Determination of the Prevalence and Virulence Properties of Aerobic Endospore-forming Bacteria Isolated from Commercial Packaged Foods in Dhaka City

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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Declaration

I, Shabnam Syeed, declare that this thesis and the work entitled **"Determination of the Prevalence and Virulence Properties of Aerobic Endospore-forming Bacteria Isolated from Commercial Packaged Foods in Dhaka City"** submitted to the Department of Mathematics and Natural Sciences (MNS), BRAC University in partial fulfillment of the requirements for the degree of Bachelor of Science in Biotechnology is a record of work carried out by me under the joint supervision of my supervisors.

I further declare that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

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Abstract

Endospore forming bacteria in packaged foods can lead to gastrointestinal disease and food spoilage upon germination in the GI tract or favourable storage conditions. In the current study,14 samples of packaged foods from 4 categories – UHT milk, milk powder, processed honey and fruit juice – were sampled for isolation of aerobic endospore forming bacteria, namely *Bacillus spp.*, with a focus on the*Bacillus cereus* group. A total of 77 isolates were found; 24 isolates were presumptively identified as *B. cereus* group, amongst which 4 isolates were classified as *B. thuringiensis* and 2 isolates were presumed to be *B.anthracis*. The remaining 52 were classified as *B. spp*. It was found that UHT milk had the lowest prevalence of endospore forming bacteria, while milk powder had the highest. All isolates were tested for production of hydrolytic exoenzymes that hydrolyze starch, casein, lipid and gelatin, while antimicrobial susceptibility of the 24 *B. cereus* isolates were tested against 25 antibiotics using disk diffusion method. *B. cereus* isolates were also screened for production of HBL toxin via discontinuous haemolysis on HBL agar. 15 out of 24 isolates showed production of HBL toxin *in vitro*, showing potential to cause diarrheal syndrome.

Keywords: *Bacillus*, *B. cereus*, B.*anthracis*, *B. thuringiensis*, packaged foods, Haemolysin BL

Chapter 1:
Introduction

1.1 Overview

Bacterial contamination of food products is an issue of great importance in the fields of both food and healthcare, as the presence of pathogenic bacteria in foodstuff can lead to serious illness. With time, food preparation and processing methods have developed to ensure that food products are sterile and safe to eat. In Bangladesh, much of the population consumes commercial packaged food on a regular, if not daily, basis. These days, packaged food products -i.e. processed honey, UHT and powdered milk, fruit juices, coffee and chocolate flavored powder, sauces, etc.- are considered safe for consumption due to the high stress conditions the material undergoes during processing [25]. While these processes may be successful in killing vegetative bacterial forms, often, they can miss out on certain highly durable bacterial structures called endospores.

Endospores are dormant forms of vegetative bacterial cells, formed only by certain genus of bacteria [11]. In unfavorable conditions, spore-forming bacteria can form these structures that their vegetative cells can remain dormant within for extended periods of time. The endospore itself does not need nutrition for survival, but it contains the parent bacteria's DNA and ribosomes, and is extremely resilient to both physical and chemical stresses. When favorable growth conditions return, the spores germinate into vegetative cells, which can then propagate into larger bacterial colonies. This regenerative ability is what makes endospore forming bacteria a dangerous food contaminant. If food processing methods cannot eliminate endospores, then the spore can easily revive on reaching the human gut after consumption of the source food and cause disease such as food poisoning or botulism.

Endospores are formed mainly by the following two genus: *Bacillus* and *Clostridium* [33]. The bacteria under genus *Bacillus* are aerobic spore forming bacteria. More specifically, they are obligate aerobes or facultative anaerobes. In contrast, *Clostridium spp.* are obligate anaerobes.

The members of *Bacillus* genus are gram positive rods, found to be ubiquitous in nature. All species of this bacteria produce endospores, but not all are pathogenic. In fact, many Bacilli are industrially important, such as *Bacillus subtilis*, which is considered a model organism for bacterial studies, and *Bacillus thuringiensis*, whose genome has been incorporated to crops to give resistance to plant pests. However, several other species are

known to be hazardous to human health. *Bacillus anthracis* is the cause of anthrax, while *Bacillus cereus* causes serious food poisoning. In cases where *Bacillus cereus* enters the bloodstream, other conditions such as bacteremia, endocarditis and respiratory tract infections can occur. Many other species such as *Bacillus megaterium* and *Bacillus coagulans* have been known to cause food poisoning and spoilage as well [8, 15].

While anthrax is the most serious, it is also rather rare. Poisoning via *Bacillus cereus* however, is quite common, but often dismissed as unimportant. Despite both diarrheal and emetic types of poisoning being caused by *Bacillus cereus*, healthcare professionals often are quick to prescribe antibiotics as a blanket treatment without determining the source of illness. As a result, there is a growing trend of antibiotic resistance seen in such cases where many drugs are simply no longer effective.

With respect to Bangladesh [38], food-borne disease, its root cause and growing antibiotic resistance of contaminant bacteria is of great importance, therefore the presence of endospore forming bacteria in processed food products in this country should be investigated.

1.2 Objectives

This study aims to find the prevalence of aerobic spore formers in commonly consumed packaged foods at retail level in Dhaka, Bangladesh, as well as to identify them and deduce their toxic potential to cause food-borne disease based on their production of diarrheal toxins, and finally to evaluate their resistance to antibiotics.

Chapter 2:
Literature Review

2.1 Endospores

The bacterial cellular structure known as an endospore was discovered by Koch in 1876 [11], described as a form of dormancy the bacterium formed as a survival strategy in unfavorable environmental conditions. They are formed by members of the Firmicutes phylum, consisting mainly of *Bacillus spp., Sporosarcina spp., Clostridium spp., Anaerobacter spp., Desulfotomaculum spp., Heliobacterium* and *Heliophilum spp*., with *Bacilli* and *Clostridia* being the most notable examples [11]. Endospores, unlike their parent vegetative cells, are metabolically inactive and have a different structural composition, which gives them their hardiness against extreme environments. Spores can easily survive radiation, sterilization, starvation and a wide range of physical and chemical stresses.

Formed at the end of the bacterial growth phase, endospores have a layered structure consisting of the outer exosporium, the spore coat, an outer membrane, which surrounds a cortex, a germ cell wall which surrounds an inner membrane, which in turn encases the core [37]. The exact structure differs among species, such as *Bacillus* sensu lato will have an exosporium, but *Bacillus* subtilis has the spore coat as the outermost layer. However each layer serves to add extra resistance and protection to the endospore. The exosporium is composed of hydrophobic glycoprotein and assists in binding to surfaces as well as evasion of innate immune cells. The spore coat is a semipermeable layer consisting of complex proteins which holds the spore together as well as germination enzymes, while also allowing transport of nutrients and preventing enzymatic degradation. The cortex is a layer of peptidoglycan perpendicular to the spore coat, which assists dormancy by reducing core water content and resisting inactivation by wet heat. Proteins responsible for cellular processes in vegetative cells are held inside the inner membrane, which also provides resistance to chemical degradation, while the core contains genetic material.

This complex structure thus enables endospores to survive conditions which normally destroy vegetative cells, such as dry and wet heat, osmotic pressures, UV radiation, exposure to chemicals, as well as extremes of pH and salinity [33]. Combined with their adherence properties and their ubiquitous presence in soil, this enables endospores to easily remain attached food processing and packaging equipment, and hence, make their way into food products themselves. An issue of increasing concern in the food industry is

the ability of endospores to survive commercial sterilization methods such as desiccation, mechanical agitation, pasteurization and even ultra-heat treatment (UHT) processes.

Spores present in food can remain dormant till the food is ingested and reaches the digestive tract, or germinate in the food product itself depending on the physiochemical characteristics [23]. For example, milk powder will have the lack of moisture and pH that keeps spores in dormant form, while honey and microwaveable foods will have storage temperatures and sugar content which allow spores to germinate before being ingested. Any combination of germination and dormancy factors may be present, that allow endospores to evade sterilization procedures and make their way into the human body [37]. In addition, contaminant spores have significantly less competition in packaged foods, because non-spore-formers are easily eliminated by commercial processes. In the case of contamination by clinically significant species such as some Bacilli and Clostridia, it poses a health risk and indicates gaps in efficient sterilization of food . Growing contamination and increasing tolerance of spore forming bacteria in industrially processed food products is thus an issue of rising concern in the food industry [12].

2.2 *Bacillus spp*

The genus *Bacillus* is comprised of aerobic and facultative anaerobic, gram-positive rod shaped bacteria that have the ability to form endospores [14]. They differ from other endospore forming microorganisms with respect to their aerobic growth and catalase synthesis. *Bacilli* are classically divided into 3 broad groups based on the shape and position of the spore and the swelling of the sporangium. Currently, 268 species and 7 subspecies are classified in the *Bacillus* genus [22], making it one of the largest and most ubiquitous. However, taxonomy and species boundaries are not clearly defined. Significant similarity is seen within groups. Several distinguishing traits are present on plasmids, which once lost, render the species nearly indistinguishable from each other.

Bacillus cereus group – *Bacillus anthracis, Bacillus cereus, Bacillus thuringiensis, B. mycoides, B. pseudomycoides and B. weihenstephanensis*

B. cereus sensu lato consists of B. anthracis, B. cereus,B. mycoides, B. thuringiensis, B. pseudomycoides and *B. weihenstephanensis,* and is defined by large gram-positive rods with motility, ellipsoidal spore and a non-distended sporangium, cells of *Bacillus cereus*

sensu lato are wider than 1μm, form large colonies compared to other groups and can produce lecithinase but cannot ferment acid from mannitol. Several members of this group have marked pathogenicity; *Bacillus cereus* is a well-known human pathogen causing emetic and diarrheal syndrome that can also cause opportunistic infections in humans, while *B. anthracis* is the causative agent of anthrax. *B. thuringiensis* is widely used as an insecticide due to formation of crystal protein. These bacteria have also been linked to food spoilage, due to the ability to secrete a range of hydrolytic enzymes. Basic classical methods used to differentiate between group 1 species are lack of haemolysis, penicillin resistance and motility by *B. anthracis*, crystal toxin formation and insect pathogenicity by *B. thuringiensis*, rhizoid growth of B. *mycoides* and psychrotolerant growth of B. weihenstephanensis However, defined phenotypic differentiation between the group members is difficult; at the genetic level, studies have shown that there is very little difference as well, with *B. cereus*, *anthracis* and *thuringiensis* being 99% in 16S rRNA sequence, leading to the proposal that they be merged into a single species.

<u>*Bacillus subtilis* group –</u> *Bacillus circulans, Bacillus coagulans, Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis, B. subtilis* subsp. *subtilis*, *B. subtilis* subsp. *spizizenii*, *B. mojavensis, B. vallismortis, B. clausii, B. atrophaeus, B. amyloliquefaciens, B. licheniformis, B. sonorensis, B. firmus, B. lentus* and *B. sporothermodurans*

Group 2 is defined by ellipsoidal spores causing swelling of the sporangium. Several of the organisms under group 2 have been known to cause food spoilage and poisoning. In particular, *B. subtilis, B. licheniformis* and *B. pumilus* are important food-borne pathogens. *B. amyloliquefaciens* has also been associated with spoilage and food poisoning in fruit juices. The subtilis group, while still being difficult to distinguish phenotypically, can yet be distinguished using molecular methods.

<u>Miscellaneous group -</u>

Bacillus sphaericus, Bacillus pasteurii

This group is characterized by swollen sporangia with circular, terminal or subterminal spores, rarely associated with human disease of food spoilage. [10]

The ubiquitous nature and ability to produce highly durable endospores allows *Bacillus spp*. to be found in and survive a range of environmental conditions, including food processing equipment, and as a result, cross-over into the food products themselves. A

range of studies in various countries have reported significant prevalence of *Bacillus spp*. in a variety of food products, including dairy items, rehydrated or powdered foods such as baby formula or milk powder, honey, fruit juice, ready to eat meats, fermented and canned foods, cheeses, etc [9, 12, 30].

Previously, only *B. cereus* was thought to be the causative agent of food poisoning, however, studies have shown that many other species such as *B. pumilis*, *B. thuringiensis*, *B. weihenstephanensis,* and *B. licheniformis*, produce a variety of toxins that are similar to those of *B. cereus*, and have been linked to disease after contaminated foods were consumed [8]. Many species of *Bacillus spp*. are commercially and industrially important, so the fact that they are now common contaminants with varying degrees of pathogenicity in even highly processed food products is a topic of growing concern in the food and health industry.

2.2.1 *Bacillus cereus*

Bacillus cereus sensu stricto, henceforth referred to as *Bacillus cereus*, is a mesophilic, neutrophilic, square ended, facultatively anaerobic, endospore forming rod, occurring either singly or in short chains. While being nearly genetically identical to *B. anthracis* and *B. thuringiensis*, *B. cereus* forms neither a capsule nor crystal proteins. It shows both swimming and swarming motility, and is resistant to penicillin and gamma-phage lysis. *B. cereus* is beta-haemolytic, another feature which distinguishes it from the non-haemolytic *B. anthracis*. In addition, *B. cereus* produces a strong lecithinase enzyme in addition to starch and casein hydrolyzing enzymes, but cannot ferment mannitol. The range of growth temperatures is optimally at temperatures 5°C to 50°C, and it can adapt to a range of environmental conditions such as salinity and nutrient deprivation, via endospore formation [10].

Some strains of *B. cereus* are useful for probiotic production. However, most *B. cereus* strains produce a range of enterotoxins, haemolysins, necrotic toxins and cytotoxins, causing 2 forms of food poisoning and various opportunistic and nosocomial human infections such as bacteremia, septicemia, respiratory tract infections and even bovine mastitis[8].

A variety of mechanisms lend *B. cereus* its pathogenicity. Its ability to grow in a wide range of environments, biofilm production and formation of durable, highly adherent endospores means that it can easily make its way to the human intestinal tract via contaminated food product, by attaching to raw materials and processing equipment, surviving processing and sterilization, and subsequently cause food poisoning via toxin production. The high stress sterilization processes eliminate non-spore-forming bacteria as well as vegetative cells easily, but still cannot destroy endospores. So *B. cereus* has much lower competition when it comes time to revive the spores and propagate vegetative cells again. Today, *Bacillus cereus* is one of the prime culprits of food related illnesses from aerobic bacteria, due to the large scale consumption of packaged, reheated and processed food products [36].

2.3 Food contamination – statistics and related species

The role of *Bacillus cereus* as an agent of foodborne illness has been well documented, causing 2 types of food poisoning through the production of several enterotoxins[18]. Additionally, in recent years, an increasing number of studies have reported that other members of *Bacillus* genus such as B. *subtilis, B. licheniformis and B. pumilus* are also responsible for a number of food poisoning outbreaks, and also for food spoilage, due to the production of a number of exoenzymes. Alarmingly, it has also been shown that *B. thuringiensis*, which is commercially used as a bioinsecticide due to its crystal protein formation, can also produce *B. cereus*-like toxins. So far there have been few reports implicating commercial strains of *B. thuringiensis* in food-borne illness. However, these statistics may be under-reported; due to the phenotypic and molecular similarities between *B. cereus* and *B. thuringiensis*, detection methods rarely differentiate between the two [15]. Crops treated with *B. thuringiensis* will have a high spore load, which can easily pass through the production line into ready to eat foods due to the high resistance of *Bacillus* endospores to commercial sterilization procedures. Therefore there may be a possibility that a proportion of *B. cereus* related food poisoning cases were actually caused by *B. thuringiensis*.

It is difficult to pinpoint the exact reason for the increasing prevalence of spore formers in food products. A number of variables are involved, including side effects of new food

ingredients, requirement to preserve food texture while still carrying out sterilization, inadequate sterilization of processing equipment, gaps in new processing and packaging methods, and varying storage conditions of packaged foods. In any case, the special resistant characteristics of endospores give them an advantage over non spore formers. Endospores can survive processes such as heating, desiccation, dehydration, drying, pasteurization, filtration, UV treatment, etc., where bacterial contaminants which cannot form endospores will not be able to survive. This enables them to persist all the way down the production chain and its various sterilization and processing techniques, through many food preparation and cooking methods, and into the human body. *B. cereus* endospores in particular, can survive extended periods of very high temperatures [8, 22]. These factors result in a finished food product that contain *Bacillus* endospores that can later grow into vegetative cells with little to no competition from other species of contaminants. While initial spore loads can be low, storage, cooking and reheating conditions can lead to increase of vegetative cells to levels hazardous for consumers.

Due to their ubiquitous nature, *Bacillus* endospores are inevitable in many different types of food; *B. cereus* infections have been reported after consumption of foods ranging from raw ingredients (vegetables, fruits, dried meats, pasta, spices), to cooked and reheated foods (Chinese meals, rice, salads, soups cold dishes), to snacks (chocolate, sweets, dried potato products) and both dry and wet packaged ready to eat foods, sauces and beverages (honey, coffee, pasteurized milk and meat products including powders, carbonated beverages, fruit juices) [9, 12, 30].

B. cereus in food typically causes 2 distinct syndromes, the emetic and the diarrheal type, with different foods associated with each. The emetic syndrome is associated more with reheated foods, rice and starchy foods, while the diarrheal type can be caused by consumption of cooked foods, dairy products, powders and protein-rich foods.

Another aspect that must be considered is the spoilage potential of endospore forming bacteria [15, 18].

2,4 Pathogenic and Toxigenic Characteristics, Effects on Human Health

2.4.1 Gastrointestinal

The infections most commonly ascribed to *B. cereus* are gastrointestinal. Globally, approximately 30% of food poisoning are associated with *Bacillus cereus*. While the symptoms are usually mild for healthy people, complications can arise, particularly in immunocompromised, young or elderly patients, leading to more severe consequences and even death. In general, food-borne illness are under-notified and under-reported, so the true degree of prevalence is uncertain.

From various clinical reports, the severity and pathogenicity of *B. cereus* infections is thought to correlate to its ability to secrete a number of protein enterotoxins, degradative exoenzymes, and cytotoxins, while also being dependent on the host's immune system and the bacterial strain's virulence. Among the most significant are Haemolysin BL (HBL), non-haemolytic enterotoxin (NHE), Cytotoxin K, Cereolysin O, Haemolysins II and III, and phospholipase C (PLC), along with many other pore formers, membrane disruptors and metalloproteases [28]. Rabbit ileal loop assays have been carried out to show that strains of *B. cereus* containing enterotoxin have vascular permeability, cause fluid accumulation and show ability to cause hemolysis in red blood cells and dermonecrosis in rabbit skin. HBL and NHE, the 2 most well-known toxins, are both tripartite proteins, and require expression of all 3 of the components for virulence to be activated, while Cytotoxin K is a single component toxin, while cereulide is a low molecular weight cyclic protein [13].

B. cereus gastrointestinal infections are of 2 types: emetic and diarrheal. The emetic syndrome is similar to the food intoxication caused by *Staphylococcus aureus*. Cereulide, a peptide toxin produced by *Bacillus cereus*, was found to be present in foods that caused emetic infections [23]. This form of food poisoning does into require live cells: only the toxin must be present in the food being consumed in order to cause illness, characterized by vomiting and nausea within 6 hours of consuming contaminated food. While diarrhea and cramps can also happen, recovery takes place within 24 hours. On the other hand, diarrheal infections have symptoms which mimic those of infections by *Clostridium perfringens*. Within 6 to 15 hours of ingesting contaminated foods, there is onset of abdominal cramps, watery diarrhea and sometimes nausea, lasting from 24 hours to

several days. This syndrome is caused by release of *Bacillus cereus* enterotoxins in the host intestinal tract. While 5 toxins are proposed as causative agents of diarrheal syndrome, only HBL, NHE and CytK have been recorded as the cause of foodborne infections [15, 18].

2.4.2 Non-gastrointestinal

B. cereus is also responsible for several non-gastrointestinal, mostly nosocomial infections. Both local and systemic infections have been recorded. In fact, *B. cereus* is one of the fastest and most destructive causes of endolphthalmitis, which is invasion of bacteria into the eye following trauma or surgery, and eventual loss of vision. Various soft-tissue and skin infection are also caused by *B. cereus* [19], including necrotizing cellulitis, and the organism is also associated with catheter use. Endocarditis and meningoencephalitis infections by *B. cereus* are rare, but such cases have a high mortality. In most cases, patients were immunosuppressed or had exposure to metallic medical equipment or machinery, supporting the idea that there is a significant persistence of *B. cereus* endospores on machinery and equipment. In several studies conducted on BALC/c mice, inhalation of varying loads of *Bacillus cereus* spores and vegetative cells led to inflammation, respiratory distress, shock-like symptoms and death. Immunocompromised patients with systemic infections of *B. cereus* were also reported to suffer from brain abscesses and hemorrhages [8].

2.5 Antibiotic resistance

As multifaceted as *B. cereus* infections are, treatment options are usually based on the antibiotic sensitivity pattern of the implicated strain. However, resistance to antimicrobial treatment is an obstacle to treatment of such infections, as different strains show varied sensitivity to different antibiotics. Generally, *B. cereus* strains are producers of ß-lactamase, and so they show resistance to ß-lactams such as penicillins, cephalosporins, ampicillin, as well as polymixin, kanamycin, colistin, bacitracin and tetracycline. In addition, antibiotic sensitivity studies in Canada have reported significant resistance of *B. cereus* strains to trimethoprim, amoxicillin and aztreonam, and intermediate resistance to nalidixic acid and cephotaxim, while showing sensitivity to vancomycin, erythromycin,

gentamycin and doxycycline. However, reports are contradictory and vary from strain to strain, as there have also been recorded cases of *B. cereus* showing resistance against erythromycin and carbapenem. Susceptibility has been reported to imipenem, chloramphenicol and ciprofloxacin, while resistance was shown against clindamycin. In a study comparing antibiotic resistance of *Bacillus cereus* from both food poisoning outbreaks and non-gastrointestinal infections, it was shown that there is a constant resistance to cephalosporins, penicillins, trimethoprim and ampicillin, whereas tetracycline, clindamycin, aminoglycosides, vancomycin, chloramphenicol and erythromycin were effective, as well as ciprofloxacin. In another study, levofloxacin, moxifloxacin, rifampin, linezolid were shown to be effective, while resistance was seen against erythromycin, trimethoprim-sulfamethoxazole and clindamycin. While antibiotics as a rule are not to be prescribed for cases of food poisoning, they are still used as secondary treatment for infections of extended duration, with the practice being more common in some countries than others. In addition, self-medication is an issue of great concern, particularly in countries such as Bangladesh where health awareness is not well spread. A 2014 study reported that 27% of 1300 survey participants admitted to self-prescribing antibiotics for various afflictions, with 36% of the cases being due to diarrhea or food poisoning-like symptoms. Unchecked use of antibiotics has been shown to lead to growing resistance in previously susceptible bacterial strains and can thus be a cause of conflicting reports in antibiotics sensitivity test reports.

2.6 Diarrheal Toxins

Of the wide variety of *Bacillus cereus* enterotoxins, 3 are implicated in the more severe, diarrheal form of infection. These are haemolysin BL (HBL), non-haemolytic enterotoxin (NHE) and cytotoxin K [23]. The 2 former are 3 component proteins that require expression of all components to carry out virulence, while cytK is a single component protein. *B. cereus* contaminated foods cause diarrhea when these toxins are released into the small intestine after consumption. Vegetative cells are not a requirement; endospores present in food can germinate into vegetative cells in the gastrointestinal tract and subsequently release these toxins in the exponential phase of growth. Studies have also shown that the toxins are stable between pH 4 to 11, but produced in pH 5.5-10. In terms

of temperature tolerance, diarrheal enterotoxins are released within 10 to 43°C. However, they are denatured at low pH, easily degraded by digestive enzymes and inactivated by exposure to temperatures of 56°C for 5 minutes. These findings support the theory that the toxins are not pre-formed in food, as is the case with the emetic toxin, but instead formed in the small intestine after ingestion of contaminated foods. It has been observed that HBL and NHE production increases in oxygen-depleted conditions such as that in the small intestine. However, endospores, due to their highly protective and layered structure, can pass unharmed through the intestinal barrier.

HBL is the most well characterized diarrheal toxin in literature, one that has been highly purified and established to be a diarrheal toxin via various animal models and haemolytic assays. It is also referred to as dermonecrotic toxin, vascular permeability factor, and fluid accumulation factor, due to the ability of the cell-free filtrate of *B. cereus* cultures to cause fluid buildup in rabbit ileal loop assays, affect the vascular permeability and cause dermonecrosis in the skin of rabbit and guinea pigs, and on intravenous injection, to kill mice. Approximately 86% of *B. cereus* strains produce Haemolysin BL, while 67% of *B. thuringiensis* strains are known to produce this toxin. Optimum production conditions are temperatures of 32-37°C and pH levels below 7. Best results were seen for small scale production of toxin in Brain Heart Infusion broth. One of the best and earliest reports of HBL were by *X*, who via immunodiffusion and western blot assay purified a tripartite protein that caused the above mentioned biological activities, along with having cytotoxicity to Chinese hamster ovary (CHO) cell lines, in vitro as well as in vivo [4, 5, 6].

The 3 components of HBL are B, a binding component, and 2 lytic components L1 and L2. The genes encoding them are hblA, hblD and hblC respectively. They are located in a single operon, and while strains can produce various combination of the components, all 3 are required for complete biological and virulent activity. Deduced nucleotide and amino acid sequences of all 3 components have been determined (GenBank accession nos. L20441, U63928, AJ237785). However, there is significant molecular heterogeneity among HBL components from different strains, leading to conflicting reports regarding their molecular weights, though the generally accepted numbers are 37.8 kDa, 38.5 kDa and 42.3 kDa respectively [6].

Commercial kits are available for the detection of HBL toxin, such as the Oxoid BCET-RPLA. It shows considerable efficacy in detection of haemolysin BL component L2, and does not detect NHE or CytK. However, as this kit only screens for presence of L2 component, and presence of all 3 components are a requirement for biological activity, it cannot be conclusively stated that the kit can select for active HBL. Besides, kits are expensive and not always available to everyone, particularly in primary level institutions. CHO or Vero cell cytotoxicity assays have much greater sensitivity and can detect active haemolysin [17, 31].

In addition, another low cost and simple detection method was developed by Beecher and Wong, namely the use of HBL agar [5]. It was found that HBL produces a characteristic discontinuous haemolysis pattern on the specific agar formulation. Instead of haemolysis beginning from the edge of the bacterial colony and a constant clearing zone being observed, on HBL agar, HBL toxin causes haemolysis a few millimeters away from the colony with a ring of intact erythrocytes in between, or shows a ring of intact erythrocytes with clear zones on either side. This pattern is transient and merges into complete haemolysis with time. This phenomenon has been proposed to be due to the different rates of diffusion of the toxin components through the agar, and the binding of the components to red blood cells. The erythrocytes must first be primed with B before lytic action can take place, but excess lytic protein can inhibit binding of B. Near the colony, lytic protein concentration is high and inhibitory to hemolysis. As the proteins diffuse further from the colony, concentration of lytic protein decreases and B can bind. Therefore, haemolysis occurs away from the bacterial colony, in areas where balanced concentrations of all 3 components are present, leaving non-haemolysed areas in between. This method for HBL detection has significant correlation with gene-specific and cell cytotoxicity mediated detection methods, and is much cheaper and able to process multiple samples at a time [5, 6].

Chapter 3:
Methods and Materials

3.1 Place of work

The entirety of this study was carried out in the laboratories of the Department of Mathematics and Natural Sciences at BRAC University.

3.2 Sample collection

14 samples of packaged food products were collected from markets in various areas of Dhaka city. Samples were collected from 4 categories: milk powder, UHT milk, packaged fruit juice and processed honey. To prevent contamination from environmental microorganisms, food packages were opened only under sterile laminar hood.

3.3 Homogenization

1g of each sample was added to 9ml of autoclaved 0.85% peptone saline diluent in falcon tubes, then thoroughly vortexed. Serial dilutions were made upto 10^{-3}.

3.4 Heat treatment

The samples were subjected to heat shock at 80°C for 10 minutes in order to kill vegetative bacterial cells and activate endospores.

3.5 Growth on Nutrient Agar

Using a glass spreader, 100µl of each tube was spread plated on nutrient agar plates. The plates were incubated at 37°C in presence of oxygen for 24 hours before unique bacterial colonies were selected for streaking on fresh nutrient agar plates for isolation of pure cultures. Streak plates of unique colonies were incubated for upto 72 hours at 37°C in order to allow for sporulation by endospore forming bacteria.

3.6 Endospore staining

Endospores were stained via modified Schaeffer-Fulton method [26] using malachite green and safranin dyes. A loopful of the culture was smeared onto a glass slide with a few drops of distilled water. The smear was heat fixed before adding 5-6 drops of malachite green over top. The slide was carefully held over a flame until the dye started to steam and bubble, at which point it was removed from the flame and allowed to cool. Cooled slides were washed thoroughly with distilled water before addition of 5-6 drops of safranin. The dye was allowed to sit for 3 minutes before the slides were again washed with distilled water and allowed to air dry. The dried stained slides were then observed

under 100x magnification of microscope with immersion oil to confirm presence or absence of endospores. Isolates were only designated negative for spore formation if there were no visible spores under microscope after 7 days of incubation.

3.7 Plating on *Bacillus Cereus* Selective Agar

Cultures that showed positive results in endospore stain were streaked onto *Bacillus Cereus* Selective agar for primary screening of isolates into *Bacillus spp* category. Culture plates were incubated at 37°C for 48 hours, then observed for colony colour and morphology, colour changes of the media and formation of egg yolk precipitate or clearing. Isolates which showed formation of white precipitate with large (turquoise to peacock) blue colonies were presumptively identified as *Bacillus cereus* sensu lato, while others were categorized as *Bacillus spp*. Further confirmatory biochemical and phenotypic tests were subsequently carried out.

3.8 Crystal staining

Isolates that were presumptive *Bacillus cereus* were stained with Coomassie Brilliant Blue [29, 35] dye to detect crystal formation in order to distinguish *Bacillus thuringiensis* from other species of *Bacillus* sensu lato. Heat fixed smears were exposed to the dye for 3 minutes before being washed, air dried and observed under 100x oil immersion for presence of darkly stained parasporal crystals alongside lighter stained spores.

3.9 Culture preservation

Isolates were routinely subcultured on nutrient agar and *Bacillus cereus* selective agar for working cultures. For long term storage, an inoculating needle was used to make several stabs of pure culture in vials of T1N1 agar. The vials were incubated at 37°C for 24 hours before being covered in sterile paraffin oil and stored at room temperature.

3.10 Biochemical tests

A range of biochemical tests were carried out in order to further classify the presumptive species of isolates bacteria. All tests were carried out according to Bergey's Manual of Systematic Bacteriology [21] using fresh, 24 hour cultures from nutrient agar plates. The following tests were performed:

3.10.1 Methyl Red

Half a loopful of culture was inoculated into a test tube containing 5ml of MRVP broth, and the tube was incubated at 37°C for 24 hours. For observation, 5 drops of methyl red dye was added to the tube without shaking. A cherry red colour indicated positive for mixed pathway fermentation of glucose, while orange indicated inconclusive, and yellow indicated negative results.

3.10.2 Voges Proskauer

Half a loopful of culture was inoculated into a test tube containing 5ml of MRVP broth, and the tube was incubated at 37°C for 24 hours. After incubation, 6 drops of Barritt's reagent A were added and the tube was shaken. After that, 6 drops of barritt's reagent B were added to the tube and observed upto 1 hour. Formation of a pink ring indicated presence of acetoin, while a brownish ring indicated negative results.

3.10.3 Indole

A loopful of culture was inoculated into a test tube containing 5ml of tryptophan broth, and the tube was incubated at 37°C for 24 hours. After addition of 5 drops of Kovac's reagent to the broth, a red colour indicated positive tryptophan hydrolysis while a yellow colour was taken as negative.

3.10.4 Citrate

Sterile slants of Simmon's Citrate Agar were prepared in vials and the surface was streaked heavily with a loopful of culture. The vials were incubated at 37°C for 48 hours and observed for bacterial growth and colour change. Growth on the slant with colour change of the medium to blue indicated positive for utiliza5tion of citrate as a carbon source, while growth without colour change was taken as a negative result.

3.10.5 Catalase

3 drops of 3% hydrogen peroxide were taken on a clean glass slide. Half a loopful of bacterial culture was mixed with the hydrogen peroxide on the slide and observed for bubble formation. Immediate, sustained formation of bubbles was taken as a positive indicator of catalase production by the sample bacterium, while slow or delayed formation of small amount of bubbles was taken as negative.

3.10.6 Oxidase

On a sterile petri dish lid, filter paper was soaked in oxidase reagent. Using a sterile inoculating loop, a heavy inoculum of bacteria was smeared onto a section of reagent soaked filter paper and observed for colour change. Changing of the smear to a pink colour indicates positive test for presence of cytochrome c oxidase enzyme, while no colour change indicates a negative result.

3.10.7 Motility and Urea

Both motility and urease tests were carried out using MIU agar. The agar was prepared first, sterilized in test tubes, and then cooled to approximately 50°C before adding 5% (v/v) of 40% syringe filtered urea solution. Once solidified, the agar was inoculated via a single stab of heavy inoculum using a sterile inoculating needle and incubated at 37°C for 24 hours. Change of the orange media to pink indicated positive for presence of urease enzyme, while spiraling growth away from the stab line was indicative of motility.

3.10.8 Growth at 0.7% NaCl

A loopful of culture was inoculated into a test tube containing 5ml of nutrient broth with 0.7% sodium chloride, and the tube was incubated at 37°C for 24 hours. Turbidity in the tube was indicative of bacterial growth.

3.10.9 Test for rhizoid growth

This test was carried out on all presumptive *Bacillus cereus* isolates to help distinguish them from *Bacillus mycoides*. Nutrient agar plates were inoculated by lightly touching an inoculating loop to a bacterial colony, then touching the loop to the surface of the agar. The plates were incubated at 30°C for 72 hours, with observation of colonies root like extensions indicating positive rhizoid growth.

3.10.10 Starch Hydrolysis

Pure cultures were streaked onto starch agar plates and incubated at 37°C for 24 hours. After incubation, the plates were flooded with gram's iodine. Clear zones around bacterial colonies against a now dark blue medium indicated positive results for hydrolysis of starch.

3.10.11 Gelatin Hydrolysis

A heavy inoculum of pure culture was stabbed into tubes containing gelatin agar. The tubes were incubated at 37°C for 24 hours, then at 4°C for 30 minutes. On tilting, if the tubes showed liquefied media, then the tubes were taken as positive for gelatin hydrolysis. Tubes which showed negative after 24 hours were incubated and observed again for 7 days before an isolate was deemed negative for gelatin liquefaction.

3.10.12 Lipid Hydrolysis

Pure cultures were streaked onto Tributyrin agar plates supplemented with Tributyrin oil, and incubated at 37°C for 24 hours. After incubation, the plates were flooded with spirit blue dye. Clear zones around bacterial colonies against a now bright blue medium indicated positive results for hydrolysis of fat.

3.10.13 Casein Hydrolysis

Pure cultures were streaked onto skim milk agar plates and incubated at 37°C for 24 hours. After incubation, the plates were observed for clear zones around bacterial colonies, indicating positive results for hydrolysis of casein.

3.10.14Haemolysis

Blood agar plates with 5% defibrinated sheep blood were streaked with pure culture and incubated at 37°C for 24 hours. The plates were then observed for clearing of media indicating beta haemolysis, greening indicating alpha haemolysis, or no change indicating gamma haemolysis.

3.11 Disc-diffusion Antibiotic Susceptibility Test

All *X* isolates taken as presumptive *Bacillus cereus* (large blue colonies on BCA, beta haemolytic on 5% SBA and positive for *X*) were tested for antibiotic susceptibility using the following *X* antibiotics.

3.11.1 Preparation of saline suspension

Half a loopful of fresh pure culture was inoculated into 9ml of 0.7% saline solution then thoroughly vortexed, to make up bacterial suspensions comparable to 0.5 MacFarland solutions.

3.11.2 Inoculation of MHA agar plates

Using a sterile cotton swab, sterile MHA plates were heavily streaked with the above prepared suspension to form a bacterial lawn. The streaking was carried out 3 times, each time rotating the plated 90 degrees. After streaking, the plates were allowed 5 minutes to absorb the inoculum before antibiotic disks were placed using sterile forceps. The plates were incubated at 37°C for 24 hours.

3.11.3 Observation of inhibition zones

After incubation, the plates were observed for zones of inhibition of bacterial growth. The diameter of clear zone around each antibiotic disk was viewed from the back of the petri dish and noted in millimeters.

3.12 Screening for HBL toxin

All *X* isolates taken as presumptive *Bacillus cereus* (large blue colonies on BCA, beta haemolytic on 5% SBA and positive for *X*) were tested for production of diarrheal toxin Haemolysin BL on HB agar as formulated by *X*.

3.12.1 Production of crude haemolysin

1 loopful of pure cultures of fresh culture of presumptive *Bacillus cereus* were inoculated into 250ml conical flasks containing 50ml BHI supplemented with 0.1% glucose. The flasks were incubated overnight in an orbital shaker at 32°C and 180 rotations per minute. After incubation, the broth culture was centrifuged at 5000xg for 20 minutes and the supernatant collected in a fresh, sterile falcon tube for storage and further work.

3.12.2 Inoculation and observation on HBL agar

HBL agar was prepared using nutrient agar, 2.5% one month old defibrinated sheep blood and 2% sheep serum. Using a micropipette, the plates were spot inoculated with supernatant collected as above, and incubated for 12 hours at 28°C. The plates were then observed for discontinuous haemolysis patterns.

Chapter 4:

Results

Primary identification of isolates was carried out via staining and plating on selective media. Illustrations of positive stains and media plates are given below. For Coomassie stain, crystal protein presence is shown by dark blue structures on the outer side of the spore body. For Malachite stain, vegetative cells are stained red while endospores are stained green. Here, both internal and external spores can be seen. For *Bacillus Cereus* Selective agar, blue colonies with white precipitate indicate *B. cereus* group, while yellow and green colonies indicate *B. spp*.

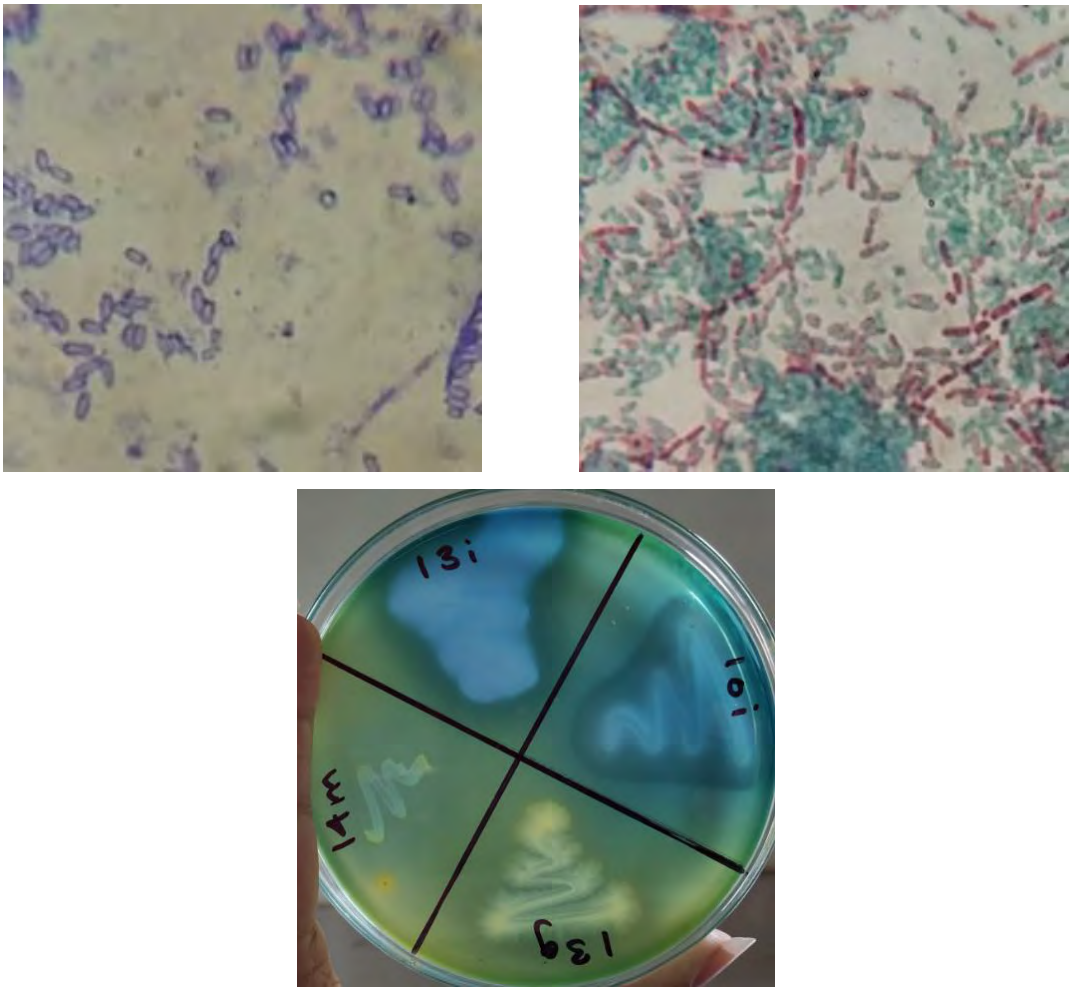

Figure 1 Results of a) Coomassie blue stain b) Malachite green stain and c) Plating on BCA

A total of 77 spore forming bacteria were isolated from all 14 samples. Amongst the 4 categories, fruit juice had the highest prevalence of endospore forming bacteria, while the lowest number of isolates were found from UHT milk. The highest number of *Bacillus*

cereus was found in UHT milk, while the highest number of *Bacillus spp*. was found in powdered milk.

Organism \ Sample	UHT milk (4)	Milk powder (3)	Processed honey (3)	Fruit juice (4)	Prevalence	
					Total	Percentage
B. alvei	0	1	0	2	3	(3.90%)
B. anthracis	1	1	0	0	2	(2.60%)
B. badius	1	0	4	0	4	(5.20%)
B. brevis	3	1	2	1	7	(9.10%)
B. cereus	8	2	4	4	18	(23.40%)
B. coagulans	0	0	0	4	4	(5.20%)
B. larvae	0	0	1	1	2	(2.60%)
B. laterosporus	0	1	1	0	2	(2.60%)
B. licheniformis	0	0	0	1	1	(1.30%)
B. macquarensis	0	0	3	1	4	(5.20%)
B. megaterium	1	2	0	0	3	(3.90%)
B. pasteurii	0	1	1	0	2	(2.60%)
B. pectinilyticus	0	0	1	0	1	(1.30%)
B. polymyxa	0	0	0	1	1	(1.30%)
B. papillae	0	0	1	0	1	(1.30%)
B. pumilus	0	2	0	1	3	(3.90%)
B. sphaericus	1	3	0	1	5	(6.50%)
B. spp	0	7	0	1	8	(10.40%)
B. subtilis	0	1	0	0	1	(1.30%)
B. thuringiensis	0	0	1	3	4	(5.20%)
Isolates in category	15	22	19	21	**77**	**100.00%**
Total percentage	(19.5 %)	(28.5)	(24.7%)	(27.3 %)		

Table 1 Prevalence of spore forming bacteria according to sample type

The above mentioned bacterial species were presumptively identified based on a series of biochemical tests. Following are the results of biochemical tests performed, and the presumptive identities of the respective isolates that were deduced from the test results:

Isolate no.	Observation on *Bacillus Cereus* Selective agar			Endospore stain		Crystal stain	Rhizoid growth	Catalase	Oxidase	Motility	Urea decomposition	Methyl-Red	Voges-Proskauer	Indole	Citrate	Growth in 0.65% NaCl	Starch hydrolysis	Casein hydrolysis	Gelatin liquefaction	Lipid Hydrolysis	Haemolysis on 5% sheep blood agar	Presumptive ID
	Colony colour	*Media colour*	*Precipitate*	*Spore shape*	*Swollen sporangium*																	
10b	Cream	Yellow	-	Oval	+	X	X	+	+	-	-	-	+	-	-	+	-	-	+	+	ɣ	B. larvae
10c	Blue	Yellow	-	Oval	+	X	X	+	+	-	-	-	+	-	-	+	-	-	+	+	ɣ	B. laterosporus
10e	Cream	Yellow	+	Oval	-	X	X	+	+	-	-	-	+	-	-	+	-	-	-	+	β	B. popillae
10f	**Blue**	**Blue**	**+**	**Oval**	**-**	**-**	**-**	+-	**-**	+	**-**	+	+	**-**	+	+	+	+	+	+	**β**	***B. cereus***
10i	Cream	Yellow	+	Circular	+	X	X	+	+	-	+	-	+	-	+	+	-	+	+	-	ɣ	B. pasteurii
10j	**Blue**	**Blue**	**+**	**Oval**	**-**	**-**	**-**	+	**-**	+	**-**	+	+	**-**	+	+	+	+	+	+	**β**	***B. cereus***
10k	**Blue**	**Blue**	**+**	**Oval**	**-**	+	**-**	+	**-**	+	**-**	+	+	**-**	+	+	+	**-**	+	+	**β**	**B.*thuringiensis***
10l	**Blue**	**Blue**	**+**	**Oval**	**-**	**-**	**-**	-+	**-**	+	**-**	+	++	**-**	+	+	+	**-**	+	+	**β**	***B. cereus***
1161	Green	Yellow	+	Oval	-	X	X	+	+	-	-	-	+	-	+	+	-	+	+	-	β	B. macquariensis
1162	Green	Yellow	+	Oval	-	X	X	+	-	+	-	+	+	-	-	+	-	+	+	+	β	B. macquariensis
11b	Green	Yellow	+	Oval	-	X	X	+	-	+	-	+	+	-	-	+	-	+	+	+	β	B. macquariensis
11e	Yellow	Yellow	+	Oval	-	X	X	+	-	+	-	-	+	-	-	+	-	+	-	+	β	B. badius
11h	Yellow	Yellow	+	Oval	-	X	X	+	-	+	-	+	+	-	-	+	-	+	+	+	ɣ	B. badius

11l	Cream	Yellow	+	Oval	-	X	X	+	+	+	-	+	+	-	+	+	-	+	+	+	β	B. badius
11o	Yellow	Yellow	+	Oval	-	X	X	+	-	-	-	-	-	-	-	+	-	-	+	+	β	B. brevis
11p	**Blue**	**Blue**	**+**	**Oval**	**-**	**-**	**-**	+	-	+	-	+	+	-	+	+	+	+	+	+	**β**	***B. cereus***
12a	Cream	Yellow	+	Oval	-	X	X	+	-	+	-	+	+	-	-	+	-	+	+	+	β	B. brevis
12b	Blue	Yellow	+	Oval	+	X	X	+	-	+	-	+	+	-	-	+	-	+	+	+	ɣ	B. pectinilyticus
12d	Cream	Yellow	-	Oval	-	X	X	+	-	+	-	-	+	-	-	+	-	+	+	-	ɣ	B. badius
13a	Cream	Yellow	-	Circular	+	X	X	+	-	+	-	+	+	-	-	+	-	+	+	-	ɣ	B. larvae
13c	Green	Yellow	-	Oval	-	X	X	+	-	+	-	+	+	-	-	+	+	+	+	+	ɣ	B. alvei
13g	Green	Yellow	+	Oval	-	X	X	+	-	+	-	+	+	-	+	+	+	-	+	-	ɣ	B. coagulans
13h	Green	Yellow	+	Oval	-	X	X	+	-	+	-	+	+	-	+	+	+	-	+	-	ɣ	B. coagulans
13i	**Blue**	**Blue**	**+**	**Oval**	**-**	**-**	**-**	+	-	+	-	+	+	-	+	+	+	+	+	+	**β**	***B. cereus***
14a	Yellow	Yellow	+	Oval	+	X	X	+	-	-	-	+	+	-	-	+	+	+	+	+	ɣ	B. alvei
14e	Yellow	Yellow	+	Oval	-	X	X	+	-	-	-	-	+	-	-	+	-	+	+	+	ɣ	*B. spp*
14f	Cream	Yellow	+	Oval	+	X	X	+	-	-	-	-	+	-	-	+	+	+	+	+	β	B. polymyxa
14j	Green	Yellow	+	Oval	+	X	X	+	-	+	-	-	+	-	+	+	+	+	+	-	ɣ	B. licheniformis
14n	**Blue**	**Blue**	**+**	**Oval**	**-**	**+**	**-**	+	-	+	-	+	+	-	+	+	+	+	+	+	**β**	**B.*thuringiensis***
14p	**Blue**	**Blue**	**+**	**Oval**	**-**	**+**	**-**	+	-	+	-	+	+	-	+	+	+	+	+	+	**β**	**B.*thuringiensis***
14q	**Blue**	**Blue**	**+**	**Oval**	**-**	**-**	**-**	+	-	+	-	+	+	-	+	+	+	+	+	+	**β**	***B. cereus***

14r	**Blue**	**Blue**	+	**Oval**	-	+	-	+	-	+	-	+	+	-	+	+	+	+	+	+	**β**	***B.thuringiensis***
14t	Green	Yellow	+	Oval	+	X	X	+	-	-	-	+-	+	-	-	+	-	+	+	-	ɣ	B. brevis
14x	**Blue**	**Blue**	+	**Oval**	-	-	-	+	-	+	-	+	+	-	+	+	+	+	+	+	**β**	***B. cereus***
14y	**Yellow**	**Blue**	+	**Oval**	-	-	-	+	-	+	-	-	+	-	+	+	+	+	+	+	**β**	***B. cereus***
15a	Yellow	Yellow	-	Oval	+	X	X	+	+	-	-	+	+	-	-	+	-	-	+	+	ɣ	B. macquariensis
15b	Yellow	Yellow	+	Oval	-	X	X	+	+	+	-	+	+	-	+	+	-	+	+	+	β	B. pumilus
15c	Cream	Yellow	-	Circular	+	X	X	+	+	+	-	-	+	-	+	+	-	+	+	+	ɣ	B. sphaericus
16a	Blue	Yellow	+	Oval	+	X	X	+	-	+	-	+	+	-	-	+	+	-	+	+	ɣ	B. coagulans
16b	Cream	Yellow	+	Oval	+	X	X	+	-	-	-	+	+	-	-	+	+	-	+	-	ɣ	B. coagulans
1d	Cream	Yellow	-	Circular	+	X	X	+	-	+	-	-	-	-	+	+	+	-	+	-	β	B. brevis
1x	**Blue**	**Blue**	+	**Oval**	-	-	-	+	-	-	-	+	-	-	+	+	-	+	+	+	**β**	***B. cereus***
1y	**Blue**	**Blue**	+	**Oval**	-	-	-	+	-	-	-	-	+	-	+	+	+	+	+	+	**β**	***B. cereus***
2e	Green	Yellow	+	Oval	-	X	X	+	+	+	-	+	+	-	+	+	-	+	+	+	β	B. brevis
2f	Green	Yellow	+	Oval	-	X	X	+	+	-	-	+-	+	-	+	+	-	+	+	+	β	B. megaterium
2g	Green	Yellow	+	Oval	-	X	X	+	+	+	-	-	+	-	-	+	-	+	+	+	β	B. badius
2i	Yellow	Yellow	-	Oval	-	X	X	+	+	+	-	-	+	-	+	+	-	+	+	-	β	B. brevis
2x	**Blue**	**Blue**	+	**Oval**	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+	**β**	***B. cereus***
2y	**Blue**	**Blue**	+	**Oval**	-	-	-	+	-	-	-	-	+	-	+	+	-	+	+	+	**ɣ**	***B. anthracis***

3a	**Blue**	**Blue**	+	**Oval**	-	-	-	+	-	+	-	+	+	-	+	+	+	+	+	+	**β**	***B. cereus***
3b	**Blue**	**Blue**	+	**Oval**	-	-	-	+	-	+	-	-	-	-	+	+	+	+	+	+	**β**	***B. cereus***
3c	**Blue**	**Blue**	+	**Oval**	-	-	-	+	-	-	-	+	-	-	+	+	-	+	+	+	**β**	***B. cereus***
3d	**Blue**	**Blue**	+	**Oval**	-	-	-	+	-	+	-	+	+	-	+	+	+	+	+	+	**β**	***B. cereus***
3e	Yellow	Yellow	-	Circular	+	X	X	+	+	+	-	+	+	-	-	+	+	-	+	+	ɣ	B. sphaericus
3f	**Blue**	**Blue**	+	**Oval**	-	-	-	-	-	-	-	+	+	-	+	+	-	+	+	+	**β**	***B. cereus***
5a	Cream	Yellow	+	Oval	-	X	X	+	-	+	-	-	+	-	+	+	-	-	+	-	ɣ	B. brevis
5c	Blue	Yellow	-	Oval	+	X	X	+	+	-	-	-	+	-	+	+	+	+	+	-	β	B. subtilis
5d	Cream	Yellow	+	Oval	-	X	X	+	+	+	-	-	+	-	+	+	+	+	+	+	β	B. megaterium
6c	Cream	Yellow	+	Oval	-	X	X	+	-	+	-	-	+	-	+	+	-	+	+	+	β	B. pumilus
6d	Cream	Yellow	+	Oval	-	X	X	+	-	+	-	+	+	-	+	+	-	+	+	+	β	*B. spp*
6e	Green	Yellow	-	Oval	-	X	X	+	-	+	-	+	+	-	+	+	-	+	+	+	β	*B. spp*
6h	Cream	Yellow	-	Oval	+	X	X	+	-	+	-	-	+	-	-	+	-	-	+	+	ɣ	B. sphaericus
6i	Green	Yellow	-	Oval	-	X	X	+	-	+	-	+	+	-	-	+	-	+	+	+	β	*B. spp*
6l	Yellow	Yellow	+	Oval	-	X	X	+	-	+	-	-	+	-	+	+	-	+	+	+	β	B. laterosporus
6m	Green	Yellow	+	Oval	-	X	X	+	+	+	-	-	+	-	-	+	-	+	+	+	ɣ	*B. spp*
6x	**Blue**	**Blue**	+	**Oval**	-	-	-	+	-	-	-	-	+	-	+	+	+	+	+	+	**ɣ**	***B. anthracis***
7b	Green	Yellow	+	Oval	-	X	X	+	-	-	+	+	+	-	+	+	-	+	+	+	β	*B. spp*

7c	Yellow	Yellow	-	Circular	+	X	X	+	-	+	+	-	+	-	-	+	-	-	+	-	ɣ	B. sphaericus
7e	Yellow	Yellow	-	Circular	+	X	X	+	+	+	+	-+	+	-	-	+	-	-	-	+	ɣ	B. sphaericus
7h	Cream	Yellow	+	Circular	+	X	X	+	-	+	-	-	+	-	-	+	-	+	+	+	ɣ	B. pasteurii
7i	Yellow	Yellow	-	Oval	-	X	X	+	-	-	-	+	+	-	+	+	-	+	+	+	ɣ	B. pumilus
7j	Cream	Yellow	+	Oval	+	X	X	+	+	+	-	-	+	-	+	+	-	+	+	-	β	*B. spp*
8a	**Blue**	**Blue**	**+**	**Oval**	**-**	**-**	**-**	+	**-**	**-**	**-**	**+**	**-**	**-**	**+**	**+**	**-**	**+**	**+**	**+**	**β**	***B. cereus***
8b	**Blue**	**Blue**	**+**	**Oval**	**-**	**-**	**-**	+	**-**	+	**-**	**+**	+	**-**	**+**	**+**	+	**+**	**+**	**+**	**β**	***B. cereus***
8g	Blue	Yellow	-	Oval	-	X	X	+	-	+	+	-	+	-	+	+	-	+	+	+	β	B. megaterium
8i	Cream	Yellow	+	Oval	-	X	X	+	-	+	-	+	+	-	+	+	-	+	-	+	ɣ	*B. spp*
8j	Cream	Yellow	+	Oval	-	X	X	+	-	-	-	-	+	-	-	+	+	+	+	-	β	B. alvei

Table 2 Results of biochemical testing and presumptive identification of respective isolates

The results of antibiotic susceptibility testing are given in the following table:

Antibiotic	*1x*	*1y*	*2x*	*2y*	*3a*	*3b*	*3c*	*3d*	*3f*	*6x*	*8a*	*8b*	*10f*	*10j*	*10k*	*10l*	*11p*	*13i*	*14p*	*14q*	*14n*	*14r*	*14x*	*14y*
amik	27	24	24	24	23	23	25	26	24	22	21	24	25	24	23	26	22	25	21	21	25	22	22	23
amox	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ampi	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
azyth	25	18	18	22	18	24	15	22	24	19	15	19	20	18	21	19	19	19	20	19	25	21	21	22
cefp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9.5	-	-	-	-	-	-	-	-
cefur	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
chlor	22	18	21	18	28	23	19	22	25	19	22	26	19	17	23	25	20	23	18	23	23	20	19	23
Cipro	31	31	28	21	26	26	32	28	30	25	28	28	30	32	29	32	25	27	27	27	28	28	27	29
clind	24	24	24	16	18	24	16	20	27	23	18	24	19	17	24	23	21	21	21	20	23	21	21	23
cot	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
doxy	31	23	21	25	22	29	33	22	32	24	24	27	28	27	31	32	24	25	24	26	26	25	24	25
eryth	24	15	14	22	23	22	26	23	25	22	25	17	17	19	14	12.5	22	22	22	21	24	23	22	23
genta	26	24	23	23	22	23	24	24	22	21	18	23	24	23	24	24	21	20	20	20	26	21	22	22
imi	38	36	36	37	34	42	40	36	42	37	38	36	42	38	42	43	33	35	35	34	42	36	36	37
levo	29	26	22	25	25	26	27	26	28	26	26	27	28	27	32	30	26	25	25	26	28	25	24	27
line	28	29	27	16	26	27	18	25	31	22	19	26	20	18	27	27	25	22	24	24	24	23	27	24
mero	27	25	25	22	23	27	28	23	29	25	27	26	27	26	30	29	23	25	24	25	27	25	25	25
moxi	26	26	25	22	24	24	28	25	28	26	26	21	28	26	30	30	24	24	24	24	26	24	24	26
nal	22	24	22	13	22	20	17	25	24	21	17	19	20	17	22	25	20	22	27	23	23	21	21	23
nitro	20	19	20	11	14	17	17	22	21	15	14	20	14	13	25	20	20	17	19	14	9	15	18	16
norfl	24	23	22	21	22	24	28	22	28	21	22	23	25	28	23	28	22	22	22	21	24	22	23	24
pen	-	-	-	**R**	-	-	-	-	-	**R**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
rifa	15	15	15	8	11	13	8	14	14	9	10	12	9	-	17	16	7	10	11	10	8.5	10	11	10
tetra	31	23	19	20	21	28	20	25	31	21	19	28	22	20	32	29	23	21	24	23	24	24	23	23
van	21	22	20	15	18	24	17	21	22	19	18	20	18	16	25	19	17	16	21	17	17	19	18	17

Table 3 Results of antibiotic susceptibility tests of presumptive *Bacillus cereus* isolates

Resistant Intermediate Susceptible

Out of 25 antibiotics tested, the isolates were seen to have complete resistance against 6: Penicillin, Rifamficin, Cotrimoxazole, Cefuroxime, Cefepime, Amoxyclav and Ampicillin. Resistance to Penicillin was seen in 2 isolates which also showed no haemolysis on 5% SBA. These were further subjected to MIC testing using Benzathine Penicillin to confirm if they could be *B. anthracis*. Results of MIC test were positive; therefore, along with the lack of motility and haemolysis, as well as characteristic staining pattern, the 2 isolates in question were taken to be *B. anthracis*. On the other hand, most of the antibiotics tested showed to be effective against B. *cereus* presumptive isolates. However, a marked degree of intermediate resistance was seen in Erythromycin and Clindamycin.

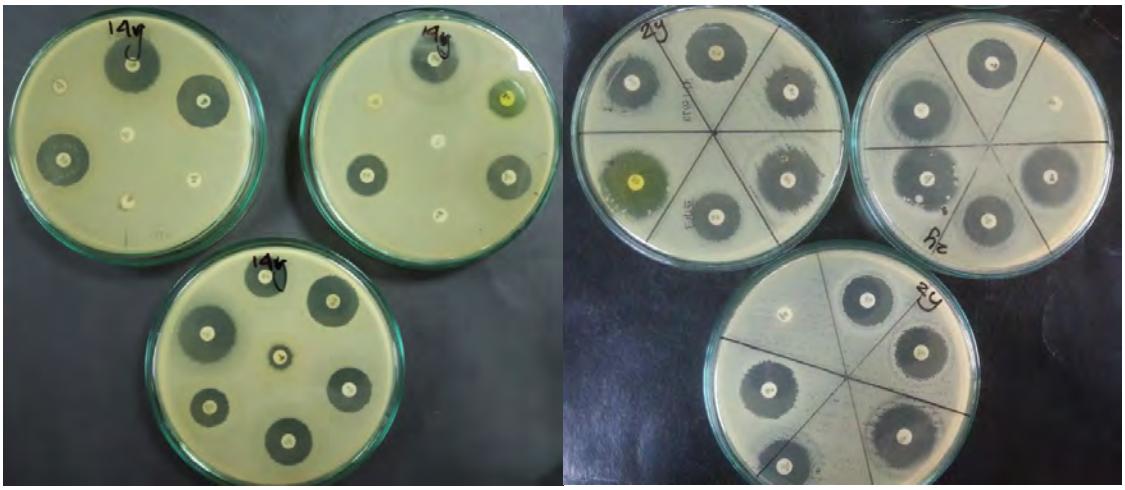

Figure 2 Antibiotic susceptibility testing via Kirby Bauer Disk Diffusion on Mueller Hinton agar

On SBA, 2 isolates (taken as being *B. anthracis*) showed no haemolysis while the remaining 22 showed complete breakdown of red blood cells.

On HBA, out of 24 isolates tested, the number of presumptive *Bacillus cereus* isolates that showed discontinuous haemolysis pattern, which indicates HBL toxin production, were 15. Of the remaining 9 isolates, 2 that showed no haemolysis on SBA were not plated on HBA, while the remaining 7 showed only continuous haemolysis on both media.

The table below shows the results of haemolysis experiments using 5% sheep's blood agar as well as HB agar. The figures below depict examples of both continuous and discontinuous haemolysis results on HB agar media.

Isolate no.	*Haemolysis on 5% SBA*	*Discontinuous haemolysis on HBA*
1x	β	-
1y	β	-
2x	ɣ	-
2y	β	x
3a	β	+
3b	β	-
3c	β	-
3d	β	+
3f	β	-
6x	ɣ	x
8a	β	-
8b	β	+
10f	β	+
10j	β	+
10k	β	+
10l	β	+
11p	β	+
13i	β	+
14p	β	+
14q	β	+
14n	β	+
14r	β	+
14x	β	+
14y	β	+

Table 4 Type of haemolysis shown on HBA by presumptive *Bacillus cereus* isolates

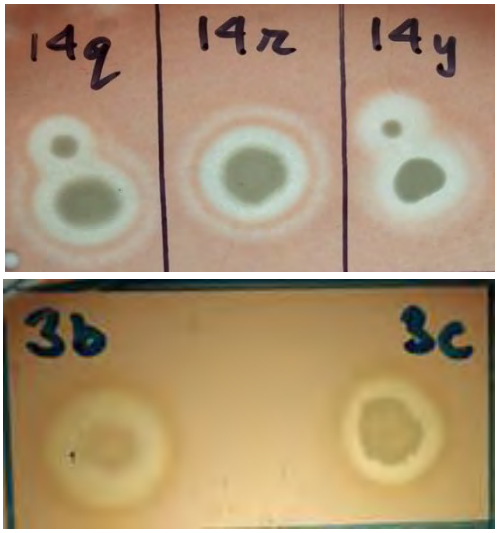

Figure 3 Detection on HBL toxin on HBA via a) discontinuous and b)continuous haemolysis

** β = beta or complete haemolysis*

**ɣ = gamma or no haemolysis*

**α = alpha or partial haemolysis*

Below are shown the images depicting exoenzyme and haemolysin production. Clear zones around bacterial colonies show a positive result while lack of clearing indicates a negative result. In the case of gelatinase test, a positive result is indicated by liquefaction of the media, showed here by movement of the media in the positive tube, while the control tube remains still when the tubes were tilted.

Figure 4 Hydrolysis on a) Tributyrin agar b) casein agar c) starch agar d), e) sheep blood agar f) gelatin medium

The table below summarises the results of the exoenzyme and haemolysis experiments.

	UHT milk	Milk powder	Processed honey	Fruit juice	Prevalence		
					Total positive	Percentage, total	Percentage, *B. cereus*
Exoenzyme activity							
Lecithinase	12	14	16	17	59	(76.6%)	X
Amylase	7	5	5	15	32	(41.5%)	X
Caseinase	13	18	13	16	60	(77.9%)	X
Gelatinase	15	20	17	21	73	(94.8%)	X
Lipase	14	17	16	14	61	(79.2%)	X
Haemolysis on 5% SBA							
β	13	13	13	9	29	(62.3%)	X
γ	2	9	6	12	0	(37.6%)	X
α	0	0	0	0	15	(0%)	X
Haemolysis on HBL agar							
Discontinuous	2	1	5	7	15	(19.5%)	(62.5%)
Continuous	6	1	0	0	7	(9.1%)	(29.2%)
Non-	1	1	0	0	2	(2.6%)	(8.3%)

Table 5 Enzymatic and haemolytic activity of *Bacillus* isolates

Chapter 5:
Discussion

Bacterial contamination of food is a common scenario in Bangladesh, where hygiene, quality control and handling regulations are not stringently followed [38]. While numerous studies have been undertaken in the past regarding the prevalence of pathogenic bacteria in foods (both packaged and unpackaged), the study of endospore forming bacteria in packaged food products has not, to the best of this author's knowledge, been carried out in Bangladesh. Nevertheless, in the case of packaged foods, one generally expects the contents to be sterile and free of contaminant pathogens. Which is why the findings of this study are a cause of concern to the food and health industry. Out of 16 samples across 4 categories, 80 individual endospore forming organisms of the genus *Bacillus* were isolated, 24 of which belonged to the pathogenic subgroup *Bacillus cereus* sensu lato; the remaining 56 were classified as *Bacillus spp*. and included members of the *Bacillus* family which are responsible for food spoilage such as B. pumilus, B. subtilis and B. licheniformis. Every category of food tested yielded at least 2 isolates of the *B. cereus* species. Further findings were 4 isolates of *B. thuringiensis* and 2 counts of *B. anthracis*. While finding *B. cereus* is itself a cause for concern, the latter two highlight other issues as well: *B. anthracis* is a potent pathogen and exposure to active cells or even spores in the appropriate concentration can be fatal; *B. thuringiensis* is used globally as a biopesticide so the presence of its spores in packaged food products is proof of the highly resistant and adhering quality of endospores, as they have survived all the way down the production line and the high-stress sterilization and processing methods that are involved.

Antibiotic susceptibility testing yielded no highly resistant profiles. However, MAR of all 24 isolates tested were all 0.28 and above, indicating that these strains have been in contact with antibiotics before. In addition, many isolates showed inhibition zones that were just on the cusp of intermediate and resistant, or intermediate and susceptible. While this may be due to handling and human error, there is a chance that this indicates growing resistance to antimicrobials. Bacilli have a natural tendency for spontaneous mutation, so resistance may be acquired that way. The high genetic similarity between most species of *Bacillus spp*. also suggests that plasmids can easily be exchanged, which can be another may for *Bacillus* to acquire antibiotic resistance if the resistance genes lie on a plasmid [39].

In addition to finding the prevalence of aerobic endospore formers, this study also attempted to evaluate the virulence and diarrheal toxin producing potential of the isolates bacteria. Haemolysin BL in the most well characterized diarrheal toxin of *Bacillus cereus*. 24 presumptive *B. cereus* isolates were screened for HBL production using HB agar, first described by Beecher and Wong [5, 7]. 15

(62.5%) of them showed the distinct discontinuous haemolysis ring on the agar plate, which indicates production of diarrheal toxin. This is a cheap and simple method for diarrheal toxin detection, and might be a good alternative for places where toxin detection kits are too expensive. Also, currently available kits only detect 1 out of 3 components of HBL, which is not always indicative of whether the bacteria will cause diarrhea in vivo, because pathogenesis requires the presence of all 3 components. In that respect, HB agar is a better alternative as it will only show the discontinuous pattern if all 3 components are present, because the erythrocytes must be primed with B component before being lysed by L1 and L2 components [17, 18]. It was also found that 4 isolates were positive for both parasporal crystal as well as HBL production. *B. thuringiensis* with the ability to cause diarrhea is an alarming idea because the use of Bt pesticide can leave endospores on crops, which can then enter the human gut on consumption and potentially cause food poisoning.

It was found that most of the isolates were positive for hydrolysis of starch, casein, lipid, gelatin and sheep blood, meaning they produce amylase, protease, lipase, gelatinase and haemolytic enzymes. Lecithinase production was also visualized on *Bacillus Cereus* Selective Agar, with precipitate formation indicating presence of phospholipases. These are all indicators of virulence, as the ability to hydrolyse various substrates enables bacteria to move about, invade and adhere to host cells and tissues, as well as to break them down for the bacteria's own use, thus damaging the host. It has also been reported that *Bacillus* species other than *B. cereus* can also cause food-poisoning. While this study did not attempt to screen non-*cereus* Bacilli for toxin production, this is another avenue that should be explored if possible, as most *Bacillus* species are considered to be non-pathogenic and thus not scrutinized or investigated for during food-related illness.

5.1 Conclusion

This study reported 80 endospore forming bacteria of genus *Bacillus* isolated from commercial packaged foods in Dhaka city. This large a number of contaminants indicate that there is a gap in food packaging and sterilization procedures here, which should be looked into because these bacteria also have the potential to cause food-borne disease as well as food spoilage. Most of the isolated organisms showed the ability to break down starch, casein, lipid and gelatin and showed complete haemolysis on sheep's blood agar, in addition to 15 isolates testing positive for haemolysin BL, a diarrheal toxin. All these factors signify that the isolated organisms have virulence potential and can cause disease in the human body. Still, presence of the above mentioned characteristics in vivo is not

a conclusive method. In order to confirm their presence, gene-based PCR and gel electrophoresis could be carried out, as well as verifying the identification of isolated species using 16S rRNA sequencing. Further work that may be done is testing of cytotoxicity using Vero cells and testing for demermonecrotis in rabbit ileal loop assays, to check whether the toxins works in vivo as well. A larger variety of samples could also be tested, in order to find a more standardized estimate of the prevalence of *Bacillus spp*. in Bangladeshi packaged foods.

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Appendices

Appendix – I Media composition

The following media was used during the study. All components were autoclaved at 121°C, 15 psi for 15 minutes unless mentioned otherwise.

Motility Indole Urease (MIU) Agar	
Ingredients	*Gms / Litre*
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

Nutrient Agar	
Ingredients	*Gms / Litre*
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Peptone saline diluent	
Ingredients	*Gms / Litre*
Peptone	1.0 g
Sodium chloride	8.5 g

Simmon's Citrate Agar	
Ingredients	*Gms / Litre*
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

Saline	
Ingredients	*Gms / Litre*
Sodium chloride	9.0

Methyl Red Voges- Proskauer Media	
Ingredients	*Gms / Litre*
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Nutrient Broth	
Ingredients	*Gms / Litre*
Nutrient Broth	13.02

Starch Agar	
Ingredients	*Gms / Litre*
Meat Extract	3.000
Peptic digest of animal tissue	5.000
Starch, soluble	2.000
Agar	15.000
Final pH (at 25°C) 7.2±0.1	

Blood Agar Base	
Components	*Gms / Litre*
Proteose peptone	15.000
HML extract #	2.500
Yeast extract	5.000
Sodium chloride	5.000
Agar	15.000
Final pH (at 25°C) 7.4±0.2	

Tributyrin Agar Base	
Ingredients	*Gms / Litre*
Peptic digest of animal tissue	5.000
Yeast extract	3.000
Agar	15.000
Final pH (at 25°C) 7.5±0.2	

Gelatin agar	
Ingredients	*Gms / Litre*
Gelatin	120.0gm
Peptone	5.0gm
Beef Extract	3.0gm
Final pH 6.8 +/- 0.2 at 25ºC.	

Mueller-Hinton Agar	
Ingredients	*Gms / Litre*
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH 7.3 ± 0.1 @ 25°C	

Skim Milk Agar	
Ingredients	*Gms / Litre*
Meat Extract	3.000
Peptic digest of animal tissue	5.000
Starch, soluble	2.000
Agar	15.000
Final pH (at 25°C) 7.2±0.1	

***Bacillus cereus* Selective Agar Base**	
Ingredients	*Gms / Litre*
Peptone	1.000
Mannitol	10.000
Sodium chloride	2.000
Magnesium sulphate	0.100
Disodium phosphate	2.500
Monopotassium phosphate	0.250
Sodium pyruvate	10.000
Bromo thymol blue	0.120
Agar	15.000
Final pH (at 25°C) 7.2±0.2	

Brain Heart Infusion Broth	
Components	*Gms / Litre*
Brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
pH 7.4 ± 0.2 @ 25°C	

Appendix II Reagents

1. Barritt's reagent

Solution A: 5 g alpha-naphthol was dissolved in 95% ethanol. The reagent was covered in aluminum foil and stored at 4°C. **Solution B:** 40 g KOH was dissolved in distilled water. Once the mixture cooled, creatine was added. Final volume was adjusted with distilled water and the reagent covered with aluminum foil was stored at 4°C.

2. Iodine solution (Gram's)

6.7 g potassium iodide was dissolved in 100 mL of distilled water. To this, 3.3 g of iodine was added, stirred, and the solution made up to 1 liter with distilled water. The reagent bottle was covered in aluminium foil and stored at room temperature.

3. Kovac's reagent

5 g para-dimethylaminobenzaldehyde was dissolved in 75 mL amyl alcohol. To this, hydrochloric acid (1M) was added to make up the final volume of 25 mL. The reagent bottle was covered with aluminium foil and stored at 4°C.

4. Malachite green (0.5%)

0.5 g malachite green was dissolved in 100 mL distilled water. The solution was stored at room temperature by covering the reagent bottle with aluminium foil.

5. Methylene blue solution (1%)

1 g of methylene blue was dissolved in 75 mL of distilled water, and then diluted to make 100 mL. The solution was filtered out and stored in reagent bottle.

6. Methyl red reagent

0.1 g methyl red was dissolved in 300 mL of 95% ethyl alcohol. To this, distilled water was added to make up the final volume of 500 mL. The reagent was covered with aluminum foil and stored at 4°C.

7. Oxidase reagent

100 mg of N, N, N1, N1-tetramethyl-p-phenyldiamine-dihydrochloride was dissolved in 10 mL distilled water. The solution was covered with aluminum foil and stored at 4°C.

8. Safranin

0.1 g of safranin was dissolved in 75 mL of distilled water. The solution was diluted to 100 mL, filtered and stored in clean reagent bottle.

9. Coomassie blue

0.25g Coomassie Blue powder was dissolved in 50ml ethanol and 7ml acetic acid, before the volume was made up to 100ml with distilled water.

Appendix III - Instruments

Instruments	**Company**
Autoclave	SAARC
Cellulose filter paper (9.0 cm)	Whatman
Colorimeter, ISO 9001	Labtronics, India
Freeze (-20°C)	Siemens
Incubator	SAARC
Hotplate stirrer	LabTech
Micropipette (10-100 µL)	Eppendorf, Germany
Micropipette (100-1000 µL)	Eppendorf, Germany
Microscope	Optima
pH meter, Model: E-201-C	Shanghai Ruosuaa, Technology company, China
Pipette (5 mL, 10 mL)	Eppendorf, Germany
Refrigerator (4ºC), Model: 0636	Samsung
Safety cabinet, Class II Microbiological	SAARC
Surgical Millipore syringe filter (0.22µm)	Millex-GS
Shaking Incubator, Model: WIS-20R	Daihan Scientific, Korea
Vortex Mixture	VWR International
Weighing balance	ADAM, EQUIPMENT™, United Kingdom
Rotary evaporator	Heidolph, Made in Germany
Shaking Incubator	Model: JSSI-1000C JS RESEARCH INC. Made in Rep. of Korea