INCIDENCE OF *CRONOBACTER* SPECIES IN FISH AND SHELLFISH

Dissertation submitted in partial fulfillment

of the requirements

for the degree of

M.F.Sc. (Post Harvest Technology)

by

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Dedicated to

My Baba, Ma and Guide

Dated: 30th June, 2018

CERTIFICATE

Certified that the dissertation entitled "**INCIDENCE OF *CRONOBACTER* SPECIES IN FISH AND SHELLFISH**"is a bonafide record of independent research work carried out by by **Mr. Sambit Kisore Das** during the period of study from August 2017 to June 2018 under our supervision and guidance for the degree of **Master of Fishery Science(Post Harvest Technology)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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DECLARATION

I hereby declare that the dissertation entitled **"INCIDENCE OF *CRONOBACTER* SPECIES IN FISH AND SHELLFISH"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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30 June, 2018 ***(Sambit Kisore Das)***

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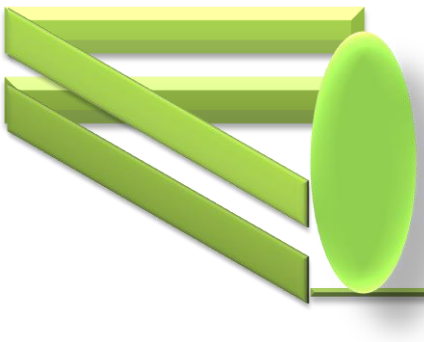
ABSTRACT

The present study investigated the presence of *Cronobacter* spp. in seafood collected from fish landing centres, fish markets and retail supermarkets from Mumbai, India. A total of 50 samples were screened, which included 32 fresh finfish, 6 shellfish, 9 dry fish and 3 water samples. Among these, 24 fresh finfish (75%), 4 shellfish (66.67%), 7 dry fish (77.78%) and all 3 water sample were shown to be positive for *Cronobacter* spp. by selective plating on Hichrome *Enterobacter Sakazakii* agar. The incidence of *Cronobacter* in dry fish was found to be higher in this study that may be attributed to the enhanced desiccation tolerance of *Cronobacter* spp. A total of 165 presumptive *Cronobacter* isolates from 38 samples were obtained, which were subjected to biochemical tests such as Gram's staining, indole, Voges Proskauer, motility, catalase, oxidase and malonate broth utilization tests, and PCR targeting 16s rDNA and ITS G and ITS IA for further confirmation. Out of these, 145 (87.87%) isolates were confirmed to be *Cronobacter* spp. by biochemical tests and 37 (22.4%) by PCR. DNA sequence analysis of the confirmed isolates revealed two of them to be *Cronobacter sakazakii* and others to be the closely related *Cronobacter malonaticus*. Screening the isolates for virulence genes showed the presence of *omp*A gene that codes for outer membrane protein, which plays an important role in the invasiveness of virulent *Cronobacter* spp. The confirmed isolates of *Cronobacter* spp. were also studied for their antibiotic susceptibility pattern by testing them against 15 commonly used antibiotics using disc diffusion method. All the isolates were found to be resistant to the 3rd generation cephalosporins, cefpodoxime and ceftazidime, and 97.29% were sensitive to kanamycin and ciprofloxacin. *Cronobacte*r isolates showed resistance to two or more antibiotics when analysed, with a less percentage of the isolates showing resistance to three or more than three antibiotics. Incidence of *Cronobacter* spp. in fresh and dried seafood reported in this study (76%) is the first of its kind from India, and continued investigations are required to understand the distribution and diversity of its occurrence in seafood.

सारांश

वर्तमान अध्ययन ने मुंबई, भारत से मछली लैंडिंग सेंटर, मछली बाजार और खुदरा सुपरमार्केट से एकत्र समुद्री भोजन में क्रोनोबैक्टर एसपीपी की उपस्थिति की जांच गेई हे । कुल 50 नमूने स्क्रीन किए गए थे, जिसमें 32 ताजा फिनफिश, 6 शेलफिश, 9 सूखी मछली और 3 पानी के नमूने शामिल थे। इनमें से 24 ताजा फिनफिश (75%), 4 शेलफिश (66.67%), 7 सूखी मछली (77.78%) और सभी 3 जल नमूने क्रोनोबैक्टर spp. के लिए सकारात्मक साबित हुए थे हाई क्रोम -एंटरोबैक्टर सकाज़ाकी agar की मदद से। शुष्क अध्ययन में क्रोनोबैक्टर की घटनाएं इस अध्ययन में अधिक थीं जो क्रोनोबैक्टर PCR की बढ़ी हुई विलुप्त होने वाली सहिष्णुता के कारण हो सकती हैं। Gram's staining, indole, Voges Proskauer, motility, catalase, oxidase और malonate broth utilization tests, जैसे जैव रासायनिक और PCR लक्ष्यीकरण 16S rRNA और ITS-G और ITS-IA परीक्षणों के अधीन 38 नमूने के कुल 165 अनुमानित क्रोनोबैक्टर प्राप्त किए गए थे। इनमें से 145 (87.87%) अलग-अलग क्रोनोबैक्टर spp. होने की पुष्टि की गई थी। बायोकेमिकल परीक्षण और PCR द्वारा 37 (22.4%) द्वारा। पुष्टि की गई डीएनए अनुक्रम विश्लेषण से पता चलता है कि उनमें से दो *Cronobacter sakazakii* और दूसरों को बारीकी से संबंधित *Cronobacter malonaticus* होने के लिए प्रकट हुए हैं। विषाणु जीन के लिए पृथक स्क्रीनिंग *ompA* जीन की उपस्थिति दिखाती है जो बाहरी झिल्ली प्रोटीन के लिए कोड करती है, जो विषाक्त क्रोनोबैक्टर एसपीपी की आक्रमण में महत्वपूर्ण भूमिका निभाती है। क्रोनोबैक्टर spp. की पुष्टि की गई। डिस्क प्रसार विधि का उपयोग कर 15 सामान्य रूप से प्रयुक्त एंटीबायोटिक्स के खिलाफ परीक्षण करके उनके एंटीबायोटिक संवेदनशीलता पैटर्न के लिए भी अध्ययन किया गया था। सभी अलगाव तीसरी पीढ़ी के सेफलोस्पोरिन, सीफोडोडॉक्सिम और सीफ्टाज़िडाइम के प्रतिरोधी पाए गए थे, और 97.2 9% कनामाइसिन और सिप्रोफ्लोक्सासिन के प्रति संवेदनशील थे। विश्लेषण के दौरान क्रोनोबैक्टर पृथक दो या दो से अधिक एंटीबायोटिक दवाओं का प्रतिरोध दिखाता है, जिसमें तीन या तीन से अधिक एंटीबायोटिक्स प्रतिरोध प्रतिरोध को अलग करता है। क्रोनोबैक्टर spp. की घटनाएं। इस अध्ययन में रिपोर्ट किए गए ताजा और सूखे समुद्री भोजन में (76%) भारत से अपनी तरह का पहला है, और समुद्री भोजन में इसकी घटना के वितरण और विविधता को समझने के लिए निरंतर जांच की आवश्यकता है.



Introduction

MATERIAL AND METHODS

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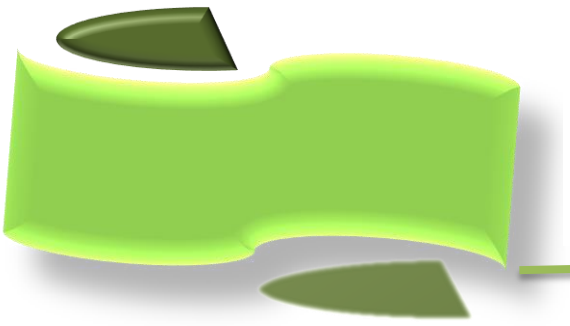

Appendix

ABBREVIATIONS

1.INTRODUCTION

Seafood is high in nutritional value, containing high-quality proteins, omega-3 fatty acids, essential micronutrients, minerals and vitamins (Mahaffey *et al.*, 2008). Globally, the seafood consumption has grown exponentially over the past few decades. Abreast this increase in demand and consumption, food safety has become a vital public-health concern. Iwamoto *et al.* (2010) stated that foodborne pathogenicity is a significant cause of the worldwide morbidity and hospitalisations that result from consuming various foods, including seafood.

Worldwide, people consume several forms of seafood that includes various species of molluscs, marine mammals, finfish, crustaceans, and fish eggs (Iwamoto *et al.,* 2010). A variety of seafood products have been reported responsible for the transmission of a variety of pathogens and contaminants from various sources to humans (Butt *et al.*, 2004). Seafood can be contaminated in numerous ways. Sources of contamination include insects, rodents, sticks, stones, straw, wood, sand and dirt. Microorganisms may enter from people working in the plant or handling the food after harvesting in landing canter, from raw products, from the food materials or ingredients or lack of proper cleaning of the food plant equipment or the food plant or the boat deck surface, storage buckets. Seafood-related disease outbreaks are caused by a wide variety of bacteria, viruses, and parasites; and can range in severity from mild gastroenteritis to life-threatening infections (Iwamoto *et al.*, 2010). Although thorough cooking kills the pathogens, it is most likely that contamination happens after the process. The post process recontamination of cooked meat can come from food handlers, from bacteria harboured in the environment or during storage (Levine and Griffin, 1993).

Generally, pathogenic bacteria and viruses are either absent or present in minimum numbers in live fish and shellfish (Gombas *et al.,* 2003; Jami *et al.*, 2014). However, given the ubiquitous nature of microorganisms, seafood can suffer microbial contamination after harvesting, at any stage of the food supply chain. The production process, in particular, provides multiple opportunities for cross-contamination and recontamination. More importantly, post-harvest microbial

contamination not only increases bacterial load but it can also introduce pathogens rarely found in the marine environment and the micro biota of live fish and shellfish.

Staphylococcus aureus, Escherichia coli, Salmonella, Listeria monocytogenes and *Vibrio parahaemolyticus* are some of the most common pathogenic bacteria found in seafood (Lipp and Rose, 1997). The epidemiology of emerging pathogens has changed in recent years due to the globalisation of the food chain. This has led to microorganisms that have not previously been thought to be foodborne becoming an essential risk to food safety and human health in the future such as *Arcobacter* spp., *Yersinia enterocolitica, Helicobacter pylori; Campylobacter, Acinetobacter, Cronobacter* etc. These pathogens contaminate and survive in, a wide range of foodstuffs including meat, water, dairy, poultry seafood, and minimally processed vegetables and ready- to eat food.

Cronobacter spp. (formerly *Enterobacter sakazakii*), a member of the *Enterobacteriaceae* family, are ubiquitous organisms, which are motile, non-spore forming, Gram-negative facultative anaerobes. They are catalase positive, oxidase negative, and generally positive for α-D-glucosidase. The International Commission for Microbiological Specifications for Foods (ICMSF) has ranked *Cronobacter* as a "Severe hazard for restricted populations, life-threatening or substantial chronic sequelae or long duration" (ICMSF, 2002). *Cronobacter* is considered more resistant to dry and osmotic stresses than other members of the *Enterobacteriaceae* (Feeney and Sleator, 2011). This may be due to the accumulation of trehalose inside the cells, which works as a protectant (Breeuwer *et al.*, 2003). It can grow at pH 4.5, at a temperature range of 6 ˚C – 45˚C, with an optimal range of 37 ˚C – 43˚C; some strains can multiply at 47˚C and slowly at household refrigeration temperatures (Iversen and Forsythe, 2004). *C. sakazakii* strains display differences in pathogenicity and have different virulence factors such as attachment to intestinal cells, survive internally in macrophages, capsule production, biofilm formation and production of enterotoxin (Iversen and Forsythe, 2004). *Cronobacter* spp. with characteristics like adherence and biofilm formation are able to survive as free-living bacteria in different environments, while invasion and the presence of some putative virulence genes (*hly* and *cpa*) should allow them to be successful opportunistic pathogens (Cruz *et al.,* 2011). Outer membrane protein A, encoded by the *ompA* gene, is one of the best-characterised virulence factors, which is required for

binding and invasion of brain endothelial cells, during pathogenesis (Nair *et al.,* 2009; Mittal *et al*.,2009; Kim *et al*.,2010).

The emergence of resistance to antimicrobials is a significant public health problem and justifies monitoring the antibiotic susceptibility of foodborne pathogens. Antibiotic treatment is considered to be the standard and preferred way to prevent the *Cronobacter* infection in humans (Depardieu *et al.*, 2007). Many studies have confirmed that antibiotics can efficiently eliminate Cronobacter strains. However, prolonged use of antibiotics is undesirable as it may result in the development of *Cronobacte*r antibiotic resistance (McMahon *et al.*, 2007).

Cronobacter spp. is considered as food safety risk which is harmful to human health, specifically in minimally processed foods (Mashoufi *et al.,* 2017) and also in new foods processed by alternative technologies (Arroyo *et al.,* 2011) It is reported from meat, sausage, minced beef, fish, shrimp, pork (dry, raw, cured), poultry, grain and seeds, soup, pasta, egg, beverages, vegetables and fruit, milk, newborn formula, powdered milk, cheese, water and environment. The great majority of the cases from *cronobacter* worldwide are reported from five countries: USA, UK, France, Belgium and the Philippines (Mashoufi *et al.,* 2017). However, literature citing occurrence of *Cronobacter* spp. in seafood, especially from Indian context is meagre. In this backdrop, the present study was carried out to determine the prevalence of *Cronobacter* spp*.* in seafood from India, with the following objectives:

- To screen fish and shellfish for the presence of *Cronobacter* species.
- To understand the potential virulence factors associated with *Cronobacter* isolates.
- To study the antibiotic sensitivity patterns of *Cronobacter s*pecies.

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8. LIST OF ABBREVIATIONS

%	Percentage
µl	Microliter
˚C	Degree Celsius
bp	Base pair
DNA	Deoxyribonucleic acid
Fig	Figure
gm	Gram
hrs	Hours
kb	Kilo base
LB	Luria-Bertani medium
min	Minute
Sec	Second
ml	Millilitre
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
rRNA	Ribosomal RNA
PIF	Powder Infant Formula
FAO	Food and Agriculture Organization
WHO	World Health Organization
ICMSF	International Commission on Microbiological Specifications
EtBr	Ethidium bromide
TAE	Tris-acetate-EDTA
TE	Tris-EDTA-buffer

9. APPENDIX

8.1. Media/ Broth composition

The media and ingredients used for the study were supplied by Himedia Laboratories Ltd. The list of the media used is given below:

A. Buffered Peptone Water

Composition

Ingredients	Gms / Litre
Proteose peptone	10.000
Sodium chloride	5.000
Disodium phosphate, anhydrous	3.500
Monopotassium phosphate	1.500
Final pH (at 25°C) 7.2±0.2	

Directions

Suspend 20.00 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

B. Cronobacter Screening Broth

Composition

Ingredients	Gms / Litre
Peptone	10.000
Meat extract	3.000
Sodium chloride	5.000
Bromocresol purple	0.004
Sucrose	10.000
Final pH (at 25°C) 7.4±0.2	

Directions

Suspend 28.0 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C)for 15 minutes. Aseptically add the contents of 1 vial of Vancomycin supplement (FD233). Mix well.

C. Vancomycin supplement

An antibiotic supplement recommended by ISO Committee for selective enrichment of *Enterobacter sakazakii* .

Composition

Per vial sufficient for 1000 ml medium

Ingredients	Concentration
Vancomycin	10mg

Directions

Rehydrate the content of 1 vial aseptically with 10 ml sterile distilled water. Mix well and aseptically add it to 1000 ml of sterile, cooled (45-50ºC) Broth

D. HiCrome *Enterobacter sakazakii* Agar

HiCrome Enterobacter sakazakii Agar is recommended for the isolation and identification of *Cronobacter sakazakii* from food and dairy products.

Composition

Ingredients	Gms / Litre
Casein enzymic hydrolysate	15.000
Papaic digest of soyabean meal	5.000
Sodium chloride	5.000
Sodium deoxycholate	0.500

Sodium thiosulphate	1.000
Chromogenic mixture	10.170
Agar	15.000
Final pH (at 25°C) 7.3±0.2	

Directions

Suspend 51.67 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and pour into sterile Petri plates.

F. Mueller Hinton Agar

Mueller Hinton Agar is used for determination of susceptibility of microorganisms to antimicrobial agents.

Composition

Ingredients	Gms / Litre
Beef, infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C) 7.3±0.1	

Directions

Suspend 38 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C. Mix well and pour into sterile Petri plates.

G. Mueller Hinton Broth

Mueller Hinton Broth is recommended to determine the susceptibility of bacteria.

Composition

Ingredients	Gms / Litre
Beef, infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Final pH (at 25°C) 7.3±0.1	

Directions

Suspend 21 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Mix well and dispense into tubes as desired. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

H. Luria-Bertani Agar

Composition

Ingredients	Gms / Litre
Casein enzymic hydrolysate	10.0
Yeast extract	5.0
Sodium chloride	10.0
Final pH @ 25°C 7.5 ± 0.2	

Directions

25 grams of the media was suspended in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Dispense as desired.

I. Motility Test Medium

Motility Test Medium is recommended for detection of bacterial motility.

Composition

Ingredients	Gms / Litre
Tryptose	10.000
Sodium chloride	5.000
Agar	5.000
Final pH (at 25°C) 7.3±0.1	

Directions

Suspend 20 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow tubed medium to cool in an upright position.

J. Barritt Reagent A (for VP test)

The reagent is used in Voges-Proskauer test for detection of action production by bacterial culture.

Composition

Ingredients	Amount
(1-Naphthol)	5.000 gm
Absolute ethanol	100.000 ml

Directions

Grow test culture in MR-VP Medium. Add 0.2 ml (2 drops) of Reagent A and 0.2 ml (2 drops) of Reagent B for 10 ml medium. Shake tubes gently for 30 seconds to 1 minute to expose the medium to atmospheric oxygen in order to oxidize the acetoin (acetylmethylcarbinol) so as to obtain a colour reaction. Allow tube to stand at least 10 to 15 minutes.

K. Barritt Reagent B (for VP test)

The reagent is used in Voges-Proskauer test for detection of action production by bacterial culture.

Composition

Ingredients	Amount
Potassium hydroxide	40.000 gm
Distilled water	100.000 ml

Directions

Grow test culture in MR-VP Medium. Add 0.2 ml (2 drops) of Reagent A and 0.2 ml (2 drops) of Reagent B for 10 ml medium. Shake tubes gently for 30 seconds to 1 minute to expose the medium to atmospheric oxygen in order to oxidize the acetoin (acetylmethylcarbinol) so as to obtain a colour reaction. Allow tube to stand at least 10 to 15 minutes.

L. Kovac's Indole Reagent

For detection of presence of indole produced by microorganisms due to tryptophan deamination

Composition

Ingredients	Amount
p-dimethylamino benzaldehyde	5.000gm
Amyl alcohol	75.000 ml
Hydrochloric acid, concentrated	25.000 ml

Directions

Add 0.2 - 0.3 ml of Kovac's reagent to 5 ml of a 24 - 48 hours old culture of the organism under investigation. Formation of a red coloured ring indicates positive indole test.

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2. REVIEW OF LITERATURE

2.1. Pathogenic *Enterobacteriaceae*

In the developed world, frequent reports of food poisoning have increased public concern about the potential presence of pathogenic organisms in food (Al-Mutairi, 2011). *Enterobacteriaceae* are recognised as some of the most important foodborne pathogens worldwide. These bacterial Infections made human society to suffer from extensive socioeconomic and health problems (Nagarjun and Rao, 2015). The name “Enterobacteriaceae” was first proposed by Rahn in1937 (Ewing *et al*., 1980) Phylogenetic studies place them in the phylum Proteobacteria, Class Gamma-proteobacteria and Order Enterobacteriales (Brenner *et al.,* 2005). *Enterobacter* species are widely dispersed in nature and existing a diverse range of environments including soil, water, domestic and food processing establishments, vegetation, vertebrate and invertebrate hosts, in the faeces of humans and animals (Cooney *et al*., 2014). The *Enterobacteriaceae* are mesophilic generally grow well, aerobically and anaerobically, at temperatures ranging between 20˚C and 37 ˚C on general laboratory media at neutral pH.

Enterobacter species are now recognized as critical nosocomial pathogens responsible for bacteremia, lower respiratory tract infections, pneumonia, skin and soft tissue infections, urinary tract infection(UTI),intra-abdominal and ophthalmic infections, endocarditis, septic arthritis, and osteomyelitis (Cooney *et al.,* 2014). These bacteria can affect people of all ages but are particularly relevant to immunocompromised individuals (Cooney *et al*., 2014). Several bacteria are recognised as important foodborne pathogens under the Enterobacteriaceae group notable examples are typhoid and non-typhoid *Salmonellae*, *Shigella dysenteriae*, *Yersinia enterocolitica* and a range of pathogenic *E. coli*, including *E. coli* associated with traveller’s diarrhoea and *E. coli* O157:H7, which has become one of the most important foodborne pathogens (Baylis *et al.,* 2011). Besides the well-known pathogens that belong to the Enterobacteriaceae family, some members that can cause infection, even if rarely such as *Klebsiella* spp., *Serratia* spp. and *Citrobacter*, *Hafnia alvei, Edwardsiella tarda* and *Cronobacter* spp. have been

implicated in human disease or can be the cause of opportunistic infections including bacteraemia, meningitis, urinary tract infections and wound infections, and are therefore important in clinical settings (Jenkins *et al.,* 2017).

2.2. History of *Cronobacter* as a new genus

Cronobacter spp. are emerging pathogens that were previously referred to as "a yellow-pigmented *Enterobacter cloaca*" until 1980, when they were designated as a new species honoring Japanese bacteriologist Ricchi Sakazakii. Iversen *and* Forsythe (2003) stated that DNA–DNA hybridization studies found no explicit generic assignment for *E. sakazakii* as it was 53–54% relevant to *Enterobacter* species. A comparison with the two type strains showed that *E. sakazakii* were 41% related to *Citrobacter freundii* and 51% associated with *Enterobacter cloacae*. It is also phenotypically closer to *Enterobacter Cloacae*. Farmer *et al.* (1980) assigned the organism to the *Enterobacter* genus. *Cronobacter* was proposed as a new genus under the family Enterobacteriaceae in the year 2007 to include the organisms formerly classified as *Enterobacter sakazakii*.

2.2.1. Taxonomy and species diversity

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma Proteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Cronobacter*

In 2008, Iversen *et al*. suggested a reclassification of *E. sakazakii* into a new genus, *Cronobacter*. It had eight different organisms including four named species, one unnamed species, and five named subspecies.

They are:*Cronobacter sakazakii*, *Cronobacter sakazakii* subspecies *sakazaki*i, *Cronobacter sakazakii* subspecies *malonaticus*, *Cronobacter dublinensis, Cronobacter dublinensis* subspecies *dublinensis*,*Cronobacter dublinensis* subspecies *lactarid* , *Cronobacter dublinensis* subspecies *lausanensis*, *Cronobacter muytjensii*, *Cronobacter turicensis*, *Cronobacter genomospecies* (a distinct species, but unnamed). The species *C. malonaticus* had been initially described as a subspecies of *C. sakazakii* by Iversen and Forsythe (2007) who could not distinguish *C*. *sakazakii* and *C. malonaticus* using 16S rDNA-sequence analysis.

Distinguishing between *C. sakazakii* and *C. malonaticus* has been problematic, and there are two primary reasons for this. First, the use of biotype profile to designate the species was not entirely robust as a few of the biotype index strains were themselves assigned the wrong species. Baldwin *et al*., 2009 Other supporting evidence which suggested that *C. malonaticus* and *C. sakazakii* are separate species were the single nucleotide polymorphisms identified for two genes, *rpoB* and *cgcA*, as reported by Stoop *et al*. (2009) and Carter *et al*. (2013)

In the year 2012–13 three new species of *Cronobacter* were expressed as *Cronobacter universalis, Cronobacter pulveris* and *Cronobacter zurichensis* (Farmer, 2015). In 2015 Farmer stated that ten species and three subspecies of *Cronobacter* have been named, described, and have standing in nomenclature:

1. *Cronobacter condimenti* (Joseph *et al*., 2012)
2. *Cronobacter dublinensis* (Iversen *et al*., 2008)

i. *Cronobacter dublinensis* subsp. *dublinensis* (Iversen *et al*., 2008)
ii. *Cronobacter dublinensis* subsp. *lactaridi* (Iversen *et al*., 2008)
iii. *Cronobacter dublinensis* subsp*. Lausannensis* (Iversen *et al.,* 2008*)*

3. *Cronobacter helveticus* (Brady *et al*., 2013)
4. *Cronobacter malonaticus* (Iversen *et al*., 2008)
5. *Cronobacter muytjensii* (Iversen *et al*., 2008)
6. *Cronobacter pulveris* (Brady *et al*., 2013)
7. *Cronobacter sakazakii* (Iversen *et al*., 2008)
8. *Cronobacter turicensis* (Iversen *et al*., 2008)
9. *Cronobacter universalis* (Joseph *et al*., 2012)

10. *Cronobacter zurichensis* (Brady *et al.,*2013*)*

The public health and virulence potential of each species within the genera are still ambiguous. *C. sakazakii*, *C. malonaticus* and *C. turicensis*, are recognised as opportunistic pathogens to humans (FAO/WHO, 2008). Although all species excluding *C. condimenti* have been related to clinical diseases, *C. sakazakii* and *C. malonaticus* isolates are liable for causing the greater number of infantile illnesses.

2.3. Detection of C*ronobacter* in food

The isolation and characterisation of *Cronobacter* spp. from food products is essential for the more keen understanding of the existence, transmission and epidemiology of these bacteria. Subsequently, it has the same ranking as more familiar food and waterborne pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* types A and B and *Cryptosporidium parvum*. (Forsythe and Stephen, 2005) All *Cronobacter* species have been associated retrospective to clinical cases of infection in either infants or adults, and therefore all species should be designated pathogenic (FAO/WHO, 2008).

However, it has been isolated from a diverse range of environments and foods. Since the organism is not part of the normal animal and human gut flora, it is probable that soil, water and vegetables are the principal sources of contaminated food. Iversen and Forsythe (2003) hypothesised that a common ecosystem for the *Cronobacter* species might be plants. This was due to physiological features such as the production of a polysaccharide capsule, production of a yellow pigment, and its desiccation resistance. Schmid *et al.* (2009) stated that the natural environment of *Cronobacter* spp. (likewise of many other representatives of the family Enterobacteriaceae), is plants. Results obtained by other scientists (Kandhai *et al*., 2010) reporting the isolation of *Cronobacter* spp. from the samples of leafy vegetables and sprouts confirm this hypothesis.

Cronobacter spp. appear in the aquatic and plant environment, in soil (Chenu and Cox*,* 2009). It also found in diverse plant-based food products, rice and other cereals seeds, and fermented products of plant origin as well as fruits.(Iversen and Forsythe, 2004; Mohammed *et al*., 2015; Park *et al*., 2016;). Berthold-Pluta *et al.* in (2017) reported the prevalence of *Cronobacter* spp. in ready-to-eat

food products of plant origin (sprouts and non-pasteurized fruit vegetable juices). Several studies have shown the existence of *Cronobacter* spp. in food production environments, including chocolate, potato flour and pasta factories (Kandhai *et al*., 2004; Drudy[b] *et al*., 2006).

A diversity of foods ranging from cheese products, herbs and spices, dried vegetables, eggs, water and milk, milk powder, beef, pork also show the presence of C*ronobacter* spp. (Iversen and Forsythe, 2004; Friedemann, 2007; Kim *et al*., 2008;). The organism has been identified from a range of foods including fermented bread, tofu, sour tea, cured meats, minced beef and sausage and less frequently from fish (Friedemann*,*2007).*E. sakazakii* has been isolated from animals (Farmer *et al*., 1985; Goullet and Picard, 1986), especially from birds, lizards, rats and piglets (Gakuya *et al*., 2001; Montgomery *et al*., 2002).*E. sakazakii* is a histamine forming microorganism in the ripening process of cheese (Roig-Sagués *et al.,* 2002; Morales *et al.,* 2004). Kandhai *et al*. (2004) also reported the presence of *Cronobacter* spp. in dust from domestic vacuum cleaners.

In the year 1950, a strain of *Enterobacteriaceae* was isolated from a “tin of dried milk” and was sent to England’s National Collection of Type Cultures (NCTC) and designated as NCTC 8155. It was re-described in the late 1970’s and fixed as *E. sakazakii* biogroup. This is the first for a strain of this organism and is also the first reported isolate of *Cronobacter* from food and a “dried milk” type of product.

E. sakazakii is a pervasive organism (Kandhai *et al*., 2004). The cause of *E. sakazakii* and vehicle of transmission is not always apparent however infant formula has been epidemiologically involved as the origin of *E. sakazakii* in several clinical cases (van Acker *et al*., 2001; Blake *et al*., 2002). The route of contamination of Powder infant formula is thought to include a broad range of dry blended raw material, together with possible environmental sources related to the production environment.

2.4. Incidence of C*ronobacter* in fish and fishery products

Cronobacter was isolated from fresh and raw shellfish in Spain by Balebona *et al.* (1990), in Switzerland in deep-frozen shrimps by Teuber (2001); from aquaculture fish in the USA by Miranda *et al*. (2003). In China it was isolated from

dried fish powder by Liu *et al.* (2006). Kim *et al*. (2008) isolated and identified *Cronobacter* from dried fish samples shrimp, anchovy, and brown seaweed (Duran and Marshall, 2005); Koonse *et al*. (2005) found that possibility of *C. sakazakii* contamination in shrimp can be high, and if shrimp is added to powdered infant formula as a dry ingredient there is a risk of *C. sakazakii* contamination. *E. sakazakii* has been isolated from smoked sardines after 12 weeks of storage after irradiation (Nketsia-Tabiri *et al*., 2003).

2.5. Detection from clinical sources

The routes of *E. sakazakii* and its vehicles of transmission are sometimes unclear. Although the organism has been detected in multiple food sources, a strong association has been found with Powdered Infant Formula (PIF). This microorganism has also been found from the hospital environment (Drudy[a] *et al.,* 2006). *E. sakazakii* has been isolated from a diverse range of clinical sources including cerebrospinal fluid, blood, bone marrow, sputum, urine, inflamed appendix, intestinal and respiratory tracts, eye, ear, wounds and faeces (Drudy[a] *et al.,* 2006). Farmer *et al.* (1980) reported that most *E. sakazakii* isolates from infected patients also originate from throat, nose, stool, gut, skin, and breast abscess. Most isolates are rare if not sporadic. However, in a 7-month period, the bacterium was isolated from the respiratory tract of 29 patients in one hospital (Gurtler *et al.,* 2005). The bacterium has been isolated from or implicated in numerous types of infections. It is of interest that a review of 17 cases of neonatal meningitis revealed that patients with *E. sakazakii* infections fared worse than those with more frequently occurring meningitis caused by other Gram-negative bacteria, including *E. cloaceae* (Willis and Robinson, 1988).

2.6. Survivability of C*ronobacter* spp. under different environmental conditions

2.6.1. Effect of temperature on survival

Cronobacter can survive over a wide temperature range. The lowest is near refrigeration temperatures (~5 °C), optimal ~37°C - 39 °C, and the maximum growth temperature is 44°C - 47 °C. The temperature growth range of *E. sakazakii* was reported by Farmer *et al*. (1980) for 57 strains, where there was no growth at 4 ˚C or 50˚C, but most strains grew at 47˚C. The generation time of *E. sakazakii* in infant formula was 40 min at room temperature. The thermal tolerance of *E. sakazakii*-strains may differ (Edelson-Mammel and Buchanan, 2005).

It has often been stated that *E. sakazakii* have a high thermal resistance in comparison to other members of the *Enterobacteriaceae* and that this may explain their high prevalence in powdered and prepared formula milk. A minimum High Temperature Short Time (HTST) pasteurization of 15 s at 71.7˚C would result in the kill of *E. sakazakii* in dried infant formula. Therefore, the organism should not survive the pasteurization process.

2.6.2. Effect of water activity

E. sakazakii has an unusual surviving ability under dry conditions (Gurtler *et al*., 2005) These bacteria are designated by high resistance to a low water activity medium (aw 0.30 to 0.83) (Lin and Beuchat, 2007). The plant environment is considered to be the usual habitat for *Cronobacter* spp. which forms a gum-like extracellular polysaccharide and resists desiccation during prolonged dry periods (Schmid *et al*., 2009). Some encapsulated *Cronobacter* spp. were still recoverable from desiccated infant formula after storage for up to 2.5 years. Jaradat *et al*. (2009) isolated *Cronobacter* from household vacuum dust in addition to isolating it from spices, indicating the strong association of the pathogen with a variety of dried food products and environmental factors.

2.6.3. Effect of pH

Dancer *et al*. (2009) reported that 72 *Cronobacter* strains were able to grow at pH 4.5, while 98.6%, 95.8% and 79.2% of these tested strains were able to grow at pH 4.3, 4.1 and 3.9, respectively. It is indicating that *Cronobacter* cells are more acid tolerant than most related enteric pathogens.

E. sakazakii has several characteristics such as biofilm formation, adherence to hydrophilic and hydrophobic surfaces, production of extracellular polysaccharides, and cell-to-cell signalling with other bacteria, which may help promote environmental endurance and the construction of a physical hurdle to conserve the cells from environmental stresses such as heat, dry conditions, antibiotics, osmotic pressure, and UV light (Lehner *et al.,* 2005) and help to the spread of *Cronobacter* spp. in food (Norberg,2012; Orieskova *et al.,* 2013,).

2.7. Virulence factors associated with pathogenicity of C*ronobacter* spp.

2.7.1. Role of OmpA

The outer-membrane protein A (OmpA) is probably the best-characterized virulence marker of *Cronobacter* (Nair *et al*., 2009; Mittal *et al*., 2009; Kim *et al*., 2010).The *ompA* gene encodes this virulence factor, and it is required, by *Cronobacter* for binding and invading the endothelial cells of the brain, leading to meningitis. OmpA has also been known to play a significant role in the adhesion and colonization of host epithelium by some Gram-negative pathogens (Dabo *et al.,* 2003; Torres and Kaper, 2003).

Cronobacter can exploit immature dendritic cells and persist within human macrophages indicates that *Cronobacter* possesses some immune evasion properties that enable them to avoid the host's immune response, and reach and penetrate the blood-brain barrier (BBB) (Mittal *et al*., 2009; Emami *et al*., 2011; Forsythe, 2015). The role of OmpA as a potential virulence factor and major fibronectin-binding protein in *C. sakazakii* was first reported by Nair *et al.* (2009) where an ompA mutant was found to be significantly attenuated in its invasiveness into cells. Hunter *et al*. (2008) observed that *C. sakazakii* invasion into human brain cells in vitro requires the expression of OmpA and an ompA-deletion mutant was less invasive than the without strain.

Mittal *et al*. (2009) showed that OmpA expression is essential for the onset of meningitis caused by *Cronobacter* in a newborn rat model as OmpA^{+}*Cronobacter* were present in high numbers in the brains of infected animals.

They also showed that OmpA expression is essential for *Cronobacter* resistance to blood and serum killing (Mittal *et al*., 2009). Although both $OmpA^{+}$ and $OmpA^{-}$ *Cronobacter* were efficiently taken up by dendritic cells, only OmpA+ *Cronobacter* resisted killing and multiplied in these cells, thus highlighting the critical role of OmpA in protecting the intracellular survival of this pathogen (Mittal *et al*., 2009).

Kim *et al.* (2010) reported that protein OmpA and OmpX are both of *C. sakazakii* essential for apical and basolateral adhesion to and invasion of host cells, as well as for movement into deeper organs, such as the spleen and liver. Other adherence mechanisms might be involved in the colonization of gastrointestinal (GI) tract cells by *Cronobacter* spp. Genes *OmpA* and *OmpX* were found in all *Cronobacter* spp., and thus cannot be responsible for the variation in pathogenicity among the different *Cronobacter* strains (Joseph *et al*., 2012). The ompA-encoded gene is thought to be universally present in *Cronobacter* although the sequences of *ompA* between various strains are different.

2.7.2. Endotoxin production

Cronobacter has also been shown to produce an endotoxin-like compound which aids in its translocation across both the blood-brain barrier in the central nervous system and the intestinal epithelial barrier in the gut. Additionally, this toxin seems to work similarly to lipopolysaccharide in that it induces an inflammatory response secondary to Toll-like receptor 4 (TLR4) activation.

The fact that historically, enterotoxin production has been a major virulence factor that marks the pathogenicity of microbes. Pagotto *et al*. (2003) were the first to describe putative enterotoxin activity in *Cronobacter*. The molecular mass of the toxin was determined to be 66 kDa, which is similar to the 62 kDa toxin of *Shigella dysenteriae* and the 65 kDa toxin of *Pseudomonas aeruginosa* with optimum activity observed at pH 6. Additionally, the action of the toxin was unaffected by holding it at 50 ˚C or 70 ˚C, and it was stable at 90 ˚C for 30 min, implying that it is a moderately heat-stable toxin. This indicates that the enterotoxin could be resistant to commercial milk pasteurization treatments and thus stays active in Powdered Infant Formula (PIF). As the endotoxin is heat stable at 100 ^{0}C, its presence in formulas

might also play an essential role in enhancing the pathogenesis of *Cronobacter* in human infants (Townsend *et al*., 2007).

2.7.3. Biofilm formation

Biofilm formation is of particular importance in the food industry because biofilms can act as a source of microbial contamination that might lead to spoilage of foods or contamination of food products undergoing processing (Lehner *et al*., 2005; Hartmann *et al*., 2010). *Cronobacter* has been reported to attach to and from biofilms on stainless steel, glass, latex, silicon, polyvinyl chloride and polycarbonate (Iversen and Forsythe*,* 2004; Lehner *et al.,* 2005). The ability of *Cronobacter* spp. to form biofilms protects from environmental stresses that impart resistance to cleaning and sanitising agents (Ravishankar and Juneja, 2003; FAO/WHO, 2006).

Capsular polysaccharides on the bacterial cell surface play a pivotal role in biofilm formation. Cruz-Cordova *et al*. (2012) reported that flagella from *C. sakazakii* are involved in biofilm formation. In addition to flagella, two hypothetical proteins (ESA_00281 and ESA_00282) were identified as possible adhesion proteins that may contribute to biofilm formation.

2.7.4. Desiccation tolerance

Genes were identified whose products could help *Cronobacter* to tolerate low water activity environments. Genes encoding osmoprotectant glycine, betaine and trehalose were found in all *Cronobacter* (Joseph *et al*., 2012). All *Cronobacter* contain genes for βcarotene production, which is believed to protect bacteria against harmful oxygen radicals (Joseph *et al.,* 2012). The identified genes include cellulose biosynthesis operons, colonic acid exopolysaccharide, the capsular biosynthetic operon, the environmental persistence capsule etc. The synergetic expression of these genes might provide resistance to desiccation stress (Grim *et al*., 2013).

2.8. Other virulence genes associated with pathogenicity

Several virulence genes and plasmids were determined and found to be specific to *Cronobacter* spp. Kothary *et al.* (2007) identified the gene locus of zinc-containing metalloprotease (*zpx*) – a protein that caused rounding of CHO cells in

tissue culture. The presence of putative *sodA* genes might provide resistance for *Cronobacter* against intracellular macrophage oxidase and acidic conditions, and may contribute to its intracellular persistence (Townsend *et al*., 2007). The genome from *C. sakazakii* was analyzed for the presence of putative virulence genes that could be associated with its pathogenesis, the following three potential virulence genes were identified: (i) gene *hly* which showed 91% identity with type III hemolysin (Hly) from *E. coli*; (ii) gene *cpa* showed 67% identity with a plasminogen activator (Cpa) from *Yersinia pestis*; and (iii) *sip* gene showed 67% identity with a siderophore interacting protein (Sip) from *E. coli* (Cruz *et al*., 2011). The genes for a haemolysin (*hly*) have been identified, but no activity has been found (Kucerova, 2010; Cruz *et al.,* 2011; Grim *et al.,* 2013). Kucerova (2010) also showed that the complete cation efflux system (cusA, cusB, cusC and cusF) and its regulatory gene *cusR* were present in strains associated with neonatal infections, but missing in the other strains, which may explain the fact that not all *Cronobacter* spp*.* are equally pathogenic and cause meningitis.

α-glucosidase activity is one of the essential biochemical characters which distinguish *E. sakazakii* from other species in the family Enterobacteriaceae and has therefore been used as a selective marker in the development of differential media. The α-glucosidase based PCR system, however, exclusively targets the gene responsible for the α-glucosidase activity in *E. sakazakiiI* is *gluA.* Within a recent study, two open reading frames have identified the gene both coding for enzymes with the potential to hydrolyze the fluorescent substrate 4-methylumbellyferryl-α-D-glucoside.

2.9. Pathogenicity of C*ronobacter*

In general *Enterobacter,* organisms are responsible for around 50% of nosocomial infections, mostly in immunocompromised patients (Leclerc *et al.,* 2001). Although there is no epidemiological evidence for a value of the infectious dose, it is reasonable to use 1000 *E. sakazakii* cells as a first approximation. This is similar to the infectious dose of the pathogenic bacteria *Neisseria meningitidis*, *E. coli* O: 157 and *L. monocytogenes*. The infectious dose can vary according to the organism's history (stress response factors), host state (healthy or immunocompromised) and food matrix.

Cronobacter sakazakii is an emanate pathogen which causes diseases in infants, children and adults, especially the aged and immunocompromised adults. *Cronobacter* causes grave infection among new-borns or young infants, with fatality rates of 40 to 80% globally (Friedemann*,* 2009). *Cronobacter* spp. have been mainly related to infections in infants. But the Foodborne Diseases Active Surveillance Network have shown that the number of infected adults above 70 years of age (37%) was higher than the number of infected infants of the below one year of age (4%) in the United States (Patrick *et al*., 2014).In adults *Cronobacter* spp. can cause pneumonia, septicemia, osteomyelitis, splenic abscesses, and wound infections (Healy *et al*., 2010). This opportunistic pathogen also causing meningitis, septicemia and enterocolitis in neonates (Bar-Oz *et al.,* 2001). Preterm, low-birth-weight or immunocompromised infants exposed to *E. sakazakii* are at particular risk (Lai*,* 2001). Survivors from *Cronobacter* infections often suffer from neurological sequelae (Gallagher and Ball, 1991; Lai, 2001).

Cronobacter spp. has been associated with bacteremia, pneumonia, conjunctivitis, and urinary tract infections. Reports indicate that older and immunocompromised adults seem to be at an increased risk of *Cronobacter* infection. *Cronobacter* is more commonly detected in adults, with most case reports describing infections related to elderly patients who were previously ill or immunocompromised. Up to 50% of adults with *Cronobacter* infection had an underlying malignancy (Lai, 2001; See *et al.,* 2007) Nosocomial infections by *Cronobacter,* such as aspiration pneumonia, urinary tract infections and conjunctivitis, are unlikely to be related to contaminated powdered infant formula (PIF), so medical equipment surfaces, water outlets and intrapersonal contacts may serve as possible sources of these infections (Friedemann, 2009; Flores *et al*., 2011).

2.10. Disease conditions caused by *Cronobacter*

It is thought that the main colonization site of *Cronobacter* is the gastrointestinal tract (GIT), where it first colonizes the mucous membranes, gastric and intestinal epithelial or endothelial tissues before their internalization within enterocytes, or translocation through the lamina propria into the systemic blood flow

and then the invasion of the brain. Successful attachment of *Cronobacter* to intestinal cells, the pathogen invades these cells in a process that ends up with the pathogen translocating across the intestinal tissue layers to enter the systemic blood flow, resulting in extra-intestinal infection, such as sepsis and meningitis (Nair and Venkitanarayanan, 2007).

2.11. Outbreaks of *Cronobacter* infection

Cronobacter infections are rare and are often underreported, especially in developing and less-developed countries (Estuningsih and Sani 2008; Friedemann 2009). The incidence of *Cronobacter* was first implicated in a case of neonatal meningitis in 1958 (Urmenyi and Franklin, 1961). Even though the incidence of documented outbreaks of *E. sakazakii* infections is low, the incidence of 16 invasive cases of infants caused by *E. sakazakii* was published between 1999 and 2002 (http://www.who.int/foodsafety/publcations/micro/mra6/en/).

In the year 1965 the second documented case of meningitis due to *E. sakazakii* occurred at the city and county hospital of Odense, Denmark. These organisms were again identified as yellow-pigmented *Enterobacter cloacae* rather than as *Enterobacter sakazakii.* In the year 1984 Muytjens and Van Druten reported eight neonatal meningitis and sepsis cases of *Enterobacter sakazakii* from the Netherlands. This was the first large series of neonatal infections to be documented and the first epidemic outbreak with a complete investigation. In this year first documented *E. sakazakii* outbreak in the United States. There were two bacteraemia cases and two additional cases of colonization in a neonatal intensive care unit.

Van Acker *et al.* (2001) reported an outbreak of *E. sakazakii* infection involving 12 infants who had necrotising enterocolitis in 1998 in Belgium. In Belgium in 2002, an infant died of *E. sakazakii*–associated meningitis after consuming a commercial PIF. In New Zealand in July 2004, a premature infant contracted *E. sakazakii* meningitis and died. Another PIF was withdrawn after a possible link to 5 cases of presumed *E. sakazakii* infection in premature infants in France in 2004 that led to the death of 2 infants.

Since then and up to July 2008, around 156 documented cases of *Cronobacter* spp. infection and at least 29 deaths from all parts of the world appeared in the published literature and reports submitted by public health organisations and laboratories (FAO/WHO, 2008).The Food and Agriculture Organization(FAO) of the United Nations/World Health Organization statistics for 2006 estimated that the annual incidence rate in the USA among low-birth-weight infants who weighed, 2500 g and were, one year old was 8.7 per 100 000 (FAO/WHO, 2006). Globally, there is no active surveillance system for tracking this pathogen; however, in their 2008 report, the WHO Expert Panel followed cases from 1961 to 2008 and found 120 recorded instances of *Cronobacter* among infants and children, three years old (FAO/WHO, 2008). Although only ~120 cases have been reported worldwide, the actual number of cases is considered far higher (Centers for Disease Control 2009; Friedemann, 2009; Teramoto *et al.,* 2010). Previously thought to be as high as 80%, the rate of mortality from microbiologically confirmed cases of *Cronobacter* has been found to be 26.9%.

See *et al*. (2007) described a case of *Cronobacter* bacteraemia with multiple splenic abscesses in a 75-year-old institutionalised woman. It was the first reported case of *Cronobacter* with splenic abscesses and was the first case in a non-immunocompromised adult. Two cases of *E. sakazakii* infections are being described from PGIMER, Chandigarh, a tertiary medical care centre. This is the first report of *E. sakazakii* infections in infants from India (Ray *et al.,* 2007).

2.12. Antimicrobial susceptibility of *Cronobacter*

Acquired antimicrobial resistance by foodborne pathogens increases infectious disease morbidity and mortality with resulting substantial socioeconomic costs. Antibiotic therapy is indicated in human cases of *Cronobacter* infection and has proven to be successful. Many researchers have demonstrated that *Cronobacter* spp. can be inactivated by antibiotics, but levels of antibiotics considered adequate today for treatment may be inadequate in the future (Depardieu *et al.,* 2007; Graziani *et al*.,2008).Bacterial cells may reduce the effect of environmental stresses through genotypic and phenotypic adaptation (Bower and Daeschel, 1999). Adaptation to these external factors can lead to increased or decreased resistance to other stresses, and this may lead to the development of

antibiotic resistance as a result of “cross-protection” (Doyle *et al*., 2006; McMahon *et al*., 2007).

This confirmed that the increased use of bacteriostatic (sub-lethal) rather than bactericidal (lethal) environmental stresses can contribute to the improvement of antibiotic resistance among food spoilage microorganisms and critical foodborne pathogens. Therefore understanding the effects of stress encountered during processing on the antibiotic susceptibility of *C. sakazakii* seems essential to assess if risks associated with *C. sakazakii* illness are to be minimized, and results could provide information useful in patient treatment.

The pathogens like *Enterobacter* species capture resistance by inactivating broad-spectrum beta-lactam antibiotics due to the production of beta-lactamases (Drudy *et al*.,2006).Other mechanisms include decreased cell permeability, active efflux, modification of drug receptor site, synthesis of resistant metabolic pathway and acquisition of plasmids and transposons(Qian *et al.,*2007). Lai *et al.* (2001) concluded that this resistance was due to an overall increasing trend of antibiotic resistance among all *Enterobacter* species in hospitals. Farmer *et al.* (1980) found all strains were susceptible to gentamicin, kanamycin, chloramphenicol, and ampicillin; 87% or over were susceptible to nalidixic acid, streptomycin, tetracycline, and carbenicillin; 71%and 67% were susceptible to sulfadiazine and colistin respectively; only 13% were susceptible to cephalothin. All strains were resistant to penicillin; only 1of over 100 strains tested showed multiple antibiotic resistance.

Although multiple antibiotic resistance (mar) operons were found in *Cronobacter* (Burgos *et al.,* 2005), the overall level of antibiotic resistance was low compared with other food-borne pathogens, and the increase of antibiotic-resistant *Cronobacter* could be a result of the overuse of antibiotics (Lee *et al*., 2012). The gain of transposable elements, the production of β-lactamases and the presence of multiple antibiotic resistance (mar) operons may allow *E. sakazakii* to be resistant to ampicillin, gentamicin and cefotaxamine (Iversen and Forsythe, 2003; Drudy *et al*., 2006). *C. sakazakii* infections have been traditionally treated with ampicillin in combination with either gentamicin or chloramphenicol. Resistance to ampicillin has emerged owing to the acquisition of transposable genetic elements and the production of β-lactamases. Molloy *et al.* (2009) investigated 51% isolates out of

total 33 isolates were resistant to antibiotic Cephalothin. Of the remaining isolates, 42% displayed intermediate sensitivity to this antibiotic, and 6% were susceptible. This is a first generation broad-spectrum cephalosporin was previously used in treating neonatal infections. Increased resistance to these antibiotics has led to the recommendation that third generation is now used to treat neonatal infections (Gurtler *et al*., 2005).

Kilonzo-nthenge *et al*. (2012) stated that some *Enterobacter* isolates could inactivate broad-spectrum penicillins and cephalosporins due to 13-lactamase production, which is increasing among strains of *C. sakazakii,* antibiotic-resistant *C. sakazakii* isolates found in domestic kitchens may have originated from processed meat products or other raw foods. Kilonzo-nthenge *et al*. (2012) studied that although *C. sakazakii* isolates were susceptible to gentamicin in previous studies but *C. sakazakii* from poultry was resistant to gentamicin. Al-Nabulsi *et al.* (2011) showed that streptomycin, gentamicin, kanamycin and ciprofloxacin are effective against both stressed and unstressed *C. sakazakii* cells, and thus these Antibiotics might be appropriate choices for patient treatment regimes. Nevertheless, Nazarowec-White and Farber (1999) found that two *Cronobacter* strains out of eight were resistant to tetracycline and chloramphenicol. Kim *et al.* (2008) described *Cronobacter* strains recovered from foods that were resistant to cephalothin or ampicillin and susceptible to tetracycline. Hochel *et al.* (2012) found that all *Cronobacter* isolates varied in their susceptibility to tetracycline.

Farm animals exposed to overuse of antibiotics for extended periods of time can develop microbial flora resistant to these agents. Resistant infections may be costly to control and can compel the use of less desirable antibiotics.

2.13. Methods for Identification of *Cronobacter* spp.

The US Food and Drug Administration described a modified method, based on work conducted by Muytjens *et al.* (1988) for detecting and isolating *Cronobacter* spp. from PIF. This method is lengthy and not specific to *Cronobacter* spp. Furthermore, this method is not useful at discriminating between *Cronobacter* and *Enterobacter* spp.

The International Standards Organization (ISO, 2006) and the International Dairy Federation (IDF) published a method in 2006 describing the isolation of

Cronobacter spp. *f*rom the milk-based powdered formula. The technique consists of pre-enriching the PIF samples in Buffered peptone water (BPW) at 37˚C overnight. The samples are then enriched in modified lauryl sulfate (addition of vancomycin) broth (mLST) at 44˚C overnight. The samples are then plated on to chromogenic agar (Druggan–Forsythe–Iversen Formulation agar DFI) at 25˚C for a duration of 48 to 72 hours. For enabling the distinction of *Cronobacter* from the background flora and confirmation of *Cronobacter* spp. Colonies formed on DFI were examined for blue-green pigmentation, which indicated constitutive expression of α-glucosidase, a characteristic of *cronobacter.*It reduced the analysis time and increase the specificity when compared to the FDA approved method. However, not all strains of *Cronobacter* can grow in mLST.

2.13.1. Pre-enrichment

The pre-enrichment step is carried out in a non-selective broth that allows stressed cells to recover from any injury that has occurred during manufacturing or preparation that makes them sensitive to selective agents. The isolation step usually involves plating out samples from the enrichment broth on to a selective diagnostic medium that allows a pathogen to be differentiated from the competitive microflora that has survived the enrichment step. This can increase the sensitivity of the method, but it has no impact on the specificity as no selective agents are present.

The International Organization for Standardization (ISO) has recommended the use of Buffered Peptone Water (BPW) as a pre-enrichment medium in methods for the isolation of a variety of Gram-negative pathogens. It was also recommended in ISO/TS22964 for the isolation of *Cronobacter* spp*.* in IFM and milk products.

2.13.2. Selective enrichment

2.13.2.1 Enterobactera Enrichment (EE) broth

One of the weaknesses of the FDA method is that it uses Enterobacteria enrichment (EE) broth. This is a general medium for the *Enterobacteriaceae* and though it inhibits the growth of Gram-positive organisms, there is nothing in this method that improves the specificity of the method for *Cronobacter* relative to the other members of the *Enterobacteriaceae*.

2.13.2.2. Lauryl Sulphate broth (Lauryl Tryptose broth (LST)

Cronobacter spp. has been reported to grow at elevated temperatures (Nazarowec-White and Farber, 1997) and this Lauryl sulphate Tryptose broth (LST) is an attractive option for enrichment of *Cronobacter*. The specificity was further enhanced by incubation at 45 °C and by the addition of 29 g/l sodium chloride (Guillaume-Gentil *et al.,* 2005). The development of this medium was based on the observation that *Cronobacter* spp. are resistant to high osmolality (Breeuwer *et al.,* 2003). Vancomycin was also added to the broth to inhibit the growth of Gram-positive organisms that are resistant to sodium lauryl sulphate and to the high salt concentration. The high salt concentration and the presence of the detergent are insufficient to inhibit the growth of *Lactobacillus* spp. (Guillaume-Gentil *et al.,* 2005). This medium has been termed modified Lauryl sulphate Tryptose (mLST) (Guillaume-Gentil *et al*., 2005). The high sodium chloride content gives the medium an osmolality of around 1200 milliosmols/kg. This is sufficient to inhibit the growth of many members of the *Enterobacteriaceae.*

Since ISO/TS 22964 (ISO-2006) was first published it has been found that mLST enrichment broth has some limitations. It has been found that some strains of *Cronobacter* spp. are inhibited by sodium lauryl sulphate (Guillaume-Gentil *et al.,* 2005; Iversen and Forsythe, 2007). There are also some strains that are not capable of growing at 45 °C (Iversen *et al.,* 2004). In mLST the *Cronobacter* population is enriched relative to *Enterobacteriaceae* (Guillaume-Gentil *et al.,* 2005), but this is not necessarily due to an increase in the numbers of *Cronobacter* and may be in some instances attributed to a decrease in the competitive background flora (Iversen and Forsythe 2007).

2.13.2.3. Cronobacter screening broth (CSB)

To circumvent the problem of sensitive *Cronobacter* strains a differential ‘screening’ broth was developed (Iversen *et al.,* 2008). This Cronobacter screening broth (CSB) is designed to encourage the growth of all *Cronobacter* strains without intentional inhibition of other *Enterobacteriaceae*.

The purpose of the broth is primarily to select for the presence of organisms that can ferment sucrose and this is indicated by a change in colour from purple to

yellow due to the incorporation of the pH indicator Bromocresol Purple. When used in isolation this broth has poor specificity with approximately 50% of known *Enterobacteriaceae* species being able to ferment sucrose, however of those that can ferment sucrose only *Cronobacter* and one other species, *E. pulveris* (Iverson *et al.,* 2008). *Cronobacter* are able to hydrolyse the chromogen 5-bromo-4-chloro-3-indolyl- α,D-glucopyranoside (XαGlc). Therefore in combination with chromogenic media, this broth has improved sensitivity and specificity over existing methods.

2.13.3. Selective plating

2.13.3.1. Chromogenic agar

The first commercially available chromogenic agar for *Cronobacter* spp. was *Enterobacter Sakazakii* Isolation Agar (ESIA).Druggan–Forsythe–Iversen Agar (DFI) was developed by Oxoid in early July 2002 and became commercially available shortly after ESIA in 2004.

The chromogenic media was developed by Druggan and Iversen in 2009 which was based on the work of Muytjens *et al*. (1984) on the α-glucosidase enzyme. Muytjens *et al*.(1984) identified this unique enzyme appears to be unique to only *Cronobacter;* as the closely-related species *Enterobacter cloacae, Enterobacter aerogenes* and *Pantoea agglomerans* were all negative for the enzyme. Media which incorporates the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α, α-glucopyranoside (X-αGlc) will result in *Cronobacter* colonies appearing blue-green on a Druggan, Fosythe and Iversen (DFI) agar plate. The α-glucosidase presents in *Cronobacter* spp*.* hydrolyzes X-αGlc to form a bromo-chloro indigo pigment. DFI is based on Tryptone Bile Agar (Anderson and Baird-Parker, 1975) using TSA as the nutrient base with XαGlc and a hydrogen sulfide detection system included differentiating weakly α-glucosidase positive *Proteus* spp.

This medium is incubated at 37 °C to ensure the growth of the temperature sensitive strains of *Cronobacter* spp. It has been noted that the high concentration of sodium deoxycholate relative to other media and the relatively low level of chromogen, coupled with endogenous sugars in the base medium, combine to reduce sensitivity for some rare strains of *Cronobacter* that are particularly susceptible to bile salts or that have weak α-glucosidase activity. During the early

evaluation work on DFI it was found that increasing the incubation temperature above 37 °C also had a detrimental effect on the production of blue-green pigment for several strains.

The limitations of DFI lead to the development of modified Druggan–Forsythe–Iversen (mDFI) Agar. A weakness of DFI was that since it was based on Trptic soya agar (TSA) many strains of *Cronobacter* spp. that produce a yellow pigment on TSA, produce yellow pigment on DFI but the colonies can then appear green rather than blue-green. The peptone composition of modified DFI was changed to prevent yellow pigment formation in *Cronobacter* colonies. The concentration of XαGlc was increased by 50% to detect weak α-glucosidase positive strains of *Cronobacter* spp. The concentration of sodium deoxycholate was reduced to increase the sensitivity of the medium to detergent sensitive *Cronobacter* strains.

2.14. Molecular Identification of *Cronobacter* spp.

As compared to biochemical and phenotypic-based tests Polymerase chain reaction PCR is becoming more routinely used as a reliable and quick method for accurately detecting food samples containing *Cronobacter* spp. (Abdesselam, 2012).

The 16S rRNA gene is widely used as target as it is a highly conserved gene, ubiquitous in all organisms and contains variable and hypervariable regions of sequence. Molecular methods based on this gene are well established as a standard method for characterization and identification of bacteria (Woo *et al*., 2003). Amplification of the 16S rRNA gene from *Cronobacter* isolates by using primer pairs Saka-1a (forward) and Saka-2b (reverse) gave PCR products with a size of 977 bp (Hassan *et al.,* 2007).

The internal transcriber spacer ITS sequence is one of the most popular targets for bacterial identification due to the variation in its sequences among bacterial species (East *et al*., 1992).For target the site of ITS of *Cronobacter* isolates using the primer pairs SG-F (forward) and SG-R (reverse) respectively gave the PCR product with a size of 282 bp.

However, several molecular means, including PCR assays that targeted the gene responsible for 16S rRNA gene sequencing and internal transcriber spacer (ITS) sequence between 16S and 23S rRNA, have been shown to be more reliable method for detection of *Cronobacter* spp (Lehner and Stephen, 2004).

Other DNA-based detection of isolates *Crononobacter* spp. Involves the detection OMPA gene wich encoding the outer membrane protein A (OmpA) by using the primer pairs OmpA1 (forward) and OmpA2 (reverse) (Nair and Venkitanarayanan, 2007) respectively gave the product size 469 bp.

3. MATERIAL AND METHODS

3.1. Sample collection

The seafood samples (fish and shellfish) used in this study were collected from different fish markets, landing centres and supermarkets located in and around North-west Mumbai (Maharashtra). In addition, coastal water samples were collected from Juhu and Versova creek and fish wash water from Versova landing centre. A total of 50 samples were used for analysis. The samples were aseptically transported to the laboratory in a chilled condition in sterile sampling bags for bacteriological analysis within one hour of collection. The details are given in tables.1-3.

3.2. Media and glassware

All microbiological media used in this study were purchased from Hi-Media Laboratories Pvt.Ltd., Mumbai. The composition and preparation of various media are given in Appendix. All the media were sterilised by autoclaving at 15 psi pressure at 121˚ C for 15 minutes. All glass wares and Petri plates used for the study were soaked in mild detergent solution for 5-10 minutes, then washed thoroughly in tap water and rinsed with distilled water. After air drying glass wares were sterilised in a hot air oven at 180°C for 2 hours and plastic Petri plates were sterilised by autoclaving at 15 psi pressure at 121˚ C for 15 minutes. All the centrifuge tubes, tips, PCR tubes etc. were sterilised by autoclaving at 15 psi pressure at 121˚C for 15 minutes.

Table 1. Samples of finfish analysed

Sampling location	Fish Sample name	Scientific name
Versova fish landing centre	Croaker	*Johnius dussumieri*
	Ribbon fish	*Trichurus lepturus*
	Bull‘s eye.	*Priacanthus hamrur*
4-Bangalow fish market	Flatfish	*Cynoglossus* spp.
	Mackerel	*Rastrelliger kanagurta*
	Bombay duck	*Harpadon nehereus*
Andheri fish market	False trevally	*Lactarius lactarius*
	Rainbow sardine	*Dussumieria acuta*
	Ladyfish	*Elops affinis*
Juhu Fishmarket	Croaker	*Johnius dussumieri*
	Golden anchovy	*Coilia dussumieri*
	Sardine	*Sardinella longiceps*
Malad fish market	Mackerel	*Rastelliger kanagurta*
	Golden anchovy	*Coilia dussumieri*
	Bombay duck	*Harpadon nehereus*
	Tiger perch	*Terapon jarbua*
	False trevally	*Lactarius lactarius*
Chakala fish market	Golden anchovy	*Coilia dussumieri*
	Bombay duck	*Harpadon nehereus*
	Flatfish	*Cynoglossus* spp.
	Sardine	*Sardinella longiceps*

Table 1.

Sampling location	Sample name	Scientific name
Dadar fish market	Sardine	*Sardinella longiceps*
	Rainbow sardine	*Dussumieria acuta*
	Coilia	*Coilia dussumieri*
	Mackerel	*Rastelliger kanagurta*
Retail supermarket	Pink perch	*Nemipterus japonicus*
	Flatfish	*Cynoglossus* spp.
	Bombay duck	*Harpadon nehereus*
	Ladyfish	*Elops affinis*
Madh-island fish market	Golden anchovy	*Coilia dussumieri*
	Mackerel	*Rastrelliger kanagurta*
	Tiger perch	*Terapon jarbua*

Total no. of fin fish samples analysed in this study: 32

Table 2. Shell fish samples analysed in this study

Sampling location	Sample name	Scientific name
4 Bangalow fish market	Crab	*Portunus pelagicus*
Andheri fish market	Squid	*Uroteuthis duvauceli*
Juhu Fishmarket	Shrimp	*Parapeneopsis stylifera*
Chakala fish market	Shrimp	*Parapeneopsis stylifera*
Dadar fish market	Yellow clam	*Meritrix meritrix*
Star-bazar supermarket	Squid	*Uroteuthis duvauceli*

Total no. of shell fish samples analysed in this study: 6

Table 3. Dry fish samples analysed

Sampling location	Dry fish Sample name	Scientific name
Malad fish market	Bombay duck	*Harpadon nehereus*
	Salted mackerel	*Rastrelliger kanagurta*
	Ribbon fish	*Trichiurus lepturus*
Dadar fish market	Golden anchovy	*Coilia dussumieri*
	Bombay duck	*Harpadon nehereus*
	Ribbon fish	*Trichiurus lepturus*
Dadar fish market	Shrimp	*Parapeneopsis stylifera*
Star-bazar supermarket	Bombay duck	*Harpadon nehereus*
	Ribbonfish	*Trichiurus lepturus*

Total no. of dry fish samples analysed in this study: 9

3.3. Sample preparation

The muscle portion along with skin and viscera of 2-5 individual fishes pooled together was used for enrichment. In the case of shrimp 4 to 5 whole individual shrimps along with shell, pooled together was used for enrichment. The shells of clam were opened with a knife and the meat portion was shucked out which was used as sample. With regard to crabs, the carapace and appendages were removed and the meat portion was used for enrichment.

3.4. Pre-enrichment, enrichment and selective plating

For pre-enrichment of *Cronobacter* spp*.,* twenty-five grams of pooled sample was aseptically weighed and macerated in a sterile stomacher bag with 225 ml sterile buffered peptone water (Hi-Media M614-500g). After adequately macerating, the homogenized sample was transferred to a conical flask and incubated for 18 to 24 hours at 37˚C. After incubation, 10 ml of the pre-enriched sample was added

aseptically to 90 ml of sterile selective enrichment Cronobacter screening broth (Hi-Media M1786-500g) supplemented with 0.9ml of Vancomycin. Incubation was done for 24 hours at 37˚C Following incubation, a loopful of culture from selective enrichment broth was streaked on to pre-dried HiCrome *Enterobacter sakazakii* Agar (Hi-media M1577-500g) plates and then incubated at 37˚C for 24 hours. *Cronobacter* spp. colonies appear as small, bluish green, circular colonies, which were considered as presumptive *Cronobacter* isolates

3.4.1. Purification of isolates

The presumptive bluish green *Cronobacter* isolates were picked from the selective plates and streaked on to Luria Britani (LB) agar plates (Hi-media M1151-500g) for purification and further analysis.

3.5. Biochemical tests for identification

The presumptive isolates of *Cronobacter* after purification were subjected to a sereis of biochemical tests for further identification and confirmation. The biochemical tests are described below:

3.5.1. Grams staining

Grams staining was done using Grams staining kit from Hi-Media (K001-1KT). Young cultures (24-36 hrs) from Luria Bertani agar plate were smeared with one drop of sterile distilled water on to the clean grease free glass slide by using a sterile loop and air dried. The smears were heat fixed and subjected to Gram's staining. First, the smear was stained with crystal violet solution for 1 min and then washed gently with tap water. After that, the smear was flooded with moderant (Gram's iodine solution) for 1 minute and washed with tap water until the excess idone solution was removed. Then, decolouriser (95% alcohol) was added and kept for 10-20 secs. After rinsing with water, staining with the counterstain safranin was done for 1 min. Finally, the slides were dried and examined under the microscope using oil immersion objective lens 100X.

3.5.2. Cytochrome oxidase test

The oxidase test (Gaby and Hadley, 1957) is used to identify bacteria that produce cytochrome C oxidase, an enzyme of the bacterial electron transport chain. When present, the cytochrome c oxidase oxidises the reagent to (indophenols) purple colour end product. When the enzyme is not present, the reagent remains reduced and is colourless. 24-36 hrs. old bacterial cultures were picked up from Luria Bertani agar using a sterile loop and spotted onto pre-moistened oxidase disk impregnated with oxidase reagent (N, N-dimethyl-p-phenylenediamine oxalate, ascorbic acid and α-naphthol, (Hi-media DD018). Development of dark purple colour within 10-30 secs shows positive reaction. A change later than 10 to 30 seconds or no change at all is considered negative reaction.

3.5.3. Catalase test

The enzyme catalase neutralises the bactericidal effects of hydrogen peroxide by breaking hydrogen peroxide (H_2O_2) into water and oxygen and the rapid formation of bubbles evidences the reaction. Gram negative presumptive *Cronobacter* bacterial culture picked up from the Luria Bertani agar by using heat sterile loop was dipped in one drop of 3% (v/v) hydrogen peroxide (H_2O_2) solution and then immediately observed for the effervescence(Chen *et al.,* 2016)

3.5.4. Motility test

3.5.4.1. Hanging-drop or Wet mount method

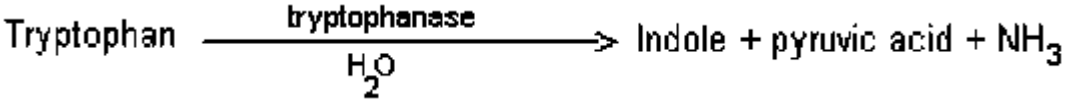
Selected presumptive positive individual colonies were picked and suspended in LB broth and incubated at 37°C for 24 hours. After incubation one drop of culture was placed on an inverted, clean, dry slide and covered with a coverslip. The slide was observed under a microscope at 40X magnification by adequately fixing the lens focus. The wet mount of the bacterial isolates were observed under the microscope simultaneously. The morphological characters like size, shape and motility were observed.

3.5.4.2. Motility test agar (Tittsler and Sandholzer,1936)

Motility Test Medium with an agar concentration of 0.4% or less is used for testing motility, which can be visualized as a diffused zone of growth flaring out from the line of inoculation. Tryptose in the medium serves as a source of essential growth nutrients required for bacterial metabolism. Sodium chloride maintains the osmotic equilibrium of the medium. A small amount of agar helps to create a semisolid medium. Bacterial motility can be observed directly by examination of the tubes which contain sterile motility test media (Hi-media M-260-500g) following incubation. Inoculation is done by aseptically stabbing the culture using a sterile loop through the centre of the medium. Incubation is done at 37°C for 18-24 hours. Non-motile organisms grow only along the line of inoculation whereas motile microorganisms grow away from the edge of inoculation or may show growth even throughout the medium.

3.5.5. Indole test (MacWilliams,2009)

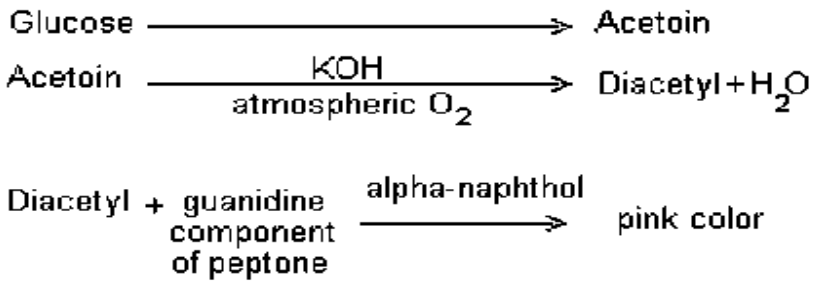
Indole is produced in tryptone broth by the enzyme systems of certain bacteria. Tryptone broth is abundant in the amino acid tryptophan, which can be used by some bacteria as a source of carbon and nitrogen as well as energy. As these enzymes increase in the tryptone broth, they degrade tryptophan into indole, pyruvic acid, and ammonia.



A tube of sterile tryptone broth or Tryptone Water (Hi-media M463-500g) is inoculated with loop full new culture from LB Plate and incubated at 37°C for 18 to 24 hours. After incubation, Kovac's reagent (Hi-media R008-100ml) is added to the tube. Kovac's reagent contains amyl alcohol, concentrated Hydrochloric acid and para-dimethylamino benzaldehyde, which react with the indole to form a red ring on the top of the broth. A red colour indicates a positive test for indole production and yellow or green colour indicates that tryptophan was not hydrolysed and is a negative test for indole production.

3.5.6. Voges Proskauer (VP) Test

VP test (Barritt,1936) detects butylene glycol producers. VP test is helpful in identifying members of the family Enterobacteriaceae. Acetyl-methyl carbinol (acetoin) is an intermediate in the production of butylene glycol. In this test two reagents, 40% potassium hydro oxide (KOH) and 5% alpha-naphthol are added to test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of alpha-naphthol to produce a red colour. Role of alpha-naphthol is that of a catalyst and a colour intensifier.



A loop full of fresh culture is inoculated into glucose phosphate broth or Methyl red- Voges Proskauer Medium (Hi-media- M070-500g) tube and incubated for at least 24 to 48 hours at 37°C temperature. After incubation 0.6 ml of alpha-naphthol or Barritt Reagent A (Hi-media- R029) is added to the test broth and shaken. 0.2 ml of 40% KOH or Barritt Reagent B (Hi-media- R030) is added to the broth and shaken. The tube is allowed to stand for 15 minutes. The appearance of brownish wine or pinkish brown colour is taken as a positive test. The negative tubes must be held for one hour, since maximum color development occurs within one hour after addition of reagents.

3.5.7. Malonate broth utilisation test (Leifson,1933 and Ewing *et al.*, 1957)

Leifson developed malonate Broth test in 1933.It is done for the differentiation of *Enterobacter* from *Escherichia* spp. based on malonate utilisation. An organism that can simultaneously utilise sodium malonate as its carbon source and ammonium sulfate as its nitrogen source produce alkalinity due to the formation of sodium hydroxide. The alkali changes the colour of the bromothymol blue indicator in the medium to light blue and finally to Prussian blue. The colour of the

medium remains unchanged in the presence of an organism that cannot utilize these substances. Also, some malonate-positive microorganisms produce only slight alkalinity that causes the results to be difficult to interpret. Therefore, these tubes should be compared with an un-inoculated malonate tube as a control.

An inoculum of a pure fresh culture from LB plate is transferred aseptically by using heat sterile loop to a sterile tube of malonate broth (Hi-media; M382). The inoculated tube is incubated at 37˚C for 24 hours, and the results are determined. Abundant growth and a change from green to blue in the medium compared with control tube indicates a positive test for growth using malonate.

3.6. Identification and characterisation of *Cronobacter* spp. using nucleic acid based methods

3.6.1. Preparation of DNA

For DNA extraction, crude lysate method followed. In a sterile 1.5 ml centrifuge tube 200 µl of sterile 1x TE (10 mM Tris pH 8.0;1 Mm EDTA) buffer was taken in which a loopful of the colony from an 18-24 hrs. old culture on LB Plate was suspended and uniformly mixed by using a vortex mixer (Abdos) in low-moderate speed for 20-30 seconds. The bacterial cells in the suspension were lysed by placing the tube on a dry bath (GeNei) maintained at 98˚C for 15 minutes followed by immediate chilling in flake ice for rapid release of DNA for 10-15 minutes. The cell debris were pelleted by centrifuging at 12,000 rpm for 2 minutes. The clear supernatant containing DNA was stored at -20˚C for further use.

3.6.2. Polymerase Chain Reaction (PCR)

In a sterile 0.2 ml PCR tube or micro centrifuge tube the following ingredients are mixed together in following order (mention in Table.5).

Table 4. Typical Composition of PCR mix

PCR master mix	Amount
Sterile MilliQ Water	16.7 µl
10x amplification DNA buffer	3 µl
dNTP mix (10Mm)	1 µl
Forward primer (10 pico moles/µl)	3 µl
Reverse primer (10 pico moles/µl)	3 µl
Taq DNA polymerase (5U/l)	0.3 µl
Template DNA	3 µl
Total volume	30 µl

PCR was performed in a final reaction volume of 30 µl containing above mixture and respective template DNA. Oligonucleotide primers for *Cronobacter species*-specific 16s gene (16s rDNA),genus-specific gene (*ITS-G* and *ITS-IA* Internal transcriber spacer sequence between 16s and 23s r RNA), and virulence gene *ompA* (outer membrane protein encoding), *hly* (hemolysin), *cpa (*plasminogen activator) were used for PCR amplification. The primer sequences are given in table 6. Amplification was done in a Simpli Amp Thermal Cycler (Applied Biosystems;

Thermofisher) and Hi-media (Prima-96). The amplification conditions are given in table7. After amplification, the PCR products were analysed by electrophoresis on1.5% agarose gel stained with ethidium bromide (8-10 µl/ml) and photographed.

Table 5. Primers used for PCR confirmation of *Cronobacter* spp.

Name of the primers	Nucleotide Sequence (5' to 3')	Target site	Product size(bp)	References
Saka 1a Saka 2b	FP: ACAGGGAGCAGCTTGCTGC RP: TCCCGCATCTCTGCAGGA	Variable regions of the 16S rRNA gene	952	Hassan *et al.(*2007)
SG-F SG-R	FP: GGGTTGTCTGCGAAAGCGAA RP: GTCTTCGTGCTGCGAGTTTG	ITS-G ITS-G and ITS-IA	282	Liu *et al.(*2006)
OmpA1 OmpA2	FP: GGATTTAACCGTTTCC RP: CGCCAGCGATGTTAGAAGA	*ompA* gene	469	Nair and Venkitanarayanan (2005)
cpa F cpa R	FP:CTAGGGCGATGATAGCTGCCTCCG RP:CTAGGGGGAACAGCCACGAGGAAA	*cpa gene*	1015	Cruz *et al. (* 2011)
hly F hly R	CTAGGGTAACGGACTGTCACAGAT CTAGGAAGAAGCGTAAGCGTCTGA	*hly gene*	880	Cruz *et al.* (2011)

Table 6. PCR condition for different primers used in this study

Gene name	Initial denaturation	Denaturation	Annealing	Extension	Final extension
16S rRNA	95˚C,4min 1cycle	95˚C,1min 30 cycle	50˚C,1min 30 cycle	72˚C,1min30s 30 cycle	72˚C,4 min 1 cycle
ITS-G ITS-G and *ITS-IA*	94°C,10 min 1cycle	94°C,10 min 30 cycle	57°C,1 min 30 cycle	72°C,1 min 30 cycle	72°C,5 min 1 cycle
ompA	94°C,2 min 1cycle	94°C,30s 30 cycle	52,1min 30 cycle	72°C,2 min 30 cycle	72°C,5 min 1 cycle
cpa	94 °C, 3 min 1cycle	94 °C,30 s, 30 cycle	62 °C for 30 s 30 cycle	72 °C,1 min 30 cycle	72 °C,10 min 30 cycle
hly	94 °C, 3 min 1cycle	94 °C,30 s, 30 cycle	52 °C for 30 s 30 cycle	72 °C,1 min 30 cycle	72 °C,10 min 30 cycle

3.6.3. Analysis of PCR products

Agarose gel electrophoresis was used to visualise the amplified DNA product. A 5 mm thick 1.5% agarose gel (prepared in 1 X TAE buffer pH-8) stained with 8-10 µl/ml ethidium bromide was mixed well and poured over the gel casting tray with combs fixed. After the gel was completely set, the comb was removed carefully and placed in the electrophoresis tank, and the buffer was added to a level of about 1 mm over the gel. Ten µl of the amplicon was mixed with 1 µl to 2 µl of 6 X tracking dye and loaded into well slots of the submerged gel and electrophoresis was carried at 100V. The DNA molecular size marker (1kb ladder) was loaded in one of the lanes and run along with the samples. Electrophoresis was stopped once the dye migrates closer to the anode (approx. after 30-35 min). The gel was then placed on UV transilluminator, observed for amplified DNA and photographed for documentation (BIO-RAD, USA) and photographed.

3.7. Purification of PCR products for sequencing

The PCR products were purified using commercial kit GENECLEAN Turbo (MP Bio medicals USA) by following the manufacturer's instructions:

Step1. The PCR products were pooled to 100 µl volume in GENECLEAN Turbo cartridge. Step 2.For adjusting the binding conditions 100 µl GENECLEAN Turbo salt solution was added. Step 3. DNA was bound by centrifuging the cartridge for 5 sec at 14000 rpm speed. Step 4. GENECLEAN Turbo cartridge was washed by adding 500 µl GENECLEAN Turbo wash solution and then centrifuged for 5 sec at 14000 rpm, followed by emptying the catch tube. Step 5. GENECLEAN Turbo cartridge was dried by centrifuging it for 4 min at 14000 rpm. Step 6. Using a new catch tube, dry GENECLEAN Turbo cartridge was inserted to bind DNA and DNA was eluted by adding GENECLEAN Turbo elution solution. This was incubated for 5 min at room temperature. After that the cartridge was centrifuged for 1 min at 14000 rpm and the purified PCR product was collected and stored in a catch tube to be used for sequencing.

3.8. Analysis of antibiotic sensitivity pattern of *Cronobacter* isolates

Cronobacter isolates were tested for determining the antibiotic susceptibility pattern by using the disk diffusion technique (Hudzicki, 2009.). The antibiotic discs used in this study are mentioned in the table.8. The antibiotic discs were purchased from Hi-media (India).

Cronobacter isolates from different sources were cultured in Muller Hinton broth (Hi-media, M391) and incubated at 37˚C for 6 to 8 hours. Muller Hinton broth was adjusted to 0.5 OD (equivalent to 10^8 cells) and then transferred to Muller Hinton agar (Hi-media, M173) plates. Antibiotic discs were dispensed on the plates and were incubated at 37˚C for 18 to 24 hours. Inhibition zones diameter was measured using measuring scale after the incubation period. An interpretative criterion (mention in the table.7) was done following the Clinical and Laboratory Standards Institute protocol given by the antibiotic discs manufacture Hi-media.

Table 7. Antibiotic discs used and interpretative criterion

Name of the antibiotic used in this study	Symbol	Disk content(mcg)	Interpretative criteria		
			Sensitive (milli-meter or more)	Intermediate(milli-meter)	Resistant (milli-meter or less)
Amoxycilin-clavulanic acid	AMC	30	19	-	19
Cefotaxime	CTX	30	26	23-25	22
Cefoxitin	CX	30	18	15-17	14
Cefpodoxime	CPD	10	21	18-20	17
Ceftazidime	CAZ	30	21	18-20	17
Chloramphenicol	C	30	18	13-17	12
Ciprofloxacin	CIP	5	21	16-20	15
Colistin	CL	10	15	-	15
Ertapenem	ETP	10	22	19-21	18
Imipenem	IPM	10	23	20-22	19
Kanamycin	K	30	18	14-17	13
Meropenem	MRP	10	23	20-22	19
Nalidixic Acid	NA	30	19	14-18	13
Piperacillin/Tazobactam	PIT	100/10	21	18-20	17
Tetracycline	TE	30	15	12-14	11

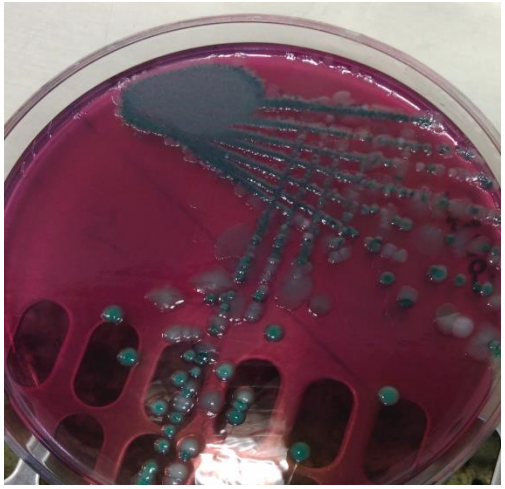
4. RESULTS

4.1. Prevalence of *Cronobacter* spp. in samples analysed

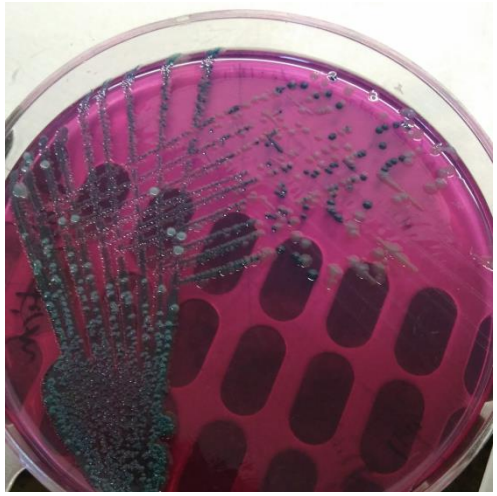
In this study, a total of 50 samples were screened for the presence of *Cronobacter* spp. which were collected from different fish markets and landing centres, and retail supermarkets located in and around the North-west Mumbai (Maharashtra). Presumptive colonies of *Cronobacter* were obtained from 38 (76%) out of these 50 samples. The samples were comprised of 32 fresh finfish, 6 shellfish, 9 dry fish and 3 water samples. Out of 32 fresh fin fish samples, 24(75%) were positive for *Cronobacter* spp. by selective plating on HiChrome *Enterobacter sakazakii* agar. A total number of 82 presumptive isolates were taken from these samples for further analysis. The details of samples positive for *Cronobacter* spp. and no of isolates taken from them are mentioned in the Table 8. Among the 6 shellfish samples analysed, 4 (66.67%) were positive for *Cronobacter* spp. by selective plating. From these positive samples, 19 presumptive isolates were chosen for further analysis, the details of which are given in Table no 10. Another 40 presumptive isolates of *Cronobacter* spp. were selected from 7 (77.78%) out of 9 dry fish samples, for biochemical and molecular analysis (Table 11.) All 3 water samples analysed showed positive for presumptive *Cronobacter* colonies, from which 24 colonies were picked (Table 12.) On the whole, 165 presumptive isolates of *Cronobacter* spp. were taken from 50 samples for further biochemical and molecular confirmatory tests.

4.1.1. Results of biochemical confirmation tests

One hundred sixty-five presumptive isolates of *Cronobacter* spp. from Hi-Chrome *Enterobacter sakazakii* selective plates were subjected to biochemical confirmatory tests that included a series of reactions. All the 82 isolates from fresh fin fish were found to be gram-negative, motile, oxidase negative, catalase positive and positive for malonate broth utilization test. Out of these, 98.78% isolates were indole negative, 12.2% VP negative and remaning 87.80% positive for VP test. The results of biochemical tests are shown in the Table.9. All the 19 presumptive isolates from shellfish samples were gram-negative, motile, oxidase negative, catalase positive and positive for malonate broth utilization test. Of these, 57.89% were indole negative and 52.63% VP negative (Table 10). Forty isolates from dry fish samples were also gram-negative, motile, oxidase negative, catalase positive, indole negative and VP positive, with 97.5% of them positive for malonate broth utilization test. From water samples, 24 presumptive isolates were taken all of which were found to be gram-negative, motile, oxidase negative, catalase positive, indole negative and VP positive with 66.67% of them positive for malonate broth utilization test.

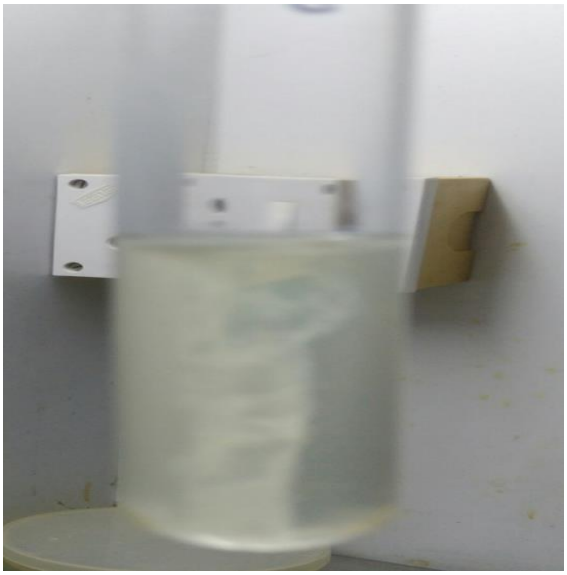


A

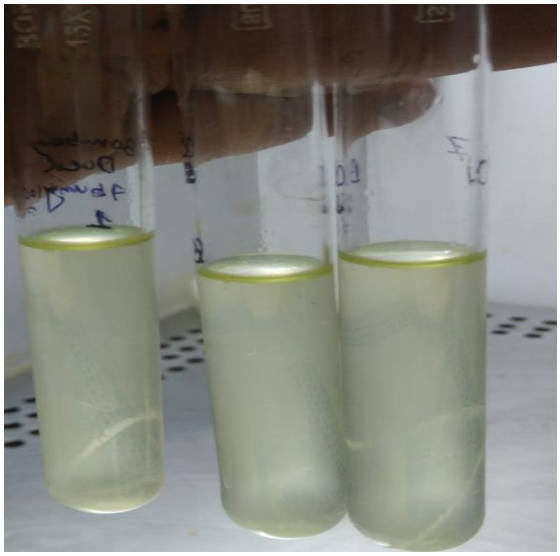


B

Fig 1. (A, B) Presumptive *Cronobacter* colonies on Hicrome *Enterobacter sakazakii* agar plate

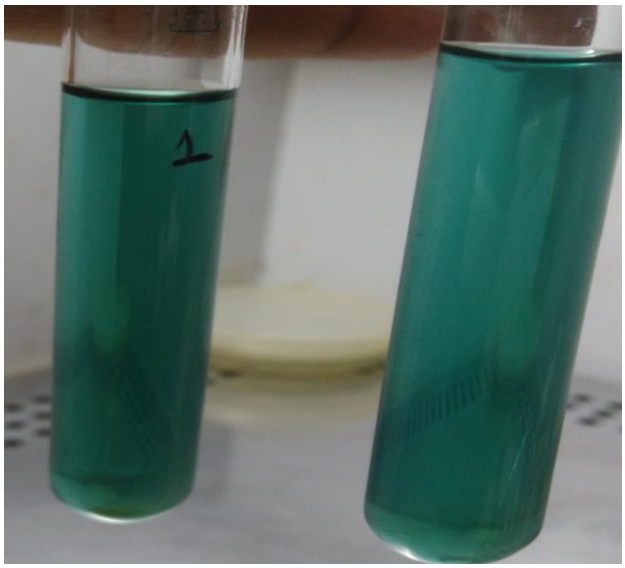


C. Motility test



D. Indole test

E. VP test



F. Malonate broth utilisation test

Fig 2. (C, D, E, and F) Biochemical tests results for presumptive *Cronobacter* isolates

Table 8. Samples positive for *Cronobacter* spp. from fresh fin fish

Number	Sampling location	Samples positive for *Cronobacter*	No. of isolates taken
1.	Versova fish landing centre	Croaker	3
2.		Bull‘s eye	3
3.	4-Bangalow fish market	Bombay duck	3
4.	Andheri fish market	False trevally	2
5.		Rainbow sardine	2
6		Ladyfish	2
7	Juhu Fishmarket	Golden anchovy	8
8		Sardine	1
9	Malad fish market	Golden anchovy	5
10		Bombay duck	8
11		Tiger perch	2
12	Chakala fish market	Golden anchovy	4
13		Bombay duck	5
14		Flatfish	1
15		Sardine	1
16	Dadar fish market	Sardine	3
17		Rainbow sardine	3
18		Mackerel	2
19	Retail supermarket	Pink perch	3
20		Flatfish	3
21		Ladyfish	2
22	Madh-island fish market	Golden anchovy	2
23		Mackerel	6
24		Tiger perch	8

Table 9. Results of biochemical confirmatory tests of *Results Cronobacter* isolates from fresh fin fish

Sample site	Name of the samples	Biochemical tests for presumptive *Cronobacter* isolates						
		Gram staining	Oxidase	Catalase	Motility	Indole	VP	Malonate
Versova fish landing centre	Croaker	-	-	+	+	-	+	+
	Ribbon fish	-	-	+	+	-	+	+
4-Bangalow fish market	Bombay duck	-	-	+	+	-	+	+
Andheri fish market	False trevally	-	-	+	+	-	+	+
	Rainbow sardine	-	-	+	+	-	+	+
	Lady fish	-	-	+	+	-	+	+
Juhu Fishmarket	Golden anchovy	-	-	+	+	-	+	+
	Sardine	-	-	+	+	-	+	+
Chakala fish market	Golden anchovy	-	-	+	+	-	-	+
	Bombay duck	-	-	+	+	-	-	+
	Tiger perch	-	-	+	+	-	+	+
	Sardine	-	-	+	+	+	-	+
Dadar fish market	Sardine	-	-	+	+	-	+	+
	Rainbow sardine	-	-	+	+	-	+	+
	Mackerel	-	-	+	+	-	+	+
Retail supermarket	Pink perch	-	-	+	+	-	+	+
	Flatfish	-	-	+	+	-	+	+
	Ladyfish	-	-	+	+	-	+	+
Madh-island fish market	Golden anchovy	-	-	+	+	-	+	+
	Mackerel	-	-	+	+	-	+	+
	Tiger perch	-	-	+	+	-	+	+

Table 10. Biochemical confirmatory tests for shell fish samples

Sample site	Samples positive for *Cronobacter* spp	No.of presumptive isolates taken	Biochemical tests for taken presumptive positive isolates						
			Gram staining	Oxidase	Catalase	Motility	Indole	VP	Malonate
Andheri fish market	Squid	2	-	-	+	+	-	-	+
Juhu Fish market	Shrimp	1	-	-	+	+	-	+	+
Chakala fish market	Shrimp	8	-	-	+	+	+	-	+
Madh-island fish market	Shrimp	8	-	-	+	+	-	+	+

Table 11. Biochemical confirmatory tests for dry fish samples

Sample site	samples positive for *Cronobacter* spp.	No.of positive isolates taken	Biochemical tests for taken presumptive positive isolates						
			Gram staining	Oxidase	Catalase	Motility	Indole	VP	Malonate
Malad fish market	Bombay duck	4	-	-	+	+	-	+	+
	Ribbon fish	8	-	-	+	+	-	+	-
Dadar fish market	Anchovy	4	-	-	+	+	-	+	+
	Bombay duck	8	-	-	+	+	-	+	+
	Ribbon fish	8	-	-	+	+	-	+	+
	Shrimp	8	-	-	+	+	-	+	+

Table 12. Biochemical confirmatory tests for water samples

Name of the sample	No.of positive isolates taken	Biochemical tests for taken presumptive positive isolates						
		Gram staining	Oxidase	Catalase	Motility	Indole	VP	Malonate
Fish wash water from Juhu fish market	8	-	-	+	+	-	+	+
Versova creek water	8	-	-	+	+	-	+	-
Versova landing canter fish wash water	8	-	-	+	+	-	+	+

4.1.2. Results of molecular confirmation of isolates by PCR

Molecular confirmation of *Cronobacter* isolates were done by PCR using genus-specific primers targeting 16s rDNA (primers Saka 1a/Saka 2b), ITS G and ITS IA, the internal transcriber spacer sequences between 16s and 23sr RNA (primers SG-F/SG-R). Seven out of 82 isolates from fresh fish (8.53%), 6 out of 19 from shellfish (31.57%), 20 out of 40 isolates from dry fish (50%) and 4 out of 24 from water samples (16.67%) were positive for *Cronobacter* spp. with the primer set SG-F/SG-R with bands at 282 bp and one isolate from dry fish (5%) samples showed positive band for using Saka 1a/ Saka 2b primer at 952 bp (Fig 3. to Fig 9.).

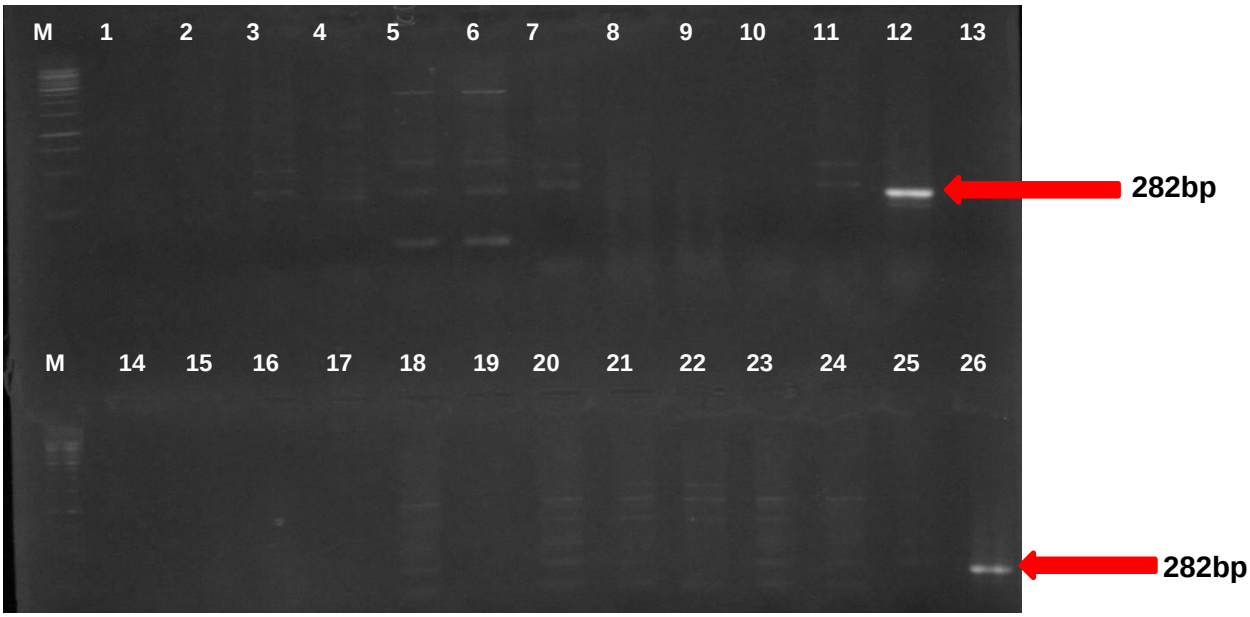


Fig 3. PCR amplification of *Cronobacter* isolates with primer set SG F/SG R

Lane-M	1 kb DNA marker
Lane-11	Mackerel from Madh-island fish market
Lanes-18,20,21 and to 23 to 25	Tiger Perch from Madh-island fish market
Lane-12 and 26	Positive isolate of *Cronobacter sakazakii* strain
Lane-13	Negative control

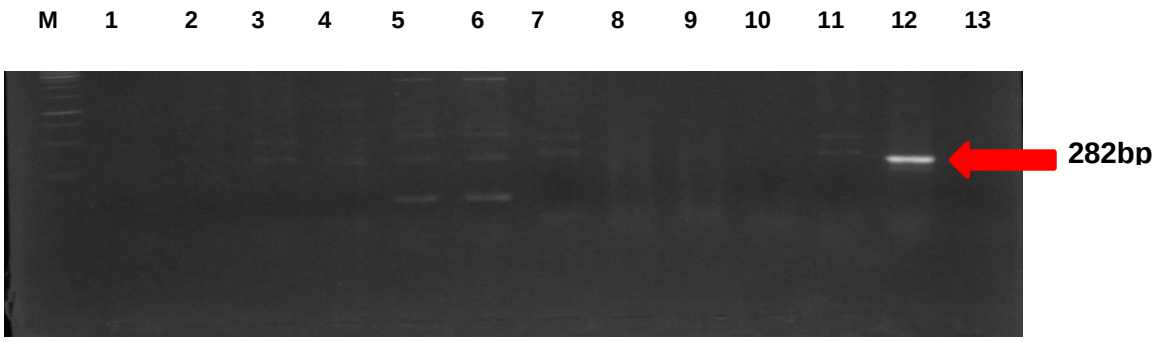


Fig 4. PCR amplification of *Cronobacter* isolates with primer set SG F/SG R

Lane-M	1 kb DNA marker
Lanes-3 and 5 to 7 and 11	Shrimp from Madh-island fish market
Lane-12	Positive isolate of *Cronobacter sakazakii* strain
Lane-13	Negative control

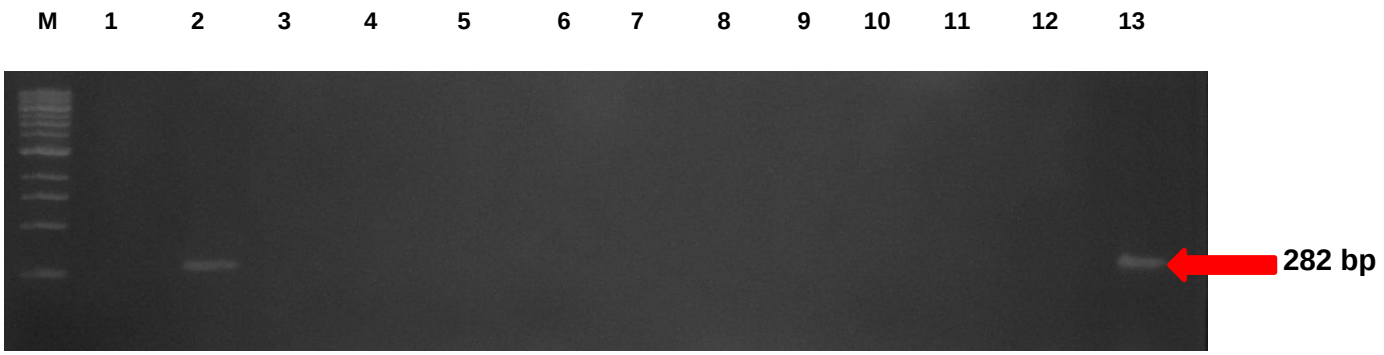


Fig 5. PCR amplification of *Cronobacter* isolates with primer set SG F/SG R

Lane-M	1 kb DNA marker
Lane-2	Shrimp from chakala fish market
Lane-13	Positive isolate of *Cronobacter sakazakii* strain

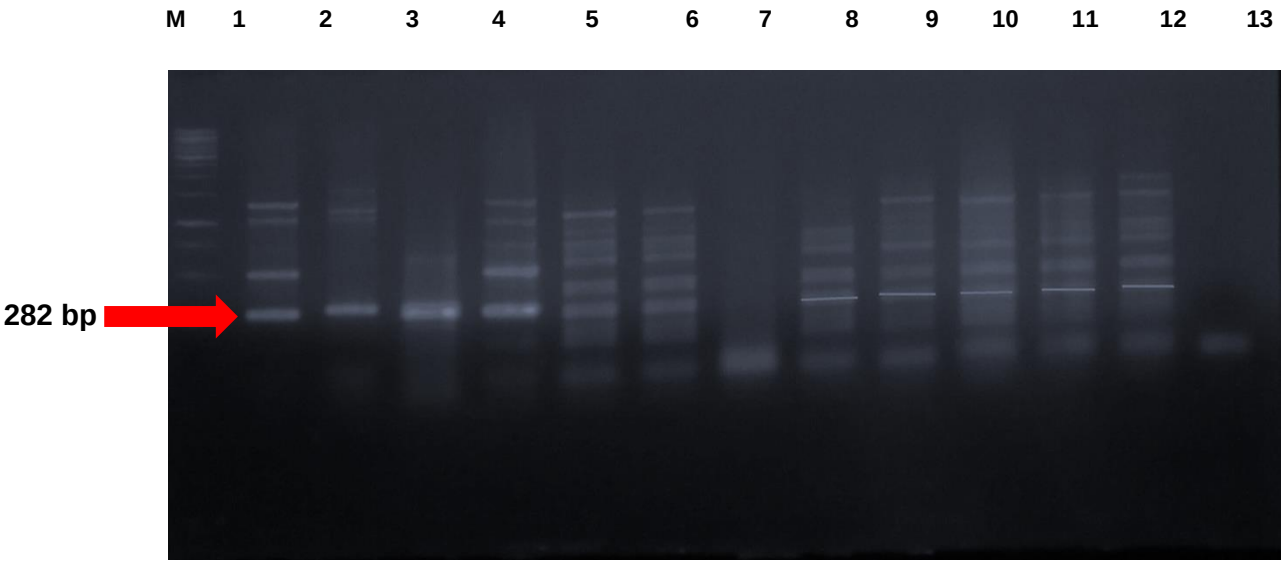


Fig 6. PCR amplification of *Cronobacter* isolates with primer set SG F/SG R

Lane-M	1 kb DNA marker
Lanes-1 to 4 and	Dry Bombay duck from Malad fish market
Lanes- 5 to 6 and 8 to12	Dry Ribbonfish from Malad fish market
Lane-13	Negative control

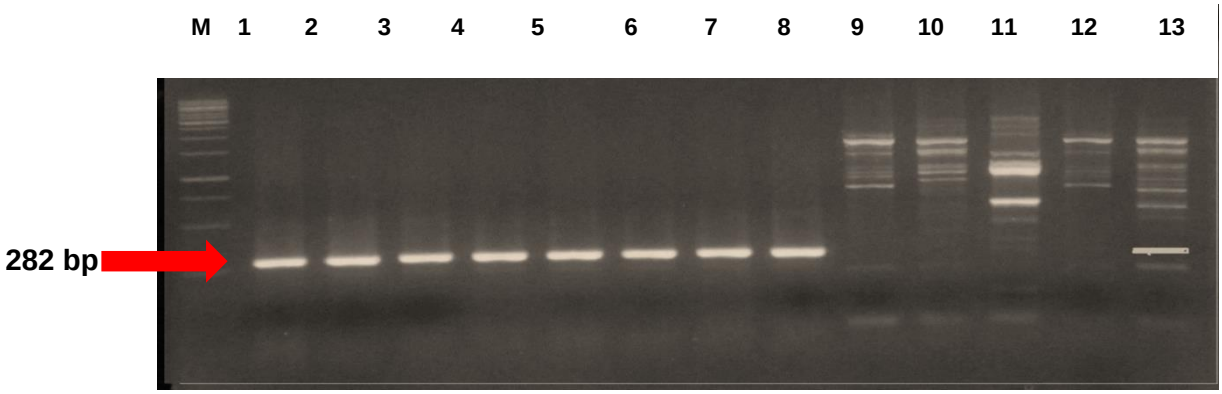


Fig 7. PCR amplification of *Cronobacter* isolates with primer set SG F/SG R

Lane-M	1 kb DNA marker
Lanes-1 to 8	Dry Bombay duck from Dadar fish market
Lane-13	Dry Ribbonfish Dadar from fish market

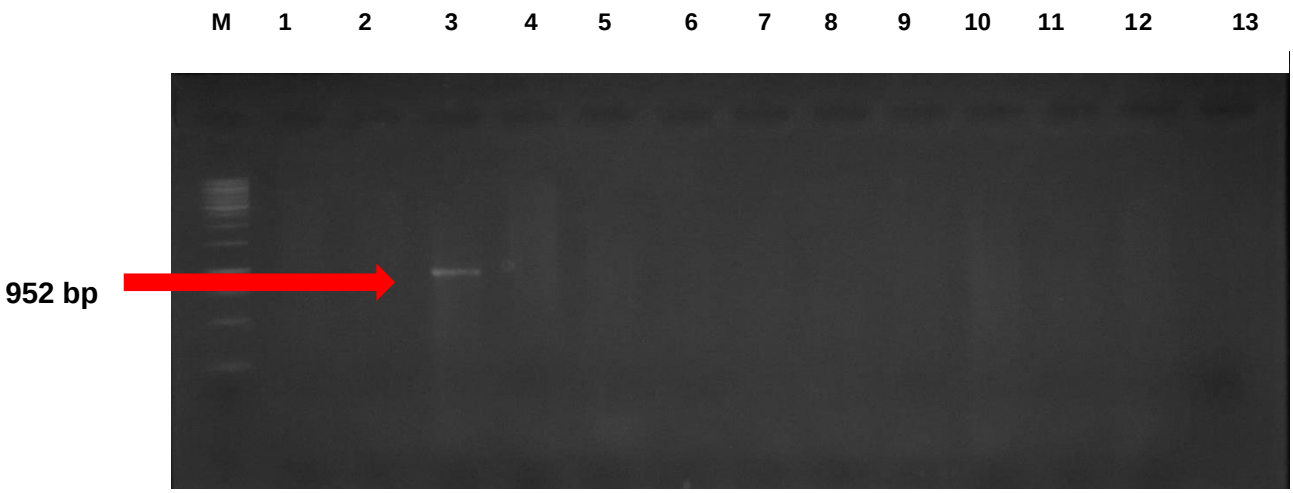


Fig 8. PCR amplification of Cronobacter isolates with primer set Saka 1a/ Saka 2b primer

Lane-M	1 kb DNA marker
Lane-3	Isolates from Malad fish market dry fish sample Bombay duck
Lane-13	Negative control

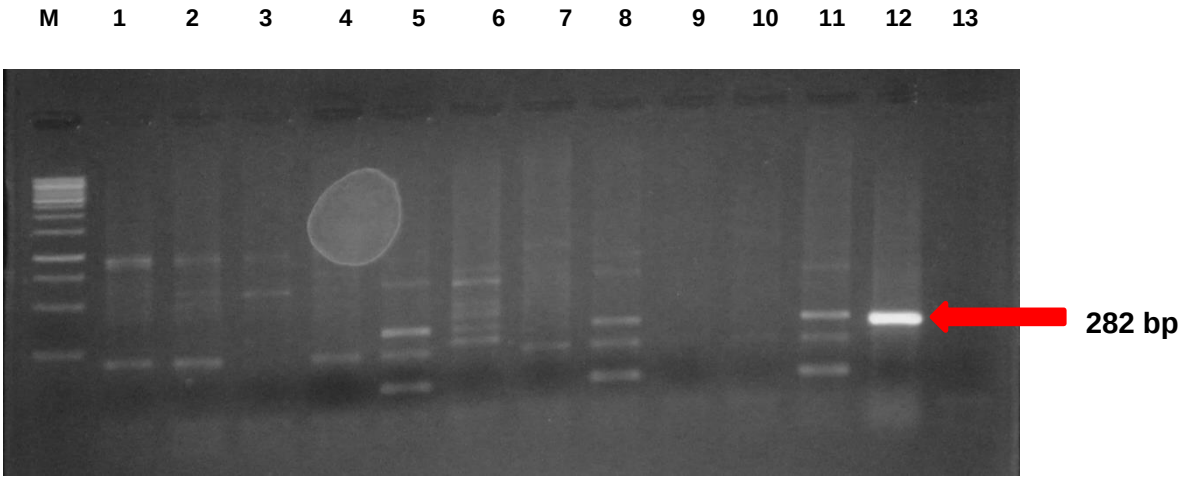


Fig 9. PCR amplification of *Cronobacter* isolates with primer set SG F/SG R

Lane-M	1 kb DNA marker
Lanes-5 to 6 and 8	Isolates from Versova creek water sample
Lane-11	Isolates from Versova landing canter fish wash water
Lane-12	Positive isolates of *Cronobacter sakazakii* strain
Lane-13	Negative control

Table 13. Biochemical and molecular confirmation of presumptive *Cronobacter* isolates

Name of the sample	No. of samples taken	No.of samples positive by selective plating (%)	No. of isolates taken	No. of isolates positive by biochemical tests (%)	No. of isolates positive by PCR (%)
Fresh fin fish	32	24(75)	82	72(87.80)	7(8.53)
Shell fish	6	4(66.67)	19	9(47.36)	6(31.57)
Dry fish	9	7(77.78)	40	40(100)	20(50)
Water	3	3(100)	24	24(100)	4(16.67)
Total	**50**	**38(76%)**	**165**	**145(87.87%)**	**37(22.42%)**

4.2. Detection of virulence genes in *Cronobacter* isolates by PCR amplification

Thirty seven isolates of *Cronobacter* that came positive by genus specific PCR were screened for the presence of virulence gene *ompA* by using the primer set ompA1/ompA2. The results of PCR amplification are given in Figure 10.

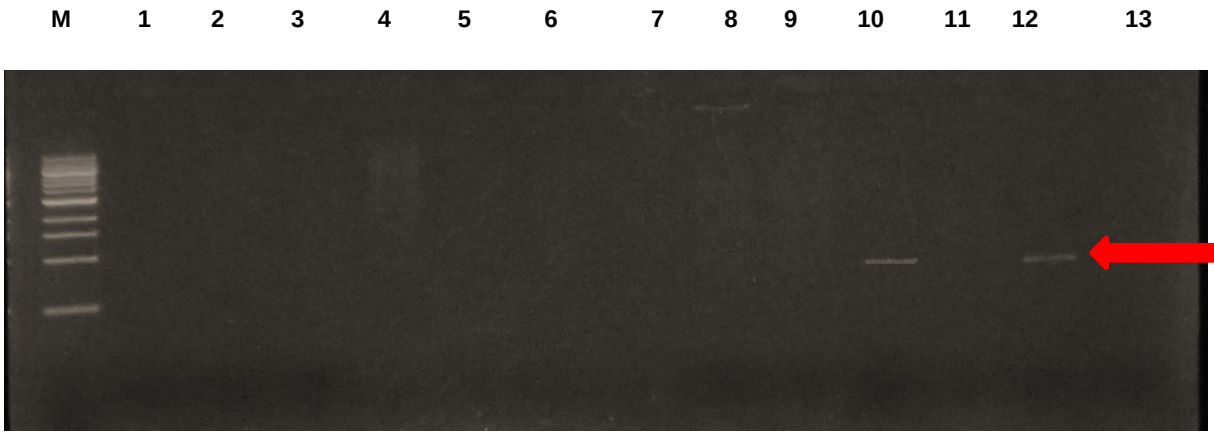


Fig 10. PCR amplification of *Cronobacter* isolates with primer set OmpA1/ OmpA2

Lane-M	1 kb DNA marker
Product size	469 bp
Lane-10	Isolates from Malad fish market dry fish sample Ribbonfish
Lane-12	Isolates from Dadar fish market dry fish sample Bombay duck

Thirty seven isolates of *Cronobacter* that came positive by genus specific PCR were screened for the presence of virulence gene *cpa* and *hly* gene by using the primer set cpa F/cpa R and hly F /hly F respectively.No isolate shown positive for presence of those gene.

4.3. BLAST (Basic local alignment search tool) analysis

The purified PCR products were sequenced by Xcelris labs, Ahmedabad. The sequence data were analysed using BLAST tool of National Centre for Biotechnology Information (NCBI). The BLAST hit showing maximum identity, minimum E-value and maximum query coverage was used as most /reliable/closest relative of the isolates from which sequences were obtained. The sequencing results are given below (Table 14):

Table 14. Results of sequencing

Sl. No.	Isolates code	Seq. length	Max score	Total score	Query cover	E value	Identity	Organism
1.	54	282	429	827	94%	4e-116	98%	*Cronobacter sakazakii*
2.	55	282	425	3871	92%	6e-115	99%	*Cronobacter malonaticus*
3.	56	282	429	4320	96%	4e-116	99%	*Cronobacter malonaticus*
4.	57	282	436	4401	93%	3e-118	99%	*Cronobacter malonaticus*
5.	58	282	438	4410	95%	7e-119	98%	*Cronobacter malonaticus*
6.	59	282	435	4366	93%	9e-118	99%	*Cronobacter malonaticus*
7.	60	282	444	840	95%	2e-120	98%	*Cronobacter sakazakii*
8	61	282	442	4390	96%	5e-120	99%	*Cronobacter malonaticus*

The isolates 54 to 61 were from dry Bombay duck sample collected from Dadar fish market. Isolate number 54 which was identified as *Enterobacter sakazakii* strain after sequencing this isolate was used as positive control to screen other isolates in PCR.

4.4. Analysis of antibiotic sensitivity pattern of *Cronobacter* isolates

The antibiotic sensitivity pattern of *Cronobacter* isolates was determined and the results are given in Tables 15 and 16.

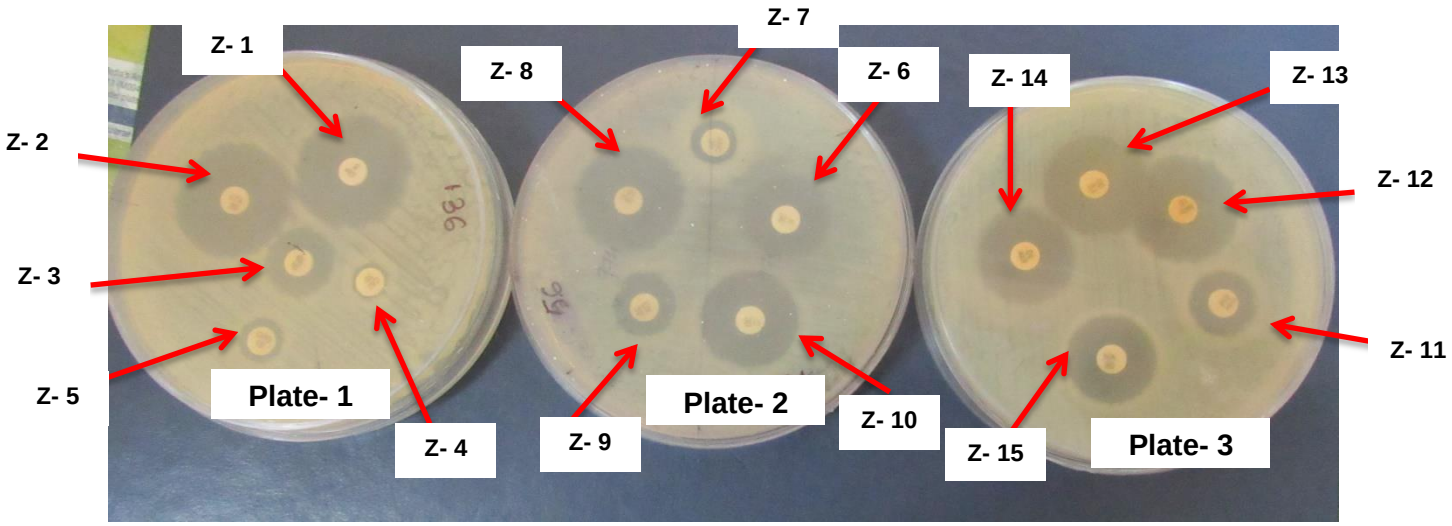


Fig 11. Inhibition zones of *Cronobacter* isolate against tested antibiotics

In Fig.11 inhibition zones of *Cronobacter* isolate (isolate no.136) from fresh finfish *Terapon jarbua* (Tiger Perch) sample from Madh-island fish market against the 15 antibiotics tested.

Plate-1

Zones	Name of the antibiotics	Inhibition zones formed by *Cronobacter* isolate
Z-1	CIP	S
Z-2	CTX	S
Z-3	CL	S
Z-4	CAZ	R
Z-5	CPD	R

Plate-2

Zones	Name of the antibiotics	Inhibition zones formed by *Cronobacter* isolate
Z-6	K	S
Z-7	AMC	R
Z-8	IPM	S
Z-9	CX	R
Z-10	C	S

Plate-3

Zones	Name of the antibiotics	Inhibition zones formed by *Cronobacter* isolate
Z-11	PIT	R
Z-12	MRP	S
Z-13	TE	S
Z-14	ETP	S
Z-15	NA	S

Z-zone; S-Sensitive; R- Resistant

Table 15. Representation of antibiotic profiles of *Cronobacter* isolates

Name of the antibiotic	Inhibition zones formed by *Cronobacter* isolates		
	Sensitive	Intermediate	Resistant
Amoxycilin-clavulanic acid	13 (35.135%)	6(16.216%)	18(48.648%)
Cefotaxime	17(45.945%)	4 (10.810%)	16 (43.213%)
Cefoxitin	4 (10.810%)	13 (35.135%)	20 (54.054%)
Cefpodoxime	-	-	37(100%)
Ceftazidime	-	-	37(100%)
Chloramphenicol	32(86.486%)	5(13.513)	-
Ciprofloxacin	36(97.297%)	-	1(2.702%)
Colistin	33(89.189%)	-	4(10.810%)
Ertapenem	22(59.459%)	4(10.810%)	11(29.729%)
Imipenem	33(89.189%)	1(2.702%)	3(8.108%)
Kanamycin	36(97.297%)	1(2.702%)	-
Meropenem	34(91.891%)	2(5.405%)	1(2.702%)
Nalidixic Acid	30(81.081)	6(16.216%)	1(2.702%)
Piperacillin/Tazobactam	4(10.810%)	8(21.621%)	25(67.567%)
Tetracycline	30(81.081%)	1(2.702%)	6(16.216)

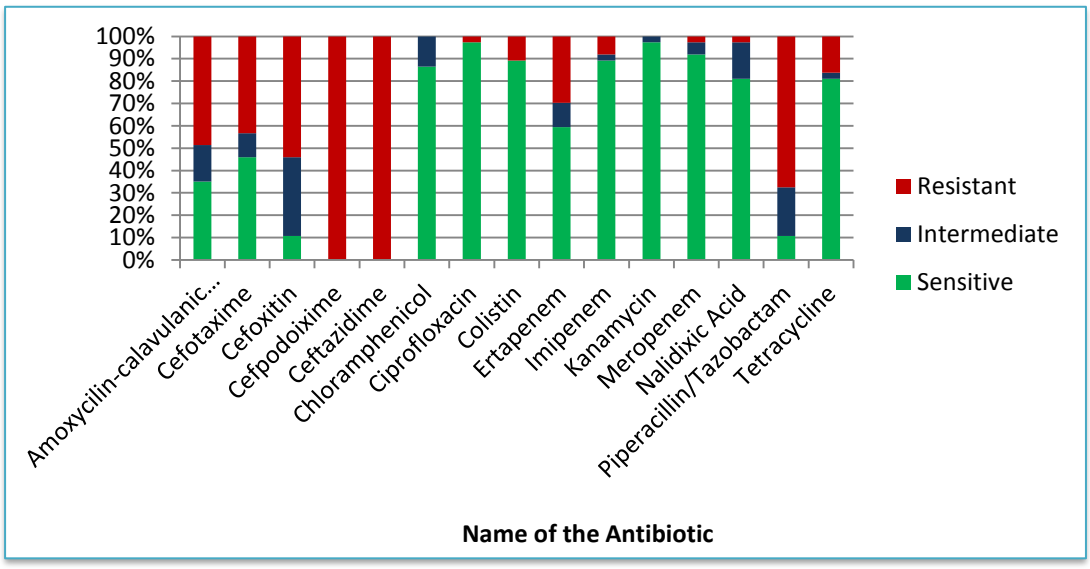


Fig 12. Antibiotic profiles of *Cronobacter* isolates

Table 16. Source wise representation of antibiotic profile of *Cronobacter* isolates

Name of the antibiotic	*Cronobacter* isolates(N=37)											
	Fish(n=7)			Shell fish(n=6)			Water*(n=4)			Dry fish(n=20)		
	S(%)	I (%)	R(%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Amoxycilin-clavulanic acid	1(14.28)	3(42.85)	2(14.28)	3(50)	1(16.67)	2(33.33)	2(50)	-	2(50%)	7(35)	2(10)	11(55)
Cefotaxime	5(71.42)	1(14.28)	1(14.28)	5(83.33)	-	1(16.67)	3(75)	-	1(25)	4(20)	3(15)	13(65)
Cefoxitin	-	3(42.85)	4(57.14)	-	2(33.33)	4(66.67)	-	1(25)	3(75)	4(20)	7(35)	9(45)
Cefpodoxime	-	-	7(100)	-	-	6(100)	-	-	4(100)	-	-	20(100)
Ceftazidime	-	-	7(100)	-	-	6(100)	-	-	4(100)	-	-	20(100)
Chloramphenicol	7(100)	-	-	5(83.33)	1	-	4(100)	-	-	16(80)	4(20)	-
Ciprofloxacin	6(85.71)	-	1(14.28)	6(100)	-	-	4(100)	-	-	20(100)	-	-
Colistin	7(100)	-	-	6100)		1(16.67)	3(75)	-	1(25)	18(90)	-	2(10)
Ertapenem	3(42.85)	1(14.28)	3(42.85)	5(83.33)	-	1(16.67)	3(75)	-	1(25)	11(55)	3(15)	6(30)
Imipenem	6(85.71)	-	1(14.28)	6(100)	-	-	3(75)	-	1(25)	18(90)	1(5)	1(5)
Kanamycin	7(100)	-		6(100)	-	-	3(75)	1(25)	-	20(100)	-	-
Meropenem	6(85.71)	-	1(14.28)	6(100)	-	-	3(75)	1(25)	-	19(100)	1(5)	-
Nalidixic Acid	6(85.71)	-	1(14.28)	5(83.33)	1(16.67)	-	2(50)	2(50)	-	17(85)	3(15)	-
Piperacillin/Tazobactam	1(14.28	1(14.28	5(71.42)	1(16.67)	1(16.67)	4(66.67)	1(25)	1(25)	2(50)	1(5)	5(25)	14(70)
Tetracycline	5(71.42)	-	2(28.571)	5(83.33)		1(16.67)	2(50)	-	2(50)	18(90)	1(5)	1(5)

S-Sensitive ; I- Intermediate; R- Resistant; N=Total no. of isolate tested; n=No. isolates tested for each sample

4.4.1. Multidrug resistance profiles of *Cronobacter* isolates

Cronobacter isolates showed resistance to two or more antibiotics when analysed, with a less percentage of the isolates showing resistance to three or more than three antibiotics.(Table.17, Fig13).The analysis included 15 antimicrobial compounds tested, such as Amoxycilin-clavulanic acid, Cefotaxime, Cefoxitin, Ciprofloxacin, Colistin, Ertapenem, Imipenem, Kanamycin, Meropenem, Nalidixic Acid, Piperacillin/Tazobactam and Tetracycline in determining the multidrug resistance profiles. Two antibiotics namely Cefpodoxime, Ceftazidime were excluded since all the isolates were resistant to them (Table.15 and 16)

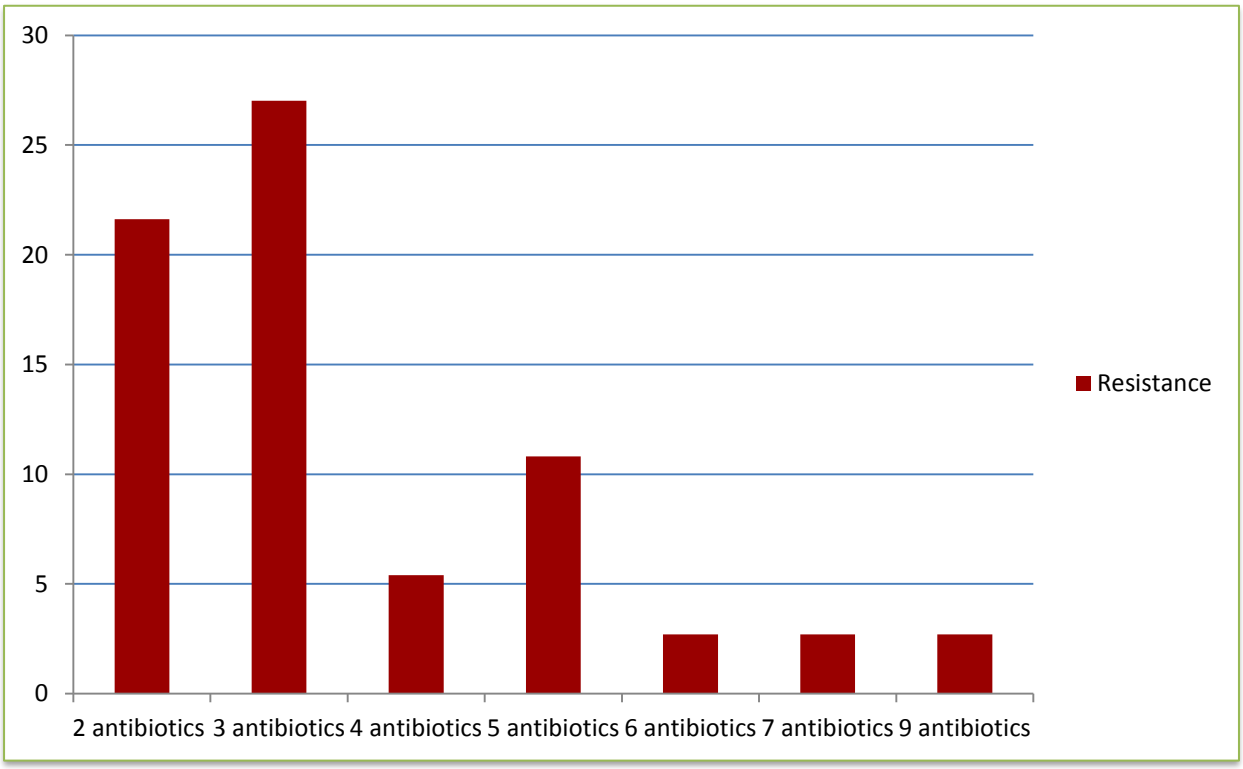


Fig 13. Multidrug resistance profiles of *Cronobacter* isolates

Table 17. Multidrug resistance profiles of *Cronobacter* isolates

		No. of isolates resistant to						
Sources	**No. of isolate tested**	**2 antibiotics (%)**	**3 antibiotics (%)**	**4 antibiotics (%)**	**5 antibiotics** (%)	**6 antibiotics** (%)	**7 antibiotics** (%)	**9 antibiotics** (%)
Dry fish	20	5(25)	1(5)	2(10)	4(20)	1(5)	-	-
Water*	4	1(25)	1(25)	-	-	-	1(25)	-
Shell fish	6	2(33.33)	3(50)	-	-	-	-	-
Fish	7	-	5(71.42)	-	-	-	-	1(14.28)
Total	37	8(21.62%)	10(27.02%)	2(5.40%)	4(10.81%)	1(2.70%)	1(2.70%)	1(2.70%)

* Versova landing canter fish wash water sample and Versova creek water sample

5. Discussion

Cronobacter spp. has been increasingly recognised as an opportunistic pathogen associated with neonatal infections resulting from the consumption of contaminated powdered infant formula (PIF) (Kandhai *et al.,* 2004). Friedemann and associates in the year 2007 stated that a broad spectrum of different kinds of food were found to be contaminated with *E. sakazakii*.,presently known as *Cronobacter sakazakii* It was isolated from plant food and food ingredients like cereal, fruit and vegetables, legume products, herbs and spices as well as from animal food sources like milk, meat and fish and products made from these foods This microorganism has been detected in raw or fresh products of animal and vegetable origin as well as in processed and prepared food such as dried, smoked, frozen, fermented, cooked or fried products, ready-to-eat and street food. Although rare, infections caused by *Cronobacter* spp*.* are often life-threatening and most frequently cause meningitis, sepsis, and necrotising enterocolitis (Hunter and Bean, 2013). But even low levels of *Cronobacter* spp. contamination could pose a health risk. The presence of *Cronobacter* in foods which are minimally processed is a potential threat to human health and highlights the need for high levels of hygiene, when handling such foods.

5.1. Distribution of *Cronobacter* spp. among the samples analysed

5.1.1. Detection of *Cronobacter* by selective plating

The highly polluted coastal water which is often used to clean fish or fish basket or deck can be one of the probable sources of contamination. In addition, the improper storage of seafood may cause penetration and multiplication of this bacterial group in fish flesh (Vaz-Pires *et al*., 2008). Sources such as the ice used for chilling fish/seafood can participate in bacterial contamination from its harvest to its delivery during handling, processing, preparation, or storage (Falcao *et al.*, 2002; Iwamoto *et al.,* 2010). Vendors and handlers (from harvesting to marketing) may be responsible for the bacteriological quality of seafood, especially if they are not fully aware of the importance of proper sanitation (Kumar *et al.,* 2005). Hence, in this study different water samples such as creek water, and fish wash water were analysed for the presence of *Cronobacter* spp.

Cronobacter also occurs in dry seafood such as dry shrimp, dry fish etc (Kim *et al.,* 2008) It was demonstrated that *Cronobacter* spp. are more desiccation resistant than other members of *Enterobacteriaceae* (Breeuwer *et al.*,2003). Drying fish in unhygienic and deplorable conditions increase the chance of contamination with *Cronobacter*. The present study also analysed dry fish samples for the presence of *Cronobacter*.

Overall, the samples comprised of finfishes, shellfish (crustaceans, cephalopods and bivalves), dry fish and water samples commonly available in this area. Given this view, a more number of finfish samples (32) were taken and a lesser number of samples in other groups such as six shellfish, nine dry fish and three water samples.

Samples were pooled from different parts of the fish because, during handling and processing of fish, the entire portions of the fish come in contact leading to contamination. Additionally, skin is likely to be contaminated with most of the pathogens when the fishes are not properly handled, or the water used for cleaning is of poor quality. In the case of shrimp whole shrimp macerated with shell was taken for analysis but for bivalves and crabs only meat portion was taken.

The samples were screened for the presence of *Cronobacter* by first pre-enriching them with buffered peptone water to enable stressed *Cronobacter* cells to recover, and then enriching using Cronobacter screening broth with vancomycin supplement allowing the multiplication of *Cronobacter* and suppressing the growth of other organisms. This was followed by plating on chromogenic agar HiCrome *Enterobacter sakazakii* agar (DFI formulation) to differentiate *Cronobacter* from other non target organisms. The presence of α-glucosidase activity is a more reliable test to distinguish *Cronobacter* spp. from most other *Enterobacteriaceae* (Muytjens *et al.,* 1984). The development of chromogenic agar, such as the DFI formulation, was based on the α-glucosidase reaction. This was a significant improvement in the isolation methodology for *Cronobacter*, as opposed to general *Enterobacteriaceae* recovery followed by speciation (Iversen *et al.,* 2004) A total of

165 representative colonies were picked based on size and shape similarity, and colour (blue-green reported by several authors and manufacture guideline) for biochemical tests. PCR for *Cronobacter*-genus-specific gene was found to be positive with the colonies featuring small point to circular smooth blue-green to deep bluish bottle green colour.

5.1.2. Biochemical confirmation of tested isolates of *Cronobacter* spp.

The genus includes facultative anaerobic, Gram-negative, oxidase negative, catalase positive, nonspore-forming rods which are generally motile, and show a positive reaction in the Voges–Proskauer test (Iversen and Forsythe 2007). For indole, the test results have been reported to be varying according to species (Jackson and Forsythe, 2016). In this study all 165 isolates were Gram-negative, oxidase negative, catalase positive and motile. Most *Cronobacter* strains are motile by means of flagella, and these also may have a role in inflammatory cytokine production by host cells (Cruz *et al*., 2011; Townsend *et al*., 2007). However,in the case of VP test, 12.2% isolates from fresh fin fish samples, 52.63% isolates from shell fish samples were shown to be VP negative. In malonate broth utilization test, 2.5% isolates from dry fish samples and 33.33% isolates from water samples were shown to be negative.One of previous study shown that *C. sakazakii* strains are negative for malonate broth utilization test (Iversen *et al.,* 2008; Chen *et al*., 2012). Forsythe (2018) in his study inferred that *C. sakazakii* strains can metabolize malonate, our study also shows most of the isolates to be positive for malonate broth utilization test. Most of the isolates were also negative for indole test. Out of 165 isolates 145 (87.87%) were biochemically confirmed as *Cronobacter* spp. Various species of *Cronobacter* are difficult to be biochemically discriminated. Farmer *et al.* (1980) used a collection of biochemical tests to describe 15 *E. sakazakii* biotypes, and Iversen *et al.,* (2006) added a 16th defined biotype, although biotypes do not accurately correlate with separate *Cronobacter* species (Baldwin *et al.*, 2009). A single biochemical test cannot be used to differentiate between the two species of *Cronobacter.* Improved speciation of strains using DNA sequencing-based methods has revealed that the earlier biotyping approach is severely flawed with no more than 50% of strains being correctly assigned to a *Cronobacter* species (Forsythe, 2015). This is in part due to the initial use of biotype

index strains which were attributed to the wrong *Cronobacter* species (Baldwin *et al.,* 2009). Owing to the unreliability in biochemical confirmatory tests, isolates that were not showing typical biochemical reactions of *Cronobacter* spp. were also used for further molecular confirmation by PCR.

5.1.3. Molecular Confirmation of *Cronobacter* isolates

Molecular techniques are considered useful tools to confirm the identified bacteria, especially when they are biochemically indiscriminatory. In this study two discriminating unique genes intended for specific detection of *Cronobacter* species, 16S rRNA and ITS sequence between 16S and 23S rRNA were used (Hassan *et al.*, 2007). The 16S rRNA gene is widely used as a target as it is a highly conserved gene, ubiquitous in all organisms and contains variable and hypervariable regions of sequence. Molecular methods based on this gene are well established as a standard method for characterisation and identification of bacteria (Woo *et al.,* 2003)

Amplification of 16S rRNA gene from the 165 presumptive isolates of *Cronobacter* spp. from 38 samples was done by using 2 primer sets. The primer set Saka-1(ACAGGGAGCAGCTTGCTGC) and Saka-2b(TCCCGCATCTCTGCAGGA) amplifies 16S rRNA with a product size of 952 bp (Hassan *et al*., 2007). Only one of the tested isolates could amplify 16S rRNA. This diversity of *E. Sakazakii* 16S rRNA could be due to the different origins of bacterial contamination. Iversen and Forsythe (2004) also observed sequence variations of the 16S rRNA gene (500 bp) and used these differences to divide *E. sakazakii* strains into four clusters.

The internal transcribed spacer (ITS) region has been used as a target for PCR-based identification and typing of many closely related bacteria (Gurtler and Stanisich, 1996). The ITS sequence is included in the rRNA operon between the 16S rRNA and 23S rRNA genes and is considered to be under less evolutionary selective pressure than the 16S and 23S coding genes because of its non-coding function and prone to greater genetic variation (Gurtler and Stanisich, 1996).It is unique for specific bacteria. Therefore, it may be useful for accurate identification of species. In this study the primer set SG-F/SG-R targeting (Internal transcriber spacer sequence between 16s and 23s r RNA appeared to be most reliable among

the tested primers, with a product size of 282 bp. Among the isolates tested, 37 (22.42%) were positive for PCR with SG-F/SG-R.

5.1.4. Prevalence of C*ronobacter* spp. in seafood samples

Cronobacter contamination can be extrinsic due to unhygienic food preparation practices. Contaminated utensil, such as spoon and blender, or various parts of domestic kitchens (sinks, countertops, dishcloths, refrigerator handles, meat drawers, and sponges) could be a source of extrinsic contamination, since *Cronobacter* have been isolated from these sites (Molloy *et al.,* 2009; Mozrova *et al.*, 2014)

In addition to primary contaminated food ingredients, *E. sakazakii*-contaminated water from environmental sources soil (Khan *et al.,*1988),water (Farmer *et a*l., 1985), waste- (Dudley *et al.,* 1980),dust and air-particles (Kandhai *et al.,* 2004) should be regarded as potential sources for contamination of animal- and plant-based food throughout the entire food production chain.

Consistent with these findings, our study has shown that the samples of finfish and shellfish collected from landing centre, local fish market, supermarket etc are contaminated with enetric pathogens. Of the 50 samples screened, presumptive isolates of *Cronobacter* (bluish green colonies on HiChrome *Enterobacter sakazakii* agar) were obtained from 38 samples, that included 24 fin fish (75%), 4 shellfish (66.67%), 7 dry fish (77.78%) and 3 water samples. A total number of 165 presumptive isolates taken from these samples were comprised of 82 isolates from fresh finfish, 19 from shellfish, 40 from dry fish and 24 from water samples. Of these, 145 isolates were confirmed biochemically and 37 by molecular confirmation to be *Cronobacter* spp. From molecular confirmation 7 out of 82 isolates from fresh fish (8.53%), 6 out of 19 from shellfish (31.57%), and 4 out of 24 from water samples (16.67%) were positive for *Cronobacter* spp. with the primer set SG-F/SG-R with bands at 282 bp and one isolate from dry fish was positive with the Saka 1a/ Saka primer with bands at 952 bp

Jaradat *et al.* in 2009 found that *Cronobacter* spp. are ubiquitous microbes found in a wide array of foods and beverages including infant formula. However, due to its thermotolerant and osmotolerant nature, the organism survives in dry foods and herbs. Additionally, *Cronobacter* strains are more thermotolerant than most other Enterobacteriaceae and remarkably resistant to osmotic stress and drying (Gurtler *et al.,* 2005). In this study nine dry fish samples were tested for the presence of *Cronobacter*. A total of 40 isolates were taken from the samples (after selective platting) of whiche twenty isolates (50%) confirmd to be *Cronobacter* species.

5.2. Presence of virulent genes in *Cronobacter* spp.

Owing to the clinical significance of *Cronobacter* particularly to infant health and immunocompromised people, the organism has been studied for a number of virulence traits. These have been identified through laboratory studies and whole-genome analysis; although there is still much confirmatory work to be undertaken. The organism has various types of adhesions. These probably enable the organism to attach to intestinal cells and also penetrate the blood-brain barrier (Jaradat *et al.,* 2014)

The outer-membrane protein A (OmpA) is probably the best-characterised virulence marker of *Cronobacter* (Nair *et al.,* 2007; Mittal *et al*., 2009; Kim *et al*., 2010). OmpA protein regulates by *ompA* gene appears to play a significant role in *Cronobacter* invasion. In vitro, it was found that OmpA plays a critical role in the attack of human intestinal epithelial cells (INT- 407), Caco-2 cells and Human brain microvascular endothelial cells (HBMECs) (Nair and Venkitanarayanan, 2007). The ompA protein encoded gene *ompA* is thought to be universally present in *Cronobacter* (Hunter and Bean, 2013). In the present study, 2 isolates of *Cronobacter* spp. from dry fish were found to harbour *ompA* gene which was amplified at a size of 469 bp.

The studies reported show that *Cronobacter* isolates can be grouped into three categories, relative to invasion ability. Only one of 23 strains displayed high-level invasion efficiency, while four strains showed an intermediate level (i.e. averaging 0.1 to 0.2%). Most isolates exhibited very low invasion efficiency (Giri *et al.,* 2012). In this study, the remaining isolates which were negative for *ompA* gene

may exhibit very low invasion efficiency. Earlier reports have suggested that ompA is somehow essential for bacterial invasion of host cells.

A plasmid-borne putative gene for the plasminogen activator (*cpa*) was identified in the *Cronobacter* isolates from plant-based products and environmental samples. The plasminogen activators are serine proteases, proposing that this gene could be engaged in exhibiting the maximum invasiveness of *Cronobacter* spp. Singh *et al.* in 2017 and Ribeiro *et al.*in 2011 showed that *cpa*, expressed by *C. sakazakii,* slowly cleaves plasminogen and increases survival in serum in comparison with a *cpa* deletion mutant. Although previous literature suggests that the serum resistance was linked to the presence of *cpa* gene. 30% of the strains used in the study was negative for the presence of *cpa* gene (Singh *et al.,* 2017). *cpa* gene was detected by Cruz *et al*. (2011) from 10 isolates of *C. sakazakii* out of 43 isolates. Isolates are mostly (9 isolates) from clinical isolates and only one isolate was obtained from dried milk. In our study no isolates of *Cronobacter* were found to positive for *cpa* gene.

The type III hemolysin, an integral outer membrane protein with hemolytic activity, is reported to be a potential virulence factor in several pathogens. Singh *et al.* (2017) studied that, all the isolates from plant-based products carried hemolysin genes (*hly*). Cruz *et al.* (2011) reported the prevalence of *hly* gene in only *Cronobacter* spp. isolated mainly from clinical sources, in 16 isolates out of 43 isolates (37%). The present study could detect *hly* gene in none of the *Cronobacter* isolates tested.

5.3. Antibiotic sensitivity pattern of *Cronobacter* isolates

Antimicrobial resistance is an increasingly emerging problem worldwide and is a critical challenge for infectious diseases management around the world (Kollef, 2003) and justifies monitoring the antibiotic susceptibility of foodborne pathogens. The emergence of antimicrobial resistance is primarily due to excessive and often unnecessary use of antibiotics in humans, animals and antibiotics are among the most commonly prescribed drugs in hospitals (Shankar *et al.,* 2003).

To test the antibiotic resistant pattern of the isolated *Cronobacter* strains from different sources, the disk diffusion assay was carried out following the instructions of the National Committee for Clinical Laboratory Standards (NCCLS). The inhibition zone was measured and interpreted according to NCCLS (guidelines given by the antibiotic discs manufacture Hi-media India) for *Enterobacteriacea*e. All 37 *Cronobacter* isolates were tested against 15 antibiotics from different groups. A representative picture of the antibiotic susceptibility pattern of *Cronobacter* (isolate number 136) from fresh finfish *Terapon jarbua* (Tiger Perch) sample from Madh-island fish market is shown in the Fig 11. Antibiotics are included combo antibiotics Amoxycilin-calavulanic acid (AMC,30µg, combination consisting of amoxicillin, a β-lactam antibiotic, and clavulanic acid, a β-lactamase inhibitor) and Piperacillin/Tazobactam (PTZ,100/10µg, containing the antibiotic piperacillin and the β-lactamase inhibitor tazobactam),2nd generation cephalosporin; Cefoxitin (CX,30µg), 3rd generation cephalosporin; Cefpodoixime (CPD,10µg), Ceftazidime (CAZ,30µg) and Cefotaxime (CTX,30µg), Chloramphenicol group antibiotic; Chloramphenicol (C,30µg), Polymixin group antibiotic; Colistin(CL,10µg),Carbapenem group antibiotics; Ertapenem (ETP,10µg), Imipenem (IMP,10µg), and Meropenem (MRP,10µg), Aminoglycoside group antibiotic; Kanamycin, Quinolone group antibiotics Nalidixic Acid (NA,30µg) and Ciprofloxacin (CIP,5µg), Polyketides group antibiotic; Tetracycline (TE,30µg).

Out of 37 *Cronobacter* isolates tested against 15 antibiotics, highest number of isolates were sensitive to Kanamycin (97.29%) and Ciprofloxacin (97.29%) followed by Meropenem (91.89%), Colistin (89.18%), Imipenem (89.18%), Nalidixic Acid (81.08%) and Tetracycline (81.08%). A less no of isolates showed sensitivity against Cefoxitin (10.81%) and Piperacillin/Tazobactam (10.81%) followed by Amoxyicillin-clavulanic acid (35.13%) Cefotaxime (45.94%) Ertapenem (59.45%)

Highest number of isolates showed intermediate sensitivity against the antibiotics Cefoxitin (35.13%), Piperacillin/Tazobactam (21.62%) followed by Nalidixic Acid (16.21%), Amoxycillin-clavulanic acid (16.21%) ,Chloramphenicol (13.51%), Ertapenem (10.81%) and Cefotaxime (10.81%). A less number of isolates showed intermediate sensitivity against Tetracycline (2.70%), Kanamycin (2.70%) Imipenem (2.70%) and Meropenem (5.40) *Cronobacter* spp. are reported to be more sensitive to some antibiotics than other *Enterobacter* species (Cui *et al*.,

2017). Xu *et al. (*2015) reported in their study that all 52 *Cronobacter* isolates were susceptible to cefotaxime, ciprofloxacin, tetracycline, and nalidixic acid. The next-highest susceptible rates were observed for antibiotics, chloramphenicol (93.0%) and gentamicin (93.0%).

All 37 *Cronobacter* isolates were resistant to the 3rd generation cephalosporins such as Cefpodoxime (100%) and Ceftazidime (100%). Many isolates were resistant to Piperacillin/Tazobactam (67.56%), Cefoxitin (54.05%), Amoxycilin-clavulanic acid (48.64%) Cefotaxime (43.21%) followed by Ertapenem (29.72%), Colistin (10.81%) and Imipenem (8.10%). A less no of isolates were resistant to Meropenem (2.70%) and Nalidixic Acid (2.70%). No isolates were found to be resistant against the antibiotics Kanamycin and Chloramphenicol. Infections caused by *Cronobacter* spp. can be successfully treated with ampicillin–gentamicin or ampicillin–chloramphenicol during therapy (Lai, 2001). Compared to other food-borne pathogens, *Cronobater* spp. is characterised by relatively low resistance to antibiotics. (Berthold-Pluta *et al*., 2017) Recently like other *Enterobacteriaceae*, *Cronobacte*r spp. were frequently found to be resistant to β-lactam derivatives, macrolides, and aminoglycosides such as rifampicin, amoxicillin-clavulanic acid, streptomycin, tetracycline, or ampicillin (Cui *et al.,* 2017). *Cronobacter* spp. have been reported to be resistant to cephalothin, cefotaxime, and streptomycin in some isolates from food samples (Chon *et al.,* 2012; Lee *et al.,* 2012). Noteworthy is that some *Cronobacter* spp. strains may be resistant to antibiotics, which results from the acquisition of transposon, synthesis of b-lactamases and possession of many antibiotic-resistant operons (Girlich *et al.,* 2001).

5.3.1. Multidrug resistance profile of *Cronobacter* isolates

Antimicrobial resistance, particularly multiple-drug resistance, is a public health concern, because it may cause failure of conventional treatment, resulting in prolonged illness and a higher risk of death. Therefore, it is necessary to establish a monitoring system for the objective evaluation of the antimicrobial resistance profile.

Out of 37 *Cronobacter* isolates total 27 isolates (72.97%) showed resistance to two or more antibiotics when analysed against 15 antibiotics. Out of 37 *Cronobacter* isolates 8 isolates (21.62%) were resistance against 2 antibiotics,10

isolates against 3 antibiotics (27.02%), 2 isolates against 4 antibiotics (5.40%),4 isolates against 5 antibiotics (10.81%), only 1 isolate (2.70%) resistant to 6,7 and 9 antibiotics. Two antibiotics namely Cefpodoxime, Ceftazidime were excluded since all the isolates were resistant to them. The multiple antibiotic resistance (mar) operons were found in *Cronobacter* (Duval and Lister, 2013) which help to adapt the bacteria and resistant them to all classes of antimicrobial drugs.

6. SUMMARY

The incidence of *Cronobacter* spp. in seafood and coastal environment in and around North-west Mumbai, India was studied in the present investigation. A total of 50 samples were screened for *Cronobacter*, which included 32 fresh finfish, 6 shellfish, 9 dry fish from different retail fish markets, landing centres and supermarkets, and 3 water samples comprising of creek water and fish wash water. Isolation of *Cronobacter* spp. was done by the modified ISO/TS 22964 method involving pre-enrichment of samples in Buffered Peptone Water (BPW), selective enrichment in Cronobacter screening broth (CSB), followed by selective plating on Hichrome *Enterobacter Sakazakii* agar. The presumptive isolates of *Cronobacter* from selective plates were further confirmed to species level by biochemical and molecular methods.

Of the 50 samples screened, presumptive isolates of *Cronobacter* (bluish green colonies on HiChrome Enterobacter sakazakii agar) were obtained from 38 samples, that included 24 fin fish (75%), 4 shellfish (66.67%), 7 dry fish (77.78%) and 3 water samples. A total number of 165 presumptive isolates taken from these samples were comprised of 82 isolates from fresh finfish, 19 from shellfish, 40 from dry fish and 24 from water samples. These isolates were subjected to a series of biochemical confirmatory tests such as Gram's staining, indole, Voges Proskauer, motility, catalase, oxidase and malonate broth utilization tests. One hundred and forty five isolates, comprising 72(87.80%) from fresh fin fish, 9 from shellfish (47.36%), all 40 from dry fish (100%) and all 24 from water samples (100%) were biochemically confirmed as C*ronobacter* spp.

Molecular affirmation of biochemically confirmed *Cronobacter* isolates were done by PCR targeting 16s rDNA and ITS G and ITS IA. Seven out of 82 isolates from fresh fish (8.53%), 6 out of 19 from shellfish (31.57%), 20 out of 40 from dry fish (50%) and 4 out of 24 from water samples (16.67%) were positive for *Cronobacter* spp. with the primer set SG-F/SG-R with bands at 282 bp and one isolate from dry fish was positive with the Saka 1a/ Saka primer with bands at 952 bp. A total of 37 isolates were confirmed to be *Cronobacter* spp. by PCR. DNA sequence analysis of the confirmed isolates revealed two of them to be *Cronobacter sakazakii* and others to be the closely related *Cronobacter malonaticus*. One of the

isolates of *C.sakazakii* was used as positive control for other PCR experiments targeting the genes. The confirmed *Cronobacter* isolates were also characterized to understand the potential virulence factors associated with them. Genes such as *omp*A coding for outer membrane protein, *cpa* coding for plasminogen inactivation factor and *hly* coding for hemolysis, were looked for by using specific PCR primers. The presence of *omp*A gene which plays an important role in the invasiveness of virulent *Cronobacter* spp was amplified in two of the tested isolates with a product size of 469 bp.

Analysis of antibiotic susceptibility pattern of confirmed *Cronobacter* isolates was done by testing them against 15 commonly used antibiotics using disc diffusion method. A higher extent of antibiotic resistance was exhibited by *Cronobacter* spp. with all of them being resistant to the 3rd generation cephalosporins, cefpodoxime and ceftazidime. However, 97.29% were sensitive to kanamycin and ciprofloxacin, whereas less number of isolates (10.81%) showed sensitivity against cefoxitin and piperacillin/tazobactam. None of the *Cronobacter* spp. tested was found resistant to kanamycin and chloramphenicol. Multidrug resistance profiling of *Cronobacter* spp. revealed that they showed resistance to two or more antibiotics when analysed, with a less percentage of the isolates showing resistance to three or more than three antibiotics.

The present investigation holds its significance as the first of its kind in an attempt to determine the incidence of *Cronobacter* spp., an emerging pathogen, from seafood in Mumbai region of West coast of India. The study reports incidence of *Cronobacter* spp. in 76% of the seafood samples analysed, which is a significant observation given that *Cronobacter* spp. is an enteric pathogen that is transmitted by the fecal-oral route in humans through contaminated food and water. Moreover, a higher incidence of *Cronobacter* spp. in dry fish (77.78%) was observed, which may be attributed to its enhanced desiccation tolerance and thereby being able to overcome the hurdle of reduced moisture content in dry fish, commonly used as a method of preservation. Besides, the antibiotic resistance observed among *Cronobacter* spp. in this study is also an important observation, adding to their virulence. Therefore, it is imperative that further studies are undertaken to characterize the *Cronobacter* spp. prevalent in fish, shellfish and coastal waters, and to explore their distribution and diversity characteristics.

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