EFFECTS OF ENVIRONMENTAL STRESSES ON THE SURVIVAL AND PRODUCTION OF DIARRHOEAL ENTEROTOXINS BY *BACILLUS CEREUS* FROM SOME FOOD SOURCES

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BY

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DEPARTMENT OF MICROBIOLOGY
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NIGERIA

OCTOBER, 2014

DECLARATION

I declare that the work reported in this Dissertation entitled "Effects of environmental stresses on the survival and production of diarrhoeal enterotoxins by *Bacillus cereus* from some food sources" has been carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Dissertation was previously presented for another degree or diploma at this or any other institution.

Sakina BELLO

Name of student	Signature	Date

CERTIFICATION

This dissertation entitled EFFECTS OF ENVIRONMENTAL STRESSES ON THE SURVIVAL AND PRODUCTION OF DIARRHOEAL ENTEROTOXINS BY *BACILLUS CEREUS* FROM SOME FOOD SOURCES by Sakina BELLO meets the regulations governing the award of the degree of Ph.D Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literacy presentation.

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DEDICATION

To my parents, Ambassador and Mrs Hamman Bello Maiha, for their endless nurturing, support and encouragement.

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ABSTRACT

This research work was conducted with the aim of studying the effects of environmental stresses on the survival and production of the Haemolysin BL (HBL) diarrhoeal enterotoxin by *Bacillus cereus* food isolates. A total of 387 food samples were purchased from street food vendors and some markets in Kaduna north and Zaria local government areas of Kaduna state. Samples which comprised of 43 samples each of unpasteurized cow milk, locally fermented milk (**nono**), raw meat, roast meat products (s**uya, soye and balangu**), uncooked rice, **Jollof** rice and boiled rice were analyzed for presence of *B. cereus*. Appropriate 10 fold dilutions of each food sample was pour plated onto sterile Mannitol Egg-yolk Polymyxin agar (MYP)(OXOID) plates and incubated at 37^{o}C for 24-48 h. Presumptive *B.cereus* isolates were biochemically characterized with Microgen^{Tm} *Bacillus* identification kits (Microgen Bioproducts). To study the effects of varying pH level, sodium chloride (NaCl) concentration (W/W) and temperature on HBL production by isolates, confirmed *B.cereus* isolates; R3MI, F5MI, Bln3g2 and F2 from raw milk, **nono, balangu** and boiled rice were selected. Mcfarland standard 1(about 8.5 log units) of each isolate was inoculated into sterile freshly prepared corresponding food samples and stored at varying NaCl concentrations (0- 2%), pH levels (6.3,6.4,6.7,6.8 for milk and 3.7, 3.8, 4.2,4.3 for **nono**), and temperature (4-10^{o}C, 26-28^{o}C, 37^{o}C). These isolates were assayed for HBL enterotoxin production using BCET–RPLA toxin detection kits (OXOID), and Polymerase Chain Reaction (PCR) studies conducted determine the presence of HBL and BceT (*bceT)* genes in the isolates. Enterotoxin producing isolates were subjected to antibacterial susceptibility tests using the Kirby-Bauer method against 10 antimicrobial agents (OXOID); Ampicillin (10µg), Amoxicillin – clavulanic acid

(20/10 µg), Cefpodoxime (10µg), Ceftriaxone (30µg), Cephalothin (30µg), Amikacin (30µg), Tetracycline (30µg), Erythromycin (30µg), Ciprofloxacin (5µg) and Trimetoprim–Sulfamethoxazole (1.25/23.75µg). A total of 47 of 109 isolates from retail food samples were confirmed as *B. cereus,* and mean *B.cereus* counts ranged from 1.1±0.2 $\log_{10}$ cfu/ml for unpasteurized milk samples to 2.1±0.3$\log_{10}$ cfu/ml for s**oye**. Counts fell within safe limits (10^2 to $<10^3$), and were not significantly different amongst producers ($P>0.05$) for all samples. Food storage studies revealed decreases in *B.cereus* cells from initial levels of 8.5 log units in boiled rice samples at NaCl levels of 1.5 and 2%, at refrigeration temperature (4 – 10^oC) for 24 h. Counts were significantly different when analyzed across NaCl concentrations, and storage period ($P<0.05$). Decreases in *B.cereus* counts from initial levels of 8.5 log units were sustained in pasteurized milk samples (pH 6.3 and 6.8) for 48 h at refrigeration temperature (4 – 10^oC), and counts were significantly different across pH levels ($P<0.05$). Isolates from pasteurized milk at pH 6.8 following 48 h of refrigerated storage tested negative (< 2ng/ml) for HBL production, whereas most rice isolates had toxin titres of ≥ 64ng/ml. PCR studies revealed that both enterotoxin and non-enterotoxin producing isolates encoded for at least one of the *hbl* genes. The *bceT* gene was detected in 70% of rice isolates, and in only about 38% of pasteurized milk isolates. Isolates from pasteurized milk at pH 6.8 following 72 h of refrigerated storage (4 – 10^oC) were sensitive to Cephalothin, in contrast to other isolates. All but 96% of *B.cereus* **balangu** isolates were sensitive to Erythromycin, while none of *B.cereus* **nono** isolates appeared to be sensitive to Tetracycline. This study has established the presence of *B.cereus in* some ready –to-eat (RTE) and uncooked foods sold in some streets and markets of Kaduna metropolis. Food

storage studies showed *B. cereus* is inhibited in boiled rice at NaCl levels above 1% at refrigeration temperature (4-10^{o}C), for up to 48 h. Results also showed sustained inhibitory effects are exerted on *B.cereus* cells at pH levels of 6.3 and 6.8 in pasteurized cow milk for up to 48 h. Findings of this study also indicate storage conditions suppress the expression of the *hbl* genes in some *B.cereus* isolates from pasteurized milk. Decreased resistance to particular antibacterial agents is also induced in these isolates following 72 h of refrigerated storage (4 – 10^{o}C). Results highlight the need for enactment and enforcement of regulations for food service operators and vendors by the appropriate agencies with a view to providing safer RTE foods in Nigeria. There is also the need to carry out more researches to understand the mechanisms of survival and pathogenicity of food pathogens in locally consumed foods. Results of such researches also need to be incorporated in mandatory enlightenment programmes for food service operators to reduce the risk of foodborne illnesses associated with RTE foods.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

Bacillus cereus is an opportunistic pathogen of humans, causing local and systemic infections, and is a frequent cause of food poisoning. This species belongs to the *B. cereus* group, which includes the closely related species, *Bacillus mycoides, Bacillus pseudomycoides, Bacillus weihenstephanensis, Bacillus thuringiensis,* and *Bacillus anthracis* (Helgason *et al.*, 2000; Ehling-Schulz *et al*., 2005; Lindbäck and Granum, 2006). *Bacillus cereus,* the type species of the "*Bacillus cereus* group", *(Senesi and Ghelardi, 2010),* is a gram positive, rod shaped bacterium capable of facultative aerobic metabolism. The organism is widely distributed in the environment, mainly in the soil, from which it is easily spread to many types of foods especially those of vegetable origin. The organism has also been isolated from meat, milks and dairy products (Notermans *et al.,* 1997; Rowan *et* al, 2001; Keith *et al.,* 2004; Werner and Hotchkiss, 2007; Prestamo et *al.,* 2007). *B. cereus* can grow over a wide temperature range (8–55 °C), but it is not well suited to tolerate low pH values (minimum 5–6), (*Senesi and Ghelardi, 2010).*

Food-Borne Diseases (FBD) or illnesses are defined as diseases of infectious or toxic nature caused by agents that enter the body through the consumption of food (WHO, 2007). More than 250 FBDs have been described. Symptoms vary widely, depending on aetiological agents, with diarrhoea and vomiting being the most common. FBDs are caused by much different disease - causing pathogens that can contaminate foods, or by toxins produced by these pathogens that are present in food (Keith *et al.*, 2004).

Almost all types of food have been associated with *B. cereus* food poisoning. *B.cereus* levels in unprocessed foods are generally too low to cause food borne poisoning, however the ability of *B. cereus* to form spores ensures its survival through all stages of food processing. Subsequent time/temperature abuse enables low levels of *B. cereus* to multiply to dangerous levels (Food Safety Authority of Ireland, 2007). It has been referred to as the major problem in convenience foods and mass catering (EFSA, 2005). This ability to form endospores enables the bacteria to tolerate adverse conditions, better than most bacterial enteropathogens do. They may proliferate in a wide range of environments, including processed and untreated foods. (Phelps and Mckillip, 2002).

A three component complex system, designated haemolysin BL (HBL) is believed to be the major diarrhoeal toxin of *B. cereus* (Burgess and Horwood, 2006). The toxin is only active when all three components are present; the B component of HBL is required to bind to the erythrocytes first, before the L component can cause lysis and apparently only trace amounts of L are required to cause haemolysis (Heinrichs *et al.,* 1993). HBL is reportedly secreted by about 45–65% of *B. cereus* strains (Thaenthanee, 2005), although Ghelardi *et al.,* (2007), have reported variations in the percentage of HBL producing strains among clinical (81%) and environmental/food isolates (43%).

Little interest was shown in anti-microbial susceptibility profiles of Bacillus species until very recently (Turnbull *et al*., 2004). This was due to a combination of reasons: the low recognition of the ability of Bacillus species other than *Bacillus anthracis* to cause infections, the increasing rarity of human anthrax in industrialized countries as a result of effective control programs, and the high susceptibility of *Bacillus anthrax* to penicillin coupled with the extreme rarity of reports of penicillin resistance (Turnball *et al*., 2004).

The relevance of the Bacillus species as food poisoning organisms and as aetiological agents in non gastrointestinal infections including local deep tissue systemic infections is however being increasingly recognized. Infections in individuals who are intravenous drug abusers or immunocompromised as a consequence of infection with human immunodeficiency virus, chemotherapy or malignancy and neonates are sometimes severe and life threatening (Rowan *et al.,* 2003; Turnball *et al.,* 2004; Deindl *et al.,* 2007; *Senesi and Ghelardi, 2010).* These and concerns about bioagression around the time of the 1991 Gulf war resulted in some examination of the effectiveness of more modern antimicrobials on *Bacillus* species.

The organism has been found to be susceptible to antibiotics like Ciprofloxacin Tetracycline, Vancomycin, Chloramphenicol, Amoxicillin - clavulanic acid (Turnball *et al.* 2004; Luna *et al.,* 2007). From the many case reports of *B. cereus* infections, the broad picture is one of resistance to penicillin, ampicillin, cephalosporins and trimethoprim (Turnball *et al.,* 2004). Varied responses also exist of the organism to some antibiotics; Luna *et al. (*2007) reported resistance of *B. cereus* to Erythromycin and Clindamycin, whereas many preceding case reports of *B. cereus* show the organism as susceptible to these antibiotics.

1.2 Statement of the Problem

In Nigeria, food vending in open places such as motor parks, offices and market places is practised without any regulations guiding the microbiological quality of such foods. Consumers and regulatory bodies most times portray a carefree attitude to the glaring

hazard of improper handling and storage of ready-to-eat foods. These practices would inadvertently expose consumers to foodborne illnesses as several pathogenic microorganisms including *B.cereus*, have been isolated from many street vended foods in some Nigerian towns and cities (Umoh and Odoba, 1999; Bello, 2002; Omemu *et al.*, 2005; Whong *et al*., 2006; Wogu *et al*., 2011).

1.3 Justification for Study

Bacillus cereus spores are extremely heat resistant, so while cooking at proper temperatures would destroy most food pathogens, including the vegetative cells of *B. cereus,* it does not destroy the spores, rather they can be activated by heat and or improper handling (Keith *et al.,* 2004). In addition, reports of some studies have shown the influence of variations in environmental conditions on the growth, survival and production of diarrhoeal enterotoxins by *Bacillus cereus* (Beuchat *et al,* 1997; Rowan *et al.,* 2001; Hikmate and Mercedec, 2002; Prestamo *et al.,* 2007). Changes in gene expression or cellular phenotype in organisms occur in some instances without any changes to the underlying DNA sequences of the organism (http://en.wikipedia.org/wiki/epigenetics, retrieved 02/09/2012), but can be associated with chemical modifications of DNA, or of the structural and regulatory proteins bound to it (Fenselfeld, 2014). These changes may remain through cell divisions for the remainder of the cell's life and may also last for multiple generations (Bird, 2007).

Extensive studies on the effects of environmental stresses on the ability of *Bacillus cereus* to survive and produce enterotoxins in food could therefore play a significant role in the quest for safe ready - to - eat foods. Knowledge of these mechanisms would enable application of measures to control *B.cereus* food transmitted infections.

1.4 Aim and Objectives

1.4.1 Aim

This research was undertaken to study the effects of environmental stresses on the survival and production of diarrhoeal enterotoxins by *Bacillus cereus.*

1.4.2 Specific objectives of study

The specific objectives of this study include to:

1. Isolate *Bacillus cereus* from selected food samples (raw and cooked meat, uncooked and cooked rice, raw cow milk and fermented cow milk).
2. Characterize presumptive *Bacillus cereus* isolates obtained from raw and cooked meat, uncooked and cooked rice, raw cow milk and fermented cow milk.
3. Grow and enumerate confirmed isolates in freshly prepared food samples incubated at varying conditions of sodium chloride concentrations, pH and temperature.
4. Determine the diarrhoeal enterotoxin producing ability of *Bacillus cereus* isolates grown at varying conditions of sodium chloride concentrations, pH and temperature.
5. Molecularly characterize enterotoxin and non-enterotoxin producing isolates.
6. Determine the susceptibility of *Bacillus cereus* isolates grown under varying conditions of sodium chloride concentrations, pH, and temperature to selected antibiotics.

1.5 Limitations of Study

Some challenges were encountered in the course of carrying out some objectives of this study, specifically during sampling from some food sellers and vendors. Skepticism and disapproval on the part of these food sellers prevented extensive sampling of samples, and also resulted in lower numbers of sampling points for some food products (**soye, balangu** and raw milk).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Bacillus cereus*

2.1.1 Description and characteristics

Bacillus cereus is a large, 1.0 x 3.0-4.0 µm, Gram-positive, rod-shaped, endospore forming, facultative anaerobic bacterium (Vilain *et al.,* 2006). It was first successfully isolated in 1969 from a case of fatal pneumonia in a male patient and was cultured from the blood and pleural fluid (Hoffmaster *et al.,* 2006). The bacterium is considered to be the type species of the *B. cereus* group bacteria, a group of five closely related species: *Bacillus mycoides, Bacillus pseudomycoides, Bacillus weihenstephanensis*, *Bacillus thuringiensis,* and *Bacillus anthracis* (Ehling-Schulz *et al*.,2005; Lindbäck and Granum, 2006). These bacteria are so closely related genetically, that 16S and 23S rRNA sequence analyses and DNA-DNA hybridization (Kaneko *et al*., 1978; Ash *et al.*, 1991; Ash *et al*., 1992), have failed to clearly separate these taxa, as such they are considered as variants of a single species.

Differences in both pathogenic properties and phenotypic traits of strains belonging to these subspecies have led to retention of their original nomenclature (Senesi and Ghelardi, 2010). While *B. anthracis* is the causative agent of the highly infectious disease anthrax (Baillie and Read, 2001), *B. cereus* is a soil bacterium behaving as a human opportunistic pathogen, and *B. thuringiensis* is an entomopathogenic bacterium used as a bio-pesticide worldwide (Hofte and Whiteley, 1989). *B. mycoides* and *B.*

pseudomycoides (Nakamura, 1998), also classified as *B. mycoides* groups 1 and 2 (Callahan et al., 2009), are environmental species that display rhizoidal growth on agar plates unlike other members of the *B. cereus* group. *B.weihenstephanensis* is distinguishable from mesophilic strains of *B. cereus* in the following characteristics: its ability to grow aerobically at 7 °C but not at 43 °C; the presence of typical signature sequences in the 16s rDNA, and in the major cold shock gene *cspA (Lechner et al., 1998).* 16s rRNA comparison reveals *Bacillus cereus* to be most related to *Bacillus anthracis*, the cause of anthrax, and *Bacillus thuringiensis*, an insect pathogen used as pesticide (DelVicchio et al., 2006). Although they have similar characteristics, they are distinguishable as *B. cereus* is most motile, *B. thuringiensis* produces crystal toxins, and *B. anthracis* is nonhaemolytic (United States Food and DrugAdministration, 2007).

B. cereus is mesophilic, with a growth temperature range of 10°C to 48°C (Schneider *et al*., 2004). It grows optimally at temperatures between 20°C and 40°C, and is capable of adapting to a wide range of environmental conditions. *B. cereus* has in recent times been divided into seven major phylogenetic groups (I to VII) with clear-cut differences in growth temperature ranges, suggesting that the genetic structure corresponds to "thermotypes" and showing the emergence of multiple psychrotrophic groups within *B. cereus* (Guinebretiere *et al.,* 2008). It has also been shown that some *B. cereus* strains contain both rDNA operons with mesophilic signatures, and psychrotolerant signatures similar to *B. weihenstephanensis* (Pruss *et al*., 1999). This suggests that intermediate forms between the two species might exist (Stenfors and Granum, 2001). *B.cereus* is reportedly able to grow over pH range of 4.3- 9.3, being frequently exposed to acidic conditions in the exudates of protons and organic acids in the plant rhizosphere

(Neumann and Martinoia, 2002) as well as to a vast array of foods at low pH where specific organic acids have been added as preservatives (Keijser *et al*., 2007).

Bacillus cereus is distributed widely in nature and is commonly found in the soil as a saprophytic organism (Vilain *et al*., 2006). The organism is also a contributor to the microflora of insects, deriving nutrients from its host, and is found in the rhizosphere of some plants (Vilain *et al*., 2006). *B. cereus* is a found in the gut microflora of invertebrates, and is an intestinal symbiont of arthropods where it exhibits filamentous growth in sow bugs, roaches, and termites (Margulis *et al*., 1998). It is also reportedly well adapted for growth in the intestinal tract of mammals as well (Stenfors-Arnesen *et al*., 2008). Arthropods consume faeces or soil with spores or cells of *B. cereus*. This intestinal stage of *B. cereus*, also known as the *Arthromitus* stage, involves the attachment of endospore fibers released from a parent cell (Margulis *et al*., 1998). After attachment of spores, they begin motile and filament growth stages and attach to the epithelium (Margulis *et* al., 1998). *B. cereus* cells are then defeacated back into the soil where they can continue to grow.

B. cereus is a facultative anaerobe so it can utilize oxygen as a terminal electron accepter, but also has methods of anaerobic respiration as a mechanism of energy release. Whole genome sequencing revealed genes encoding for metabolic enzymes such as NADH dehydrogenases, succinate dehydrogenase, complex III, non-proton-pumping cytochrome bd quinol oxidases, and proton-pumping oxidases such as cytochrome c oxidase and cytochrome aa3 quinol oxidase (Duport *et al* ., 2006). In aerobic respiration, reducing equivalents produced from glycolysis and the Krebs cycle are reoxidized by the electron transport chain, creating a proton motive force and ATP by ATP synthase

(Duport *et al*., 2006). In anaerobic respiration, *B. cereus* utilizes fermentation to generate energy. Fermentation recycles NAD+ by reducing pyruvate and produces lactate and ethanol (Duport *et al*., 2006). ATP is generated by substrate level phosphorylation. *B. cereus* can metabolize a variety of compounds including carbohydrates, proteins, peptides and amino acids for growth and energy. Some of the major products produced from carbon sources such as sucrose or glucose during anaerobic respiration include L-lactate, acetate, formate, succinate, ethanol, and carbon dioxide (Mols *et al*., 2007). During nitrate respiration, nitrate reductase converts nitrate into nitrite which is converted to ammonium by nitrite reductase (Mols *et al*., 2007).

Bacillus cereus cell structure consists of an inner membrane and a thick peptidoglycan which functions to maintain cell shape (Ticknor *et al*., 2001). The peptidoglycan of some *B. cereus* strains are unique with only a few oligomers present, the cross-linked muropeptides are dimers, and many of the muropeptide lack the N-acetly group (Severin *et al*., 2004). These distinguishing features affect cell surface charge which contributes to the attachment of an outer capsule or an S-layer in pathogenic strains. Clinical isolates of *B. cereus* have a glycoprotein S-layer over its peptidoglycan which consists of proteinaceous paracrystalline arrays and covers the cell surface. The S-layer is involved in the virulence of *B. cereus* and functions to promote interactions with human polymorphonuclear leucocytes (Mignot *et al*., 2001 b). It also enables *B. cereus* to adhere to laminin, type I collagen, fibronectin, and fibrinogen of the epithelium, and thus has a role in increasing interaction between *B.cereus* and its host (Mignot *et al*., 2001b). In addition, this proteinaceous layer enhances its resistance to radiation.

B. cereus is motile by means of flagella and exhibits two types of motility: swimming and swarming, depending on the enivronment. Single cells exhibit swimming motility by means of short flagellated rods (Senesi *et al*., 2002). On the other hand, swarming is a collective movement of swarm cells with flagellum that is observed to be three to four times longer, and also forty times more flagellated than single swimming cells (Senesi *et al*., 2002).

2.1.2 Endospore formation in *Bacillus cereus*

An endospore is a dormant, tough, and non-reproductive structure produced by certain bacteria from the Firmicute phylum. The name "endospore" is suggestive of the bacterium changing internally to a spore or seedlike form (*endo* means within), but it's not a true spore (not an offspring). The endospore becomes important when the bacterium is experiencing an environment that is deleterious to the usual vegetative state of the bacterium, including when the bacterium is getting dried out (desiccated). When a bacterium detects environmental conditions are becoming unfavourable it may start the process of endosporulation, which takes about eight hours. The DNA is replicated and a membrane wall known as a spore septum begins to form between it and the rest of the cell. The plasma membrane of the cell surrounds this wall and pinches off to leave a double membrane around the DNA, and the developing structure is now known as a forespore. Calcium dipicolinate is incorporated into the forespore during this time. Next the peptidoglycan cortex forms between the two layers and the bacterium adds a spore coat to the outside of the forespore. Sporulation is now complete, and the mature endospore will be released when the surrounding vegetative cell is degraded.

Endospores enable the survival of a bacterium through periods of environmental stress. When the environment returns to favourable state, the endospore can reactivate itself to the vegetative state. Not all, nor even most, types of bacteria can change to the endospore form. Examples of those that can do so include *Bacillus* and *Clostridium* species. The endospore consists of the bacterium's DNA and part of its cytoplasm, surronded by a very tough outer coating (http:/en.wikipedia.org/wiki/endospore, retrieved 16/03/2008).

Endospores can survive without nutrients. They are resistant to ultraviolet radiation, desiccation, high temperature, and chemical disinfectants. Heating at 100°C for 5 minutes however reportedly results in cellular damage to the membranes and ribosomes (Logan and Rodrigez-Diaz, 2006; Majcher *et al*., 2008). Common anti-bacterial agents that work by destroying vegetative cell walls are ineffective on endospores. Endospores are commonly found in soil and water, where they may survive for long periods of time (http:/en.wikipedia.org/wiki/endospore, retrieved 16/03/2008). *B. cereus* spore formation occurs when nutrients are scarce within the environment and germinate into vegetative cells once they are available (Wijnands *et al*., 2006). Therefore, spore structure is important to the survival of this bacterium. *B. cereus* spores consist of an inner core surrounded by the inner membrane, and outer cortex surrounded by the outer membrane with an additional exterior coat (Kutima and Foegeding, 1987). The spore coat is made of proteins and small amounts of lipids and carbohydrates which contribute to its resistance to oxidizing agents and chemicals by blocking toxic molecules (Pol *et al*., 2001. In addition, the outer spore structure allows them to be heat and γ-radiation resistant (Pol *et al*., 2001). Spore germination is commonly in response to L-alanine which stimulates

germination events including hydrations of spores, loss of Ca2+ and dipicolinic acid, and metabolism (Pol *et al*., 2001).

The position of the endospore differs among bacterial species and is useful in identification. The main types within the cell are terminal, subterminal, and centrally placed endospores. Terminal endospores are seen at the poles of cells, whereas central endospores are more or less in the middle. Subterminal endospores are those between these two extremes, usually seen far enough towards the poles but close enough to the center so as not to be considered either terminal or central. Lateral endospores are seen occasionally

Examples of bacteria having terminal endospores include *Clostridium tetani*, the pathogen that causes the disease tetanus. Bacteria having a centrally placed endospore include *Bacillus cereus*, and those having a subterminal endospore include *Bacillus subtilis*. Sometimes the endospore can be so large the cell can be distended around the endospore, this is typical of *Clostridium tetani*. The arrangement of spore layers from the outermost to innermost layer is as follows: exosporium, spore coat, spore cortex and core wall.

Endospores are resistant to most agents that would normally kill the vegetative cells they are formed from. Household cleaning products generally have no effect, nor do most alcohols, quaternary ammonium compounds or detergents. Alkylating agents however, such as ethylene oxide, are effective against endospores. While resistant to extreme heat and radiation, endospores can be destroyed by burning or by autoclaving. Endospores are able to survive boiling at 100°C for hours, although the longer the number of hours the

fewer that will survive. An indirect way to destroy them is to place them in an environment that reactivates them to their vegetative state. They will germinate within a day or two with the right environmental conditions, and then the vegetative cells can be straightforwardly destroyed. This indirect method is called Tyndallization. It was the usual method for a while in the late 19th century before the advent of inexpensive autoclaves. Prolonged exposure to high energy radiation, such as x-rays and gamma rays, will also kill most endospores.

Reactivation of the endospore occurs when conditions are more favourable and involves activation, germination, and outgrowth. Even if an endospore is located in plentiful nutrients, it may fail to germinate unless activation has taken place. This may be triggered by heating the endospore. Germination involves the dormant endospore starting metabolic activity and thus breaking hibernation. It is commonly characterised by rupture or absorption of the spore coat, swelling of the endospore, an increase in metabolic activity, and loss of resistance to environmental stress. Outgrowth follows germination and involves the core of the endospore manufacturing new chemical components and exiting the old spore coat to develop into a fully functional vegetative bacterial cell, which can divide to produce more cells (http://en.wikipedia.org/wiki/endospore, retrieved 16/03/2008).

2.2 Microbial Food Poisoning

Food poisoning is defined as an illness caused by the consumption of food or water contaminated with bacteria and/or their toxins, or with parasites, viruses, or chemicals (Gamarra *et al*., 2009). Bacteria are responsible for approximately 75% of the outbreaks of food poisoning and for 80% of the cases with a known cause in the United States (Hughes and Angulo, 1996). As many as 1 in 10 Americans has diarrhoea due to food-borne infection each year.

The symptoms, varying in degree and combination, include abdominal pain, vomiting, diarrhoea, and headache; more serious cases can result in life-threatening neurologic, hepatic, and renal syndromes leading to permanent disability or death. Most of the illnesses are mild and improve without any specific treatment. Some patients have severe disease and require hospitalization, aggressive hydration, and antibiotic treatment. In children younger than 5 years, attacks range from 2-3 illnesses per child per year in developed countries; attacks are at least 5 times more common in developing countries. In underdeveloped countries, acute diarrhoeal diseases are responsible for 1 billion cases per year and 4-6 million deaths per year. (Gamarra *et al*., 2009).

Worldwide, diarrhoeal illnesses are among the leading causes of death. Travelers to developing countries often encounter food poisoning in the form of traveler's diarrhoea or "Montezuma's revenge" (http/www.emedecinehealth.com, retrieved 03/12/2008). Additionally, there are possible new global threats to the world's food supply through terrorist actions using food toxins as weapons. Increased virulence of known pathogens

has caused deadly outbreaks such as the *E. coli* STEC outbreak in Germany in 2011(Cunha, 2011)

Initially, food-borne diseases were estimated to be responsible for 6-8 million illnesses and as many as 9000 deaths each year (Hughes and Angulo, 1996; Smith, 1998). However, the change in food supply, the identification of new food-borne diseases, and the availability of new surveillance data have changed the morbidity and mortality figures. A study from the US Centers for Disease Control and Prevention (CDC) reports that food-borne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5000 deaths in the United States each year. Identified pathogens account for an estimated 14 million illnesses, 60,000 hospitalizations, and 1800 deaths. *Salmonella*, *Listeria*, and *Toxoplasma* organisms are responsible for 1500 deaths. Unidentified pathogens account for the remaining 62 million illnesses, 265,000 hospitalizations, and 3200 deaths. Overall, food-borne diseases appear to cause more illnesses but fewer deaths than previously estimated (CDC FoodNet Report, 2009).

According to a 2009 CDC study on food-borne disease outbreaks (for the year 2006), there were 1270 such outbreaks, or 27,634 cases, reported within 48 states (USA), with 11 deaths resulting (CDC, 2009). However, most cases of food poisoning occur sporadically, rather than as part of an outbreak. The etiologic agent was confirmed for more than 620 outbreaks, with *Norovirus* accounting for 54% of the outbreaks and a total of 11,879 cases. *Salmonella* was the second most frequent cause, accounting for 18% of the outbreaks and 3,252 cases. One of the 11 deaths resulted from a mushroom toxin; the rest were associated with bacteria (CDC, 2009). A food borne disease outbreak is defined by the following two criteria; Similar illness, often gastrointestinal, in a minimum of 2 people, and evidence of food as the source (Gamarra *et al.,*

2009). The pathogenesis of diarrhoea in food poisoning according to Gamarra *et al.* (2009) is classified broadly into either non inflammatory and inflammatory types:

2.2.1 Non inflammatory diarrhoea

This is caused by the action of enterotoxins on the secretory mechanisms of the mucosa of the small intestine, without invasion. This leads to large volume watery stools in the absence of blood, pus, or severe abdominal pain. Occasionally, profound dehydration may result. The enterotoxins may be either preformed before ingestion or produced in the gut after ingestion. Examples of organisms which produce such enterotoxins include *Vibrio cholerae,* enterotoxic *Escherichia coli , Clostridium perfringens, Bacillus cereus,* Staphylococcus organisms , *Giardia lamblia, Cryptosporidium,* rotavirus, norovirus (genus *Norovirus*, previously called Norwalk virus), and Adenovirus.

2.2.2 Inflammatory diarrhoea

This is caused by the action of cytotoxin on the mucosa, leading to invasion and destruction. The colon or the distal small bowel commonly is involved. The diarrhoea usually is bloody; mucoid and leukocytes are present. Patients are usually febrile and may appear toxic. Dehydration is less likely than with non nflammatory diarrhea because of smaller stool volumes. Fecal leukocytes or a positive stool lactoferrin test indicates an inflammatory process, and sheets of leukocytes indicate colitis. Sometimes, the organisms penetrate the mucosa and proliferate in the local lymphatic tissue, followed by systemic dissemination. Examples of such organisms include *Campylobacter jejuni, Vibrio parahaemolyticus,* enterohemorrhagic and enteroinvasive *E.coli, Yersinia enterocolitica, Clostridium difficile, Entamoeba histolytica, Salmonella* and *Shigella* species In some types of food poisoning (e.g Staphylococci, *B cereus*), vomiting is caused

by a toxin acting on the central nervous system. The clinical syndrome of botulism results from the inhibition of acetylcholine release in nerve endings by the botulinum (Gamarra *et al.,* 2009).

2.3 *Bacillus cereus* Food Poisoning

As a soil bacterium, *B. cereus* can spread easily to many types of foods such as plants, eggs, meat, and dairy products, and is known for causing 25 % of food-borne intoxications due to its secretion of emetic toxins and enterotoxins (United States Food and Drugs Administration, 2007). *B.cereus* is known to be able to produce five enterotoxins and an emetic toxin, which can be heat-stable or heat-labile depending on the strain (From *et al*., 2005; Logan and Rodrigez-Diaz, 2006).

The symptoms of food-borne disease caused by *B. cereus* are generally mild and self-limiting, but in rare instances they can also be life-threatening, as was shown in 1998 when a food-poisoning outbreak in France, which was attributable to *B. cereus*, caused the deaths of three persons (Lund *et al*., 2000). In addition to food poisoning, *Bacillus cereus* is an opportunistic human pathogen and is occasionally associated with infections, causing periodontal diseases and other more serious infections (Hoffmaster *et al*., 2006). In immunucompromised individuals, *B.cereus* infections can be life threatening (Le Scanff *et al*., 2006). Immunocompromised patients are susceptible to bacteraemia, endocarditis, meningitis, pneumonia, and endophthalmitis (Wijnands *et al*., 2006).

Symptoms associated with emetic syndrome include nausea and vomiting within half an hour to six hours after ingestion of food and also lasts for about twenty-four hours Wijnands *et al*., 2006). The cereulide peptide toxin associated with emetic syndrome is more resistant to acidic conditions and remains active at 121 °C (Wijnands *et al*., 2006).

The emetic syndrome is thought to be caused by cereulide peptide toxin which is secreted during stationary phase (Granum *et al.,* 1997). This toxin has a ring structure, dodecadepsipeptide, which consists of four amino acids, repeating three times, and oxy acids (Granum *et al.,*1997) .

Worldwide, diseases caused by *B. cereus* are commonly found in places where there is improper food handling (Logan and Rodrigez-Diaz, 2006). Food poisoning occurs when food is left without refrigeration for several hours before it is served. Remaining spores of contaminated food from heat treatment grow well after cooling and are the source of food poisoning. However, a majority of reported outbreaks were linked to the consumption of heat-treated foods and frequently occurred in restaurants and catering establishments. Failure in refrigeration was frequently suspected (EFSA, 2005)

2.3.1 *B.cereus* diarrhoeal syndrome

B.cereus diarrhoeal syndrome result from production of enterotoxins in the gastrointestinal tract during vegetative growth of the organism (Ankolekar, 2009). The dosage of ingested *B.cereus* cells leading to diarrhoeal syndrome is 10^5–10^7 cfu/g/ml of ingested food (Wijnands *et al.,* 2006). An infective dose of 10^4–10^9 cfu/g/ml has also been documented (Logan and Rodrigez –Diaz, 2006). In children and infants, lower dosages of 10^5 and 10^3 cfu/g/ml respectivelyof *B.cereus* cells could lead to illness (Becker *et al*., 1994). The likelihood of viable *B. cereus* cells colonizing and causing diarrhoea in its host is however reportedly largely dependent on whether spores or vegetative cells are ingested, on the type of the ingested food and the level of stomach acidity (Clavel *et al.,* 2004). The mechanism of the diarrhoeal syndrome generally involves *B. cereus* spores

and/or vegetative cells passing through the stomach, reaching the small intestine, germinating, growing, and producing enterotoxins. Vegetative cells are believed to be very susceptible to stomach conditions, and will therefore hardly reach the small intestine.

However, during food consumption the pH in the stomach may reach values of around 4.5 – 5.0. At such values also vegetative cells may survive the conditions in the stomach, and subsequently reach the small intestine intact. Under certain circumstances, short residence times in the stomach or protective compounds may help vegetative cells to pass the gastric barrier. In the small intestine these vegetative cells may grow and thus also contribute to the onset of disease (Wijnands *et al*., 2006). The diarrhoeal type of poisoning is frequently associated with raw, processed and dried food, such as beef sea foods, salads, dairy products and ready meals. The bacterium has been isolated from 50% of dried beans and cereals and from 25% of dried foods such as spices, seasoning mixes and potatoes.

Diarrhoeal type of *B. cereus* food poisoning is attributed to heat labile enterotoxins. Symptoms of diarrhoeal syndrome include watery diarrhoea, abdominal cramps, and pain ccurring 6-15 hours after ingestion which may last for twenty-four hours (United States Food and DrugAdministration, 2007). Virulence factors associated with diarrhoeal syndrome include Haemolysin BL (HBL), Non-Haemolytic Enterotoxin (NHE), cytotoxin K (cytK) and enterotoxin T (bceT) (Kramer and Gilbert, 1989; Beecher *et al.*, 1995b; Fermanian and Wong, 2000; McKillip, 2002; Lund *et al.*, 2000; Lindbäck *et al.*, 2004; Fagerlund *et al.*, 2004; Schoeni and Wong, 2005).

2.3.1.1 *Haemolysin BL (HBL)*

Haemolysin BL (HBL) is believed to be the major diarrhoeal toxin of *B. cereus* (Burgess and Horwood, 2006). The three protein components L_1, L_2 and B, constitute a hemolysin. The B component is for binding to the targeted intestinal cells, while L_1 and L_2 are lytic components following which colloidal osmotic lysis occurs (Beecher *et al*., 1995; Beecher and Wong, 1997). The toxin is only active when all three components which are secreted independently are present; hence all the components together are necessary for the expression haemolysis to occur (Schoeni and Wong, 2005). It has been proposed that the three HBL proteins bind to erythrocytes independently and the membrane-associated HBL components form a membrane attack complex that causes lysis by a colloid osmotic mechanism (Beecher and Wong, 1997). Excess of the L_1 component however inhibits binding of the B component to cells. Near the source, colony or well, L_1 diffuses rapidly and accumulates to inhibitory levels before cells are primed. Hemolysis begins in a zone in which the B concentration is high enough to prime cells that are then rapidly lysed by any concentration of L_1 and L_2 (Beecher and Wong, 1997). The hemolytic enterotoxin HBL is encoded by the *hbl*CDA operon. The B and L (L_1 and L_2) components are encoded in the genes *hblA, hblD* and *hblC*, respectively (Ryan *et al*., 1997).

2.3.1.2 *The Non –Haemolytic enterotoxin (Nhe)*

The non - hemolytic enterotoxin (Nhe) is composed of NheA, NheB and NheC. Similar to the HBL enterotoxin, the three genes encoding the Nhe components constitute an operon. Separate expression showed that all three components are required for biological activity (Todar, 2008). The enterotoxin was first isolated from a large food poisoning outbreak in Norway (Lund *et al*., 1996). Nhe is said to be produced by almost 100% of *B. cereus* strains (Moravek *et al*., 2006), and is reported to be cytotoxic against Vero cells (Lindback *et al*., 2004) through osmotic lysis (Fagerlund *et al*., 2008). Haemolytic activity of the Nhe enterotoxin has been documented,

although at a level comparatively lower than that of the the HBL (Fagerlund *et al*., 2008). Common structural features have been identified with the B component of the HBL (Madegowda *et al*., 2008) and the B and C components of Nhe ((Fagerlund *et al*., 2008). NheB is said to be the binding component of the enterotoxin complex, and an increase in the concentration of NheC results in a decrease in Nhe toxicity (Lindback *et al*., 2004).

2.3.1.3 *Enterotoxin T (BceT)*

A single component protein T (BceT) was identified by Agata *et al.,* in 1995 and exhibited cell cytotoxicity, positive vascular permeability and fluid accumulation in mouse ileal loop. It has however been recently suggested that the *bce*T gene product does not possess biological activity and cannot contribute to food-borne diseases (Choma and Granum, 2002) and seems to be a cloning artifact (Hansen *et al.*2003). Evidence of the existence of a third single component enterotoxin of *B*. *cereus* was first discovered by Granum *et al.,* (1996). The role of this enterotoxin is yet to be known, but it has sequence homology to a cell wall hydrolase from *B. subtilis* (Margot *et al.*, 1998).

2.3.1.4 *Cytotoxin K (CytK)*

CytK was first identified as the only cytotoxic, necrotic, and hemolytic toxin of the *B. cereus* strain NVH391/98, which was involved in a severe outbreak of food poisoning occurring in France in 1998(Lund *et al*., 2000). Amino acid sequencing showed a similarity of CytK to *B.cereus* Haemolysin II (Lund *et al*., 2000). This enterotoxin can occur in two forms as CytK 1 and CytK 2 (Senesi and Ghelardi, 2010) and has been shown to be highly cytotoxic against Vero cells (Lindback *et al*., 2004), with variations to cytotoxity linked to variations in the levels of toxin expression (Senesi and Ghelardi, 2010).

2.4 Hurdle Technology and its Significance in Food Safety

Factors used for food preservation are called ‘hurdles’ and there are numerous hurdles that have been applied for food preservation. These preservative factors act as hurdles against microorganisms by inhibiting their growth, as so serve as essential control measures for particular food borne pathogens (Pujol *et al*., 2012). Potential hurdles for use in the preservation of foods can be divided into physical, physicochemical, microbially derived and miscellaneous hurdles (Leistner and Gorris, 1995). Among these hurdles, the most important have been used for centuries and are as either ‘process’ or ‘additive’ hurdles including high temperature, low temperature, water activity , acidity, redox potential (Eh), competitive microorganisms (e.g. lactic acid bacteria) and preservatives (e.g. nitrite, sorbate, sulphite) (Leistner, 1995).

To ensure the stability and safety of foods, each hurdle should be applied in the proper manner. However, the high strength or concentration of any single hurdle could adversely affect food quality such as loss of nutrients, texture, color, etc. Therefore, if manufacturers are to achieve a product that accommodates consumer preferences while remaining safe and stable, they must achieve synergies through the combined use of these or other hurdles which, while they may not prevent the growth of pathogenic or spoilage microorganisms on their own, will do so collectively (Sun-Young and Kang, 2004; Pujol *et al*., 2012). An important current trend is toward the use of procedures that deliver food products that are less “heavily” preserved, higher in quality, perceived as being more “natural”, contain less additives, and are nutritionally healthier. Some new and “emerging” techniques aim to meet some of these objectives. Most of these techniques act by inactivating microbes (Sun-Young and Kang, 2004).

In recent years, the concept of combining several factors has been developed by Leistner (Leistner, 1995) and others into the '*hurdle effect*'. From an understanding of the hurdle effect, hurdle technology has been derived (Leistner, 1985), which has the goal not just to understand why a certain food is safe and stable, but to improve the microbial quality of the food by an optimization and intelligent modification of the hurdles present. It employs the intelligent combination of different hurdles or preservation techniques to achieve multi-target, mild but reliable preservation effects (Sun-Young and Kang, 2004).

Microorganisms react homeostatically to stress factors. When their environment is disturbed by a stress factor, they usually react in ways that maintain some key element of their physiology constant. Preservative factors functioning as hurdles can disturb one or more of the homeostasis mechanisms, thereby preventing microorganisms from multiplying and causing them to remain inactive or even die. The success of hurdle technology depends on ensuring metabolic exhaustion. Most stress reactions of microorganisms are active processes, and this often involves the expenditure of energy, e.g. to transport protons across the cell membrane, to maintain high cytoplasmic concentrations of 'osmoregulatory' or 'compatible' solutes. Restriction of the availability of energy is then a sensible target to pursue. This probably forms the basis of many the successful, empirically derived, mild combination preservation procedures exemplified by hurdle technology (Gould, 1995). Environmental stresses can however provide varying results, because some bacteria may become more resistant or even more virulent under stresses through stress reactions such as synthesis of protective stress shock proteins (Leistner, 2000). However, the use of different stresses at the same time (combination treatment) may also prevent the synthesis of those protective proteins because simultaneous exposure to different stresses will require energy-consuming synthesis of several or at least much more

protective stress shock proteins which in turn may cause the microorganisms to become metabolically exhausted (Leistner, 2000). There are however some cases where application of the hurdle concept for food preservation may inhibit outgrowth, but induce prolonged survival of microorganisms in foods (Uyttendaele *et al*., 2001).

A number of promising hurdles have been identified for their effectiveness in killing microorganisms in foods, but the application of the idea in the food industry has been largely restricted to the meat sector (Sun-Young and Kang, 2004). Recent studies however, emphasize a much wider potential application, e.g., in bakery products, fish, and dairy products. More specifically, the concept was introduced into mild processing of fruits and vegetable and there is only limited information available about the effect of combined hurdles in these types of foods (Sun-Young and Kang, 2004).

2.5 Effects of Environmental Factors on Enterotoxin Production by Pathogenic Bacteria

Environmental stresses are external factors that have adverse effects on the physiological welfare of bacterial cells, leading to reduction in growth rate, inhibition and/or death, at individual cell or population levels (McMahon et al., 2007). Environmental stresses could include extremes of temperature, pH, and depletion of nutrients (Archer, 1996) and the presence of toxic or inhibitory compounds, including antibiotics (McDowell, 2004).

Many modern food preservation processes aim to extend the lag phase of bacteria present in food by the application of one or more environmental stresses (or hurdles) to slow or prevent bacterial growth (McMahon *et al*., 2007). However, absence of growth does not necessarily mean absence of metabolic and/or genetic activity, and stress-inhibited bacteria act to reduce the impact of environmental stresses by making phenotypic and genotypic adaptations (Storz and Hengge-Aroniz, 2000). Mild heat treatment, low pH and cold storage are examples of hurdles used to preserve food. In order to cope with these environmental dynamics, microorganisms have developed stress adaptation strategies that lead to the possibility that bacteria overcome harsher conditions for a variety of stresses (Desriac *et al*., 2013). A common strategy that bacteria use to counter stressful conditions is to activate a specific alternative sigma factor, which leads to the transcription of a set of genes or regulon which then protect the cell against adverse conditions (Kazmierczak *et al*., 2005). In *B. cereus*, the alternative sigma factor is activated in several stress conditions such as ethanol, NaCl exposure or H_2O_2 and acid shock (Desriac *et al*., 2013).

Levels of bacterial toxins in enterotoxigenic organisms, like other cellular components such as enzymes are determined by a number of factors like nutrient availability or growth stage, consequently, in order not to waste resources, these are only synthesized when they are needed (Rajkovic, 2006). The expression of virulence genes by bacterial pathogens is thus controlled by regulatory mechanisms as a response to changes in the environment (Casadevall and Pirofski, 1999; Liang *et al.*, 2005). These regulatory mechanisms allow either positive (activator genes) or negative (repressor genes) regulation or combination. The most common type of regulation occurs at the

transcription stage (Rajkovic, 2006). Changes in gene expression or cellular phenotype in organisms occur in some instances without any changes to underlying DNA sequences of the organism (Wikipedia, 2013). This phenomenom, known as epigenetics, focuses on ways in which heritable traits can be associated not with changes in nucleotide sequence, but with chemical modifications of DNA, or of the structural and regulatory proteins bound to it (Fenselfeld, 2014). These changes may remain through cell divisions for the remainder of the cell's life and may also last for multiple generations (Bird, 2007).

Some researches have highlighted the role of physiological welfare in the expression of virulence genes by *B. cereus*. A study conducted by Havie *et al.*, (2005), revealed that *B. cereus fur* (Ferric Uptake Regulator) gene, a transcriptional regulator that is responsible for bacterial iron uptake and metabolism, was shown to reduce virulence in the pathogenic *fur* mutant. Results in this investigation revealed a decrease in the regulation of iron uptake with three times more intercellular iron in the *fur* mutant than in the wild-type, resulting in a greater amount of oxidizing free radicals. The virulence of *B. cereus fur* mutant was measured in an insect infection which revealed the significance of iron metabolism regulation in bacterial pathogens by its reduced virulence. This study gave an insight to the importance of the *fur* gene in the regulation of iron concentrations for cell growth, survival, and pathogenesis. The reduced virulence of *B. cereus fur* mutants in this experiment demonstrates the potential of the *fur* gene to be a good target for antimicrobial drugs as a conserved protein among pathogenic bacteria (Havie *et al*., 2005).

The role of the flagellar export complex on the expression of the HBL enterotoxin has also been documented. Failure to secrete intracellularly-synthesized HBL was demonstrated in *B. cereus* natural isolates lacking flagella (Ghelardi *et al*., 2007). A significant reduction in the amount of secreted HBL was reported for a *B. cereus* mutant lacking FlhF, a signal recognition particle (SRP)-like GTPase involved in the regulation of the number and arrangement of flagella on the bacterial surface (Salvetti *et al*., 2007). This mutant was characterized by a marked reduction in the number of flagella (1–3 per cell) in comparison to wild-type (10–12 per cell) (Salvetti *et al*., 2007).

HBL production by *B. cereus* is highly controlled, as a wide variety of signals and proteins act as regulators of *hbl* gene transcription (Senesi and Ghelardi, 2010). Expression of the *hbl* genes has been shown to be regulated by the proteins PlcR, ResD, Fnr, and CcpA (Agaisse *et al*., 1999; Duport *et al*., 2006; Zigha *et al*., 2007; Esbelin *et al*., 2009; Messaoudi *et al*., 2010); expression of most virulence factors is however activated by the pleiotropic transcriptional regulator PlcR (phospholipase C regulator) except in *B. anthracis* (Agaisse *et al*., 1999). HBL genes are part of a virulence regulon that is controlled by the pleiotropic regulator PlcR, whose expression is autoinduced and starts shortly before the onset of the stationary phase of growth (Lereclus *et al*., 1996; Gohar *et al*., 2002). In *B.cereus sensu stricto* and *B. thuringiensis*, PlcR activity peaks at the onset of the stationary growth phase and is dependent on the presence of PapR, a small signaling peptide that acts as a quorum-sensing effector (Slamti and Lereclus, 2002).

The ability of the bacteria to communicate, sense and respond to population density is termed ‘‘quorum sensing” (QS). Quorum sensing is widely recognized as an efficient mechanism to regulate expression of specific genes responsible for communal behavior in bacteria (Gonzalez and Keshavan, 2006). Many bacterial physiological functions such as luminescence, virulence, motility, sporulation, biofilm formation, etc., are regulated by QS systems (Gram *et al*., 2002). Quorum sensing enables bacteria to co-ordinate and respond quickly to environmental changes, such as the availability of nutrients, other microbes or toxins in their environment (Lal, 2009). Some pathogenic and spoilage bacteria are able to regulate phenotypic characteristics as a function of cell density under the control of chemical signal molecules (Gram *et al*., 2002), called autoinducers. At low population densities, basal-level expression of an autoinducer synthase gene results in the production of small amounts of autoinducer signal molecules that diffuse out of the cell and are immediately diluted in the surrounding environment.

An increase in bacterial population results in the gradual accumulation of autoinducers in and around the cells. The autoinducer specifically activates a transcriptional regulator protein by binding to it. Activated regulators then interact with target DNA sequences and enhance or block the transcription of quorum-sensing-regulated genes, resulting in the synchronous activation of certain phenotypes in a bacterial population (Gonzalez and Keshavan, 2006). Most quorum sensing signals are small organic molecules or peptides. Gram-negative bacteria employ N-acyl homoserine lactones (AHLs), alkyl quinolones (AQs) and fatty acidmethyl esters. Gram-positive bacteria use peptides like the autoinducing peptides (AIPs). AHL-mediated quorum sensing is one of the best characterized cell-to-cell communication mechanisms and more than 70 bacterial species

are known to produce AHL-type quorum-sensing signals, with many producing multiple AHLs (Lal, 2009).

In recent times, a number of researches have focused on mechanisms through which quorum sensing could be inhibited or intercepted as a way of suppressing bacterial pathogenesis or food spoilage. It has been hypothesized that if QS systems regulate bacterial mechanisms in food spoilage, then inhibition of the communication underlying the QS systems could be a good strategy to reduce or prevent the spoilage reactions (Abolghait *et al*., 2011). This phenomenom is referred to as "quorum quenching". Research has focused on inhibiting QS using bacterial biosensors and indicators (Choo *et al*., 2006). QS inhibitory activity of food of animal origin such as poultry meat-derived fatty acids, raw cow milk and she-camel's milk have been reported (Widmer *et al*., 2007; Abolghait *et al*., 2011).

2.6 The Health Impact of Street Food Consumption in Nigeria

"Street food" as defined by WHO (1996), refers to "a wide variety of ready-to -eat (RTE) foods and beverages that are sold and sometimes prepared in public places, and may be consumed where it was purchased or taken away and eaten elsewhere". Besides offering business opportunities for developing entrepreneurs, the sale of street foods can make a sizeable contribution to the economies of developing countries (Rane, 2011). In Nigeria, street foods enjoy wide patronage around public places such as offices, schools, markets, cars/bus stations and a host of other public places. It has been documented that four out of every five Nigerians visit fast food centers at least once a day (Wogu *et al*., 2011). This wide patronage ensues from the fact that they are usually easily accessible and

reasonably affordable, even by low income earners. A variety of foods such as boiled, ***jollof***, and fried rice, soups, bean cakes, fried foods, roast or barbecued meat (**suya, balangu, tsire, kilishi**), **moi moi**, as well as beverages like cereal based drinks **(kunun zaki, kunun aya**), traditional fermented milk (**nono**), sorrel leaves drink (**zobo**), and a good number of others, are vended in public places.

Street foods are perceived to be a major public health risk due to lack of basic infrastructure and services, difficulty in controlling the large numbers of street food vending operations because of their diversity, mobility and temporary nature (Ghosh, 2007; deSousa, 2008). Several pathogenic microorganisms such as *Staphylococcus aureus, Escherichia coli* and *Bacillus cereus* have been isolated from street vended foods (Umoh and Odoba, 1999; Bello, 2002; Mensah et al., 2002; Omemu *et al.*, 2005; Whong *et al*., 2006; Abdalla *et al*., 2009; Wogu *et al*., 2011). Studies by FAO (1995) recorded poor knowledge practices in food handling in an assessment of microbial contamination of food sold by vendors. In addition, other factors such as vending location, quality of raw materials, utensils and equipment, food preparation method as well as personal hygiene of vendors and food handlers all impact on the safety of street vended foods (Rane, 2011).

Some street vendors operate under conditions unsuitable for the preparation and selling of food (Barro *et al*., 2006). A study conducted by Muinde *et al*., (2005) in Kenya, revealed that 85% of the vendors prepared foods like fish, fruit salads, roasted maize and chips in unhygienic conditions, given that garbage and dirty waste were conspicuously close to the stalls. Some researchers have equally noted that vendors used poor quality water for food preparation, washing and drinking. Studies carried out in different regions of Asia,

Africa and South America have frequently pointed the unavailability of potable water for various activities at the vending site as a major concern. Due to the shortage of clean potable water, many vendors tend to re-use water, especially for cleaning utensils and used dishes (Dawson, 1991). Holding foods at high ambient temperatures for long periods of time have been reported to be a major contributor to the occurrence of food poisoning outbreaks (Adesiyun and Balbirsingh, 1996). High counts of foodborne pathogens such as *Escherichia coli, Bacillus cereus* and *Clostridium perfringes* have been isolated from foods held at high ambient temperature for long periods (Bryan, 1995).

According to surveys carried out in 2006 and 2007 in Nigeria by the Department of Public Health of the Federal Ministry of Health, there were more than two million recorded cases of foodborne diseases in the country with the number of death put at over five hundred (Agu, 2011). Some of these cases could be linked to consumption of contaminated street vended foods. Also in 2008, the Consumer Protection Council's, CPC, survey on street vended foods within the Federal Capital Territory, FCT, was to say the least, tragic, as it also gave a similar scenario that presupposes that the situation could be worse in the hinterland where most facilities such as portable water and electricity are irregular (Agu,2011).

Reducing the risks of foodborne illness from street foods would require controlling the steps in food preparation and sale that may contribute to the contamination, growth and survival of the microbes responsible for food borne illness. Appropriate regulations should be prepared, and incorporated into existing food regulations. FAO has implemented and supported several projects which aimed at improving various aspects of

the street food sector in countries like Bolivia, Colombia, Ecuador, India, Zaire, etc. (Rane, 2011).

2.7 Control Measures Against *Bacillus cereus* Food Poisoning

The CDC estimates that 97% of all cases of food poisoning result from improper food handling; 79% of cases result from food prepared in commercial or institutional establishments (Malek *et al*., 2009) and 21% of cases result from food prepared at home. The most common causes are as follows: (1) leaving prepared food at temperatures that allow bacterial growth, (2) inadequate cooking or reheating, (3) cross-contamination, and (4) infection in food handlers. Cross-contamination may occur when raw contaminated food comes in contact with other foods, especially cooked foods, through direct contact or indirect contact on food preparation surfaces. (Malek *et al*., 2009).

Effective control measures depend on destruction by a heat process and temperature control to prevent spore germination and multiplication of vegetative cells in cooked, ready-to-eat foods. Measures to reduce or eliminate the threat of food poisoning by *B. cereus* include: 1). Avoid preparing food too far in advance of planned service, 2). Avoid holding cooked foods at room temperature, 3). Use quick chill methods to cool foods below 7.2 ºC (45ºF) within 4 h of preparation; store in shallow pans/small quantities with the food less than 4 inches (10.2 cm) deep; if food is especially thick (e.g., refried beans), store no more than 3 inches [7.6 cm] deep, 4). Hold/store hot foods above 60ºC (140ºF) until served, and 5). Reheat foods rapidly to 74ºC (165ºF) or above (Kramer and Gilbert, 1989; Reed, 1994).

According to the following United States based instituitions, National Institute of Health (NIH), the National Institutes of Allergy and Infectious Diseases, and the National Food Processors Association, the suggestions below are good examples of how to destroy *B. cereus* (Schneider *et al*., 2004)

-Steaming under pressure, roasting, frying, and grilling foods can destroy the vegetative cells and spores.

- The diarrhoeal toxin can be inactivated by heating foods for five minutes at 133ºF (56 ºC), while foods need to be heated to 259ºF (126 ºC) for more than 90 minutes to inactivate the the emetic toxin. Reheating foods until they are steaming is not enough to kill the emetic toxin.

In meat processing facilities, the following measures could effectively prevent contamination and toxin formation:

-Assure current Good Manufacturing Practices (cGMP) are being used in the slaughter houses and processing units.

- Apply approved treatments of carcasses to remove faecal bacteria.

- Use proper cleaning and disinfection of food contact surfaces (FCS) with hypochlorite or approved sanitizers.

- Utilize a heat process to destroy the vegetative cells and a rapid cooling process to prevent the spores from germinating.

-Keep hot foods above 60ºC and cold foods below 4ºC to prevent the formation of spores.

- wash hands, utensils, FCSs with hot soapy water after they touch raw meat or before food preparation, and after using the bathroom.

- cook beef and beef products thoroughly

-Properly refrigerate leftovers.

The major control measures are however to control temperature, and to establish HACCP system. Only heat treatments used for canning of low acid foods will ensure a complete destruction of spores of *B. cereus*. The number of spores in other processed foods must be kept as low as possible by proper cleaning and disinfection of equipment. Rapid cooling is necessary to prevent germination and growth of *B. cereus* spores. Low pH (below 4.5), reduction in a_w (below 0.92) would inhibit *B. cereus*. In other cases, refrigeration below 4°C is necessary to prevent growth of all types of *B. cereus*, including psychrotrophic strains. However, below 10°C, lag time and generation times are very significantly increased, particularly whenever other factors (i.e. pH, aw, nutrient content of the food) are not optimum for *B. cereus* (EFSA, 2005).

2.8 Antibacterial Susceptibility of *Bacillus cereus*

Microbial resitance to antimicrobials has over the years increasingly become a major concern to health professionals, as the success of treatment of illnesses associated with microorganisms depends to a large extent on their sensitivity to these antimicrobials. Before the discovery of antimicrobial drugs or antibiotics, microbial infections were like death sentences and thus resulted in the loss of many lives. Since its discovery, antibiotics have been critical in the fight against infectious disease caused by bacteria and other microbes (Todar, 2013). Antimicrobial chemotherapy has been a leading cause for the dramatic rise of average life expectancy in the twentieth century (Todar, 2013).

Resistance to antimicrobials occur in part because bacteria and other microbes that cause infections are remarkably resilient and have developed several ways to resist antibiotics and other antimicrobial drugs. Environmental conditions also appear to play a part in the

ability of microbes to resist the effects of antimicrobial drugs. Environmental stresses induced by detergents, organic solvents, dyes, food components, preservatives, and antibiotics, induce the multiple antibiotic resistance operon (McMahon *et al*., 2007), which regulates the expression of a large number of genes (Alekshun and Levy, 1997; Alekshun and Levy, 1999) including those coding for at least one broad-specificity efflux pump (the *arcAB* efflux pump) (Rickard *et al*., 2004)), which are more strongly expressed under conditions of environmental stress (Ma *et al*., 1995).

Bacterial resistance to antibacterial agents may also be attributed to the problem of increasing and irrational use, and misuse of existing antibiotics in human and veterinary medicine and in agriculture (WHO, 2012; Todar, 2013). It is believed that bacterial cells sense the actions of antibiotics as just another form of environmental stress (McDowell, 2004), and sublethal concentrations of antibiotics have been shown to induce stress hardening (Blackburn and Davies,1994) and cross-protection (Hastings *et al*., 2004). Such environmental stresses can induce resistance to antibiotics by a range of means (McMahon *et al*., 2007).

Foodborne bacterial pathogens that are resistant to a variety of antibiotics have become a major health concern (Roy *et al*., 2007). Over the years, a number of studies have shown that *B. cereus* strains isolated from different food sources have resistant strains against some antibiotics (Antwerpen *et al.*, 2007; Roy *et al.*, 2007; Whong and Kwaga, 2007; Brown and Jang, 2008; Dikbas, 2010). In general, most *B. cereus* isolates are resistant to penicillins and cephalosporins as a consequence of β-lactamase production (Bottone, 2010). In a study of minimum inhibition concentrations (MICS) of selected antibiotics for *Bacillus cereus,* Turnball *et al.,* (2004) observed no group trends were apparent among

the different categories of *B. cereus* isolates from food poisoning incidents, non gastrointestinal infections, food and environmental specimens not associated with illness. Resistance of *B. cereus* to erythromycin, tetracycline, and carbapenem has also been reported (Kiyomizu *et al*., 2008; Savini *et al.*, 2009). There are however variations regarding the sensitivity of the organism to some antibiotics; many case reports of *B. cereus* infections concluded that resistance to penicillin, ampicillin, cephalosporins, and trimethoprim was constant, while susceptibility to clindamycin, erythromycin, chloramphenicol, vancomycin, the aminoglycosides, and tetracycline was usually observed. Susceptibility to ciprofloxacin was uniform, and it has been shown to be highly effective in the treatment of *B. cereus* wound infections (Turnball *et al*., 2004). Luna *et al*. (2007) however documented small populations of *B. cereus* isolates showing resistance to clindamycin, erythromycin, and trimethoprim-sulfamethoxazole.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sampling Area

The sampling area for this study included some parts of Kaduna metropolis (Plate I), specifically Kaduna north (Unguwar rimi, Unguwar sarki and Unguwar dosa districts), Kaduna south (Tudun wada district) and Zaria town (Kwangila area) (Plate II). Kaduna state with a population of about 6 million (2006 census), is said to be one of the most densely populated states of Nigeria. Kaduna and Zaria are regarded as the two urban centres of the state. The state occupies a central position in the map of the Federal Republic of Nigeria and occupies a landmass of about 30,118Km^2. The city of Kaduna with a population of 1.5 million people accommodates people of diverse culture (Kaduna state ministry for culture and tourism, 2010). Food samples were purchased from sellers/ vendors on some streets and markets in the study area where the samples are popularly consumed and easily accessible. Proximity to laboratory facilities was also an added factor in the selection of sampling areas.

3.2 Sample Size

In order to establish an appropriate sample size of food samples to be analysed for isolation of *Bacillus cereus* in this study, the formula below was used as described by Naing *et al*., (2006).

$$n = \frac{z^2pq}{d^2}$$

Where;

n = number of samples

z = 1.96 critical value for *95%* confidence interval.

p = Expected proportion (where there is no fixed prevalence rate, statistics recommends a high prevalence of 50% , i.e P=0.5)

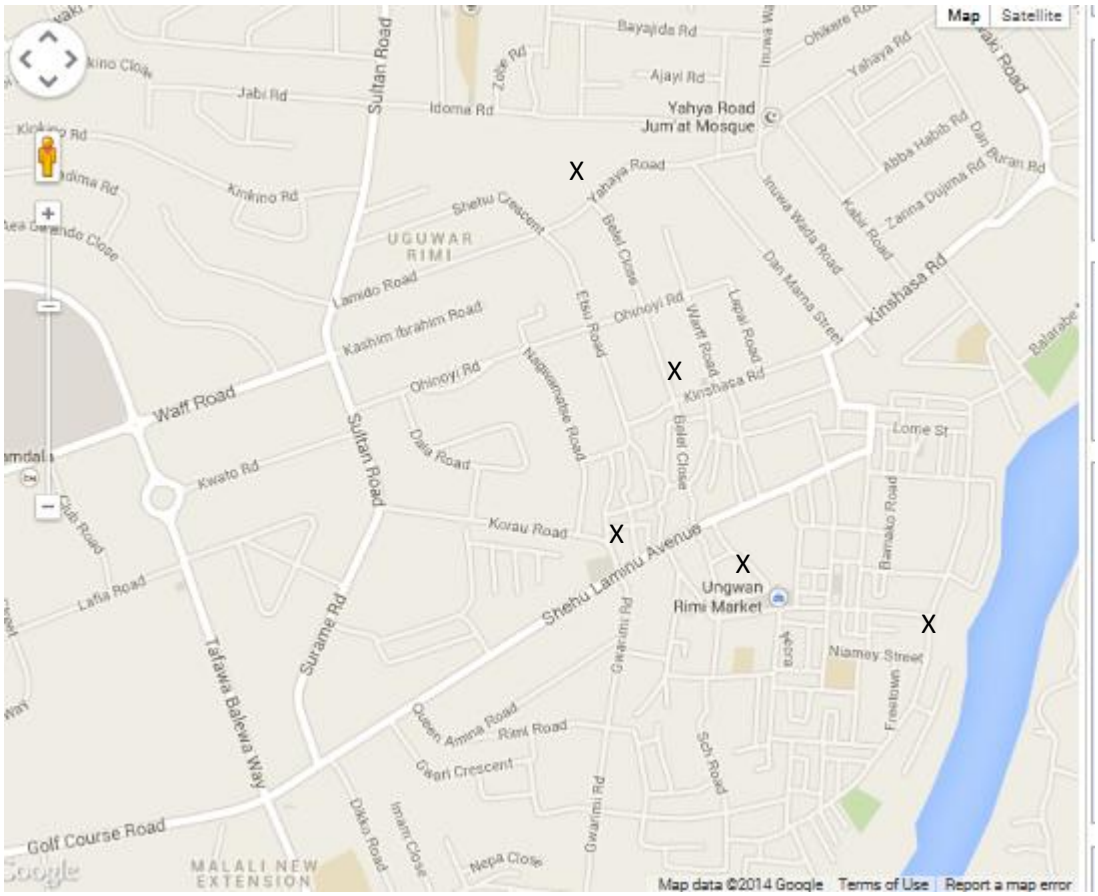


Plate I: Map of Kaduna Metropolis Showing Location of Some Sampling Areas (X)

Source: http://www.maps-streetview.com/Nigeria/Kaduna/roadmap.php. Retrieved 24/08/2014

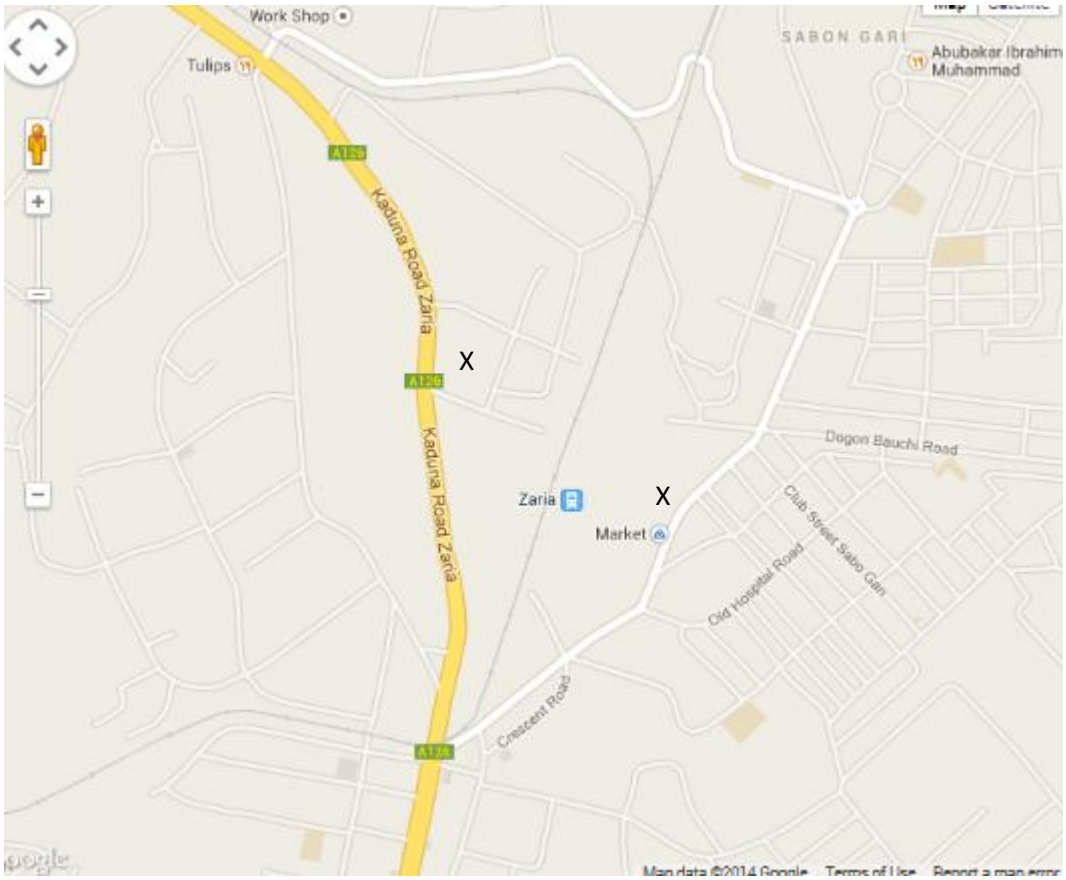


Plate II: Map of Zaria Metropolis Showing Location of Some Sampling Areas (X)

Source: http://www.maps-streetview.com/Nigeria/Kaduna/roadmap.php. Retrieved 24/08/2014

q = 1 - p

d = Allowable error of *5%*

$$= \frac{(1.96)^2 \times 0.5\ (1- 0.5)}{0.05^2}$$

= 384.16

Therefore, a total of 387 food samples were subjected to *Bacillus cereus* isolation. This was equally distributed to give a total number of 43 samples each from the following food items; raw meat, **balangu** (roast meat), **soye** (spiced hawked roast meat), **suya** (spiced roast meat), uncooked rice, **jollof** rice, plain boiled rice, raw cow milk, and **nono** (locally fermented milk).

3.3 Sample Collection

Food samples were purchased from 10 producers/vendors of each of the food samples above (3.1), with the exception of **balangu** (8 producers), **soye** (6 producers) and raw milk (9 producers). Samples were collected in sterile containers and transported in ice pack containers to the laboratory for analysis. Raw meat and uncooked rice were purchased from Unguwar rimi (Plate I), Unguwar shanu, Kasuwar barci (Tudun wada) and Samaru markets, while **suya, balangu, jollof** rice, plain boiled rice, and **nono** were purchased from Suya spots, local restaurants and Fulani milk maids at Unguwar Rimi, Unguwar Sarki, Unguwar dosa and Tudun wada. Raw milk was purchased at milking points from Fulani herdsmen settled on undeveloped plots and land around the fringes of Unguwar rimi area. All **soye** samples were purchased from Kwangila area of Zaria town (Plate II) where it is readily available and highly patronized owing to the busy nature of the area.

3.4 Preparation of Samples for Analyses

3.4.1 Serial dilution of samples

Food samples which included raw meat, **balangu, soye, suya,** uncooked rice, **jollof** rice, plain boiled rice, raw milk and **nono** were first homogenized by blending 25g/25ml of each sample in 225 ml of sterile 0.1% buffered peptone water for 5 to 10 seconds. Liquid samples were homogenized by manual shaking in 500ml conical flasks. With the aid of sterile pipettes, the food homogenates were then subjected to 10-fold serial dilutions in sterile 0.1% peptone water by aseptically diluting 1ml of the homogenate through a series of five 9ml sterile dilution blanks in test tubes. Dilutions 10^{-1}, 10^{-3}, and 10^{-5} were then used for enumeration of *B.cereus* as described below.

3.5 Enumeration of *Bacillus cereus*

Dilutions 10^{-1}, 10^{-3}, and 10^{-5} of serially diluted food samples were pour plated on sterile plates of Mannitol Egg-yolk Polymyxin Agar (MYP) (OXOID, U.K.), a highly selective *Bacillus cereus* medium developed by Holbrook and Anderson, (1980). MYP plates of the food samples were incubated at 37°C for 24-48 h. Flat, white granular colonies that appeared surrounded by zones of clearance on pink or red backgrounds on the MYP plates were counted and recorded as presumptive *Bacillus cereus* (MFLP-42, Http:// www.hc-sc.gc.ca. Retrieved 27/7/2007). Colonies were streaked on nutrient agar slants, incubated at 37°C for 24 to 48 h, and stored at 4°C until required for characterization.

3.6 Biochemical Characterization of Presumptive *Bacillus cereus* Isolates

Stored presumptive *B.cereus* isolates obtained from food samples in section 3.5 which were catalase positive, Gram positive with central or subterminal spores which did not

distend the cell, were characterized using a Microgen® Bacillus ID identification kit (Microgen Bioproducts, U.K.). This kit is a miniaturized biochemical identification system designed to identify mesophilic Bacillus species and related genera associated with food and beverage spoilage and food poisoning. The identification system comprises twenty four (24) biochemical substrates (Appendix X) specifically selected and formulated to provide accurate and efficient identification of Bacillus species.

A suspension of 18-24 h culture of each isolate to be identified was prepared equivalent to Mcfarland Standard 2, in the suspending medium provided with the kit. A volume of 100µl of inoculum was inoculated into each of the 24 wells of test strip for each isolate. The arginine well of each test strip was then overlaid wih mineral oil before the strips were incubated at 30ºC for 24 h. Results of all sugar fermentation tests, citrate and urease tests were read and recorded at the end of 24 h as instructed by the manufacturer. Test strips were then further incubated for another 24 h, following which Kovac's reagent, Voges Proskauer reagents and Nitrate reagents provided with the kit were added to the appropriate wells for Indole test,Voges Proskauer test and nitrate reduction test respectively. Results were recorded on report forms provided with the identification kits. Results recorded on the report forms generated an octal code for each isolate. These codes were then analyzed using the Microgen Identification System Software ((Microgen Bioproducts, U.K.).

3.7 Growth and Enumeration of Confirmed Isolates at Conditions of Varying Sodium Chloride Concentrations, pH, and Temperature.

3.7.1 Standardization of B. *cereus* inoculum.

Confirmed *B. cereus* isolates R3M1, F5M1, F2 and Bln3g2 isolated from raw cow milk, **nono**, plain boiled rice and **balangu** respectively were standardized prior to inoculation in food samples. Portions of 18 to 24 h cultures of each isolate were transferred into sterile physiological saline to make Mcfarland standards 1 of the test isolates giving an approximate cell count of 3×10^8 cfu/ml. The inocula were used immediately after preparation.

3.7.2 Growth of *B. cereus* isolates at varying sodium chloride (NaCl) concentrations and temperature.

B.cereus isolates F2 and Bln3g2 were inoculated into cooked local parboiled rice and **balangu** respectively as described below:

Local parboiled rice was purchased from Unguwar Rimi market in Kaduna, cleaned by manually picking out dirt and husks. The rice was then milled into grits with a local milling machine to increase surface area for bacterial growth. Nine hundred grams (900g) of washed, milled rice was placed in a small aluminium pot and soaked with 900ml of boiled cooled water (0% NaCl) , for about 5 min to soften the grits, this was then cooked over low heat until the grits were soft and all the water was absorbed. About 100g of the cooked grits was placed in each of 9 sterile glass bottles and tyndallized at 100^0C for 5 min. Accordingly, four other sets of 900g of the rice grits were each soaked and cooked in 0.5, 1, 1.5, and 2% (W/V) of NaCl solutions ,put in sterile glass jars and also heat treated as above. Following tyndallization, sterile cooked rice grits in each jar was then

inoculated with 1 ml suspension of an approximate cell count of 3×10^8 cfu/ml of isolate F2.

Balangu (saltless) was purchased from a suya spot in Kwangila, Zaria. In a washed, dried mortar, the **balangu** pieces were pulverized using a pestle to produce a thread-like sample, this was done to increase surface area for bacterial growth as was done for the rice samples. The samples were then treated with varying concentrations of NaCl solutions as was done for rice samples, placed in sterile glass jars, and sterilized as the rice samples above. Sterile **balangu** in each bottle was then inoculated with 1 ml suspension of an approximate cell count of 3×10^8 cfu/ml of isolate Bln3g2, as was done for boiled rice samples.

The cooked rice and **balangu** samples were then divided into three sets, each set consisting of triplicate samples at NaCl concentration levels of 0, 0.5, 1, 1.5 and 2%. One set was incubated at room temperature for 24 h, one at 37^oC for 24 h, and one at refrigeration temperature for 72 h.

3.7.3 Growth of B. *cereus* isolates at varying pH levels and temperature.

B.cereus isolates R3MI and F5M1 were inoculated into pasteurized fresh cow milk and **nono** as described below:

Fresh cow milk was obtained from the dairy processing unit of National Animal Production and Research Institute (NAPRI) in Shika, Kaduna State. This was then tyndallized at 72^0C for 15 min (HTST) in a Grant JB series (Grant Instruments, U.K.) water bath. This was done by placing a sterile thermometer into the sample until it attained the temperature of 72^0C which was then maintained for 15 min. Following

tyndallisation, 0.1 ml of the sample was then spread on sterile nutrient agar plate after the three day heat treatment, and incubated for 24 h at 37^0C to test for sterility. One hundred and fifty millilitres (150ml) of the pasteurized milk was then dispensed into each of four sterile 250ml conical flask. The milk in each of these conical flasks had its pH adjusted to 6.3, 6.4, 6.7 and 6.8 respectively, using 0.1M each of lactic acid and sodium hydroxide. The pH in each case was monitored with a JENWAY 3150 series (JENWAY, U.K.) pH meter. Lactic acid was used to lower and sodium hydroxide to increase pH levels as required. These pH levels were selected to include the minimum and maximum levels recorded for retail samples analyzed for presence of *B.cereus* (Appendix VIII), as well as one point below, and above, these levels. Twenty millilitre (20ml) aliquots of milk sample from each conical flask were then dispensed into each of 3 sterile glass bottles. These bottles were each inoculated with1 ml suspension of an approximate cell count of 3×10^8 cfu/ml of *B. cereus* isolate R3M1 and refrigerated for 72 h.

Nono was purchased from Samaru market, Zaria, and tyndallized by heat treating at 63^oC for 30 min for a period of three days to sterilize the sample. A lower temperature was adopted to avoid coagulation of the fermented milk. The sample was then checked for sterility after which 200ml of sterile **nono** was then dispensed in each of four 250ml conical flasks. The pH levels were adjusted to 3.7, 3.8, 4.2 and 4.3 as per the pasteurized milk above. These levels were selected using the same criteria adopted for pasteurized milk. Twenty millilitre (20ml) aliquots of **nono** sample from each conical flask were then dispensed in each of 9 sterile glass bottles. The samples were then inoculated with 1 ml suspension of an approximate cell count of 3×10^8 cfu/ml of *B. cereus* isolate F5M1, as was done for pasteurized milk samples above. Following inoculation, samples were

divided into three sets, each set consisting of triplicate samples at pH levels of 3.7, 3.8, 4.2 and 4.3. One set was kept at optimum temperature (37^{o}C) for 24 h, one at room temperature for 24 h and the last set at refrigeration temperature for 72 h.

3.7.4 Enumeration of *B.cereus* in food samples during storage

Counts of *B.cereus* for each of the food samples stored at different temperatures were determined at 24 h, for samples stored at room temperature and at 37^{o}C (optimum temperature). Food samples stored at refrigeration temperature had their counts determined at 24, 48 and 72 h respectively. Ten-fold serial dilutions of food samples were carried out as described in Section 3.4. One millilitre (1ml) of appropriate dilutions of each sample was then plated on sterile nutrient agar (OXOID, U.K.) plates. The plates were incubated at 37^{o}C for 24 h. Counts were then recorded, and isolates stored on nutrient agar slants under refrigeration temperature.

3.8 Determination of Production of Diarrhoeal Enterotoxin by *B. cereus* Grown at Varying pH Levels, Temperature and Sodium Chloride Concentration.

B.cereus isolates obtained from food samples in section 3.7 were subjected to assay for production of HBL diarrhoeal enterotoxin using BCET-RPLA toxin detection kits (Oxoid, U.K.) following the manufacturer's instruction as described below:

One loopful of *B.cereus* isolates was inoculated into Brain heart infusion (BHI) broth (OXOID, U.K.), and incubated at 37^{o}C for 18 h. One millilitre (1ml) of each culture fluid was then dispensed into a sterile Eppendorf centrifuge tube, and centrifuged at 3000rpm for 20 min at 4 ^{o}C using a TGL-16G series refrigerated centrifuge (Ningbo biocotek, China). Culture filtrates were retained and assayed for enterotoxin content in V- well microtitre plates (TITERTEK, U.S.A). The microtitre plates were arranged so that each row consisted of 8 wells; two such rows were used

for each test sample. Using an Eppendorf micropipette, 25µl of diluent (phosphate buffered saline) was dispensed in each well of the 2 rows except the first well in each row. Twenty five microlitres (25µl) of test sample was then added to the first and second wells of both rows. Using a diluter (DYNATEK LABS, U.S.A.) and starting at the second well of each row, 25µl was picked and dilutions performed along each of the rows. This was stopped at the 7th well leaving the last well containing only the diluent. To each well in the first row, 25µl of sensitised latex [latex sensitized with specific *B.cereus* anti-enterotoxin (rabbit igG)] was added; while to each well in the second row 25µl of latex control (latex suspension sensitized with non-immune rabbit globulin was added. To mix the contents of each well, the plates were agitated by hand. A toxin control (lyophilized *B.cereus* enterotoxin) provided in the kit was used alongside test samples to provide a reference for positive patterns, and confirm the correct working of the test latex. The plates were then left undisturbed on a vibration- free surface at room temperature for 20 to 24 h. Each plate was then examined against a black background for agglutination, and toxin titres (concentrations) recorded from the last dilutions that gave detectable antigen-antibody reaction. The sensitivity of this test in detecting enterotoxin is 2ng/ml in test extracts.

3.9 Molecular Characterization of Enterotoxin and Non-enterotoxin Producing *B.cereus* Isolates.

Enterotoxin and non enterotoxin producing isolates from section 3.7 were subjected to assay for the presence of HBL and BceT enterotoxins DNA sequences by PCR as described by Rowan *et al*., (2001). DNA sequences of diarrhoeagenic genes *hblD*, *hblC*, *hblA*, and *bceT* were used to design primers that were used amplify to segments of the genes where present in DNA extracts from the test isolates.

3.9.1 Extraction of DNA from *B.cereus* isolates.

DNA extraction from *B.cereus* isolates was carried out using ZR fungal/bacterial DNA miniprep™ DNA extraction kits (ZYMO RESEARCH, South Africa) as described below:

Isolates were inoculated into sterile Lauria- Bertani (LB) broth (MERCK, Germany) and incubated at 37°C for 24 h. One millilitre (1ml) of each test broth was placed in a sterile 1.5ml Eppendorf tube and centrifuged at 3000rpm for 5 min at 4°C to separate the cells from the culture filtrates. This was done twice, to obtain sizeable amounts of bacterial cells. The bacterial cells were then resuspended in 200µl of sterile distilled water, and transferred into a ZR BashingBead lysis tube. The tube was then vortexed at 5000rpm for 5 min to disrupt the bacterial cells, following which it was centrifuged at 10,000rpm for 1 min at 4°C. Four hundred microlitres (400µl) of the supernatant in the bashing tube was transferred into a Zymo-Spin™ IV Spin filter in a collection tube and centrifuged at 7,000rpm for 1 min. One thousand two hundred microlitres (1200µl) of Fungal/Bacterial DNA binding buffer was added to the filtrate in the collection tube above. Eight hundred microlitres (800µl) of this mixture was added to a Zymo-spin™ 11C column in a collection tube and centrifuged at 10,000rpm for 1 min. The flow from this collection tube was discarded and the step repeated. Two hundred microlitres (200 µl) of DNA Pre-Wash Buffer was added to the Zymo-spin™ 11C column in a new collection tube and centrifuged at 10,000 rpm for 1 min. This was followed by adding 500µl of Fungal/Bacterial DNA Wash Buffer to the Zymo-spin™ 11C column, and centrifuging at 10,000rpm for 1 min. The Zymo-spin™ 11C column was then transferred to a clean 1.5 ml microcentrifuge tube. One hundred microlitres (800µl) of DNA Elution Buffer was

added directly to the column matrix and centrifuged at 10,000rpm for 30 s to elute the DNA, which was then stored by freezing until required.

3.9.2 Amplification of diarrhoeagenic genes from DNA extracts

DNA extracts obtained from *B.cereus* isolates were analyzed for presence of genes that encode for the HBL diarrhoeagenic genes *hblD* (439bp), *hblC* (339bp), *hblA* (873bp) as well as the *bceT* (428bp) enterotoxins by PCR amplificaton. Primers for the test genes were designed by Inqaba Biotechnical Industries Limited, Pretoria, South Africa.

DNA extracts from test isolates were prepared for amplification following the producers' instructions described below:

A 50 µl reaction mixture was prepared for each DNA extract, for DNA amplification comprising of DreamTaq™ PCR Master Mix (2X) (FERMENTAS Inc, Canada), forward and reverse primers, nuclease- free water and DNA template as outlined below:

Dreamtaq™ PCR master mix (2X)	25 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
Template DNA	7 µl
Nuclease- free water	to 50µl (16 µl)

The DreamTaq™ master mix (2X) was gently vortexed and centrifuged briefly after thawing. Components were then added into UV sterilized PCR tubes in a laminar flow cabinet equipped with an UV lamp (LABCAIRE, U.K.) with the aid of UV sterilized pipette tips. Tubes were then gently vortexed, and amplification was carried out in a PCR thermal cycler (TECHNE, U.K.) as described in the PCR programmes in table 3.1.

One percent (1%) Seakem® LE agarose (BMA products, U.S.A.) was prepared (by boiling for about 2 min) into which Tris-acetate buffer (TAE) and ethidium bromide were added. This was then poured into a gel tray and allowed to set to make a gel. PCR products were then loaded into set Agarose gel wells layered with TAE buffer, and attached to an electrophoresis machine (CONSORT, Belgium) for 30 min to allow for movement of the PCR products across the gel. A marker ladder of 100bp was also loaded alongside amplicons to aid in measurement of target amplicons. Following electrophoresis, the gel was viewed under a UV lamp (VILBERLOURMAT TFX, Germany) to verify for presence of amplicons coding for the presence of HBL and BceT genes.

3.10 Antibacterial Susceptibility of Enterotoxin Producing *B.cereus* Isolates

Enterotoxin producing *B.cereus* isolates from section 3.8 were subjected to antimicrobial susceptibility tests by the disc diffusion method (Agwa *et al.*, 2012). Isolates were tested for their sensitivity to ten antimicrobial agents using filter paper discs impregnated with specified concentrations of the antimicrobial agents (OXOID, U.K.). These included Amikacin (30µg) Amoxycillin-Clavulanic acid (20/10µg), Ampicillin (10µg), Cefpodoxime (10 µg), Ceftriaxone (30 µg), Cephalothin (30 µg), Ciprofloxacin (5 µg), Erythromycin (30 µg), Tetracycline (30 µg), and Trimethoprim-Sulfamethoxazole (1.25/23.75 µg). Isolates were tested for their sensitivity to these antimicrobials agents following the manufacturer's instruction described below:

Table 3.1: Primers and PCR Programmes for Amplification of Genes Encoding for the HBL and BceT Diarrhoeal Enterotoxins in *B. cereus*

Genes	Primer sequence	Expected	PCR	Reference

		amplicons	programme	
hblA	5´GCTAATGTAGTTTCACCTAGCAAC3´ 3´AATCATGCCACTGCGTGGACATATAA5´	873bp	1	Rowan *et al*., 2001
hblC	5´AATAGGTACAGATGGACAGG3´ 3´GGCTTTCATCAGGTCATACTC5´	339bp	2	
hblD	5´ AATCAAGACCTGTCACGAAT3´ 3´ CACCAATTGACCATGCTAAT5´	439bp	3	
bceT	5´TTACATTACCAGGACGTGCTT3´ 3´TGTTTGTGATTCTAATTCAGG5´	428bp	4	

Programme	Cycling procedure
1	36 times (94^{o}C, 30 s; 63 ^{o}C, 60 s; 72 ^{o}C, 60 s)
2	36 times (94 ^{o}C, 30 s; 62 ^{o}C, 60 s; 72 ^{o}C, 60 s)
3	36 times (94 ^{o}C, 30 s; 54 ^{o}C, 60 s; 72 ^{o}C, 60 s)
4	36 times (94 ^{o}C,30 s; 58 ^{o}C, 60 s; 72 ^{o}C, 60 s)

Isolated colonies from 18-24 h nutrient agar plates were carefully picked and transferred into tubes containing sterile physiological saline. Each tube was then calibrated to make a

suspension equivalent to 0.5 Mcfarland standards. A sterile swab stick was then immersed in each tube, and rotated against the side of the tube to remove excess fluid. Each swab stick was then used to swab the entire surface of a sterile Mueller-Hinton Agar (MERCK, Germany) plate in three different directions. Test discs were then carefully applied onto each plate, which was then incubated aerobically at 37^{o}C for 16-18h.

Plates were observed for zones of growth inhibition (areas showing no obvious growth detectable by the naked eye) following the incubation period and the diameters of the zones were measured to the nearest millimeter for each isolate. The responses of test isolates to the antimicrobial agents were then interpreted according to the Clinical and Laboratory Standards Institute methodology (CLSI M100- S17, 2011) as either resistant, intermediate or sensitive.

CHAPTER 4

4.0 RESULTS

4.1 Mean Counts and Prevalence of *B. cereus* in Food Samples

Mean counts of *B.cereus* isolated from uncooked and cooked meat products are shown in Table 4.1. Counts in these products ranged from <1.4± 0.0 $\log_{10}$ cfu/g to 0.9 ± 0.1 $\log_{10}$ cfu/g for uncooked meat. **balangu** had counts ranging from <1.4± 0.0 $\log_{10}$ cfu/g to 1.1 ± 0.1 $\log_{10}$ cfu/g. Counts for **soye** and **suya** ranged from 0.4 ± 0.7 $\log_{10}$ cfu/g to 1.3± 0.7 $\log_{10}$ cfu/g and <1.4± 0.0 $\log_{10}$ cfu/g to 0.7 ± 0.9 $\log_{10}$ cfu/g respectively (see Appendices 1-IV for more details). Statistical analysis (see Appendix XXVIII for more details) using the one-way ANOVA showed no significant differences (P 0.05)n *B.cereus* count for all meat samples at 5% level.

The recovery frequencies of presumptive *B.cereus* isolated from uncooked and cooked meat are also shown in Table 4.1. *B.cereus* was isolated from 9 (21%) of uncooked meat samples, while 14 (23%) samples of **balangu** had the organism isolated from them. **Soye** and **suya** had recovery frequencies of 19 (44%) and 11 (26%) respectively.

Mean counts of *B. cereus* isolated from uncooked and cooked rice are also shown in Table 4.1. *B.cereus* counts ranged from <1.4 ± <0.0 $\log_{10}$ cfu/g to 1.0 ± 1.0 $\log_{10}$ cfu/g for uncooked rice, <1.4 ± 0.0 $\log_{10}$ cfu/g to 0.5 ± 0.7 $\log_{10}$ cfu/g for plain boiled rice and <1.4 ± 0.0 $\log_{10}$ cfu/ml to 1.3 ± 0.9 $\log_{10}$ cfu/g for **jollof** rice (see Appendices V-VI for more details). The one-way ANOVA showed no significant differences (P 0.05)n *B.cereus* counts for all cooked and uncooked rice samples at 5% level. The recovery frequency of presumptive *B. cereus* isolated from rice samples is also shown in Table 4.1. *B.cereus* was isolated from 15 (35%) of uncooked rice samples; 6 (14%) of boiled rice had the

Table 4.1: Presumptive *B. cereus* Counts (cfu/ml/g) of Retail Food Samples

Food item(n=43)	No. and percent (%) positive for *B.cereus*	*B.cereus* counts (log $_{10}$ cfu/ml/g)**	
		Mean *B. cereus* count	Range of mean *B.cereus* count

Uncooked meat	9(21)	0.4±0.3	<1.4± 0.0 to 0.9 ± 0.1
Balangu	14(23)	0.5± 0.4	<1.4± 0.0 to 1.1 ± 0.1
Soye	19(44)	0.9±0.3	<1.4± 0.0 to 1.1 ± 0.1
Suya	11(26)	0.4±0.2	<1.4± 0.0 to 0.7 ± 0.9
Uncooked rice	15(35)	0.5±0.3	<1.4 ± 0.0 to 1.0 ± 1.0
Jollof rice	8(19)	0.2±0.4	<1.4 ± 0.0 to 1.3 ± 0.9
boiled rice	6(14)	0.1±0.2	<1.4 ± 0.0 to 0.5 ± 0.7
Raw milk	6(14)	0.1±0.2	<1.4 ± 0.0 to 0.4 ± 0.6
Nono	21(49)	0.8±0.3	0.3 ± 0.7 to 1.3 ± 0.8

Key:
n: number of samples per food item
cfu/ml/g: colony forming units per milliliter/gram of food sample
**: counts below lower limits of 25

organism recovered from them, while 8 (19%) of **jollof** rice samples also had *B.cereus* isolated from them.

Mean counts of *B.cereus* isolated from raw and fermented milk **(nono)** are also shown in Table 4.1. *B.cereus* counts ranged from 0.3 ± 0.7 $\log_{10}$ cfu/ml to 1.3 ± 0.8 $\log_{10}$ cfu/ml for **nono**, and <1.4 ± 0.0 $\log_{10}$ cfu/ml to 0.4 ± 0.6 $\log_{10}$ cfu/ml for raw milk (see Appendices VIII-IX for more details). Statistical analysis using the one-way ANOVA showed no significant differences in *B.cereus* count and for all raw milk and **nono** samples at 5% level (P□0.05).

Recovery frequencies of presumptive *B.cereus* isolated from raw milk and **nono** are also shown on Table 4.1. Twenty-one (49%) of **nono** samples analyzed had *B.cereus* recovered from them, while only 6 (14%) of raw milk analyzed had *B.cereus.*

4.2 Biochemical Characterization of Presumptive *B. cereus* Isolates

Table 4.2 shows results of the biochemical characterization of stored presumptive *B.cereus* isolates obtained from retail food samples. Forty-seven of a total of 109 isolates were confirmed as *B.cereus* by Microgen™ Bacillus ID kits (Appendix XXII-XXVII). Table 4.3 shows prevalence and mean counts of the confirmed isolates where in 13 were obtained from **nono**, 7 from **balangu**, 5 from **suya**, 3 from **soye***,* 6 from uncooked rice, 6 from **jollof** rice, 4 from boiled rice, 3 from raw milk and 13 from **nono**. None of the presumptive isolates from uncooked meat was acceptably characterized as *B.cereus* by the Microgen™ Bacillus ID kits used.

Table 4.2: Biochemical Characterization of Presumptive *B. cereus* Isolates Obtained From Food Samples

Tests	Number Positive (n = 109)	Percentage Positive (%)

Gram reaction	109	100
Catalase	109	100
Indole	23	21
ONPG	47	43
Nitrate	107	98
Arginine Dihydrolase	99	91
Citrate Utilisation	61	56
Voges Proskauer	103	94
Fermentation of:		
Arabinose	12	11
Cellobiose	26	24
Inositol	14	13
Mannitol	30	28
Mannose	37	34
Raffinose	3	3
Rhamnose	10	9
Salicin	63	58
Sorbitol	11	10
Sucrose	82	75
Xylose	35	32
Adonitol	16	15
Galactose	36	33
Methyl-D-Mannoside	1	1
Methyl-D-Glucoside	7	6
Inulin	0	0
Melezitose	3	3
Trehalose	101	93

Key:

n : Total number of samples analyzed

ONPG: Ortho-nitrophenol

Table 4.3: Prevalence and Mean Counts of Confirmed *B.cereus* in Retail Foods

Food item (n= 43)	No. and percentage (%) positive	Mean *B.cereus* counts ($\log_{10}$ cfu/ml/g)**
Raw meat	0(0)	0
Balangu	7(16)	1.5±0.3
Soye	3(7)	2.4±0.3
Suya	5(12)	1.4±0.3
Uncooked rice	5(12)	1.6±0.4
Jollof rice	8(19)	1.3±0.3
Boiled rice	4(9)	1.2±0.2
Raw milk	3(7)	1.1±0.2
Nono	13(30)	1.6±0.3

Key:

cfu/ml/g: colony forming units per milliliter/gram
**: counts below lower limits of 25
n: number of samples per food item.

4.3 Survival of *B.cereus* Isolates During Storage Under Varying NaCl Concentrations and Temperature

4.3.1 Survival of *B.cereus* isolates during storage under varying NaCl and temperature in boiled rice.

Figure 1 shows mean *B.cereus* counts in boiled rice during storage at refrigeration, room and elevated temperatures at different NaCl concentrations (see Appendices X-XI for more details). Mean *B.cereus* counts increased from initial levels of 8.5 $\log_{10}$ cfu/g to 10.3 ± 0.2 $\log_{10}$ cfu/g, 9.1 ± 0.2 $\log_{10}$ cfu/g and 9.6 ± 0.6 $\log_{10}$ cfu/g at 0, 0.5 and 1% NaCl in boiled rice stored at refrigeration temperature (4-10^{o}C) for 24 h. Counts decreased from the above initial levels, to 6.3 ± 0.2 $\log_{10}$ cfu/g and 8.2 ± 1.6 $\log_{10}$ cfu/g in rice samples at 1.5 and 2% NaCl respectively, stored at the same time-temperature combination. Mean counts dropped from their levels at 24 h in all rice samples at 48 h storage at refrigeration temperature (4-10^{o}C), except at 1.5% NaCl, where counts increased from 6.3 ± 0.2 $\log_{10}$ cfu/g at 24 h, to 7.6 ± 0.6 $\log_{10}$ cfu/g at 48 h. Mean counts at 72 h during refrigeration temperature (4-10^{o}C) storage increased for all samples, from their levels at 48 h, with the highest count being 13.1 ± 0.1 $\log_{10}$ cfu/g at 0% NaCl (control samples).

Mean counts decreased from initial levels of 8.5 $\log_{10}$ cfu/g to 8.4 ± 1.1 $\log_{10}$ cfu/g, 8.4 ± 1.2 $\log_{10}$ cfu/g and 7.1 ± 0.3 $\log_{10}$ cfu/g at NaCl concentrations of 0, 0.5 and 1.5% respectively in boiled rice stored at room temperature (26 - 28^{0}C). Counts however increased from initial levels of 8.5 $\log_{10}$ cfu/g to 9.0 ± 1.0 $\log_{10}$ cfu/g and 8.8 ± 0.1 $\log_{10}$ cfu/g in NaCl concentrations of 1 and 2% respectively in samples stored at the same temperature. At elevated temperature (37^{0}C), there were noticeably lower counts from

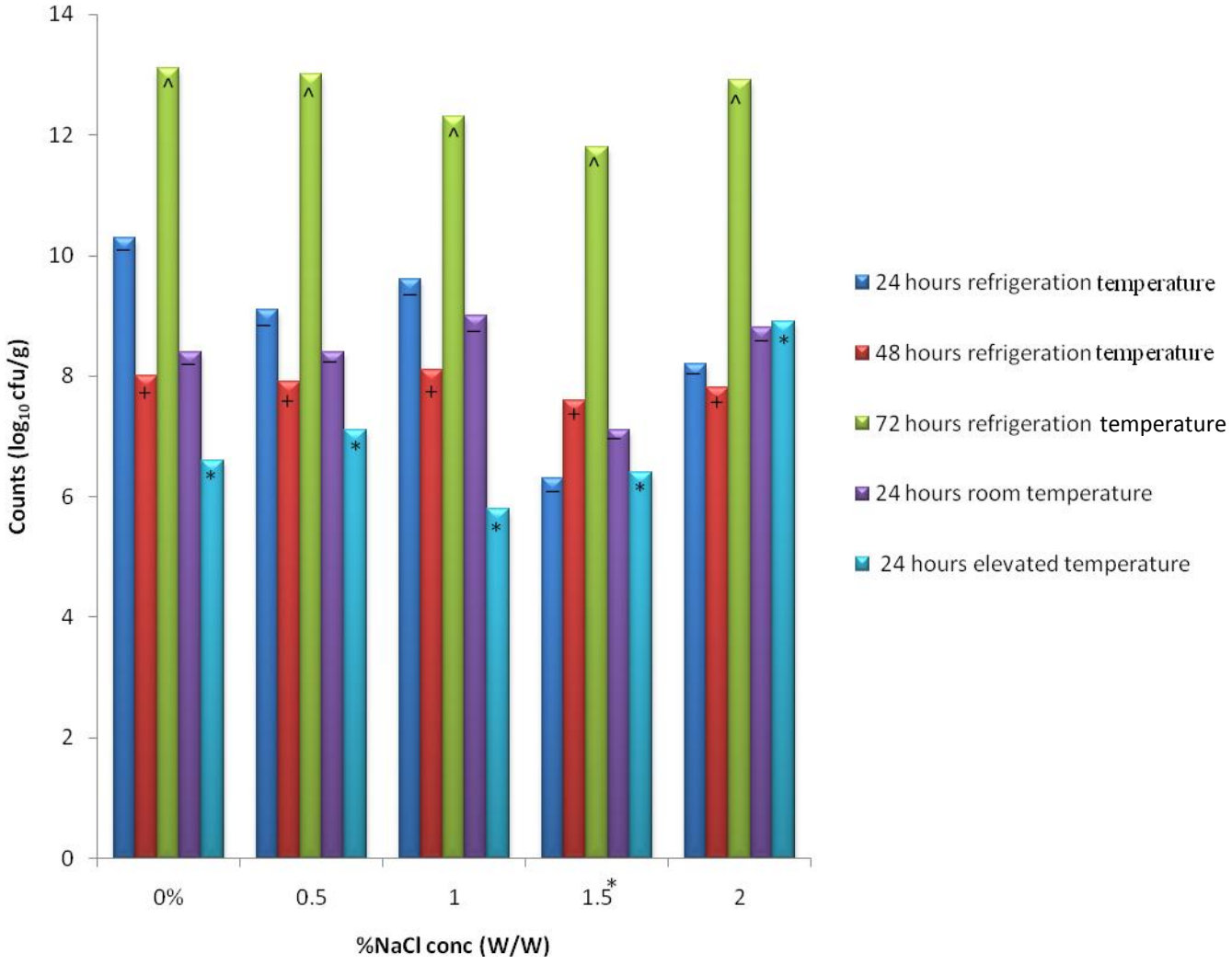


Fig 1: *B. cereus* counts in boiled rice during storage at refrigeration (4- 10^{o}C), room (26-28^{o}C), and elevated (37^{o}C) temperatures.

Mean counts of triplicate samples. Counts across storage conditions and NaCl concentrations with the same symbols are not significantly different (P>0.05)

initial levels of 8.5 $\log_{10}$ cfu/g in all rice samples, except at 2% NaCl, where counts still remained at 8.5 $\log_{10}$ cfu/g.

Statistical analysis using one-way ANOVA revealed significant differences at 5% level (P 0.05), in *B.cereus* counts in boiled rice samples during storage across the different temperatures used. Lowest mean counts were recorded for samples stored at elevated temperature of 37^{o}C, while highest value was recorded for samples stored at refrigeration temperature (4-10^{o}C) for 72 h. Similarly, significant differences (P 0.05)were recorded for the above samples across the different NaCl concentrations used, with lowest counts for samples at 1.5% NaCl concentration.

4.3.2 Survival of *B.cereus* isolates during storage under varying NaCl concentrations and temperature in balangu

Figure 2 shows mean *B. cereus* counts in **balangu** during storage at refrigeration temperarure, room and elevated temperatures (see Appendices XII-XIII for more details). Counts increased from initial levels of 8.5 $\log_{10}$ cfu/g in all samples stored at refrigeration temperature (4-10^{o}C) for 24 h except at 0.5% NaCl, where counts decreased to 8.1± 0.1 $\log_{10}$ cfu/g. Counts then increased from their levels at 48 h except at 1 and 1.5% NaCl where levels decreased from 12.2± 0.1 $\log_{10}$ cfu/g and 12.3±0.1 $\log_{10}$ cfu/g to 11.3± 0.1 $\log_{10}$ cfu/g and 10.2±0.2 $\log_{10}$ cfu/g respectively. Counts decreased in all samples at 72 h of storage at refrigeration temperature (4-10^{o}C) from their levels at 48 h, with a lowest count of 9.5± 0.2 $\log_{10}$ cfu/g at 1.5% NaCl. Mean *B.cereus* counts in **balangu** increased from initial levels of 8.5 $\log_{10}$ cfu/g at all NaCl concentrations (Fig 2), with the highest counts of 14.3 $\log_{10}$ cfu/g at 1% NaCl when stored at room temperature (26 – 28^{o}C).

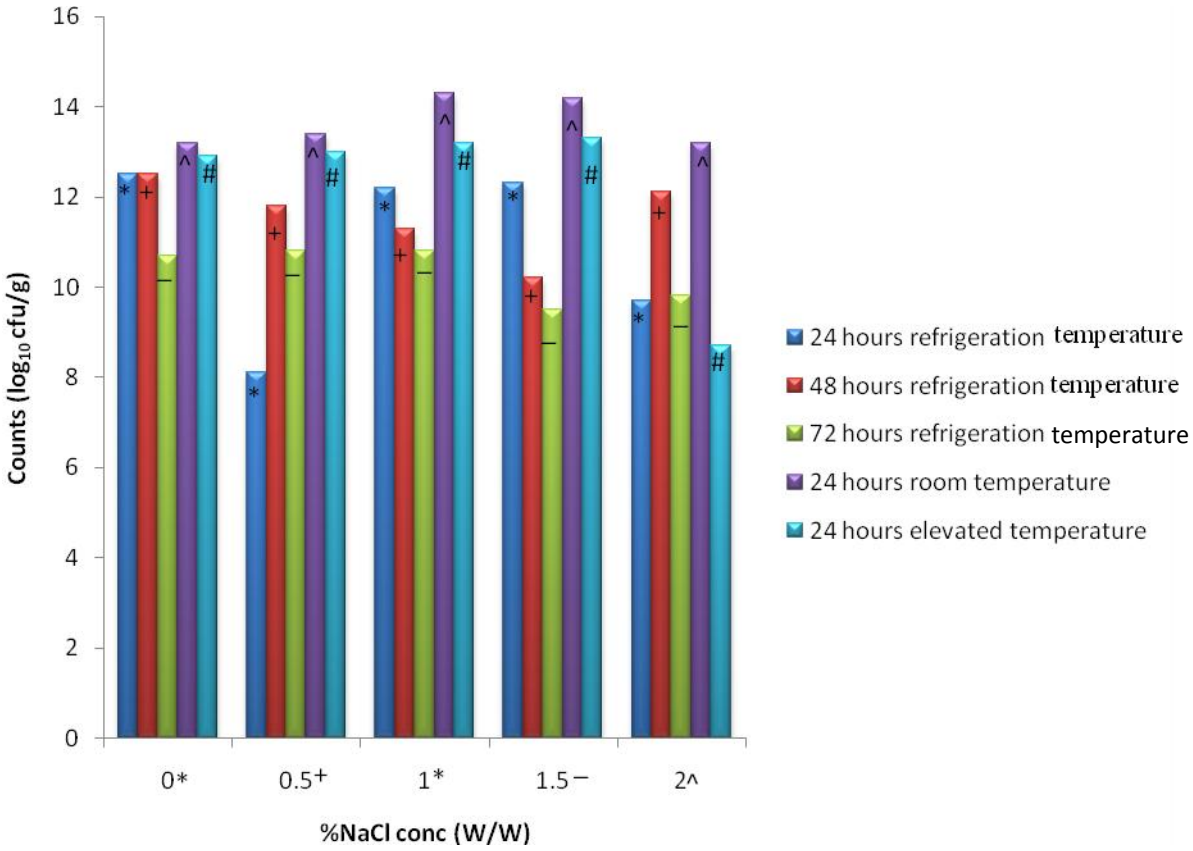


Fig 2: *B.cereus* counts in **balangu** at different concentrations of NaCl during storage at refrigeration (4-10^{o}C), room (26 -28^{o}C) and elevated (37^{o}C) temperatures.

Mean counts of triplicate samples. Counts across storage conditions and NaCl concentrations with the same symbols are not significantly different (P>0.05)

Counts also increased from 8.5 log $_{10}$ cfu/g in all samples stored at elevated temperature (37^{o}C). There was however noticeable lower count values for samples stored at 2% NaCl.

Significant differences were also recorded for **balangu** samples at 5% level, in *B.cereus* counts across the different temperatures, with lowest and highest means recorded for samples at 72 h of refrigerated storage (4-10^{o}C) and samples stored at room temperature (26-28^{o}C) respectively. *B.cereus* counts in the samples across the different NaCl concentrations used also showed significant differences at 5% level where lowest counts were recorded for samples at 2% NaCl. Highest levels were recorded for control samples (0%) and samples at 1%.

4.4 Survival of *B.cereus* Isolates During Storage Under Varying pH Levels and Temperature

4.4.1 Survival of *B.cereus* isolates during storage under varying pH levels and temperature in nono

Mean *B. cereus* counts decreased for all **nono** samples at 24 h of storage from initial levels of 8.5± $\log_{10}$ cfu/ml to a lowest of 6.9±0.3 $\log_{10}$ cfu/ml at pH 4.2 during refrigeration temperature (4-10^{o}C) (Fig 3). Counts further decreased at 48 h for all samples to a lowest of 6.3± 0.1 $\log_{10}$ cfu/ml at pH 3.8. At 72 h counts increased from their levels at 48 h in all samples except at pH 3.7, where counts decreased from 7.0± 0.2 $\log_{10}$ cfu/ml to 6.2 ± 0.6 $\log_{10}$ cfu/ml. Fig 3 also shows mean *B.cereus* counts in **nono** samples stored at room and elevated temperatures at different pH levels. Mean counts decreased from initials level of 8.5 $\log_{10}$ cfu/ml

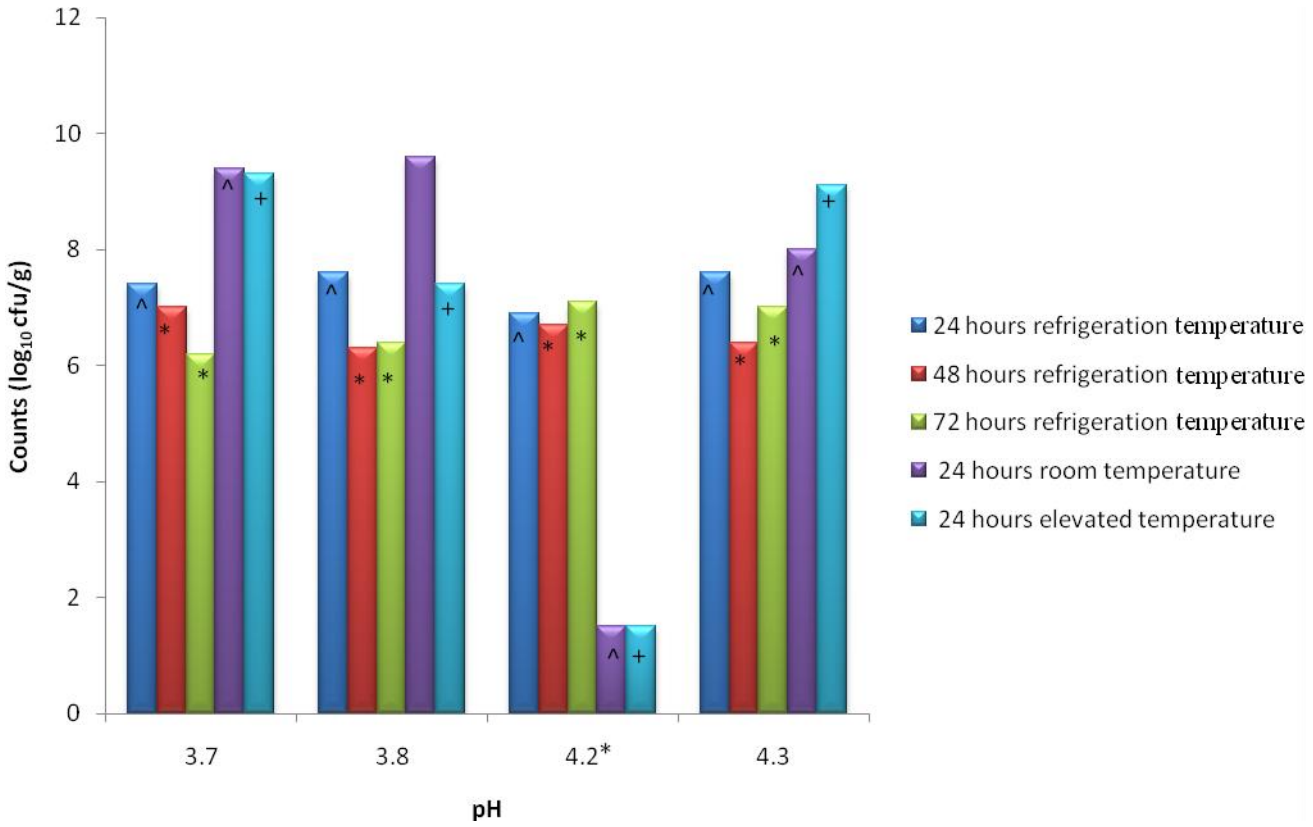


Fig 3: *B.cereus* counts in **nono** at different pH levels during storage at refrigeration (4-10^{o}C) room (26-28^{o}C) and elevated (37^{o}C) temperatures.

Mean counts of triplicate samples. Counts across storage conditions and pH levels with the same symbols are not significantly different (P>0.05)

except at pH 3.7, to a lowest of 1.5±0.4 $\log_{10}$ cfu/ml at pH 4.2 (control) for samples stored at room temperature (26-28^{o}C). At elevated temperature however (37^{o}C), Mean *B. cereus* counts increased for all samples from initial levels of 8.5 $\log_{10}$ cfu/ml to a highest level of 9.6± 0.4 $\log_{10}$ cfu/ml except at pH 4.2 (see Appendices XIV-XV for more details).

Statistical analysis using one-way ANOVA revealed significant differences at 5% level (P 0.05), in *B.cereus* counts in **nono** samples during storage across the different temperatures used. Lowest mean counts were recorded for samples stored at refrigeration temperature (4-10^{o}C) for 48 and 72 h, while highest value was recorded for samples stored at room temperature (26-28^{o}C) and at refrigeration temperature (4-10^{o}C) for 24 h. Similarly, significant differences were recorded for the above samples across the different pH levels used ($P<0.05$), with lowest counts for samples at pH 4.2, and highest counts at pH 3.8.

4.4.2 Survival of *B.cereus* isolates in pasteurized cow milk at varying pH levels during storage at refrigeration temperature

Mean *B.cereus* counts decreased from initial levels of 8.5± $\log_{10}$ cfu/ml in all pasteurized milk samples (Fig 4), except at pH 6.3 after 24 h at refrigeration temperature (4-10^{o}C) storage, where mean counts still remained at 8.5± 0.3 $\log_{10}$ cfu/ml. Mean counts then decreased at 48 h of storage from their level at 24 h to 6.6 ± 0.3 $\log_{10}$ cfu/ml and 7.2 ± 0.2 $\log_{10}$ cfu/ml at pH 6.3 and 6.8 respectively. Mean counts however increased from 8.2 ± 0.2 $\log_{10}$ cfu/ml and 8.7 ± 0.5 $\log_{10}$ cfu/ml for pH 6.4 and 6.7(controls) respectively

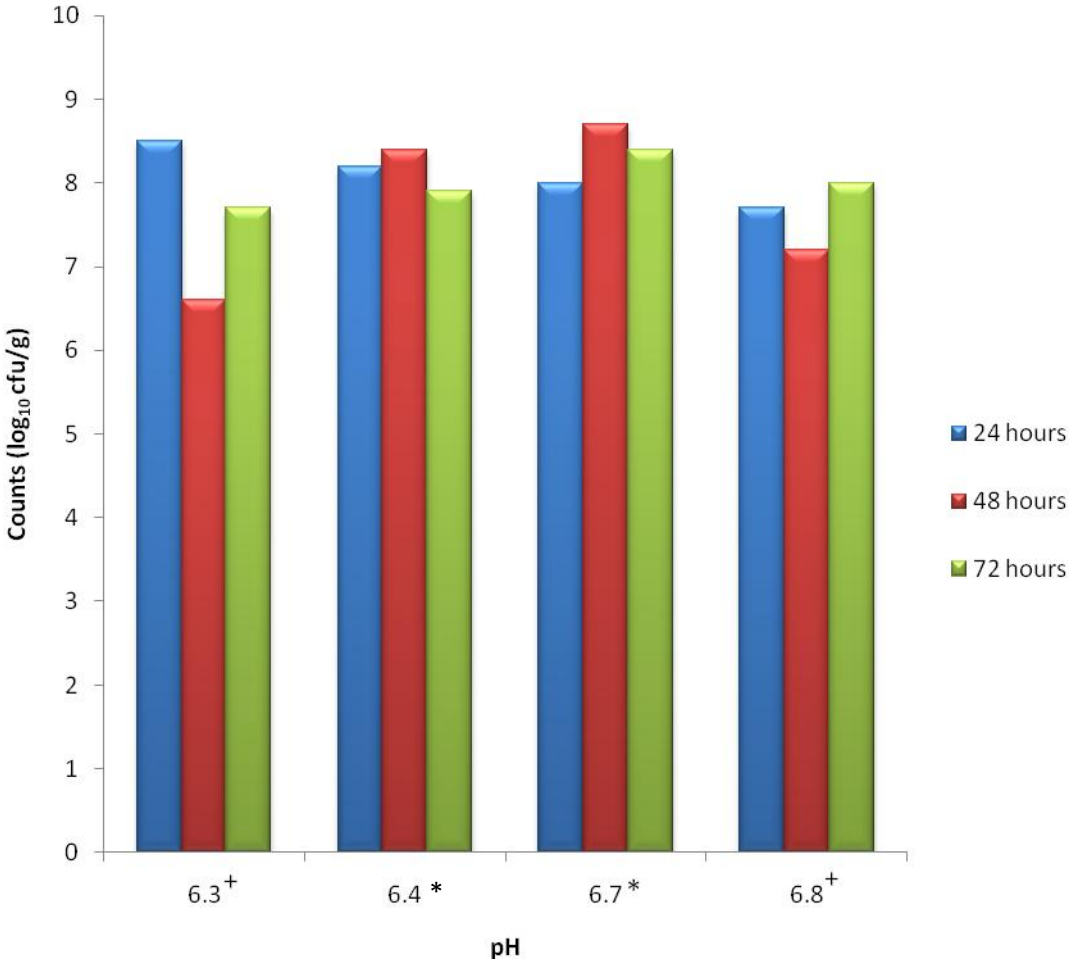


Fig 4: *B.cereus* counts in pasteurized milk during storage at refrigeration temperature (4-10^{o}C) for 72 h.

Mean counts of triplicate samples. Counts not significantly different across all storage conditions (P>0.05), and at pH levels with the same symbols.

at the same period of storage. At 72 h of storage at refrigeration temperature (4-10^{o}C) , mean *B. cereus* counts decreased from their levels at 48 h from 8.2 ± 0.5 $\log_{10}$ cfu/ml and 8.0± 0.5 $\log_{10}$ cfu/ml to 7.9 ± 0.4 $\log_{10}$ cfu/ml and 8.4± 0.5 $\log_{10}$ cfu/ml at pH 6.4 and 6.7 respectively. There were however increases in counts from 6.6 ± 0.3 $\log_{10}$ cfu/ml and 7.2± 0.2 $\log_{10}$ cfu/ml at pH 6.3 and 6.8 respectively, at the same period of storage (see Appendix XVI for more details).

No significant differences were recorded for pasteurized milk samples at 5% level (P 0.05) in *B.cereus* counts across the different storage periods at refrigeration temperature (4-10^{o}C). *B.cereus* counts in the samples across the different pH levels used were however significantly different (P 0.05) at 5% level where lowest counts were recorded for samples at pH 6.3 and 6.8. Highest levels were recorded for control samples (pH 6.4 and 6.7).

4.5 Production of Diarrhoeal Enterotoxins by *B. cereus* Isolates Grown Under Varying pH levels, NaCl Concentrations and Temperature.

4.5.1 Production of diarrhoeal enterotoxins by *B. cereus* isolates grown under varying NaCl concentrations and temperature .

Results of diarrhoeal enterotoxin production by isolates grown under combined effects of NaCl concentration and temperature with time in boiled rice are shown in Table 4.4. Highest enterotoxin titres of ≥64 ng/ml were recorded for isolates at all levels of NaCl concentrations at optimum temperature (37^{o}C). Similarly high toxin titres of ≥64 ng/ml were recorded for all isolates grown at room temperature (26-28^{o}C). Highest enterotoxin titres of ≥64 ng/ml were recorded for samples at all levels of NaCl concentrations during

Table 4.4: Combined Effects of NaCl Concentration, Temperature and Storage Time on Enterotoxin Production by *B.cereus* Isolates From Boiled Rice

Temperature	NaCl	Storage	Counts	Toxin Titre	Correlation	P**

(^{0}C)	Concentration (%)	Time (h)	(log_{10}cfu/ml)	(ng/ml)*	coefficient (r)	
37	0	24	6.6±1.0	≥64		0.96
	0.5		7.1±0.8	≥64		
	1		5.8±0.3	≥64		
	1.5		6.4±0.2	≥64		
	2		8.5±0.1	≥64		
26-28	0	24	8.4±1.1	≥64		
	0.5		8.4±1.2	≥64		
	1		9.0±1.0	≥64		
	1.5		7.1±0.3	≥64		
	2		8.8±0.1	≥64		
4-10	0	24	10.3±0.2	≥64		
	0.5		9.1±0.2	≥64		
	1		9.6±0.6	≥64		
	1.5		6.3±0.2	≥64		
	2		8.2±1.6	≥64		
4-10	0	48	8.0±0.2	≥64	-0.63	
	0.5		7.9±0.3	≥64		
	1		8.1±0.6	32		
	1.5		7.6±0.6	≥64		
	2		7.8±0.2	≥64		
4-15	0	72	13.1±0.1	32	0.28	
	0.5		13.1±0.1	32		
	1		12.3±0.0	32		
	1.5		11.8±1.5	32		
	2		12.9±0.6	≥64		

Key:

cfu/ml: colony forming units per milliliter of triplicate samples (initial inoculum levels of 8.5 log_{10}cfu/ml)

*: Sensitivity at 2ng/ml

**: level of significance at $P<0.05$ for toxin titres at all storage periods,

24 and 48 h of refrigerated storage in boiled rice. Titres were however observed to reduce to32 ng/ml at 72 h for most of the samples.

Results of diarrhoeal enterotoxin production by *B.cereus* isolates grown under combined effects of NaCl concentration and temperature in **balangu** are shown in Table 4.5. Enterotoxin titres ranged from 16ng/ml for isolates from **balangu** at 2% NaCl concentrations, to ≥64 ng/ml for isolates at lower NaCl concentrations during storage at optimum temperature (37^{o}C). Similarly, a lower toxin tire of 32 ng/ml was recorded for isolates from 2% NaCl concentration during storage at room temperature ((26-28^{o}C). High positive correlations of 0.99 and 0.96 were recorded for samples at optimum temperature (37^{o}C) and room temperature (26-28^{o}C) respectively. No toxin (2ng/ml) was recorded for control samples stored at room temperature at (26-28^{o}C) for 24 h. Toxin titres of ≥64 ng/ml were recorded for isolates from challenge samples (0.5-2%) at the end of 24 h refrigerated storage (4-10^{o}). There appeared to be positive correlations between toxin titres and counts with values increasing from 0.44 to 0.98 from 48 to 72 h respectively. Toxin titres for isolates from control samples increased from 32ng/ml at 24 h to 64ng/ml at 48 and 72 h respectively.

4.5.2 Production of diarrhoeal enterotoxins by *B. cereus* isolates grown under varying pH levels and temperature.

Table 4.6 shows results of diarrhoeal enterotoxin production tests by *B.cereus* isolates grown in **nono** at different pH levels and time-temperature combinations. Enterotoxin titres of ≥64 ng/ml were recorded for isolates from all **nono** samples during the 72 h refrigerated storage (4-10^{o}C) period. Isolates from samples stored at room temperature (26-28^{o}C) had titre values ranging from 4ng/ml at pH 3.7 and 16ng/ml at pH 3.8.

Table 4.5: Combined Effects of NaCl Concentration, Temperature and Storage Time on Enterotoxin Production by *B.cereus* Isolates From **Balangu**

Temperature (^{0}C)	NaCl Concentration (%)	Storage Time (h)	Counts (log_{10}cfu/ml)	Toxin Titre (ng/ml)*	Correlation coefficient (r)	P**
37	0	24	12.9±0.5	≥64	0.99	0.11
	0.5		13.0±0.0	32		
	1		13.2±0.1	≥64		
	1.5		13.3±0.0	≥64		
	2		8.7±0.1	16		
26-28	0	24	13.2±0.1	□ 2	0.96	
	0.5		13.4±0.2	≥64		
	1		14.3±0.1	≥64		
	1.5		14.2±0.3	≥64		
	2		13.2±0.1	32		
4-10	0	24	12.5±0.3	32	-0.44	
	0.5		8.1±0.1	≥64		
	1		12.2±0.1	≥64		
	1.5		12.3±0.1	≥64		
	2		9.7±0.5	≥64		
	0	48	12.5±0.9	≥64	0.44	
	0.5		11.8±0.7	4		
	1		11.3±0.1	32		
	1.5		10.2±0.2	32		
	2		12.1±1.4	≥64		
	0	72	10.7±0.2	≥64	0.98	
	0.5		10.8±0.3	≥64		
	1		10.8±0.4	≥64		
	1.5		9.5±0.2	32		
	2		9.8±0.4	32		

Key:

cfu/ml: colony forming units per milliliter of triplicate samples (initial inoculum levels of 8.5 log_{10}cfu/ml)

*: Sensitivity at 2ng/ml

**: level of significance at $P<0.05$ for toxin titres at all storage periods,

Table 4.6: Combined Effects of pH, Temperature and Storage Time on Enterotoxin Production by *B.cereus* Isolates From **nono**

37	3.7	24	9.3±0.3	8	0.52	0.54
	3.8		9.6±0.4	8		
	4.2		1.5±0.2	NVI		
	4.3		8.1±0.2	≥64		
26-28	3.7	24	9.4±0.3	4	0.71	
	3.8		9.7±1.7	16		
	4.2		1.5±0.4	NVI		
	4.3		9.3±0.6	8		
4-10	3.7	24	7.4±0.3	≥64		
	3.8		7.6±0.7	≥64		
	4.2		6.9±0.3	≥64		
	4.3		7.6±0.8	≥64		
	3.7	48	7.0±0.2	≥64		
	3.8		6.3±0.1	≥64		
	4.2		6.7±0.3	≥64		
	4.3		6.4±0.2	≥64		
	3.7	72	6.2±0.6	≥64		
	3.8		6.4±0.3	≥64		
	4.2		7.1±0.4	≥64		
	4.3		7.0±0.5	≥64		

Key:

cfu/ml: colony forming units per milliliter of triplicate samples (initial inoculum levels of 8.5 $\log_{10}$cfu/ml)

*: Sensitivity at 2ng/ml

**: level of significance at P<0.05 for toxin titres at all storage periods

NVI: no viable isolate

A high positive correlation (r=0.71) between toxin titre and counts at the end of storage period was recorded for these isolates. Samples stored at elevated temperature of 37^{o}C had toxin titre values of ≥64ng/ml for isolates at pH 4.3, and 8 ng/ml for isolates at pH 3.7 and 3.8. A medium, but positive correlation (r = 0.52) between toxin titre and counts at the end of storage period was also recorded for these isolates.

Toxin titer values for *B.cereus* in pasteurized milk during refrigerated storage (Table 4.7) were comparatively lower than those recorded for isolates from **Nono** samples; titer values of ≥64 ng/ml were recorded only for samples at pH 6.3 at 24 h of storage and at pH 6.7 at 48 h of storage. Values ranged from ≤2 to32 ng/ml for other samples, with samples at pH 6.8 at 48 h of storage having the lowest value of ≤ 2 ng/ml. There appeared to be a positive correlation between toxin titre and counts for isolates over the 72 h storage period with values ranging from 0.40 at 24 h to 0.71 at 72 h.

Non-specific agglutination or auto agglutination was not observed in this assay, as no agglutination was observed with non immune sera. Non-uniform distribution of agglutination usually encountered in this type of assay was however observed, such that wells with higher titres had higher agglutination levels than wells with lower titres.

4.6 Molecular Characterization of Enterotoxin and Non- enterotoxin Producing Isolates.

Results of detection of diarrhoeagenic genes HBL and BceT in *B.cereus* isolates grown in food samples at different pH, NaCl and time-temperature combinations are shown in Plates III-VI, and Tables 4.8 and 4.9.

Table 4.7: Combined Effects of pH and Storage Time on Enterotoxin Production by *B.cereus* Isolates From Pasteurized Cow Milk During Refrigeration

Temperature (^{0}C)	pH	Storage Time (h)	Counts (log_{10}cfu/ml)	Toxin Titre (ng/ml)*	Correlation coefficient (r)	P**
4-10	6.3	24	8.5±0.3	≥64	0.40	0.11
	6.4		8.2±0.5	4		
	6.7		8.0±0.5	32		
	6.8		7.7±0.2	32		
	6.3	48	6.6±0.3	4	0.70	
	6.4		8.4±0.5	8		
	6.7		8.7±0.1	≥64		
	6.8		7.2±0.2	□ 2		
	6.3	72	7.7±0.4	16	0.71	
	6.4		7.9±0.4	2		
	6.7		8.4±0.5	2		
	6.8		8.0±0.3	8		

Key:

cfu/ml: colony forming units per milliliter of triplicate samples (initial inoculum levels of 8.5 log_{10}cfu/ml)

*: Sensitivity at 2ng/ml

**: level of significance at P<0.05 for toxin titres at all storage periods,

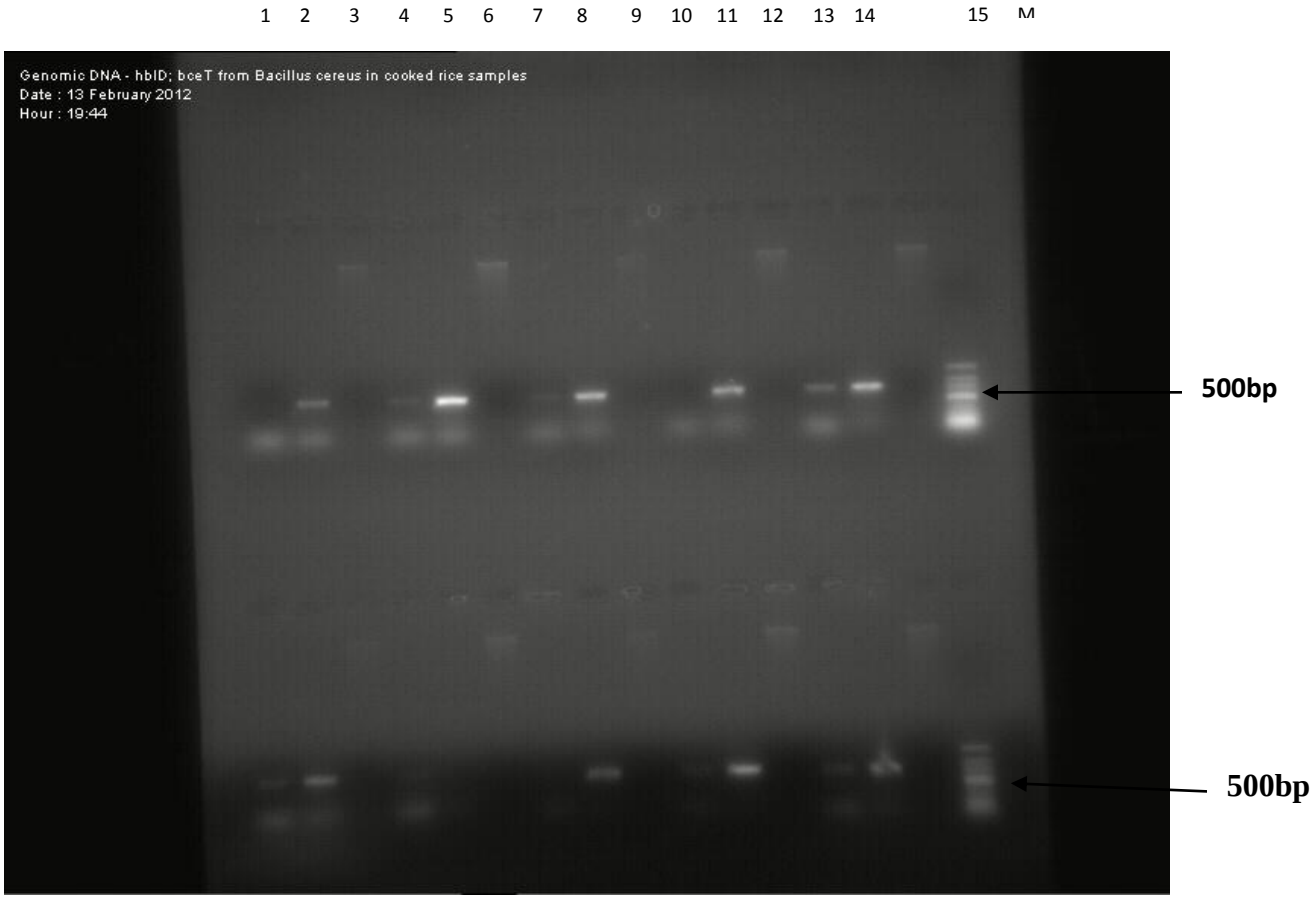


Plate III: Genomic DNA and amplicons encoding for *hblD* (439bp) and *bceT* (428bp) genes in *B.cereus* from boiled rice.

Key:

Lanes 4 and 5, row 1: *bceT* and *hblD* in isolate from rice at 0% NaCl elevated temperature (37^{o}C) for 24 h

Lane 10, row 1: *hblD* only in isolate grown in rice at 0% NaCl at room temperature (26-28^{o}C) for 24 h

Lanes 12 and 13, row 1: *bceT* and *hblD* genes in unchallenged isolate from retail boiled rice

Lane 4, row 2: *bceT* only in isolate grown in rice at 1 % NaCl at refrigeration temperature (4-10 ^{o}C) for 48 h

M: Ruler

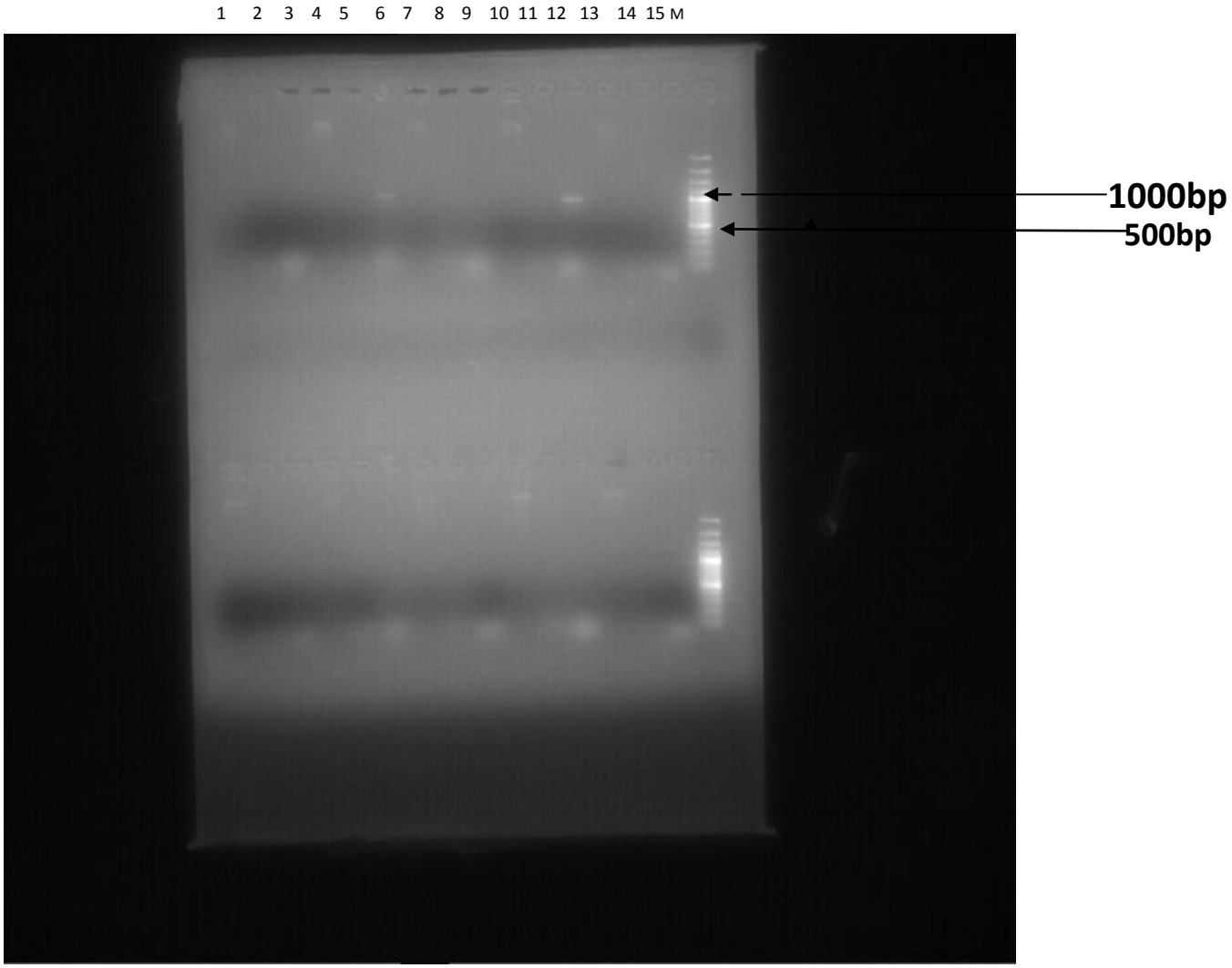


Plate IV: Genomic DNA and amplicons encoding for *hblC* (339bp) and *hblA* (873bp) genes in *B.cereus* from boiled rice

Key:

Lane 6, row1: *hblA* gene in isolate from boiled rice at 0% NaCl concentration at room

temperature (26- 28^{o}C)

Lane 12, row1: *hblA* gene in isolate from boiled rice at 0.5% NaCl concentration at elevated temperature (37^{o}C)

Lanes 11 and 12, row 2: *hblC* and *hblA* absent in isolate grown at 1% NaCl at refrigeration temperature (4-10 ^{o}C) for 48 h

M: Ruler

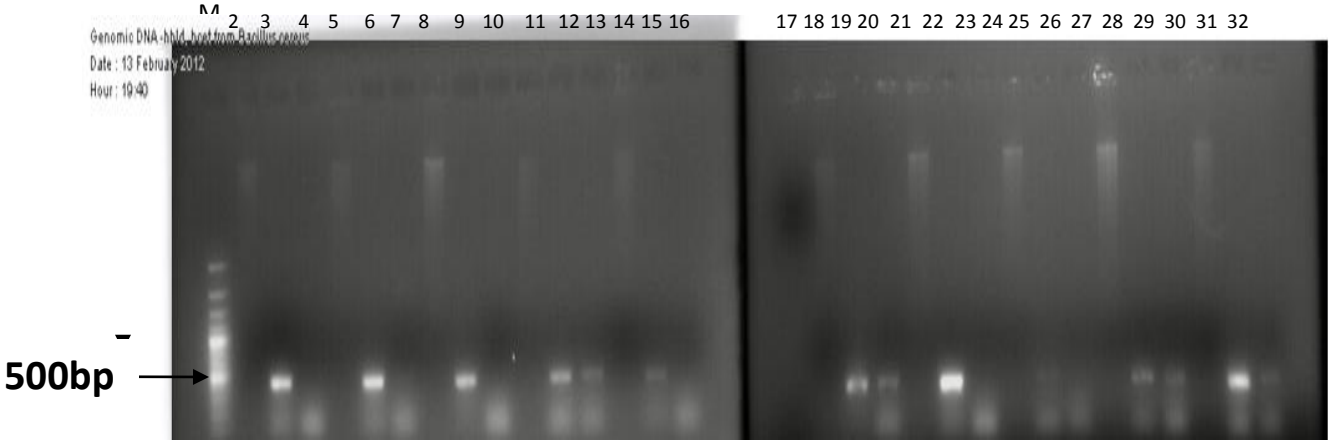


Plate V: Genomic DNA and amplicons encoding for *hblD* (439bp) and *bceT* (428bp) genes in *B.cereus* from pasteurized milk during storage at refrigeration temperature(4-10 ^{o}C)

Key:

M: Ruler

Lane 2: Genomic DNA from unchallenged isolate (R3M1) obtained from unpasteurized milk

Lane 3: *hblD* gene in R3M1

Lane 4: *bceT* absent in R3M1

Lanes 12 and 13: *hblD* gene and *bceT* genes in R3M1 from pasteurized milk at pH 6.4 during refrigeration for 48 h.

Lanes 28 and 29: *hblD* and *bceT* genes in R3M1 from pasteurized milk at pH 6.8 during refrigeraton for 48 h.

Lanes 31 and 32: *hblD* and *bceT* genes in R3M1 from pasteurized milk at pH 6.8 during refrigeraton for 72 h.

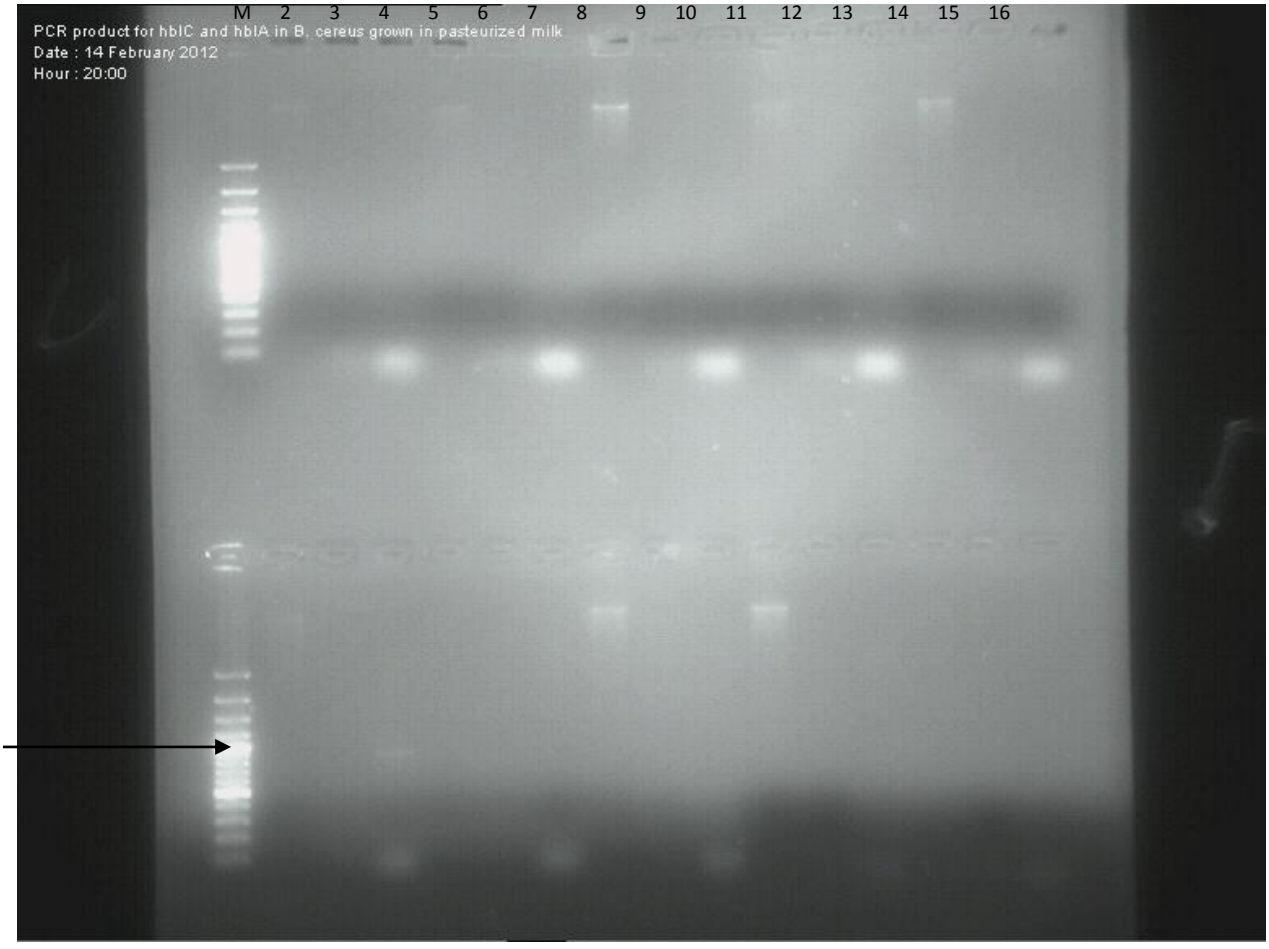


Plate VI: Genomic DNA and amplicons encoding for *hblC* (339bp) and *hblA* (873bp) genes in *B.cereus* from pasteurized milk during refrigeration

Key:

M: Ruler
Lane 4, row 2: *hblA* gene in isolate from pasteurized milk at pH 6.7 at refrigeration temperature for 48 h only

Table 4.8: Diarrhoeagenic Genes Detected by PCR in *B.cereus* Isolate F2R From Boiled Rice at Varying NaCl Concentrations, Temperature and Storage Time

0	26-28	24	≥64	+	-	+	-
0	4-10	24	≥64	+	-	-	+
0.5	37	24	≥64	+	- +	+	
0.5	4-10	24	≥64	+	-	-	-
0.5	4-10	48	≥64	+	-	-	+
1	4-10	48	32	-	-	-	+
2	4-10	24	≥64	+	-	-	+
2	4-10	48	≥64	+	-	-	-
2	4-15	72	≥64	+	-	-	+
Unchallenged isolate	NA	NA	≥64	+	-	-	+

Key:

*: toxin titre sensitivity at 2ng/ml
PCR: Polymerase Chain Reaction
hblD: haemolysin BL D gene
hblC: haemolysin BL C gene
hblA: haemolysin BL A gene
bceT: *B.cereus* enterotoxin T gene
NA: not applicable for isolate

Table 4.9: Diarrhoeagenic Genes Detected by PCR in *B.cereus* Isolate R3M1 From Pasteurized Milk at Varying pH During Refrigeration*

6.3	24	≥64	+	-	-	-
6.4	24	4	+	-	-	-
6.4	48	8	+	- -	+	
6.4	72	2	+	-	-	-
6.7	48	≥64	+	-	+	+
6.8	24	32	-	-	-	-
6.8	48	□ 2	+	-	-	+
Unchallenged isolate	NA	8	+	-	-	-

Key:
*: refrigeration temperature of 4-10^{o}C
**: toxin titre sensitivity at 2ng/ml
PCR: Polymerase Chain Reaction
hblD : haemolysin blD gene
hblC: haemolysin blC gene
hblA: haemolysin blA gene

bceT: *B.cereus* enterotoxin T gene
NA: not applicable for isolate

The unchallenged *B.cereus* isolate from retail rice (F_2R) showed the presence of only *hblD* and *bceT* genes (Plate III). The isolates when grown in boiled rice at 0% NaCl at room temperature (26-28 ^{o}C) for 24 h, 0.5% NaCl at refrigeration temperature (4-10 ^{o}C) for 24 h and at 2% NaCl for 48 h at refrigeration temperature (4-10 ^{o}C) did not appear to possess the *bceT* gene (Table 4.8). The *hblA* gene (in addition to the *hblD* gene) was also detected in isolates from boiled rice at 0% NaCl following storage for 24 h at room temperature (Plate IV). A Similar observation was made for isolates from boiled rice at 0.5% NaCl following storage at elevated temperature (37^{o}C) (Plate IV). It was observed that the *hblA*, *hblD* and *bceT* genes were all detected by PCR for isolates grown at 0.5% NaCl and at elevated temperature (37) ^{o}C for 24 h (Table 4.8), and which was not the case with isolates from other storage conditions. The *hblC* was not detected by PCR in these isolates irrespective of storage condition (Table 4.8).

The unchallenged isolates from raw milk (R3M1) had only the *hblD* gene (Plate V). The same gene was also detected in all other isolates grown in milk samples except isolates from milk at

pH 6.8 following 24 h of storage at refrigeration temperature (4-10 ^{o}C) (Table 4.9). All Isolates at 48 h refrigeration temperature (4-10 ^{o}C), irrespective of pH level had the *bceT* gene, in addition to the *hblD* gene (Table 4.9). Isolates from milk at pH 6.7 following 48 h of storage at refrigeration temperature (4-10 ^{o}C), had the *hblA* (Plate VI) and *bceT* genes. The *hblC* was also not detected by PCR in pasteurized milk isolates irrespective of storage conditions (Table 4.9).

4.7 Antibiograms of Diarrhoeal Enterotoxin Producing *B.cereus* Isolates Obtained From Foods Stored at Varying pH Levels, NaCl Concentrations and Temperature.

Tables 4.10- 4.13 show results of susceptibility tests for diarrhoeal enterotoxin *B. cereus* isolates obtained from foods stored at varying pH, NaCl and temperature (see appendix XVII-XX for more details). All isolates (100%) were found to be resistant to amoxyclav and ampicillin. Similarly, all isolates (100%) were found to be resistant to cefpodoxime. Isolates were also all resistant to trimethoprim - sulfamethoxazole (100%).

Ninety-six percent (96%) of boiled rice isolates were resistant to ceftriaxone (Table 4.10). Isolates from rice at 0.5% NaCl stored for 24 h refrigeration exhibited intermediate response to ceftriaxone. Similarly, about 17% of isolates from boiled rice exhibited intermediate response to tetracycline, while the rest were all sensitive (Table 4.10).

Isolates from **balangu** (Table 4.11) were all sensitive to tetracycline, except for isolates from control samples stored at 37^{o}C for 24 h which showed intermediate response to the antibiotic. Ninety- six percent of **balangu** isolates were resistant to erythromycin, where as all other food isolates did not appear to be resistant.

Only 33% and 18% of isolates from **nono** and pasteurized milk samples (Tables 4.12 and 4.13) respectively, were resistant to tetracycline.Cephalothin had 91% resistant isolates from pasteurized milk (Table 4.13). Isolates from milk at pH 6.8 after 72 h refrigeration were the only isolates that were sensitive to cephalothin. None of the isolates in this study (0%) was resistant to amikacin and ciprofloxacin.

Table 4.10: Antibacterial Susceptibility of Diarrhoeal Enterotoxin Producing *B.cereus* Boiled Rice Isolates

Amoxicillin-Clavulanic acid (20/10)	24 (100)	0 (0)	0 (0)
Ampicillin (10)	24 (100)	0 (0)	0 (0)
Cefpodoxime (10)	24 (100)	0 (0)	0 (0)
Ceftriaxone (30)	23 (96)	1(4)	0 (0)
Cephalothin (30)	24 (100)	0 (0)	0 (0)
Amikacin (30)	0 (0)	0 (0)	24 (100)
Tetracycline (30)	0 (0)	4 (17)	20 (83)
Erythromycin (30)	0(0)	0 (0)	24 (100)
Ciprofloxacin (5)	0 (0)	0 (0)	24 (100)
Trimethoprim/Sulfamethoxazole (1.25/23.75)	24 (100)	0 (0)	0 (0)

Key:

*Figures in brackets are amount of antibiotic incorporated in discs.

Table 4.11: Antibacterial Susceptibility of Diarrhoeal Enterotoxin Producing *B.cereus* **Balangu** Isolates

Amoxicillin-Clavulanic acid (20/10)	25 (100)	0 (0)	0 (0)
Ampicillin (10)	25 (100)	0 (0)	0 (0)
Cefpodoxime (10)	25 (100)	0 (0)	0 (0)
Ceftriaxone (30)	25 (100)	0 (0)	0 (0)
Cephalothin (30)	25 (100)	0 (0)	0 (0)
Amikacin (30)	0 (0)	0 (0)	25 (100)
Tetracycline (30)	0 (0)	1 (4)	24 (96)
Erythromycin (30)	24(96)	0 (0)	1 (4)
Ciprofloxacin (5)	0 (0)	0 (0)	25 (100)
Trimethoprim/Sulfamethoxazole (1.25/23.75)	25 (100)	0 (0)	0 (0)

Key:

*Figures in brackets are amount of antibiotic incorporated in discs.

Table 4.12: Antibacterial Susceptibility of Diarrhoeal Enterotoxin Producing *B.cereus* **Nono** Isolates

Amoxicillin-Clavulanic acid (20/10)	18 (100)	0 (0)	0 (0)
Ampicillin (10)	18 (100)	0 (0)	0 (0)
Cefpodoxime (10)	18 (100)	0 (0)	0 (0)
Ceftriaxone (30)	18 (100)	0 (0)	0 (0)
Cephalothin (30)	18 (100)	0 (0)	0 (0)
Amikacin (30)	0 (0)	0 (0)	18 (100)
Tetracycline (30)	6 (33)	12 (67)	0(0)
Erythromycin (30)	0(0)	0 (0)	18(100)
Ciprofloxacin (5)	0 (0)	0 (0)	18 (100)

Trimethoprim/Sulfamethoxazole (1.25/23.75)	18 (100)	0 (0)	0 (0)

Key:

*Figures in brackets are amount of antibiotic incorporated in discs.

Table 4.13: Antibacterial Susceptibility of Diarrhoeal Enterotoxin Producing *B.cereus* Pasteurized Milk Isolates

Antibacterial agent (Potencyh in µg)*	No. and (%) Resistant	No. and (%) intermediate	No. and (%) Sensitive
Amoxicillin-Clavulanic acid (20/10)	11(100)	0 (0)	0 (0)
Ampicillin (10)	11 (100)	0 (0)	0 (0)
Cefpodoxime (10)	11 (100)	0 (0)	0 (0)
Ceftriaxone (30)	11 (100)	0 (0)	0 (0)
Cephalothin (30)	10 (91)	0 (0)	1(9)
Amikacin (30)	0 (0)	0 (0)	11 (100)
Tetracycline (30)	2(18)	2(18)	7 (64)
Erythromycin (30)	0(0)	0 (0)	11 (100)
Ciprofloxacin (5)	0 (0)	0 (0)	11 (100)

Trimethoprim/Sulfamethoxazole (1.25/23.75)	11(100)	0 (0)	0 (0)

Key:

*Figures in brackets are amount of antibiotic incorporated in discs.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Prevalance of *B.cereus* in Retail Food Samples

The results of this study showed low prevalence rates of *B.cereus* in raw (0%) and processed meat (<20%) following biochemical characterization of presumptive isolates. This low prevalence rate in raw meat which were sampled in the morning, could be attributed to the nature and population of the microbial associations (Mossel *et al.,* 1995) that exist on meat samples during slaughtering. Meat can be contaminated during processing through contact with the skin of animals, feet and intestinal contents of the animal, floor, equipment and bleeding of the animal and subsequently be distributed via cut or raw meat intended for further processing (Badr, 2004; Abdalla *et al*., 2009a; Abdalla *et al.,* 2009b). As a result, raw meat could contain diverse groups of microorganisms, amongst which are the enterococci and lactobacilli which are frequently encountered (Nychas *et al*., 2007).

Bacillus (which includes the *B.cereus)*, though not frequently encountered, is known to occur on raw meat (Nychas *et al*., 2007). In microbial associations, antagonism between microorganisms frequently occurs and reports have been documented of antagonistic activity of bacteriocins from Lactobacillus against *B. cereus* (Arokiyamary and Sivankumar, 2012), as well as Enterocins from enterococci (Wilaipun *et al.,* 2004). In addition, the incidence of *B. cereus* has been shown to be higher in uncooked and processed ground beef than in raw meat (Nortje *et al*., 1999; Mosupye and Von Holg, 2000). The findings of this study agree with that of Hassan *et al*. (2010), where in a related study of microbial contamination of raw meat and its environment in retail shops in Karachi, 84% of meat and environmental samples reportedly had bacterial pathogens isolated from them, including *B. subtilis*; no *B. cereus* was reportedly isolated from these samples.

The prevalence rate of *B. cereus* for processed meat samples in this study were 7% for **soye** (roasted hawked meat), 12% for **suya** and 16% for **balangu** (roasted meat). These low prevalence rates are nevertheless of significance with respect to the safety of these products. These low incidences could be attributed to a number of factors: the possibly very low number (or none) of *B. cereus* cells on the raw meat used as raw material (as shown by this study), and the effects of processing techniques and handling of the finished products by the producers. Hawking of finished products as is done for **soye** in open air crowded areas for example, and the vendor physically touching the products provide channels for contamination of the products with a multitude of organisms, including the ubiquitous soil organism, *B.cereus*.

The microbial association that would be expected to exist, may not promote proliferation of *B. cereus*, as these associations may include lactic bacteria acid bacteria (LAB), which are considered as being naturally found in meat and meat products (Olaoye and Ntuen, 2011).

Several representatives of the genus may typically dominate the microbial population especially of vacuum packages and processed meat products (Holzapfel, 1998; Conter *et al*., 2005). In such situations LAB may produce metabolites antagonistic towards *B. cereus*, but not to Gram-negative bacteria which are protected by their outer membrane, which prevents bacteriocins from reaching the plasma membrane (Abee *et al*., 1995). In other words, **soye** would be more likely to cause illnesses resulting from Gram-negatives such as *E. coli*, than it would from *B. cereus.* Meat-borne LAB produce a range of bacteriocins that are generally active towards other LAB (contributing to the competitiveness of the producing strain) and food-borne gram-positive pathogens such as *L.monocytogenes, S.aureus, Cl. perfringens,* and *B.cereus.* (Noonpakdee *et al*., 2003).

Lower prevalence rates in **soye** could be attributed to differences in the methods used during sale of the products, as **soye** is hawked around in crowded areas, exposing the product to post-processing contamination, resulting in microbial associations likely to suppress *B. cereus* growth. **Suya** and **balangu** are not hawked, but sold at "**suya** spots" where they are produced. In addition, post processing contamination at these **suya** spots would be remedied by acts of reheating the products before sale which would likely destroy most bacteria, while *B.cereus* spores, which are more resistant to heat could survive the reheating process.

B. cereus counts for **soye** samples in this study had mean counts within acceptable limits of 10^2 to $<10^3$ for *B. cereus* in ready to eat foods (NSW Food Authority, 2009). Counts for both **balangu** and **suya** samples in this study were also all within expected microbiological ($<10^2$) levels that presented no food safety concern (NSW food authority, 2009) at the times of sampling, which were after mean storage time of 2±0.1h for **balangu** and 3±0.5h for **suya**. Whong *et al*. (2006)

also reported isolating *B.cereus* from ***suya*** samples sold in Zaria*,* although at a higher prevalence rate of 85%.

Result obtained from the prevalence study of *B.cereus* in raw milk and **nono** showed a much more lower prevalence in raw milk (14%), than **nono** (49%). The much higher prevalence in **nono** may be explained by likely contamination from utensils like calabashes, wooden spoons and straw mats, unclean udder and milking environments littered with cow dung, as well as the storage environment during fermentation, and methods employed at sale. Similar results were observed by Bello (2002), where HACCP of traditional processing of uncontrolled fermented milk revealed hazards associated with most steps of the production. An important observation made at that time was the addition of water to the fermented milk, which was then agitated by shaking vigorously to separate fat globules from the fermented milk. In addition during sale of the product, small wooden calabashes or plastics exposed to open air were repeatedly dipped into the product to serve consumers. These practices could contaminate the products with more *B. cereus*, as such would explain the higher frequency in **nono**.

Results of this study agree with those of Whong *et al*. (2006) who recorded a higher prevalence rate of 93.3% of the organism in locally fermented milk sold in Zaria. Uzeh *et al.* (2006b) also reported the isolation of *B.cereus* in uncontrolled fermented milk from.
B.cereus counts in both raw and fermented milk samples in this study were within expected microbiological levels for ready- to- eat meals (NSW Food Authority, 2009).

Insignificant differences in these counts ($P>0.05$) for both raw and fermented milk samples may be attributed to similarities in methods employed during milking, proximity of milking environments to each other which could result in having similar microbial associations. It has been hypothesized that differences in feeding and housing strategies for cows may influence the

microbial quality of milk (Torkar and Teger, 2008). A number of studies have also reported the isolation of microorganisms, including pathogens from raw milk (Zelalem and Bernard, 2006; Mubarack *et al*., 2010; Tassew and Seifu, 2011).

Prevalence study of *B.cereus* in dry season samples of local unbranded, uncooked rice samples in this study showed a rather low frequency (14%) of the organism in the samples. This is inspite of the fact that *B. cereus* is widely distributed in the environment, mainly in the soil from which it spreads to food (Keith *et al*., 2004; Prestamo *et al*., 2007). This low frequency could however be attributed to techniques employed during post harvest processing such as drying, husking and polishing, which would have reduced the number of *B. cereus* in the rice samples (Sarrias *et al*., 2002). Another possible reason could be an expected low moisture content resulting from the dry weather, which would likely not support growth of microorganisms.

The prevalence rate in this study contradicts that of Ankolekar *et al*. (2009), where *B. cereus* constituted 83% of *Bacillus* species isolated from raw rice from retail food stores in the U.S. Similarly, Tahir *et al*. (2012) reported 100% prevalence of *B. cereus* in uncooked and cooked unbranded rice samples. Whong *et al.* (2006) also reported the presence of the organism in 76.7% of uncooked rice from retail outlets in Zaria. In addition, mean *B. cereus* counts for uncooked rice samples in this study were relatively low (1.5±0.4 $\log_{10}$ Cfu/g) compared to those obtained by Whong *et al*. (2006) (4.1±3.4) and Ankolekar *et al*. (2009) (3.9 to 460 cfu/g). Tahir *et al*. (2012) reported lowest *B.cereus* counts of 0.922 x 10^1 cfu/ml in unbranded rice samples.

Results from this study revealed low prevalence rates of *B. cereus* in cooked rice sampled from local food centers. The low prevalence rates could be as a result of the heat processing effect of boiling which would kill vegetative cells. It could also be due to the low prevalence rates of the organism in raw uncooked rice. Additional contamination could however have occurred during

processing, from added ingredients such as herbs and spices (ICMSF, 2005) especially in Jollof rice samples. Similarly, low levels of hygiene employed in cleaning storage vessels and serving utensils, as well as methods used during sale would serve to contribute to presence of the organism in cooked rice. The practice of storing in insulated food boxes, which are frequently opened during sale, would lower temperatures to levels that could promote growth and multiplication of *B. cereus* spores. Some strains of *B.cereus* are able to grow at temperatures as high as 55^0C (Choma *et al*., 2000; Borge *et al*., 2001) and mean storage temperatures for samples in this study were 43 ± 2.6°C and 45 ± 2.1°C for plain boiled rice and Jollof rice after mean storage periods of 4 ± 0.5 h and 3 ± 0.3 h respectively. Variations in time-temperature during cooking and storage, as well as level of hygienic practices used could play significant roles in determining microbiological quality of ready- to- eat- foods (Little *et al*., 2001; Abdalla *et al*., 2008a).

The findings of this study agree with those of Nichols *et al*. (1999); Mensah *et al*. (2002), Whong *et al.* (2006) and Wogu *et al*. (2011) who all documented isolating *B. cereus* from cooked rice samples. Levels of counts for cooked rice samples in this study fell within acceptable limits (10^2 to $<10^3$) for ready –to–eat foods (NSW food authority, 2009) at the time of sampling.

5.2 Effects of pH, NaCl and Temperature on Survival of *B.cereus* in Foods

5.2.1 Effects of NaCl concentration and temperature on survival of *B.cereus* in boiled rice

B.cereus counts in cooked rice during refrigerated storage exhibited similar patterns across samples at lower NaCl concentrations (0 – 1%) over the 72 h storage period; at the end of 24 h, it was observed that counts in control samples increased by about 2 log units, while an increase of about 1 log unit was observed at 0.5 and 1%. At 1.5 and 2%, bactericidal effects were

observed, especially at 1.5%. This pattern seems to infer increase in bactericidal effects, as NaCl concentration is increased during low temperature storage for 24 h. Results of this study however contradict that of Adenuga (2004), where NaCl concentrations ≥ 12% had little effect on growth of *B.cereus* in carbohydrate and protein rich foods.

Decreases in counts (by about 2 log units) from their levels at 24 h was also observed for samples at NaCl levels of up to 1%, following 48 h of storage at refrigeration temperature. This drop in counts could be attributed to a likely decrease in pH, which is usually associated with breakdown of foods with high levels of carbohydrate (Mossel *et al*., 1995), as cells metabolise growth substrate. This may explain why counts increased in samples at 1.5% (by about 2 log units), which initially had lowest counts; it would be expected that pH levels in these samples be higher, as such would be more likely to allow for growth of *B.cereus* cells. In addition, change in composition of the growth medium would alter previous inhibitory effects of the samples on *B.cereus* cells, as death of cells is sometimes followed by autolysis releasing a variety of components back into the medium (Mossel *et al*., 1995). It has been documented that Bacilli reverse plasmolytic effects of reduced water activity, by accumulation or production of intracytoplasmic compatible solutes. These solutes balance most of the osmolality in the surrounding medium and as such retain most of the water, otherwise lost due to diffusion, in the cytoplasm. Compatible solutes include carbohydrates and amino acids, which can replace water in a biochemical system without seriously affecting its function (Mossel *et al*., 1995; Kuhlman and Bremer, 2002; Motta *et al*., 2004). Other solutes such as glutamate, glutamine, trehalose, ectoines and ɣ-amino butyrate have been associated with adaptation to low a_w (Csonka, 1989; Csonka and Hanson, 1991; Severin *et al*., 1992).

Dramatic increases in counts (by about 5 log units) in all samples observed after 72 h was accompanied by stronger off-odours than was observed at 48 h, and sliminess for samples at 2% NaCl. This could also again be attributed to changes in growth media as a result of autolysis of dead cells which could alter previous inhibitory effects of the samples on *B.cereus* cells. Decreases in *B.cereus* (by about 2 log units) observed in control and challenge samples at elevated (37^0C) and room temperatures (26-28^oC) were accompanied with changes in sensory attributes of samples from loose, cream coloured grits to wet, darker and foul smelling grits, an indication of microbial activity. These changes were more pronounced with samples at 37^0C. This agrees with the findings of Ezeonu and Okonkwo (2013), who reported *B.cereus* growing best and producing extracellular proteins at this temperature. Bacillus species synthesise exoenzymes such as proteases, nucleases, amylases and lipases (Balcazar-Reyes *et al*., 2012) with which they use to degrade organic compounds in order to obtain carbon sources for their metabolic processes. As a result of proteolytic, lipolytic, and saccharolytic activities of *Bacillus* species in foods, undesirable changes in colour, texture, consistency and odour develop in such foods. Metabolites produced as a result of such activities ultimately adversely affect the survival of these organisms in closed systems (Mossel *et al*., 1995). This may account for the comparatively lower counts observed for samples at elevated temperature of 37^oC, which is within the optimum range for mesophilic bacteria, where changes in sensory attributes were more pronounced. A slower accumulation of metabolites due to inhibitory effects at higher NaCl (1%) would have resulted in slower decreases in cell counts, hence the higher levels observed in comparison to samples at lower NaCl concentrations.

It was however noted that NaCl concentration of 1.5% had the highest inhibitory effect (more than that at 2% NaCl) on *B.cereus* in all samples, irrespective of storage temperature. Counts in these samples all decreased by about 2 log units. Similarly,

samples at 2% NaCl, irrespective of storage temperature exerted a somewhat bacteriostatic effect that resulted in similar level of counts (8.2 – 8.8$\log_{10}$ Cfu/ml) in all samples.

The variations observed in the above survival patterns corroborate the significant differences ($P<0.05$) in *B.cereus* counts when analyzed across the different NaCl concentrations, as well as at the different storage periods used in this study.

5.2.2 Effects of NaCl concentration and temperature on survival of *B.cereus* in Balangu

Survival patterns of *B.cereus* in **balangu** during storage at refrigeration, at different NaCl concentrations appeared very different from those observed with rice samples studied under the same conditions, as counts in **balangu** were significantly higher at 24 h of storage. NaCl concentration of 0.5% appeared to have had the most inhibitory effect at 24 h, as counts appeared unchanged. However, there appeared to be a general pattern of increases in *B.cereus* counts in samples at NaCl concentrations ≤ 2%, suggesting non-inhibitory effects of these levels of NaCl and low temperature used.

Nutrient exhaustion could explain decrease in counts observed at 48 and 72 h for samples which had high counts (>12 $\log_{10}$ cfu/ml) at 24 h. It has been reported that in most batch systems, stationary phase is attained when there are 8 $\log_{10}$ – 10$\log_{10}$ cfu/ml, depending on the type of substrate (Mossel *et al.*, 1995). To this effect, it was noted that NaCl concentrations which had initially inhibited growth (0.5 and 2%) allowed for increases in *B.cereus* counts after 48 h. In addition, changes in growth medium resulting from lysis of cells at 24 h, along with a rich supply of amino acids in growth media would provide compatible solutes that could enable *B.cereus* cells in these samples reverse plasmolytic effects of a reduced a_w (as earlier discussed).

Results of this study also showed that high temperatures were less inhibitory on *B.cereus* cells in **balangu,** than refrigeration temperature (4-12 ^{o}C), although room temperature (26-28^{o}C) appeared to be less inhibitory than elevated temperature of 37^{0}C, after 24 h of storage. *B.cereus* counts for samples at elevated temperature of 37^{0}C were observed to be at the same levels (about 13 log units) including the control sample, except at 2% where bactericidal effect was observed. This suggests NaCl concentrations 2% may not s**p**press growth of *B.cereus* in **balangu** samples at 37^{0}C, while growth may be inhibited at 2% at elevated temperature of 37^{0}C.

The significant differences in *B.cereus* counts in samples when analyzed across different NaCl concentrations, as well as at the different temperatures used ($P<0.05$), corroborates the different survival patterns observed.

5.2.3 Effects of pH and temperature on survival of *B.cereus* in Nono

Survival patterns of *B.cereus* in **nono** samples at different pH levels during refrigeration appeared to be similar with those observed for pasteurized milk samples in this study. However, *B.cereus* counts for **nono** samples were observed to be slightly lower than those for pasteurized milk. *B.cereus* counts dropped consistently in all **nono** samples over 48 h and further at 72 h at pH 3.7, suggesting that the pH level at that temperature resulted in gradual death of the *B.cereus* cells. *B.cereus* has been shown to be affected by low levels of acidity by rapidly dying in yoghurt as acidity of fermented milk increased (Hassan *et al.,* 2010). In addition, bacteriocins which could have been produced during uncontrolled fermentation of milk would exert additional inhibitory effects on *B.cereus* cells. The broad spectrum bacteriocin, Enterocin AS-48 has been documented to decrease cell counts of toxigenic psychrotrophic strains of *B.cereus* in food systems consisting of boiled rice, commercial infant rice-based gruel in whole milk stored at 37^{o}C, 15^{o}C and 6^{o}C (Grande *et al*., 2006). Mohankumar and Murugalatha (2011), documented

resistance of bacteriocins from lactobacilli isolated from raw cow milk to boiling for 10 min. Abdelbasset and Djamila (2008) also reported stability of inhibitory activity of bacteriocins following heat treatment at 95^{o}C and even at 120^{o}C for 20 min, and activity at pH range 3-10 for bacteriocins (obtained from uncontrolled fermented milk) that inhibited *B.cereus*. The bacteriocins were more active at lower pH, and activity decreased with alkalinity.

Lactoferrins, another antimicrobial substance associated with milk, could also have exerted additional inhibitory effects on *B. cereus* in fermented milk samples. Lactoferrins have been shown to remain active even at low pH (Valenti and Antonini, 2005), and has demonstrated remarkable resistance to proteolytic degradation by trypsin and tryspin-like enzymes (Iyer and Lonnerdal, 1993) and also to pepsin, lipase and catalase (Abdelbasset and Djamila, 2008). Increase in *B.cereus* counts at 72 h could have been as a result of rise in pH of the fermented milk samples due to lysis of dead cells, and activity of "fitter" cells (Mossel *et al*., 1995). These would have led to a subsequent decrease (Abdelbasset and Djamila, 2008) or loss of antimicrobial properties of the fermented milk samples.

The survival pattern of *B.cereus* in **nono** samples stored at refrigeration (4-10^{o}C), room (26-28^{o}C) and elevated (37^{o}C) temperatures at 24 h observed in this study exhibited a rather irregular pattern. The Results suggested higher temperatures (26-28^{o}C and 37^{o}C) allowed for increases in counts, while bactericidal effects were observed for samples at refrigeration temperature (4-10^{o}C) at the same pH levels of 3.7. Bactericidal effects were also observed for refrigerated samples (4-10^{o}C), as well as samples at room temperature (26-28^{o}C) at pH 3.8- 4.3. It was also observed elevated temperature favoured growth the most at all pH levels except at 4.2. A number of factors, including bacterial strains and environmental factors such as temperature have been shown to affect the antimicrobial activity of organic acids in microbial cultures

(Buchana and Edelson, 1996; Conner and Kotrola, 1995; Cheng *et al*., 2003). Growth of *B.cereus* has been reported in yoghurt milk at 31^{o}C, although as the pH dropped the growth rate declined, and ceased at pH 5.7 (Robinson *et al*., 2002). Lactic acid, like other organic acids are either present as the acid in its undissociated form at low pH, or as the ion salt at higher pH. In its undissociated form, it is more permeable to the cell membrane, where upon entry into the cytoplasm, disrupts cellular functions (Lὺck and Jager, 1997). Undissociated acid molecules are uncharged and lipophilic; hence they penetrate plasma membranes and enter cells (Mols and Abee, 2011).

Decreases in counts observed at pH 4.3 during storage at lower temperature as opposed to that observed for 37^{o}C, suggest that the combined conditions of likely increases in ions salts of organic acids with increased pH, as well as low temperature, exerted bactericidal effects. To this effect, it was observed that refrigerated samples appeared to exert the most inhibitory effects on *B.cereus* cells, as lowest counts were observed in comparison to other samples. Similar findings have been reported by Conner and Kotrola, (1995), where effects of organic acids were observed to be less inhibitory on *E. coli* 0157:H7 at temperatures of 25^{o}C and 37^{o}C, than at 10^{o}C. The growth of *B.cereus* at low pH levels observed in this study contradicts report of minimum survival pH of the organism as 4.5 (Food Safety Network, 2012). Uzeh *et al.* (2006) also reported isolating *B.cereus* in locally fermented milk at pH as low as 2.87.

It was also observed in this study that samples at pH 4.2 had a bactericidal effect that resulted in reduction of *B.cereus* cells by about 4 log units, during storage at room and elevated temperatures for 24 h. Colonies were also observed to be much smaller, appearing as pinpoint colonies. Storage at refrigeration temperature at the same pH had less bactericidal effect, resulting in reduction of cells by only 2 log units. These observations therefore suggest that

B.cereus cells are killed at pH 4.2 in uncontrolled fermented milk, at both low and elevated temperatures. This would explain why *B.cereus* counts were significantly different across the pH levels, with samples at pH 4.2 having the lowest mean counts ($P<0.05$). Similarly, the significant difference in counts when analyzed across different temperatures could be explained by the increased bactericidal effect observed in refrigerated samples.

5.2.4 Effects of pH and temperature on survival of *B.cereus* in pasteurized milk

Observations made during the storage of pasteurized milk at varying pH levels at refrigeration temperature (4-10^{o}C) revealed either a bactericidal or bacteriostatic effect on *B.cereus* cells in all samples at the end of 24 h. Increases in cell counts were then observed with increasing storage period in these samples.

Milk is protected to different extents against microbial contaminations by natural inhibitory systems including the lactoperoxidase/thiocyanate/hydrogen peroxide (LP) system, lactoferrins, Lysozymes, immunoglobulins and free fatty acids (El agamy *et al*., 1992; Losnedahl *et al*., 1998; Kappeler, 1999; Adleroval *et al*., 2008). Interest has arisen in the ability of milk to kill bacteria, and in how this knowledge can be applied to control mastitis, human health and functional foods for people (Abolghait *et al*., 2011). Lactoferrin has the potential of inhibiting the growth of bacteria, and even kill them by depriving them of iron and has been shown to be bacteriostatic against a wide range of microorganisms including *Bacillus* species (Losnedahl *et al.*, 1998). The biocidal effects of different antimicrobial components of milk appear to add-up in a synergistic manner, as the total antimicrobial effect in milk was reported to be greater than the sum of the individual contributions of antimicrobial proteins (Clare and Swaisgood, 2000). Results obtained in this study suggest that the temperature range and the milk medium irrespective of the pH of

the milk samples inhibited or killed the initial population of the inoculum after 24 h, and also further at 48 h at pH extremes of 6.3 and 6.8.

Increase in counts at 48 h in control samples, and at 72 h for challenge samples may be attributed to the likely loss of activity of some of the antimicrobial properties of the milk medium due to autolysis of dead cells, as well as increasing numbers of *B. cereus* cells breaking down milk components. Bacilli are known for their abilities to secrete large amounts of alkaline proteases having significant proteolytic activity and stability at considerably high pH and temperatures (Pastor *et al*., 2001; Abou-Elela *et al*., 2011). Breakdown of milk would result in production of metabolites such as amines, amino acids; the release of these metabolites would affect the ionic strength and concentration of metal ions of the milk medium to levels which could reduce lysozyme activity. High concentrations (>50mmol/l) of cations such as Na^+, K^+, NH_4^+ has been shown to reduce lysozyme activity (Priyardarshani and Kanshal, 2003). The resulting increase in pH (Mossel *et al.*, 1995; Sumana *et al*., 2009) would permit growth of previously inhibited *B.cereus* cells in the medium. A number of studies have reported survival of *B. cereus* at refrigerated temperatures less than 10^0C (van Netten *et al*., 1990; Vaisainin *et al*., 1991; Valik *et al*., 2003; Sadek *et al*., 2006; Bellow *et al*., 2007). Brillard *et al*. (2010) reported the presence of several genes in *B.cereus* that enable the organism to grow successfully under restrictive conditions of cold habitats.

The likely exhaustion of nutrients in both milk media after 72 h, as well as increased pH levels would explain the decrease in counts observed. Unlike in control milk samples, increase in counts in samples at pH extremes of 6.3 and 6.8 after 72 h could be attributed to a slower increase in pH which permitted growth, lower levels of inhibitory metabolites , as well as higher levels of available nutrients owing to suppressed growth -during the initial storage period. These

variations in counts would explain the significant difference in *B.cereus* counts (P 0.05) across the samples when analyzed across pH levels.

5.3 Diarrhoeal Enterotoxin Production by *B.cereus* Isolates Grown Under Varying NaCl Concentrations, pH Levels and Temperature

5.3.1 Effects of NaCl concentration and temperature on production of diarrhoeal enterotoxins by *B.cereus* isolates grown in boiled rice and balangu.

Differences in temperature/NaCl concentration did not appear to significantly affect cross reactivity between cell free culture filtrates from *B.cereus* isolates grown in rice and immune rabbit globulins in this study. Toxin titres for isolates were generally the same following refrigerated storage for 24 h and 48 h (≥64ng/ml) as such did not appear to be affected by temperature and NaCl concentration. This implies that these conditions did not suppress toxin production in these isolates. Similar observations were made for *B.cereus* cells following storage in boiled rice at elevated temperatures (26-28^{o}C and 37^{o}C) at NaCl concentration of 0-2% (W/W); samples at elevated temperatures however showed pronounced changes in sensory attributes indicative of prolific microbial activity. It appeared exposure to apparent stressful conditions as a result of prolific growth, resulted in high toxin titres from surviving cells. Lower titres were however recorded for all isolates (32ng/ml) irrespective of NaCl concentration, following 72 h of refrigerated storage where there were significant increases in counts from 48 h, except for isolates at 2% NaCl.

Expression of HBL genes has been shown to be regulated by a number of proteins, such as the PlcR (Phospholipase C Regulator) (Agaisse *et al.*, 1999). It has been documented that the PlcR, a

transcriptional regulator, controls most known virulence factors in *B.cereus* (Gohar *et al.*, 2002). Its transcription is auto induced or activated by PapR, an auto inducer peptide that accumulates inside the bacteria when high density cells are reached (Slamti *et al*., 2002). It is repressed by the sporulation factor SpoOA (Lereclus *et al.,* 2000), as accumulation of metabolites adversely affect vegetative cells. The stationary phase of growth is induced in bacteria under conditions of starvation or accumulation of toxic metabolites (Mossel *et al.*, 1995). The PlcR and PapR therefore function as a quorum sensing system, a phenomenon wherein bacteria communicate, sense and respond to cell density. It has also been proven that plcR- strains are not able to produce the enterotoxin HBL, while their PlcR+ equivalent did show HBL production (Salamitou *et al*., 2000).

Decreased toxin titres observed with boiled rice isolates after 72 h could be attributed to the likely inactivation of the PlcR (Phospholipase C Regulator) responsible for HBL secretion. This inactivation could occur due to nutrient exhaustion and accumulation of metabolites as cell counts increased, particularly as temperature range during the storage period exceeded refrigeration temperature (4-15^{o}C). The low positive correlation (0.28) between toxin titre and counts corroborates this likely effect of cell density on toxin expression. Furthermore, it was noted that toxin titre was higher for isolates from boiled rice at 2% NaCl concentration, where unlike in other samples, counts steadily dropped over 48 h and increased at 72 h. This would have resulted in more available nutrients and less toxic metabolites for isolates in these samples. To this effect, it was noted that the unchallenged isolate had toxin titer >64ng/ml.

Toxin titres for *B.cereus* isolates from **balangu** sample portrayed a rather irregular pattern for almost all isolates except for isolates at 0.5% and 1% NaCl concentration. With these isolates toxin titres appeared to decrease and then increase following 48 h and 72 h at refrigeration

temperature (4-10^{o}) respectively. It was also observed that except for the isolates from control sample (0% NaCl) at 24 h room temperature that showed no cross reactivity , all other isolates, irrespective of time, temperature and NaCl exhibited some level of agglutination. This infers that the *B.cereus* isolates in this study are able to produce HBL diarrhoeal enterotoxin during storage of **balangu** at low and elevated temperature at NaCl concentrations of 0-2%. This was also observed for boiled rice isolates. Pearson's coefficient of correlation established almost perfect positive correlations (0.96 and 0.99) between toxin titres and cell counts at elevated temperatures (26-28^{o}C and 37^{o}C) respectively, following 24 h of storage. During refrigeration storage however, there was a medium positive correlation (0.44) at 48 h as cell counts decreased or increased from levels at 24 h ; thus higher titres were observed with isolates from samples with lower cell counts at 24 h, highlighting the effects of environmental stress (from decreasing nutrients and increasing metabolites) on toxin production. It was noted that toxin titres, generally appeared higher for isolates grown in rice, than those recorded for isolates from **balangu**.

The report of one study has revealed the role of intercellular iron quantity on virulence in *B.cereus.* It was observed that mutant cells which had three times more intercellular iron, which resulted in greater amount of oxidizing free radicals, had reduced virulence when compared to wild type *B.cereus.* (Havie *et al.*, 2005). The lower levels of toxin titres observed with isolates from **balangu** a meat product, may be attributed to higher amounts of intercellular iron with these isolates; iron contents of meat is shown to be higher than that of rice. This could result in differences in level of oxidizing free radicals that could reduce virulence. Differences in toxin titres did not, however, appear to be significant ($P>0.05$) for the isolates across the samples irrespective of temperature, time and NaCl concentration.

5.3.2 Effects of pH and temperature on production of diarrheaol enterotoxins by *B.cereus* isolates grown in pasteurized milk and nono.

Cross reactivity between immune rabbit globulins and cell free BHI culture filtrates obtained from *B.cereus* isolates grown in refrigerated pasteurized milk, showed toxin titres that correlated positively with counts. There was a general pattern of toxin production gradually increasing for isolates, under conditions of slow increases in cell density. These observations infer a positive impact of cell count on HBL production by *B.cereus* from milk during storage at refrigeration temperatures (4-10^{o}C). As earlier stated, this may also be attributed to activation of the PlcR by the PapR, as cell density increases. No cross reactivity was observed with isolates from milk at pH 6.8 after 48 h refrigeration, indicating a toxin titre level of □ 2ng/ml. This is however of significance, as it could imply enterotoxin production could be suppressed in strains from pasteurized milk at this pH, time/temperature combination. In addition, it highlights the possibility of heritable traits of HBL toxin suppression under these conditions, especially as Cueppens *et al.*(2012), reported spores as low as 4.0 log spores/g showing excellent survival and viability throughout gastrointestinal passage simulation.

Increases in toxin titres were recorded in test samples (pH 6.3 and 6.8), as cell population increased following 72 h of storage. Over time, *B.cereus* cells may have adapted by way of general stress response mechanisms, which have been described to be involved in cross protection of *B.cereus* cells exposed to stress conditions (den Besten *et al.*, 2010). The resulting increases in cell density as cells adapted may have resulted in accumulation of PapR, activating the PlcR.

Cell free BHI culture filtrates from both unchallenged as well as from *B.cereus* isolates grown in **nono** generally exhibited high toxin titres with immune rabbit globulins. Toxin titres for all

refrigerated samples were ≥ 64ng/ml, whereas lower titres were recorded for isolates at higher storage temperatures (where counts were considerably higher at 24 h of storage). This could suggest that higher temperatures at these pH levels suppressed toxin production ability of *B. cereus* in **nono** samples; toxin titres in isolates from samples at pH 3.8 and room temperature (26-28^{o}C), for example, were observed to be considerably higher (about twice the level) than those for isolates at the same pH at 37^{o}C. Higher optimal growth temperatures would have led to accumulation of toxic metabolites and nutrient exhaustion associated with closed systems. This was evident by the more pronounced changes in sensory attributes of these samples. This could have resulted in inactivation or repression of the PlcR responsible for HBL secretion, as well as to a host of other environmental signals (that arise due to environmental changes) which modulate virulence factor expression. Higher positive correlation coefficient (r) of 0.71 between toxin titres and lower cell counts for samples at room temperature (26-28^{o}C) than that for samples at 37^{o}C (0.51) corroborates the effect of increased bacterial activity on HBL toxin expression in **nono** samples in this study. To this effect, it was observed that sensory changes associated with microbial activity were more evident in samples at 37^{o}C. As with isolates from pasteurized milk samples, no significant differences in toxin titres from isolates in all fermented milk samples were observed (P 0.05).

5.4 Distribution of *hbl* and *bceT* Genes in Diarrhoeal Enterotoxin and non- diarrhoeal Enterotoxin Producing Isolates.

Molecular analysis by PCR showed a negative correlation between expression of the L_2 component of the *hbl* diarrhoeal enterotoxin, and presence of *hbl* genes in both enterotoxin and non enterotoxin producing *B.cereus* isolates in this study. Although enterotoxin producing

isolates were shown by PCR to encode for at least one of the HBL genes, none of the isolates appeared to simultaneously encode for all three genes.

It was observed that storage conditions affected the number and type of *hbl* and *bceT* genes detected by PCR in isolates; in the unchallenged rice isolate (F2R), for example, the *hblD* and *hblA* genes were detected by PCR following storage at NaCl of 0.5% for
24 h at 37^{o}C, while following storage at refrigeration for 24 h, only the *hblD* gene was detected by PCR. The same phenomenon was also observed with *B.cereus* isolates from milk; variations were observed in the distribution of HBL genes by PCR for the isolates under different culture conditions. The *hblC* gene (which encodes for the L_2 component of the HBL gene) was not detected in all isolates by PCR, while the *hblD* gene was detected in almost all isolates.

The results of this study also showed a higher prevalence of the *bceT* gene in rice isolates, than was observed for milk isolates. Gitahi *et al.,* (2009) also documented results which suggested a higher incidence of *bceT* in rice than was observed for isolates from milk and cheese. Gitahi *et al.* (2009) additionally reported the presence of the *hblD* genes in isolates from milk and rice. Similar to this study also, none of the *B.cereus* isolates from milk and rice appeared to harbor all three *hbl* genes, in contrast to this study however, the *hblC* was observed in both milk and rice samples.

Whilst studies have documented variations in the distribution of diarrhoeagenic genes in *B.cereus* isolates from environmental and food samples (Rowan *et al.*, 2003; Gitahi *et al.*, 2009; Kim *et al.*, 2011), no reports were found in the course of this study that suggested varying growth environment affected the number and type of diarrheal enterotoxin genes detected by PCR in the same *B.cereus* strain from food samples, as was observed in this study.

It was also observed in this study that non-enterotoxin producing isolates also encoded for the HBL gene. *B.cereus* isolates from milk samples at pH 6.8, following refrigeration for 48 h, failed to secrete the L_2 component of the HBL complex despite encoding for the *hblD* gene, inferring that expression of the gene was suppressed. HBL expression in *B.cereus* isolates is reportedly regulated by a number of proteins which induce or repress HBL secretion in response to environmental factors. Its production is highly controlled as these proteins act as regulators of its transcription (Senesi and Ghelardi 2010). The combined effects of high pH and low temperature on the isolates in this study may have induced repression of the HBL genes as earlier discussed.

Variations in genetic distribution of the genes encoding for diarrhoeal enterotoxin in test isolates in this study did not appear to determine the levels of toxin titres observed for the isolates when analysed for presence of the L_2 component of the HBL complex. Multiple genes were encoded by isolates which exhibited low, as well as higher toxin titre values. It was also noted in this study that *B.cereus* isolates from rice samples at 1% NaCl did not appear to harbor any of the *hbl* genes, after 48 h refrigeration. This is inspite of a relatively lower toxin titre which nevertheless inferred the ability of the isolate to produce diarrhoeal enterotoxins. A similar finding has been documented by Banerjee *et al*. (2011). Lopez and Alippi (2010) and Banerjee *et al.* (2011) also documented variations in the distribution of HBL genes in *B.cereus* clinical isolates. The non detection of all three *hbl* genes in some isolates has been attributed to polymorphism among genes (Hansen and Hendriksen, 2001), a phenomenon where multiple forms of a single gene exist in an organism.

Interestingly, none of the isolates appeared to harbour the *hblC* gene, which encode for the L_2 component of the HBL complex. Guinebretiere *et al*. (2002) also reported high polymorphism in

hbl genes in food-associated *B. cereus* strains than diarrhoeal strains, as *B.cereus* isolates which tested negative for *hbl* genes with PCR, had the genes confirmed by southern blotting. The southern analysis showed that genetic heterogeneity among *B.cereus* strains, particularly environmental strains, were more truly associated with sequence polymorphism, than with the lack of the genes composing the *hbl* operon. Genes contained in an operon are either expressed together or not at all. Several genes must be both co-transcribed and co-regulated to define an operon. Polymorphism may therefore explain why isolates in this study which had positive results for the L_2 component of the HBL enterotoxin with the BCET-RPLA assay, did not appear to encode for some of the *hbl* genes by PCR.

5.5 Antibiograms of Diarrhoeal Enterotoxin Producing *B.cereus* Isolates Obtained From Foods Stored at Varying pH, NaCl and Temperature.

The results of sensitivity tests on *B.cereus* isolates from food stored under varying pH, NaCl and temperature conditions revealed resistance of all isolates to ampicillin, an extended spectrum penicillin, as well as to amoxyclav (amoxicillin-clavulanic acid), a penicillin plus β-lactamase inhibitor combination. This agrees with the findings of Dikbas (2010), who also documented 100% resistance of *B. cereus* isolates from different food sources to penicillins. Similar findings have also been reported by Schlegelova *et al.* (2003); Whong *et al*. (2006); Merens *et al*. (2008); Ankolekar *et al.* (2009) and Banerjee *et al*. (2011). In this study, variations in pH, NaCl and temperature conditions did not appear to impact on response of isolates to the penicillins. *Bacillus cereus* has long been noted as a producer of a potent broad spectrum β-lactamase, which negatively affects the activity of penicillins and cephalosporins (Andrews and Wise, 2002). Consequently, results of this study showed all isolates being resistant to cefpodoxime a broad

spectrum (third generation) cephalosporin. Whong *et al*. (2006) also documented resistance of 56.7% of food isolates to cefotaxime, another broad spectrum cephalosporin. Isolates in this study were also resistant (96%) to ceftriaxone, another broad spectrum cephalosporin.

Findings of this study also revealed all isolates being resistant to cephalothin, a narrow spectrum (first generation) cephalosporin, except for the isolate from pasteurized milk at pH 6.8 stored at refrigeration temperature for 72 h, which appeared sensitive. Interestingly, isolates from this particular milk sample tested negative for the presence of the L_2 component of the diarrhoeal enterotoxin HBL complex following 48 h of refrigerated storage. This may infer that the combined effects of pH and temperature not only suppressed the ability of the isolates to express the HBL diarrhoeal enterotoxin at 48 h, but may also have suppressed the ability of surviving isolates to resist the effects of this particular antibacterial agent at 72 h. Resistance to β- lactam drugs is usually due to synthesis of β-lactamases, decreased affinity of penicillin binding proteins, or decreased uptake (Nester et al., 2009). Furthermore, it has been documented that broad spectrum cephalosporins are less active than the narrow spectrum agents against Gram positive cocci (Yao and Moellering JR, 2007). This may probably explain the sensitivity of these *B.cereus* milk isolates to cephalothin (a narrow spectrum agent), and their resistance to ceftriaxone and cefpodoxime (broad spectrum agents).

All isolates were sensitive to amikacin, an aminoglycoside, as well as to ciprofloxacin a fluoroquinolone. These also agree with the findings of Whong *et al*. (2006); Dikbas (2010); Banerjee *et al*. (2011) and Hafiz *et al.* (2012). Variations were however observed in the patterns of susceptibility of isolates to tetracycline, with respect to storage condition. Whilst none of the isolates grown at various NaCl concentrations (0- 2%) showed resistance to the antibacterial agent, some isolates from **nono** and pasteurized milk samples were resistant to the

antimicrobial agent, and it was also noted that resistance to tetracycline was exhibited by isolates from challenge samples where counts decreased over time during refrigerated storage (fermented milk at pH 3.7 and pasteurized milk at pH 6.3). In a related study, McMahon *et al*. (2007) documented increased antibiotic resistance in food pathogens when incubated under reduced pH (<5.0), as well as continued expression of this resistance after stress removal. The researchers established significant differences between the patterns of changes in susceptibility induced by thermal stress and the patterns induced by pH and NaCl stresses. Resistance to tetracyclines occur as a result of active efflux of drug from the cell, altered ribosomal target site that prevents binding, or production of modifying enzymes that inactivate the drug (Yao and Moellering JR, 2007). Shobana *et al*. (2008) also reported 85.90% of *B.cereus* food isolates showing resistance to the antibacterial agent.

Varied responses were also observed for erythromycin, as isolates from boiled rice, locally fermented milk and pasteurized cow milk were all sensitive to the antibacterial agent. However, all but one isolate from **balangu** (isolate from **balangu** at 1% NaCl, stored at 37^{o}C for 24 h) were resistant to the agent. Schlegelova *et al*. (2003) and Agwa *et al.* (2012) also documented varied responses in *B.cereus* isolates to erythromycin with respect to food type. Resistance to trimetoprim - sulfamethoxazole (agents which target folate biosynthesis in bacteria) observed for all isolates in this study is consistent with findings from other researches (Turnball *et al.,* 2004; Luna *et al.,* 2007).

A number of reasons could account for the apparent multidrug resistance exhibited by some isolates in this study, as well as the differences in susceptibility observed for particular isolates with respect to growth conditions. Simm *et al*. (2012) documented the up regulation of genes that encode for multidrug resistance protein in *B. cereus* isolates under stressful conditions. The

study however also revealed that multidrug resistance protein is expressed during growth of *B.cereus* under non-stressful conditions where it may have a role in the normal physiology of the bacteria.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The following conclusions are drawn from the findings of this research work;

Results have established the presence of *B.cereus in* some ready –to-eat (RTE) and uncooked foods sold in some streets and markets of Kaduna metropolis, although within acceptable limits, but nevertheless of significance in RTE foods. Findings of previous researchers are supported by results of this study, which also showed presence of *B. cereus* in ready –to-eat foods sold in some parts of Zaria.

Results of food storage studies suggest *B. cereus* is inhibited in boiled rice at NaCl levels above 1% at refrigeration temperature (4-10^{o}C), for up to 48 h. In contrast, *B. cereus* cells are not however inhibited in **balangu** at levels up to 2% at temperatures between 4-37^{o}C.

Sustained inhibitory effects are exerted on *B.cereus* cells at pH levels of 6.3 and 6.8 in pasteurized cow milk for up to 48 h, while in locally fermented milk (**nono**); inhibitory effects are exerted on *B.cereus* cells at pH level of 3.7 during refrigerated storage for
72 h.

Findings of this study also indicated that *B.cereus* isolates from boiled rice at NaCl concentrations of 0-2% during storage at elevated (37^{o}C), room (26-28^{o}C) temperatures for 24 h, and at refrigeration for 72 h could produce the HBL diarrhoeal enterotoxin, and exposure to apparent stressful conditions as a result of prolific growth resulted in high toxin titres.

B.cereus isolates from **balangu** at NaCl concentrations of 0-2% during storage at elevated (37^{o}C) and room (26-28^{o}C) temperatures for 24 h and at refrigeration temperatures (4-10^{o}) for 72 h could also produce the HBL diarrhoeal enterotoxin. In contrast to isolates from rice, periods of prolific growth resulted in lower toxin production.

HBL diarrhoeal enterotoxin production was suppressed in *B.cereus* isolates from milk at pH 6.8 following 48 h of refrigerated (4-10^{o}C) storage. In toxin producing isolates, toxin production increased following periods of increases in cell density in refrigerated milk. The above findings significantly highlight the possibility of sustained heritable traits of toxin suppression in *B.cereus* isolates as a result of some environmental conditions during food storage.

Results also indicated that a higher prevalence of the *bceT* gene is detected by PCR in isolates from rice than in isolates from milk, supporting the findings of previous researchers. This study also suggests varying environmental conditions could contribute to polymorphism of *hbl* and *bceT* genes in the same *B.cereus* strain in food samples.

Antibacterial sensitivity tests in this study suggest that decreased resistance to cephalothin, a first generation cephalosporin, could be induced in isolates following suppression of HBL production in milk isolates. In addition, results also reaffirm the findings of previous researchers who documented sustained responses to antibacterial agents even after removal of stress factors. Multidrug resistance was observed with some isolates in this study, re-affirming previous findings.

6.2 Recommendations

The following recommendations could be made following the findings of this research;

1. There is the need for enactment and enforcement of regulations for food service operators and vendors by the appropriate agencies with a view to providing safer RTE foods in Nigeria. Food service operators should for example be required to have some basic qualification, as well as operating licenses. The wide patronage of RTE foods by Nigerians also highlights the need for a wider outreach of effective control measures by the appropriate government agencies.

2. There is also the need to carry out researches to understand the mechanisms of survival and pathogenicity of foodborne pathogens in locally consumed foods. Government agencies, educational institutions as well as standards organizations need to support and partake in such rather time consuming and financially tasking, yet very important researches which could reveal simple, effective measures that would aid in the control of foodborne illnesses.

3. Furthermore, findings of such researches should be exploited by food industries in Food product development programmes. This could serve as platforms that could ultimately provide significant contributions towards reducing the risks of food- borne diseases. Findings of such researches should also be conveyed to food service operators, vendors and consumers. These need to be enlightened on the need and use of simple and effective techniques in food preparation and processing methods that could reduce the risk of foodborne illnesses associated with consumption of RTE foods.

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Appendix I: Presumptive *B.cereus* Counts of Retail Raw Meat

Producer	Sample	*B.cereus* count (cfu/g)
1	M1T1	$<2.5 \times 10^1$(est)
	M1T2	$<2.5 \times 10^1$(est)
	M1T3	$<2.5 \times 10^1$(est)
	M1T4	$<2.5 \times 10^1$(est)
2	M2T1	$<2.5 \times 10^1$(est)
	M2T2	4.0×10^1(est)
	M2T3	6.0×10^1(est)
	M2T4	$<2.5 \times 10^1$(est)
3	M3T1	$<2.5 \times 10^1$(est)
	M3T2	$<2.5 \times 10^1$(est)
	M3T3	$<2.5 \times 10^1$(est)
	M3T4	$<2.5 \times 10^1$(est)

4	M4T1	<2.5 x10^1(est)
	M4T2	3.0 x10^1(est)
	M4T3	<2.5 x10^1(est)
	M4T4	<2.5 x10^1(est)
5	M5T1	<2.5 x10^1(est)
	M5T2	<2.5 x10^1(est)
	M5T3	<2.5 x10^1(est)
	M5T4	1.0 x10^1(est)
6	M6T1	<2.5 x10^1(est)
	M6T2	<2.5 x10^1(est)
	M6T3	4.0 x10^1(est)
	M6T4	<2.5 x10^1(est)
	M6T5	2.0 x10^1(est)
7	M7T1	<2.5 x10^1(est)
	M7T2	<2.5 x10^1(est)
	M7T3	<2.5 x10^1(est)
	M7T4	<2.5 x10^1(est)
	M8T1	<2.5 x10^1(est)
8	M8T2	1.0 x10^1(est)
	M8T3	<2.5 x10^1(est)
	M8T4	<2.5 x10^1(est)
	M8T5	<2.5 x10^1(est)
9	M9T1	<2.5 x10^1(est)
	M9T2	<2.5 x10^1(est)
	M9T3	<2.5 x10^1(est)
	M9T4	2.0.x10^1(est)
	M9T5	1.0 x10^1(est)
10	M10T1	<2.5 x10^1(est)
	M10T2	<2.5 x10^1(est)
	M10T3	<2.5 x10^1(est)
	M10T4	<2.5 x10^1(est)

Key: Mt : Raw (uncooked meat), (est): Estimated counts on plates with no colony or counts less than 25 where colonies where formed.

Appendix II: Presumptive *B.cereus* Counts of Retail **Balangu**

Producer	**Sample**	***B.cereus* count (cfu/g)**
1	Bln1g1	<2.5 x10^1(est)
	Bln1g2	<2.5 x10^1(est)
	Bln1g3	<2.5 x10^1(est)
	Bln1g4	<2.5 x10^1(est)
	Bln1g5	<2.5 x10^1(est)

2	Bln2g1	<2.5 x10^{1}(est)
	Bln2g2	<2.5 x10^{1}(est)
	Bln2g3	<2.5 x10^{1}(est)
	Bln2g4	3.0 x10^{1}(est)
	Bln2g5	4.0 x10^{1}(est)
3	Bln3g1	8.0 x10^{1}(est)
	Bln3g2	4.0 x10^{1}(est)
	Bln3g3	<2.5 x10^{1}(est)
	Bln3g4	<2.5 x10^{1}(est)
	Bln3g5	6.0 x10^{1}(est)
4	Bln4g1	6.0 x10^{1}(est)
	Bln4g2	<2.5 x10^{1}(est)
	Bln4g3	<2.5 x10^{1}(est)
	Bln4g4	<2.5 x10^{1}(est)
	Bln4g5	<2.5 x10^{1}(est)
	Bln4g6	<2.5 x10^{1}(est)
5	Bln5g1	<2.5 x10^{1}(est)
	Bln5g2	<2.5 x10^{1}(est)
	Bln5g3	1.0 x10^{1}(est)
	Bln5g4	<2.5 x10^{1}(est)
	Bln5g5	<2.5 x10^{1}(est)
6	Bln6g1	<2.5 x10^{1}(est)
	Bln6g2	<2.5 x10^{1}(est)
	Bln6g3	<2.5 x10^{1}(est)
	Bln6g4	<2.5 x10^{1}(est)
	Bln6g5	1.0 x10^{1}(est)
7	Bln7g1	3.0 x10^{1}(est)
	Bln7g2	2.0 x10^{1}(est)
	Bln7g3	<2.5 x10^{1}(est)
	Bln7g4	7.0 x10^{1}(est)
	Bln7g5	<2.5 x10^{1}(est)
	Bln7g6	<2.5 x10^{1}(est)
8	Bln8g1	<2.5 x10^{1}(est)
	Bln8g2	3.0 x10^{1}(est)
	Bln8g3	<2.5 x10^{1}(est)
	Bln8g4	2.0 x10^{1}(est)
	Bln8g5	5.0 x10^{1}(est)
	Bln8g6	<2.5 x10^{1}(est)

Key: Blng: **Balangu**, (est) : Estimated counts on plates with no colony or counts less than 25 where colonies where formed.

Appendix III : Presumptive *B.cereus* Counts of Retail **Soye**

Producer	Sample	*B.cereus* counts (cfu/g)
1	S1Y1	$<2.5 \times 10^1$(est)
	S1Y2	$<2.5 \times 10^1$(est)
	S1Y3	3.2×10^2
	S1Y4	$<2.5 \times 10^1$(est)
	S1Y5	$<2.5 \times 10^1$(est)
	S1Y6	1.8×10^2 (est)
	S1Y7	$<2.5 \times 10^1$(est)
	S1Y8	6.0×10^1 (est)
2	S2Y1	$<2.5 \times 10^1$(est)
	S2Y2	4.0×10^1(est)
	S2Y3	4.1×10^2
	S2Y4	1.2×10^2(est)
	S2Y5	$<2.5 \times 10^1$(est)
	S2Y6	3.3×10^2
	S2Y7	$<2.5 \times 10^1$(est)
3	S3Y1	$<2.5 \times 10^1$(est)
	S3Y2	3.5×10^2
	S3Y3	1.5×10^2(est)
	S3Y4	$<2.5 \times 10^1$(est)
	S3Y5	$<2.5 \times 10^1$(est)
	S3Y6	$<2.5 \times 10^1$(est)
	S3Y7	1.3×10^2(est)
4	S4Y1	1.1×10^2(est)
	S4Y2	9.0×10^1(est)
	S4Y3	2.2×10^2(est)
	S4Y4	3.9×10^2
	S4Y5	$<2.5 \times 10^1$(est)
	S4Y6	$<2.5 \times 10^1$(est)
	S4Y7	$<2.5 \times 10^1$(est)
5	S5Y1	2.5×10^1(est)
	S5Y2	5.0×10^1(est)
	S5Y3	$<2.5 \times 10^1$(est)
	S5Y4	$<2.5 \times 10^1$(est)
	S5Y5	$<2.5 \times 10^1$(est)
	S5Y6	$<2.5 \times 10^1$(est)
	S5Y7	$<2.5 \times 10^1$(est)
6	S6Y1	$<2.5 \times 10^1$(est)

	S6Y2	1.7 x10^2(est)
	S6Y3	1.4 x10^2(est)
	S6Y4	<2.5 x10^1(est)
	S6Y5	9.0 x10^1(est)
	S6Y6	<2.5 x10^1(est)
	S6Y7	<2.5 x10^1(est)

Key: SY: **Soye** (Roasted hawked meat), est: Estimated counts on plates with no colony or counts less than 25 where colonies where formed.

Appendix IV: Presumptive *B.cereus* Counts of Retail **Suya**

Producer	Sample	*B.cereus* count *(*cfu/g*)*
1	S1S1	<2.5 x10^1(est)
	S1S2	6.0 x10^1(est)
	S1S3	1.0 x10^1(est)
	S1S4	<2.5 x10^1(est)
2	S2S1	<2.5 x10^1(est)
	S2S2	2.0 x10^1(est)
	S2S3	<2.5 x10^1(est)
	S2S4	<2.5 x10^1(est)
3	S3S1	<2.5 x10^1(est)
	S3S2	<2.5 x10^1(est)
	S3S3	<2.5 x10^1(est)
	S3S4	5.0 x10^1(est)
4	S4S1	<2.5 x10^1(est)
	S4S2	<2.5 x10^1(est)
	S4S3	8.0 x10^1(est)
	S4S4	<2.5 x10^1(est)
5	S5S1	<2.5 x10^1(est)
	S5S2	<2.5 x10^1(est)
	S5S3	<2.5 x10^1(est)
	S5S4	2.0 x10^1(est)
6	S6S1	<2.5 x10^1(est)
	S6S2	<2.5 x10^1(est)
	S6S3	<2.5 x10^1(est)
	S6S4	<2.5 x10^1(est)
7	S7S1	<2.5 x10^1(est)
	S7S2	<2.5 x10^1(est)
	S7S3	<2.5 x10^1(est)
	S7S4	3.0 x10^1(est)
	S7S5	4.0 x10^1(est)
8	S8S1	3.0 x10^1(est)

	S8S2	<2.5 x10^1(est)
	S8S3	<2.5 x10^1(est)
	S8S4	<2.5 x10^1(est)
	S8S5	<2.5 x10^1(est)
9	S9S1	<2.5 x10^1(est)
	S9S2	1.0 x10^1(est)
	S9S3	<2.5 x10^1(est)
	S9S4	<2.5 x10^1(est)
10	S10S1	<2.5 x10^1(est)
	S10S2	<2.5 x10^1(est)
	S10S3	<2.5 x10^1(est)
	S10S4	2.0 x10^1(est)
	S10S4	<2.5 x10^1(est)

Key: SS: **Suya** , (est): Estimated counts on plates with no colony or counts less than 25 where colonies where formed.

Appendix V: Presumptive *B.cereus* Counts of Retail Uncooked Rice

Producer	Sample	B.cereus count (cfu/g)
1	R1B1	2.0 x10^1(est)
	R1B2	2.0 x10^1(est)
	R1W3	1.5 x10^2
	R1W4	<2.5 x10^1(est)
	R1W5	<2.5 x10^1(est)
2	R2B1	5.0 x10^1(est)
	R2W2	1.8 x10^2(est)
	R2W3	<2.5 x10^1(est)
	R2B4	4.0 x10^1(est)
	R2W5	<2.5 x10^1(est)
3	R3B1	3.0 x10^1(est)
	R3W2	6.0 x10^1(est)
	R3W3	<2.5 x10^1(est)
	R3B4	<2.5 x10^1(est)
4	R4W1	<2.5 x10^1(est)
	R4W2	3.0 x10^1(est)
	R4W3	<2.5 x10^1(est)
	R4B4	2.0 x10^1(est)
5	R5W1	1.0 x10^1(est)
	R5B2	<2.5 x10^1(est)
	R5B3	<2.5 x10^1(est)
	R5B4	<2.5 x10^1(est)
6	R6W1	<2.5 x10^1(est)
	R6B2	2.0 x10^1(est)
	R6W3	<2.5 x10^1(est)
	R6W4	<2.5 x10^1(est)

7	R7W1	<2.5 x10^1(est)
	R7B2	<2.5 x10^1(est)
	R7W3	<2.5 x10^1(est)
	R7B4	<2.5 x10^1(est)
8	R8W1	<2.5 x10^1(est)
	R8B2	3.0 x10^1(est)
	R8W3	<2.5 x10^1(est)
	R8W4	<2.5 x10^1(est)
9	SMR9B1	<2.5 x10^1(est)
	SMR9B2	<2.5 x10^1(est)
	SMR9B4	<2.5 x10^1(est)
	SMR9B4	3.0 x10^1(est)
	SMR9B5	<2.5 x10^1(est)
10	SMR10W1	1.0 x10^1(est)
	SMR10B2	<2.5 x10^1(est)
	SMR10B4	<2.5 x10^1(est)
	SMR10B4	<2.5 x10^1(est)

Key: RB: Brown Rice (uncooked), RW: White Rice (Uncooked), SMRB: Samaru Brown Rice (uncooked), SMRW: Samaru White Rice (Uncooked), (est): Estimated counts on plates with no colony or counts less than 25 where colonies where formed.

Appendix VI: Presumptive *B.cereus* Counts of Retail **Jollof** Rice

Producer	Sample	*B.cereus* count (cfu/g)	Storage time at sampling (h)	Temp at sampling (^oC)
1	L1	<2.5 x10^1(est)	1	47
	L2	<2.5 x10^1(est)	$2^1/_2$	47
	L3	<2.5 x10^1(est)	3	50
	L4	<2.5 x10^1(est)	4	45
2	M1	1.0 x10^1(est)	1	48
	M2	<2.5 x10^1(est)	$2^1/_2$	43
	M3	<2.5 x10^1(est)	4	37
	M4	<2.5 x10^1(est)	4	44
	M5	<2.5 x10^1(est)	4	51
3	N1	<2.5 x10^1(est)	3	38
	N2	<2.5 x10^1(est)	$2^1/_2$	51
	N3	<2.5 x10^1(est)	4	36
	N4	<2.5 x10^1(est)	2	48

4	P1	<2.5 x10^1(est)	2	38
	P2	<2.5 x10^1(est)	3	45
	P3	1.0 x10^1(est)	3	51
	P4	<2.5 x10^1(est)	3	50
5	Q1	4.0 x10^1(est)	4	44
	Q2	<2.5 x10^1(est)	3	52
	Q3	7.0 x10^1(est)	3	48
	Q4	2.0 x10^1(est)	3	53
	Q5	1.0 x10^1(est)	4	42
6	R1	<2.5 x10^1(est)	4	40
	R2	<2.5 x10^1(est)	3	48
	R3	<2.5 x10^1(est)	3	45
	R4	<2.5 x10^1(est)	3	45
7	S1	<2.5 x10^1(est)	4	38
	S2	<2.5 x10^1(est)	3	39
	S3	<2.5 x10^1(est)	3	41
	S4	<2.5 x10^1(est)	3	45
8	T1	<2.5 x10^1(est)	4	42
	T2	<2.5 x10^1(est)	3	37
	T3	<2.5 x10^1(est)	3	44
	T4	<2.5 x10^1(est)	3	48
9	U1	2.0 x10^1(est)	3	41
	U2	<2.5 x10^1(est)	4	42
	U3	1.0 x10^1(est)	3	44
	U4	<2.5 x10^1(est)	4	48
10	V1	<2.5 x10^1(est)	3	48
	V2	<2.5 x10^1(est)	3	38
	V3	<2.5 x10^1(est)	3	46
	V4	<2.5 x10^1(est)	4	45
	V5	<2.5 x10^1(est)	3	-

Key : L-V: **Jollof** rice, (est): Estimated counts on plates with no colony or counts less than 25 where colonies where formed.

Appendix VII: Presumptive *B.cereus* Counts of Retail Boiled Rice

Producer	sample	*B.cereus* counts (cfu/g)	Storage time at sampling (h)	Temp at sampling (^{o}C)
1	A1	<2.5 x10^1(est)	1	48
	A2	<2.5 x10^1(est)	$2^1/_2$	53
	A3	<2.5 x10^1(est)	2	44
	A4	<2.5 x10^1(est)	$3^1/_2$	42

2	B1	<2.5 x10^1(est)	1	40
	B2	<2.5 x10^1(est)	$2^1/_2$	47
	B3	3.0 x10^1(est)	3	42
	B4	<2.5 x10^1(est)	3	37
	B5	<2.5 x10^1(est)	3	36
3	C1	<2.5 x10^1(est)	3	35
	C2	<2.5 x10^1(est)	$2^1/_2$	45
	C3	<2.5 x10^1(est)	3	39
	C4	<2.5 x10^1(est)	4	49
4	D1	1.0 x10^1(est)	3	41
	D2	<2.5 x10^1(est)	$2^1/_2$	46
	D3	<2.5 x10^1(est)	3	50
	D4	<2.5 x10^1(est)	3	39
5	E1	1.0 x10^1(est)	3	45
	E2	<2.5 x10^1(est)	3	50
	E3	<2.5 x10^1(est)	4	42
	E4	<2.5 x10^1(est)	4	49
	E5	<2.5 x10^1(est)	4	46
6	F1	<2.5 x10^1(est)	3	38
	F2	1.0 x10^1(est)	4	48
	F3	<2.5 x10^1(est)	3	44
	F4	3.0 x10^1(est)	4	46
	F5	<2.5 x10^1(est)	4	40
7	G1	<2.5 x10^1(est)	3	45
	G2	<2.5 x10^1(est)	3	50
	G3	<2.5 x10^1(est)	4	42
	G4	<2.5 x10^1(est)	4	49
8	H1	<2.5 x10^1(est)	3	41
	H2	<2.5 x10^1(est)	4	39
	H3	<2.5 x10^1(est)	3	38
	H4	<2.5 x10^1(est)	4	35
9	J1	<2.5 x10^1(est)	3	50
	J2	<2.5 x10^1(est)	3	46
	J3	<2.5 x10^1(est)	4	32
	J4	<2.5 x10^1(est)	4	39
10	K1	<2.5 x10^1(est)	4	39
	K2	<2.5 x10^1(est)	4	41
	K3	2.0 x10^1(est)	4	43
	K4	<2.5 x10^1(est)	4	46

Key: A-K: boiled plain rice, (est): Estimated counts on plates with no colony or counts less than 25 where colonies where formed

Appendix VIII: Presumptive *B.cereus* Counts of Retail Raw Milk

Producer	Sample	pH	*B. cereus* count (cfu/ml)
1	R1M1	6.4	$<2.5 \times 10^1$(est)
	R1M2	6.5	$<2.5 \times 10^1$(est)
	R1M3	6.6	$<2.5 \times 10^1$(est)
	R1M4	6.6	$<2.5 \times 10^1$(est)
	R1M5	6.5	$<2.5 \times 10^1$(est)
2	R2M1	6.5	$<2.5 \times 10^1$(est)
	R2M2	6.5	$<2.5 \times 10^1$(est)
	R2M3	6.4	$<2.5 \times 10^1$(est)
	R2M4	6.5	$<2.5 \times 10^1$(est)
	R2M5	6.5	$<2.5 \times 10^1$(est)
3	R3M1	6.5	1.0×10^1(est)
	R3M2	6.6	$<2.5 \times 10^1$(est)
	R3M3	6.4	$<2.5 \times 10^1$(est)
	R3M4	6.5	$<2.5 \times 10^1$(est)
	R3M5	6.6	$<2.5 \times 10^1$(est)
4	R4M1	6.4	$<2.5 \times 10^1$(est)
	R4M2	6.4	$<2.5 \times 10^1$(est)
	R4M3	6.6	$<2.5 \times 10^1$(est)
	R4M4	6.5	$<2.5 \times 10^1$(est)
5	R5M1	6.6	1.0×10^1(est)
	R5M2	6.6	1.0×10^1(est)
	R5M3	6.4	$<2.5 \times 10^1$(est)
	R5M4	6.4	$<2.5 \times 10^1$(est)
6	R6M1	6.4	3.0×10^1(est)
	R6M2	6.4	1.0×10^1(est)
	R6M3	6.5	$<2.5 \times 10^1$(est)
	R6M4	6.4	$<2.5 \times 10^1$(est)
	R6M5	6.6	$<2.5 \times 10^1$(est)
7	R7M1	6.5	$<2.5 \times 10^1$(est)
	R7M2	6.4	$<2.5 \times 10^1$(est)
	R7M3	6.5	$<2.5 \times 10^1$(est)
	R7M4	6.6	$<2.5 \times 10^1$(est)
	R7M5	6.4	2.0×10^1(est)
8	R8M1	6.6	$<2.5 \times 10^1$(est)
	R8M2	6.4	$<2.5 \times 10^1$(est)
	R8M3	6.4	$<2.5 \times 10^1$(est)
	R8M4	6.6	$<2.5 \times 10^1$(est)
	R8M5	6.4	$<2.5 \times 10^1$(est)

9	R9M1	6.4	$<2.5 \times 10^1$(est)
	R9M2	6.5	$<2.5 \times 10^1$(est)
	R9M3	6.5	$<2.5 \times 10^1$(est)
	R9M4	6.6	$<2.5 \times 10^1$(est)
	R9M5	6.7	$<2.5 \times 10^1$(est)

Key: RM: Raw cow milk, (est): Estimated counts on plates with no colony or counts less than 25 where colonies where formed.

Appendix IX: Presumptive *B.cereus* Counts of Retail N**ono**

Producer	sample	*B. cereus* count (cfu/ml)	Temp (^oC)	pH
1	F1M1	5.0×10^1(est)	28	3.8
	F1M2	$<2.5 \times 10^1$(est)	30	3.9
	F1M3	3.0×10^1(est)	30	4.0
	F1M4	$<2.5 \times 10^1$(est)	31	3.9
	F1M5	$<2.5 \times 10^1$(est)	32	4.0
2	F2M1	$<2.5 \times 10^1$(est)	31	3.9
	F2M2	2.0×10^1(est)	30	3.9
	F2M3	$<2.5 \times 10^1$(est)	30	4.1
	F2M4	1.0×10^1(est)	31	3.8
	F2M5	$<2.5 \times 10^1$(est)	31	4.0
3	F3M1	3.0×10^1(est)	31	3.8
	F3M2	$<2.5 \times 10^1$(est)	31	4.2
	F3M3	5.0×10^1(est)	29	3.9
	F3M4	7.0×10^1(est)	28	3.9
4	F4M1	$<2.5 \times 10^1$(est)	30	3.8
	F4M2	$<2.5 \times 10^1$(est)	31	4.0
	F4M3	$<2.5 \times 10^1$(est)	30	4.0
	F4M4	4.0×10^1(est)	30	3.8
5	F5M1	3.0×10^1(est)	31	3.9
	F5M2	1.0×10^1(est)	30	4.0
	F5M3	$<2.5 \times 10^1$(est)	30	3.8
	F5M4	$<2.5 \times 10^1$(est)	31	3.9
	F5M5	$<2.5 \times 10^1$(est)	32	4.0
6	F6M1	$<2.5 \times 10^1$(est)	31	4.0
	F6M2	7.0×10^1(est)	30	3.8
	F6M3	2.0×10^1(est)	30	4.2
	F6M4	$<2.5 \times 10^1$(est)	31	4.0
7	F7M1	1.0×10^1(est)	31	3.8
	F7M2	$<2.5 \times 10^1$(est)	32	4.1
	F7M3	6.0×10^1(est)	31	4.1
	F7M4	3.0×10^1(est)	30	3.8
8	F8M1	$<2.5 \times 10^1$(est)	32	4.0
	F8M2	5.0×10^1(est)	29	3.8
	F8M3	$<2.5 \times 10^1$(est)	31	3.8
	F8M4	8.0×10^1(est)	30	3.9

9	F9M1	3.0 x10^1(est)	30	3.9
	F9M2	8.0 x10^1(est)	31	3.8
	F9M3	<2.5 x10^1(est)	30	4.1
	F9M4	<2.5 x10^1(est)	29	4.1
10	F10M1	<2.5 x10^1(est)	30	4.0
	F10M2	7.0 x10^1(est)	31	3.8
	F10M3	<2.5 x10^1(est)	30	3.9
	F10M4	1.0 x10^2(est)	31	4.0

Key: FM : Fermented milk , (est) : Estimated counts on plates with no colony or counts less than 25 where colonies where formed.

Appendix X: *B.cereus* Counts in Boiled Rice Samples Stored for Twenty- Four Hours at Room and Elevated Temperatures* at Different NaCl Concentrations

NaCl concentration (%)	Sample	Counts (cfu/g) at Room Temperature (^{o}C)	Counts (cfu/ml) at Elevated Temperature (^{o}C)
0	A	1.5 x 10^9	1.1 x 10^6
	B	4.1 x 10^9	1.0 x 10^6
	C	6.8 x 10^8	6.8 x 10^7
0.5	A	1.4 x 10^6	2.0 x 10^6
	B	2.3x 10^9	2.0 x 10^7
	C	5.0 x 10^8	6.5 x 10^7
1	A	1.3 x 10^8	3.5 x 10^5
	B	1.0 x 10^9	6.6 x 10^5
	C	8.5 x 10^8	1.4 x 10^6
1.5	A	1.8 x 10^7	3.3 x 10^6
	B	1.4 x 10^7	2.3 x 10^6
	C	6.5 x 10^6	1.7 x 10^6
2	A	7.6 x 10^8	7.8 x 10^8
	B	5.7 x 10^8	9.3 x 10^8
	C	6.7 x 10^8	8.5 x 10^8

Key:

*: Room temperature range of 26-28^{o}C, elevated temperature of 37^{o}C.

A: Boiled rice sample A

B: Boiled rice sample B

C: Boiled rice sample C

Appendix XI: *B.cereus* Counts in Boiled Rice Samples During Storage at Refrigeration Temperature* at Different NaCl Concentrations

0	A	2.5×10^{10}	7.3×10^{7}	1.4×10^{13}
	B	1.9×10^{10}	2.0×10^{8}	1.0×10^{13}
	C	1.4×10^{10}	7.7×10^{7}	1.4×10^{13}
0.5	A	1.1×10^{9}	3.8×10^{7}	5.1×10^{13}
	B	2.2×10^{9}	1.2×10^{8}	1.6×10^{13}
	C	1.2×10^{9}	7.6×10^{7}	1.4×10^{13}
1	A	1.3×10^{10}	5.6×10^{8}	2.2×10^{12}
	B	1.9×10^{9}	7.0×10^{7}	2.3×10^{12}
	C	1.1×10^{10}	4.5×10^{7}	2.0×10^{12}
1.5	A	1.3×10^{6}	1.1×10^{7}	1.3×10^{10}
	B	2.3×10^{6}	1.6×10^{8}	4.7×10^{12}
	C	2.0×10^{6}	5.2×10^{7}	4.7×10^{12}
2	A	1.7×10^{9}	1.1×10^{8}	2.2×10^{13}
	B	2.1×10^{6}	6.8×10^{7}	2.0×10^{12}
	C	1.4×10^{9}	3.7×10^{7}	1.7×10^{12}

Key:

*: Refrigeration temperature of 4-10^{o}C

A: Boiled rice sample A

B: Boiled rice sample B

C: Boiled rice sample C

Appendix XII: *B.cereus* Counts in **Balangu** Samples Stored for Twenty- Four Hours at Room and Elevated Temperatures* at Different NaCl Concentrations

NaCl concentration (%)	Sample	Counts (cfu/g) at room Temperature (^{o}C)	Counts (cfu/g) at Elevated Temperature (^{o}C)
0	A	1.6×10^{13}	1.8×10^{13}
	B	1.8×10^{13}	2.3×10^{12}
	C	1.6×10^{13}	1.7×10^{13}
0.5	A	2.0×10^{13}	2.2×10^{13}
	B	$2.2x\ 10^{13}$	2.0×10^{13}
	C	4.5×10^{13}	2.1×10^{13}
1	A	1.9×10^{14}	1.3×10^{13}
	B	1.7×10^{14}	1.5×10^{13}
	C	3.1×10^{14}	1.5×10^{13}
1.5	A	1.7×10^{14}	2.1×10^{13}
	B	1.8×10^{14}	2.1×10^{13}
	C	1.8×10^{14}	2.2×10^{13}
2	A	1.5×10^{13}	4.9×10^{8}
	B	1.4×10^{13}	7.0×10^{8}

	C	1.6×10^{13}	4.5×10^{8}

Key:

*: Room temperature range of 26-28^{o}C, elevated temperature of 37^{o}C
A: **Balangu** sample A
B: **Balangu** sample B
C: **Balangu** sample C

Appendix XIII: *B.cereus* Counts in **Balangu** Samples During Storage at Refrigeration Temperature* at Different NaCl Concentrations

0	A	2.1×10^{12}	1.2×10^{13}	4.4×10^{10}
	B	2.4×10^{12}	2.6×10^{11}	8.5×10^{10}
	C	2.6×10^{12}	8.0×10^{12}	3.1×10^{10}
0.5	A	1.0×10^{8}	1.4×10^{12}	1.2×10^{11}
	B	1.4×10^{8}	1.0×10^{11}	3.7×10^{10}
	C	1.5×10^{8}	1.7×10^{12}	5.0×10^{10}
1	A	1.4×10^{12}	2.2×10^{11}	1.5×10^{11}
	B	2.2×10^{12}	2.1×10^{11}	2.3×10^{10}
	C	1.4×10^{12}	2.0×10^{11}	8.1×10^{10}
1.5	A	2.4×10^{12}	1.6×10^{10}	4.3×10^{9}
	B	2.0×10^{12}	9.0×10^{9}	1.5×10^{9}
	C	2.1×10^{12}	2.7×10^{10}	4.3×10^{9}

2	A	9.6×10^{9}	1.1×10^{13}	$2.7x\ 10^{9}$
	B	1.0×10^{10}	$2.9x\ 10^{10}$	8.5×10^{9}
	C	1.7×10^{9}	7.6×10^{12}	1.2×10^{10}

Key:

*: Refrigaration temperature 4-10^{o}C.
A: **Balangu** sample A
B: **Balangu** sample B
C: **Balangu** sample C

Appendix XIV: *B.cereus* Counts In **Nono** Samples Stored for Twenty- Four Hours at Room and Elevated Temperatures* at Different pH Levels

pH level	Sample	Counts (cfu/ml) at room temperature (^{o}C)	Counts (cfu/ml) at elevated temperature (^{o}C)
3.7	A	5.3×10^{9}	7.1×10^{8}
	B	1.9×10^{9}	3.0×10^{9}
	C	1.6×10^{9}	3.5×10^{9}
3.8	A	5.5×10^{7}	1.6×10^{9}
	B	6.5×10^{10}	9.5×10^{10}
	C	4.9×10^{10}	3.2×10^{10}
4.2	A	7.0×10^{1}	2.0×10^{1}
	B	1.0×10^{1}	5.0×10^{1}

	C	5.0×10^1	3.0×10^1
4.3	A	7.5×10^7	5.0×10^8
	B	2.4×10^8	7.7×10^9
	C	1.1×10^8	2.2×10^9

Key:

*: Room temperature range of 26-28°C, elevated temperature of 37°C
A: **Nono** sample A
B: **Nono** sample B
C: **Nono** sample C

Appendix XV: *B.cereus* Counts in **Nono** Samples During Storage at Refrigeration Temperature* at Different pH Levels

pH level	Sample	Counts (cfu/ml) at 24 h	Counts (cfu/ml) at 48 h	Counts (cfu/ml) at 72 h
3.7	A	2.5×10^7	1.1×10^7	3.8×10^6
	B	5.3×10^7	6.2×10^6	2.5×10^5
	C	1.2×10^7	1.0×10^7	2.3×10^6
3.8	A	6.5×10^6	1.5×10^6	4.8×10^6

	B	1.2×10^8	2.8×10^6	2.3×10^6
	C	9.8×10^7	2.7×10^6	1.7×10^6
4.2	A	2.2×10^7	2.4×10^6	5.8×10^6
	B	7.0×10^6	4.4×10^6	2.5×10^7
	C	5.5×10^6	9.1×10^6	2.0×10^7
4.3	A	5.5×10^6	3.8×10^6	2.4×10^6
	B	1.7×10^8	2.5×10^6	2.0×10^7
	C	1.1×10^8	1.7×10^6	1.7×10^7

Key:

*: Refrigeration temperature range of 4-10^{o}C.
A: **Nono** sample A
B: **Nono** sample B
C: **Nono** sample C

Appendix XVI: *B.cereus* Counts in Pasteurized Milk Samples During Storage at Refrigeration Temperature* at Different pH Levels

pH level	Sample	Counts (cfu/ml) at 24 h	Counts (cfu/ml) at 48 h	Counts (cfu/ml) at 72 h

6.3	A	4.6×10^{8}	1.8×10^{6}	3.9×10^{7}
	B	1.6×10^{8}	4.8×10^{6}	1.5×10^{8}
	C	3.6×10^{8}	7.2×10^{6}	2.5×10^{7}
6.4	A	4.7×10^{7}	8.3×10^{8}	5.1×10^{7}
	B	3.9×10^{8}	1.0×10^{8}	5.2×10^{7}
	C	1.5×10^{8}	2.7×10^{8}	2.6×10^{8}
6.7	A	3.2×10^{7}	6.1×10^{7}	1.1×10^{9}
	B	2.6×10^{8}	5.3×10^{8}	1.0×10^{8}
	C	1.0×10^{8}	5.4×10^{8}	2.1×10^{8}
6.8	A	5.0×10^{7}	1.2×10^{7}	2.0×10^{7}
	B	2.9×10^{7}	9.6×10^{6}	5.9×10^{7}
	C	6.5×10^{7}	2.3×10^{7}	8.1×10^{7}

Key:

*: Refrigeration temperature range of 4-10^{o}C.
A: Pasteurized milk sample A
B: Pasteurized milk sample B
C: Pasteurized milk sample C

AppendixXVII: Antibiograms of Enterotoxin Producing *B.cereus* Isolates From Boiled Rice at Varying NaCl Concentrations and Temperature

Food sample	Diameter of Zone of inhibition (mm) to antibiotic (potency in µg)									
	AMC(30)	AK (30)	SXT (25)	E(30)	CPD(10)	KF(30)	TE(30)	CIP(5)	CRO(30)	AMP(10)
0% NaCl at 24 h optimum temperature	8 (R)	28(S)	0(R)	36(S)	0 (R)	8(R)	26(S)	36(S)	12 (R)	0(R)
0% NaCl at 24 h room temperature	9(R)	28(S)	0(R)	34(S)	0(R)	8(R)	24(S)	34(S)	10(R)	0(R)
0% NaCl at 24 h refrigeration	9(R)	28(S)	0(R)	30(S)	0(R)	7(R)	24(S)	32(S)	12(R)	0(R)
0% NaCl at 48 h refrigeration	12(R)	27(S)	0(R)	34(S)	0(R)	10(R)	22(S)	30(S)	9(R)	0(R)
0% NaCl at 72 h refrigeration	10(R)	29(S)	0(R)	31(S)	0(R)	11(R)	24(S)	32(S)	10(R)	0(R)
0.5% NaCl at 24 h optimum temperature	8(R)	28(S)	0(R)	30(S)	0(R)	0(R)	22(S)	32(S)	0(R)	8(R)
0.5% NaCl at 24 h room temperature	8(R)	28(S)	0(R)	32(S)	0(R)	8(R)	22(S)	34(S)	0(R)	8(R)
0.5% NaCl at 24 h refrigeration	9(R)	29(S)	0(R)	32(S)	0(R)	8(R)	24(S)	28(S)	14(I)	0(R)
0.5% NaCl at 48 h refrigeration	*	*	*	*	*	*	*	*	*	*
0.5% NaCl at 72 h refrigeration	11(R)	29(S)	0(R)	31(S)	0(R)	11(R)	20(S)	30(S)	8(R)	0(R)
1% NaCl at 24 h optimum temperature	9(R)	28(S)	0(R)	30(S)	0(R)	8(R)	24(S)	36(S)	10(R)	8(R)
1% NaCl at 24 h room temperature	9(R)	26(S)	0(R)	30(S)	0(R)	7(R)	24(S)	34(S)	10(R)	0(R)
1% NaCl at 24 h refrigeration	8(R)	28(S)	0(R)	32(S)	0(R)	8(R)	24(S)	32(S)	8(R)	0(R)
1% NaCl at 48 h refrigeration	14(R)	26(S)	0(R)	34(S)	0(R)	11(R)	19(S)	30(S)	9(R)	7(R)
1% NaCl at 72 h refrigeration	11(R)	28(S)	0(R)	30(S)	0(R)	11(R)	22(S)	32(S)	10(R)	0(R)
1.5% NaCl at 24 h optimum temperature	8(R)	28(S)	0(R)	31(S)	0(R)	8(R)	24(S)	34(S)	12(R)	7(R)
1.5% NaCl at 24 h room temperature	8(R)	29(S)	0(R)	32(S)	0(R)	8(R)	22(S)	30(S)	12(R)	0(R)
1.5% NaCl at 24 h refrigeration	7(R)	26(S)	0(R)	30(S)	0(R)	8(R)	18(I)	26(S)	7(R)	0(R)
1.5% NaCl at 48 h refrigeration	14(R)	26(S)	0(R)	32(S)	0(R)	11(R)	19(S)	28(S)	7(R)	0(R)
1.5% NaCl at 72 h refrigeration	10(R)	27(S)	0(R)	30(S)	0(R)	10(R)	22(S)	30(S)	8(R)	0(R)
2% NaCl at 24 h optimum temperature	8(R)	28(S)	0(R)	30(S)	0(R)	8(R)	24(S)	34(S)	10(R)	0(R)
2% NaCl at 24 h room temperature	8(R)	28(S)	0(R)	30(S)	0(R)	7(R)	22(S)	34(S)	8(R)	0(R)
2% NaCl at 24 h refrigeration	7(R)	26(S)	0(R)	31(S)	7(R)	7(R)	16(I)	26(S)	0(R)	0(R)
2% NaCl at 48 h refrigeration	12(R)	29(S)	0(R)	32(S)	0(R)	10(R)	16(I)	26(S)	7(R)	0(R)
2% NaCl at 72 h refrigeration	10(R)	28(S)	0(R)	30(S)	0(R)	11(R)	18(I)	28(S)	8(R)	8(R)

Key: R: resistant, I: intermediate, S: susceptible, AMC: amoxicillin-clavulanic acid, AK: amikacin, SXT: trimethoprim-sulfamethoxazole, E: erythromycin, CPD: cefpodoxime, KF: cephalothin, TE: tetracycline, CIP: ciprofloxacin, CRO: ceftriaxone, AMP: ampicillin, optimum temperature 37^{o}C, room temperature 26-28^{o}C, Refrigeration temperature 4-10^{o}C, *: no viable isolate.

Appendix XVIII: Antibiograms of Diarrhoeal Enterotoxin Producing *B.cereus* Isolates From **Balangu** at Varying NaCl Concentrations and Temperature

Key: R: resistant, I: intermediate, S: susceptible, AMC: amoxicillin-clavulanic acid, AK: amikacin, SXT: trimethoprim-sulfamethoxazole, E: erythromycin, CPD:

Food sample	Antibiotic (potency in μg)									
	AMC(30)	AK (30)	SXT (25)	E(30)	CPD(10)	KF(30)	TE(30)	CIP(5)	CRO(30)	AMP(10)
0% NaCl at 24 h opt temperature	7(R)	23(S)	0(R)	22(R)	0(R)	0(R)	17(I)	26(S)	0(R)	0(R)
0% NaCl at 24 h room temp	0(R)	27(S)	0(R)	22(R)	0(R)	0(R)	22(S)	30(S)	0(R)	0(R)
0% NaCl at 24 h refrigeration	0(R)	26(S)	0(R)	21(R)	0(R)	0(R)	22(S)	31(S)	7(R)	0(R)
0% NaCl at 48 h refrigeration	0(R)	23(S)	7(R)	21(R)	0(R)	0(R)	23(S)	31(S)	0(R)	0(R)
0% NaCl at 72 h refrigeration	0(R)	26(S)	0(R)	21(R)	0(R)	0(R)	23(S)	29(S)	7(R)	0(R)
0.5% NaCl at 24 h optimum temperature	0(R)	26(S)	0(R)	22(R)	0(R)	0(R)	22(S)	32(S)	7(R)	0(R)
0.5% NaCl at 24 h room temp	0(R)	24(S)	0(R)	23(R)	0(R)	0(R)	22(S)	31(S)	7(R)	0(R)
0.5% NaCl at 24 h refrigeration	0(R)	26(S)	0(R)	22(R)	0(R)	0(R)	23(S)	29(S)	0(R)	0(R)
0.5% NaCl at 48 h refrigeration	9(R)	23(S)	7(R)	22(R)	0(R)	0(R)	23(S)	31(S)	0(R)	0(R)
0.5% NaCl at 72 h refrigeration	0(R)	24(S)	0(R)	21(R)	0(R)	0(R)	22(S)	31(S)	8(R)	0(R)
1% NaCl at 24 h optimum temperature	9(R)	26(S)	0(R)	28(S)	0(R)	0(R)	24(S)	31(S)	8(R)	0(R)
1% NaCl at 24 h room temperature	0(R)	26(S)	0(R)	23(R)	0(R)	0(R)	22(S)	32(S)	0(R)	0(R)
1% NaCl at 24 h refrigeration	0(R)	26(S)	0(R)	21(R)	0(R)	0(R)	22(S)	31(S)	0(R)	0(R)
1% NaCl at 48 h refrigeration	7(R)	26(S)	0(R)	23(R)	0(R)	0(R)	23(S)	30(S)	0(R)	0(R)
1% NaCl at 72 h refrigeration	0(R)	26(S)	0(R)	21(R)	0(R)	0(R)	23(S)	28(S)	0(R)	0(R)
1.5% NaCl at 24 h optimum temperature	0(R)	26(S)	0(R)	23(R)	0(R)	0(R)	22(S)	31(S)	0(R)	0(R)
1.5% NaCl at 24 h oomtemperature	0(R)	26(S)	0(R)	23(R)	0(R)	0(R)	22(S)	30(S)	0(R)	0(R)
1.5% NaCl at 24 h refrigeration	0(R)	26(S)	7(R)	21(R)	0(R)	0(R)	22(S)	31(S)	0(R)	0(R)
1.5% NaCl at 48 h refrigeration	0(R)	24(S)	0(R)	22(R)	0(R)	0(R)	23(S)	31(S)	0(R)	0(R)
1.5% NaCl at 72 h refrigeration	0(R)	23(S)	0(R)	22(R)	0(R)	0(R)	24(S)	30(S)	0(R)	0(R)
2% NaCl at 24 h optimum temperature	0(R)	23(S)	7(R)	21(R)	0(R)	0(R)	22(S)	32(S)	0(R)	0(R)
2% NaCl at 24 h room temperature	9(R)	26(S)	0(R)	22(R)	0(R)	0(R)	22(S)	30(S)	0(R)	0(R)
2% NaCl at 24 h refrigeration	0(R)	26(S)	0(R)	22(R)	0(R)	0(R)	22(S)	29(S)	0(R)	0(R)
2% NaCl at 48 h refrigeration	0(R)	23(S)	0(R)	22(R)	0(R)	0(R)	23(S)	31(S)	0(R)	0(R)
2% NaCl at 72 h refrigeration	0(R)	23(S)	0(R)	22(R)	0(R)	0(R)	23(S)	30(S)	0(R)	0(R)

cefpodoxime, KF: cephalothin, TE: tetracycline, CIP: ciprofloxacin, CRO: ceftriaxone, AMP: ampicillin, optimum temperature 37^{o}C, room temperature 26-28^{o}C, Refrigeration temperature 4-10^{o}C.

Appendix XIX: Antimicrobial Susceptibility of Diarrhoeal Enterotoxin Producing *B.cereus* Isolates From **Nono** During Storage at

	AMC(30)	AK(30)	SXT(25)	E(30)	CPD(10)	KF(30)	TE(30)	CIP(5)	CRO(30)	AMP(10)
pH 3.7 at 24 h optimum temperature	0(R)	25(S)	0(R)	30(S)	0(R)	0(R)	13(R)	28(S)	0(R)	0(R)

Varying pH and Temperature

Food sample	Antibiotic (potency in µg)									
pH 3.7 at 24 h room temperature	0(R)	25(S)	0(R)	29(S)	0(R)	0(R)	14(R)	26(S)	0(R)	0(R)
pH 3.7at 24 h refrigeration	0(R)	26(S)	0(R)	34(S)	0(R)	0(R)	16(I)	28(S)	0(R)	0(R)
pH 3.7at 48 h refrigeration	0(R)	22(S)	0(R)	28(S)	0(R)	0(R)	11(R)	26(S)	0(R)	0(R)
pH 3.7at 72 h refrigeration	0(R)	22(S)	0(R)	25(S)	7(R)	0(R)	11(R)	28(S)	0(R)	0(R)
pH 3.8 at 24 h optimum temperature	0(R)	23(S)	0(R)	30(S)	0(R)	0(R)	14(I)	28(S)	0(R)	0(R)
pH 3.8 at 24 h room temperature	0(R)	25(S)	0(R)	30(S)	0(R)	0(R)	18(R)	27(S)	0(R)	0(R)
pH 3.8 at 24 h refrigeration	0(R)	25(S)	0(R)	32(S)	0(R)	0(R)	12(R)	28(S)	0(R)	0(R)
pH 3.8 at 48 h refrigeration	0(R)	25(S)	0(R)	28(S)	7(R)	0(R)	16(I)	27(S)	0(R)	0(R)
pH 3.8 at 72 h refrigeration	0(R)	24(S)	0(R)	28(S)	0(R)	0(R)	16(I)	27(S)	0(R)	0(R)
pH 4.2 at 24 h optimum temperature	*	*	*	*	*	*	*	*	*	*
pH 4.2 at 24 h room temperature	*	*	*	*	*	*	*	*	*	*
pH 4.2 at 24 h refrigeration	0(R)	25(S)	0(R)	32(S)	0(R)	0(R)	17(I)	27(S)	0(R)	0(R)
pH 4.2 at 48 h refrigeration	0(R)	22(S)	0(R)	28(S)	0(R)	7(R)	16(I)	26(S)	0(R)	0(R)
pH 4.2 at 72 h refrigeration	0(R)	22(S)	0(R)	26(S)	0(R)	0(R)	16(I)	26(S)	0(R)	0(R)
pH 4.3 at 24 h optimum temperature	0(R)	25(S)	0(R)	32(S)	0(R)	0(R)	16(I)	27(S)	0(R)	0(R)
pH 4.3 at 24 h room temperature	0(R)	22(S)	0(R)	30(S)	0(R)	0(R)	17(I)	28(S)	0(R)	0(R)
pH 4.3 at 24 h refrigeration	0(R)	26(S)	0(R)	32(S)	0(R)	0(R)	17(I)	26(S)	0(R)	0(R)
pH 4.3 at 48 h refrigeration	0(R)	23(S)	0(R)	27(S)	7(R)	0(R)	17(I)	27(S)	0(R)	0(R)
pH 4.3 at 72 h refrigeration	0(R)	22(S)	0(R)	25(S)	0(R)	0(R)	16(I)	26(S)	0(R)	0(R)

Key: R: resistant, I: intermediate, S: susceptible, AMC: amoxicillin-clavulanic acid, AK: amikacin, SXT: trimethoprim-sulfamethoxazole, E: erythromycin, CPD: cefpodoxime, KF: cephalothin, TE: tetracycline, CIP: ciprofloxacin, CRO: ceftriaxone, AMP: ampicillin, optimum temperature 37^{o}C, room temperature 26-28^{o}C, Refrigeration temperature 4-10^{o}C, *: no viable isolate.

Appendix XX: Antimicrobial Susceptibility of Diarrhoeal Enterotoxin Producing *B.cereus* Isolates From Pasteurized Milk During Refrigeration Storage at Varying pH Levels

	AMC(30)	AK (30)	SXT (25)	E(30)	CPD(10)	KF(30)	TE(30)	CIP(5)	CRO(30)	AMP(10)
pH 6.7 at 24 h refrigeration	11(R)	27(S)	0(R)	34(S)	0(R)	8(R)	24(S)	29(S)	8(R)	0(R)
pH 6.7 at 48 h refrigeration	8(R)	28(S)	0(R)	29(S)	0(R)	11(R)	21(S)	32(S)	7(R)	0(R)
pH 6.7 at 72 h refrigeration	15(R)	27(S)	0(R)	29(S)	0(R)	9(R)	26(S)	29(S)	8(R)	0(R)
pH 6.8 at 24 h refrigeration	0(R)	27(S)	0(R)	34(S)	0(R)	0(R)	27(S)	31(S)	7(R)	0(R)
pH 6.8 at 48 h refrigeration	*	*	*	*	*	*	*	*	*	*
pH 6.8 at 72 h refrigeration	18(R)	28(S)	0(R)	25(S)	0(R)	22(S)	24(S)	29(S)	7(R)	0(R)
pH 6.3 at 24 h refrigeration	14(R)	30(S)	0(R)	34(S)	0(R)	14(R)	14(I)	38(S)	0(R)	0(R)
pH 6.3 at 48 h refrigeration	13(R)	24(S)	0(R)	32(S)	0(R)	11(R)	13(I)	31(S)	7(R)	0(R)
pH 6.3 at 72 h refrigeration	11(R)	25(S)	0(R)	29(S)	0(R)	10(R)	16(I)	28(S)	7(R)	0(R)
pH 6.4 at 24 h refrigeration	12(R)	28(S)	0(R)	34(S)	0(R)	9(R)	24(S)	28(S)	14(R)	0(R)
pH 6.4 at 48 h refrigeration	8(R)	28(S)	0(R)	31(S)	0(R)	0(R)	16(I)	31(S)	7(R)	0(R)
pH 6.4 at 72 h refrigeration	11(R)	25(S)	0(R)	32(S)	0(R*)	8(R)	23(S)	29(S)	10(R)	0(R)

Key: R: resistant, I: intermediate, S: susceptible, AMC: amoxicillin-clavulanic acid, AK: amikacin, SXT: trimethoprim-sulfamethoxazole, E: erythromycin, CPD: cefpodoxime, KF: cephalothin, TE: tetracycline, CIP: ciprofloxacin, CRO: ceftriaxone, AMP: ampicillin, optimum temperature 37^{o}C, room temperature 26-28^{o}C, Refrigeration temperature 4-10^{o}C, *: non-enterotoxin producing isolate.

Appendix XXI : Biochemical Responses of Presumptive *B.cereus* Isolates From **Nono** to Microgen Bacillus Identification kits

Sample no.	Sample name	Fermentation of sugars at 48 h incubation																				Physiological tests				
		ARA	CEL	INO	MAN	MNS	RAF	RHA	SAL	SOR	SUC	TRE	XYL	ADO	GAL	MDM	MDG	INU	MLZ	IND	ONPG	ARG	CIT	VP	NIT	Isolate
1	F5M2	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	*B.cereus* group
2	F2M4	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	*B.cereus* group
3	F7M4	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
4	F1M3	-	-	-	-	-	-	-	+	-	+	+	-	-		-	-	-	-	-	-	+	+	+	+	*B. cereus* group
5	F3M4	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	*B.licheniformis*
6	F1M1	-	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	*B. firmus*
7	F3M3	-	-	+	+	+	-	-	+	-	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	*B.licheniformis*
8	F8M2	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B. cereus* group
9	F2M2	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
10	F3M1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	*B.licheniformis*
11	F5M1	-	+	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
12	F9M2	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
13	F10M4	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
14	F10M2	-	+	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	-	-	-	+	-	+	+	*B.cereus* group
15	F8M4	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
16	F6M2	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B. cereus* group
17	F9M1	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
18	F7M3	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
19	F4M4	+	-	-	+	+	-	-	+	-	+	+	+	+	+	-	-	-	+	-	+	+	+	+	+	*B.licheniformis*
20	F6M3	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	*B.cereus* group
21	F7M1	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group

Key: ARA: Arabinose, CEL: Cellobiose, INO: Inositol, MAN: Mannitol, MNS: Mannose, RAF: Raffinose, RHA: SAL: Salicin, SOR: Sorbitol, SUC: Sucrose, TRE: Trehalose, XYL: Xylose, ADO: Adonitol, GAL: Galactose, MDM: Methyl-D-Mannose, MDG: Methyl-D-Glucoside, INU: Inulin, MLZ: Melezitose, IND: Indole

prodution, ONPG: Ortho-nitrophenol, ARG: Arginine hydrolysis, CIT: Citrate utilization, VP: Voges-Proskauer test, NIT: Nitrate reduction.
FM: locally fermented milk (**nono**)

Appendix XXII : Biochemical Responses of Presumptive *B.cereus* Isolates From Raw Milk and Raw Meat Samples to Microgen Bacillus Identification Kits

Sample no.		Fermentation of sugars at 48 h incubation																			Physiological tests						
		ARA	CEL	INO	MAN	MNS	RAF	RHA	SAL	SOR	SUC	TRE	XYL	ADO	GAL	MDM	MDG	INU	MLZ	IND	ONPG	ARG	CIT	VP	NIT	ISOLATE
22	R5M2	-	-	+	+	+	-	-	+	-	+	+	-	+	+	-	-	-	-	+	+	+	+	+	+	*B.licheniformis*
23	R3M1	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
24	R6M2	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
25	R5M1	-	-	+	+	+	-	-	+	-	+	+	-	+	+	-	-	-	-	+	+	+	+	+	+	*B.licheniformis*
26	R7M1	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus group*
27	R6M1	+	+	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	+	-	+	+	*B.firmus*
28	M8T2	-	-	-	-	+	-	-	-	-	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	*B.firmus*
29	M4T2	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	*B. firmus*
30	M2T2	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	+	-	+	+	+	+	*B.firmus*
31	M9T4	-	-	-	-	+	-	-	+	-	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	*B.licheniformis*
32	M9T5	-	-	-	-	+	-	-	-	-	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	*B.firmus*
33	M6T4	-	-	-	-	+	-	-	+	-	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	*B.licheniformis*
34	M2T3	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	*B.firmus*
35	M5T4	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	*B.firmus*
36	M6T3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	*B.licheniformis*

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RM: raw milk, MT: raw meat

AppendixXXIII : Biochemical Responses of Presumptive *B.cereus* Isolates From **suya, jollof** and Boiled Rice Samples to Microgen Bacillus Identification Kits

Sample no.	Sample name	Fermentation of sugars at 48 h																				Physiological tests						
		ARA	CEL	INO	MAN	MNS	RAF	RHA	SAL	SOR	SUC	TRE	XYL	ADO	GAL	MDM	MDG	INU	MLZ	IND	ONPG	ARG	CIT	VP	NIT	Isolate
37	S10S4	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	-	-	+	-		+	+	+	*B.cereus* group
38	S4S3	-	-	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	+	-	+	+	+	+	*B.cereus* group
39	S1S3	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	*B.brevis*
40	S5S4	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	B.*cereus* group
41	S8S1	-	+	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	B.*cereus* group
42	S1S2	-	-	+	-	+	-	-	+	-	+	+	-	-	+	-	-	-	-	+	-	+	+	+	+	B.*cereus* group
43	S3S4	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	B.*cereus* group
44	S9S2	-	-	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	B.*cereus* group
45	S7S5	-	-	-	-	+	-	+	+	-	+	+	-	-	-	-	-	-	-	+	-	+	+	+	+	B.*laterosporus*
46	S2S2	-	+	-	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	B.*cereus* group
47	S7S4	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	-	-	+	-	+	+	+	+	*B.cereus* group
48	K3	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
49	Q3	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	+	-	+	+	*B,cereus* group
50	E1	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
51	F4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	*B.brevis*
52	B3	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	+	-	+	+	*B.cereus* group
53	F2	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B. cereus* group

54	M1	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
55	D1	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
56	Q4	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	+	-	*B.brevis*
57	Q5	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
58	Q1	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	+	+	*B.cereus* group
59	P3	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	*B.cereus* group
60	U3	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	B.*cereus* group
61	U1	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	B.*cereus* group

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SS: ***Suya:*** B,D,E,F: boiled rice, K,M,P,Q,: **Jollof** rice

Appendix XXIV : Biochemical Responses of Presumptive *B.cereus* Isolates From **Balangu** to Microgen Bacillus Identification Kits

Sample no	Sample name	Fermentation of sugars at 48 h incubation																				Physiological tests						
		ARA	CEL	INO	MAN	MNS	RAF	RHA	SAL	SOR	SUC	TRE	XYL	ADO	GAL	MDM	MDG	INU	MLZ	IND	ONPG	ARG	CIT	VP	NIT	Isolate
62	Bln2g5	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+	-	+	+	*B.firmus*
63	Bln7g4	+	-	-	-	+	-	-	-	-	+	+	+	-	+	-	-	-	-	-	-	+	+	-	+	*B.firmus*
64	Bln3g2	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	*B.cereus* group
65	Bln2g4	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B. cereus* group
66	Bln8g2	-	-	+	+	+	-	-	+	-	-	-	+	+	+	-	-	-	-	-	-	+	+	+	+	*B. licheniformis*
67	Bln8g4	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	*B. cereus* group
68	Bln3g1	-	+	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	+	-	+	+	*B. firmus*
69	Bln5g3	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	-	+	+	+	*B. firmus*
70	Bln7g2	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
71	Bln8g5	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	*B.brevis*
72	Bln4g1	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	*B.cereus* group
73	Bln3g5	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	*B.cereus* group
74	Bln6g5	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
75	Bln7g1	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	*B.brevis*

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Blng: **Balangu**

Appendix XXV : Biochemical Responses of Presumptive *B.cereus* Isolates From Ucooked Rice to Microgen Bacillus Identification Kits

Sample no.		Fermentation of sugars at 48 h incubation																				Physiological tests					
		ARA	CEL	INO	MAN	MNS	RAF	RHA	SAL	SOR	SUC	TRE	XYL	ADO	GAL	MDM	MDG	INU	MLZ	IND	ONPG	ARG	CIT	VP	NIT	Isolate
76	R2W2	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B. cereus* group
77	R3B1	+	+	-	+	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	*B.licheniformis*
78	R3W2	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B. cereus* group
79	R4B4	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	*B. brevis*

80	R2B4	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	*B. cereus* group
81	SMR10W1	+	+	+	+	+	-	-	+	+	+	+	+	-	-	-	+	-	-	-	+	+	+	-	+	*B.licheniformis*
82	R1W3	+	+	-	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	+	+	-	+	+	*B.licheniformis*
83	R8B2	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	+	*B. brevis*
84	R1B2	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus group*
85	R1B1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	*B. brevis*
86	R2B1	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	+	*B. brevis*
87	SMR9BR4	-	-	-	+	-	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	*B.licheniformis*
88	R5W1	-	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	*B. firmus*
89	R6B2	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
90	R4W2	-	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	*B. firmus*

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RB: unbranded parboiled rice, RW: unbranded white rice, SMRBR: unbranded samaru parboiled rice.

Appendix XXVI : Biochemical Responses of Presumptive *B.cereus* Isolates From **Soye** to Microgen Bacillus Identification Kits

Sample no.		Fermentation of sugars at 48 h incubation																			Physiological tests					
		ARA	CEL	INO	MAN	MNS	RAF	RHA	SAL	SOR	SUC	TRE	XYL	ADO	GAL	MDM	MDG	INU	MLZ	IND	ONPG	ARG	CIT	VP	NIT	Isolate
91	S2Y6	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
92	S3Y7	-	+	+	+	+	-	+	+	+	-	+	+	+	+	-	-	-	-	-	+	+	-	+	+	*B.licheniformis*
93	S1Y6	-	+	-	+	+	-	-	+	-	+	+	-	-	-	-	+	-	-	-	-	+	+	+	+	*B. laterosporus*
94	S5Y1	-	+	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	-	-	+	+	-	+	+	*B.licheniformis*
95	S2Y2	+	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	-	+	-	+	+	+	+	+	*B.licheniformis*
96	S6Y2	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	-	+	+	*B.licheniformis*

97	S3Y2	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	*B. firmus*
98	S1Y8	-	+	+	+	+	-	+	+	-	-	+	+	+	+	-	-	-	-	+	+	+	+	+	+	*B.licheniformis*
99	S1Y3	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	+	-	+	+	+	+	*B. firmus*
100	S6Y5	+	-	-	-	-	-	-	+	-	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	*B.licheniformis*
101	S4Y1	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
102	S2Y3	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	-	+	-	+	+	*B.cereus* group
103	S4Y2	-	+	-	+	+	-	-	-	+	+	+	-	-	-	-	+	-	-	-	+	-	+	+	+	*B. subtilis*
104	S5Y2	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	+	+	+	+	+	*B. brevis*
105	S4Y3	+	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	-	+	-	+	+	+	+	+	*B.licheniformis*
106	S3Y3	-	+	-	+	+	-	-	+	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	*B. laterosporus*
107	S2Y4	-	+	-	+	+	-	+	+	+	-	+	+	+	+	-	-	-	-	-	+	+	-	+	+	*B.licheniformis*
108	S6Y3	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	*B.licheniformis*
109	S4Y4	-	+	-	+	+	-	-	+	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	*B. laterosporus*

Key: ARA: Arabinose, CEL: Cellobiose, INO: Inositol, MAN: Mannitol, MNS: Mannose, RAF: Raffinose, RHA: SAL: Salicin, SOR: Sorbitol, SUC: Sucrose, TRE: Trehalose, XYL: Xylose, ADO: Adonitol, GAL: Galactose, MDM: Methyl-D-Mannose, MDG: Methyl-D-Glucoside, INU: Inulin, MLZ: Melezitose, IND: Indole prodution, ONPG: Ortho-nitrophenol, ARG: Arginine hydrolysis, CIT: Citrate utilization, VP: Voges-Proskauer test, NIT: Nitrate reduction.
SY: **Soye**

Appendix XXVII : Plates of Some Sampling Points*, Food Samples and Laboratory Assays of *B.cereus* Isolates



Plate VII: Raw meat during sale in an open air market



Plate VIII: Raw meat slices during **Suya** production



Plate IX: Staked meat being coated with spiced groundnut cake



Plate X: Roasting process in **suya** and **balangu**

*Arrows in plates show possible sources of contamination at sampling points.



Plate XI: **Soye** during sale near a bus station

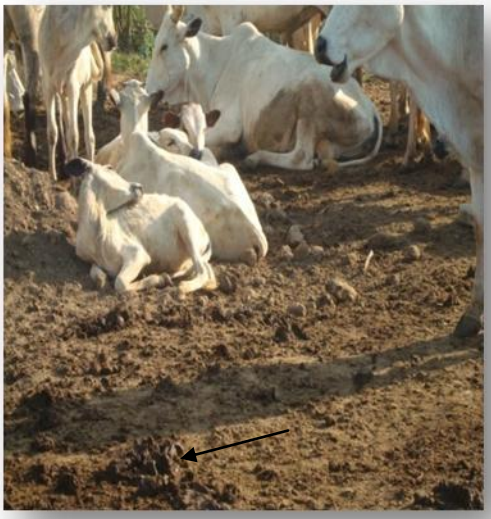


Plate XII: Cattle herd in an outdoor open air housing system, observe the cow dung littering the area (arrow)

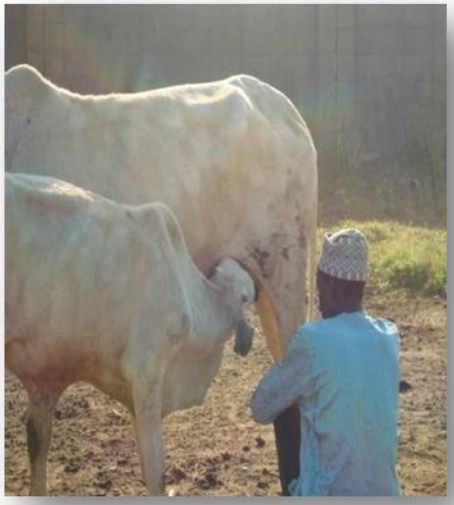


Plate XIII: Initiation of milk flow by calf prior to milking



Plate XIV: Hand milking in process



Plate XV: **Nono** during sale

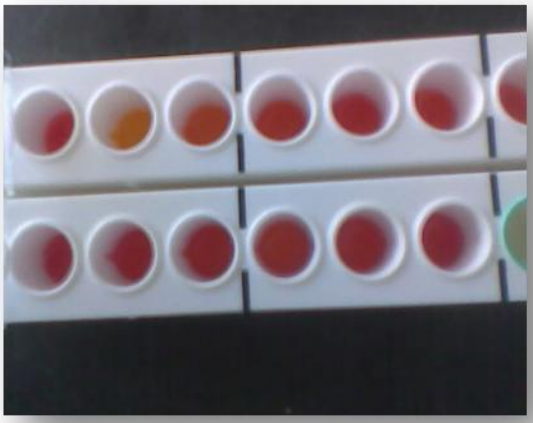




Plate XVI: Carbohydrate fermentation tests showing pink negative wells (left), and yellow positive wells (right) in Microgen biochemical characterization test kits

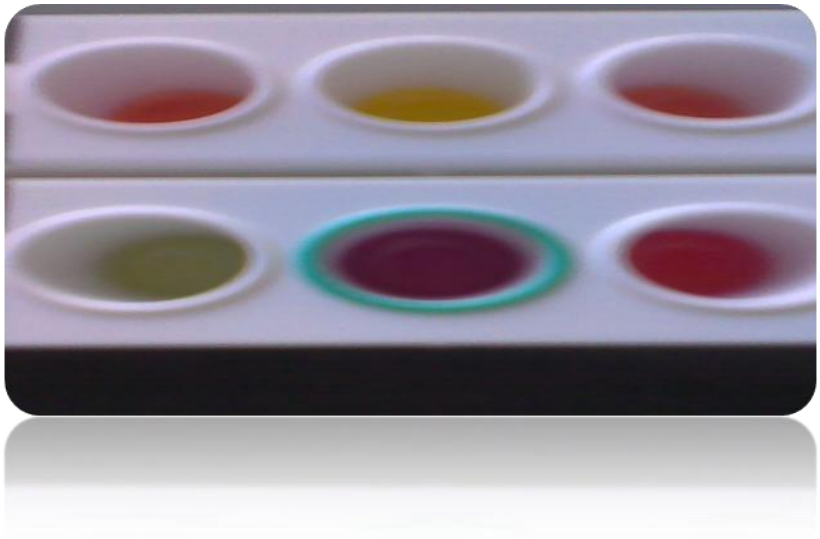


Plate XVII: Microgen test kit showing VP positive pink well (centre) and citrate utilization negative yellow well (extreme left)





Plate XVIII : Boiled rice grits (left) and **balangu** (right) prior to inoculation with *B. cereus* cells

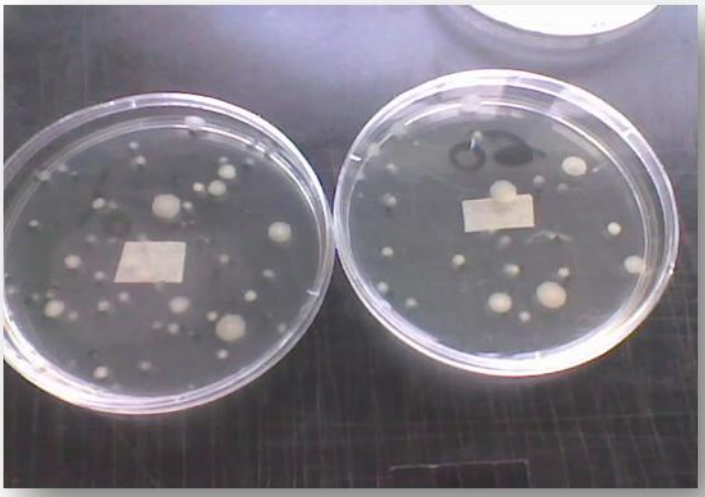


Plate XIX: *B.cereus* cells from **nono** at pH 3.7(left) and 4.3 (right) following 48 h refrigeration.



Plate XX: BCET-RPLA toxin detection kit

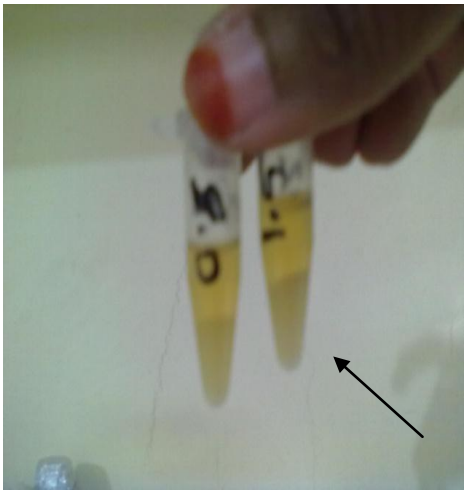


Plate XXI : *B.cereus* cells (arrow) in BHI broth following centrifugation

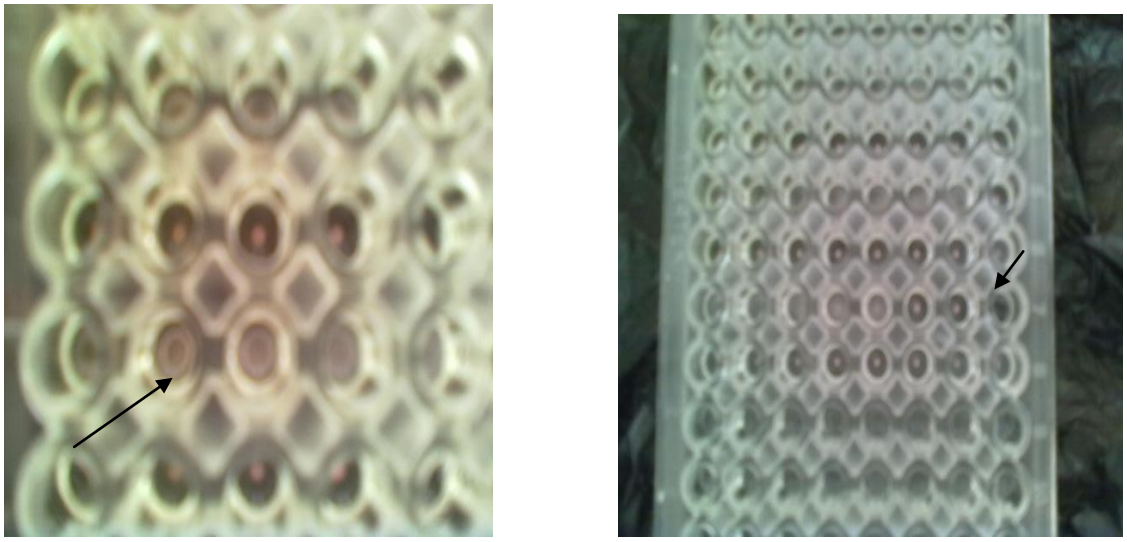


Plate XXII: Microtitre plates showing positive (left arrow) and negative (right arrow) responses of some *B.cereus* isolates to enterotoxin detection test using the BCET-RPLA toxin detection kit.



Plate XXIII: Extracted DNA in PCR tubes before amplification



Plate XXIV: PCR products inoculated into Agarose gell wells

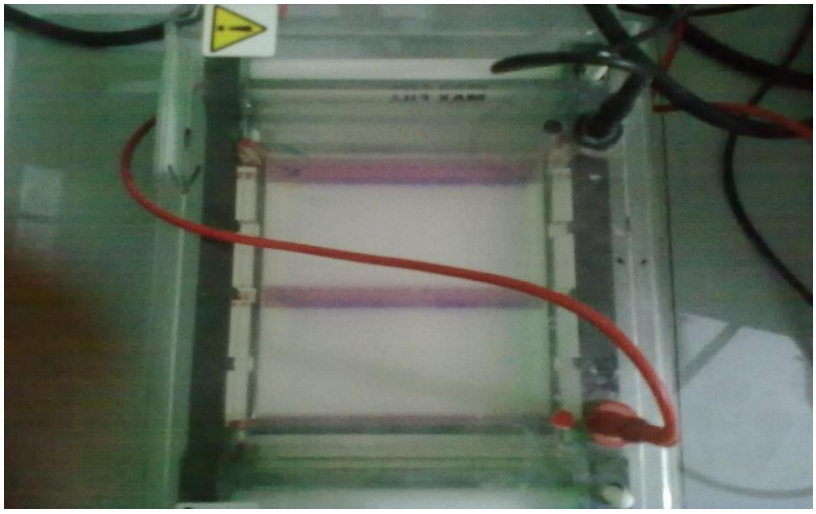


Plate XXV: PCR products in Agarose gell wells during electrophoresis

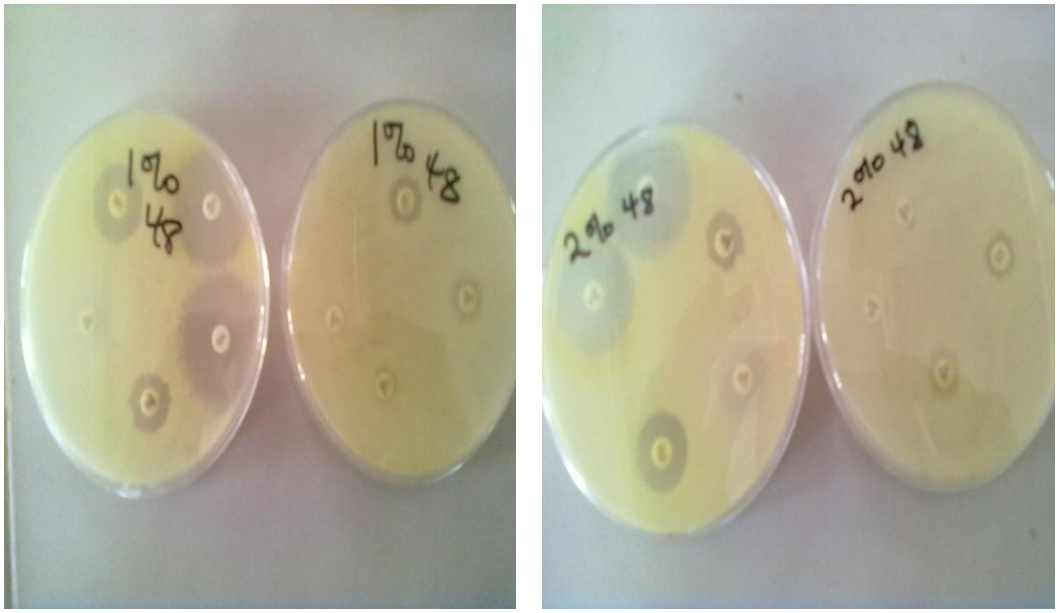


Plate XXVI: Antibacterial sens-itivity responses of *B.cereus* isolates from 48 h refrigerated boiled rice at 1% and 2% NaCl concentrations.

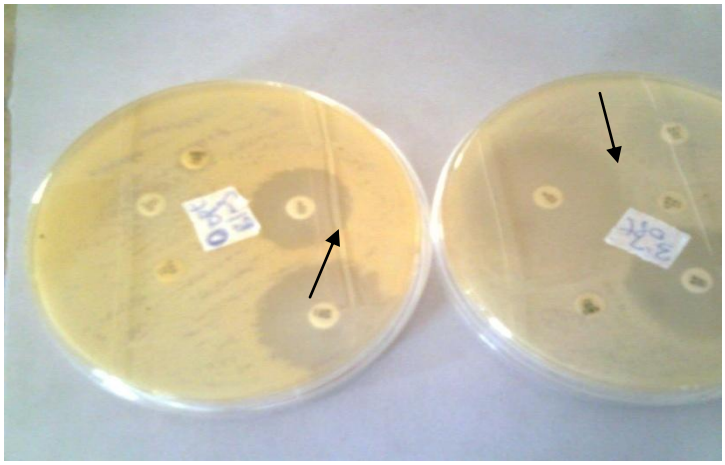


Plate XXVII: *B.cereus* isolates from **balangu** (left arrow) more resistant to Erythromycin than isolates from **nono** (right arrow) under the same storage conditions.

Appendix XXVIII: Statistical analysis of data

Analysis to Compare *Bacillus cereus* Counts from Different Producers of Hawked Roasted Meat (Soye)

The following inferential statistical analysis would be used to compare *Bacillus cereus* **c**ounts across different producers of **soye**. The one-way ANOVA at the 5% level of significance would be employed for each analysis. Hence, we want to investigate whether *Bacillus cereus* **c**ounts are the same from different producers of **soye**. The results of the one-way ANOVA are presented below:

Analysis for *Bacillus cereus* counts from different producers:

H_0: There is no significant difference in the *Bacillus cereus* ***c***ounts from different producers of **soye**

H_1: There is significant difference in the *Bacillus cereus* counts from different producers of **soye**

Results:

Using the two-way ANOVA decision criterion, reject H_0 in each case if $p<0.05$ otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and one-way ANOVA tables:

Table 1: Descriptive Statistics for *Bacillus cereus* counts from different producers of Soye.

					95% Confidence Interval for Mean	
Producers	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound
Producer 1	8	0.83	1.15	0.41	0.00	1.79
Producer 2	7	1.26	1.22	0.46	0.13	2.38
Producer 3	8	0.85	1.18	0.42	0.00	1.84
Producer 4	8	1.11	1.20	0.43	0.11	2.12
Producer 5	7	0.39	0.69	0.26	0.00	1.02
Producer 6	7	0.90	1.12	0.42	0.00	1.94
Total	45	0.89	1.08	0.16	0.57	1.22

The summary table above shows the Descriptive Statistics of the *Bacillus cereus* **c**ounts from different producers of **soye**. The mean plot follows immediately.

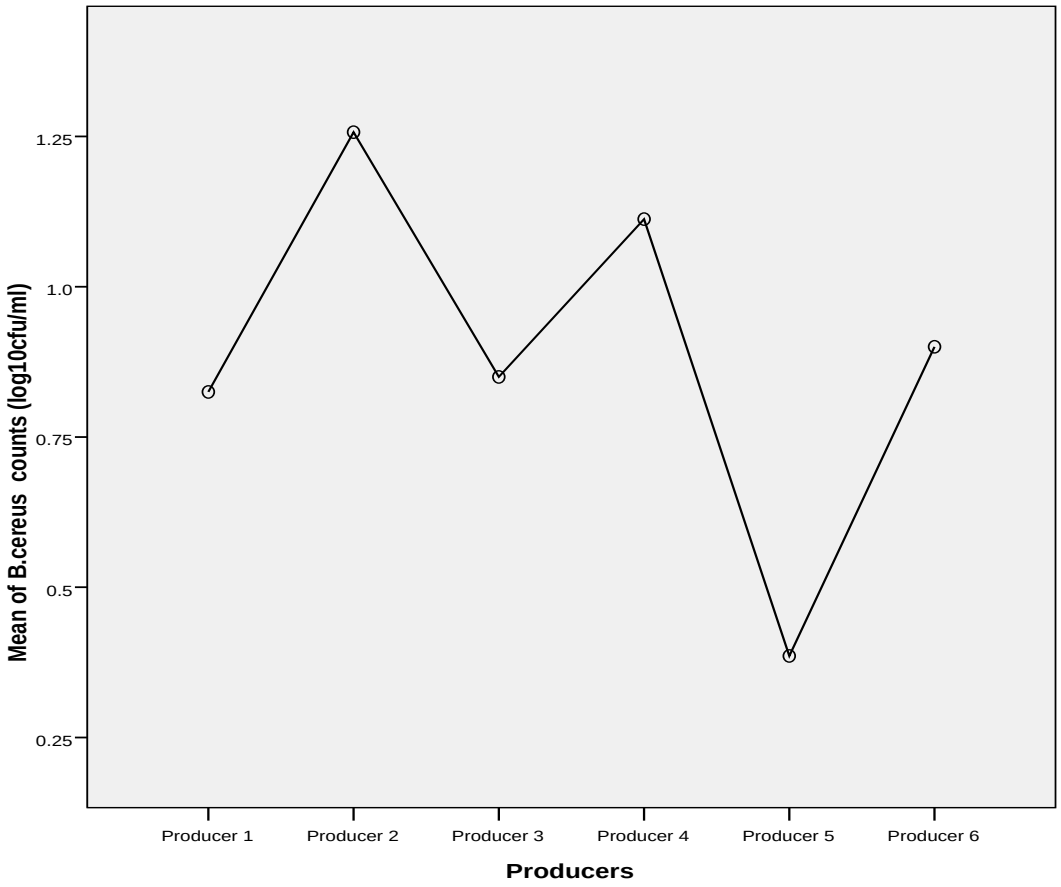


Table 2: ANOVA for different producers of Soye.

Counts	Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Bacillus cereus	Between Groups	3.167	5	0.633	0.509	0.768
	Within Groups	48.549	39	1.245		
	Total	51.716	44			

Results:

From the ANOVA Table above, since *p=0.768>0.05* for *Bacillus cereus* counts, we therefore accept the null hypothesis and conclude that there is no significant difference in the *Bacillus cereus* counts from different producers of **soye**.

Analysis to compare *Bacillus cereus* counts from different producers of uncooked Rice

The following inferential statistical analysis would be used to compare *Bacillus cereus* **c**ounts across different producers of uncooked rice. The one-way ANOVA at the 5% level of significance would be employed for each analysis. Hence, we want to investigate whether *Bacillus cereus* **c**ounts are the same from different products of uncooked rice. The results of the one-way ANOVA are presented below:

Analysis for *Bacillus cereus* counts from different producers:

- **H_0:** There is no significant difference in the *Bacillus cereus* **c**ounts from different producers of uncooked rice
- **H_1:** There is significant difference in the *Bacillus cereus* **c**ounts from different producers of uncooked rice

Results:

Using the two-way ANOVA decision criterion, reject H_0 in each case if $p<0.05$ otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and one-way ANOVA tables:

Table 3: Descriptive Statistics for *Bacillus cereus* counts from different producers of uncooked Rice

					95% Confidence Interval for Mean	
Producers	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound
Producer 1	5	0.96	0.95	0.42	0.00	2.14
Producer 2	5	1.10	1.03	0.46	0.00	2.38
Producer 3	4	0.83	0.96	0.48	0.00	2.35
Producer 4	4	0.70	0.81	0.41	0.00	1.99
Producer 5	4	0.25	0.50	0.25	0.00	1.05
Producer 6	4	0.33	0.65	0.33	0.00	1.36
Producer 7	4	0.00	0.00	0.00	0.00	0.00
Producer 8	4	0.38	0.75	0.38	0.00	1.57
Producer 9	4	0.38	0.75	0.38	0.00	1.57
Producer 10	5	0.20	0.45	0.20	0.00	0.76

Total	43	0.53	0.76	0.12	0.29	0.76

The summary table above shows the Descriptive Statistics of the *Bacillus cereus* **c**ounts from different producers of uncooked rice. The mean plot follows immediately.

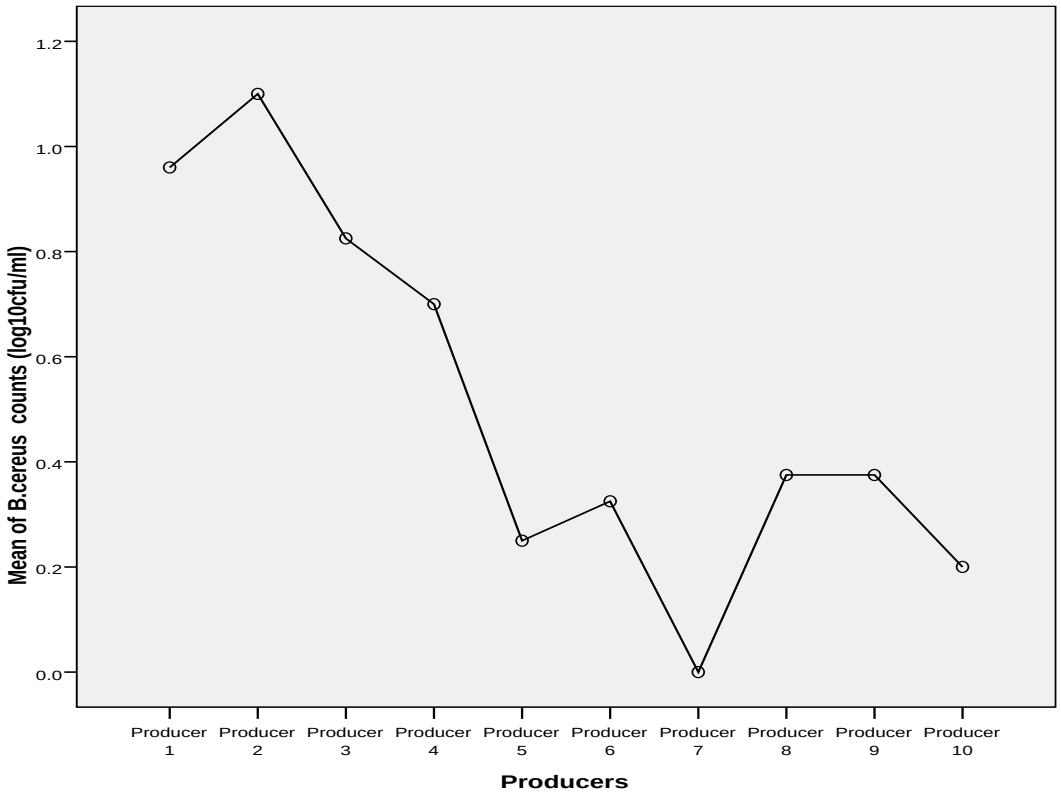


Table 4: ANOVA for different producers of uncooked Rice

Counts	Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Bacillus cereus	Between Groups	5.355	9	0.595	1.045	0.427
	Within Groups	18.792	33	0.569		
	Total	24.147	42			

Results:

From the ANOVA Table above, since *p=0.427>0.05* for *Bacillus cereus* counts, we therefore accept the null hypothesis and conclude that there is no significant difference in the *Bacillus cereus* counts from different producers of uncooked ri

ce.

Analysis to compare *Bacillus cereus* counts from Different Producers of Boiled Rice

The following inferential statistical analysis would be used to compare *Bacillus cereus* counts across different producers of boiled rice. The one-way ANOVA at the 5% level of significance would be employed for each analysis. Hence, we want to investigate whether *Bacillus cereus* **c**ounts are the same from different producers of boiled rice. The results of the one-way ANOVA are presented below:

Analysis for *Bacillus cereus* counts from different producers:

H_0: There is no significant difference in the *Bacillus cereus* counts from different producers of boiled rice

H_1: There is significant difference in the *Bacillus cereus* counts from different producers of boiled rice

Results:

Using the two-way ANOVA decision criterion, reject H_0 in each case if $p<0.05$ otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and one-way ANOVA tables:

Table 5: Descriptive Statistics for *Bacillus cereus* counts from different producers of boiled Rice

					95% Confidence Interval for Mean	
Producers	N	Mean	Std. Deviation	Std. Error	Lower Bound	Upper Bound
Producer 1	4	0.35	0.70	0.35	0.00	1.46
Producer 2	5	0.30	0.67	0.30	0.00	1.13
Producer 3	4	0.35	0.70	0.35	0.00	1.46
Producer 4	4	0.35	0.70	0.35	0.00	1.46
Producer 5	5	0.28	0.63	0.28	0.00	1.06
Producer 6	5	0.50	0.71	0.32	0.00	1.38
Producer 7	4	0.35	0.70	0.35	0.00	1.46
Producer 8	4	0.35	0.70	0.35	0.00	1.46
Producer 9	4	0.35	0.70	0.35	0.00	1.46
Producer 10	4	0.33	0.65	0.33	0.00	1.36
Total	43	0.35	0.61	0.09	0.16	0.54

The summary table above shows the Descriptive Statistics of the *Bacillus cereus* **c**ounts from different producers of boiled rice. The mean plot follows immediately.

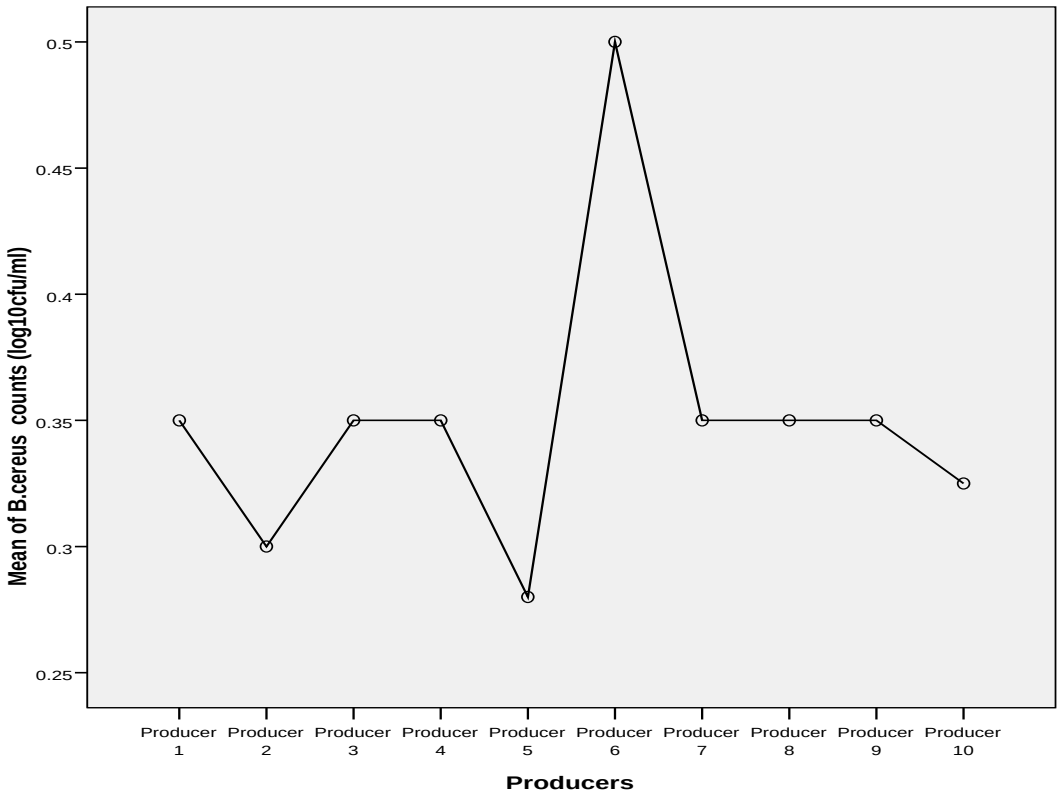


Table 6: ANOVA for different producers of boiled Rice

Counts	Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Bacillus cereus	Between Groups	0.152	9	0.017	0.036	0.999
	Within Groups	15.455	33	0.468		
	Total	15.607	42			

Results:

From the ANOVA Table above, since *p=0.999>0.05* for *Bacillus cereus* counts, we therefore accept the null hypothesis and conclude that there is no significant difference in the *Bacillus cereus* counts from different producers of boiled Rice.

Analysis to compare *Bacillus cereus* counts from different samples of raw (unpasteurized) Milk

The following inferential statistical analysis would be used to compare *Bacillus cereus c*ounts across different samples of raw milk. The one-way ANOVA at the 5% level of significance would be employed for each analysis. Hence, we want to investigate whether *Bacillus cereus* **c**ounts are the same from different producers of raw milk. The results of the one-way ANOVA are presented below:

Analysis for *Bacillus cereus* counts from different producers:

- **H_0:** There is no significant difference in the *Bacillus cereus* **c**ounts from different producers of raw milk
- **H_1:** There is significant difference in the *Bacillus cereus* **c**ounts from different producers of raw milk

Results:

Using the two-way ANOVA decision criterion, reject H_0 in each case if $p<0.05$ otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and one-way ANOVA tables:

Table 7: Descriptive Statistics for *Bacillus cereus* counts from different producers of raw (unpasteurized) Milk

Producers	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Producer 1	4	0.35	0.70	0.35	0.00	1.46
Producer 2	4	0.35	0.70	0.35	0.00	1.46
Producer 3	4	0.35	0.70	0.35	0.00	1.46
Producer 4	4	0.35	0.70	0.35	0.00	1.46
Producer 5	6	0.60	0.67	0.27	0.00	1.30
Producer 6	7	0.36	0.63	0.24	0.00	0.94
Producer 7	5	0.26	0.58	0.26	0.00	0.98
Producer 8	5	0.28	0.63	0.28	0.00	1.06
Producer 9	5	0.56	0.77	0.34	0.00	1.51
Total	44	0.39	0.62	0.09	0.20	0.58

The summary table above shows the Descriptive Statistics of the *Bacillus cereus* Counts from different producers of raw milk. The mean plot follows immediately.

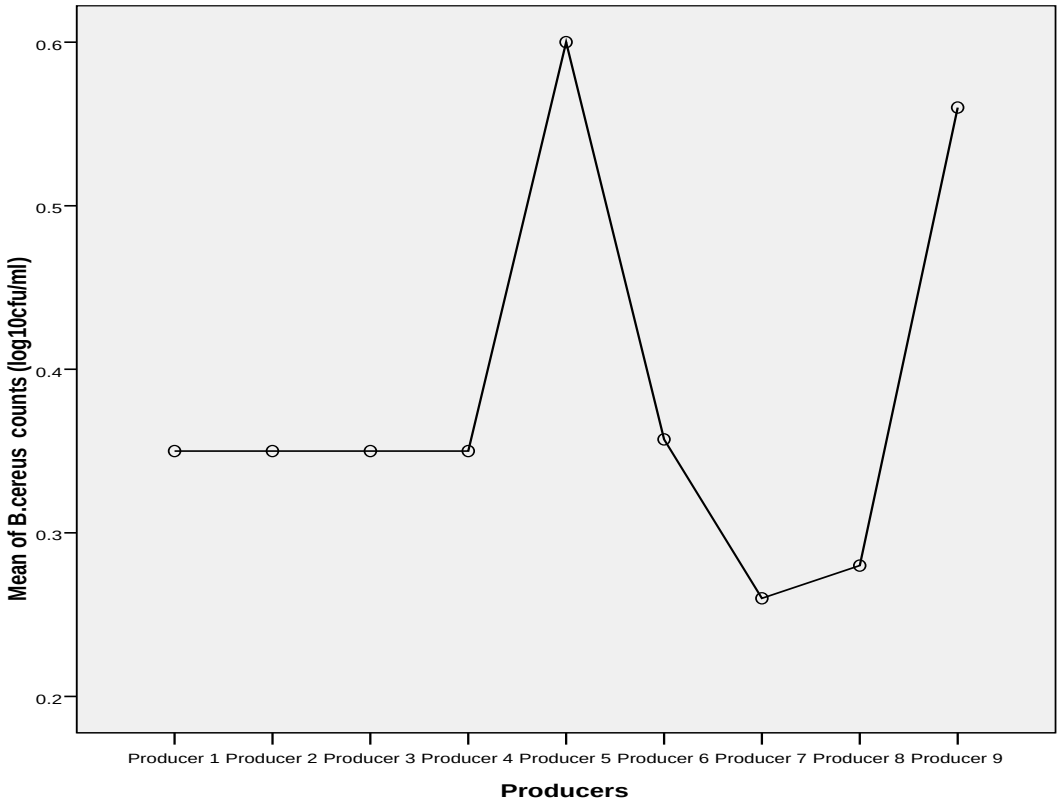


Table 8: ANOVA for different producers of raw (unpasteurized) Milk

Counts	Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Bacillus cereus	Between Groups	0.587	8	0.073	0.163	0.994
	Within Groups	15.729	35	0.449		
	Total	16.316	43			

Results:

From the ANOVA Table above, since *p=0.992>0.05* for *Bacillus cereus* counts, we therefore accept the null hypothesis and conclude that there is no significant difference in the *Bacillus cereus* counts from different producers of raw milk.

Analysis to compare *Bacillus cereus* Counts from Different Producers of Nono

The following inferential statistical analysis would be used to compare *Bacillus cereus* counts across different producers of **nono**. The one-way ANOVA at the 5% level of significance would be employed for each analysis. Hence, we want to investigate whether *Bacillus cereus* counts are the same from different producers of **nono**. The results of the one-way ANOVA are presented below:

Analysis for *Bacillus cereus* counts from Different Producers:

H_0: There is no significant difference in the *Bacillus cereus* counts from different producers of **nono**

H_1: There is significant difference in the *Bacillus cereus* counts from different producers of **nono**

Results:

Using the two-way ANOVA decision criterion, reject H_0 in each case if *p<0.05* otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and one-way ANOVA tables:

Table 9: Descriptive Statistics for *Bacillus cereus* counts from Different Producers of Nono

	N	Mean	Std.	Std. Error	95% Confidence Interval for

Producers			Deviation		Mean	
					Lower Bound	Upper Bound
Producer 1	5	0.64	0.88	0.39	0.00	1.73
Producer 2	5	0.46	0.64	0.29	0.00	1.25
Producer 3	4	1.25	0.84	0.42	0.00	2.59
Producer 4	5	0.32	0.72	0.32	0.00	1.21
Producer 5	5	0.50	0.71	0.32	0.00	1.38
Producer 6	4	0.78	0.92	0.46	0.00	2.24
Producer 7	4	1.08	0.79	0.39	0.00	2.33
Producer 8	4	1.25	0.86	0.43	0.00	2.62
Producer 9	4	0.85	0.99	0.50	0.00	2.43
Producer 10	4	1.30	0.90	0.45	0.00	2.74
Total	44	0.81	0.81	0.12	0.56	1.06

The summary table above shows the Descriptive Statistics of the *Bacillus cereus C*ounts from different producers of **Nono.** The mean plot follows immediately.

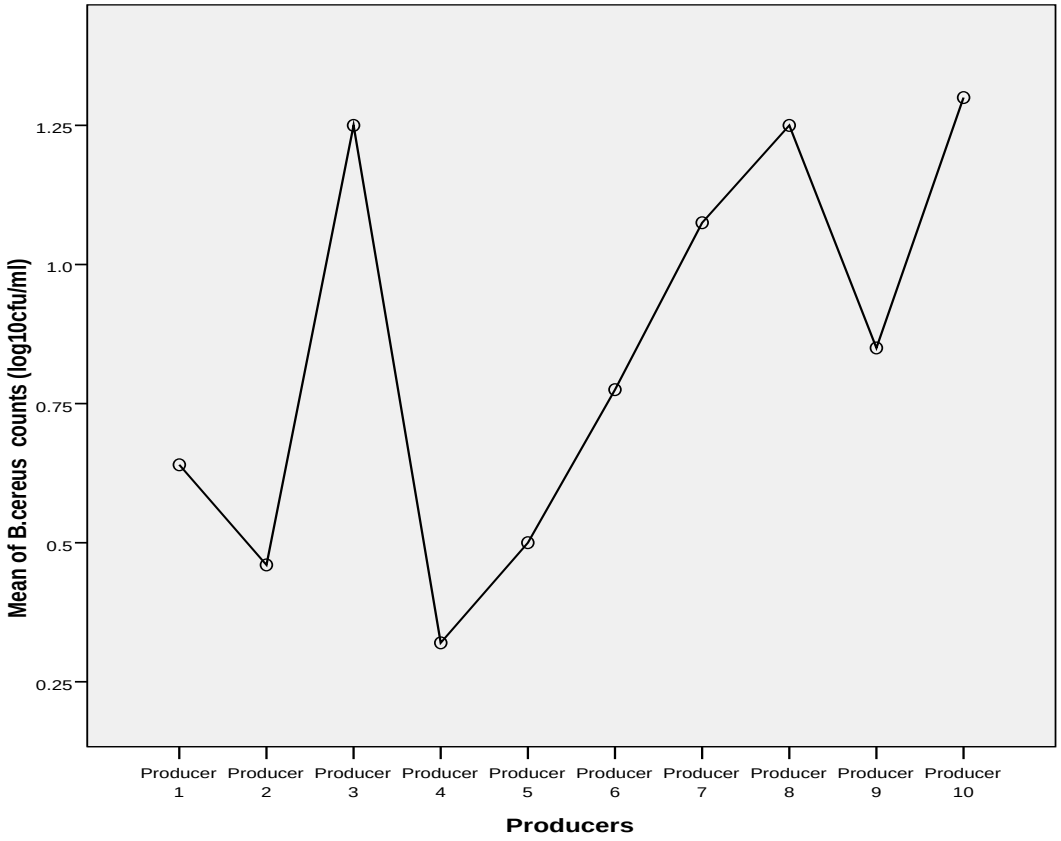


Table 10: ANOVA for Different Producers of nono

Counts	Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Bacillus cereus	Between Groups	5.239	9	0.582	0.864	0.566
	Within Groups	22.917	34	0.674		
	Total	28.156	43			

Results:

From the ANOVA Table above, since p=0.566>0.05 for *Bacillus cereus* counts, we therefore accept the null hypothesis and conclude that there is no significant difference in the *Bacillus cereus* counts from different producers of **nono**

Analysis to compare *Bacillus cereus* Counts in Boiled Rice During Storage at Different Storage Conditions and NaCl Concentrations

The following inferential statistical analysis would be used to compare the *Bacillus cereus* counts across five different storage conditions and five different NaCl concentrations in boiled rice. The two-way ANOVA at the 5% level of significance would be employed for the analysis. Hence, we want to investigate whether *Bacillus cereus* counts at optimum temperature (37^{o}C), room temperature, refrigerated for 24 h, 48 h and 72 h are the same. On the other hand, we want to investigate whether *Bacillus cereus* counts at 0, 0.5, 1, 1.5 and 2% salt concentration are the same. The results of the two-way ANOVA are presented below:

Analysis for Temperatures:

- **H_0:** There is no significant difference in the *Bacillus cereus* counts at different storage conditions in boiled rice
- **H_1:** There is significant difference in the *Bacillus cereus* counts at different storage conditions in boiled rice

Analysis for NaCl Concentrations:

- **H_0:** There is no significant difference in the *Bacillus cereus c*ounts at different NaCl concentrations in boiled rice
- **H_1:** There is significant difference in the *Bacillus cereus c*ounts at different NaCl concentrations in boiled rice

Results:

Using the ANOVA decision criterion, reject H_0 in each case if $p<0.05$ otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and two-way ANOVA tables:

Table 11: Descriptive Statistics for *Bacillus cereus* counts in Boiled Rice at Different Storage Conditions and NaCl Concentrations

Temperature	NaCl concentration (%)	N	Mean	SD
Optimum temperature	0.0%	3	6.63	1.012

	0.5%	3	7.13	0.764
	1.0%	3	5.80	0.300
	1.5%	3	6.37	0.153
	2.0%	3	8.93	0.058
	Total	15	6.97	1.214
Room temperature	0.0%	3	8.53	0.833
	0.5%	3	8.40	1.179
	1.0%	3	9.00	0.954
	1.5%	3	7.07	0.252
	2.0%	3	8.83	0.058
	Total	15	8.37	0.969
Refrigerated for 24 h	0.0%	3	10.27	0.153
	0.5%	3	9.13	0.153
	1.0%	3	9.47	0.473
	1.5%	3	6.27	0.153
	2.0%	3	8.20	1.646
	Total	15	8.67	1.563
Refrigerated for 48 h	0.0%	3	8.03	0.231
	0.5%	3	7.87	0.252
	1.0%	3	8.10	0.608
	1.5%	3	7.63	0.603
	2.0%	3	7.80	0.200
	Total	15	7.89	0.396
Refrigerated for 72 h	0.0%	3	13.07	0.058
	0.5%	3	13.00	0.265
	1.0%	3	12.30	0.000
	1.5%	3	11.83	1.501
	2.0%	3	12.93	0.551
	Total	15	12.63	0.791

The summary table above shows the Descriptive Statistics of the *Bacillus cereus* counts at five different storage conditions and at five different NaCl concentrations in boiled rice.

Table 12: Two-Way ANOVA for Boiled Rice

Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Temperature	284.486	4	71.122	157.256	0.000
NaCl Concentration	23.107	4	5.777	12.773	0.000

Temp * NaCl	33.182	16	2.074	4.585	0.000
Error	22.613	50	0.452		
Total	363.389	74			

Conclusion:

From the Two-Way ANOVA Table above, since *p=0.000<0.05* in all, we therefore reject the null hypothesis in each case and conclude that there is significant difference in the *Bacillus cereus* counts at different storage conditions in boiled rice. On the other hand, there is significant difference in the *Bacillus cereus* counts at different NaCl concentrations in boiled rice. We hereby apply the Duncan Test to investigate real differences between temperature and NaCl concentrations.

Table 13: Duncan Multiple Range Test for the *Bacillus cereus* Counts at Different Storage Conditions in Boiled Rice

		Subsets			
Temperatures	N	1	2	3	4
Optimum temperature	15	6.97			
Refrigerated for 48 h	15		7.89		
Room temperature	15			8.37	
Refrigerated for 24 h	15			8.67	
Refrigerated for 72 h	15				12.63
Means for groups in homogeneous subsets are displayed					

Using the result of the Duncan multiple-range test in the table above, in the first subset, we can deduce that boiled rice at optimum temperature has the least *Bacillus cereus* counts, followed by boiled rice refrigerated for 48 h in the second subset, then boiled rice at room temperature and refrigerated for 24 h in the third subset. In the fourth subset, the highest *Bacillus cereus* counts come from boiled rice refrigerated for 72 h. This is shown by the mean plot below.

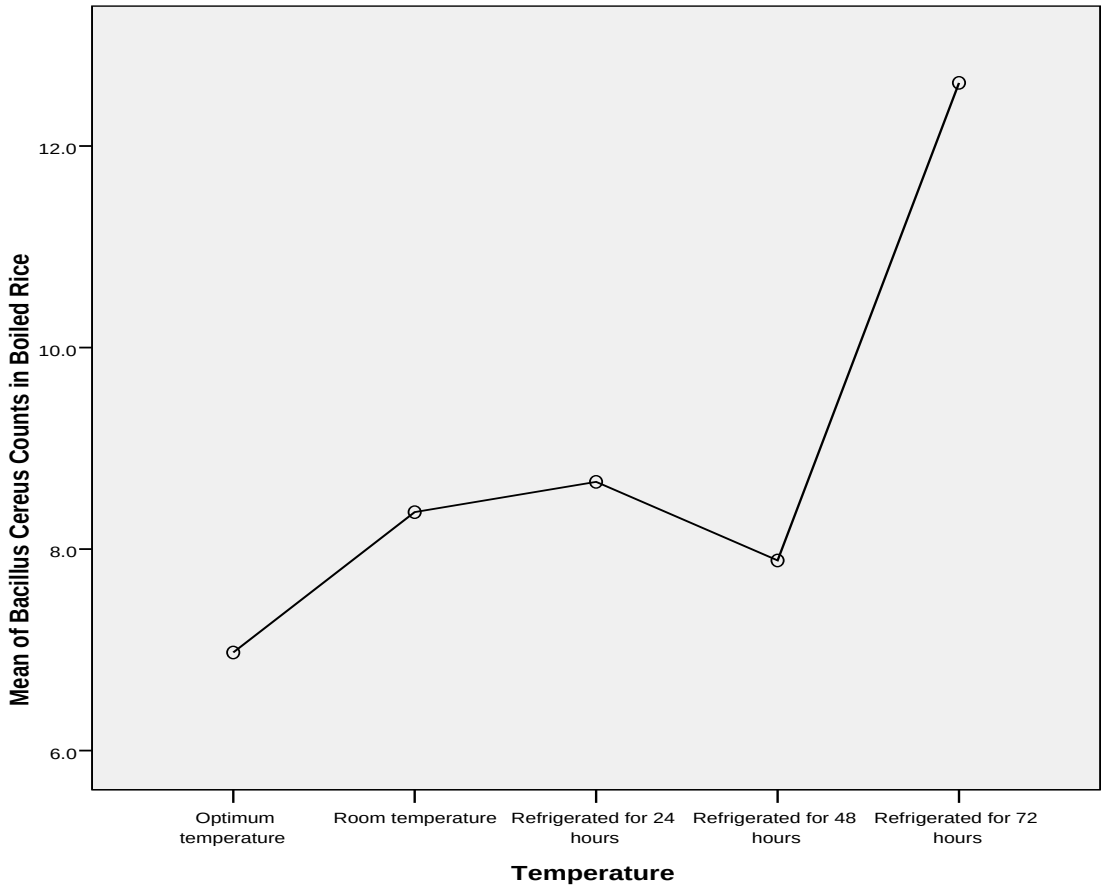
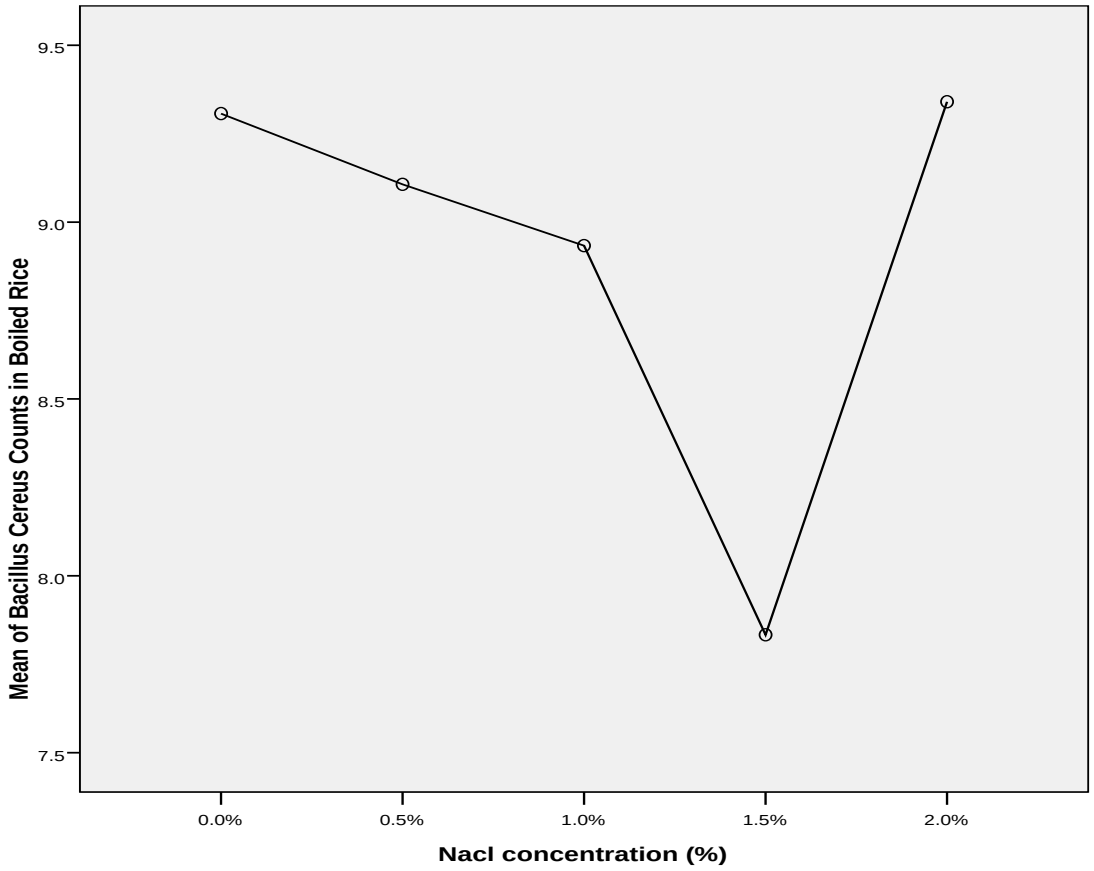


Table 14: Duncan Multiple Range Test for the *Bacillus cereus* counts at different NaCl Concentrations in Boiled Rice

		Subsets	
NaCl Concentrations	N	1	2
1.5%	15	7.83	
1.0%	15		8.93
0.5%	15		9.11
0.0%	15		9.31
2.0%	15		9.34

Similarly, using the result of the Duncan multiple-range test in the table above, in the first subset, we can deduce that boiled rice at 1.5% NaCl concentration has the least *Bacillus cereus* counts. All the other NaCl concentrations fall in the second subset. That is, 1.0, 0.5, 0.0, and 2.0

NaCl concentrations have higher counts and they have the same level of *Bacillus cereus* counts. This is shown by the mean plot below.



Analysis to Compare the *Bacillus cereus* Counts for Different Storage conditions and Different Salt Concentrations in Balangu

The following inferential statistical analysis would be used to compare the *Bacillus cereus* counts across five different storage conditions and five different salt concentrations in **balangu**. The two-way ANOVA at the 5% level of significance would be employed for the analysis. Hence, we want to investigate whether *Bacillus cereus* counts at optimum temperature (37^{o}C), room temperature, refrigerated for 24 h, 48 h and 72 h are the same. On the other hand, we want to

investigate whether *Bacillus cereus* counts at 0, 0.5, 1, 1.5 and 2% salt concentration are the same. The results of the two-way ANOVA are presented below:

Analysis for Temperatures:

H_0: There is no significant difference in the *Bacillus cereus* counts at different Storage conditions in **balangu**

H_1: There is significant difference in the *Bacillus cereus* counts at different Storage conditions in **balangu**

Analysis for NaCl Concentrations:

H_0: There is no significant difference in the *Bacillus cereus* counts at different NaCl concentrations in **balangu**

H_1: There is significant difference in the *Bacillus cereus* counts at different NaCl concentrations in **balangu**

Results:

Using the ANOVA decision criterion, reject H_0 in each case if $p<0.05$ otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and two-way ANOVA tables:

Table 15: Descriptive Statistics for *Bacillus cereus* Counts for Different storage conditions and Different Salt Concentrations in Balangu

Temperature	NaCl concentration (%)	N	Mean	SD
Optimum temperature	0.0%	3	12.93	0.462
	0.5%	3	13.30	0.000
	1.0%	3	13.17	0.058

	1.5%	3	13.30	0.000
	2.0%	3	8.70	0.100
	Total	15	12.28	1.867
Room temperature	0.0%	3	13.20	0.100
	0.5%	3	13.43	0.231
	1.0%	3	14.33	0.153
	1.5%	3	14.27	0.058
	2.0%	3	13.17	0.058
	Total	15	13.68	0.545
Refrigerated for 24 h	0.0%	3	12.50	0.265
	0.5%	3	8.10	0.100
	1.0%	3	12.20	0.100
	1.5%	3	12.33	0.058
	2.0%	3	9.73	0.462
	Total	15	10.97	1.833
Refrigerated for 48 h	0.0%	3	12.47	0.929
	0.5%	3	11.80	0.693
	1.0%	3	11.33	0.058
	1.5%	3	10.20	0.200
	2.0%	3	12.13	1.415
	Total	15	11.59	1.072
Refrigerated for 72 h	0.0%	3	10.67	0.208
	0.5%	3	10.80	0.265
	1.0%	3	10.93	0.252
	1.5%	3	9.47	0.231
	2.0%	3	9.80	0.361
	Total	15	10.33	0.649

The summary table above shows the Descriptive Statistics of the *Bacillus cereus* counts at five different storage conditions and at five different NaCl concentrations in **balangu**.

Table 16: Two-Way ANOVA for balangu

Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Temperature	99.607	4	24.902	142.242	0.000
Nacl Concentration	29.405	4	7.351	41.991	0.000

Temp * NaCl	83.830	16	5.239	29.928	0.000
Error	8.753	50	0.175		
Total	221.595	74			

Conclusion:

From the Two-Way ANOVA Table above, since *p=0.000<0.05* in all, we therefore reject the null hypothesis in each case and conclude that there is significant difference in the *Bacillus cereus* counts at different storage conditions in **balangu**. On the other hand, there is significant difference in the *Bacillus cereus* counts at different NaCl concentrations in **balangu**. We hereby apply the Duncan Test to investigate real differences between temperature and NaCl concentrations.

Table 17: Duncan Multiple Range Test for the *Bacillus cereus* counts at different storage conditions in Balangu

		Subsets				
Temperatures	N	1	2	3	4	5
Refrigerated for 72 h	15	10.33				
Refrigerated for 24 h	15		10.97			
Refrigerated for 48 h	15			11.59		
Optimum temperature	15				12.28	
Room temperature	15					13.68
Means for groups in homogeneous subsets are displayed						

Using the result of the Duncan multiple-range test in the table above, in the first subset, we can deduce that **balangu** refrigerated for 72 h at has the least *Bacillus cereus* counts, followed by **balangu** refrigerated for 24 h in the second subset, then **balangu** refrigerated for 48 h in the third subset. In the fourth subset, we have **balangu** kept at optimum temperature. Again, in the fifth subset, the highest *Bacillus cereus* counts come from **balangu** at room temperature. This is shown by the mean plot below.

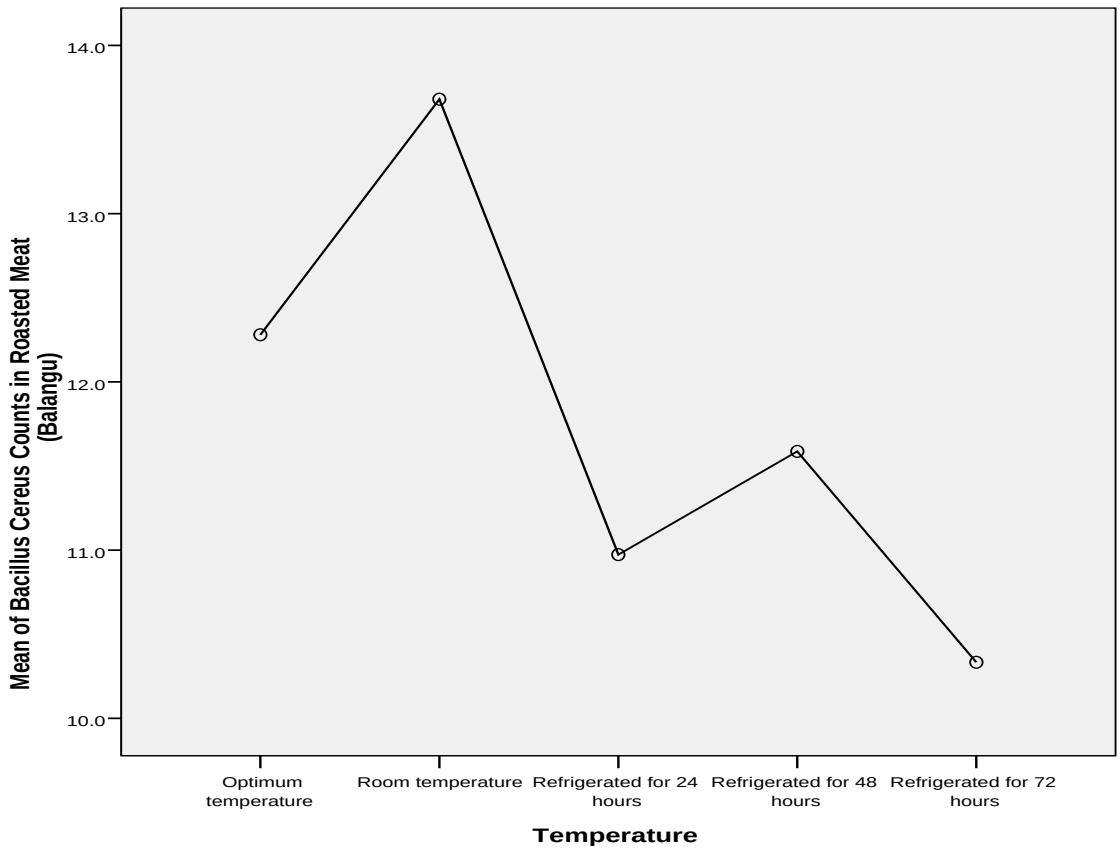
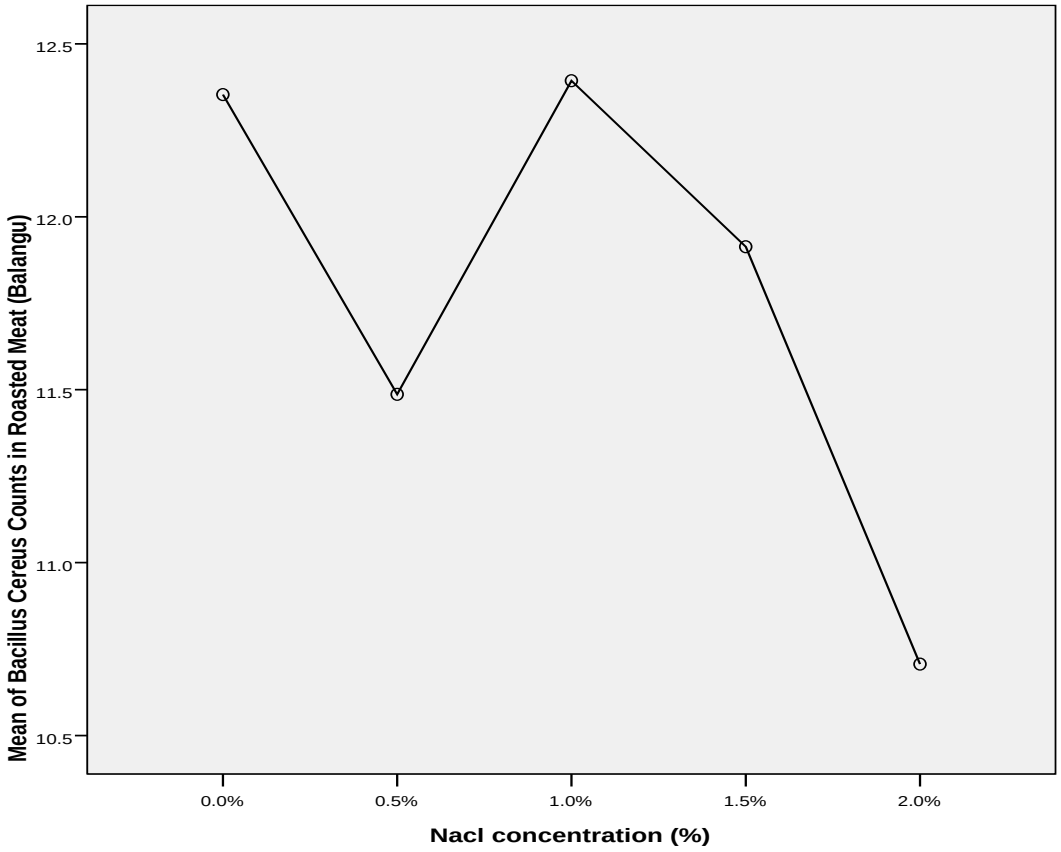


Table 18: Duncan Multiple Range Test for the *Bacillus cereus* Counts at different NaCl Concentrations in Balangu

		Subsets			
NaCl Concentrations	N	1	2	3	4
2.0%	15	10.71			
0.5%	15		11.49		
1.5%	15			11.91	
0.0%	15				12.35
1.0%	15				12.39

Similarly, using the result of the Duncan multiple-range test in the table above, in the first subset, we can deduce that **balangu** at 2.0% NaCl concentration has the least *Bacillus cereus* counts, followed by **balangu** at 0.5% NaCl concentration in the second subset, then **balangu** at 1.5% NaCl concentration in the third subset. In the fourth subset, we have **balangu** kept at 0.0

and 1.0% NaCl concentrations with the highest *Bacillus cereus* counts. This is shown by the mean plot below.



Analysis to Compare the *Bacillus cereus* Counts for Different Storage Conditions and Different pH Values in Nono

The following inferential statistical analysis would be used to compare the *Bacillus cereus* counts across five different storage conditions and four different pH values in **nono**. The two-way ANOVA at the 5% level of significance would be employed for the analysis. Hence, we want to investigate whether *Bacillus cereus* counts at optimum temperature (37^{o}C), room temperature, refrigerated for 24 h, 48 h and 72 h are the same. On the other hand, we want to investigate

whether *Bacillus cereus* counts at 3.7, 3.8, 4.2 and 4.3 pH values are the same. The results of the two-way ANOVA are presented below:

Analysis for storage conditions in Nono:

- **H_0:** There is no significant difference in the *Bacillus cereus* counts at different Storage conditions in **nono**
- **H_1:** There is significant difference in the *Bacillus cereus* counts at different Storage conditions in **nono**

Analysis for pH Values in Nono:

- **H_0:** There is no significant difference in the *Bacillus cereus* counts at different pH values in **nono**
- **H_1:** There is significant difference in the *Bacillus cereus* counts at different pH values in **nono**

Results:

Using the ANOVA decision criterion, reject H_0 in each case if $p<0.05$ otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and two-way ANOVA tables:

Table 19: Descriptive Statistics for *Bacillus cereus* counts for Different storage conditions and Different pH Values in Nono

Temperature	pH Values	N	Mean	SD
Optimum temperature	pH 3.7	3	9.30	0.346
	pH 3.8	3	9.57	0.404
	pH 4.2	3	1.50	0.200
	pH 4.3	3	8.10	0.265
	Total	12	7.12	3.446
Room temperature	pH 3.7	3	9.40	0.265
	pH 3.8	3	9.70	1.735
	pH 4.2	3	1.50	0.436
	pH 4.3	3	9.33	0.603
	Total	12	7.48	3.701
Refrigerated for 24 h	pH 3.7	3	7.40	0.300
	pH 3.8	3	7.63	0.723
	pH 4.2	3	6.93	0.321
	PH 4.3	3	7.63	0.814
	Total	12	7.40	0.583
Refrigerated for 48 h	pH 3.7	3	6.97	0.153
	pH 3.8	3	6.33	0.115
	pH 4.2	3	6.67	0.306
	pH 4.3	3	6.40	0.200
	Total	12	6.59	0.315
Refrigerated for 72 h	pH 3.7	3	6.17	0.586
	pH 3.8	3	6.43	0.252
	pH 4.2	3	7.17	0.321
	pH 4.3	3	6.97	0.493
	Total	12	6.68	0.559

The summary table above shows the Descriptive Statistics of the *Bacillus cereus* counts at five different storage conditions and at four different pH values in **Nono**.

Table 20: Two-Way ANOVA for Nono

Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Temperature	7.909	4	1.977	6.247	0.001
PH	106.423	3	35.474	112.083	0.000
Temp * PH	170.516	12	14.210	44.896	0.000
Error	12.660	40	0.317		
Total	297.508	59			

Conclusion:

From the Two-Way ANOVA Table above, since *p=0.001* and *p=0.000 <0.05* in all, we therefore reject the null hypothesis in each case and conclude that there is significant difference in the *Bacillus cereus c*ounts at different storage conditions in **nono**. On the other hand, there is significant difference in the *Bacillus cereus* counts at different pH values in **nono**.

Table 21: Duncan Multiple Range Test for the *Bacillus cereus* counts at different storage conditions in Nono

		Subsets		
Temperatures	N	1	2	3
Refrigerated for 48 h	12	6.59		
Refrigerated for 72 h	12	6.68		
Optimum temperature	12		7.12	
Refrigerated for 24 h	12			7.40
Room temperature	12			7.48
Means for groups in homogeneous subsets are displayed				

Using the result of the Duncan multiple-range test in the table above, in the first subset, we can deduce that **nono** refrigerated at for 48 and 72 h had the least *Bacillus cereus* counts. Again, in the third subset, the highest *Bacillus cereus* counts come from **nono** refrigerated for 24 h and same, kept at room temperature. This is shown by the mean plot below.

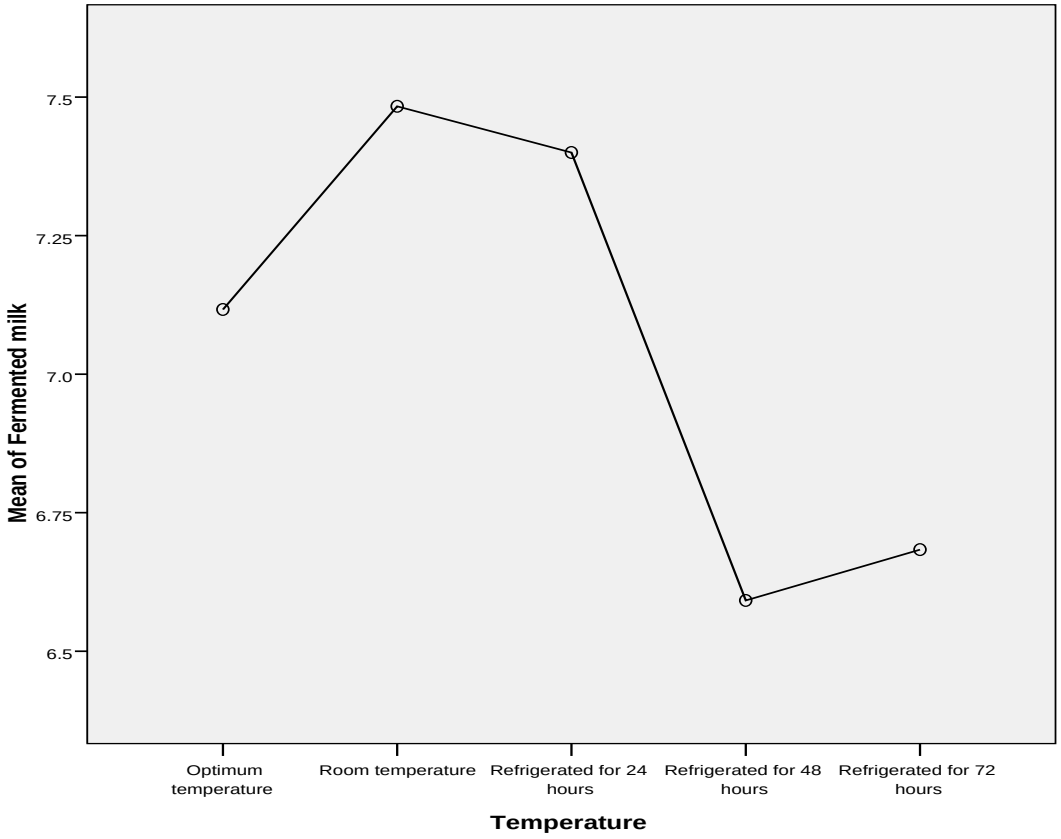
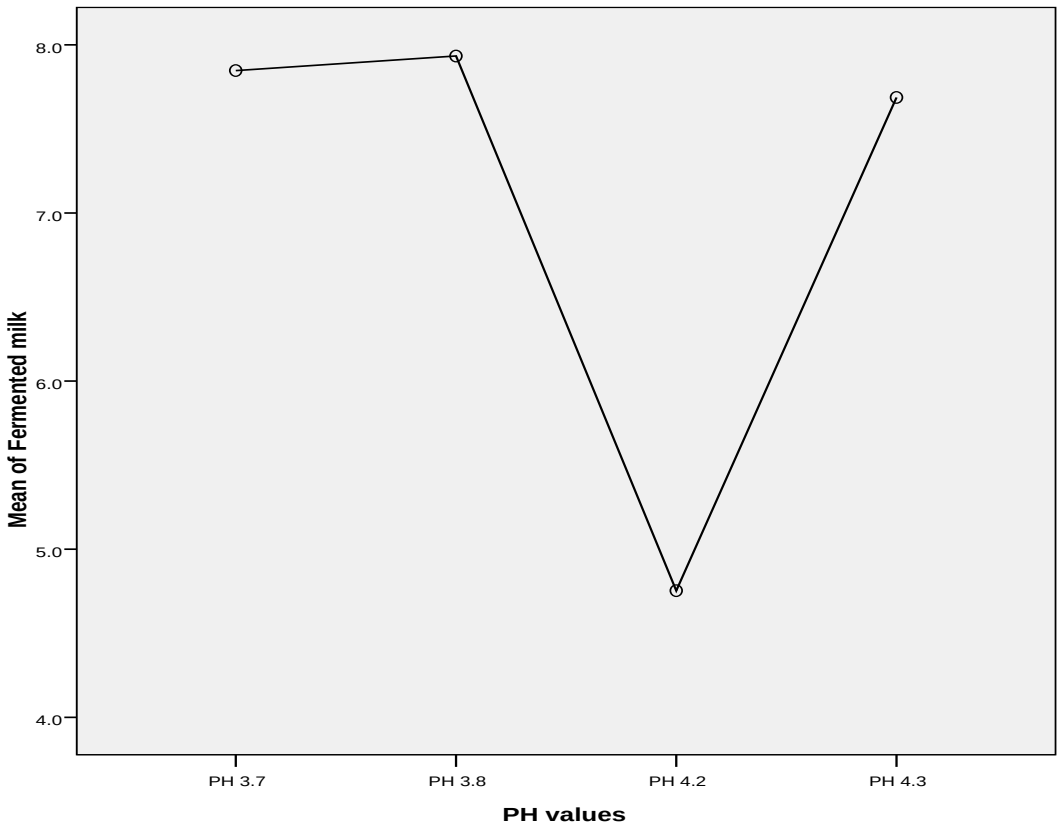


Table 22: Duncan Multiple Range Test for the *Bacillus cereus* counts at different pH values in Nono

		Subsets	
pH Values	N	1	2
pH 4.2	15	4.75	
pH 4.3	15		7.69
pH 3.7	15		7.85
pH 3.8	15		7.93

Similarly, using the result of the Duncan multiple-range test in the table above, in the first subset, we can deduce that **Nono** at pH of 4.2 has relatively less *Bacillus cereus* counts. In the second subset, **Nono** at pH of 4.3, 3.7 and 3.8 had higher *Bacillus cereus* counts. This is shown by the mean plot below.



Analysis to Compare the Toxin Titres of *Bacillus cereus in* Boiled Rice at Different Storage conditions.

The following inferential statistical analysis would be used to compare the Toxin titre of *Bacillus cereus* across five different storage conditions in boiled rice. The analysis of covariance ANCOVA at the 5% level of significance would be employed for the particular analysis. Hence, we want to investigate whether *Bacillus cereus* counts and the Toxin titres at optimum temperature (37^{o}C), room temperature, refrigerated for 24 h, 48 h and 72 h are the same or not.

Analysis for Toxin Titre:

- **H_0:** There is no significant difference in the Toxin titre of boiled rice at different storage conditions
- **H_1:** There is significant difference in the Toxin titre of boiled rice at different storage conditions

Analysis for *Bacillus cereus* counts with Toxin Titre as Covariate:

H_0: There is no significant difference in the *Bacillus cereus* counts of boiled rice at different storage conditions regardless of the status of Toxin titre

H_1: There is significant difference in the *Bacillus cereus* counts of boiled rice at different storage conditions regardless of the status of Toxin titre

Results:

Using the ANCOVA decision criterion, reject H_0 in each case if $p<0.05$ otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and ANCOVA tables:

Table 23: Descriptive Statistics of Toxin Titre in Boiled Rice at Different storage conditions

Toxin Titre	N	Mean	Std. Dev.	Std. Error
Optimum temperature for 24 h	5	64.00	0.00	0.00
Room temperature for 24 h	5	64.00	0.00	0.00
Refrigerated for 24 h	5	64.00	0.00	0.00
Refrigerated for 48 h	5	57.60	14.31	6.40
Refrigerated for 72 h	5	38.40	14.31	6.40
Total	25	57.60	13.06	2.61

Table 24: Descriptive Statistics of *Bacillus cereus* counts in Boiled Rice at Different storage conditions

Bacillus cereus counts	N	Mean	Std. Dev.	Std. Error
Optimum temperature for 24 h	5	6.96	1.18	0.53
Room temperature for 24 h	5	8.34	0.74	0.33
Refrigerated for 24 h	5	8.70	1.54	0.69
Refrigerated for 48 h	5	7.88	0.19	0.09
Refrigerated for 72 h	5	12.62	0.55	0.25
Total	25	8.90	2.18	0.44

Table 25: Toxin Titre ANCOVA for Boiled Rice

Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	94.982	5	18.996	19.324	0.000
Toxin Titre	0.002	1	0.002	0.002	0.962
Bacillus cereus	47.372	4	11.843	12.047	0.000
Error	18.678	19	0.983		
Total	113.660	24			

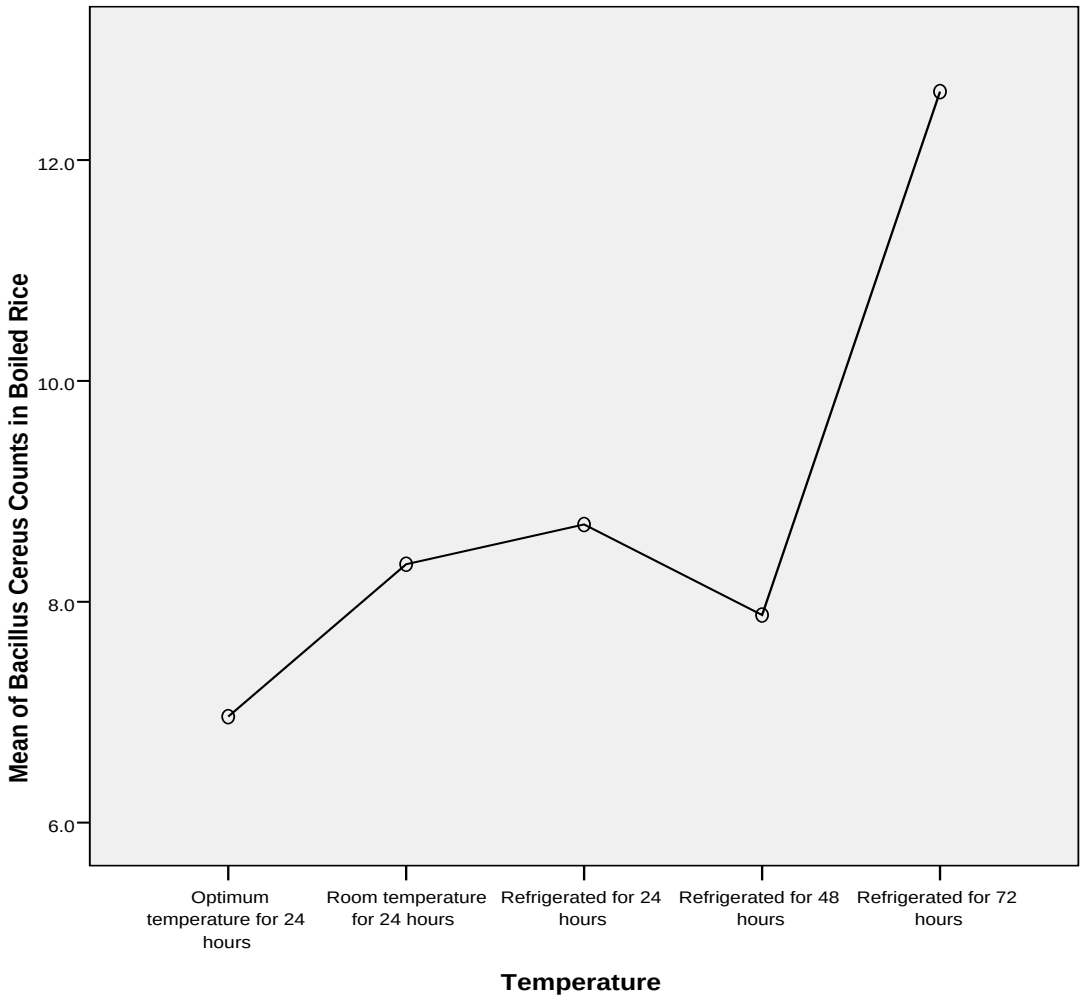
Conclusion:

The descriptive statistics were displayed in the descriptive tables above. From the ANCOVA Table above, since *p=0.962>0.05* for Toxin Titre, we therefore accept the null hypothesis for Toxin Titre and conclude that there is no significant difference in the Toxin titre of boiled rice at different storage conditions. On the other hand, since *p=0.00<0.05* for *Bacillus cereus* counts; we therefore reject the null hypothesis for *Bacillus cereus* counts and conclude that there is significant difference in the *Bacillus cereus* counts of boiled rice at different conditions regardless of what Toxin titre might be. We hereby apply the Duncan multiple-range test to investigate underlying differences between *Bacillus cereus* counts at various conditions with Toxin Titre as covariate.

Table 26: Duncan Multiple Range Test for the *Bacillus cereus* Counts at Different storage conditions in Boiled Rice with Toxin Titre held Constant

		Subsets		
Temperatures	N	1	2	3
Optimum temperature for 24 h	5	6.96		
Refrigerated for 48 h	5	7.88		
Room temperature for 24 h	5		8.34	
Refrigerated for 24 h	5		8.70	
Refrigerated for 72 h	5			12.62
Means for groups in homogeneous subsets are displayed				

Using the result of the Duncan multiple-range test in the table above, in the first subset, we can deduce that boiled rice at optimum temperature and boiled rice refrigerated for 48 h have the least *Bacillus cereus* counts, followed by boiled rice at room temperature and refrigerated for 24 h in the second subset, then boiled rice at refrigeration for 72 h in the third subset. This is shown by the mean plot below.



Analysis to Compare the Toxin Titre of *Bacillus cereus* in Balangu at Different Storage Conditions

The following inferential statistical analysis would be used to compare the Toxin titre as well as the *Bacillus cereus* counts across five different storage conditions in **balangu**. In other words, we also want to compare the *Bacillus cereus* counts using the Toxin titre as covariate. The analysis of covariance ANCOVA at the 5% level of significance would be employed for the particular analysis. Hence, we want to investigate whether *Bacillus cereus* counts and the Toxin titre at optimum temperature (37^{o}C), room temperature, refrigerated for 24 h, 48 h and 72 h are the same or not. We also want to compare the *Bacillus cereus* counts across five different storage conditions while holding Toxin titre (covariate) constant. The results of the ANCOVA are presented below:

Analysis for toxin titre:

- **H_0:** There is no significant difference in the Toxin titre of **balangu** at different storage conditions
- **H_1:** There is significant difference in the Toxin titre of **balangu** at different conditions

Analysis for *Bacillus cereus* counts with toxin titre as covariate:

- **H_0:** There is no significant difference in the *Bacillus cereus* counts of **balangu** at different storage conditions regardless of the status of Toxin titre
- **H_1:** There is significant difference in the *Bacillus cereus* counts of **balangu** at different storage conditions regardless of the status of Toxin titre

Results:

Using the ANCOVA decision criterion, reject H_0 in each case if $p<0.05$ otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and ANCOVA tables:

Table 20: Descriptive Statistics of Toxin Titre in Balangu at Different storage conditions

Toxin Titre	N	Mean	Std. Dev.	Std. Error
Optimum temperature for 24 h	5	57.60	14.31	6.40
Room temperature for 24 h	5	35.20	28.62	12.80
Refrigerated for 24 h	5	57.60	14.31	6.40
Refrigerated for 48 h	5	39.20	25.36	11.34
Refrigerated for 72 h	5	51.20	17.53	7.84
Total	25	48.16	21.31	4.26

Table 21: Descriptive Statistics of *Bacillus cereus* counts in Balangu at different storage conditions

Bacillus Cereus Counts	N	Mean	Std. Dev.	Std. Error
Optimum temperature for 24 h	5	12.22	1.97	0.88
Room temperature for 24 h	5	13.66	0.55	0.24
Refrigerated for 24 h	5	10.96	1.97	0.88
Refrigerated for 48 h	5	11.58	0.89	0.40
Refrigerated for 72 h	5	10.32	0.62	0.28
Total	25	11.75	1.71	0.34

Table 22: Toxin Titre ANCOVA for Balangu

Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	37.703	5	7.541	4.466	0.007
Toxin Titre	4.868	1	4.868	2.883	0.106
Bacillus Cereus	37.445	4	9.361	5.544	0.004
Error	32.080	19	1.688		
Total	69.782	24			

Conclusion:

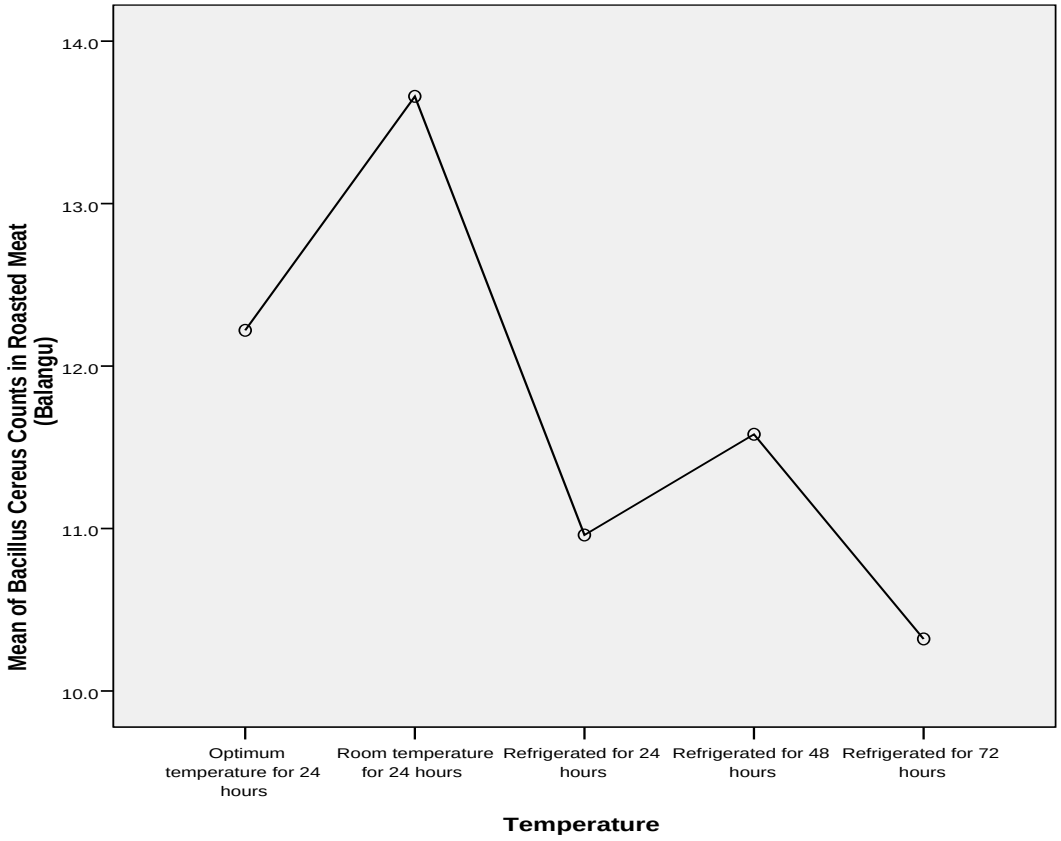
The descriptive statistics were displayed in the descriptive tables above. From the ANCOVA Table above, since *p=0.106>0.05* for Toxin Titre, we therefore accept the null hypothesis for Toxin Titre and conclude that there is no significant difference in the Toxin titre of **balangu** at different storage conditions. On the other hand, since *p=0.007<0.05* for *Bacillus cereus* counts; we therefore reject the null hypothesis for *Bacillus cereus* counts and conclude that there is

significant difference in the *Bacillus cereus c*ounts of **balangu** at different storage conditions regardless of what Toxin titre might be. We hereby apply the Duncan multiple-range test to investigate underlying differences between *Bacillus cereus* counts at various conditions Toxin Titre as covariate.

Table 23: Duncan Multiple Range Test for the *Bacillus cereus* counts at different storage conditions in Balangu with Toxin Titre held Constant

		Subsets	
Temperatures	N	1	2
Refrigerated for 72 h	5	10.32	
Refrigerated for 24 h	5	10.96	
Refrigerated for 48 h	5	11.58	
Optimum temperature for 24 h	5		12.22
Room temperature for 24 h	5		13.66
Means for groups in homogeneous subsets are displayed			

Using the result of the Duncan multiple-range test in the table above, in the first subset, we can deduce that **balangu** Refrigerated for 72 h, Refrigerated for 48 h and **balangu** Refrigerated for 24 h have the least *Bacillus cereus* counts, followed by **balangu** at room temperature and **balangu** at optimum temperature in the second subset. This is shown by the mean plot below.


Mean of Bacillus Cereus Counts in Roasted Meat (Balangu)
14.0
13.0
12.0
11.0
10.0
Optimum temperature for 24 hours
Room temperature for 24 hours
Refrigerated for 24 hours
Refrigerated for 48 hours
Refrigerated for 72 hours
Temperature

Analysis to Compare the Toxin Titre of *Bacillus cereus* in Pasteurized Milk at Different Storage Periods

The following inferential statistical analysis would be used to compare the Toxin titre as well as the *Bacillus cereus* counts across three different storage periods in pasteurized milk. The analysis of covariance ANCOVA at the 5% level of significance would be employed for this particular analysis. Hence, we want to investigate whether *Bacillus cereus* counts and the Toxin titres of *Bacillus cereus* from pasteurized milk refrigerated for 24 h, 48 h and 72 h are the same or not. We also want to compare the *Bacillus cereus* counts across the three different storage periods while holding Toxin titre (covariate) constant. The results of the ANCOVA are presented below:

Analysis for Toxin titre:

- **H_0:** There is no significant difference in the Toxin titre of pasteurized milk at different storage periods
- **H_1:** There is significant difference in the Toxin titre of pasteurized milk at different storage periods

Analysis for *Bacillus cereus* counts with Toxin titre as covariate:

- **H_0:** There is no significant difference in the *Bacillus cereus* counts of pasteurized milk at different storage periods regardless of the status of Toxin titre
- **H_1:** There is significant difference in the Bacillus Cereus Counts of pasteurized milk at different storage periods regardless of the status of Toxin titre

Results:

Using the ANCOVA decision criterion, reject H_0 in each case if $p<0.05$ otherwise accept H_0 at the 5% level of significance. The computations are obtained using the SPSS as employed herein for data analysis using the following descriptive and ANCOVA tables:

Table 24: Descriptive Statistics of Toxin Titre in Pasteurized Milk at Different storage periods

Toxin titre	N	Mean	Std. Dev.	Std. Error
Stored at refrigeration for 24 h	4	33.00	24.52	12.26
Stored at refrigeration for 48 h	4	19.00	30.18	15.09
Stored at refrigeration for 72 h	4	7.00	6.63	3.32
Total	12	19.67	23.40	6.75

Table 25: Descriptive Statistics of *Bacillus cereus* counts in Pasteurized Milk at Different storage periods

Bacillus cereus counts	N	Mean	Std. Dev.	Std. Error
Stored at refrigeration for 24 h	4	8.10	0.34	0.17
Stored at refrigeration for 48 h	4	7.73	0.99	0.50
Stored at refrigeration for 72 h	4	8.00	0.29	0.15
Total	12	7.94	0.59	0.17

Table 26: Toxin Titre ANCOVA for Pasteurized Milk

Sources of variation	Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.325	3	0.442	1.399	0.312
Toxin Titre	1.023	1	1.023	3.241	0.109
Bacillus cereus	0.386	2	0.193	0.612	0.566
Error	2.525	8	0.316		
Total	3.849	11			

Conclusion:

The descriptive statistics were displayed in the descriptive tables above. From the ANCOVA Table above, since *p=0.109>0.05* for Toxin Titre, we therefore accept the null hypothesis for Toxin Titre and conclude that there is no significant difference in the Toxin titre of *Bacillus cereus* from pasteurized

milk at different storage periods. Similarly, since *p=0.312>0.05* for *Bacillus cereus* counts; we also accept the null hypothesis for *Bacillus cereus* counts and conclude that there is no

significant difference in the *Bacillus cereus* counts of pasteurized milk at different storage periods regardless of what Toxin titre might be. This is shown by the mean plot below.

