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# IMPACT OF STORAGE CONDITIONS AND SODIUM CHLORIDE ON THE PRODUCTION OF HAEMOLYSIN BL ENTEROTOXIN BY *Bacillus cereus* ISOLATES FROM BOILED RICE AND ROASTED BEEF

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## ABSTRACT
Haemolysin bl (HBL) is one of three enterotoxins produced by *Bacillus cereus* that result in food infections. Therefore, this study evaluated the effects of varying storage temperature, storage duration and sodium chloride concentration on HBL enterotoxin production by *B. cereus* isolates recovered from boiled rice and roasted beef. *B. cereus* isolates were inoculated into cooked rice and roasted beef (inoculum size of 8.5 $\log_{10}$ CFU/mL) treated with varying sodium chloride concentrations (0. 0.5, 1, 1.5, 2 % w/w), and stored for 24-72 h at 4-15 ℃, and 24 h at 26-28 ℃, and 37 ℃. Highest enterotoxin titres of ≥64 ng/ml were recorded from rice isolate at all levels of NaCl concentration at elevated (37 ºC) and room temperature (26-28 ºC). At 72 h refrigerated storage, enterotoxin titres reduced to 32 ng/ml in most rice samples. Lower enterotoxin titres (16 and 32 ng/ml) were recorded from roasted beef at 2% NaCl concentrations during storage at 37 ºC and room temperature (26-28 ºC), respectively. Negative correlations (r) were observed between toxin titre (ng/ml) and counts for rice isolates at refrigerated storage at 48-72h (-0.63 and 0.28). There were positive correlations (r) between toxin titres and counts for roasted beef at higher storage temperatures, than was observed at refrigerated storage, with values increasing from 0.44-0.98, 48-72 h, respectively. Results suggest temperature, storage duration and NaCl concentration influenced toxin titres. Food type appeared to influence toxin titres, as rice isolates produced higher toxin titres than roasted beef isolates under same elevated storage temperature.

**Keywords:** *Bacillus cereus*, Haemolysin bl, Boiled rice, Roasted beef, Sodium chloride, Temperature

## INTRODUCTION
Microbial contamination of food products remains a critical global public health concern, posing severe risks to human health and placing a heavy economic burden on the food industry (Swarnakar *et al.*, 2024). Among the primary bacterial pathogens responsible for foodborne outbreaks, *Bacillus cereus (B. cereus.)* is a major culprit. *Bacillus cereus* is a Gram-positive, facultative anaerobic, spore-forming rod ubiquitously distributed in soil, dust, plant surfaces, and the rhizosphere (Komprda *et al.*, 2025). Due to the pervasive nature and intrinsic stress resistance of its endospores, *B. cereus* easily enters the food production chain via crops and raw materials, leading to widespread contamination across a broad range of food matrices (De Bellis *et al.*, 2015; Komprda *et al.*, 2025). *B. cereus* is the cause of 1.4-12% of global foodborne outbreaks, a figure that may be understated, as per Grutsch *et al.* (2018). In 2024, the European Food Safety Authority (EFSA) reported 127 foodborne outbreaks that were attributed to *B. cereus* toxins. This represents the highest number of recorded cases of illness for this pathogen, with 3,315 cases of illness, 49 hospitalizations, and nine deaths, accounting for 1.9% of all reported outbreaks (EFSA & European Centre for Disease Prevention and Control (ECDC), 2025).

As a prominent member of the *Bacillus cereus sensu lato* group, which encompasses genetically closely related species such as *B. mycoides* and *B. thuringiensis* (Skriver *et al.*, 2026). *B. cereus* causes two distinct types of food poisoning: an emetic syndrome induced by the pre-formed, heat- and acid-stable toxin cereulide, and a diarrheal syndrome caused by heat-labile enterotoxins (Komprda *et* al., 2025). The etiology of the diarrheal syndrome depends on the survival of ingested spores or vegetative cells through the hostile, low-pH environment of the stomach to reach the small intestine alive, where enterotoxins are subsequently produced (Tirloni *et al*., 2017).

Hemolysin BL (HBL) was selected as the target enterotoxin for this study due to its critical role as a primary virulence factor in *Bacillus cereus*-induced diarrheal syndrome. Unlike single-component toxins, HBL is a tri-component enterotoxin (B, $L_1$, $L_2$) that exhibits potent cytotoxic, dermonecrotic, and hemolytic activities, directly causing intestinal epithelial damage and fluid accumulation (Senesi & Ghelardi, 2010). Furthermore, the high prevalence of HBL-producing strains in food poisoning outbreaks makes it a key target for understanding the pathogenic mechanism and bioactivity of *B. cereus* enterotoxins.

To mitigate microbial proliferation in ready-to-eat (RTE) meals, which are intrinsically prone to pathogen survival and contamination, standard food safety protocols recommend adhering to the "40–60" rule, maintaining

food temperatures strictly below 40°C or above 60°C (Kameník *et al.*, 2025). However, foodborne pathogens frequently encounter sub-lethal stress along the food chain. Recent evidence demonstrates an intimate link between stress resistance and virulence regulation in *B. cereus* (Meng *et al*., 2025). Foodborne pathogens must have developed defence mechanisms to withstand the severe environmental and processing conditions, such as hunger and acidic stomach conditions, that they encounter along the food chain and right before they enter the gut. Indeed, it has been demonstrated that microbial pathogenesis largely depends on bacteria's resistance to oxidative stress and other challenges not posed by the stomach's low pH. In addition to highlighting the intricacy and fine-tuning of bacterial gene expression control systems, this suggests a close link between virulence and stress resistance (Guillén *et al.*, 2021).

Important unanswered issues regarding bacterial physiology and the variability of microorganisms both within and between species are raised by how microorganisms modify their metabolism and cell machinery in response to environmental changes (e.g., nutrient availability, temperature, pH, salinity, inhibitors, etc. (Gonzalez and Aranda, 2023). Although *B. cereus* has typically been linked to complex foods that have rice as an ingredient, other rice-based goods and farinaceous foods, such as pasta and noodles, can also be a source of contamination and contribute to *B. cereus* poisoning (Rodrigo *et al.*, 2021). Due to the widespread consumption of rice, microbiological contamination in cooked rice-based meals is a global concern (Owusu *et al.*, 2023). Microorganisms may contaminate rice during growing, harvesting, milling, storage, or processing. During cooking, heat-resistant *Bacillus cereus* spores survive the heating process. If the cooked rice is subsequently kept under improper storage conditions, it provides a favourable environment for bacterial growth. Under these favourable conditions, the surviving spores germinate into vegetative cells, which rapidly multiply. If this sequence of events occurs, *B. cereus* may produce toxins that cause foodborne illness (Bursová *et al.*, 2024).

Additionally, cooked rice is frequently served alongside popular ready-to-eat street foods such as roasted beef a widely consumed mutton product traditionally prepared over a coal or wood fire, particularly in northern Nigeria, with salt and spices added to taste (Abdullahi *et al.*, 2019; Ribah *et al.*, 2022). Since ancient times, sodium chloride (NaCl), also referred to as salt, has been used as a food preservative (Kim *et al*., 2021). The food matrix itself can substantially influence microbial growth and virulence factor expression, as differences in nutrient composition, water activity, and pH between carbohydrate-rich and protein-rich substrates create distinct physicochemical environments for bacterial proliferation (Skriver *et al.*, 2026). However, the interaction between sub-inhibitory NaCl concentrations, storage temperatures, and the regulation of virulence factors like HBL enterotoxin in contrasting food matrices (carbohydrate-rich rice vs. protein-rich meat) remains insufficiently characterized. Against the above backdrop, this study evaluated the combined effect of sodium chloride concentration and storage conditions on HBL toxin production by *Bacillus cereus* isolates recovered from boiled rice and roast beef.

## MATERIALS AND METHODS

### Standardisation of *B. cereus* Inoculum

Presumptive *Bacillus cereus* isolates from retail rice and roasted beef were characterised biochemically for confirmation using a Microgen® Bacillus ID identification kit (Microgen Bioproducts, U.K. Confirmed *B. cereus* isolates F2 and $Bln_3g_2$, isolated from plain boiled rice and roasted beef, respectively, were selected as the representative isolates from each food matrix, as these isolates exhibited the highest percentage probability (99%) with the Microgen identification system software for *Bacillus cereus.* Isolates where standardised before inoculation in food samples by transferring portions of 18 to 24 h cultures of each into sterile physiological saline to make McFarland standard 1 of the test isolates, giving an approximate cell count of $3\times10^8$ cfu/ml. The inocula were used immediately after preparation (Bello *et al*., 2020)

### Growth and Enumeration of *B. cereus* Isolates at Varying Sodium Chloride (NaCl) Concentrations and Temperatures

*B. cereus* isolates F2 and $Bln_3g_2$ were inoculated into cooked local parboiled rice and roasted bee, respectively, as described by Bello *et al.* (2020). 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 (900 g) of washed, milled rice was placed in a small aluminium pot and soaked with 900 ml of boiled, cooled water (0% NaCl to serve as the control sample) 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 100 g of the cooked grits was placed in each of 9 sterile glass bottles and tyndallized by heating in water to 100 $^oC$ holding for 15 min, followed by incubating at room temperature (25-29 $^oC$ ). This process was repeated for three consecutive days. Accordingly, four other sets of 900 g of the rice grits were each soaked and cooked in 0.5, 1, 1.5, and 2% (W/W) NaCl solutions, put in sterile glass jars, and also heat-treated as above. Following tyndallization, sterile cooked rice grits in each jar were then inoculated with 1 mL suspension of an approximate cell count of $3\times10^8$ cfu/ml of isolate F2.

Roasted beef (salt-less and spice-less) made from mutton meat was purchased from a suya spot in Kwangila, Zaria. In a washed, dried mortar, the roasted beef pieces were pulverised 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 the rice samples, placed in

sterile glass jars, and sterilised as the rice samples above. Sterile roasted beef weighing 100 g was placed in each bottle and then inoculated with 1 mL suspension of an approximate cell count of $3\times10^8$ cfu/ml of isolate $Bln_3g_2$, as was done for boiled rice samples.

Samples at NaCl concentration levels of 0, 0.5, 1, 1.5, and 2% (3 sets for both rice and roasted beef samples were incubated at room temperature for 24 h, at 37 °C for 24 h, and at refrigeration temperature for 72 h. These time-temperature combinations represent what is observed in household settings where refrigerated food samples are typically stored and consumed over longer periods. At the end of each storage period, food homogenates were made of each food sample using 25 g of food sample in 225 mL of sterile 0.1% buffered peptone water. Dilutions $10^{-5}$, $10^{-7}$, $10^{-9}$ and $10^{-11}$ of 10 - fold serial dilutions of the food homogenates were pour-plated on sterile nutrient agar (Oxoid, UK) plates and incubated at 37 °C for 24 h for enumeration of counts. Colonies were counted with the aid of a digital colony counter, calculated, and expressed as colony-forming units per gram (Cfu/g) of sample.

**Determination of HBL Diarrhoeal Enterotoxin Titers**

Cell-free culture filtrates were prepared from Brain Heart Infusion (BHI) broth extracts of inoculated food samples. HBL enterotoxin titers were determined using the BCET-RPLA toxin detection kit (Oxoid, UK), which specifically targets the $L_2$ subunit of the HBL complex (detection limit: 2 ng/mL). Two-fold serial dilutions of the cell-free filtrates were prepared in V-bottom microtiter plates and mixed with sensitized latex beads according to the manufacturer's guidelines. After incubation for 20–24 h at room temperature, agglutination patterns were recorded to establish enterotoxin titers (ng/mL).

**Statistical Analysis**

Pearson product-moment correlation coefficients (r) were computed to determine relationships between bacterial density ($\log_{10}$ CFU/mL) and toxin titers (ng/mL) at the end of each storage period for each set of samples. Analysis of Covariance (ANCOVA, SPSS 27) was employed to determine for significant differences (at 5% level of significance) in toxin titres of test isolates.

**RESULTS AND DISCUSSION**

The combined effects of sodium chloride (NaCl) concentrations (0–2% w/w), storage temperatures (4–15, 26–28, and 37 °C), and incubation periods (24– 72 h) on *Bacillus cereus* proliferation and diarrhoeal enterotoxin expression in boiled rice and roasted beef are presented in Tables 1 and 2.

**Boiled Rice Matrix**

In the boiled rice substrate, maximum enterotoxin production (64 ng/ml) was consistently observed across all NaCl levels (0–2%) following 24 h of incubation at optimum (37°C and ambient (26–28 °C) temperatures (Table 1). The level of enterotoxin titres was high at 64 ng/ml up to 48 h at (4–10 °C) for all the treatments. Despite an increase in bacterial count, the level of enterotoxin titres decreased to 32 ng/ml for most of the samples at 72 h (4–10 °C). A strong positive association for the correlation analysis was shown in 24 h at (37 °C) (r = 0.96, weak correlation was shown in 72 h at cold storage (r = 0.28).

The persistence of high hemolysin bl (HBL) diarrhoeal enterotoxin titres (64 ng/mL) across varying combinations of sodium chloride (NaCl) concentration (0–2 %w/w) and storage temperature (4–37 °C) revealed phenotypic resilience of *Bacillus cereus* in plain boiled rice ready-to-eat (RTE) food vehicles. The lack of significant suppression of antigenic cross-reactivity between cell-free culture filtrates and immune rabbit globulins suggestsub-lethal osmotic stress (0–2% NaCl) may not impair HBL enterotoxin synthesis or alter its structural antigenicity. Storage temperature is a paramount extrinsic parameter governing microbial growth kinetics and food safety (Jeon *et al.,* 2025). Outbreaks associated with *B. cereus* arise almost exclusively due to temperature abuse during the storage of growth-supportive food products (Charpe *et al.,* 2019). At elevated storage temperatures (26–28 and 37 °C), rapid vegetative population growth coincided with marked organoleptic deterioration. This indicates that the physiological stress encountered during rapid population expansion triggers robust virulence factor expression.

In the present study, an elevated level of enterotoxin titer (64 ng/ml) at 24 and 28 h of storage in the refrigerator (4–15 °C) shows that the combination of salting and refrigeration was not sufficient to prevent HBL secretion. Enterotoxin and emetic toxin can be produced by Bacillus cereus, and eating food tainted with endospores can result in Bacillus foodborne diseases (Wang *et al.,* 2014). The complex process of enterotoxin gene expression depends on the interaction of multiple transcriptional regulator proteins, which in turn react to a range of signals, including the availability of carbohydrates and nitrogen (CcpA, CodY), the cell's energy status (CodY), oxygen status (ResD, Fnr), phase transition (SinR), or the quorum sensing peptide PapR (PlcR) (Jessberger *et al.,* 2020). Several proteins, including PlcR (Phospholipase C Regulator), have been shown to control the expression of HBL genes. The majority of known virulence factors in B. cereus are regulated by the transcriptional regulator PlcR (Gohar *et al.,* 2002). PapR, an auto-inducer peptide that builds up inside the bacteria when high-density cells are attained, auto-induces or activates its transcription (Slamti & Lereclus, 2002). It is repressed by the sporulation factor SpoOA (Lereclus *et al.,* 2000).

The result of the study shows a decrease in enterotoxin titre (32 ng/mL) in boiled rice after 72 h despite a high bacterial count (11.8–13.1 $\log_{10}$ CFU/mL). A possible inactivation of PlcR may explain decreases observed in toxin titres despite the high bacterial count. 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). The observed decrease in toxin titers after 72 h in boiled rice isolates may be attributable to cell density-dependent regulatory

mechanisms. In *Bacillus cereus*, the expression and secretion of Hemolysin BL (HBL) are mediated by the PlcR (Phospholipase C Regulator) transcriptional network (Senesi & Ghelardi, 2010). It is possible that nutrient depletion and metabolic waste accumulation—accelerated by cell proliferation at temperatures above refrigeration (4–37 ºC)- led to the downregulation or inactivation of PlcR activity, a phenomenon known to occur during late stationary phase and nutrient transition (Gohar *et al*., 2008). This potential cell density-dependent suppression of toxin expression is consistent with the weak positive correlation (r = 0.28) observed between toxin titer and cell counts. 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 also noted that the unchallenged isolate (NaCl) had a toxin titer >64ng/ml.

**Table 1: Combined effects of NaCl concentration, temperature and storage time on enterotoxin production by *B. cereus* isolates from boiled rice**

| Temperature (℃) | NaCl Concentration (%) | Storage Time (h) | Counts ($log_{10}$ cfu/ml) | Toxin Titre (ng/ml)* | Correlation coefficient (r) |
|---|---|---|---|---|---|
| **37** | 0 (control) | 24 | 6.6±1.0 | ≥64 | |
| | 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 (control) | 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 (control) | 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 | |

Values represent mean colony-forming units per milliliter (CFU/mL) of triplicate samples). *Assay detection sensitivity limit is 2 ng/Ml

**Table 2: Combined effects of NaCl concentration, temperature and storage time on enterotoxin production by *B. cereus* isolates from roasted beef**

| Temperature (°C) | NaCl Concentration (%) | Storage Time (h) | Counts ($log_{10}$cfu/ml) | Toxin Titre (ng/ml)* | Correlation coefficient (r) |
|---|---|---|---|---|---|
| **37** | 0 (control) | 24 | 12.9±0.5 | ≥64 | 0.99 |
| | 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 (control) | 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 (control) | 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 | |
| **4-10** | 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 | |
| **4-15** | 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 | |

Values represent mean colony-forming units per milliliter (CFU/mL) of triplicate samples *Assay detection sensitivity limit is 2 ng/mL.

**Roasted Beef Matrix**

In the protein-rich roasted beef substrate, enterotoxin titres displayed higher variability across environmental parameters (Table 2). At 37 °C, enterotoxin concentrations ranged from 16 ng/ml at 2% NaCl after 24 h to 64 ng/ml at lower salinity levels after 24 h. Similarly, at ambient temperature (26–28 °C, isolates subjected to 2 NaCl yielded a lower toxin titre (32 ng/ml) relative to lower salt concentrations (64ng/ml). Strong positive correlations between bacterial counts and enterotoxin production were revealed at 37 °C (r = 0.99) and 26–28 °C (r = 0.96). During refrigerated storage (4–10 °C), enterotoxin titres in challenge roasted beef samples (0.5–2% NaCl) reached 64 ng/ml within 24 h. Between 48 h and 72 h of cold storage, the correlation between bacterial density and enterotoxin levels shifted from a moderate positive value (r = 0.44) to a strong positive association (r = 0.98). In unchallenged control samples (0% NaCl), toxin titres increased progressively from 32 ng/ml at 24 h to 64 ng/ml at 48 h and 72 h.

Toxin titres for *B. cereus* isolates from roasted beef samples 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 after 48 and 72 h at refrigeration temperature (4-10 °C), respectively. It was also observed that, except for the isolates from the control sample (0% NaCl) at 24 h at room temperature that showed no cross-reactivity, all other isolates, irrespective of time, temperature, and NaCl, exhibited some level of agglutination. This implies that the *B. cereus* isolates in this study produce HBL diarrhoeal enterotoxin during storage of roasted beef at low and elevated temperatures 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. 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 roasted beef. 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. Reduced levels of HBL secretion caused by oxidative stress may be the cause of the lower enterotoxin content in isolates of roasted beef.

**CONCLUSION**

The study's findings indicate that, despite seemingly har sh storage conditions, *B. cereus* isolates from boiled ric

e produce higher levels of toxins compared to isolates fr om roast beef. This suggests that *B. cereus* may produce HBL toxin in response to environmental stress, with pr oduction levels closely regulated by the type of food.

**Conflict of interest:** The authors declare no financial, personal, or professional conflicts of interest that could inappropriately influence or bias the content and findings presented in this study.

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