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Antibiotic susceptibility and selected toxin genes profiles among *Bacillus cereus* isolated from raw bovine milk from a milk Collection Center in Kawempe division, Kampala district.

BY

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Declaration

I, **BARIYANGA Jean Damascene** declare that this dissertation is my original compilation and that none of its section(s) is/are plagiarized. I further declare that this dissertation has never been submitted to any university for the award of any degree.

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Dedication

To my beloved wife Mrs Patricie Munganyinka, daughter Irene Isingizwe and my son Guillaume Niyubahwe for their patience and encouragement during the entire study. To my late grandmother Thacienne Nyiranzamuye (RIP) who catered for my childhood. To uncle Nsekanabo Joel for financial support.

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List of abbreviations

BHI	Brain heart infusion
ces	Cereulide synthetase
CFU	Colony-forming unit
CLSI	Clinical Laboratory Standards Institute
COVAB	College of Veterinary Medicine, Animal Resources and Biosecurity
Cyt-K	Cytotoxicity- K
DNA	Deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
F/R	Forward/Reverse
Hbl	Hemolysin bl
HCl	Hydrochloride
KB	Kirby-Bauer
KCCA	Kampala Capital Council Authority
MAAIF	Ministry of Agriculture, Animal Industries and Fisheries
MHA	Mueller Hinton Agar
MCC	Milk collection centre
mM	Milli-molar
NaOH	Sodium hydroxide
Nhe	Non-Hemolytic enterotoxins
PEMBA	Polymyxin-egg yolk mannitol-bromothymol agar
pH	Potential in hydrogen
qPCR	Quantitative Polymerase Chain Reaction
RT-PCR	Real time-Polymerase Chain Reaction
sl	*Sensu lato*
STs	Sequence types
TE	Tris- ethylenediaminetetraacetic acid
UBOS	Uganda Bureau of Statistics
UK	United Kingdom
UNBS	Uganda National Bureau of Standards
UNCST	Uganda National Council for Science and Technology

USA	United States of America

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Abstract

Bacillus cereus causes food intoxication hence a burden to public health. *Bacillus cereus* is also among the top five foodborne pathogens, especially in starchy foods and raw cow milk. The pathogenicity of the *B. cereus* is associated with the potential to produce diarrheal and emetic toxins. The pathogen has been associated with clinical infections, whose management could be challenged by antimicrobial resistance. There is scanty information on the contamination levels of raw bovine milk with *B. cereus*, its toxigenic potential, and its susceptibility to antibiotics in Uganda. This study aimed to determine the proportion of raw bovine milk contaminated with *B. cereus* sold within Kawempe division, Kampala as well as its toxigenic potential and antibiotic susceptibility profiles. In the present study, 30 raw bovine milk samples were collected from a milk collection centre in the Kawempe division, Kampala . From the samples where growth occurred, five isolates were selected for further characterization. Isolates were identified and confirmed using gram-staining, citrate, catalase, oxidase and motility. Antibiotic susceptibility profiles were determined by the Kirby-Bauer technique, while multiplex real-time PCR allowed the detection of toxigenic genes (*hblD*, *nheA*, *cytK 1* and *ces*). Of the 30 milk samples, eight (26.7%) were contaminated with *B. cereus*. All the 40 isolates that were selected for antibiotic sensitivity testing, exhibited susceptibility to gentamycin, tetracycline, chloramphenicol and ciprofloxacin, but all were resistant to ß-lactam products including Ampicillin and Penicillin.
The *hblD*, *nheA*, *cytK1* and *ces* were encountered in 82.5 % (33/40), 85 % (34/40), 0%, and 0% of the *B. cereus* isolates respectively. Contamination of milk with *B. cereus* and the presence of the diarrheal toxin genes, *hblD* and *nheA*, implies that there is a potential risk of associated food-borne illnesses, especially where raw bovine milk is consumed. The *cyt*K1, a diarrheal toxin-encoding gene was absent. In this research, the *ces* was absent hence no potential for emesis. Thus, there is a need for wider surveillance of contamination of especially the raw bovine milk.

CHAPTER ONE | INTRODUCTION

1.1 Background

Bacillus cereus is a Gram-positive, facultative aerobic, rod-shaped, and spore-forming bacterium and is common in soil and raw plants. It is frequently associated with either raw or processed starchy foods, such as rice and other foods like soy, beef; milk, as well as dairy products (Khudor et al., 2012; Rodrigo et al., 2021). The bacteria are commonly associated with foodborne illnesses but have also been incriminated as nosocomial pathogens and in a multitude of other clinical conditions (Bottone, 2010). Food poisoning as a result of *B. cereus* contamination may manifest itself in two different ways, that is, emetic and diarrheic syndromes (Griffiths & Schraft, 2017).

Among the foodborne outbreaks caused by microorganisms, *Bacillus cereus'* contribution is estimated to be 1.4%–12% worldwide (Grutsch et al., 2018). In the European Union, it is reported as the second most important cause, probably due to the severity of the associated symptoms (Jovanovic et al., 2021). Previous studies reported that the incidence of diarrheal or emetic outbreaks may be influenced by country-specific dietary habits, with the emetic syndrome occurring in Japan or Great Britain, while the diarrheal syndrome is slightly more prevalent in Europe or the United States of America (Dietrich, Jessberger, Ehling-schulz, et al., 2021; Jessberger, 2018; Jessberger et al., 2020).

In Africa, i.e., in Sudan, data concerning *B. cereus* was reported together with other *Bacillus spp*. to be responsible for milk spoilage (Reem & Basit, 2011), while in Ethiopia, the prevalence of milk contaminated with *B. cereus* was 15.4% (Kassa et al., 2017). In Rwanda, in the study on subclinical mastitis, the raw milk samples used were contaminated with other bacteria and the contribution to the contamination was 10.3%(Mpatswenumugabo et al., 2017). In a study done in Kawempe division Kateregga et al. (2019) reported that 52% of the unpasteurized milk sold within different locations of Kawempe were contaminated with *Salmonella*. Moreover, in Uganda, a study on bacterial contamination in soured milk was done and revealed that 89.1% of the samples showed significant bacterial contamination. The isolated microorganisms were *E. coli* (47.1%), *Klebsiella* spp (28.4%) *Shigella* Spp (11.6%), *Salmonella* Spp (7.1%) and *Enterobacteria faecalis* (5.8%) (Kamurasi et al., 2018). In the greater Luwero, 70% of the raw bovine milk samples were contaminated with *Listeria monocytogenes* 70% of the samples involved in the study (Mukasa et al., 2016).

Various environmental and temperature signals stimulate the production of several distinct toxins, which in addition to sporulation ability and resistance to some antibiotics, contribute to the virulence of *B. cereus* (Shawish & Tarabees, 2017). Furthermore, resistance to antibiotics and disinfectants is of clinical significance and may be the reason for a higher contamination rate by *B. cereus* compared to other foodborne pathogens in foodstuffs including dairy products (Saeed et al., 2021; Zhao et al., 2020). Beta-lactamases production by bacteria from different food items (including pasteurized milk, frozen food, and ready-to-eat food) may lead to antibiotic resistance to ß-lactam-containing antibiotics as reported in China, Thailand and South Korea (Gao et al., 2018; Guo et al., 2021; Sornchuer & Tiengtip, 2021; Yim et al., 2015).

The emetic syndrome is caused by a heat-stable toxin, cereulide encoded on the *ces* gene locus and is mostly produced before the bacteria are ingested, which may complicate diagnosis based on microbial detection. Cereulide is also resistant to extreme pH conditions and proteases. For diarrheal syndrome, *B. cereus* toxins such as non-hemolytic enterotoxin (*Nhe*), hemolysin BL (*Hbl*), cytotoxic K-1,2 (*CytK*) encoded by *nheABC, hblCDA, cytK-1* and *cytk-2*, respectively, are often involved in food poisoning (Alipour & Mahdavi, 2020*;* Glasset et al., 2021*;* Granum & Ma, 2013*;* Khudor et al., 2012). Diarrhoea occurs after ingestion of live *B. cereus* that eventually multiplies within the intestines and produces enterotoxins (Jessberger et al., 2020).

In Uganda, fragmented studies on the prevalence of foodborne pathogens in different foodstuffs and/or antibiotic susceptibility profiles have been conducted, but there is no systematic surveillance. Pathogens commonly considered include *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, and *Listeria monocytogenes* (Baluka et al., 2015; Mukasa et al., 2016). Kasozi and others (2016) reported *B. cereus* contamination in 100% of the analyzed dry-salted fish obtained from the West Nile region of Uganda. However, information on the occurrence of foodborne *B. cereus* and the toxin encoding genes they harbour is still scanty, yet it is important for the management of clinical cases, food safety and hygiene. Hence, ascertaining the availability of safe raw bovine milk will lead to the consumption of essential nutrients and will result in healthy consumers. The milk lacks safety if contaminated with *B. cereus* with toxigenic potential together with the antimicrobial resistance profile.

1.2 Problem statement

Milk for consumption should be free from pathogens like *B. cereus* due to its role in foodborne outbreaks, its contribution is estimated to be 1.4%–12% worldwide raising concerns about consumer safety who may suffer from foodborne diseases affecting people as a result of

foodborne bacteria. Moreover, commonly isolated bacteria including *E. coli* (47.1%), *Klebsiella* spp (28.4%), *Shigella* spp (11.6%), *Salmonella* spp (11.6%), and *Enterobacteria faecalis* were reported to contaminate soured milk in Uganda (Kamurasi et al., 2018), but the contribution of any kind of milk contamination with *B. cereus* in Uganda was not well reported, and this bacterium is known for its toxigenic potential of causing foodborne syndromes like diarrheal and emetic. The milk contamination may lead to poor safety that can cause milk rejection, hence the loss of milk rejection by the milk collection centre, causing the economic losses to the farmers and influencing food insecurity. Moreover, cooking can denature the toxigenic genes but the cereulide which is emetic toxin is resistant to high temperatures, pH and proteases. These put consumers at risk due to the consumption of unsafe food. There is a lack of systematic surveillance for the *B. cereus* contamination of raw bovine milk in Uganda. Fragmented research of food contamination concentrated on some bacteria but little on *Bacillus cereus.* Hence, there is a paucity of information on the proportion of raw bovine milk contaminated with *B. cereus* in Uganda, as well as on toxin genes possessed by these microorganisms. There is also a lack of data on antibiotic susceptibility profiles of *B. cereus* yet they are of clinical significance in addition to being foodborne pathogens. I determined the proportion of raw bovine milk contaminated with B. *cereus*, tested for the isolates' antibiotic susceptibility and established its toxin gene profiles to illustrate the milk contamination caused by *B. cereus*.

1.3 General objective

To determine the proportion of raw bovine milk contaminated with *B. cereus* as well as the isolates' toxigenic potential and antibiotic susceptibility profiles from a milk collection Centre in Kawempe, Kampala district.

1.4 Specific objectives

1. To determine the proportion of raw bovine milk contaminated with *B. cereus* from Kawempe division, Kampala district.
2. To determine the antibiotic susceptibility profiles of *B. cereus* isolated from raw bovine milk from Kawempe division, Kampala district.
3. To establish selected toxin encoding gene profiles in *B. cereus* isolated from raw bovine milk from Kawempe division, Kampala district.

1.5 Research questions

1. What is the proportion of raw bovine milk contaminated with *B. cereus* from Kawempe division, Kampala district?
2. What are the antibiotic susceptibility profiles of *B. cereus* isolated from raw bovine milk sold within Kawempe division, Division, Kampala district?
3. What is the frequency of selected toxin genes possessed by presumptive *B. cereus* isolated from raw bovine milk sold within Kawempe division, Kampala district?

1.6 Study justification

In Uganda, no reports on milk contamination with *B. cereus*, its toxigenic potential and its antibiotic susceptibility profile. Researchers reported in Kenya that milk contaminated with *B. cereus* harboring the toxin genes in 1999. In Rwanda, other bacteria with *B. cereus* contaminated the bacteria together with *B. cereus* in subclinical mastitis with the same other in 2017, which led to milk spoilage. But, in Uganda, this microorganism caused the contamination of dried fish in 2016. Milk contamination causes foodborne diseases like diarrhea and emesis influenced by *B. cereus* contamination and toxin genes. This study intended to determine the proportion of raw bovine milk contaminated with *B. cereus,* the isolates' toxigenic potential and antibiotic susceptibility profiles from a milk collection Centre in Kawempe division, Kampala, to inform on the milk safety sold to the public.

1.7 Study significance

This study is the first to report on the contamination of raw bovine milk by *B. cereus* together with their food poisoning potential and antimicrobial susceptibility in Uganda. The data will help the scientist and academicians to go further on other gaps shown from by this report.

This is particularly important for the management of the safety of milk from Uganda. The results generated from this study form a basis for guiding personnel along the milk value chain. The report will also guide the policymakers to ensure the safety of raw bovine milk for human consumption and the entire dairy industry.

1.8 Conceptual framework

The conceptual framework (Fig. 1) shows the association between milk safety concerning the presence of the enterotoxins produced by *B. cereus*. Moreover, the antibiotic susceptibility exhibited by the *B. cereus* also contributes to milk safety.

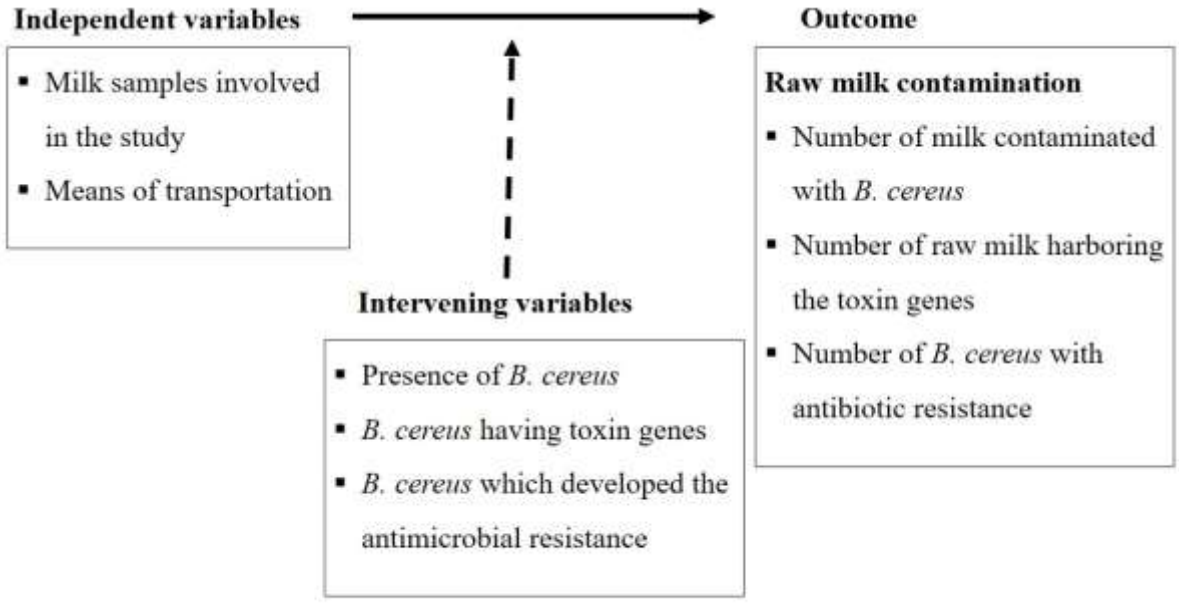

Figure 1: Conceptual framework

CHAPTER TWO | LITERATURE REVIEW

2.1 Prevalence of *B. cereus* and its toxin encoding genes in various foodstuffs

Bacillus cereus is a Gram-positive bacterium that harbours emetic and diarrheic genes. These bacteria may also express genes which are not toxic (Gdoura et al., 2019; Chica et al., 2021). Some of the *B. cereus* generate the emetic toxin cereulide, which may lead to fatal infections (Elise et al., 2019).

Bacillus cereus is of global public health concern with the potential to cause food intoxications that are associated with emetic and diarrheal disease syndromes (Abdeen et al (2020). *Bacillus cereus* was listed in different previous studies as number one together with *Listeria monocytogenes* and *Staphylococcus aureus* which were reported to cause food poisoning outbreaks and are often implicated in ready-to-eat food (Batchoun et al., 2011; Hwang & Park, 2015; Wu et al., 2016; Yang et al., 2016). *Bacillus cereus* was considered the 2nd and 3rd most serious foodborne pathogen in France and Europe respectively (Glasset et al., 2018, 2021). In France, from 2007-2014, *B. cereus* outbreaks of foodborne illnesses due to contamination levels of 10^5 CFU/g in implicated foods occurred (Glasset et al., 2016). New Zealand has more strict regulatory limits set at 10^2–10^3 CFU/g which was the acceptable level while in CFU/g is $\geq 10^4$ as potentially hazardous as reported by (Yu et al., 2019) and (NSW, 2009). In A review by Rodrigo et al. (2021) revealed that 94 of 173 rice samples in the United States of America were contaminated with *B. cereus*.

In Rwanda, *Bacillus* spp. were reported to contribute 10.3% of the raw milk contamination (Mpatswenumugabo et al., 2017) . The proportions of *B. cereus* isolates contamination in milk and Ras-cheese collected from local markets in Menoufia Governorate, Egypt was found to be 6.9% and 8.5%, respectively (Abdeen et al., 2020). The *nheA* gene is reported to be the most common enterotoxin encoding gene in the *B. cereus* isolates from different sources (Arslan et al., 2014; Chica et al., 2020; Glasset et al., 2016; Chica et al., 2021; Sornchuer & Tiengtip, 2021). Furthermore, the genes including *cytK*, *hblD*, *hbl* and *ces* were detected in 55.5%, 33.3%, 33.3%, and 22.2% of *B. cereus* isolated from the milk powder and ras-cheese respectively (Abdeen et al., 2020).

In Africa, *B. cereus* contamination levels as high as 4.5×10^4 CFU/ml were found in the slurry in Ouagadougou, in Burkina Faso; and toxin genes, *Hbl* and *Nhe*, were detected in 72% and 38% of the isolates respectively (Humblot et al., 2012). In Tunisia, toxin genes were encountered in *B. cereus* from contaminated foodstuffs including cereals, and included *nheA*,

nheC, *nheB*, *hblC*, *hblD*, *hblA* and *hblB* at 98.9%, 97.7%, 86.8%, 54.6%, 54.6%, 29.9% and 14.9%, respectively. In Tunisia, the gene coding for *Nhe* toxins among *B. cereus* isolated from different foodstuffs including dairy products was more commonly encountered than that for *hbl* toxins (Gdoura et al., 2019). In Zambia, reports of *B. cereus* together with *Bacillus anthracis* contributing to the death of humans exist (Ogawa et al., 2015). In Sudan, *B. cereus* was found to be responsible for milk spoilage in 11% of all samples tested during a study conducted by Reem & Basit (2011). The proportion of raw milk contaminated with *B. cereus* was found to be 15.4% in Ethiopia (Kassa et al., 2017). The proportion of dry-salted Pebbly fish contaminated with *B. cereus* was 100% in the West Nile of Uganda (Kasozi et al., 2016). Information on raw milk contamination status with *B. cereus* in Uganda was not encountered.

2.2 Virulence of *B. cereus*

Bacillus cereus has highly variable virulence, with some strains being mild and others being harmful (Elise et al., 2019). This inconsistency makes it problematic to report the severity of *B. cereus* and frequently leads to confusion and mishandling of the related risks (Ramarao et al., 2020). However, the description of *B. cereus'* virulence potential is the main problem for the agro-food industries and hospitals (Awadelkarim & Suleiman, 2014; Reem & Basit, 2011; Thorsen et al., 2011).

Virulent *B. cereus* strains can produce varying types and quantities of toxins. The toxins contain degradation enzymes, cytotoxic factors, haemolysins, proteases, and cell-surface proteins (Ramarao et al., 2020). The sporulation can also contribute to the virulence of *B. cereus.* Gastrointestinal factors such as temperature, atmospheric composition, oxidation-reduction potential, pH, agitation consistency, carbohydrate availability, anions and cation concentrations can also contribute to the virulence of *B. cereus* (Ceuppens et al., 2011). Consequently, consumption of foods containing very high numbers of *B. cereus* (*sensu lento*), i.e., above 10^5 CFU/g, may lead to diarrhoeal illness in humans within a period of four to 16 hours (Germany Federal Institute of risk assessment, 2020).

2.3 Antibiotic susceptibility of *B. cereus*

Bacillus cereus is of clinical significance, hence determining its susceptibility to antimicrobial agents is critical for the management of cases and outbreaks (Forghani et al., 2014; Ikeda et al., 2015). A high number of the strains are sensitive to aminoglycosides, gentamicin, chloramphenicol, erythromycin, and ciprofloxacin (Nga Ombede et al., 2020; Sornchuer &

Tiengtip, 2021). Moreover, some strains of *B. cereus* with resistance to tetracycline and clindamycin were reported (Yim et al., 2015; Zhang et al., 2017).

Some strains of *Bacillus cereus* are susceptible to various antibiotics. Sensitivity to Imipenem has been reported to range from 98.15% to 100% (Zhang et al., 2017; Zhao et al., 2020). While the sensitivity to tetracycline was (98.15%), ciprofloxacin (94.44%), trimethoprim-sulfamethoxazole (85.18%), erythromycin (83.33%), and kanamycin (83.33%) (Zhao et al., 2020). By contrast, *B. cereus* resisted drugs containing β-lactam ampicillin (98%), oxacillin (92%), penicillin (100%), amoxicillin (100%), and cefepime (100%) (Kwarteng et al., 2017).

2.4 Phenotypic and genotypic identification of *B. cereus* isolates and associated toxin encoding genes

The description of the toxigenic potential of *Bacillus* isolates, and the safety requirements for toxin analysis in different foodstuffs, depend on the presence of reliable analytical tests (Ceuppens et al., 2011). Cytotoxicity approaches for enterotoxin analysis depend on microscopic examination of particular toxin-induced cell impairments (Miller et al., 2018). Antibody-based kits, some of which are commercially available, are component-specific and consequently identify only one of the enterotoxin complexes (Germany Federal Institute of Assessment, 2020). Regarding the diarrheal toxin encoding genes, the ileal-loop movement forecasts the ability to produce human diarrhoea. This assay has usually been taken as a decisive test of the enterotoxin effect. Assessing the cytotoxic effect of some enterotoxin like *nheA* is often used because these toxins are alleged to cause diarrhea by destroying epithelial cells in the small intestine (Granum & Ma, 2013).

Bacteria are commonly identified based on their phenotypic characteristics, which is not entirely reliable for the identification and differentiation of isolates (Manzano et al., 2010). The genetic resemblance between *B. cereus*, *B. thuringiensis*, and *B. anthracis* has been explored and proved that the use of various molecular techniques is more accurate than the phenotypic diagnostic tools. The molecular techniques include DNA-DNA reassociation; multilocus enzyme electrophoresis (MEE) to compare the allozyme patterns of 10–20 genes; Pulsed Field Gel Electrophoresis (PFGE), Randomly Amplified Polymorphic DNA (RAPD)-PCR, repetitive extragenic palindromic (rep)-PCR, and Microarrays and Real-Time PCR (Manzano et al., 2010). The use of molecular methods leads to improvement in *B. cereus* diagnostics and patient care (Ramarao et al., 2020).

The genotypic methods are less time-consuming, useful for determining phylogenetic relationships between microbial isolates, and are less reliant on bacterial growth variables (Wei et al., 2018; Zhao et al., 2020). Molecular methods using conventional PCR are of importance in the specific detection of *B. cereus* isolates from culture and on microbial DNA extracted from food (Sánchez-Chica et al., 2021).

The most important advantage of real-time PCR is that it can monitor the progress of the PCR reaction as it occurs, showing a fluorescence graph on the screen, and the ability to precisely monitor the amount of amplicon at each cycle. Moreover, real-time PCR allows highly accurate quantification of the amount of starting material in the samples, and it also has an increased dynamic range of detection. Amplification and detection occur in a single tube, eliminating post-PCR manipulations (Cattani et al., 2016; Wei et al., 2018).

CHAPTER THREE | MATERIALS AND METHODS

3.1 Study procedures

This study involved isolation and identification of *B. cereus*, detection of selected toxin-encoding genes harboured, and determination of sensitivity to commonly used antibiotics. Raw milk samples were obtained from a milk collection centre located in Kawempe Division, Kampala district in February 2022. The selected toxin-encoding genes included those that were reported elsewhere, i.e., *nheA*, *hblD*, *cyt-K1* and *ces*. All isolates were also subjected to antibiotic susceptibility tests. Figure 2 illustrates the study flow process.

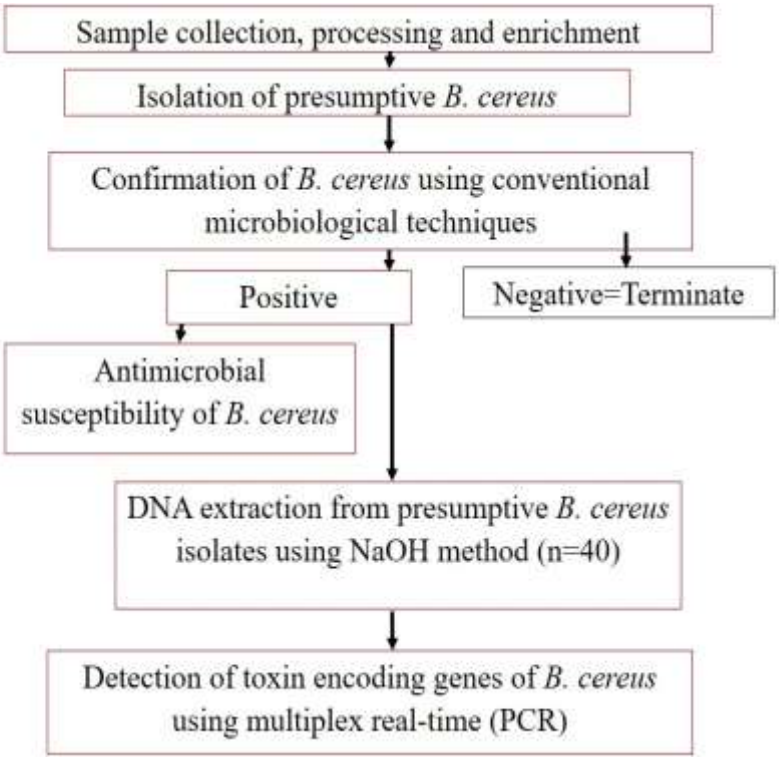

Figure 2: Flow process illustrating each step conducted during this study

3.2 Study area target population

Kawempe division is one of the five divisions that make Kampala Capital city. The coordinates of the division are 00 23N, 32 33E (Latitude: 0.3792; Longitude: 32.5574). The target population will be the milk transporters who supply the milk to the milk collection center in Kawempe division.

3.3 Sample size determination

The sample size was determined using a formula reported by Thrusfield (2005). A 6.9% prevalence as previously reported in a study by Abdeen et al. (2020) was used for sample size determination. Hence, the sample size was calculated as follows:

$$n = \frac{(1.96)^2 \, Pexp \, (1-Pexp)}{d^2} = \frac{(1.96)^2 \, 0.069 \, (1-0.069)}{(0.05)^2}$$

n = 98.7 ~ 99

Where n = required sample size, Pexp = expected proportion, d^2 = desired absolute precision at 95% Confidence Interval. According to the above formula, 99 raw bovine milk samples were estimated to be collected, however, one in three was sampled, only 30 milk samples were analysed.

3.4 Sample collection

The simple random sampling was used. One in three cans was chosen for sampling as long as long as the transporter supplied the milk to the milk collection center on the bicycle regardless of the milk origin. I chose to take samples from the milk transporters before the milk is pasteurized. The sampling took place at Kawempe milk Collection Centre, Kampala. About half a litre of raw bovine milk was bought from milking cans of transporters at a milk collecting Centre. The milk was placed in a sterile container (autoclaved), which was immediately closed and transported to the Laboratory. About half a litre of raw bovine milk was bought from a milk collecting centre. The milk was placed in a sterile container, which was immediately closed and transported to the Microbiology Laboratory at the College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University. Regarding sample transportation, the journey from the MCC was 15 minutes and was transported at room temperature.

3.5 Sample preparation

From each sample, 10 ml of raw bovine milk were homogenized in 90 ml of peptone water (pH 7.0). The mixture was incubated overnight at 37°C to aid the recovery of *B. cereus*.

3.6 Identification of *B. cereus* isolates

From the overnight Peptone water broth, subcultures were made on Polymyxin-egg yolk mannitol-bromothymol agar (Oxoid: Basingstoke, UK). The plates were incubated at 35 °C for 48 h and *B. cereus* suspected colonies were selected based on appearance, for example: dry

peacock blue surrounded by a visible translucent zone of precipitate due to lecithin hydrolysis. Gram staining was carried out to reveal Gram-positive, rod-shaped spore-forming cells, typical of *Bacillus spp*. additionally, selected biochemical tests included citrate, catalase, oxidase and motility. Sheep Blood agar (Thermo Fisher Scientific, UK) was used to detect hemolysis while Trypticase soy agar (Sigma-Aldrich, USA) was used for colony purification of *B. cereus* growth.

3.7 Antibiotic susceptibility testing

The antibiotic susceptibility test (AST) was performed using the Kirby-Bauer disc diffusion method as described by Yim et al. (2015). Mueller-Hinton agar (Merck, Darmstadt, Germany) were allowed to warm at room temperature, ensured MHA to have 4mm depth. The discks were then removed from the refrigerator, equilibrated at room temperature leaving them to settle for 15 minutes. The MHA was inoculated with the isolate using a sterile cotton tipped into the suspension and antibiotic discs were applied. Six antibiotics (Oxoid, Basingstoke, UK) that are bacteriostatic were evaluated. The antibiotics included ampicillin (10 mg), penicillin G (10 μg), gentamicin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), and ciprofloxacin (5 μg). The AST results on the isolates were reported as susceptible, intermediate susceptible, or resistant according to the standard guidelines from the Clinical Laboratory Standard Institute (2020) on performance.

3.8 Detection of selected toxigenic genes

The detection of selected toxigenic genes of *B. cereus* involved extraction of DNA, and optimization of PCR, after which multiplex RT-PCR was performed. As explained as follow:

3.8.1 Genomic DNA extraction

The genomic DNA extraction was performed on overnight brain heart infusion (BHI) cultures following a protocol described by Lefimil et al. (2013): From the bacterial culture, 100 µL were placed at the centre of a 4cm Whatman filter paper No. 4), which was then air-dried at 25°C for one hour. Two 3cm diameter discs were punched using a Harris 3.0-mm Micro Punch (Whatman Biosciences Ltd.) and discharged into 1.5 ml Eppendorf tubes. Two hundred (200) mM NaOH was added and incubated at 37°C for 30 minutes. The solution was discarded, and then the filter paper disc was washed twice in 200 µL of Tris- ethylenediaminetetraacetic acid (TE) buffer (10 mM Tris–HCl and 0.1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0). After the removal of the TE buffer, the disk was subsequently transferred into a 0.2 ml PCR

tube and then air-dried at 37°C. One hundred microliters of nuclease-free water (Qiagen, Germany) was added and the extracted DNA was stored at -20°C for future use.

3.8.2 Optimization of primers working conditions

The Primers that were used are presented in Table 1. Singleplex conventional PCR was used to optimize RT-PCR conditions as described by Wehrle et al., (2010). The amplification conditions included a cycle at 95ºC for 1 minute, followed by 45 cycles at 95ºC for 15 seconds, 60ºC for 30 seconds and 72ºC for 1 minute using DNA Engine Dyad® Cycler (PTC-0221, Bio-Rad Laboratories Inc.). The PCR products were run on, 2 % agarose (Bio Tolls Inc. Japan), stained in 0.5% Ethidium [Sigma-Aldrich, USA), and visualized on an ultraviolet trans-illuminator (Wagatech, UK), for fragment size determination against a 50 bp molecular marker (Hyper Ladder, New England, UK).

Table 1: Primer sequences, annealing temperature and expected amplification product size

Gene	Primer	Primer sequence [5'-3']	Annealing T°	Product size (bp)
hblD	mp3L1R1for	F: AGT TAT TGC AGC TAT TGG AGG	60°C	14
	mp3L1R1rev	R: GTC CAT ATG CTT AGA TGC TGT GA		8
nheA	mp3AR2for	F: TTCAAATTCAAAAGAATGTTGAAGAAGG	60°C	11
	mp3AR2rev	R: GATTTGTTTGCTTATTCATTTCATCAC		1
cyt	mp4CytKfor	F: GCTTTGTATAAGCAACTTGGATAG	60°C	17
K1	mp4CytKrev AGC	R: AGCCTCTGTAACACCAAGC		6
Ces	ces_SYBR_F	F: CAC GCC GAA AGT GAT TAT ACC AA	60°C	38
	ces_SYBR_R CAC	R: CAC GAT AAA ACC ACT GAG ATA GTG		9

3.8.3 Multiplex real-time PCR for detection of selected *B. cereus* toxin genes

Multiplex real-time PCR and primers whose sequences are presented in Table 1, were adopted from Wehrle et al., (2010). For each of the genes detected in the real-time PCR, each primer had its unique melt curve temperature value number (Wehrle et al., 2010). A final 20 µl reaction mixture contained 10 µl Luna qPCR SYBR Mastemix, 2 µl of template DNA, 0.01 mM of forward and reverse primers hblD F/R, nheA F/R, cytK1 F/R, and ces F/R primers, and nuclease-free water (Qiagen, Germany). The amplification was performed using RT-PCR (Thermo Fisher Scientific, UK). The conditions were: one cycle at 95ºC for 1 minute, followed by 45 cycles at 95ºC for 15 seconds, 60ºC for 30 seconds and 72ºC for 1 minute with subsequent heating to 72ºC for 1 minute.

3.9 Data analysis and interpretation

Data (number of raw bovine milk contaminated with *B. cereus*, diameters of inhibition zones by antibiotic disks, and number of *B. cereus* isolates harbouring the selected toxin genes) were entered, screened for errors and stored in Microsoft Excel 2010. Proportions of raw bovine milk contaminated with *B. cereus,* toxin genes profiles and antibiotic susceptibility were calculated using Microsoft Excel. The proportion of milk samples contaminated with *B. cereus*

was calculated by taking the number of positive samples (numerator) over the total number of samples analyzed (denominator) and then multiplied by 100. Regarding the antimicrobial susceptibility profile, the numerator was the number of susceptibilities while the denominator was the total number of isolates tested. In the toxin gene profile, the numerator was the number of positive samples (with corresponding melt curve) over the denominator which was the total number of DNA samples of all isolates analyzed.

3.10 Ethical approval

This study obtained clearance from the College of Veterinary Medicine, Animal Resources and Biosecurity, reference Principal/COVAB/62/'22 and was registered by the Uganda National Council for Science and Technology (reference NS356ES)(Appendix 2).

3.11 Quality control and Biosafety measures

Isolation of *B. cereus* was performed in the biosafety level two (BSLII) because these organisms are classified under *B. cereus sensu lato* that also contains *Bacillus anthracis,* which is very pathogenic and fatal and is also found in the environment. This study had no positive controls, I evaluated my findings based on the size of the PCR products as reported by previous researchers including Fricker et al. (2007) and Wehrle et al. (2010)**.**

CHAPTER FOUR: RESULTS

4.1 Proportion of *B. cereus* in raw bovine milk from the Kawempe divison, Kampala district

From a total of 30 milk samples analyzed, eight (8) samples (26.7%) were contaminated with *Bacillus cereus.*

4.2 Antibiotic susceptibility profiles of *B. cereus*

All 40 (five from each positive milk sample) isolates analyzed were susceptible to gentamycin, ciprofloxacin, chloramphenicol and tetracycline. While all of them were resistant to ß-lactam antibiotics including ampicillin and penicillin (Figure 3). Determination of susceptible and resistant strain to a given antimicrobial followed the guidelines in CLSI (2020).

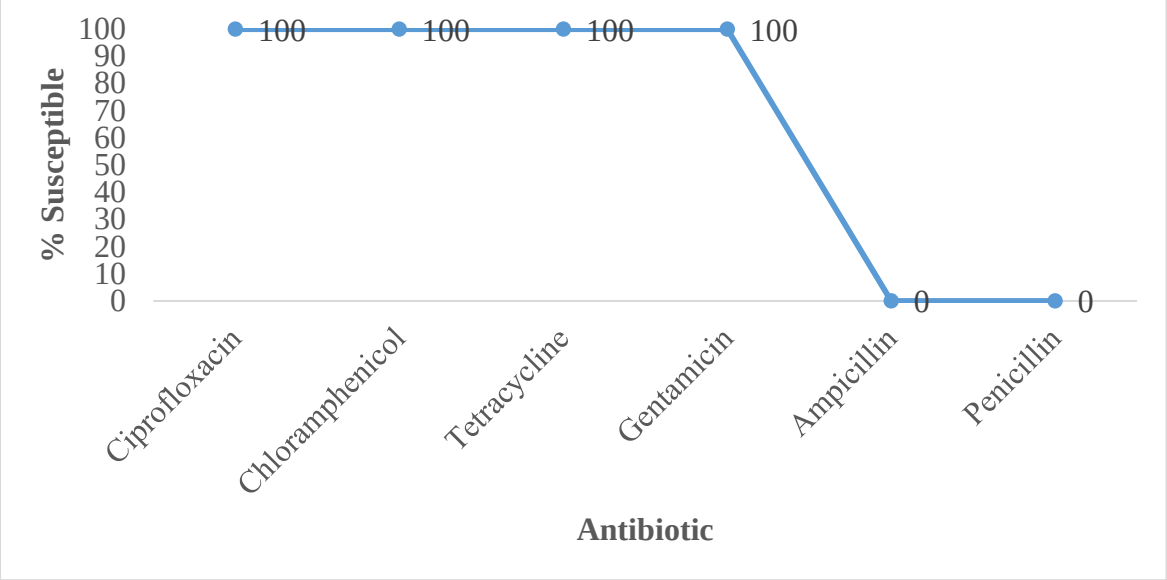

Figure 3: Antibiotic susceptibility profiles of *B. cereus* isolated from raw bovine milk

4.3 Presence of selected toxin encoding genes

Results of a singleplex conventional PCR to optimize the PCR working conditions are presented for some samples (Figure 4). Some representative isolates harbouring the genes analysed for, that is, *hblD* (about 148 base pairs) and *nheA* (about 111 bp) amplification and the absence of *cytK1* are indicated. There were no positives. The findings were evaluated based on the size of the PCR products as reported by previous researchers (Wehrle et al., 2010).

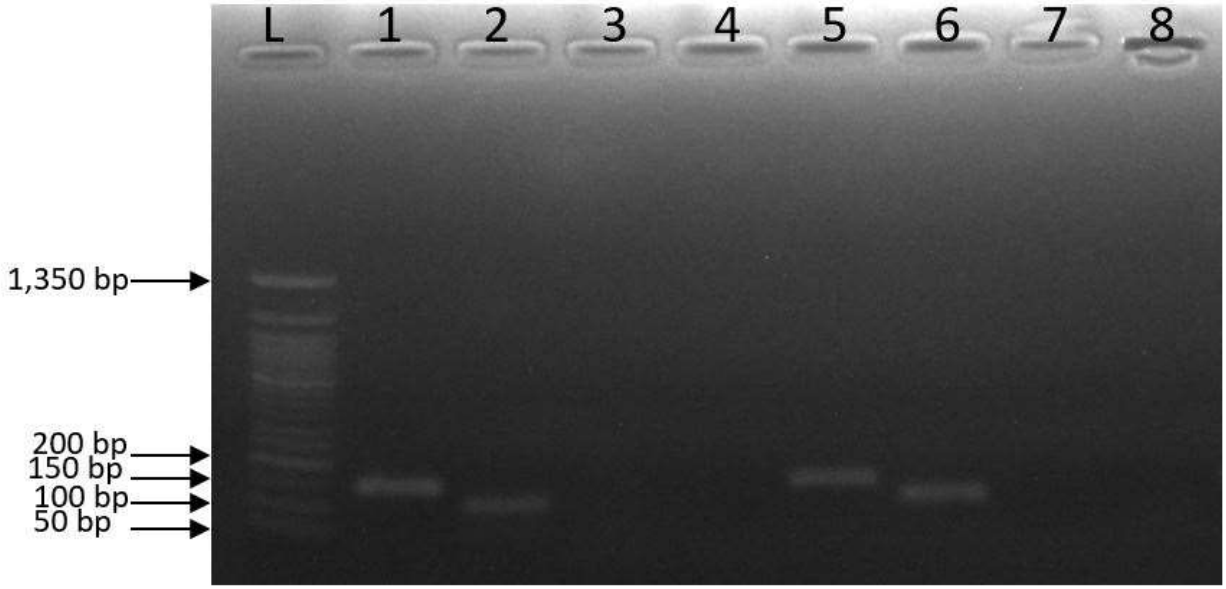

Figure 4: Singleplex conventional PCR for the toxin genes: L, 50 bp DNA ladder (New England Biolabs inc., UK), lane 1, isolate 9 positive for *hblD*=148 bp, lane 2, *nheA*, 111 bp, lane 3, negative for the *cytK1*, lane 4, negative for the ces, lane 5, positive for the *hblD*, lane 6, positive for the *nheA*, lane 7, negative for the *cytK1*, lane 8, negative for the *ces*

Multiplex real-time PCR detected 85 % (34) and 82.5 % (33) of the 40 isolates that harboured the *nheA* and *hbl*D gene respectively (Figure 6). The genes *cytK1* and *ces* were not detected in any of the isolates analysed. The multiplex real-time PCR results for toxin gene detection are summarized in Table 2.

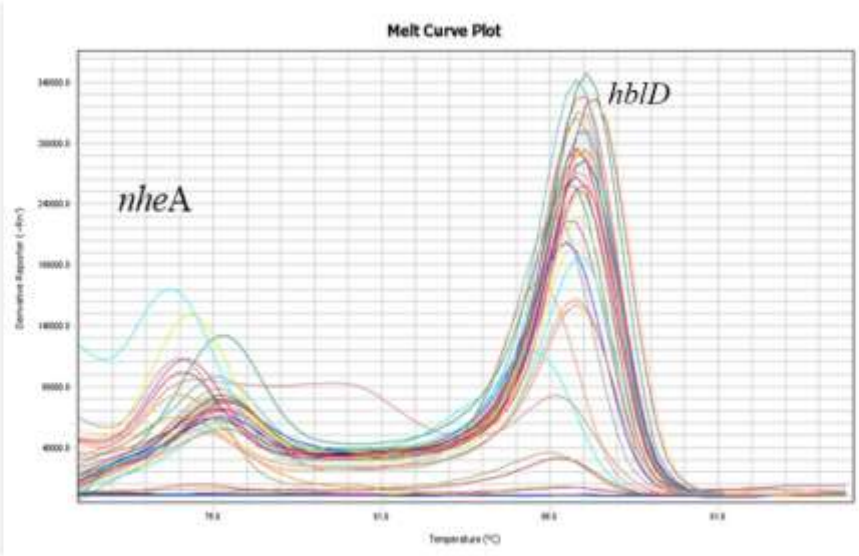

Figure 5: Melt curve for *nheA* and *hblD*

The toxigenic gene profiles of bacterial isolates from the various milk samples are presented in Table 2. Five (5) isolates had *hblD* only; six (6) had *nheA* only; while 28 had both *hblD* and *nheA.* None of the isolates harboured *cytK1* and *ces* genes.

Table 2*: Bacillus cereus* isolates harbouring selected toxin genes after multiplex RT-PCR

Sample ID	**Isolate ID**	**Gene profiles**	
		hblD	***nheA***
KRBM2	KRBM2_1	+	-
	KRBM2_2	+	+
	KRBM2_3	+	+
	KRBM2_4	+	+
	KRBM2_5	-	+
KRBM4	KRBM4_1	+	+
	KRBM4_2	+	+
	KRBM4_3	+	+
	KRBM4_4	+	+
	KRBM4_5	+	+
KRBM12	KRBM12_1	-	+
	KRBM12_2	+	-
	KRBM12_3	+	-
	KRBM12_4	+	+

	KRBM12_5	+	+
KRBM15	KRBM15_1	+	+
	KRBM15_2	+	+
	KRBM15_3	+	+
	KRBM15_4	+	+
	KRBM15_5	+	+
KRBM20	KRBM20_1	+	+
	KRBM20_2	+	+
	KRBM20_3	+	+
	KRBM20_4	+	+
	KRBM20_5	+	+
KRBM26	KRBM26_1	+	+
	KRBM26_2	+	+
	KRBM26_3	-	+
	KRBM26_4	-	+
	KRBM26_5	+	+
KRBM28	KRBM28_1	+	+
	KRBM28_2	-	-
	KRBM28_3	-	+
	KRBM28_4	-	+
	KRBM28_5	+	+
KRBM29	KRBM29_1	+	+
	KRBM29_2	+	+
	KRBM29_3	+	-
	KRBM29_4	+	-
	KRBM29_5	+	+

KRBM (Kawempe raw bovine milk),

+: positive meaning that the gene was detected,

-: meaning that the gene was not detected

CHAPTER FIVE: DISCUSSION

The present study revealed a 26.7% proportion of *B. cereus* contamination of raw bovine milk sold at a milk collection centre within Kawempe division. The raw milk contamination in our settings could be that the containers cleanness, habit of adulteration by adding more water, which is often unboiled, can contribute to high contamination levels. The transfer of *B. cereus* from soil to raw bovine milk may also be related to the cleanness of the milk transportation cans from the farm to the milk collection centre. But boiling the milk before consumption can prevent the foodborne diseases caused by bacteria like *B. cereus*. However, *B. cereus* are spore formers that can withstand high temperatures and the pH. The level of contamination with *B. cereus* could be that some countries with lower counts are having lower ambient temperatures, some are developed countries likely to have different animal husbandry systems as well as milking techniques. Milking machines if well maintained hygienically often have low microbial contamination compared to our farmers who carry out hand milking without observing milking hygiene. The proportion from our study was higher than what was reported in earlier studies elsewhere. In Rwanda, 10.3% of the milk was contaminated with *Bacillus* spp.(Mpatswenumugabo et al., 2017). In Spain for example, 5.3% of the milk and milk products were contaminated with *B. cereus* (Mosso et al., 1989), 6.9% of milk powder and 8.5% of Ras-cheese in Egypt (Abdeen et al., 2020). Also, in Ethiopia 15.4% of raw milk, respectively were reported to be contaminated with *B. cereus* (Kassa et al., 2017). However, higher prevalence of *B. cereus* were found in raw milk in Guinea (33.3%), in Senegal (35.2%) and in western Iran (97.5 %) (Hempen, 2017, Alipour Banaei & Mahdavi, 2020).

All *B. cereus* isolated from the current study were sensitive to gentamycin, tetracycline, chloramphenicol and ciprofloxacin. This could be because *B. cereus* is usually a contaminant from the environments where they have not been exposed to antibiotics, which is a major driver for the development of drug resistance. Moreover, the broad range spectrum of antibiotic susceptibility of *B. cereus* gives a sight of what can be used to treat gastrointestinal illnesses caused by this microorganism since it has clinical significance for humans (Kwarteng et al., 2017). However, the current study revealed that all the 40 *B. cereus* isolates were resistant to both ampicillin and penicillin G. The *B. cereus* resistance to β-lactam antibiotics may be due to its ability to produce β-lactamases (Bottone, 2010). The data on the antibiotic susceptibility of the isolates from animal products also raises concerns about the continuous spread of antimicrobial resistance to humans. So, this study contributes to existing information on where

to start controlling antimicrobial resistance which is among the 10 global public health threats, which is in agreement with findings by Ikeda et al. (2015); Khudor et al. (2012); Shawish & Tarabees (2017); Yim et al. (2015**).**

Toxigenic genes *hblD* and *nheA* were detected in the majority of the isolates. The quantity of some enterotoxins production is built on numerous factors, such as the number of vegetative bacteria and spores in the food, in addition to the conducive environment with required ambient temperature and nutrients (Alipour Banaei & Mahdavi, 2020). The diarrheal encoding toxin gene profile established for raw bovine milk raises public health concerns for people, especially those who may risk drinking unboiled milk. The detection of *hblD* in isolates from raw cow milk is in close agreement with previous studies by Alipour Banaei & Mahdavi (2020) and Yu et al. (2020). This study is also in agreement with the previous studies which stated that *nheA* is the most common enterotoxin harboured by *B. cereus* isolates (Abdeen et al., 2020; Arslan et al. (2014). It is reported that the expression of *hblD* genes by *B. cereus* is influenced by the food nutrient contents (carbohydrates and/or proteins), i.e., milk and dairy products, and an appropriate temperature for bacterial growth (Carlin et al., 2010). In contrast, the current findings are higher than 67% and 33.3% as reported by Gao et al. (2018), Abdeen et al. (2020).

In this study *ces* and *cytK1* were not detected in any of the isolates analyzed. Failing to detect ces may be associated with the number of samples and the test used because the *ces* is often produced in its environment. This result agrees with different studies including that of Chica et al. (2020), Samapundo et al. (2011), Ankolekar (2009) and Arslan et al. (2014) who did not detect the *ces* gene in *B. cereus*. The *ces* gene codes for the production of cereulide, which causes emesis due to ingesting food that was contaminated by specific bacteria (Agata et al., 2002; Jääskeläinen et al., 2004). The cereulide is heat stable once produced in the food which may cause public health concerns (Dietrich et al., 2021; Tempelaars et al., 2011). However, a proportion of 7.7 % of the *ces* gene in the isolates from different food items was reported (Batchoun et al., 2011). For the case of the *CytK1* gene, this was not detected, which is in agreement with a study by Glasset et al. (2018). The diarrheal enterotoxin *cytK1*gene is rare but if detected it is associated with serious diarrheal outbreaks (Glasset et al., 2016; Glasset et al., 2021; Jovanovic et al., 2021; Chica et al., 2021). This information can be used for risk and safety assessment not only in Kawempe division. This study has a limitation on data on which farm the milk was generated from. The study did not go for the contributing factors to the milk contamination by *B. cereus* like poor milking personnel hygiene practice, transport cans cleanness and the cow health status

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- The raw bovine milk samples collected from transporters were found to be contaminated with *B. cereus* causing public health concerns for the consumers who may risk taking the uuboiled milk.
- The results of this study show that diarrheal genes are more common than the emetic toxin encoding gene, which were not detected at all.
- This study revealed that there is a resistance to the β-lactams ampicillin and penicillin, but also a broad spectrum of the antimicrobials (gentamycin, tetracycline, chloramphenicol and ciprofloxacin) with high effects on the *B. cereus*.

6.2 Recommendations

- The sample size and source of the samples did not represent the amount of milk supplied to Kawempe division in general or even give a picture of what is in Kampala district or in the country, there is a need for a bigger study to overcome this.
- *Bacillus cereus*, being spore formers and therefore withstand high temperatures, to revive the bacteria, I incubated at 37°C which supports the multiplication of other bacteria which may be the reason for lower detection and isolation. There is a need for a study that considers higher temperatures that favour the growth of this microorganism to give a clear picture.
- This study targeted only four toxigenic genes (*hblD*, *nheA*, *cyt-K1* and *ces*) that were commonly reported by previous researchers and in various geographical regions, so there is a need to screen for other genes to get a complete picture within the local settings.
- Regarding the antibiotic susceptibility test–six drugs were tested and only two of them were resisted, there is also a need for a bigger study on more antibiotics to establish the wider resistance profiles not only on penicillin and its derivatives but also on other antibiotics to elucidate on the cross-resistance.
- Since the raw bovine milk samples used in this study were taken directly at the milk collecting centre, It is advised to introduce significant hygiene practices such as specific

hazard analysis of critical control points from the farm to the table to thus reduce potential public health issues to the consumers in Kawempe division. The public health authorities should add *B. cereus* on the priority on bacteria that are the milk contaminants and are of public health concerns.

- Once diarrhea is reported to the health practitioners, *B. cereus* should be added among the suspects of the causative agents and include it in diagnosis.

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Appendices:

Appendix 1: Antibiotic Susceptibility Tests (Disk diffusion)

	CN	CIP	AMP	TE	C	P
Lab ID	Z	Z	Z	Z	Z	Z
1	25	32	9	24	32	12
2	28	31	8	25	31	12.5
3	29	26	0	23.5	30.5	11
4	24	28.5	0	22	26.5	11
5	28	31	0	24	31	13
6	26	30	0	22	31	11
7	27	30	0	23	32	11
8	29	34	9	26	36	10
9	30	32	0	23	33	11
10	31	32	7	21	32	11
11	27	33	0	23	28	11
12	26	29	0	24	29	10
13	25	32	0	23	31	12.5
14	24	27	0	22	30.5	12
15	29	26	0	23.5	33	13
16	25	31	0	24	32	11
17	24	28	0	25	26.5	11
18	22	20	0	23	25	11
19	22	20	11	23	28	13
20	23	29	11	24	29	11
21	23	29	10	24	33	14
22	23	27	0	24.5	28	0
23	27	32.5	0	23.5	30.5	0
24	23	28	0	23	30.5	0
25	24	29	0	22	29	11
26	27	32	9	23	35	14
27	24	29	9	22	31	12
28	25	31	16	26	31	16
29	27	31	0	28	32	0
30	24	31	17	28	34	14
31	27	28	16	28	32	13
32	23	30	16	30	34	15
33	25	27	15	27	28	14
34	23	30	16	26	30	15
35	25	32	16	25	32	14
36	28	30	9	22	31	0
37	24	29	16	23.5	30	14
38	27	31	15	27	32	11

39	26	28	16	26	28	16
40	23	27	17	25	29	15

Appendix 2: Proof of registration from the Uganda National Council for Science and Technology

Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: NS356ES **22 June 2022**

BARIYANGA Jean Damascene
Makerere University/College of Veterinary Medicine, Animal Resources and Biosecurity(COVAB)
Kampala

Re: Research Approval: **TOXIN GENE PROFILING AND ANTIBIOTIC SUSCEPTIBILITY OF Bacillus cereus ISOLATED FROM RAW BOVINE MILK SOLD WITHIN KAMPALA CAPITAL CITY**

I am pleased to inform you that on **22/06/2022**, the Uganda National Council for Science and Technology (UNCST) approved the above referenced research project. The Approval of the research project is for the period of **22/06/2022** to **22/06/2023**.

Your research registration number with the UNCST is **NS356ES**. Please, cite this number in all your future correspondences with UNCST in respect of the above research project. As the Principal Investigator of the research project, you are responsible for fulfilling the following requirements of approval:

1. Keeping all co-investigators informed of the status of the research.
2. Submitting all changes, amendments, and addenda to the research protocol or the consent form (where applicable) to the designated Research Ethics Committee (REC) or Lead Agency for re-review and approval **prior** to the activation of the changes. UNCST must be notified of the approved changes within five working days.
3. For clinical trials, all serious adverse events must be reported promptly to the designated local REC for review with copies to the National Drug Authority and a notification to the UNCST.
4. Unanticipated problems involving risks to research participants or other must be reported promptly to the UNCST. New information that becomes available which could change the risk/benefit ratio must be submitted promptly for UNCST notification after review by the REC.
5. Only approved study procedures are to be implemented. The UNCST may conduct impromptu audits of all study records.
6. An annual progress report and approval letter of continuation from the REC must be submitted electronically to UNCST. Failure to do so may result in termination of the research project.

Please note that this approval includes all study related tools submitted as part of the application as shown below:

No.	Document Title	Language	Version Number	Version Date
	Project Proposal	English	DEFENDED PROPOSAL ON 6TH DECEMBER, 2021	
1	Approval Letter	English		
2	Administrative Clearance	English		

Yours sincerely,

Hellen Opolot
For: Executive Secretary
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