# Identification of *Bacillus cereus* and *Cronobacter sakazakii* on food products consumed by infants and children in Bangladesh

By

Maysha Mahjabeen Hassan Medha
17126035
Suraya Siraj
17126031
Fariya Hossain
17126011

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelor of Science in Microbiology

Department of Mathematics and Natural Sciences
Brac University
August, 2022

## Declaration

It is hereby declared that

1. The thesis **titled "Identification of *Bacillus cereus* and *Cronobacter sakazakii* on food products consumed by infants and children in Bangladesh"** submitted is our own original work while completing the degree of Bachelors in Science in Microbiology at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

**Student's Full Name & Signature:**

**Maysha Mahjabeen Hassan Medha**
Student ID: 17126035

**Suraya Siraj**
Student ID: 17126031

**Fariya Hossain**
Student ID: 17126011

# Approval

The thesis titled "Identification of *Bacillus cereus* and *Cronobacter Sakazakii* on food products consumed by infants and children" was submitted by

1. Maysha Mahjabeen Hassan Medha (17126035)
2. Suraya Siraj (17126031)
3. Fariya Hossain (17126011)

Of Summer, 2022 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology on 25 August 2022.

**Examining Committee:**

Supervisor:
(Member)

_____

Fahim Kabir Monjurul Haque, PhD
Assistant Professor, Microbiology Program,
Department of Mathematics and Natural Sciences
Brac University

Program Coordinator:
(Member)

_____

Mahbubul Hasan Siddiquee, PhD
Associate Professor, Microbiology Program,
Department of Mathematics and Natural Sciences
Brac University

Departmental Head:
(Chair)

_____

Prof. A. F. M Yusuf
Chairperson, Mathematics and Natural Science
Brac University

## Abstract

Dried manufactured food products like baby formulas are often contaminated with various pathogenic bacteria. *Bacillus cereus*, which is widely known for foodborne intoxication, is a frequently observed contaminant. On the other hand, *Cronobacter sakazakii* is an occasional contaminant but it causes severe neurological infections, including sepsis in infants and children. The purpose of this study was to investigate the prevalence of *B. cereus* and *C. sakazakii* in Powdered Infant Formula (PIF), Powdered Follow-Up Formula (PFUF), Child Complementary foods, and regular Milk Powders. Additionally, the study also focused on determining the infection risks associated with these products. A total of 26 samples commonly found in Bangladesh were analyzed. The approach consisted of inoculation in enrichment media and enumeration was done in chromogenic media. The pathogens were identified by colony morphology. *B. cereus* was detected in 54% (n = 14) of the total samples, and *C. sakazakii* was detected in 19.23% (n = 5) of the samples. Also, the antimicrobial susceptibility patterns were tested with the isolates of *B. cereus* and *C. sakazakii*. The findings of this thesis study emphasize that Bangladesh requires extensive research in this sector for the microbiological safety of infant and children formula.



**Keywords:** *Cronobacter Sakazakii*; *Bacillus cereus;* PIF Contamination; FUF Contamination; Infant Infection; Antibiotic-Resistant.

## Acknowledgement

First of all, we want to express our sincere gratitude to our supervisor, **Dr. Fahim Kabir Monjurul Haque**, for his guidance, encouragement, and patience throughout the entire thesis journey. Without his assistance, we might not have been able to complete our thesis successfully.

We would like to convey our respect and gratitude towards our chairman **Prof. A. F. M Yusuf** for his dedication to the advancement of our department.

Additionally, we would like to extend our thankfulness and appreciation to our course coordinator, **Dr. Mahbubul Hasan Siddiquee**, for leading us throughout our university life and inspiring us to pursue a career in microbiology.

Also, we would like to convey our gratitude and appreciation toward **Dr. Mahboob Hossain** for his guidance and encouragement throughout our university life.

We also want to convey our gratitude and admiration towards our faculty **Akash Ahmed** for guiding and motivating us with his wit and expertise in our university life.

We are deeply grateful to our senior **Sumaiya Sultana** for helping us with her assistance during our thesis.

## Dedication

We would like to dedicate our thesis to our families for encouraging and being with us as we complete our undergraduate thesis successfully.

# Table of Contents

## List of Tables

# List of Figures

## List of Acronyms

- PIF=Powdered Infant Formula
- FUF= Follow Up Formula
- PFUF=Powdered Follow-Up Formula
- CSB=Chromogenic Screening Broth
- TNTC=Too numerous to count
- TFTC=Too few to count
- CLSI= Clinical and Laboratory Standards Institute
- FAO= Food and Agricultural organization
- WHO= World health organization
- FDA= Food and Drug Administration

# Chapter 1

## 1.1 Introduction

Food consumption during the early stages of childhood and infancy plays a significant role in the overall growth and development of the immune system. Children between the ages of 0 to 2 years globally are fed a variety of commercially prepared foods and dietary formulas. The commercially available dietary dried foods have a high potential for microbial contamination since they comprise a large number of ingredients from various sources (Kim et al., 2011). Several studies have reported the microbial presence of both gram-positive and gram-negative bacteria in infant and child food formulas. In this study, the microbiological safety of several food products -Powdered Infant Formula (PIF), Powdered Follow-Up Formula (PFUF), dried Milk Powder, and other Complementary food products targeting *Bacillus cereus* and *Cronobacter sakazakii* consumed by infants and young children were investigated. The presence of Gram-positive bacteria *B. cereus* in rehydrated manufactured foods is common but its toxin can cause both local and systematic infections in infants and children. On the contrary, the incidence of contamination of Gram-negative bacteria *C. sakazakii* is less but the mortality rate from its infection is significantly high. Therefore, intensive continuous monitoring and proper hygiene practice are required to avoid such microbial contamination.

## 1.2 Objectives of the Study

The major objectives of this study is given below-

- Identification of *B. cereus* and *C. sakazakii* from powdered formulas, complementary foods and milk powders.
- Comparison of contamination between different market areas in Dhaka city.
- Comparison of contamination between different types of collected samples
- Determination of Antibiotic susceptibility pattern of the isolates.

## 1.3. General characteristics of *Bacillus cereus*

It is recognized that *Bacillus cereus* sensu lato (or *Bacillus cereus* group) consists of eight distinct species: *Bacillus anthracis, Bacillus pseudomycoides, Bacillus mycoides, Bacillus thuringiensis, Bacillus weihenstephanensis, Bacillus cytotoxicus, Bacillus toyonensis* and *Bacillus cereus*. Even with 16S rDNA sequencing, many of these species cannot be distinguished. In the presence of oxygen, *B. cereus* produces resistant endospores and can be classified as Gram-positive, aerobics or facultative anaerobic, spore-former, motile, pathogenic, and opportunistic bacteria. The bacteria *B. cereus* is widely distributed in the environment, particularly in soil, where spores persist even under adverse conditions. Despite its ability to grow within a wide range of temperatures (8–55°C), *B. cereus* cannot tolerate low pH values (maximum 5–6) or low water contents (0.95 minimum water activity) (Stenfors Arnesen et al., 2008).

In addition to glucose, fructose, and trehalose, the organism is still unable to metabolize pentose and most sugar alcohols. Certain strains use sucrose, salicin, maltose, mannose, glycerol, m-inositol, and lactose. Among the most active are those that hydrolyze starch, casein, and gelatin (Te Giffel & Beumer, 1998).

### 1.3.1 Phylogeny of *Bacillus cereus*

**The organism:** *Bacillus cereus*
**Taxonomy**

**Domain:** Bacteria
**Kingdom:** Bacteria
**Phylum:** Bacillota
**Class:** Bacilli
**Order:** *Bacillales*
**Family:** *Bacillaceae*
**Genus:** *Bacillus*
**Species:** *Bacillus cereus*

### 1.3.2 *Bacillus cereus* and its virulence factors:

*Bacillus cereus* food poisoning can be classified into two types. The first type is emetic toxin which causes vomiting. Second is the endotoxin which causes diarrohea. Only a small percentage of cases report both types of symptoms, possibly due to both types of toxins being produced. Enterotoxins produced in the foods can still cause intoxication. However, to cause food poising by enterotoxins the number of *B. cereus* cells present needs to be at least two orders of higher magnitude. Therefore, such products would be unacceptable to consumers. Table 1 summarizes the characteristics of the two types of *B. cereus* food poisoning**.**

*Table 1*

| Table 1 | **Diarrheal syndrome** | **Emetic syndrome** |
|---|---|---|
| **Dose of infection** | $10^5$–$10^7$ (total) | $10^5$–$10^8$ (cells $g^{-1}$) |
| **Production of toxins** | In the small intestine of the host | Preformed in foods |
| **Toxin type** | Protein | Cyclic peptide |
| **Period of incubation** | 8–16 h (occasionally >24 h) | 0.5–5 h |
| **Infection duration** | 12–24 h (occasionally several days) | 6–24 h |
| **Signs and symptoms** | Abdominal pain, watery diarrhea and occasionally nausea | Nausea, vomiting, and malaise, (sometimes followed by diarrhea, due to additional enterotoxin production) |
| **Involved foods** | Meat products, soups, vegetables, puddings/sauces, and milk/milk products | Fried and cooked rice, pasta, pastry ,and noodles |

**Table 1: Characteristics of the two types of disease caused by *B. cereus***

## 1.4 General Characteristics of *Cronobacter sakazakii*

*Cronobacter sakazakii* is a rod shaped peritrichous flagellated bacteria and does not form spores. Despite having a limited supply of oxygen, the organism is categorized as facultative anaerobic since it thrives without it. The temperature for growth ranges from 6 to 45 degrees Celsius, with an optimum range for multiplication being 37 to 43 degrees Celsius. Additionally, it may endure low-moisture conditions for up to 12 months, such as in newborn formula with a water activity of 0.30 to 0.83 (Henry & Fouladkhah, 2019).

There are high levels of *C. sakazakii* found in domestic and industrial environmental samples, ranging from 9–44%. Among the factors that influence *C. sakazakii* survival is its thermal resistance characteristic. Powdered infant formula can be reconstituted using lukewarm water with a temperature range of 52 to 58 degrees Celsius. Figure 1 describes a traditional suitable culture of this bacteria by Tryptic soy agar *C. sakazakii*, which exhibits a yellow pigmented morphology. Researchers have found that non-*sakazakii* strains of *Cronobacter*, such *as malonaticus, turicensis, universalis, dublinensis, muytjens, and condimenti*, may cause morbidity and life-threatening complications.

*Figure 1*

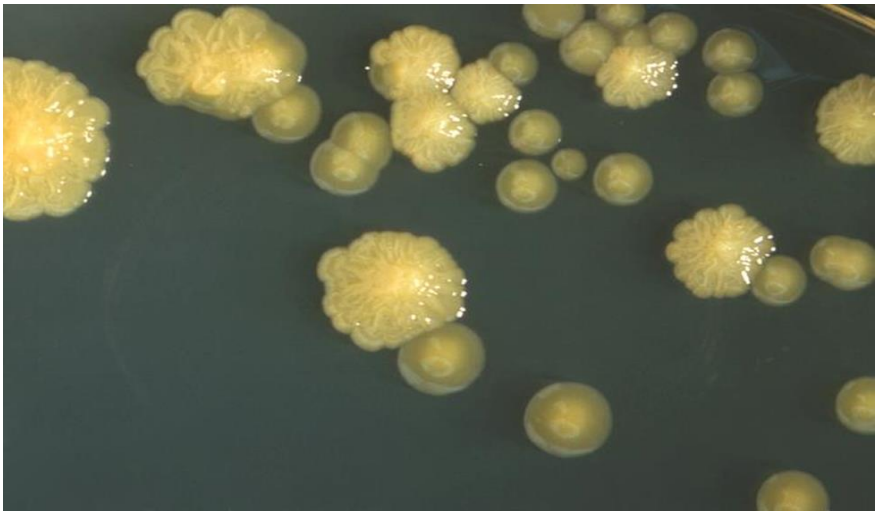

*Figure 1 incubation of C.sakazakii on trypticase soy agar for three days at 25°C Results in colonies of C. sakazakii this bacteria on a Petri dish. Source: Public Health Image Library, Center for Disease Control, Dr. J.J. Farmer (1978).*

### 1.4.1 Phylogeny of *Cronobacter sakazakii*

**The organism:** *Cronobacter sakazakii*

**Taxonomy**

**Domain:** Bacteria
**Kingdom:** Bacteria
**Phylum:** Pseudomonadota
**Class:** Gammaproteobacteria
**Order:** *Enterobacterales*
**Family:** *Enterobacteriaceae*
**Genus:** *Cronobacter*
**Species:** *Cronobacter sakazakii*

### 1.4.2 Pathogenicity and Virulence factors of *Cronobacter sakazakii*

The main cause of infections associated with *Cronobacter sakazakii* is foodborne illness. The route of *C. sakazakii* infections is the digestive tract through which it enters inside the host via mouth and then travels to the intestine to cause infection. Using fibronectin as an adhesive, the bacterium attaches to intestinal epithelial cells (Figure 2-A). In the host body, fibronectin is found in the blood plasma and on the surface of the cell. As it binds to both cells, it contributes to wound healing by connecting the two cells together. Intestinal epithelial cells contain fibronectin that allows the pathogen to bind more strongly to them. A newborn's underdeveloped tight junctions between cells also make it possible for the bacteria to bypass the epithelium (Figure 2-B). As a result, such invaders cannot enter the bloodstream through these junctions (Henry & Fouladkhah, 2019).

By attaching to target cells, *C. sakazakii* enters the bloodstream, replicates, and multiplies. OmpA and OmpX are outer membrane proteins (Omp) produced by the bacterium, which are used to invade intestinal cells (Figure 2-A). Through exocytosis - the transport of vesicles containing cellular components occurs from an interior location to an exterior location .The

pathogen invades the intestinal cell and passes throughout it before being released into the bloodstream. Infecting the endothelial cells that make up the blood-brain barrier is the next step for *Cronobacter sakazakii* after entering the bloodstream (Figure 2-C). As a result, meningitis is caused, in which membranes surrounding the brain become inflamed, causing brain cells to die. This secondary invasion is mediated by OmpA and OmpX, but their mechanisms remain unclear. By engulfing and digesting bacteria inside macrophages, *C. sakazakii* is also capable of evading the host's immune system. An enzyme known as superoxide dismutase is believed to be produced by *C. sakazakii*. To help digest bacteria, macrophages produce reactive oxygen species (ROS) that this enzyme detoxifies. As a result of the ROS not being able to degrade the pathogen, it survives and multiplies. It is also believed that the pathogen produces unknown enterotoxins (Henry & Fouladkhah, 2019).

*Figure 2*

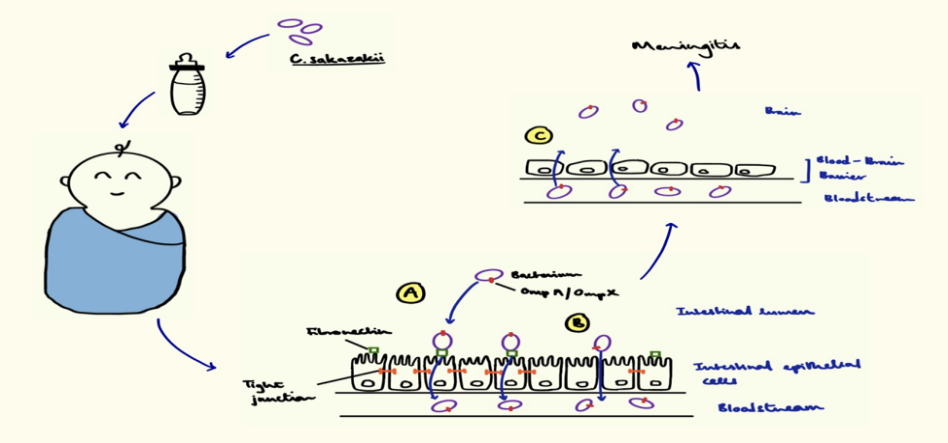


**Figure 2. *Cronobacter sakazakii* infection pathway and virulence mechanisms.**

An overview of the actions of fibronectin and virulence factors OmpA and OmpX on pathogen invasion of intestinal epithelium. B) Lack of tight junctions causes pathogens to enter the bloodstream. C) Infection of brain cells by pathogens crossing the blood-brain barrier. Source: Fiona Chan Pak Choon **(**Henry and Fouladkhah, 2019**).**

# Chapter 2

## 2.1 Literature review

In the baby formula sector, *B. cereus* contamination of milk products is a major concern (Lesley et al., 2017). These milk products can facilitate the growth and enterotoxin production of *B. cereus* when they are reconstituted and kept at room temperature for an extended period (Lesley et al., 2017). Although the spores do not grow in certain forms of food when stored at room temperature for a long time may induce germination, and vegetative cells may multiply and create toxins (Di Pinto et al., 2013).Furthermore, *B. cereus* psychrotrophic strains can develop and produce toxins at storage temperatures above 6 degrees Celsius (Reyes et al., 2007). Infants and toddlers who consume these products frequently have a higher chance of developing food poisoning. *B. cereus* was responsible for 1 to 33% of foodborne illnesses, according to a 2005 investigation by the European Food Safety Authority (EFSA) (Sandra et al., 2012).

The contamination of *C. sakazakii* in the powdered formula is a major issue because this bacterium can survive and remain in environments with low humidity and dryness (O'Brien et al., 2009). Several outbreaks and epidemiological cases have been linked with *C. sakazakii* infections occasionally. In most cases, the method of Cronobacter infection transmission related to children's infections has not yet been established. However, several research studies into many occurrences suggested that the most likely sources of contamination were the equipment used to produce the rehydrated PIF and FUF products, which functioned as the transmission method. For instance, one study revealed that *C. sakazakii* has been found in several processing units and utensils of a PIF manufacturing facility, such as blenders, dryers, and vacuums (Iversen et al., 2008). The misuse of reconstitution of the formula's temperature was a frequent contributor to documented *Cronobacter* epidemics in France (Caubilla-Barron et al., 2007). This emphasizes the temperature regulation necessary to stop microbiological

growth especially since newborns lack immune capacity, in formulas as they do not have proper gut flora (Townsend and Forsythe 2007).

According to FAO/WHO report (2008) and Codex 2020, the products used in both infant and follow-up formulas are non-sterile. Hence, both toxins of *B. cereus* and opportunistic pathogen *C. sakazakii* can get around this condition and cause fatal diseases in children.

## 2.2 Outbreaks of *Bacillus Cereus*

*B. cereus* food poisoning incidents increased by 122.2% in Europe in 2011, according to data from the EFSA. The presence of *B. cereus* in PIF and PFUF is common but still very alarming. It is commonly acknowledged that powdered infant formula products may contain low amounts (100 CFU/g) of this bacterium; as such, the manufacturer should manage and monitor the product as necessary (*Final Assessment Report Application A454 Bacillus Cereus Limits in Infant Formula*, 2004). Since 1950, several outbreaks from a range of foods have been reported in Europe, including milk, and ice cream. Either a thermos table emetic enterotoxin or a thermo sensitive diarrhea genic enterotoxin is to blame for the food poisoning. Several outbreaks from 2007 to 2014 in France were associated with *B. cereus.* As many as 14 incidents of food poisoning linked to milk-based products have been documented as of 2014. From milk and milk products, 85% of enter toxigenic *B. cereus* has been isolated. Furthermore, *B. cereus*-related foodborne illness outbreaks and sporadic cases, including newborn and toddler cases, have been well documented in China.

## 2.3 Outbreaks of *Cronobacter sakazakii*

Several outbreaks and epidemiological cases have been linked with *C. sakazakii* infections occasionally. According to the International Commission for Microbiological Specifications

for Foods (ICMSF), the bacterium poses a severe hazard for restricted populations, and is considered threatening over longer duration.

The link between *C. sakazakii* and newborns was first noted in a 1961 case report by Urmenyi and Franklin, who documented two infant meningitis fatalities attributable to this bacterium. The cause of meningitis is subsequently identified as this bacterium. The United Nations Microbiological Food Standards Commission, categorized *C. sakazakii* by MSF as a life-threatening agent, placing it in the same class as pathogens like *Listeria monocytogenes* and *Clostridium botulinum.* Since the first two instances of *Cronobacter* spp. infection was documented (in 1958 in a hospital in St. Albans, England, where two newborns perished from meningitis), the bacteria have spread worldwide be recognized as the source of uncommon but deadly newborn condition septicemia, necrotizing meningoencephalitis, meningitis brain infarctions, and necrotizing enterocolitis. Studies suggests that several infections in neonatal intensive care unit are associated with *C. sakazakii.*

According to CDC reports, in 1994 thirteen neonates were infected and three of them died. In July 1998 in Belgium, twelve neonates contracted severe diseases due to *C. sakazakii* infection. Also, in 2001 two twins died due to contamination of *C. sakazakii* in PIF. Subsequently, in 2002 in Belgium an infant died due to meningitis infection caused by *C. sakazakii* contamination in PIF. Moreover, in the year 2004, two separate outbreaks were reported by WHO in intensive care units of the hospitals of New Zealand and France connected to PIF contamination. These outbreaks caused the death of infants in both countries. In Mexico in the years 2008 and 2010 respectively *C. sakazakii* outbreaks were observed. Additionally in the year 2008, a total of 5 babies died due to this infection (Jackson et al., 2015).Furthermore, in the year 2016, a 21 days baby was lost due to contamination of *C. sakazakii* in pasteurized human milk (Simth, 2006).

## 2.4 *Bacillus cereus* and *Cronobacter sakazakii* contamination in infants and baby food found in Bangladesh

In Bangladesh, around 9% of breastfeeding children under the age of 6 months consume infant formula (BDHS 2007). Data on the presence of microbial contamination in infant formula consumed among infants in Bangladesh are still not studied properly. Currently, not much data is available on the quantity and quality of newborn and subsequent children formulas consumed by Bangladeshi babies. No outbreaks have been reported linked to *Bacillus cereus* and *Cronobacter sakazakii* contamination.

Few investigations have been conducted on *B. cereus* contamination in milk powder, PFUF and liquid FUF .Only one study reported the presence of pathogenic *B. cereus* on cereal based PFUF. Only 2 studies have been conducted so far on the presence of *C. sakazakii* contamination on PIF and normal Milk powder. The study investigated on dried milk powder reported the qualitative presence of *C. sakazakii*. The study conducted on PIF had only 1 positive result out of 32 PIF samples (Hoque et al., 2010).

However, several reports from various hospitals in Bangladesh indicate the presumption of the presence of these two pathogens. Thus, extensive research studies are required for investigations of possible microbial contaminations on these commercial products. In Bangladesh, very few percentages of dried powdered children's formula are manufactured locally.

# Chapter 3:

## 3.1 Method and Materials

The entire work flow chart of this study briefly given below:

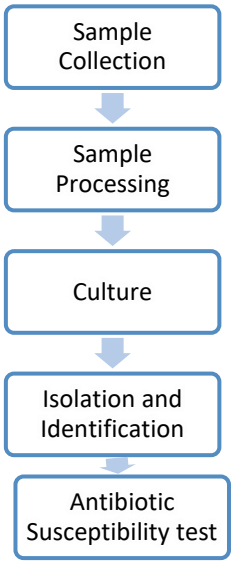


*Figure 3*

**Figure 3- Work flow chart**

## 3.2 Sample Collection

Local and commercially available powdered food products for infants and children were bought for this study after a week of Market analysis. A survey was done to shortlist the brand's name which is consumed mostly in Bangladesh, especially Dhaka city was in major consideration. Samples were collected from eight areas of Dhaka city (Table-4). Additionally, some samples were also collected from popular online platform stores. Out of 26 samples collected, only 4 were manufactured in Bangladesh. The remaining samples were imported into Bangladesh from several countries, and packing was completed in various regions of Bangladesh. This is to emphasize that some samples were formulated in one country and

produced in another. The majority of the products are shipped in large quantities. From there, the goods are locally packed for distribution throughout Bangladesh.

The samples collected (table-4) are suitable for consumption from zero to two years old. The types of collected samples are PIF, PFUF (cereal-based and milk formulas), complementary food products for young children (custard, semolina), and milk powders.

## 3.3 Sample Processing:

All 26 samples were processed and reconstituted for microbiological culture by the international standard methods. For both the pathogens *B. cereus* and *C. sakazakii*, the samples were processed in the same manner but for the culture of *C. sakazakii,* additional enrichment broth is required.

The laminar airflow and the sample containers were cleaned with 70% (wt/vol) ethanol to ensure an aseptic environment. Then 25 gm. of each sample was added to every 225 ml of autoclaved buffered peptone water (pH 7.2) at 45° C. The flasks were swirled by hand to homogenize the mixture until the powder was dissolved. All the 26 flasks were kept in shaker incubator at 37° C, 100 rpm for 24 hours.

### 3.4.1 *Bacillus cereus* Culture

HiCrome$^{TM}$ *Bacillus* Agar was utilized for this purpose. This agar was formulated by Mossel et al. and contains ingredients like peptone and HM extract that give nitrogenous compounds for the microorganism's selective growth. Additionally, the presence of the chromogenic mixture which is broken down by the enzyme beta-glucosidase found in *B .cereus* helps in visualizing the colonies. For the selective growth of *B. cereus* polymyxin B supplement was added to the media. In this study, 10.947µl supplement was added for 2600 ml media (n=26). The overnight pre-enrichment peptone buffer culture was inoculated on the media plates by

serial dilution from $10^{-2}$ to $10^{-5}$. The pre-enrichment broth was mixed in the plates by the spread plate method. After plating, the media plates were kept for incubation at 37° C for 24 hours.

### 3.4.2 Isolation and Identification of *Bacillus cereus*

After 24 hours of incubation, the visible colonies were observed and isolated on Nutrient Agar media and were kept for incubation at 37° C for 24 hours. After 24 hours, isolates of *B. cereus* was picked randomly from the positive samples.

### 3.5.1 *Cronobacter sakazakii* Culture

For the culture of *C. sakazakii,* two consecutive procedures were followed. In the first procedure, a pre-enrichment Peptone buffer broth was prepared for sample processing. After overnight incubation, 1µl of pre-enrichment culture was transferred to 100 ml autoclaved Chromogenic Screening Broth (CSB). This was done at a ratio of 1:100 for every sample according to the protocol mentioned by Iversen et al., (2008). after the addition of pre-enrichment culture to the enrichment broth CSB, vancomycin (5µg) supplement was added. CSB was incubated (in a shaker incubator) at 40°C, 100 rpm for 24 hours. Iversen et al. formulated Cronobacter Screening Broth particularly to be utilized with a chromogenic medium. It enables the identification of samples that are likely to be *Cronobacter* positive before the selective plating process and the release of *Cronobacter*-negative products at least 24 hours earlier than with other conventional techniques. This is accomplished by adding sucrose and the pH indicator bromocresol purple.

*Table 2*

| Ingredients | gm./100 ml |
|---|---|
| Peptone | 2 gm. |
| Meat Extract | 0.6 gm. |
| Sodium Chloride | 1 gm. |
| Bromocresol Purple | 0.0008 gm. |
| Sucrose | 2 gm. |

**Table 2- Preparation of Chromogenic Screening Broth (CSB)** (According to oxford, UK- CM1121).

The overnight culture of the enrichment broth CSB was observed and inoculated into selective media. For the culture purpose, HiCrome agar *Enterobacter sakazakii* was used. This agar is formulated with various compounds like nitrogenous and carbonaceous substances, casein enzymatic hydrolysate, and papaic digest of soya bean meal. This combination of various elements offers vital growth for the bacteria. Additionally, it contains sodium chloride aids in preserving the medium's osmotic balance and sodium deoxycholate prevents the growth of the surrounding gram-positive bacteria. The purpose of this media-containing chromogenic mixture is used to break down the glucosidase enzyme present in *Cronobacter* species. Due to this chromogenic substrate development of blue-green colonies. The presence of deep strong blue colonies belongs to the *Cronobacter* species.100µl of overnight enrichment CSB culture was inoculated into Hi-Chrome *C.sakazakii* media. Then carefully mixed with spreader by spread plate technique. The media plates were kept at 38°C for 72 hours. For confirmation of the better results, certain samples were kept for 86 hours.

### 3.5.2 Isolation and Identification of *Cronobacter sakazakii*

The identification was done in two steps. Firstly, after 24 hours of the new enrichment broth CSB, the color of the broth was observed. Secondly, after 72-86 hours of incubation, the visible colonies were observed. Then the selected colonies were isolated on Nutrient Agar media and kept for incubation at 37° C for 24 hours. After 24 hours, isolates of *C. sakazakii* were picked from the positive samples.

# Chapter 4:

## Antibiotic Susceptibility testing

Antibiotic susceptibility testing was done to determine the susceptibility pattern of *C.sakazakii* and *B.cereus* isolates. This step is important for conducting clinical trials and understanding the overall growth phase of the pathogen. In specific clinical situations, such as the treatment of bacterial endocarditis with bacteremia patients, the need of an antibiotic to kill germs is crucial (Fung-Tomc et al., 2000).

Antibiotic diameter determination was done according to CLSI 2020 protocol.

*Table 3*

| Antimicrobial Class | Antimicrobial Agents (disk content) | Sensitive | Intermediate | Resistant |
|---|---|---|---|---|
| Aminoglycosides | Amikacin (30µg) | 17≥ | 15-16 | 14≤ |
| | Netilmicin (30µg) | 15≥ | 13-14 | 12≤ |
| | Kanamycin (30µg) | 18≥ | 14-17 | 13≤ |
| | Gentamycin (30µg) | 15≥ | 14 | 12≤ |
| Fluor quinolones | Ciprofloxacin (5µg) | 21≥ | 11-15 | 15≤ |
| Sulfonamides | Sulfamethoxazole (25µg) | 16≥ | 14-22 | 10≤ |
| Macrolides | Erythromycin (15µg) | 23≥ | 13-15 | 13≤ |
| Tetracycline | Doxycycline (30µg) | 16≥ | 15-18 | 13≤ |
| | Tetracycline (30µg) | 19≥ | 15-18 | 14≤ |
| Glycopeptides | Vancomycin (30µg) | 17≥ | 15-16 | 19≤ |
| Lipopeptides | Colistin (10µg) | 17≥ | 12-16 | 11≤ |
| Lincosamides | Clindamycin ( 2 µg) | 4≥ | 15-20 | 21≤ |
| β-lactam | Ampicillin (25µg), | 15≥ | 12-14 | 11≤ |
| | Penicillin G (30µg) | 28≥ | 20-22 | 19≤ |
| | Piperacillin tazobactam (10µg) | 21≥ | 18-20 | 21≤ |
| | Ceftazidime (10µg) | 21≥ | 18-20 | 21≤ |
| | Ceftriaxone (30µg) | 23≥ | 20-22 | 23≤ |
| Phenicols | Chloramphenicol (30µg) | 18≥ | 13-17 | 17≤ |
| Carbapenem | Imipenem (10µg) | 23≥ | 20-22 | 23≤ |
| | Meropenem (10µg) | 23≥ | 20-22 | 23≤ |

**Table 3: Antibiotic Diameter Determination Chart. Source: CLSI 2020**

## 4.1 Determination of Antibiotic Susceptibility Profile of *Bacillus cereus*:

The Kirby-Bauer method was used to determine *B. cereus's* antibiotic sensitivity test .The known antibiotics for *B. cereus* were selected and tested. Eleven antibacterial drug diffusion discs were used on *B. cereus* isolates. A total of 19 isolates from the nutrient agar plates were

used for this purpose. From table 3, the green and purple marked set of antibiotics were used. The antibiotic discs were obtained from HI Media Company.

The *B. cereus* isolates were inoculated in Luria Broth (LB) and incubated for, 24 h at 37°C. After 24 hours incubation *B. cereus* isolates were drawn a lawn on Mueller-Hilton agar plates with the help of sterile swabs. The plates were kept at the room temperature for 5 minutes. Then the diffusion discs with antibacterial drugs were distributed on the plates with the help of forceps and then incubated for 24 hours at 37°C. The zones of inhibition were measured using a millimeters scale rule, and the results were evaluated. According to CLSI 2020 (table-3), the results were categorized as either resistant or sensitive.

## 4.2 Determination of Antibiotic Susceptibility Profile of *Cronobacter sakazakii:*

The Kirby-Bauer method was used to determine *C. sakazakii's* antibiotic sensitivity profile .The known antibiotics for *C. sakazakii* were selected and tested. Fourteen antibacterial drug diffusion discs were used on *C. sakazakii* isolates. A total of 12 isolates from the nutrient agar plates were used for this purpose. From table- 3, the blue and purple marked set of antibiotics were used .The majority antibiotic discs were obtained from HI media, only Imipenem and Ceftazidime discs were used from Lofichem-company.

The *Cronobacter sakazakii* isolates were inoculated in Luria Broth (LB) and incubated for 24 hours at 37°C. After 24 hours incubation *C. sakazakii* isolates were streaked on Mueller-Hilton agar plates with the help of sterile swabs. The plates were kept at the room temperature for 5 minutes. Then the diffusion discs with antibacterial drugs were distributed on the plates with the help of forceps and then incubated for 24 hours at 37°C. The zones of inhibition were

measured using a millimeters scale rule, and the results were evaluated. According to CLSI 2020 (table-3), the results were categorized as either resistant or sensitive.

# Chapter 5:

## RESULT

*Table 4*

| SI NO. | Sample Code | Age Group (in months) | Sample type | Food type | Collection area | Manufacturing Area | Packaging Area | *B. cereus* result | *C. sakazakii* result | Both |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 203 | 0 to 6 months | PIF | Milk powder | Chaldal | Belgium | Narshingdi | - | - | ✓ |
| 2 | 201 | 4+ | Complementary food | Custard | Chaldal | EU (UK) | London | ✓ | - | - |
| 3 | 702 | 4 to 6 | Complementary food | Custard | Gulshan 2 | EU(Dublin) | Middle Badda | - | - | - |
| 4 | 703 | 6+ | PFUF | Cereal based | Chaldal | Belgium | Narshingdi | - | - | - |
| 5 | 302 | 6 to 24 | Complementary food | Semolina | Mohakhall | Shirajganj | Shirajganj | - | - | - |
| 6 | 101 | 6 to 24 | PFUF | Cereal based | North Badda | Switzerland | Gazipur | - | - | ✓ |
| 7 | 303 | 6 to 24 | PFUF | Cereal based | Gulshan 1 | Belgium | Nasirabad | - | - | ✓ |
| 8 | 102 | 6 to 24 | PFUF | Milk powder | North Badda | Switzerland | Gazipur | - | - | - |
| 9 | 202 | 6 to 24 | PFUF | Cereal based | Chaldal | Belgium | Narshingdi | ✓ | - | - |
| 10 | 605 | 10 to 12 | PFUF | Cereal based | Gulshan 1 | Switzerland | Gazipur | ✓ | - | - |
| 11 | 301 | 12 to 14 | PFUF | Cereal based | Gulshan 1 | Switzerland | Gazipur | - | - | - |
| 12 | 604 | 12+ | PFUF | Milk powder | Mirpur DOHS | Philippines | Gazipur | - | - | - |
| 13 | 401 | 12+ | dried dairy product | full cream milk powder | Mirpur 2 | Australia | Chittagong | ✓ | - | - |
| 14 | 402 | 12+ | dried dairy product | full cream milk powder | Mohakhali | New Zealand | Narayanganj | - | ✓ | - |
| 15 | 403 | 12+ | dried dairy product | full cream milk powder | Mohakhali | New Zealand | Narayanganj | ✓ | - | - |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 16 | 404 | 12+ | dried dairy product | full cream milk powder | North Badda | Dubai, UAE | Dubai, UAE | - | - | - |
| 17 | 405 | 12+ | dried dairy product | full cream milk powder | Mohakhali | New Zealand | Narayanganj | - | - | - |
| 18 | 406 | 12+ | dried dairy product | full cream milk powder | Mohakhali | Denmark | Gazipur, | ✓ | - | - |
| 19 | 501 | 12+ | dried dairy product | full cream milk powder | North Badda | Australia | Chittagong | ✓ | - | - |
| 20 | 502 | 12+ | dried dairy product | full cream milk powder | North Badda | Australia | Chittagong | ✓ | - | - |
| 21 | 503 | 12+ | dried dairy product | full cream milk powder | North Badda | New Zealand | Narayanganj | - | - | ✓ |
| 22 | 504 | 12+ | dried dairy product | full cream milk powder | North Badda | Gazipur | Gazipur | ✓ | - | - |
| 23 | 601 | 12+ | dried dairy product | full cream milk powder | Banani | Naraynganj | Narayanganj | - | - | - |
| 24 | 602 | 12+ | dried dairy product | full cream milk powder | Banani | Narshingdi | Narshingdi | ✓ | - | - |
| 25 | 603 | 12+ | dried dairy product | full cream milk powder | Dhanmondi | Narshingdi | Narshingdi | - | - | - |
| 26 | 701 | 12+ | dried dairy product | Instant full cream mill powder | Chaldal | New Zealand | Narayanganj | - | - | - |

## 5.1 Result of *Bacillus cereus*

The appearance of blue-flat colonies after the incubation period on the media plate indicated the presence of *Bacillus cereus*. 14 samples indicated the presence of *B. cereus*.

*Figure 4*

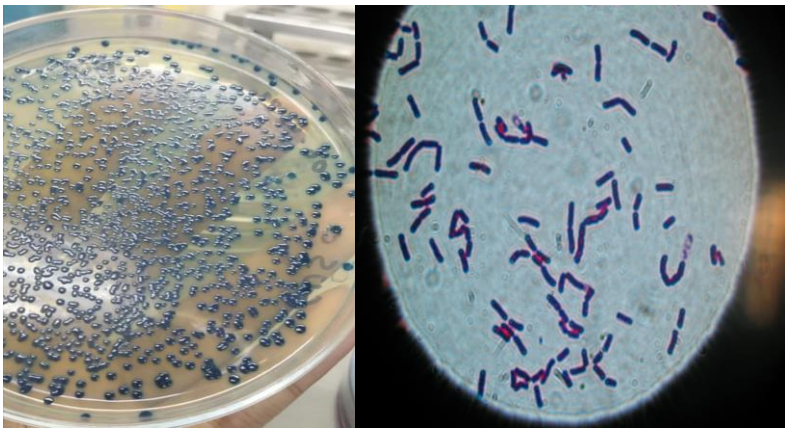

**Figure 4 (a). Colony *of B. cereus,* (b).Gram staining of *B. cereus*. Source-BCCDC labs.**

The sample analysis result from table-4 for *B. cereus* incidence in collected samples-

- Out of 2 PIF samples 1 sample had presence of *B. cereus*
- Out of 3 complementary foods for infant and babies 1 had positive result for *B. cereus*
- Out of 7 PFUF 4 samples were contaminated with *B. cereus*
- Out of 14 full cream milk powder 8 samples were contaminated with *B. cereus*

## 5.2 Result of *Cronobacter sakazakii*

The appearance of small blue-green colonies after the incubation period on the media plate indicated the presence of *Cronobacter sakazakii*. 5 samples indicated the presence of *C. sakazakii*.

The sample analysis result from table-4 for *C. sakazakii* incidence in collected samples-

- Out of 2 PIF samples 1 sample had presence of *C. sakazakii*
- Out of 3 complementary foods for infant and babies 0 contamination was for *C. sakazakii*
- Out of 7 PFUF 2 samples were contaminated with *C. sakazakii*
- Out of 14 full cream milk powder 2 samples were contaminated with *C. sakazakii*

## 5.3 Antimicrobial Susceptibility Result of *Bacillus Cereus:*

The nineteen isolates of *B. cereus* was incubated for 24 hours with disc diffusion techniques containing eleven antibiotic discs. The zones of inhibition of the *B. cereus* isolates were measured using a millimeters scale rule. According to CLSI 2020, the results were categorized as either resistant or sensitive.

*Table 5*

| Antimicrobial class | Antibiotic Discs | Sensitive | Resistant | Intermediate |
|---|---|---|---|---|
| **Aminoglycosides** | Gentamicin(GEN) | 15 (78.95%) | 4 (21.052%) | 0 (0%) |
| **Fluoroquinolones** | Ciprofloxacin(CIP) | 18 (94.74%) | 1 (5.26%) | 0 (0%) |
| **Sulfonamides** | Sulfamethoxazole(COT) | 18 (94.74%) | 1 (5.26%) | 0 (0%) |
| **Macrolides** | Erythromycin(E) | 1 (5.26%) | 17 (89.47%) | 1 (5.26%) |
| **Glycopeptides** | Vancomycin(VA) | 15 (78.95%) | 4 (21.052%) | 0 (0%) |
| **Tetracycline** | Tetracycline(TE) | 11 (57.89%) | 8 (42.11%) | 0 (0%) |
| **Lincosamides** | Clindamycin(CD) | 3 (15.78%) | 16 (84.21%) | 0 (0%) |
| **Phenicols** | Chloramphenicol(C) | 15 (78.94%) | 4 (21.052%) | 0 (0%) |
| **Rifamycin** | Rifampicin(RIF) | 7 (36.84%) | 8 (42.11%) | 4 (21.052%) |
| **β-lactam** | Ampicillin(AMP) | 15 (78.94%) | 3 (15.78%) | 1 (5.26%) |
| | Penicillin G(P) | 19 (100%) | 0 (0%) | 0 (0%) |

**Table- 5 Antimicrobial test patterns of *B. cereus* in percentage**

The *B. cereus* isolates obtained from PIF, PFUF & other dried and complementary products indicated that most of them were resistant to erythromycin, clindamycin and rifampicin (from

table-5). Also, few isolates isolated from powdered infant formula (milk and cerelac) and dried powder multi drug resistance properties. Most bacterial isolates showed (from table-5) resistance to antibiotic groups to macrolides, lincosamides and rifamycin. Additionally penicillin G compared to other antimicrobial drugs showed full sensitive pattern towards *B.cereus.*

## 5.4 Antimicrobial Susceptibility Result of *Cronobacter Sakazakii:*

The twelve isolates of *C. sakazakii* were incubated for 24 hours with disc diffusion techniques containing fourteen antibiotic discs. The zones of inhibition of the *C. sakazakii* isolates were measured using a millimeters scale rule. According to CLSI 2020, the results were categorized as either resistant or sensitive.

*Table 6*

| Antimicrobial class | Antibiotic Discs | Sensitive | Resistant | Intermediate |
|---|---|---|---|---|
| **Aminoglycosides** | Amikacin(AK) | 11 (91.67%) | 1 (8.33%) | 0 (0%) |
| | Netilmicin(NET) | 11 (91.67%) | 0 (0%) | 1 (8.33%) |
| | Gentamicin(GEN) | 12 (100%) | 0 (0%) | 0 (0%) |
| | Kanamycin(k) | 1 (8.33%) | 11 (91.67%) | 0 (0%) |
| **Fluoroquinolones** | Ciprofloxacin(CIP) | 12 (100%) | 0 (0%) | 0 (0%) |
| **Sulfonamides** | Sulfomethoxazole(COT) | 12 (100%) | 0 (0%) | 0 (0%) |
| **Macrolides** | Erythromycin(E) | 4(33.33%) | 2 (16.67%) | 6 (50%) |
| **Tetracyclines** | Doxycycline (DO) | 12 (100%) | 0 (0%) | 0 (0%) |
| **Lipopeptides** | Colistin(CL) | 1(8.33%) | 9(75%) | 2 (16.67%) |
| **β-lactam** | Ceftazidime(CAZ) | 4 (33.33%) | 7 (58.33%) | 1(8.33%) |
| | Ceftriaxone(CTR) | 6 (50%) | 5 (41.67)% | 1 (8.33%) |
| | Tazobactum (PIT) | 6 (50%) | 6(50%) | 0 (0%) |
| **Carbapenem** | Imipenem(IMI) | 11 (91.67%) | 1 (8.33%) | 0 (0%) |
| | Meropenem(MRP) | 12 (100%) | 0 (0%) | 0 (0%) |

**Table 6- Antimicrobial test pattern of *C. sakazakii***

The *C. sakazakii* isolates obtained from PIF, FUF & other dried and complementary products indicated that most of them were resistant to kanamycin, colistin and ceftazidime (From table-

6). In table-6 most bacterial isolates showed resistance to antibiotic groups to aminoglycosides, lipopeptides and β-lactam. Additionally, Gentamicin, ciprofloxacin, doxycycline and meropenem showed full sensitivity towards *C. sakazakii*

# CHAPTER 6

## 6.1 Discussion

In this study, a total of 26 samples, including 2 varieties of PIF, 7 varieties of PFUF(including both cereal and milk based formulas), 3 varieties of small child complementary foods, and 14 varieties of milk powder were analyzed from different areas of Dhaka city. Among them 14 samples were contaminated by *Bacillus cereus* and 5 samples were contaminated by *Cronobacter sakazakii*. To concise, 4 samples had presence of both the pathogens. In our findings, 54% were positively contaminated with *Bacillus cereus a*nd 19.23% were contaminated with *Cronobacter sakazakii.*

Among the collected samples the incidence of contamination from “Mohakhali” and “North Badda” market areas were highest compared to other areas. *B. cereus* contamination was found much in normal milk powder and PFUF compared to PIF. Whereas, *C. sakazakii* contaminations were found on 2 FUF cereal-based product, 1 PIF and 2 full cream milk powder.

The possible contamination in these samples could be due to both intrinsic and extrinsic factors. *C. sakazakii* contamination can hamper health of neonates, infants and children adversely. Temperature changes in reconstituted dried foods favor the optimum growth of this pathogen. Furthermore, as mentioned earlier this bacteria is plant-based based reservoir and occasionally found on rice wheat-based based formulas. Similarly, temperature fluctuation in the milk and

cereal- based induce the germination of spores. Though contamination of *Bacillus cereus* is common and accepted up to less than 100 CFU/g. If the limits exceed it can cause threatening infections including sepsis. *B. cereus* usually forms two types of spores depending on temperature. The slow germinating spores are heat resistant and can cause fatality rate contamination.

Most of the samples were contaminated with *B. cereus* the possible reason can be due to the presence of spores. Moreover, the bacterial load on the culture plates exceeded the accepted range given by FDA/WHO. Most of the results were concluded as TNTC.

Out of 5 samples contaminated by *C. sakazakii*, 2 samples cereal based formula, 1 powered milk infant form follow-up- formula, and 2 milk powder for children above one year. The cereal-based formulae were fruits mixed and wheat based, which is fed to infants above 6 months to 12 months. The antimicrobial result of *C. sakazakii* indicated that most of them were susceptible to the antibiotics but the highest incidence of resistance was seen towards kanamycin and colistin. On the other hand, *B. cereus* isolates were mostly resistant to clindamycin and erythromycin.

Certain limitations were in this study due to less number of samples and unavailability of proper data regarding PIF and FUF consumption in Bangladesh. This research can be extensively enriched if more types of samples are collaborated including clinical samples and improved laboratory conditions.

**6.2 Prevention:**

Utilizing proper hygiene and good handling practices according to the existing recommendations can reduce such infections. Medical personnel must promote nursing as the ideal method of supplying nourishment to newborns because PIF is not a sterile product and a baby may be more susceptible, being prone to infection (Mullane et al., 2006).There is no active

surveillance program for *Cronobacter* spp. infections, despite the fact that the prevalence of immune compromised newborns and children, as well as infants with low birth weight, is higher in nations like Bangladesh than in more developed ones. Therefore, it is crucial for public health specialists in Bangladesh to actively raise medical staff understanding of *Cronobacter* spp. and *Bacillus* spp infections.

### 6.3 Conclusion:

According to the study, most isolates were found to be sensitive to the antibiotics, which is certainly a positive finding when it comes to risk assessment, but for a few antibiotics, intermediate and resistance ratios were found, which can pose a risk. It is possible to increase the resistance ratio by expanding the sample collection area. The findings could also be extended to determine the quality of baby foods by testing pathogenic strains of *C. sakazakii* and *B. cereus*. To ensure microbiological safety of these products consumed by fragile consumers should be monitored strictly. Both the extrinsic and intrinsic microbial contamination should be checked to reduce infection rate. To conclude, Bangladesh requires further research and investigations to determine if *C. sakazakii* and *B. cereus* exist in baby food, since these bacteria can be dangerous, and also have a potential to cause outbreaks.

## References


1. Caubilla-Barron, J., Hurrell, E., Townsend, S., Cheetham, P., Loc-Carrillo, C., Fayet, O., Prère, M. F., & Forsythe, S. J. (2007). Genotypic and phenotypic analysis of *Enterobacter sakazakii* strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France. *Journal of Clinical Microbiology*, *45*(12), 3979–3985. https://doi.org/10.1128/JCM.01075-07
2. Di Pinto, A., Bonerba, E., Bozzo, G., Ceci, E., Terio, V., & Tantillo, G. (2013). Occurence of potentially enterotoxigenic *Bacillus cereus* in infant milk powder. *European Food Research and Technology*, *237*(2), 275–279. https://doi.org/10.1007/s00217-013-1988-8
3. *Final Assessment Report Application a454 Bacillus Cereus Limits in Infant Formula*. (2004). *February*, 1–62.
4. Fung-Tomc, J. C., Gradelski, E., Valera, L., Kolek, B., & Bonner, D. P. (2000). Comparative killing rates of fluoroquinolones and cell wall-active agents. *Antimicrobial Agents and Chemotherapy*, *44*(5), 1377–1380. https://doi.org/10.1128/AAC.44.5.1377-1380.2000
5. Henry, M., & Fouladkhah, A. (2019). Outbreak history, biofilm formation, and preventive measures for control of *Cronobacter sakazaki*i in infant formula and infant care settings. *Microorganisms*, *7*(3). https://doi.org/10.3390/microorganisms7030077
6. Hoque, A., Ahmed, T., Shahidullah, M., Hossain, A., Mannan, A., Noor, K., Nahar, K., Ilias, M., & Ahmed, D. (2010). Isolation and molecular identification of *Cronobacter* spp. from powdered infant formula (PIF) in Bangladesh. *International Journal of Food Microbiology*, *142*(3), 375–378. https://doi.org/10.1016/j.ijfoodmicro.2010.07.019
7. Iversen, C., Mullane, N., McCardell, B., Tall, B. D., Lehner, A., Fanning, S., Stephan, R., & Joosten, H. (2008). Cronobacter gen. nov., a new genus to accommodate the

biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., Cronobacter malonaticus sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensi*i sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter genomospecies* 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. ... *International Journal of Systematic and Evolutionary Microbiology*, *58*(6), 1442–1447. https://doi.org/10.1099/ijs.0.65577-0

8. Jackson, E. E., Flores, J. P., Fernández-Escartín, E., & Forsythe, S. J. (2015). Reevaluation of a suspected *Cronobacter sakazakii* outbreak in Mexico. *Journal of Food Protection*, *78*(6), 1191–1196. https://doi.org/10.4315/0362-028X.JFP-14-563
9. Kim, S. A., Oh, S. W., Lee, Y. M., Imm, J. Y., Hwang, I. G., Kang, D. H., & Rhee, M. S. (2011). Microbial contamination of food products consumed by infants and babies in Korea. *Letters in Applied Microbiology*, *53*(5), 532–538. https://doi.org/10.1111/j.1472-765X.2011.03142.x
10. Lesley, M. B., Ernie, S. R., Kasing, A., & Son, R. (2017). Detection of *Bacillus cereus* in formula milk and ultra high temperature (UHT) treated milk products. *International Food Research Journal*, *24*(3), 985–989.
11. Mullane, N. R., Murray, J., Drudy, D., Prentice, N., Whyte, P., Wall, P. G., Parton, A., & Fanning, S. (2006). Detection of *Enterobacter sakazakii* in dried infant milk formula by cationic-magnetic-bead capture. *Applied and Environmental Microbiology*, *72*(9), 6325–6330. https://doi.org/10.1128/AEM.03056-05
12. O'Brien, S., Healy, B., Negredo, C., Anderson, W., Fanning, S., & Iversen, C. (2009). Prevalence of *Cronobacter* species (*Enterobacter sakazakii*) in follow-on infant formulae and infant drinks. *Letters in Applied Microbiology*, *48*(5), 536–541. https://doi.org/10.1111/j.1472-765X.2009.02562.x
13. Reyes, J. E., Bastías, J. M., Gutiérrez, M. R., & Rodríguez, M. de la O. (2007).

Prevalence of *Bacillus cereus* in dried milk products used by Chilean School Feeding Program. *Food Microbiology*, *24*(1), 1–6. https://doi.org/10.1016/j.fm.2006.04.004

14. Sandra, A., Afsah-Hejri, L., Tunung, R., Tuan Zainazor, T. T. C., Tang, J. Y. H., Ghazali, F. M., Nakaguchi, Y., Nishibuchi, M., & Son, R. (2012). *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat cooked rice in Malaysia. *International Food Research Journal*, *19*(3), 829–836.
15. Simth, B. (2006). Notes from the Field. *Social Marketin Quarterly*, *12*(2), 70–72. https://doi.org/10.1080/15245000600678562
16. Stenfors Arnesen, L. P., Fagerlund, A., & Granum, P. E. (2008). From soil to gut: *Bacillus cereus* and its food poisoning toxins. In *FEMS Microbiology Reviews* (Vol. 32, Issue 4, pp. 579–606). https://doi.org/10.1111/j.1574-6976.2008.00112.x
17. Te Giffel, M. C., & Beumer, R. R. (1998). Isolation, identification and characterization of *Bacillus cereu*s from the dairy environment. In *Wageningen University and Research.* (Vol. 123, Issue 21).
18. Townsend, S. and Forsythe, S.J. (2008) the neonatal intestinal microbial flora, immunity, and infections. In *Enterobacter sakazakii* ed. Farber, J. and Forsythe, S.J. pp. 61–100 Washington, DC: ASM Press.
19. World Health Organization (2004). *Enterobacter sakazakii* and other microorganisms in powered infant formula. MRA Series 6