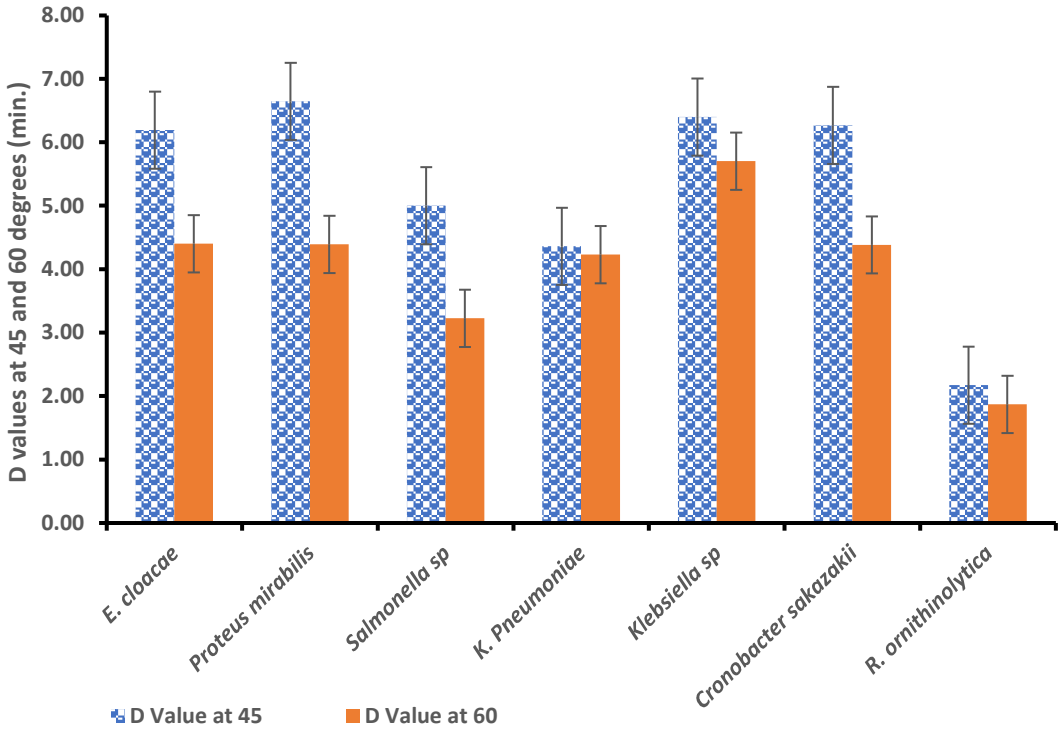

Fig. 4: Thermotolerance of Enterobacteriaceae isolates at 45°C and 60°C

Table 1: Antibiotic susceptibility pattern (%) of Enterobacteriaceae isolates from Infant Food

Isolates	**No.**	**SXT**	**CH**	**SP**	**CPX**	**AM**	**AU**	**CN**	**PEF**	**OFX**	**S**
Proteus mirabilis	3	33	67	33	100	100	67	100	100	100	33
Salmonella enterica	6	83	83	50	83	83	17	50	100	100	100
Raoultella. ornithinolytica	1	0	0	0	0	0	0	100	100	100	100
Cronobacter. sakazakii	1	0	0	0	100	0	100	100	100	100	100
Klebsiella spp	7	57	57	71	71	86	43	43	100	100	86
Enterobacter spp.	5	60	60	60	80	60	60	80	80	80	80
Klebsiella pneumoniae	7	86	57	71	86	100	57	86	100	100	100

Key: SXT= Septrin 30µg, CH= Chloramphenicol 30µg, SP= Sparifloxacin 10µg, CPX= Ciprofloxacin 30µg, AM= Amoxacillin 30µg, AU= Augmentin 10µg, CN= Gentamycin 30µg, PEF= Pefloxacin 30µg, OFX= Tarivid (Ofloxacin) 10µg, S= Streptomycin 30µg

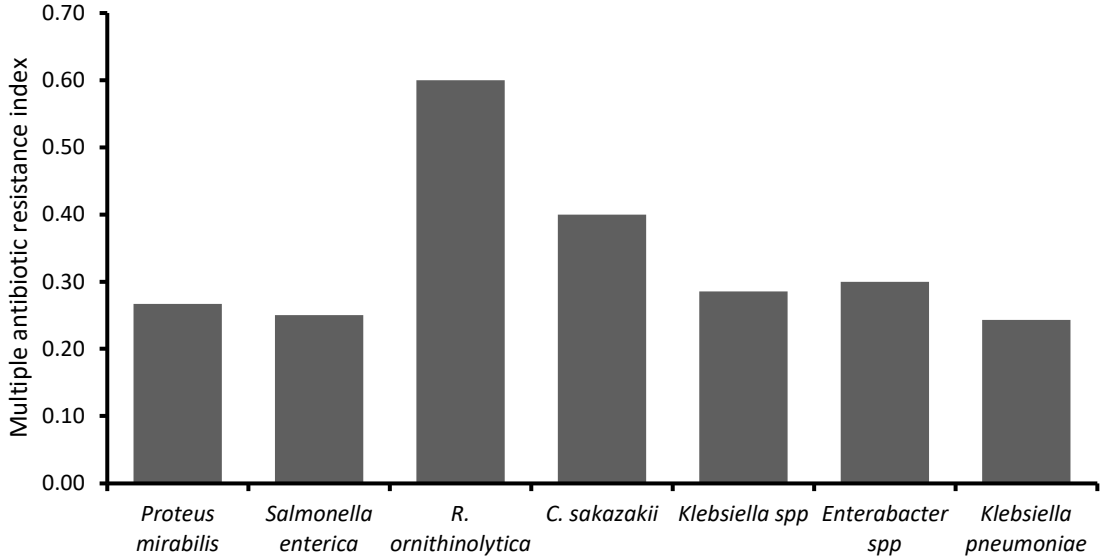

Fig. 5: Multiple antibiotic resistance index of Enterobacteriaceae isolates

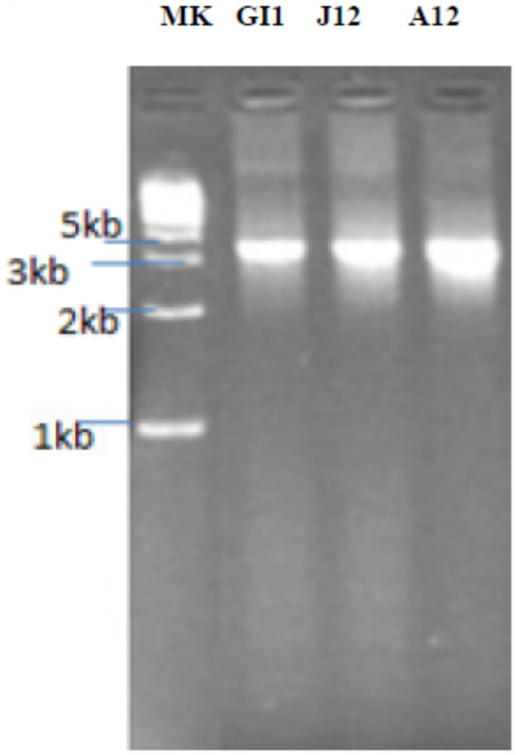

Plate 1: Plasmid profile of isolates with the highest MAR index
Key: GI1 = *Raoultella ornithinolytica*, JI2 = *Cronobacter sakazakii*, AI2 = *Enterobacter cloacae*

Discussion

Acid tolerance assays were conducted to assess the capacity of Enterobacteriaceae strains to withstand the acidic conditions of the stomach and at a pH of 4.5, *R. ornithinolytica* showed an increase in viability instead of a decline. After 1 hour of incubation, it is shown that

R. ornithinolytica requires a longer period of exposure to an acid-stressed environment in order for its growth to be affected. The viability of *C. sakazakii* strain decreased at 0-1 hour, but then increased after 1 hour of exposure. This trend would suggest that the strain has the ability to adapt favourably to the acid-stressed environment and resume growth. When exposed for 1-5 hours at a pH of 3.0, *Cronobacter sakazakii* exhibited the maximum level of viability. *Enterobacter* spp exhibited a decrease in viability after being exposed for 1 hour, followed by an increase in viability at 3 hours. This observation would also suggest the strain's capacity to adapt favorably in an acidic environment. This phenomenon may emanate from a synergistic interplay of genetic and physiological factors, which is commonly observed in acidophilic microbes (Nwagu *et al*., 2020). *Cronobacter sakazakii* has been documented to display atypical resilience to growth conditions that are affected by acid, and it is capable of thriving at pH levels as low as 4.5. However, the specific pH tolerance may differ among strains and the type of acid involved (Bai *et al*., 2019).

Bile serves as a crucial antibacterial agent within the human digestive system. The ability of pathogens to tolerate bile salts is crucial for their survival in the small intestine. Bacterial proliferation is impeded by the presence of bile, which permeates the duodenal segment of the small intestine. The sensitivity of bacteria cell membranes to bile salts is due to the composition of lipids and fatty acids which are sensitive to bile salts (Nwagu *et al*., 2020). In this study, *K. pneumonia*e exhibited a remarkable level of tolerance to bile, with a viability rate of over 95% after being incubated for 1-5 hours in a bile concentration of 0.3%. All the isolates exhibited growth in the presence of bile salt content, with viabilities exceeding 80%. The findings align with a study by Fakruddin *et al*. (2014), which indicated that all Enterobacteriaceae, including species from the genera *Enterobacter, Klebsiella,* and *Citrobacter* (isolated from various dietary sources), had the ability to withstand bile salt concentrations. Furthermore, members of the Enterobacteriaceae family are frequently found in the digestive system of warm-blooded mammals. These data would suggest that when these organisms are ingested with food, they have the ability to withstand the acidic and bile-filled conditions of the digestive system, which is necessary for them to reach and live in the intestines and cause an infection.

The thermo-tolerance investigations showed that *Proteus mirabilis* had the greatest D-value of 6.64 minutes at 45°C, whereas *Klebsiella* spp had the highest D-value of 5.70 minutes at 60°C. Nevertheless, *Cronobacter sakazakii* demonstrated its ability to withstand high temperatures, specifically 45°C for 6.27 minutes and 60°C for 4.38 minutes. Nazarowec-white and Farber (1997) found that the D-values at 60°C for clinical and food isolates were 3.06 ± 0.12min and 2.15 ± 0.07min, respectively. Nevertheless, these findings contradict the research conducted by Iversen *et al*. (2004), which documented D-values ranging from 0.20 ± 0.06 to 1.80 ± 0.82 for *Coronobacter* spp at a temperature of 60°C. Several variables can impact the thermal tolerance of bacteria. These factors include the physiological condition of the organism, the temperature at which the inoculum grows, and the composition of the heating menstrum (such as fat content, solids and sugar concentration). Additionally, the method

used for bacterial recovery is also important (Nazarowec-White and Farber, 1997).

According to Amalaradjou and Venkitanarayanan (2011), *Cronobacter sakazakii* has been found to have a high level of tolerance to heat. This trait provides a competitive edge, enabling it to survive in situations when infant food is improperly reconstituted or prepared. In a study conducted by Shi *et al*. (2017), it was shown that exposure to desiccation stress led to a considerable reduction in the heat resistance of *C. sakazakii* in reconstituted newborn formula. In contrast, Chen and Jiang (2017) discovered that desiccated *Salmonella* cells in poultry litter exhibited heightened heat tolerance compared to non-desiccated cells of *Salmonella* species. This investigation documented the ability to withstand high temperatures, specifically 45°C (with a duration of 5.00 minutes) and 60°C (with a duration of 3.23 minutes). According to their statement, the *rpos* gene played a role in protecting dehydrated *Salmonella* cells from high temperatures. According to Yang *et al*. (2015), the heat resistance of *C. sakazakii* was reduced by desiccation in comparison to non-stressed cells. It was hypothesized that the drying process could cause bacteria to become metabolically exhausted, resulting in reduced ability to withstand unfavorable environments. Values ranging from 0.11 to 0.13 minutes have been observed for the D value of *K. pneumoniae* in human milk and has been documented by Iversen and Forsythe (2004). Additionally, a D value of 6.56 minutes at 55°C has been observed for *E. coli* present in whole milk. A log decrease of microorganisms is often necessary in many thermal processes to ensure process control. Nevertheless, the possibility of food items being contaminated during post-processing remains a concern.

Antimicrobial resistance is a significant issue in the field of human medicine. The antibiotic sensitivity profile of the Enterobacteriaceae isolates indicated that majority (96.67%, 86.67%, and 70%) of the isolates exhibited sensitivity to fluoroquinolone (ofloxacin), macrolide (streptomycin), and aminoglycoside (gentamycin) accordingly. The resistance pattern of the isolates revealed that 56.67% and 43.33% exhibited resistance to quinolones (namely ciprofloxacin and sparfloxacin) and Chloramphenicol, respectively. Approximately 36.67% of the isolates exhibited resistance to β-lactams and sulphonamide antibiotics. The findings align with the findings of Hassan *et al*. (2011), who showed that the sensitivity range of Enterobacteriaceae isolates to β-lactams and aminoglycosides antibiotics was 45-82% and 55-71%, respectively. The isolates in this study exhibited a sensitivity range of 63.33% and 70% to these antibiotics, respectively. Nevertheless, these findings were at variance with the findings of Raheem and Mohammed (2021) who documented that all *K. pneumoniae* isolates showed resistance to majority of the antibiotics utilized and Wojcicki *et al*. (2022), who observed that a notable proportion (47 out of 53) of *Salmonellae* displayed resistance to antibiotic drugs utilized in their research. This study indicated that 25% and 15.71% of *Salmonella enterica* and *K. pneumoniae* isolates respectively exhibited antibiotic resistance. All the isolates examined in this investigation had a multiple antibiotic resistance (MAR) index above the threshold of 0.20, while, *R. ornithinolytica* had the highest MAR index, which was 0.60. The detection of

plasmids in the multiple antibiotic-resistant isolates, as indicated by the plasmid profile, could also be of health concern, considering the potential of microorganisms to spread resistance genes through horizontal gene transfer, especially in cases where antibiotic resistance is plasmid-borne.

Conclusion

The study has provided valuable and relevant information on the presence of multiple antibiotic-resistant bacteria isolates, with a high level of tolerance to heat, bile salts and acid stress in food products meant for infant consumption. These data suggest a high possibility of survival of these organisms in improperly reconstituted infant foods and as such, when these organisms are ingested with food, they have the ability to withstand the acidic and bile-filled conditions of the digestive system, which is necessary for them to survive and cause an infection.

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