

Isolation and Identification of *Bacillus cereus* Associated with some Ready-to-Eat Soybean Curds (Awara) sold in Kano Metropolis, Kano-Nigeria

Yakubu Abubakar Bala^{1*}, Adamu Abdullahi Shehu¹, Ahmed Faruk Umar², Farida Isah Elhassan¹, Yahaya Ubah Ya'u³, Sadisu Farouk Umar¹ & Hudu Sabo⁴

¹Department of Microbiology, Aliko Dangote University of Science and Technology, Wudil

²Department of Microbiology, Abubakar Tafawa Balewa University, Bauchi

³Department of Science Lab. Technology, Aliko Dangote University of Science & Technology, Wudil

⁴University Health Services, Aliko Dangote University of Science and Technology, Wudil

Received: 05 / 09 / 2026

Accepted: 09 / 10 / 2026

Published: 25 / 10 / 2026

Abstract: This study isolates and identifies the *Bacillus cereus* obtained from some ready-to-eat soybean curds (*Awara*) sold in Kano metropolis, Kano-Nigeria. A total of two hundred (200) samples of *Awara* were purchased from different locations of Kano metropolis, in which twenty-five (25) samples were purchased from each Local Government of the metropolis by using random sampling method. The samples were blended, serially diluted and subjected to microbiological analyses using; aerobic plate counts to determine *Bacillus* counts. Discrete colonies were further subjected to Gram's staining and standard biochemical tests for identification. The result of *Bacillus* counts showed that the samples contained mean counts ranging from $2.10 \times 10^3 \pm 137.84\text{cfu/g}$ to $1.61 \times 10^4 \pm 556.23\text{cfu/g}$. The overall results of this study indicated that, all the mean aerobic mesophilic *bacillus* counts are within the standard limits of satisfactory for WHO and FAO of 10^3 to 10^5cfu/g . Biochemical characterization of the bacterial isolates obtained from the study revealed the presence of *Bacillus cereus*, *B. thuringiensis*, *B. subtilis* and *B. Megaterium*. The findings revealed the occurrence of *B. cereus* in ready-to-eat *Awara* samples to be 11.5%, with differences in contamination levels among sampling locations.

Keywords: *Awara*, *B. cereus*, Contamination, Kano Metropolis, Ready-to-eat.

Cite this article: Bala, Y. A., Shehu, A. A., Umar, A. F., Elhassan, F. I., Ya'u, Y. U., Umar, S. F. & Sabo, H. (2026). Isolation and Identification of *Bacillus cereus* Associated with some Ready-to-Eat Soybean Curds (*Awara*) sold in Kano Metropolis, Kano-Nigeria. *MRS Journal of Multidisciplinary Research and Studies*, 3(10), 42-47.

Introduction

Bacillus cereus which is categorized as an important bacterial species belongs to the genus *Bacillus* is described as large Gram-positive rods, facultative anaerobic, motile, strongly beta-hemolytic, have the ability to form spores in unfavorable conditions and widely distributed in nature. It is usually found in soil and implicated in several foods (Abayneh *et al.*, 2019), induced food poisoning symptoms in human and the level of contamination of meat products with *B. cereus* constitutes serious problems for consumers (Tawab *et al.*, 2020). *B. cereus* contamination depends upon the production of several exogenous enzymes such as hemolysins, phospholipases, proteases and the ability to form biofilms, in addition to the presence of toxin-encoding genes which play an important role in its pathogenicity (Tirloni *et al.*, 2020).

Various toxins produced by *B. cereus* play an important role in food safety as it cause health problems to consumers as a result of their ability to spoil food and cause diseases (Owusu-Kwarteng *et al.*, 2017), these enterotoxins act on the epithelial cells of the small intestine through damaging its plasma membrane and causing diarrhea as a result of these tissue destructive proteins (Ali *et al.*, 2017). Abdominal pain and diarrhea are *B. cereus* food

poisoning symptoms which appears 8 - 16 hours subsequently to contaminated food consumption, then disappears generally within 12 - 24 hours. On the other hand, more serious cases necessitating hospitalization have been defined; endocarditis; meningitis in addition to fatal cases were recorded (Alimi *et al.*, 2016). Foodborne outbreaks of *B. cereus* are generally benign and spontaneously resolved, however, also sometimes it may cause hospitalization of immunosuppressed persons or even death may occur (Ali *et al.*, 2017).

Materials and Method

Study Area

The study area, Kano Metropolis lies between latitude $11^{\circ} 05' \text{ N}$ to $12^{\circ} 07' \text{ N}$ and longitude $8^{\circ} 23' \text{ E}$ to $8^{\circ} 47' \text{ E}$ and altitude 472 meters above sea level. Kano Metropolis bordered by Minjibir Local Government Area on the North-East and Gezawa Local Government Area to the East, while Dawakin Kudu Local Government Area to the South-East, Madobi and Tofa Local Government Areas to the South-West. Kano Metropolis is the second largest industrial and commercial center in Nigeria after Lagos and it experiences rapid population growth rate, the

population of metropolis at the year 2000 is 1.6 million. In 2003, the population rose to 2.3 million (approximately) and in 2006 (NPC, 2006) to 2.8 million, which was projected to 4.6 million in 2022. Based on the estimated annual population growth rate of 3.14%, the population of Kano metropolis was projected population of 5.2 million inhabitants as in 2026 (WPR, 2026). The area covers almost 499 square kilometers, with a population density of 1000 inhabitants per km² within the Kano closed-settled zone compared to the national average of 267 inhabitants per km²; It is also one of the most crowded. The city also has a large migrant workers population which has been increasing at the rate of 30 to 40 percent per annum. It has also through time become a cosmopolitan city with all the ethnic groups across the city.

Sampling Sites

The sampling site comprises the six-core urban Local Government (Dala, Fagge, Gwale, Kano Municipal, Nassarawa and Tarauni) and two peri-urban Local Governments (Kumbotso and Ungogo). Residential uses dominate most part of the study area, however other land uses such as commercial, institutional, and educational are all located within the areas. Dakata, Sharada and Bompai functioned as industrial layout. Industries such as steel rolling, packaging, beverages processing etc are found in this site. Commercial activities happened to be growing very fast in the site. There are many big and international markets such as Sabon Gari, Kantin Kwari, Singer market, Dawanau and Yankaba markets in the sampling sites (Maiwada, 2000)

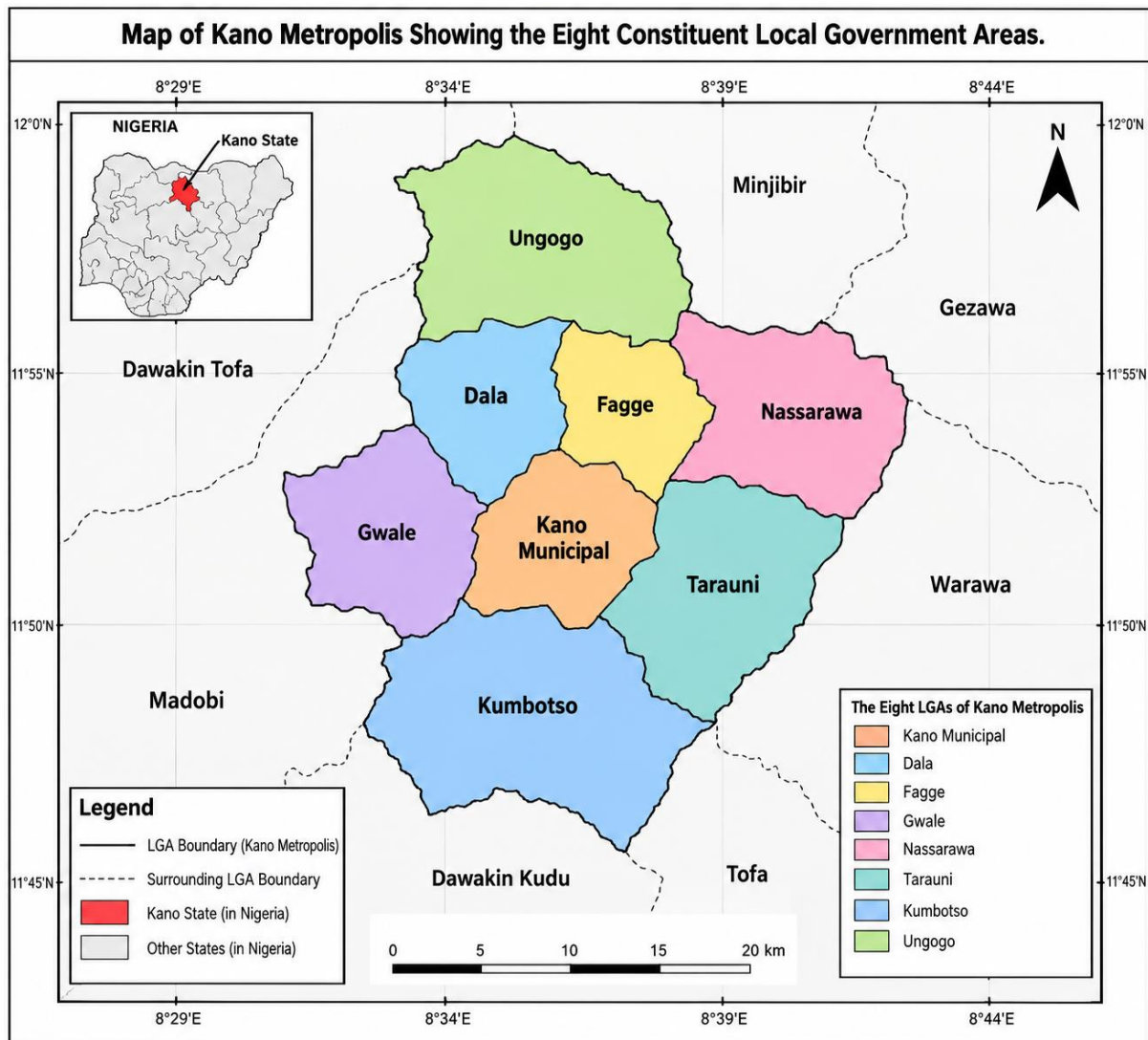


Figure 1: Map of Kano Municipal

Sample Size

Formula for calculating sample size was used to obtain the required size of sample to be analyzed.

$$N = \frac{Z^2 \times P \times (1-P)}{E^2}$$

Where N= Number of Sample

Z= is the Z-score =1.96 at 95% confidence interval

P = Estimated proportion of the population = 11.26% = 0.1126 (Aruwa and Akinsoye, 2015)

E= Margin Error = 0.05% at 5% margin error

$$N = \frac{(1.96)^2 \times 0.1126 \times (1-0.1126)}{(0.05)^2}$$

N= 153.56

Approximately N= 154

31% of the calculated result was added to obtain approximately a total of 200 samples

Collection of Samples

A total of two hundred (200) ready-to-eat *Awara* samples were purchased randomly from various selling points in Kano Metropolis (eight Local Governments of Kano Metropolis) by following a procedure adopted by Bintu *et al.* (2015), in which 25 samples were purchased from different selling point of each Local Government Area within the period of the research. Approximately 50g of ready-to-eat Soybean curds were collected from each selected vendor using sterile forceps and immediately transferred into a sterile, labeled sample bag. The samples were collected directly from the portions of Soybean curds that were being offered for sale to consumers. Where several pieces of Soybean curds from the same batch were available, portions were collected different parts of the batch to obtain a representative sample. Samples from different vendors were collected separately to prevent cross-contamination. Each collected sample was assigned unique identification code indicating the sampling location and sample number. Information including sample code, sample location, date and time of collection, vendor code and display condition was recorded in the sample collection form

Samples transportation

The *Awara* samples were weighed, marked clearly, collected separately in clean rubber container were transported in a sterile cool container to the laboratory of Microbiology, Aliko Dangote University of Science and Technology (ADUSTECH), Wudil. The samples were subjected to bacteriological examination. The time limits for carrying out bacteriological analysis after collecting the samples were 1 to 2 days (Bintu *et al.*, 2015).

Preparation of the Control Sample

Soybean seed (8.0kg) was cleaned, sorted, and washed with clean water and left for soaking in water for six hours. The soaked soybeans were drained, washed with water, and hulled using mortar and pestle. Grinding took place immediately after the beans were cleaned. 3 liters of water were added to obtain slurry which was mixed with a small amount of water and then filtered using a muslin cloth to extract the soymilk and leaves the residue. To further extract the soymilk, the residue was mixed with water again and filtered. The soy extract was subjected to boiling at about 98°C with constant stirring for about 20mins. Effluent from previously prepared *Awara* was left to ferment and then used as a coagulant. A 3ltrs of coagulant was added to the boiling soymilk and allowed to curdle until the whey separated visibly from the curd. The mixture (curd & whey) was then poured into a clean porous cloth and pressed by application of pressure to separate the whey from the curd thereby forming a soybean curd (*Awara*). The soybean curd (*Awara*) was then cut into desired sizes and shapes whereby some portions were fried in hot vegetable oil, while some portions were dried under sun and air to form dried pellets and then fried in hot vegetable oil (Agbara *et al.*, 2018)

Bacteriological Analysis

Sample processing

Twenty-five grams of *Awara* were homogenized in 225ml of 0.1% bacteriological peptone water and incubated at 37°C for 2h. Later, after incubation, serial dilutions were made and 1mL

from each diluted sample was spread onto the surface of Mannitol Egg-yolk Polymyxin (MYP) (Bintu *et al.*, 2015)

Isolation of *Bacillus cereus* species

The plates containing the Mannitol Egg-yolk Polymyxin (MYP) were incubated at 37°C for 48 h for counting of colonies on plates. The colonies with pink color (mannitol-negative), surrounded by heavy precipitate indicating lecithinase activity and showing distinct β -hemolytic on blood agar were assumed to be *B. cereus* (FDA, 2020). If reactions are not clear, the plates were incubated for additional 24h before counting colonies. Plates that contain estimated 15-150 pink colonies (MYP), lecithinase-producing colonies were selected. The bottom of plates was marked into zones with a pen to facilitate counting and colonies that are typical of *B. cereus* were counted. This was the presumptive plate counts of *B. cereus*. At least 5 presumptive positive colonies from the MYP plates were picked and one colony transferred to a nutrient agar slant for storage. Typical colonies grown on MYP were confirmed by using biochemical testing (FDA, 2020)

Confirmation of *B. cereus* Isolates

Confirmatory tests for *B. cereus* isolates were employed by following a method described by FDA, 2020. Exactly 5 eosin pink, lecithinase-positive colonies were picked from MYP agar plates and transferred to nutrient agar slants. Slants were incubated for 24 h at 30°C. Gram-stained smears were prepared from slants and examine microscopically. *B. cereus* was appeared as large Gram-positive bacilli in short-to-long chains; spores are ellipsoidal, central to sub terminal and do not swell the sporangium. A 3mm loopful of culture was transferred from each slant to 13 × 100mm tube containing 0.5 ml of sterile phosphate-buffered dilution water then the following confirmatory media where inoculated.

Phenol red glucose broth

A 3 mL broth was inoculated with 2 mm loopful of culture. Tubes were incubated anaerobically for 24h at 35°C in GasPak anaerobic jar. The tubes were shaken vigorously and observed for growth as indicated by increased turbidity and color change from red to yellow, which indicates that acid has been produced anaerobically from glucose. A partial color change from red to orange/yellow may occur, even in uninoculated control tubes, due to a pH reduction upon exposure of media to CO₂ formed in GasPak anaerobic jars (FDA, 2020).

Nitrate broth

A 5 ml broth was inoculated with 3 mm loopful of culture. The tubes were incubated for 24h at 35°C. To test for nitrite, 0.25ml each of nitrite test reagents A and C was added to each culture. An orange color, which develops within 10 min, indicates that nitrate has been reduced to nitrite (FDA, 2020).

Modified VP medium

A 5ml medium was inoculated with 3 mm loopful of culture and the tubes were incubated for 48 ± 2h at 35°C. Test for production of acetylmethyl-carbinol by pipetting 1ml culture into a test tube and adding 0.6 ml alpha-naphthol solution and 0.2 ml 40% potassium hydroxide. The tubes were shaken and few drops' crystals of creatine were added. Observe results after holding for 1h at room temperature. Test is positive if pink or violet color develops (FDA, 2020).

Motility test

The test organisms were aseptically stabbed into two test tubes with the aid of sterile inoculating wire loop. Another tube without a test organism was also stabbed with a sterile inoculating loop served as control tube, all the tubes were incubated at 35°C for 24hr to 48 hours, After the period of incubations, the growth pattern for characteristics that spreading from the stabbed lines were carefully observed. Reddish to purplish colour extending diffusely from the stabbed line indicates the organisms are Motile. While a reddish to a purplish colour only along the stabbed line indicates the organisms are no motile (Aznar *et al.*, 2015).

Gram Staining

Thin smears were made on clean, grease-free glass slides, which were then fixed by passing them over a Bunsen flame. A crystal violet solution was applied to cover the smears for 30 seconds and then washed with distilled water. Next, Lugol's iodine was applied to the surface for 30 seconds. Acetone was used as a

decolorizer and applied briefly. Safranin solution was applied on the surface for 30 seconds, washed and allowed to air dry at room temperature. The stains were observed under a microscope after oil immersion was applied onto the slides. Consequently, a red stain indicated Gram-negative and a purple stain indicated Gram-positive bacteria (Makawi *et al.*, 2021).

Results

Mean Counts of Aerobic Mesophilic Bacteria and *Bacillus* sp

This study, Isolation and Identification of *Bacillus cereus* associated with some ready-to-eat Soybean curds (*Awara*) sold in Kano Metropolis, Nigeria, Table 1 revealed that mean *Bacillus* sp counts ranged from $2.10 \times 10^3 \pm 137.84\text{cfu/g}$ in KMC to $1.61 \times 10^3 \pm 556.23\text{cfu/g}$ in Fagge. Other locations recorded $1.29 \times 10^4 \pm 391.10\text{cfu/g}$ (Nasarawa), $1.36 \times 10^3 \pm 439.32\text{cfu/g}$ (Gwale), $1.19 \times 10^3 \pm 403.73\text{cfu/g}$ (Dala), $5.63 \times 10^3 \pm 108.54\text{cfu/g}$ (Tarauni), $5.20 \times 10^3 \pm 111.36\text{cfu/g}$ (Ungoggo) and $3.77 \times 10^3 \pm 111.27\text{cfu/g}$ (Kumbotso). No *Bacillus* sp. detected in the control sample.

Table 1: Mean Counts of Aerobic Mesophilic Bacteria and *Bacillus* sp Obtained from *Awara* Samples sold at Kano Metropolis, Kano-Nigeria

S/N	Site	Mean <i>Bacillus</i> sp counts (cfu/g)	WHO Limit
1	Nasarawa	$1.29 \times 10^4 \pm 391^a$	1.0×10^4
2	Gwale	$1.36 \times 10^4 \pm 439^b$	
3	Fagge	$1.61 \times 10^4 \pm 556^b$	
4	Dala	$1.19 \times 10^4 \pm 403^b$	
5	Tarauni	$5.63 \times 10^3 \pm 108^a$	
6	Ungoggo	$2.20 \times 10^3 \pm 111^b$	
7	Kumbotso	$3.77 \times 10^3 \pm 111^b$	
8	KMC	$2.10 \times 10^3 \pm 137^b$	
9	Control	Nil	

Values are in mean $\pm$ standard error of mean and values with different superscripts differ significantly at p (0.05). Key: KMC = Kano Municipal

Morphological and biochemical characteristics of the bacterial isolates

Table 2 shows that four different species of *Bacillus* were identified from the analyzed samples in the sampling location (Kano Metropolis), these species are; *Bacillus cereus*, *B. thuriensis*, *B. subtilis* and *B. Megaterium*. The identification was achieved through microscopic, cultural and biochemical characteristic of the bacterial isolates

Table 2: Microscopic, Cultural and Biochemical Characteristics of Bacteria Isolated from *Awara* at Kano Metropolis- Kano Nigeria

Cultural Characteristics of the isolate	GR	NIT	MVP	MOT	LCT	PRGB	Suspected Organism
Pink colony, often rough, granular and dry with irregular or feathery edges	+	+	+	+	+	+	<i>Bacillus cereus</i>
Creamy white to pinkish-white colony, large dry and matte-surfaced colony, often 2-7mm in diameter	+	+	+	+	+/-	+	<i>B. thuriensis</i>
Generally yellow, creamy or white colonies	+	+	+	+	-	-	<i>B. subtilis</i>
Creamy white to pale, sometime turning yellowish or brownish with prolong incubation	+	+	+	+	-	+	<i>B. Megaterium</i>

Key: GR = Gram reaction, - =Negative, + =Positive, MVP= Modified Voges Proskauer, NIT= Nitrate test, CIT = Citrate Utilization test, MOT = Motility test, LCT= Lecithinase test and PRGB = Phenol Red Glucose Broth

Frequency of occurrence of bacterial isolates

Table 3 shows that four different species of *Bacillus* were identified from the analyzed samples in the sampling location (Kano Metropolis), these species are; *Bacillus cereus*, *B. thuriensis*, *B. subtilis* and *B. Megaterium*. The identification was achieved through microscopic, cultural and biochemical characteristic of the bacterial isolates

Table 3: Frequency of occurrence of *Bacillus Cereus* isolated from the Awara samples sold in the study area

S/N	Site	Samples analyzed	Samples contaminated by <i>B. cereus</i>	Percentage (%)	p-value
1	Nasarawa	25	3	12	0.01400*
2	Gwale	25	8	32	
3	Fagge	25	4	16	
4	Dala	25	2	8	
5	Tarauni	25	0	0	
6	Ungoggo	25	0	0	
7	Kumbotso	25	2	8	
8	KMC	25	4	16	
9	Total	200	23	11.5	

Key: * = The result is not statistically significant ($X^2 = 10.93$, and $df=7$)

KMC = Kano Municipal and *B. cereus* = *Bacillus cereus*

Discussion

This study revealed that mean *Bacillus* sp counts in the study area ranged from $2.10 \times 10^3 \pm 137.84$ cfu/g to $1.61 \times 10^4 \pm 556.23$ cfu/g. These falls within the acceptable limit of World Health Organization (WHO, 2020) of 10^3 - 10^5 cfu/g, similarly Food and Agricultural Organization (FAO, 2019) guidelines indicate the acceptable limit for *B. cereus* to be 10^3 - 10^5 cfu/g for ready to eat foods. *Bacillus cereus* is a spore-forming bacterium commonly found in soil and various food products, including Awara. The potential for *Bacillus cereus* to produce heat-stable toxins poses a significant risk of foodborne illnesses if contaminated products are consumed (Sonu *et al.*, 2014). The present study focuses on the sectional Cross sectional studies on *Bacillus cereus* among Awara samples sold in Kano Metropolis, Kano State-Nigeria. The 11.56% occurrences of *Bacillus cereus* in the Awara samples revealed light contamination. This observation corroborates to the findings of several researchers (Ugwu *et al.*, 2009; Odeyele *et al.*, 20214; Ocholi, 2018) who evaluated microbial contamination in foods. However, the occurrence (11.5%) observed in this study was lower than the highest occurrence of (34%) reported by Ocholi (2018). This variation could be attributed to sources of contamination and storage conditions.

The cultural, morphological and biochemical characteristics of *Bacillus cereus* in this study conform to the features reported by several researchers (Ugwu *et al.*, 2009; Odeyele *et al.*, 20214; Ocholi, 2018) who isolated and characterized *Bacillus* species in grains and processed product of soybean (Awara). The ability of the bacterial isolates to utilize common sugars such as glucose, sucrose, lactose and maltose, indicates their high enzyme-producing potentials. This ability had been described by other

researchers (Ugwu *et al.*, 2009; Odeyele *et al.*, 20214; Ocholi, 2018).

The isolation of *Bacillus* spp. across all the sampling locations highlights their ubiquity in the environment and their ability to survive food processing conditions due to spore formation. The predominance of *Bacillus cereus* among the isolates is particularly significant, given its ability to produce heat-stable emetic toxins and heat-labile enterotoxins. Ishaq *et al.* (2018) reported high counts of *Bacillus* spp. in Awara samples from Kaduna Metropolis, attributing their persistence to post-frying contamination and improper storage.

Conclusion

This study, Isolation and identification of *Bacillus cereus* associated with ready-to-eat Awara sold in Kano metropolis. A total of 200 samples were analyzed for aerobic mesophilic bacterial counts, with particular emphasis on *Bacillus* species. This study concluded that the mean *Bacillus* sp counts ranged from $2.10 \times 10^3 \pm 137.84$ cfu/g to $1.61 \times 10^3 \pm 556.23$ cfu/g and the *B. cereus* species isolated from the analyzed samples are: *Bacillus cereus*, *B. thuriensis*, *B. subtilis* and *B. Megaterium*. The overall occurrence of *B. cereus* was 11.5%, with variation across different sites. The detection of these organisms suggests fecal contamination, poor personal hygiene of vendors, cross-contamination and inadequate temperature control. In contrast, the control sample showed absence of *Bacillus* species, confirming that contamination likely occurred during post-processing handling and vending.

Acknowledgment

We are grateful to all our study participants who join the study voluntarily. We are grateful to Technologists and laboratory attendant of Microbiology Laboratory Aliko Dangote University of

Science and Technology, Wudil for providing enabling environment, resources and techniques for this study. We really salute their wonderful efforts.

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