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Artículo Original

Enterotoxigenic profile, biofilm production, and antimicrobial resistance of *Bacillus cereus* isolated from rice-based food marketed in southern Mexico

Perfil enterotoxigénico, producción de biopelícula y resistencia antimicrobiana de *Bacillus cereus* aislado de arroz comercializado al sur de México

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ABSTRACT

Bacillus cereus is responsible for food poisoning worldwide, thus, the characterization of strains isolated from food, in this case rice, is essential. Therefore, the objective of this study was to identify the toxigenic profile, lytic enzymes, antimicrobial resistance, and biofilm production of *B. cereus* strains isolated from rice. The genetic profile of toxins and biofilm-related genes of strains was determined by endpoint PCR. Biofilm was visualized by safranin staining. Evaluation of lytic enzymes was determined in culture media. Psychrophiles were monitored by the growth of the strains at refrigeration temperature. The GTG5 technique was used to determine the genetic diversity of the strains. Antimicrobial resistance of the strains was validated by minimum inhibitory concentration. The strains of *B. cereus s.l.* from rice contained genes for enterotoxins and genes associated with biofilm production. The strains did not have the cereulide gene. The strain isolated from fried rice was the only one that contained the Hbl toxin gene and the *Eps2* operon. The same strain was the only one that did not produce biofilm, had intermediate sensitivity to erythromycin, was amylase positive, had high lecithinase activity, and grew at refrigeration temperature.

Keywords: *Bacillus cereus*; rice; virulence.

RESUMEN

Bacillus cereus es responsable de intoxicaciones alimentarias a nivel mundial, siendo importante la caracterización de cepas aisladas de alimentos, en este caso, de arroz. Por lo tanto, el objetivo de este estudio fue identificar el perfil toxigénico, enzimas líticas, resistencia antimicrobiana y producción de biopelículas en cepas de *B. cereus* aisladas de arroz. El perfil genético de toxinas y los relacionados a biopelículas fue determinado por PCR en punto final. La biopelícula fue visualizada por tinción con safranina. La evaluación de enzimas líticas fue determinada en medios de cultivo. Los psicrófilos fueron monitoreados por crecimiento de cepas en temperatura de refrigeración. La técnica de GTG5 fue usada para determinar la diversidad genética de las cepas. La resistencia a antimicrobianos fue validada por concentración mínima inhibitoria. Las cepas de *B. cereus s.l.* de arroz contenían los genes de enteroxinas y genes asociados a producción de biopelículas. Las cepas no contienen el gen de la cereulida. La cepa aislada de arroz frito es la única que contiene el gen de la toxina Hbl y el operón *eps2*. Esta misma cepa no produce biopelícula, tiene sensibilidad intermedia a eritromicina, es amilasa positiva, tiene alta actividad lecitinolítica y crece en temperaturas de refrigeración.

Palabras clave: *Bacillus cereus;* arroz; virulencia.

INTRODUCTION

The *Bacillus cereus* group, or *B. cereus sensu lato* (*B. cereus s.l.*), are Gram-positive bacteria with a low GC content that belong to the Firmicutes phylum. It is a group that includes bacillus-shaped, spore-forming, aerobic to facultative anaerobic bacteria, with peritrichous flagella involved in locomotion, including at least eight highly related species: *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus* and *B. toyonensis* (Enosi Tuipulotu *et al*., 2021; Liu *et al*., 2015).

The species of the *B. cereus* group are endemic soil bacteria that occupy diverse ecological habitats due to the formation of endospores resistant to heat, UV radiation, acids, and desiccation, for which the bacteria can persist in a dormant state (Ehling-Schulz *et al*., 2019). In addition to spore production, *B. cereus* resists adverse environmental conditions due to the formation of biofilms on abiotic surfaces and living tissues (Duport *et al*., 2016). In this sense, it has been reported that *B. cereus* can produce biofilms in pipes and food storage systems, favoring the systematic contamination of food products (Majed *et al*., 2016; Shemesh and Ostrov, 2020; Wijman *et al*., 2007).

In addition to soil, species have been isolated from fresh and frozen foods, invertebrates, and plants (Ehling-Schulz *et al*., 2019). In this sense, it is estimated that *B. cereus* is responsible for 1.4%-12% of all food poisonings worldwide (Grutsch *et al*., 2018). *B. cereus* is responsible for two types

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of gastrointestinal syndromes. The emetic type is mainly characterized by nausea and emesis, which appears one hour after consumption of contaminated food and is clinically indistinguishable from *Staphylococcus aureus* enterotoxin poisoning (Stenfors Arnesen *et al*., 2008). The diarrheal type of food poisoning is also associated with various foods. The disease mainly manifests in diarrhea and abdominal cramps, like *Clostridium perfringes* food poisoning, and occurs after approximately 8 to 16 h after consumption (Jessberger *et al*., 2020; Stenfors Arnesen *et al*., 2008).

The diarrhoeal syndrome is associated with different enterotoxins produced after germination of spores and growth of vegetative cells, ingested through contaminated food and activated in the small intestine (Schoeni and Wong, 2005, Stenfors Arnesen *et al*., 2008). *B. cereus* produces three pore-forming enterotoxins: Nhe (a three-component non-haemolytic enterotoxin), Hbl (a three-component haemolytic enterotoxin) and, the single-component cytotoxin CytK. The emetic syndrome results from poisoning caused by a toxin called cereulide, which is found in food (Schoeni and Wong, 2005). Cereulide is a dodecapeptide composed of alpha amino and alpha hydroxy acids, structurally related to the potassium ionophore valinomycin. Cereulide is produced by a peptide synthetase called ces, which represents a new type of non-ribosomal peptide synthetase (NRPS) (Ehling-Schulz *et al*., 2004).

Epidemiological data show that rice, pasta, cakes, and noodles are associated with emetic syndrome, whereas vegetables, meat, and dairy products have been associated with diarrheal syndrome (Enosi Tuipulotu *et al*., 2021). In Mexico, there is currently no health legislation related to the presence of *B. cereus* in foods, but the presence of *B. cereus* in vegetables (Castulo-Arcos *et al*., 2022; Flores-Urbán *et al*., 2014), ice cream (Adame-Gomez *et al*., 2019), artisanal cheeses and eggs (Adame-Gómez *et al*., 2020b; Cruz-Facundo *et al*., 2022; 2023) has been reported. Rice is a product consumed in Mexico in different preparations, including fried rice and milk dessert with rice. In a preliminary study, *B. cereus* was isolated from 5% of rice samples, with a higher frequency in rice desserts (8.5%). However, not only the isolation of the microorganism is essential, but also the molecular characterization of different virulence factors, are needed. Therefore, the objective of this study is to identify the toxigenic profile, lytic enzymes, antimicrobial resistance, and biofilm production of *B. cereus* strains isolated from rice.

MATERIAL AND METHODS

Bacterial strains

In this study, six strains previously characterized as *B. cereus s.l.* (Cano- Ponce and Ramirez- Peralta, 2023) are included, based on the isolation and presumptive identification in MYP agar and the amplification of the *gyrB* gene as a confirmatory test (Wei *et al*., 2018). The strains are named according to unique laboratory strain codes such as B629, B630, B631, B632, B633, B634. The B629 strain was isolated from fried rice and the rest of the strains were from rice-based desserts.

Bacterial DNA extraction

For DNA extraction, 1 mL of bacterial liquid culture was centrifuged for 10 minutes at 10,000 rpm. The pellet was re-suspended in 200 µL of lysis solution (10 mM Tris-HCl, 1 mM EDTA pH 8.0, and 1 mg/mL lysozyme) and incubated for 30 minutes at 37°C. Afterwards, 250 µL of phenol-chloroform-isoamyl alcohol (ratio 25:24:1) were added and homogenized by inversion. Then, it was centrifuged at 10,000 rpm for 5 min, 200 µL of the aqueous phase was recovered to avoid contamination with organic phase (chloroform/ phenol phase) and mixed with 1 mL of cold absolute ethanol. Finally, the solution was centrifuged at 10,000 rpm for 10 min. The supernatant was completely removed, and the DNA was resuspended in 20 µL of TE buffer (Cruz-Facundo *et al*., 2023).

Enterotoxigenic profile

Detection of the *B. cereus* toxin genes was performed by PCR using conserved regions of the *nheABC* (NA2-F AAGCI-GCTCTTCGIATTC, NB1-R ITIGTTGAAATAAGCTGTGG), *hblACD* (HD2-F GTAAATTAIGATGAICAATTTC, HA4-R AGAATAGGCATT-CATAGATT) operons, which encode non-hemolytic enterotoxins and hemolysin BL, respectively; and the genes *ces* (CesF1- GGTGACACATTATCATATAAGGTG, CesR2- GTAAGCGA-ACCTGTCTG-TAACAACA) and *cytK* (P1-cytK CAAAACTCATC-TATGCAATTATGCAT, P3-cytK ACCAGTTGTATTAA-TAACGG-CAATC), which encode for emetic toxin and cytotoxin, respectively (Ehling-Schulz *et al*., 2006; Ołtuszak-Walczak *et al*., 2006). For each PCR, the mixture contained the following: 25 µL of REDTaq DNA Polymerase Ready Mix (Sigma-Aldrich, St. Louis, MO, USA), 11 µL of sterile MiliQ water, 10 to 20 ng of genomic DNA, and 0.02 µM of each oligonucleotide. The conditions for the *nhe, hbl*, and *ces* genes were 1 cycle at 94°C for 5 minutes, 25 cycles at 94°C for 30s, 49°C for 1 minute, 72°C for 1 minute, and one cycle at 72°C for 5 minutes. For the *cytK* gene it was one cycle at 94°C for 2 minutes, 35 cycles at 94°C for 30 seconds, 52°C for 1 minute, 72°C for 30 seconds, and one cycle at 72°C for 10 minutes. The *B. cereus* strains used as positive controls, were ATCC14579 for *hbl* and cytK genes, and BC133 for the *nhe* gene. The latter strain was previously isolated and characterized in the laboratory from dairy formula (Adame-Gómez *et al*., 2020a).

Determination of *B. cereus* biofilms

Biofilm determination was performed in glass and polyethylene tubes, as well as 96-well polystyrene plates. The tubes and plates were filled with 200 µL of Brain Heart Infusion (BHI) broth supplemented with 1% dextrose and inoculated with 20 µL of 24 h liquid cultures of the strains ($6x10^6$ UFC/mL). The tubes and plate were incubated at 37°C for 48 h. Later, the cultures were removed from the tubes and plates, which were then washed three times with PBS 1X (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2). Biofilms were stained with 200 µL of safranin for 30 min. Then, tubes and plate were washed three times with PBS 1X and distained with absolute alcohol for 10 minutes. The absorbance of the alcohol from safranin staining was determined at 550 nm (Adame-Gómez *et al*., 2020a).

Determination of genes involved in *B. cereus* biofilm formation

The genes involved in biofilm formation were amplified by endpoint PCR using the following oligonucleotides for each of the genes of interest: *sipW* (sipW- F AGATAATTAGCAACGCGATCTC, sipW-R AGAAATAGCGGAATAACCAAGC), tasA (tasA-F AGCAGCTTTAGTTGGTGGAG, tasA- R GTAACTTATCGCCTTGGAATTG), *calY* (calY-F AGGTATCGGGAGTTCATCAG, calY R CAGCTTCTTGGTTGGCATTG), and *eps2* (eps2-F TGTTTTGAGCGGATTTGTTTTGT, eps2-R GATTGCTCTGCCAATGTCTTT) (Caro-Astorga *et al*., 2014; 2020). For each PCR, the mixture contained the following: 25 µL of REDTaq DNA Polymerase Ready Mix (Sigma-Aldrich, St. Louis, MO, USA), 11 µL of sterile MiliQ water, 10 to 20 ng of genomic DNA, and 0.02 µM of each oligonucleotide. The conditions for the *sipW*, *tasA*, and *calY* genes were 1 cycle at 94°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 61°C for 45 seconds, 72°C for 45 seconds, and a cycle of 72°C for 5 minutes. For the *eps2* operon, it was one cycle at 94°C for 2 minutes, 35 cycles at 94°C for 30 seconds, 52°C for 30 seconds, 72°C for 90 seconds, and one cycle at 72°C for 10 minutes. The *B. cereus* ATCC14579 strain was used as a positive control (Cruz-Facundo *et al*., 2023).

Determination of *B. cereus* extracellular enzymes and growth at low temperatures

The determination of extracellular enzymes was carried out by inoculating 2 µL of a 24-h culture on different agar plates: BHI with 1% starch agar, 5% casein agar, 5% gelose blood agar, and 10% MYP agar of egg yolk emulsion. The agar plates were incubated at 30°C for 24 h, and the 5% blood agar plates were incubated at 10°C for 10 days. After incubation, 30 µL of lugol were added to demonstrate amylolytic activity on 1% starch agar. For proteolytic and lecitinolytic activity, the presence or absence of hydrolysis zones was observed in 5% casein agar and 10% egg yolk emulsion agar (Claus and Berkeley, 1986). To estimate the lecithinase activity, the ratio was estimated by zone diameter of colony, and zone diameter of halo. In the case of psychotropic capacity, growth/non-growth at 10°C of the strains was monitored for ten days by visual observation, considering the day on which growth was observed as the detection time (DT) (Berthold-Pluta *et al*., 2019; Cruz-Facundo *et al*., 2023).

Genetic diversity of *B. cereus*

The phylogenetic relationship between the strains of the *B. cereus* group was determined using the repetitive palindromic element PCR technique (rep-PCR) using GTG^5 primers (GTGGTGGTGGTGGTG) with the following reaction conditions: initial denaturation at 95°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 40°C for 2 minutes, 72°C for 4 minutes and a final extension of 72°C for 5 minutes (De Jonghe *et al*., 2008). Electrophoresis was performed in 2% agarose gels at 90V for 120 minutes. The gels were stained with Midori Green (Nippon Genetics, Germany) and visualized with LED light.

The DICE similarity coefficient was calculated to establish the genetic distances of the profiles. The genetic distance matrix was analyzed by the UPGMA method. A dendrogram was made with the analyzed data using NTSYS 2.0 software.

Antimicrobial resistance in *B. cereus*

Broth microdilution testing was performed for each strain using Mueller-Hinton, broth in accordance with CLSI guideline M45: ED3 (Hindler and Richter, 2016) . A total of ten antibiotics (Oxoid, UK) were tested: ampicillin (0.12 - 16 µg/mL), ciprofloxacin (0.5 - 4 µg/mL), clindamycin (0.2 - 4 µg/mL), gentamicin (2 -500 µg/mL), tetracycline (2-16 µg/mL), trimethoprim (0.5 - 9.5 µg/mL), kanamycin (2-500 µg/mL), vancomycin (1-64 µg/mL), erythromycin (0.5 - 8 µg/mL) and chloramphenicol (0.5 - 32 µg/mL). The *B. cereus s.l.* inoculum was prepared from a 24 h culture by first adjusting the culture concentration to 0.5 McFarland of the standard turbidity scale (1×10^8 CFU/mL). The final inoculum concentration in the microplate was 5×10^5 CFU/mL. Each plate included a positive control (MH broth with inoculum without antibiotic) and a negative control (MH broth without inoculum with antibiotic). The microplates were incubated at 30°C for 24 h. Ten µL of a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution were added to verify growth and incubated for 30 min at 30°C in the dark. Formazan production indicated the presence of viable cells.

RESULTS AND DISCUSSION

In Mexico, the annual per capita consumption of rice is 8.5 kg, considered the second crop with the highest expenditure among mexican families (SAGARPA, 2017). However, *B. cereus*, a pathogen associated with foodborne diseases worldwide, has been isolated in rice (Dietrich *et al*., 2021; Enosi Tuipulotu *et al*., 2021). Rice and its derivatives serve as an ideal growth medium for bacteria, particularly *B. cereus*, due to their compositional and chemical characteristics (pH close to 7, approximately 79% carbohydrates, 7% protein, and 2% fat). These properties, combined with the resilience of *B. cereus* spores, which can survive in dehydrated grains and withstand the temperatures typically applied during processing, enable the bacterium to proliferate under a variety of conditions (Jaquette and Beuchat, 1998). Therefore, in this study, strains of *B. cereus s.l.* were characterized and isolated from different rice-based preparations.

In the different studies of *B. cereus* in rice, strains with the same toxigenic profiles have not been isolated, and even the frequencies change depending on the type of rice and country (Ankolekar *et al*., 2009; Kim *et al*., 2009; 2014; Park *et al*., 2009), which is why studying the diversity of *B. cereus* strains in Mexico is essential. A total of six strains of the *B. cereus* s.l. group previously isolated from rice were characterized. All these strains were positive for the non-hemolytic toxin gene (*nhe*) and negative for the cereulide toxin gene (*ces*). Only one strain (B629) was positive for the hemolytic toxin BL gene (*hbl*) and negative for the cytotoxin K gene (*cytK*) (Table 1) (Figure 1).

In this study, a remarkable occurrence of the non-hemolytic toxin gene (*nheABC*) with a 100% frequency was

Table 1. Toxin-coding genes identified in the *B. cereus s.l.* strains from this study.
Tabla 1. Genes de las toxinas identificadas en las cepas de *B. cereus s.l.* de este estudio.

Strain	*hbl*	*nhe*	*cytK*	*ces*
B629	+	+	-	-
B630	-	+	+	-
B631	-	+	+	-
B632	-	+	+	-
B633	-	+	+	-
B634	-	+	+	-

identified in the analyzed strains. Globally, non-hemolytic toxin genes are frequently reported, often reaching values of up to 100%. In Mexico, several food products, including cheese, ice cream, eggs, and vegetables, have shown a high prevalence of non-hemolytic toxin genes (Adame-Gomez *et al*., 2019; 2020b; Castulo-Arcos *et al*., 2022; Cruz-Facundo *et al*., 2022; 2023); it is important to note that these findings do not exclude the possibility that these strains are circulating in rice, in which, the frequency of non-hemolytic toxin genes show a range from 47.3% to 100%. The variability in these results is suggested to be related to the specific type of rice analyzed. For example, in ready-to-eat rice-based foods, a frequency of 47.3% was observed (Chen *et al*., 2022) while frequencies approaching 100% were identified in different types of rice, including white rice, brown rice, black rice, hard rice, and glutinous rice (Ankolekar *et al*., 2009; Kim *et al*., 2009; 2014; Park *et al*., 2009).

The frequencies of Hbl toxin genes were low (16.6%) compared to the global reported frequencies for these toxin genes (40-70%) (Jessberger *et al*., 2020). As for Mexico, the frequencies are low (Adame-Gomez *et al*., 2019; 2020b; Castulo-Arcos *et al*., 2022; Cruz-Facundo *et al*., 2022; 2023) as in this study, which does not rule out a regional circulation of *B. cereus* strains in different food products. Regarding rice, the reported frequencies of Hbl toxin genes are high compared to this study. The lowest frequencies for the Hbl toxin are found in strains isolated from rice-based ready-to-eat foods (36.3 %), and the highest frequencies are found in white and brown rice (83-100%) in China and Korea. In the United States, even when it was isolated from white and brown rice, the frequency of the Hbl toxin was low (56.6%), so it is not surprising that not only the type of product, but also the geographic region, is important in the frequency of enterotoxins. In this sense, the regionalization of diarrheal and emetic syndromes with the dietary habits of each country has even been described (Enosi Tuipulotu *et al*., 2021; Kotiranta *et al*., 2000).

Another virulence factor, in addition to spore formation and toxin production by *B. cereus*, is the ability to form biofilms. Biofilms are bacterial communities that, in the case of *B. cereus*, have been described in food production areas, becoming a problem because, on one hand, they favor systematic contamination of food. On the other hand, the formation of biofilms prevents the eradication of the microorganisms trapped in it due to the characteristics of resistance to disinfectant agents (Majed *et al*., 2016).

Regarding biofilm formation, strains B631, B632, B633, and B634 exhibit substantial biofilm production on glass material in contrast to strain B629 ($p = 0.0211$, $p = 0.0445$, $p = 0.0211$, $p = 0.0216$). Notably, three strains (B632, B633, B634) show the ability to form biofilms on polystyrene. It is noteworthy that none of the strains form biofilms on polyethylene as shown in Figure 2.

In this investigation, prolific biofilm production was observed exclusively on glass material, in contrast to minimal production on polyethylene and, to a lesser extent, polystyrene. The variation in biofilm production on different materials is consistent with previous literature which attribute such differences to the hydrophobic properties and

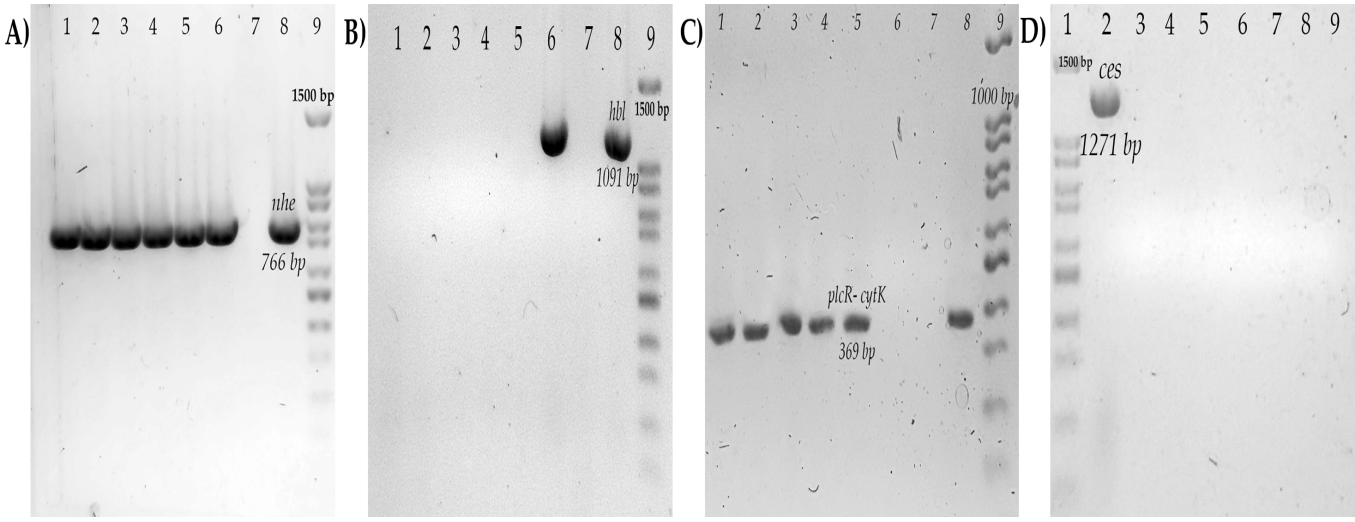

Figure 1. Gel electrophoresis of PCR products from toxin profile of *B. cereus s.l.* A) *nheABC*, B) *hblABD*, C) *plcr- cytK*. D) *ces*. Lane 1. B634, Lane 2. B633, Lane 3. B632, Lane 4. B631, Lane 5. B630, Lane 6. B629, Lane 7. Negative control, Lane 8. Positive control *B. cereus* ATCC 14579 (*hbl*+, *plcr- cytK*+).
Figura 1. Electroforesis en gel de los productos de PCR del perfil de toxinas de *B. cereus s.l.* A) *nheABC*, B) *hblABD*, C) *plcr- cytK*. D) *ces*. Carril 1. B634, Carril 2. B633, Carril 3. B632, Carril 4. B631, Carril 5. B630, Carril 6. B629, Carril 7. Control negativo, Carril 8. Control positivo *B. cereus* ATCC 14579 (*hbl*+, *plcr- cytK*+).

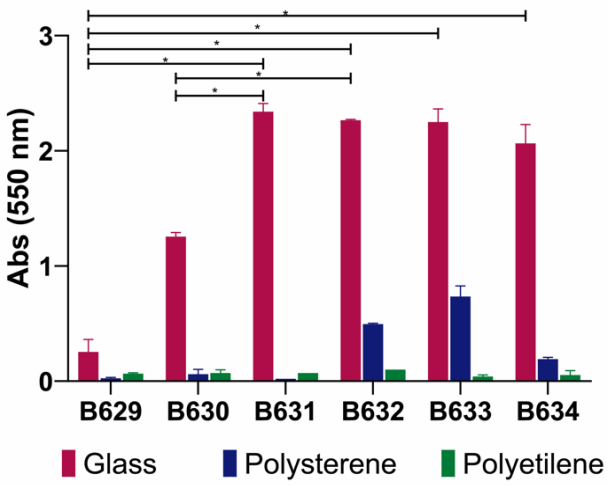

Figure 2. Biofilm production of *B. cereus s.l.* strains in different materials to 37°C for 48h. Safranin absorbance data from two independent assays are shown, represented as mean and standard deviation. The p values were calculated from the ANOVA statistical test with Tukey posthoc.
Figura 2. Producción de biofilm de las cepas de *B. cereus s.l.* en diferentes materiales a 37°C por 48h. Se muestran los datos de absorbancia de safranina de dos ensayos diferentes, representados como media y desviación estándar. El valor de p fue calculado por la prueba estadística de ANOVA con posthoc de Tukey.

surface roughness of the materials(Adame-Gómez *et al*., 2020a; De-la-Pinta *et al*., 2019). In addition, the presence of trace elements, particularly iron, has been implicated in biofilm production. Hayrapetyan *et al*. (2015) reported increased biofilm production on stainless steel, attributed to iron ions, compared to polystyrene. Within our research group, previous studies have indicated higher biofilm production on PVC compared to other materials (Adame-Gómez *et al*., 2020a; Cruz-Facundo *et al*., 2022). It's worth noting that discrepancies between our results and those of this study may be related to the different origins of the strains. Hayrapetyan *et al*. (2016) relate the origin of the isolation to the adaptation capacity of the strains to environments with different iron conditions.

Regarding the genes related to biofilm production, only strain B629 has the *eps2* operon. All strains have the *sipW-tasA-calY* operon genes (Figure 3). In *B. cereus*, the participation of different genes in the production of biofilms has been described; these genes are grouped in the *sipW-tasA-calY* and *eps2* operons (Caro-Astorga *et al*., 2014; 2020). SipW is a peptidase that participates in the maturation of the tasA protein, which functions in the formation of amyloid-type fibers, which are part of a mature biofilm (Caro-Astorga *et al*., 2014). CalY fulfills a dual function during biofilm production, participating as an adhesin on glass but not polystyrene in the early stages of biofilm formation and forming fibers during the late phases (Candela *et al*., 2019). *Eps2* is an operon that include genes related to the extracellular matrix, the main component of biofilms (Caro-Astorga *et al*., 2020). The diverse functions these proteins fulfill explain why most of the strains in this study produce abundant biofilms on glass, except for strain B629, which, despite having all the genes related to biofilms, visually produces a biofilm that easily detaches from the air-liquid interface and therefore is not evident by safranin staining. Dogsa *et al*. (2013) showed that the width of the biofilm is related to the culture medium and to the presence of the *tasA* and *epsA* genes. The absence of one of these genes drastically reduces the thickness of the biofilm, and in the absence of both, the strain does not produce a biofilm. For B629, the production of a thin biofilm could be explained by presence of a non-functional *tasA* gene, which is reflected in a thin biofilm that easily detaches from the tube. Despite the detailed information about the participation of

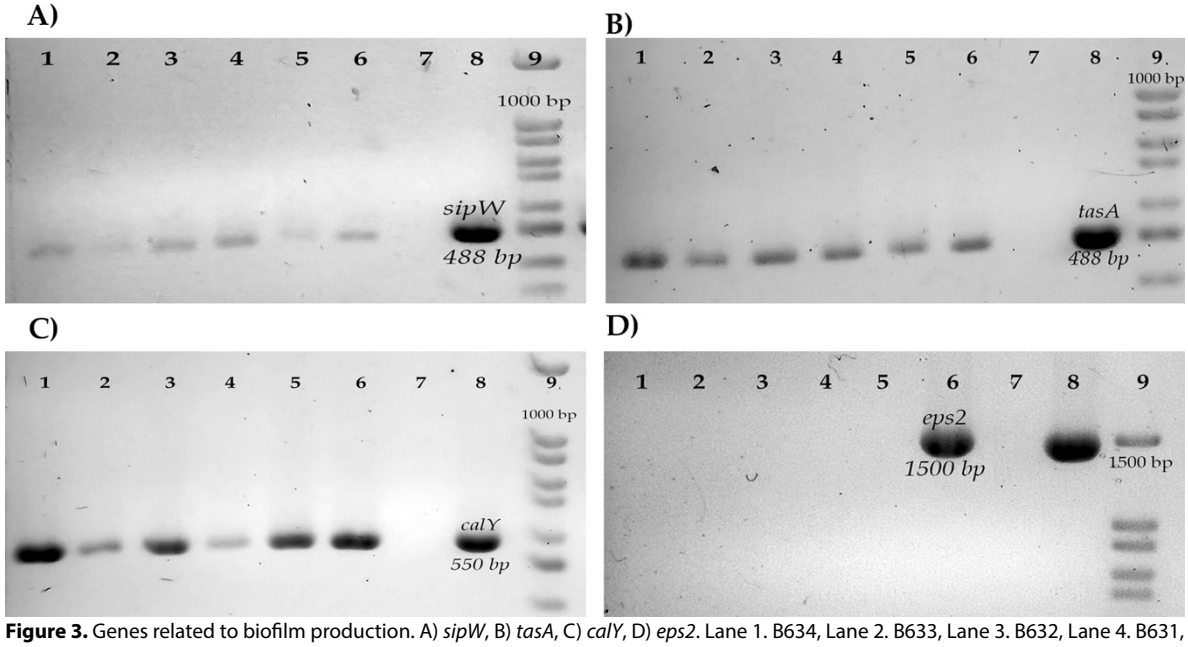

Figure 3. Genes related to biofilm production. A) *sipW*, B) *tasA*, C) *calY*, D) *eps2*. Lane 1. B634, Lane 2. B633, Lane 3. B632, Lane 4. B631, Lane 5. B630, Lane 6. B629, Lane 7. Negative control, Lane 8. Positive *B. cereus* ATCC 14579 control.
Figura 3. Genes relacionados a la producción de biopelículas. A) *sipW*, B) *tasA*, C) *calY*, D) *eps2*. Carril 1. B634, Carril 2. B633, Carril 3. B632, Carril 4. B631, Carril 5. B630, Carril 6. B629, Carril 7. Control negativo, Carril 8. Control positivo *B. cereus* ATCC 14579.

the *sipW-tasA-calY* and *eps2* operons in biofilm production (Caro-Astorga *et al*., 2020; 2014), few epidemiological studies include them in molecular characterization (Cruz-Facundo *et al*., 2023). Since biofilm production is part of the resistance mechanisms and systematic contamination of food products (Enosi Tuipulotu *et al*., 2021), not only enterotoxigenic and enzymatic profiles should be considered, but also those genetic profiles associated with biofilm production, which are included in this study.

B. cereus not only produces enterotoxins, but also a significant number of extracellular enzymes with degradative activity, such as phospholipases, proteases, chitinases, and amylases (Ivanova *et al*., 2003). This enzymatic profile is related to the ability of *B. cereus* to spoilage food and as a nutrient assimilation mechanism (Arslan *et al*., 2014). In this sense, the B629 strain is the only amylolytic strain in the study, and coincides with being the only strain isolated from fried rice. Strains isolated from sweet rice are capable of producing proteases, which can have a significant impact on the decomposition of the product, since it has been described that proteases are capable of gelatinizing milk (Chen *et al*., 2003). All strains produce proteases and strains in different proportions produce lecithinase. Only one strain can grow at refrigeration temperatures (B629) (Table 2). In this sense, strains of *B. cereus* are divided into two groups: psychotrophic and mesophilic. In this study, it is reported that strain B629 is a psychotrophic strain capable of growing in 5 days at a temperature of 10°C, and that the other strains, since they cannot grow at refrigeration temperatures, could be considered mesophilic strains. The presence of psychrotrophic strains, such as B629, impact the food safety of food products that are preserved in the cold, including rice.

Table 2. Lytic enzymes and cold tolerance of *B. cereus s.l.* strains.
Tabla 2. Enzimas líticas y tolerancia al frío de las cepas de *B. cereus s.l.*

Strain	*Amylase*	*Protease*	*Lecithinase*	Growth in low temperatures (DT)
B629	+	+	0.71	5
B630	-	+	0.52	-
B631	-	+	0.60	-
B632	-	+	0.50	-
B633	-	+	0.50	-
B634	-	+	0.60	-

Detection time (DT): the day on which growth was observed
Tiempo de detección (DT): El día en donde se observa crecimiento

Genetic diversity techniques, such as GTG'5, have allowed strains to be grouped according to different characteristics, including growth temperature. Regarding the genetic diversity of the *B. cereus s.l.* group, strains were grouped into three clusters. In the first cluster, two clones were identified; the first clone includes strain B629 isolated from fried rice, the only strain that grows at refrigeration temperatures and is amylase positive, and is also the only one positive for the toxin BL gene (*hbl*); the second clone includes strains B632 and B633, which share most of the characteristics with the clones belonging to the other clusters (Figure 4).

The GTG'5 technique allowed the identification of two clones, one with tolerance to refrigeration temperatures, amylolytic, not a biofilm producer, and isolated from fried rice. Furthermore, the technique allowed us to separate this clone from three remaining clones isolated from sweet rice with similar genotypic characteristics, including the profile of toxins and genes associated with biofilm production. This is evidence that the strains circulating in fried rice are different from the strains found in sweet rice, which allows us to estimate that the risk of poisoning also depends on the product consumed. For example, emetic strains have been linked to the consumption of products such as pasta and rice, while the consumption of meat and vegetables has been linked to enterotoxigenic strains (Enosi Tuipulotu *et al*., 2021).

Regarding antimicrobial resistance, it was found that all strains are resistant to penicillin, ceftriaxone, clindamycin, and trimethoprim. The strains were susceptible to kanamycin,

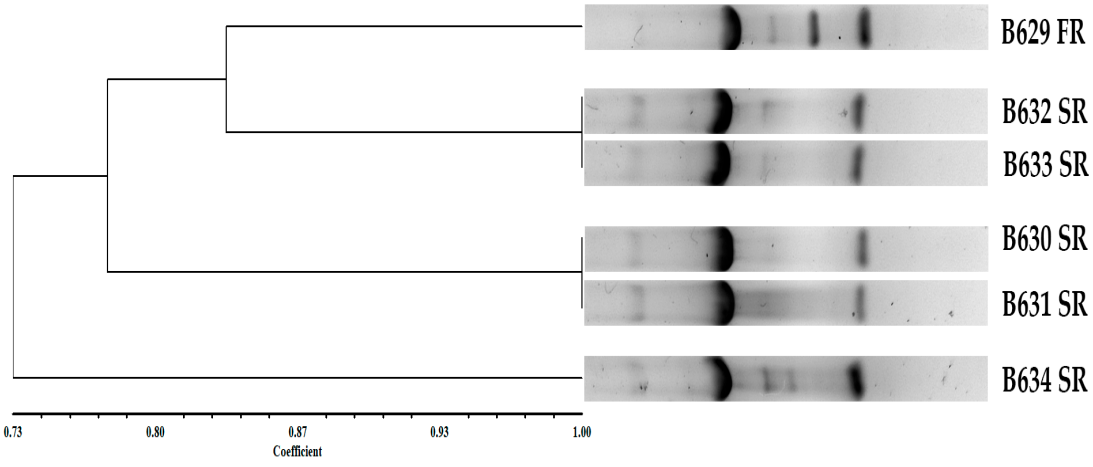

Figure 4. Dendrogram obtained by GTG-5' of *B. cereus s.l.* strains. Six *gyrB*-positive rice strains were grouped, three clusters and four clones. The DICE test was used, and the strains were grouped by hierarchical cluster. A DICE coefficient >0.8 was used to cluster the strains. FR: Fried rice. SR: Sweet rice.
Figura 4. Dendrograma obtenido por GTG-5' de las cepas de *B. cereus s.l.* Se agruparon seis cepas positivas para *gyrB*, tres grupos y cuatro clones. Se utilizó la prueba DICE y las cepas fueron agrupados por grupo jerárquico. Un coeficiente DICE >0.8 fue usado para agrupar las cepas. FR: Arroz frito. SR: Arroz dulce.

gentamicin, vancomycin, tetracycline, chloramphenicol, and ciprofloxacin. It is important to note that only B629 has intermediate sensitivity to erythromycin (Table 3).

Regarding antibiotic resistance, strains of *B. cereus s.l.* from this study were resistant to beta-lactams, which has been reported in other studies (Chen *et al*., 2022; Fraccalvieri *et al*., 2022; Park *et al*., 2009; Perera and Ranasinghe, 2012). Fraccalvieri *et al*. (2022) demonstrated that the main mechanism of resistance to beta-lactams is mediated by the beta-lactamases BLA-1 and BLA-2, since all their beta-lactam resistant strains presented these genes. In addition to resistance to beta-lactams, high resistance to trimethoprim has also been reported in other studies in strains of *B. cereus s.l.* isolated from rice and cheese (Chen *et al*., 2022; Cruz-Facundo *et al*., 2023). It is important to mention the reports related to the high susceptibility to different groups of antimicrobials such as aminoglycosides, glycopeptides, tetracyclines, and phenicol's (Chen *et al*., 2022; Cruz-Facundo *et al*., 2023; Park *et al*., 2009) which is also consistent with what was reported in these strains. However, the presence of a strain with intermediate sensitivity to erythromycin in this study (B629) should not be underestimated.

Table 3. Antimicrobial resistance of *B. cereus s.l.* strains from rice.
Tabla 3. Resistencia a antimicrobianos de las cepas de *B. cereus s.l.* obtenidas de arroz.

Antibiotic	Sensible (≤ 2μg/ mL)	Intermediate (>2- <16 μg/ mL)	Resistant (≥ 16 μg/ mL)
Penicillin			6 (100%)
Ceftriaxone			6 (100%)
Erythromycin	5 (83.4%)	1 (16.6%)	
Clindamycin			6 (100%)
Ciprofloxacin	6 (100%)		
Tetracycline	6 (100%)		
Chloramphenicol	6 (100%)		
Vancomycin	6 (100%)		
Gentamycin	6 (100%)		
Kanamycin	6 (100%)		
Trimethoprim			6 (100%)

CONCLUSIONS

The *Bacillus cereus sensu lato* (*B. cereus s.l.*) strains isolated from rice exhibit enterotoxigenic profiles, indicating their potential to cause food poisoning. Of particular note is the finding that strains isolated from sweet rice have a propensity to form biofilms on glass, and exhibit a distinct enterotoxigenic profile compared to the strain isolated from fried rice. The unique characteristics identified in the strain isolated from fried rice underscore the importance of further investigation and warrant an expansion of the sample size, specifically within this rice type. This expansion aims to systematically explore the presence of strains with similar metabolic and virulence characteristics.

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CONFLICTS OF INTEREST

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper

REFERENCES

Adame-Gomez, R., Castro Alarcón, N., Vences-Velázquez, A., Rodríguez-Bataz, E., Santiago-Dionisio, M.C. and Ramírez-Peralta, A. 2019. Prevalencia de cepas del grupo de *Bacillus cereus* productoras de biopelicula en helados comercializados en México. https://doi.org/10.5281/ZENODO.3520530

Adame-Gómez, R., Cruz-Facundo, I.-M., García-Díaz, L.-L., Ramírez-Sandoval, Y., Pérez-Valdespino, A., Ortuño-Pineda, C., Santiago-Dionisio, M.-C. and Ramírez-Peralta, A. 2020a. Biofilm production by enterotoxigenic strains of *Bacillus cereus* in different materials and under different environmental conditions. Microorganisms 8, 1071. https://doi.org/10.3390/microorganisms8071071

Adame-Gómez, R., Muñoz-Barrios, S., Castro-Alarcón, N., Leyva-Vázquez, M.-A., Toribio-Jiménez, J. and Ramírez-Peralta, A., 2020b. Prevalence of the strains of *Bacillus cereus* Group in artisanal mexican cheese. Foodborne Pathog. Dis. 17, 8-14. https://doi.org/10.1089/fpd.2019.2673

Ankolekar, C., Rahmati, T. and Labbé, R.G. 2009. Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in U.S. rice. Int. J. Food Microbiol. 128, 460-466. https://doi.org/10.1016/j.ijfoodmicro.2008.10.006

Arslan, S., Eyi, A. and Küçüksarı, R. 2014. Toxigenic genes, spoilage potential, and antimicrobial resistance of *Bacillus cereus* group strains from ice cream. Anaerobe 25, 42-46. https://doi.org/10.1016/j.anaerobe.2013.11.006

Berthold-Pluta, A., Pluta A., Garbowska, N. and Stefańska, I. 2019. Prevalence and toxicity characterization of *Bacillus cereus* in food products from Poland. Foods 8, 269. https://doi.org/10.3390/foods8070269

Candela, T., Fagerlund, A., Buisson, C., Gilois, N., Kolstø, A.-B., Økstad, O.A., Aymerich, S., Nielsen-Leroux, C., Lereclus, D. and Gohar, M. 2019. CalY is a major virulence factor and a biofilm matrix protein. Mol. Microbiol. 111, 1416-1429. https://doi.org/10.1111/mmi.14184

Cano-Ponce, M. and Ramirez- Peralta, A., 2023. Aislamiento de *B. cereus* en arroz comercializado en el sur de México. Tlamati Press.

Caro-Astorga, J., Álvarez-Mena, A., Hierrezuelo, J., Guadix, J.A., Heredia-Ponce, Z., Arboleda-Estudillo, Y., González-Munoz, E., de Vicente, A. and Romero, D. 2020. Two genomic regions encoding exopolysaccharide production systems have complementary functions in *B. cereus* multicellularity and host interaction. Sci. Rep. 10, 1000. https://doi.org/10.1038/s41598-020-57970-3

Caro-Astorga, J., Pérez-García, A., de Vicente, A. and Romero, D., 2014. A genomic region involved in the formation of adhesin fibers in *Bacillus cereus* biofilms. Front. Microbiol. 5, 745. https://doi.org/10.3389/fmicb.2014.00745

Castulo-Arcos, D.A., Adame-Gómez, R., Castro-Alarcón, N., Galán-Luciano, A., Santiago Dionisio, M.C., Leyva-Vázquez, M.A., Perez-Olais, J.-H., Toribio-Jiménez, J. and Ramirez-Peralta, A. 2022. Genetic diversity of enterotoxigenic *Bacillus cereus* strains in coriander in southwestern Mexico. PeerJ 10, e13667. https://doi.org/10.7717/peerj.13667

Chen, J., Zhang, J., Zhan, L., Chen, H., Zhang, Z., Huang, C. and Yue, M. 2022. Prevalence and antimicrobial-resistant characterization of *Bacillus cereus* isolated from ready-to-eat rice products in Eastern China. Front. Microbiol. 13, 964823. https://doi.org/10.3389/fmicb.2022.964823

Chen, L., Daniel, R.M. and Coolbear, T. 2003. Detection and impact of protease and lipase activities in milk and milk powders. Int. Dairy J. 13, 255-275. https://doi.org/10.1016/S0958-6946(02)00171-1

Claus, D. and Berkeley, C. 1986. The genus *Bacillus*, in: Bergey's Manual of Systematic Bacteriology. The Williams & Wilkins Co, Baltimore, pp. 1105-1139.

Cruz-Facundo, I., Adame-Gómez, R., Vences-Velázquez, A., Rodríguez-Bataz, E., Muñoz-Barrios, S., Pérez-Oláis, J. and Ramírez-Peralta, A. 2022. *Bacillus cereus* in eggshell: Enterotoxigenic profiles and biofilm production. Braz. J. Poult. Sci. 24, eRBCA-2021-1535. https://doi.org/10.1590/1806-9061-2021-1535

Cruz-Facundo, I.-M., Toribio-Jiménez, J., Castro-Alarcón, N., Leyva-Vázquez, M.-A., Rodríguez-Ruíz, H.-A., Pérez-Olais, J.-H., Adame-Gómez, R., Rodríguez-Bataz, E., Reyes-Roldán, J., Muñoz-Barrios, S. and Ramírez-Peralta, A. 2023. *Bacillus cereus* in the artisanal cheese production chain in southwestern Mexico. Microorganisms 11, 1290. https://doi.org/10.3390/microorganisms11051290

De Jonghe, V., Coorevits, A., Vandroemme, J., Heyrman, J., Herman, L., De Vos, P. and Heyndrickx, M. 2008. Intraspecific genotypic diversity of *Bacillus* species from raw milk. Int. Dairy J. 18, 496-505. https://doi.org/10.1016/j.idairyj.2007.11.007

De-la-Pinta, I., Cobos, M., Ibarretxe, J., Montoya, E., Eraso, E., Guraya, T. and Quindós, G. 2019. Effect of biomaterials hydrophobicity and roughness on biofilm development. J. Mater. Sci. Mater. Med. 30, 77. https://doi.org/10.1007/s10856-019-6281-3

Dietrich, R., Jessberger, N., Ehling-Schulz, M., Märtlbauer, E. and Granum, P.E. 2021. The food poisoning toxins of *Bacillus cereus*. Toxins 13, 98. https://doi.org/10.3390/toxins13020098

Dogsa, I., Brloznik, M., Stopar, D. and Mandic-Mulec, I. 2013. Exopolymer diversity and the role of levan in *Bacillus subtilis* biofilms. PloS One 8, e62044. https://doi.org/10.1371/journal.pone.0062044

Duport, C., Jobin, M. and Schmitt, P. 2016. Adaptation in *Bacillus cereus*: From stress to disease. Front. Microbiol. 7. https://doi.org/10.3389/fmicb.2016.01550

Ehling-Schulz, M., Fricker, M. and Scherer, S. 2004. *Bacillus cereus*, the causative agent of an emetic type of food-borne illness. Mol. Nutr. Food Res. 48, 479-487. https://doi.org/10.1002/mnfr.200400055

Ehling-Schulz, M., Guinebretiere, M.-H., Monthán, A., Berge, O., Fricker, M. and Svensson, B. 2006. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiol. Lett. 260, 232-240. https://doi.org/10.1111/j.1574-6968.2006.00320.x

Ehling-Schulz, M., Lereclus, D. and Koehler, T.M. 2019. The *Bacillus cereus* group: *Bacillus* species with pathogenic potential. Microbiol. Spectr. 7, 7.3.6. https://doi.org/10.1128/microbiolspec.GPP3-0032-2018

Enosi Tuipulotu, D., Mathur, A., Ngo, C. and Man, S.M. 2021. *Bacillus cereus*: Epidemiology, virulence factors, and host-pathogen interactions. Trends Microbiol. 29, 458-471. https://doi.org/10.1016/j.tim.2020.09.003

Flores-Urbán, K.A., Natividad-Bonifacio, I., Vázquez-Quiñones, C.R., Vázquez-Salinas, C. and Quiñones-Ramírez, E.I. 2014. Detection of toxigenic *Bacillus cereus* strains isolated from vegetables in Mexico City. J. Food Prot. 77, 2144-2147. https://doi.org/10.4315/0362-028X.JFP-13-479

Fraccalvieri, R., Bianco, A., Difato, L.M., Capozzi, L., Del Sambro, L., Simone, D., Catanzariti, R., Caruso, M., Galante, D., Normanno, G., Palazzo, L., Tempesta, M. and Parisi, A. 2022. Toxigenic genes, Pathogenic potential and antimicrobial resistance of *Bacillus cereus* group isolated from ice cream and characterized by whole genome sequencing. Foods Basel Switz. 11, 2480. https://doi.org/10.3390/foods11162480

Grutsch, A.A., Nimmer, P.S., Pittsley, R.H., Kornilow, K.G. and McKillip, J.L. 2018. Molecular pathogenesis of *Bacillus* spp., with emphasis on the dairy industry. Fine Focus 4, 203-222. https://doi.org/10.33043/FF.4.2.203-222

Hayrapetyan, H., Muller, L., Tempelaars, M., Abee, T. and Nierop Groot, M. 2015. Comparative analysis of biofilm formation by *Bacillus cereus* reference strains and undomesticated food isolates and the effect of free iron. Int. J. Food Microbiol. 200, 72-79. https://doi.org/10.1016/j.ijfoodmicro.2015.02.005

Hayrapetyan, H., Siezen, R., Abee, T. and Nierop Groot, M. 2016. Comparative genomics of iron-transporting systems in *Bacillus cereus* strains and impact of iron sources on growth and biofilm formation. Front. Microbiol. 7, 842. https://doi.org/10.3389/fmicb.2016.00842

Hindler, J.A. and Richter, S.S., 2016. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria: M45, 3rd edition. ed, Documents / Clinical and Laboratory Standards Institute. Clinical and Laboratory Standards Institute, Wayne, PA.

Ivanova, N., Sorokin, A., Anderson, I., Galleron, N., Candelon, B., Kapatral, V., Bhattacharyya, A., Reznik, G., Mikhailova, N., Lapidus, A., Chu, L., Mazur, M., Goltsman, E., Larsen, N., D'Souza, M., Walunas, T., Grechkin, Y., Pusch, G., Haselkorn, R., Fonstein, M., Ehrlich, S.D., Overbeek, R. and Kyrpides, N. 2003. Genome sequence of *Bacillus cereus* and comparative analysis with *Bacillus anthracis*. Nature 423, 87-91. https://doi.org/10.1038/nature01582

Jaquette, C.B. and Beuchat, L.R. 1998. Survival and growth of psychrotrophic *Bacillus cereus* in dry and reconstituted infant rice cereal. Journal of food protection, 61(12), 1629-1635. https://doi.org/10.4315/0362-028x-61.12.1629

Jessberger, N., Dietrich, R., Granum, P.E. and Märtlbauer, E. 2020. The *Bacillus cereus* food infection as multifactorial process. Toxins 12, 701. https://doi.org/10.3390/toxins12110701

Kim, B., Bang, J., Kim, H., Kim, Y., Kim, Byeong-sam, Beuchat, L.R. and Ryu, J.-H. 2014. *Bacillus cereus* and *Bacillus thuringiensis* spores in Korean rice: Prevalence and toxin production as affected by production area and degree of milling. Food Microbiol. 42, 89-94. https://doi.org/10.1016/j.fm.2014.02.021

Kim, S.K., Kim, K.-P., Jang, S.S., Shin, E.M., Kim, M.-J., Oh, S. and Ryu, S. 2009. Prevalence and toxigenic profiles of *Bacillus cereus*

isolated from dried red peppers, rice, and sunsik in Korea. J. Food Prot. 72, 578-582. https://doi.org/10.4315/0362-028X-72.3.578

Kotiranta, A., Lounatmaa, K. and Haapasalo, M. 2000. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect. 2, 189-198. https://doi.org/10.1016/s1286-4579(00)00269-0

Liu, Y., Lai, Q., Göker, M., Meier-Kolthoff, J.P., Wang, M., Sun, Y., Wang, L. and Shao, Z. 2015. Genomic insights into the taxonomic status of the *Bacillus cereus* group. Sci. Rep. 5, 14082. https://doi.org/10.1038/srep14082

Majed, R., Faille, C., Kallassy, M. and Gohar, M. 2016. *Bacillus cereus* biofilms-same, only different. Front. Microbiol. 7, 1054. https://doi.org/10.3389/fmicb.2016.01054

Ołtuszak-Walczak, E., Walczak, P. and Modrak, R. 2006. Detection of enterotoxic *Bacillus cereus* producing hemolytic and non hemolytic enterotoxins by PCR test. Pol. J. Microbiol. 55, 113-118.

Park, Y.-B., Kim, J.-B., Shin, S.-W., Kim, J.-C., Cho, S.-H., Lee, B.-K., Ahn, J., Kim, J.-M. and Oh, D.-H. 2009. Prevalence, genetic diversity, and antibiotic susceptibility of *Bacillus cereus* strains isolated from rice and cereals collected in Korea. J. Food Prot. 72, 612-617. https://doi.org/10.4315/0362-028X-72.3.612

Perera, M.L. and Ranasinghe, G.R. 2012. Prevalence of *Bacillus cereus* and associated risk factors in chinese-style fried rice available in the city of Colombo, Sri Lanka. Foodborne Pathog. Dis. 9, 125-131. https://doi.org/10.1089/fpd.2011.0969

SAGARPA. 2017. Arroz Mexicano. (Federal), Planeacion agricola nacional 2017. 2030. SAGARPA, Ciudad de Mexico.

Schoeni, J.L. and Wong, A.C.L. 2005. *Bacillus cereus* food poisoning and its toxins. J. Food Prot. 68, 636-648. https://doi.org/10.4315/0362-028x-68.3.636

Shemesh, M. and Ostrov, I. 2020. Role of *Bacillus* species in biofilm persistence and emerging antibiofilm strategies in the dairy industry. J. Sci. Food Agric. 100, 2327-2336. https://doi.org/10.1002/jsfa.10285

Stenfors Arnesen, L.P., Fagerlund, A. and Granum, P.E. 2008. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol. Rev. 32, 579-606. https://doi.org/10.1111/j.1574-6976.2008.00112.x

Wei, S., Chelliah, R., Park, B.-J., Park, J.-H., Forghani, F., Park, Y.-S., Cho, M.-S., Park, D.-S. and Oh, D.-H. 2018. Molecular discrimination of *Bacillus cereus* group species in foods (lettuce, spinach, and kimbap) using quantitative real-time PCR targeting groEL and gyrB. Microb. Pathog. 115, 312–320. https://doi.org/10.1016/j.micpath.2017.12.079

Wijman, J.G.E., de Leeuw, P.P.L.A., Moezelaar, R., Zwietering, M.H. and Abee, T. 2007. Air-liquid interface biofilms of *Bacillus cereus*: formation, sporulation, and dispersion. Appl. Environ. Microbiol. 73, 1481-1488. https://doi.org/10.1128/AEM.01781-06