Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

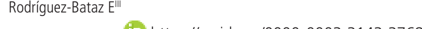
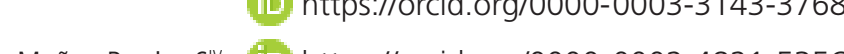
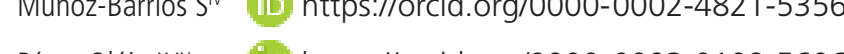
ISSN 1516-635X 2022 / v.24 / n.2 / 001-010

http://dx.doi.org/10.1590/1806-9061-2021-1535

Original Article

Bacillus Cereus in Eggshell: Enterotoxigenic Profiles and Biofilm Production

Author(s)

Cruz-Facundo IM[I] https://orcid.org/0000-0002-8836-3011
Adame-Gómez R[I] https://orcid.org/0000-0002-1375-2485
Vences-Velázquez A[II] https://orcid.org/0000-0001-8810-1401
Rodríguez-Bataz E[III]  https://orcid.org/0000-0003-3143-3768
Muñoz-Barrios S[IV]  https://orcid.org/0000-0002-4821-5356
Pérez-Oláis JH[V]  https://orcid.org/0000-0003-0109-5606
Ramírez-Peralta A[I] https://orcid.org/0000-0002-7037-6412

[I] Universidad Autónoma de Guerrero, Laboratorio de Investigación en Patometabolismo Microbiano, Chilpancingo, Guerrero, México.
[II] Universidad Autónoma de Guerrero, Laboratorio de Investigación en Inmunobiología y Diagnóstico Molecular. Microbiano, Chilpancingo, Guerrero, México.
[III] Universidad Autónoma de Guerrero, Laboratorio de Investigación en Parasitología, Chilpancingo, Guerrero, México.
[IV] Universidad Autónoma de Guerrero, Laboratorio de Investigación en Inmunotoxigénomica, Chilpancingo, Guerrero, México.
[V] Universidad Autónoma Metropolitana, Unidad Cuajimalpa, Departamento de Ciencias Naturales. Ciudad de México, México.

Mail Address

Corresponding author e-mail address
Arturo Ramírez-Peralta
Laboratorio de Investigacion en Patometabolismo Microbiano. Universidad Autonoma de Guerrero, Chilpancingo, 39000, Mexico.
Phone: +052 747 189 6780
Email: ramirezperaltauagro@gmail.com

Keywords

Bacillus cereus, eggshell, biofilm.

Submitted: 26/June/2021
Approved: 02/December/2021

ABSTRACT

A study was carried out with the objective of determining the presence of *Bacillus cereus* in eggshells commercialized in Mexico, the enterotoxigenic profile of the isolated strains, and the production of biofilms in different materials as well as in the eggshell. 1000 chicken eggs from four commercial brands were collected from markets and supermarkets located in the city of Chilpancingo, Mexico. *Bacillus cereus* was isolated from the eggshell. The molecular identification was by amplification of the *gyrB* gene and the enterotoxigenic profiles by the amplification of the *cytK, ces, nheABC,* and *hblABD* genes, in addition to the amplification of the *tasA* and *sipW* genes associated with the production of biofilms. In different materials and in eggshells, the production of biofilms was evaluated. The microbiological and molecular analysis of *B. cereus* yielded a frequency of 5.5% (55/1000), this was higher in brand III (11.6%, p=0.0001) and white eggshell (7.6%, 38/500, $p \leq 0.001$) and by marketing source, it was similar between market (5.2% / 26/500) and supermarket (5.8%, 29/500). The most common was the toxigenic profile A (23/55). Biofilm production is high in PVC in relation to other materials (p<0.0001), and the frequency of the related genes *tasA* and *sipW* was 72.7% and 40% respectively; the highest production was related to the *tasA* gene; in eggshell, most of the strains (54/55) were able to produce biofilm. Strains of *B. cereus* with toxigenic potential circulate and persist in this product, which shows the need for sanitary regulation in the country.

INTRODUCTION

Eggs are used as an economical food source either as table eggs or eggshells, liquid, frozen or dehydrated products (Salfinger & Tortorello, 2015). Eggs and their products provide an important source of nutrition (Howard *et al.*, 2012) as well as being a functional part of other foods such as ice-cream, desserts, meats, breakfasts, and seafood (Kone *et al.*, 2013). Mexico is the main consumer of fresh eggs worldwide; by the end of 2019, it was estimated that the per capita consumption of Mexican is 22.8 Kg of eggs per day (Sader, 2019). Regarding production during 2019, the laying flock of Mexican poultry farms obtained 2,949,782,315 tons of eggs for fresh consumption (SIAP, 2019). Considering the egg as an important part of the diet in Mexico.

Due to the wide use of eggs in the food chain, the sanitary quality of the product is imperative. In this sense, it has been described that contamination by eggshell bacteria can occur in a variety of routes, such as in egg formation in the hen's reproductive system (Howard *et al.*, 2012) or due to poor hygiene and handling conditions; eggs can also become contaminated after breeding when exposed to contaminated conditions due to the inevitable accumulation of important microorganism in animal waste (Gentry & Quarles, 1972;

Quarles *et al.*, 1970); this contamination could be a risk for food safety, increasing the risk of disease in humans (Bencardino *et al.*, 2017). The shell microflora is remarkably heterogeneous, with Gram-positive microorganisms being the most prevalent, probably due to their ability to tolerate stressful conditions (Siriporn, 2015). Regarding the last point, *Bacillus cereus* is a Gram-positive microorganism that can be found in multiple stressful environments due to the formation of endospores that resist heat, dehydration, and other physical agents (Drobniewski, 1993). Therefore, its presence in the eggshell has been previously reported (Kone*et al.*, 2013; Siriporn, 2015). *B. cereus* being considered as an important microorganism transmitted by food; in this sense, various studies refer to the presence of *B. cereus* spores in raw milk and milk powders (Benahmed *et al.*, 2020; Pretorius & Buys, 2021). Furthermore, the production of biofilms on surfaces of dairy production has been described (Alonso & Kabuki, 2019). Its presence in dairy products as well as in other foods is important because it is related to its ability to produce a wide range of virulence factors that can cause disease in humans of short and moderate duration (Stenfors *et al.*, 2008); these poisonings could not only be caused by the consumption of the egg, but by its usefulness in the production of other foods; therefore, the presence of microorganisms in chickens and poultry products, including eggs, potentially increases the entry of these microorganisms into the food chain, generating food poisoning (EFSA, 2011).

It is important not only to point out the presence of *B. cereus* in food, but also to characterize the main virulence factors associated with the two types of food poisoning that it causes. *B. cereus* produces the emetic toxin (*ces*operon), which causes emetic syndrome and has been characterized as a small ring-shaped peptide (Ehling-Schulz *et al.*, 2004). In addition, it produces three enterotoxins that have been implicated in diarrheal syndrome: pore-forming hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe), and cytotoxin K (CytK) (Beecher & Macmillan, 1991; Lund *et al.*, 2000; Lund & Granum, 1996). Finally, *B. cereus* has been described as capable of producing biofilms in a variety of environments associated with food production (Evans *et al.*, 2004; Gunduz & Tuncel, 2006; Storgårds *et al.*, 2006; Marchand *et al.*, 2012; Alonso & Kabuki, 2019) and that biofilm production could be a systematic contamination mechanism (Rajkovic *et al.*, 2008); However, it has been reported that it can produce biofilms directly in food and that it could also be a mechanism of persistence in food (Elhariry, 2011). Currently, Mexico has no regulation which indicates the presence of this microorganism in these types of foods, but it has a history of the presence of the *B. cereus* in its products and its importance in the production chain. Therefore, the aim of this study was to expose the presence of *B. cereus*, the enterotoxigenic profile of the isolated strains, and the production of biofilms in different materials including the eggshell in eggs commercialized in Mexico.

MATERIAL AND METHODS

Collection of samples

A total of 1000 chicken eggs representing four commercial brands (250 eggs per brand, 125 brown eggs, and 125 white eggs) were collected from January to September 2019 from markets and supermarkets located in Chilpancingo City, Mexico. Those four brands represent some of the most popular brands in Mexico, two brands are commonly sold in supermarkets and two in markets. In supermarkets and markets, the eggs were stored at room temperature in unit tray packs (30 eggs per pack). A tray pack of each brand was collected each week from each store and transported to the laboratory (Laboratorio de Investigación en Patometabolismo Microbiano, Chilpancingo, México) at room temperature in a container. The experiments were carried out on the samples arrived at the laboratory; 10 eggs from each tray pack were randomly selected until the number of samples of each brand was completed.

Isolation and identification of *Bacillus cereus* in eggshell

The eggs were placed into a sterile plastic bag and 1 mL of brain heart infusion broth (BHI) was added, they were immediately rubbed manually for half an hour in order to recover all the microorganisms present in the eggshell. Once the time had elapsed, we spread 100 µL of the BHI broth on Mannitol Egg Yolk (MYP) Agar (Bioxon, México), and incubated under aerobic conditions at 30ºC for 24 h. We considered as suspicious colonies of *B. cereus*, the pink colonies with an opaque halo and confirmed by beta hemolysis in trypticase soy agar supplemented with sheep blood.

Molecular identification and enterotoxigenic profiles

From bacterial cultures, a thermal shock was performed to obtain the chromosomal DNA. In brief, cells from one colony were suspended in sterile water,

heated to 95 ° C for 3 minutes, and placed on ice for 15 minutes. After centrifugation, the supernatant was used as a template for the molecular identification, the enterotoxigenic profile and genes that possibly participated in the production of the biofilm.

The differentiation of *B. cereus* group was targeted on *gyrB* gene (Wei *et al.*, 2018) and the toxin gene profiles from *cytK*, *ces*, *nheABC* y *hblABD* gene; *tasA* and *sipW* genes were also included due to previous reports of their participation in biofilm production (Caro-Astorga *et al.*, 2015). The reaction mixes (50 µL) contained the following: 25 µL of REDTaqReadyMix DNA polymerase (Sigma-Aldrich), 11µL of sterile Milli-Q water, 0.5 µL of the genomic DNA template (concentration about 10–20 ng/µL), and 0.02 µM of each primer. The PCR cycling conditions and primers are shown in Table 1. A strain of *B. subtilis* was used as a negative control, this strain was previously characterized in the laboratory. *B. cereus* ATCC 14579 (diarrheagenic) and VK4 strain (emetic) were used as control strains.

Electrophoresis was performed on 2% agarose gels at 80V for 120 minutes. The gels were stained with Midori Green (Nippon Genetics, Düren, Germany) and visualized with LED light. A 100 bp molecular weight marker was used in all electrophoresis (CSL-MDNA, Cleaver Scientific Ltd, Warwickshire, England, UK).

Production of biofilm in different materials

Prior to the determination of static biofilm in polyvinyl chloride (PVC), the PVC coupons were placed inside glass tubes. The coupons were pretreated to remove dust and other organic components. The biofilms were generated in brain heart infusion broth (BHI). Each glass tube containingthe PVC coupon was filled with 1 mL of BHI. The broths were inoculated with 5% volume of a 24 h culture (1 mL). The tubes were incubated at 30ºC in aerobiosis, for 48 h under static conditions. For the determination in glass and polyethylene, the procedure was similar without the PVC coupons and using tubes of each respective material. In the case of polystyrene, 96-well microplates were used, which were filled with 200 µL of the BHI. The incubation time was the same in all cases.

Next, the biofilm formation was measured by performing safranin assay. After incubation, the growth was analyzed by removing 200 µL of culture and reading it at an absorbance of 600 nm. Then, in the case of PVC, the coupons were carefully washed three times by dipping them in phosphate buffer saline (PBS) (Life Technologies, Carlsbad, CA, USA) using sterile tweezers. Because the PVC coupons were in glass tubes, they were considered as another material (PVC glass). In the case of the glass and polyethylene tubes, as well as the polystyrene plates, the medium

Table 1 – Polymerase chain reaction cycling conditions and primer sequences.

Gene	Primer sequences	PCR cycling conditions	Reference
gyrB	F- GCC CTG GTA TGT ATA TTG GAT CTA C R- GGT CAT AAT AAC TTC TAC AGC AGG A	Initial denaturation of 2 minutes at 94ºC, followed by 30 cycles at 94ºC for 30s at, 52ºC for one minute and 72ºC for 30 s, and final elongation at 72ºC for 10 minutes.	(Wei *et al.*, 2018)
nheABC	F- AAG CIG CTC TTC GIA TTC R- ITI GTT GAA ATA AGC TGT GG	Initial denaturation of 5 minutes at 94ºC, followed by 30 cycles at 94ºC for 30 s, 49ºC for one minute and at 72ºC for one minute, and final elongation at 72ºC for five minutes	(Ehling-Schulz *et al.*, 2006)
hblABD	F-GTA AAT TAI GAT GAI CAA TTT C R- AGA ATA GGC ATT CAT AGA TT		
ces	F- TTG TTG GAA TTG TCG CAG AG R-GTA AGC GGA CCT GTC TGT AAC AAC	Initial denaturation of 2 minutes at 94ºC, followed by 30 cycles at 94ºC for 30s at, 52ºC for one minute and 72ºC for 30 s and a final elongation at 72ºC for 10 minutes	
cytK-plcR	P1- CAA AAC TCT ATG CAA TTA TGC AT P3- ACC AGT TGT ATT AAT AAC GGC AAT C	Initial denaturation of 2 minutes at 94ºC, followed by 30 cycles at 94ºC for 30s at, 52ºC for one minute and 72ºC for 30 s, and final elongation at 72ºC for 10 minutes.	(Oltuszak-Walczak & Walczak, 2013)
tasA	F- AGC AGC TTT AGT TGG TGG AG R-GTA ACT TAT CGC CTT GGA ATTG	Initial denaturation of 5 minutes at 94C, followed by 40 cycles at 94ºC for 30 s, 59ºC for 45 s and 72ºC for 45 s, and final elongation at 72ºC for five minutes	(Caro-Astorga *et al.*, 2015)
sipW	F- AGA TAA TTA GCA ACG CGA TCTC R- AGA AAT AGC GGA ATA ACC AAGC	Initial denaturation of 5 minutes at 94ºC, followed by 40 cycles at 94ºC for 30 s, 54ºC for 45 s, and 72ºC for 45 s, and a final elongation at 72ºC for five minutes	

*I: inosine.

was removed and they were washed three times with PBS. The adhered biofilm was stained with a 0.1% safranin solution (BD Difco, Franklin Lakes, NJ, USA) for 30 minutes. The coupons, as well as the glass and polyethylene tubes and the polystyrene plates, were washed again three times with PBS and were incubated with 70% ethanol for 30 minutes to release the biofilm- bound to safranin. The solubilized safranin was quantified by absorbance at a wavelength of 492 nm. The safranin assays were repeated in three independent experiments. The culture medium without inoculum was used as a negative control. To determine the specific biofilm formation (SBF) the formula proposed by Niu & Gilbert (2016) was used.

Production of biofilm in eggshells

White eggshells were disinfected and carefully cut into 1cm^2 coupons and immersed in tubes with 1mL of sterile BHI broth, determining the biofilm production in the same way as the PVC material. In this case, it was not discolored with ethanol and only the differences in staining between the negative control (eggshell not inoculated, but stained with safranin) and the samples were visually checked.

Statistical analysis

Relative proportions were performed with STATA v program. 12 using the chi- square test or Fisher's exact test. The effects of the material on biofilm formation by *B. cereus* strains were compared using Kruskal Wallis test with Dunn's post hoc test. Statistical significance was considered when the p value was less than <0.05.

RESULTS

Determined by microbiological analysis and molecular identification, the frequency of *B. cereus* was 5.5% (55/1000). The frequency was compared with the commercial brand of the egg, the color of the eggshell, and the source of commercialization. There is a higher frequency of *B. cereus* in brand III of 11.6% (p=0.0001) (Table 2).

Table 2 – Frequency of *B. cereus* in eggshells by brand.

Microorganism	Brand I	Brand II	Brand III	Brand IV	p=
	n (%) n=250				
B. cereus	7 (2.8)	12 (4.8)	29 (11.6)	7(2.8)	0.0001

Also, when comparing by the color of the eggshell, with 500 eggs processed for each color, we found a higher frequency of *B. cereus* in white eggshell with 7.6% (p=0.003) (Table 3).

Table 3 – *B. cereus* frequency in eggshell by eggshell color.

Microorganism	Eggshell color: Brown	Eggshell color: White	p=
	n (%) n=500		
B. cereus	17 (3.4)	38 (7.6)	0.003

At the same time, the *B. cereus* frequency was compared by the origin of commercialization; finding similar frequencies between market (5.2% / 26/500) and supermarket (5.8%, 29/500).

Of the 55 strains, 8 enterotoxigenic profiles were determined (Figure 1, table 4), the most common being the toxigenic profile A (23/55) which includes genes of the three enterotoxins, only two strains were classified in the H profile (2 / 55,) which refers to strains without any gene encoding toxins.

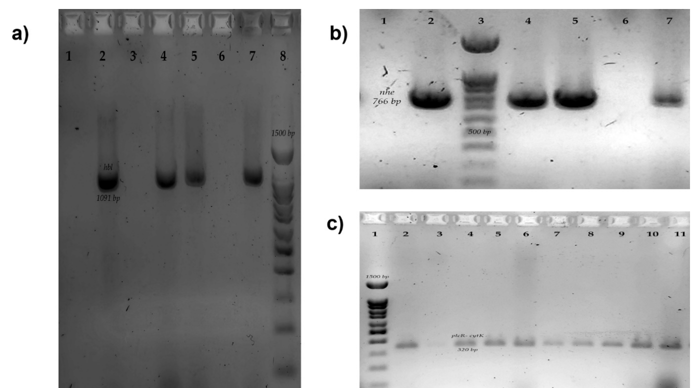


Figure 1 – Molecular identification of toxins genes of *B. cereus*. In a) amplification of hemolitic BL gene (*hbl*, 1091bp), 1: negative control (*B. subtilis*), 2: positive control (*B. cereus* ATCC14579), 4,5,7: positive strain, 3,6: negative strains, 8: molecular weight market of 100 bp. In b) amplification of no-hemolitic enterotoxin gene (*nhe*,766bp) 1: Control negative (*B. subtilis*), 2: positive control (*B. cereus* ATCC14579), 3: molecular weight market of 100 bp, 4,5, 7: Positive strains, 6: negative strain. In c) amplification of cytotoxin K gene (*cytk*-PlcR, 320bp) 1: molecular weight market of 100 bp, 2: positive control (*B. cereus* ATCC14579), 3: negative control (*B. subtilis*), 4 to 11 positive strains.

Table 4 – Toxigenic profiles of *B. cereus* strains isolated from eggshells.

Toxin profile	*nhe*	*hbl*	*ces*	*cytK*	*n strains*
A	+	+	-	+	23
B	-	-	-	+	5
C	+	-	-	-	0
D	+	-	-	+	2
E	-	+	-	+	7
F	+	+	-	-	15
G	-	+	-	-	1
H	-	-	-	-	2

Biofilm production by *B. cereus* was determined in different materials, observing a higher production in PVC in relation to other materials such as glass (p<0.0001), polystyrene (p<0.0001), and polyethylene (p<0.0001) (Figure 2)

A high frequency of genes related to biofilm production was observed, 72.7% for *tasA* and 40% for *sipW* (Figure 3). The production of biofilms in

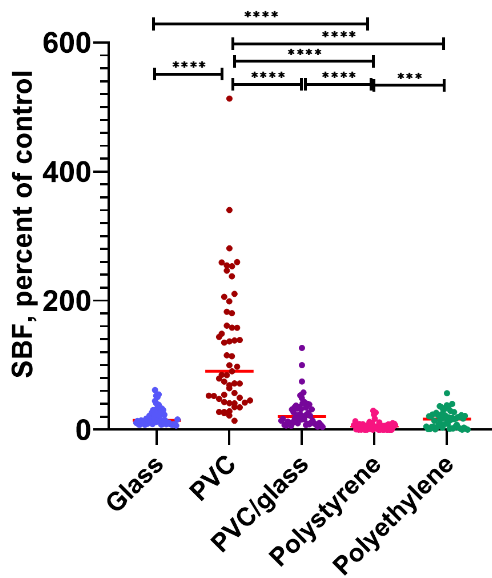


Figure 2 – Biofilm production of *B cereus* strains from eggshell isolation in different materials. light blue circles, glass; red circles, PVC; purple circles, PVC/glass; pink circles polystyrene; green circles, polyethylene * $p \leq 0.05$, **** $p \leq 0.0001$. In the X axis are the materials and, in the Y, the specific biofilm formation (SBF). Kruskal Wallis post hoc Dunn's.

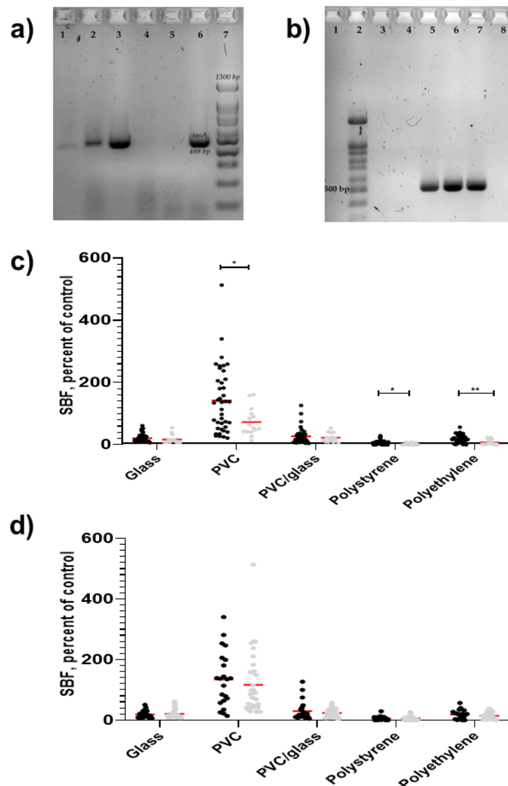


Figure 3 – Biofilm production of *B. cereus* according to the presence of the *tasA* and *sipW* genes. In a) Agarose gel electrophoresis of the PCR products of *tasA*, 7: molecular weight market of 100 bp, 6: positive control (*B. cereus* ATCC14579), 5: negative control, 4: negative strain, 3 to 1: positive strains. In b) Agarose gel electrophoresis of the PCR products of sipW, 2: molecular weight market of 100 bp, 5: positive control, 1: negative control, 3,4: negative strains, 6,7: positive strains. In c) biofilm production of B. cereus strains according to *tasA*+ and *tasA*- gene in different materials. Black circles are positive strains to tasA and gray circles are negative strains *tasA*. In d) biofilm production of B. cereus strains according to *sipW*+ and *sipW*- gene in different materials. Black circles are positive strains to *sipW* and gray circles are negative strains *sipW*. * $p \leq 0.05$, **** $p \leq 0.0001$. In the X axis are the materials and, in the Y, the specific biofilm formation (SBF). Sidak's multiple comparisons test.

different materials was related to the presence of these two genes; *tasA* positive strains were found to produce a higher number of biofilms compared to negative strains in PVC ($p=0.017$), polystyrene ($p=0.03$), and polyethylene ($p=0.002$).

It was observed that most of the 54/55 strains are capable of producing biofilms on the surface of the eggshell (Figure 4).

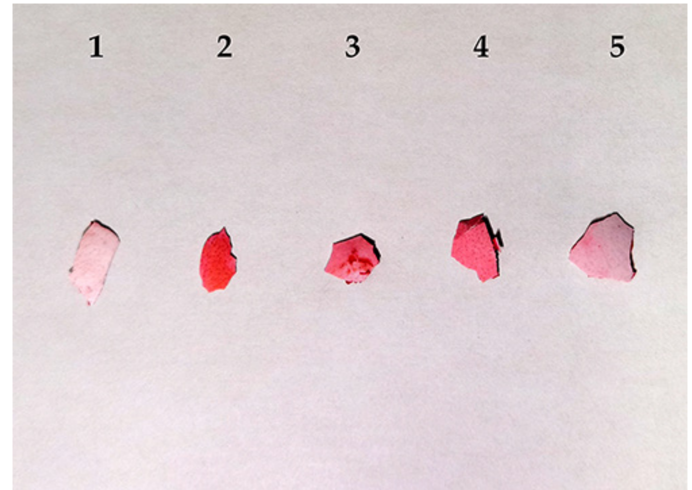


Figure 4 – Production biofilm of *B. cereus* strains on eggshell. 1: negative control, 2 to 5: *B. cereus* strains from eggshell isolation. The biofilm producing strains were observed when safranin's red color was kept on the egg's surface. The experiments were made to 30°C on Brain heart infusion broth.

DISCUSSION

Eggs are important in human nutrition, due to their low cost, nutritional content and because of their incorporation in a large number of products (Howard *et al.*, 2012; Kone *et al.*, 2013; Salfinger & Tortorello, 2015). Therefore, the egg in all its presentations must be monitored regarding the sanitary quality of the product because natural contamination by pathogens such as *Salmonella* has been reported (Howard *et al.*, 2012), contamination in laying by microorganisms of the gastrointestinal and environmental tract, as well as contamination associated with poor hygienic practices has been reported (Quarles *et al.*,1970, Gentry & Quarles, 1972).

B. cereus is a widely distributed microorganism in nature, whose presence has been detected in poultry feed (Mahami *et al.*, 2019), poultry slaughter facilities (Lues *et al.*, 2007; Liang *et al.*, 2013) and in poultry meat (Smith *et al.*, 2004; López *et al.*, 2015; Osman *et al.*, 2018); therefore, it is not surprising that it can be found in products such as the egg. This study did not survey farms, but different egg brands that are marketed in Mexico, isolating this microorganism in all the brands analyzed. A higher frequency of the microorganism in the product could be explained

by contamination deriving from the farm, and its association with different risk factors such as the lack of cleaning and draining of the drinking fountains, the hygiene of the sanitary fence, the type of disinfection and the concentration of dust (Kone *et al.*, 2013). In the case of other microorganisms, the time that the egg spends in contact with other eggs as well as with the hen, which contributes to the content of the shell microbiome (Trudeau *et al.*, 2020) or the diet of chickens, which affects the gut microbiome and therefore that of the shell (Smith *et al.*, 2000) is associated to contamination. For the total count of microorganisms in the eggshell, the cage system used has also been analyzed; theeggs produced by hens in a free system had a higher CFU count than the eggs obtained from hens in conventional systems (Gentry & Quarles, 1972; De Reu *et al.*, 2005; Samiullah & Chousalkar, 2014). Together, these data could explain the abundance of *B. cereus* in all brands and, on the other hand, the differences in the percentage of contaminated eggs between brands in this study.

Also, there are differences in contamination by eggshell color, being *B. cereus* more frequent in eggs with white hells, in this sense, it has been reported that the brown pigment of the egg has antibacterial activity against microorganisms such as *Staphylococcus aureus* and *B. cereus* (Ishikawa *et al.*, 2010), which could explain the low prevalence in brown eggs hells in our study. Finally, no differences were found regarding the final point of sale, noting that at this point they arrive in containers, which are generally covered and protected from environmental pollutants. Therefore, we deduce that the product contamination occurs before reaching the markets and supermarkets; Because contamination should not only be considered during the laying of the eggs but also in transport or preparation for sale; in this sense, the egg can be contaminated by any surface on which the egg comes in contact with. Water, packaging material, insects, hands, broken shells, dust, are the main sources of contamination (Board & Tranter, 1995). Even egg washing before hatching is performed to reduce the number of bacteria present in the eggs, which is considered routine practice in the United States, Australia, and Japan (Hutchison *et al.*, 2004) although it has also been described that it favors the penetration of microorganisms (Gole *et al.*, 2014).

A key characteristic of our study is that it was not only based on the isolation of the microorganism, but the molecular characterization of virulence factors was carried out. In this study, we show the high toxigenic potential of the strains because 53/55 strains contain at least one gene for a *B. cereus* enterotoxin; Furthermore, the A profile that contains the genes of the three enterotoxins was the most frequent in the population studied, similar to previous studies of *B. cereus* in food (Park *et al.*, 2009; Chon *et al.*, 2012; Hwang & Park, 2015; Adame-Gómez *et al.*, 2018; Adame-Gómez *et al.*, 2020; Adame-Gómez *et al.*, 2020).

The enterotoxin gene *nhe* was reported more frequently in our study, which has been previously reported in strains isolated from food, including chicken samples (Gaviria *et al.*, 2000; Hansen & Hendriksen, 2001; Guinebretière *et al.*, 2002; Smith *et al.*, 2004; Ankolekar *et al.*, 2009; Chaves *et al.*, 2012; Chon *et al.*, 2012; Ouoba *et al.*, 2008) and silo tanks (Ehling-Schulz *et al.*, 2006). In this study, no strains with the cereulide gene (*ces*) were found, which coincides with different studies that found a low frequency of emetic strains (Altayar & Sutherland, 2006; Ouoba *et al.*, 2008; Chon *et al.*, 2012; Jessberger *et al.*, 2020), in addition, a higher frequency of emetic syndrome has been reported in countries such as Japan and the United Kingdom (Kramer *et al.*, 1989). In turn, negative emetic strains have been described as containing the three enterotoxin genes (Ehling-Schulz *et al.*, 2005), which is also observed in this study. Also, the characterization of emetic strains can not only be carried out from *ces* gene, which is important for the search for other genes such as *bceT* or *EM1* (Guinebretière *et al.*, 2002; Ouoba *et al.*, 2008; Chon *et al.*, 2012). It is important to note that *B. cereus* is not only a toxigenic microorganism; *B. cereus* has been described as having an enzyme profile that gives it the ability deteriorate food products such as milk. Benahmed *et al* (2020) described that 78% of their strains can produce lecithinase; while Mehta *et al* (2019) show that *Bacillus* strains can produce different levels of lipase. Both enzymes capable of degrading major components of the egg yolk; Therefore, it is not surprising that this microorganism can deteriorate the egg yolk if it is able to adhere to the eggshell and colonize the egg yolk.

The persistence of *B. cereus* in the eggshell may be related to the adhesive characteristics and environmental resistance of the spores (Drobniewski, 1993); However, in recent years, particular emphasis has been placed on the study of biofilm production by *B. cereus* as a mechanism of persistence and contamination of various food products (Rajkovic *et al.*, 2008; Majed *et al.*, 2016) affecting the shelf life of the products and their safety. The production of biofilms has even been reported under conditions like the production of food associated with poultry (Iñiguez-

Moreno *et al.*, 2019) and milk (Alonso & Kabuki, 2019). In this study, we determined the production of biofilms by *B. cereus* strains in different materials; the included materials are commonly used in the food industry as part of conveyor belts (PVC) or as packaging (CFR, 2020); furthermore, we decided to include different materials since it has previously been reported that biofilm production is differential according to the type of material used (Wijman *et al.*, 2007; Hayrapetyan *et al.*, 2015).

In this study, high biofilm production on PVC was found, which has previously been reported by our group with strains isolated from other food sources (Adame-Gómez *et al.*, 2020); These production differences by the material may be related to the physicochemical characteristics of the materials (De-la-Pinta *et al.*, 2019) or to the own requirements or the genetic profile of the microorganisms to generate biofilm (Hayrapetyan *et al.*, 2016; Caro-Astorga *et al.*, 2020). For example, in glass and in polystyrene, the presence of extracellular DNA has been described as a requirement to favor the production of biofilms (Vilain *et al.*, 2009). On the other hand, we found that strains positive for *tasA* produce a greater quantity of biofilms in PVC, polystyrene, and polyethylene, grouped as positive or negative for *tasA*. This gene is paralogue to *tasA* in *Bacillus subtilis* (Caro-Astorga *et al.*, 2015), which codes for an amyloid-type protein of the same name and has been reported as the most abundant structural component of *B. subtilis* floating biofilms (Romero *et al.*, 2011); which could support the theory that *tasA*-positive *B. cereus* strains are indeed producing a greater number of biofilms.

In this study, we report for the first time that the strains of *B. cereus* produce biofilms in the eggshell, which reaffirms that this could be the persistence mechanism in the product. The production of biofilms in the eggshell has been reported for bacteria of the *Salmonella* genus (Pande *et al.*, 2016), which with other physical and nutritional factors of the shell favor its permanence and gradually the invasion towards the yolk (Gole *et al.*, 2014). Therefore, it is important to define the molecular characteristics for this process to be carried out and to show that the microorganism can pass through the shell and the mechanisms by which it does so.

This study reaffirms the circulation of *B. cereus* strains in the product, their toxigenic potential and their persistence characteristics in the product; which highlights the need for a sanitary regulation of this microorganism in Mexico in this type of product.

FUNDING

PFCE 2018. Secretaria de Educación Pública

REFERENCES

Adame-Gómez R, Cruz-Facundo I-M, García-Díaz L-L, Ramírez-Sandoval Y, Pérez-Valdespino A, Ortuño-Pineda C, *et al.* Biofilm production by enterotoxigenic strains of Bacillus cereus in different materials and under different environmental conditions. Microorganisms 2020;8(7):1071.

Adame-Gómez R, Muñoz-Barrios S, Castro-Alarcón N, Leyva-Vázquez M-A, Toribio-Jiménez J, Ramírez-Peralta A. Prevalence of the Strains of Bacillus cereus Group in Artisanal Mexican Cheese. Foodborne Pathogens and Disease 2020;17(1):8–14.

Adame-Gómez R, Guzman-Guzman K del A, Vences-Velazquez A, Leyva-Vazquez MA, Muñoz-Barrios S, Ramirez-Peralta A. Prevalence and genetic diversity of the strains of Bacillus cereus groups in food for infants and young children in Mexico. African Journal of Microbiology Research 2018;12(30):730–735.

Altayar M, Sutherland AD. *Bacillus cereus* is common in the environment but emetic toxin producing isolates are rare. Journal of Applied Microbiology 2006;100(1):7–14.

Alonso VPP, Kabuki DY. Formation and dispersal of biofilms in dairy substrates. International Journal of Dairy Technology 2019;72:472-478.

Ankolekar C, Rahmati T, Labbé RG. Detection of toxigenic *Bacillus cereus* and *Bacillus thuringiensis* spores in U.S. rice. International Journal of Food Microbiology 2009;128(3):460–466.

Beecher DJ, Macmillan JD. Characterization of the components of hemolysin BL from *Bacillus cereus*. Infection and Immunity 1991;59(5):1778–1784.

Benahmed M, Leguerinel I, Moussa-Boudjemaa B. Biodiversity, spoilage capacity and heat resistance of mesophilic aerobic spores isolated from milk powders marketed in Algeria. International Journal of Dairy Technology 2020;73: 771-780.

Bencardino D, Vitali LA, Petrelli D. High prevalence of clonally diverse spa type t026 *Staphylococcus aureus* contaminating rural eggshells. Journal of Medical Microbiology 2017;66(8):1196–1201.

Board R, Tranter H. Egg science and technology. 4th ed. Boca Raton: Food Products Press; 1995.

Caro-Astorga J, Álvarez-Mena A, Hierrezuelo J, Guadix JA, Heredia-Ponce Z, Arboleda-Estudillo Y, *et al.* Two genomic regions encoding exopolysaccharide production systems have complementary functions in *B. cereus* multicellularity and host interaction. Scientific Reports 2020;10(1):1000.

Caro-Astorga J, Pérez-García A, de Vicente A, Romero D. A genomic region involved in the formation of adhesin fibers in *Bacillus cereus* biofilms. Frontiers in Microbiology 2015;5.

CFR - Code of Federal Regulations. Title 21— food and drugs: 21 Food and Drugs § 177. 2020. Available from: https://www.ecfr.gov/cgi-bin/text-idx?SID=068f4a3c3eab145db047823da54b935e&mc=true&tpl=/ecfrbrowse/Title21/21cfr177_main_02.tpl

Chaves JQ, Cavados C de FG, Vivoni A. M. Molecular and toxigenic characterization of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from commercial ground roasted coffee. Journal of Food Protection 2012;75(3):518–522.

Chon JW, Kim JH, Lee S-J, Hyeon JY, Seo KH. Toxin profile, antibiotic resistance, and phenotypic and molecular characterization of *Bacillus cereus* in Sunsik. Food Microbiology 2012;32(1):217–222.

De Reu K, Grijspeerdt K, Heyndrickx M, Zoons J, De Baere K, Uyttendaele M, *et al.* Bacterial eggshell contamination in conventional cages, furnished cages and aviary housing systems for laying hens. British Poultry Science 2005;46(2):149–155.

De-la-Pinta I, Cobos M, Ibarretxe J, Montoya E, Eraso E, Guraya T, *et al.* Effect of biomaterials hydrophobicity and roughness on biofilm development. Journal of Materials Science. Materials in Medicine 2019;30(7):77.

Drobniewski FA. *Bacillus cereus* and related species. Clinical Microbiology Reviews 1993;6(4):324–338.

EFSA. Panel on Biological Hazards (BIOHAZ). Scientific Opinion on a quantitative estimation of the public health impact of setting a new target for the reduction of *Salmonella* in broilers. EFSA Journal 2011;9(7).

Ehling-Schulz M, Guinebretiere MH, Monthan A, Berge O, Fricker M, Svensson B. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiology Letters 2006;260(2):232–240.

Ehling-Schulz M, Svensson B, Guinebretiere MH, Lindbäck T, Andersson M, Schulz A, *et al.* Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. Microbiology 2005;151(1):183–197.

Ehling-Schulz M, Fricker M, Scherer S. *Bacillus cereus*, the causative agent of an emetic type of food-borne illness. Molecular Nutrition & Food Research 2004;48(7):479–487.

Elhariry HM. Attachment strength and biofilm forming ability of *Bacillus cereus* on green-leafy vegetables: Cabbage and lettuce. Food Microbiology 2011;28(7):1266–1274.

Evans JA, Russell SL, James C, Corry JEL. Microbial contamination of food refrigeration equipment. Journal of Food Engineering 2004;62(3):225–232.

Gaviria Rivera AM, Granum PE, Priest FG. Common occurrence of enterotoxin genes and enterotoxicity in *Bacillus thuringiensis*. FEMS Microbiology Letters 2000;190(1):151–155.

Gentry RF, Quarles CL. The measurement of bacterial contamination on egg shells. Poultry Science 1972;51(3):930–933.

Gole VC, Chousalkar KK, Roberts JR, Sexton M, May D, Tan J, *et al.* Effect of egg washing and correlation between eggshell characteristics and egg penetration by various *Salmonella Typhimurium* Strains. PLoS ONE 2014;9(3):e90987.

Guinebretière MH, Broussolle V, Nguyen-The C. Enterotoxigenic profiles of food-poisoning and food-borne *Bacillus cereus* strains. Journal of Clinical Microbiology 2002;40(8):3053–3056.

Gunduz GT, Tuncel G. Biofilm formation in an ice cream plant. Antonie van Leeuwenhoek 2006;89(3–4):329–336.

Hansen BM, Hendriksen NB. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. Applied and Environmental Microbiology 2001;67(1):185–189.

Hayrapetyan H, Siezen R, Abee T, Nierop Groot M. Comparative genomics of iron-transporting systems in *bacillus cereus* strains and impact of iron sources on growth and biofilm formation. Frontiers in Microbiology 2016;7, 842.

Hayrapetyan H, Muller L, Tempelaars M, Abee T, Nierop Groot M. Comparative analysis of biofilm formation by *Bacillus cereus* reference strains and undomesticated food isolates and the effect of free iron. International Journal of Food Microbiology 2015;200, 72–79.

Howard ZR, O'Bryan CA, Crandall PG, Ricke SC. *Salmonella Enteritidis* in shell eggs: current issues and prospects for control. Food Research International 2012;45(2):755–764.

Hutchison ML, Gittins J, Sparks AWN, Humphrey TJ, Burton C, Moore A. An assessment of the microbiological risks involved with egg washing under commercial conditions. Journal of Food Protection 2004;67(1):4–11.

Hwang JY, Park JH. Characteristics of enterotoxin distribution, hemolysis, lecithinase, and starch hydrolysis of *Bacillus cereus* isolated from infant formulas and ready-to-eat foods. Journal of Dairy Science 2015;98(3):1652–1660.

Iñiguez-Moreno M, Gutiérrez-Lomelí M, Avila-Novoa MG. Kinetics of biofilm formation by pathogenic and spoilage microorganisms under conditions that mimic the poultry, meat, and egg processing industries. International Journal of Food Microbiology 2019;303:32–41.

Ishikawa S, Suzuki K, Fukuda E, Arihara K, Yamamoto Y, Mukai T, Itoh M. Photodynamic antimicrobial activity of avian eggshell pigments. FEBS Letters 2010;584(4):770–774.

Jessberger N, Dietrich R, Granum PE, Märtlbauer E. The Bacillus cereus Food Infection as Multifactorial Process. Toxins 2020;12(11):701.

Kone AZ, Jan S, Le Marechal C, Grosset N, Gautier M, Puterflam J, Baron F. Identifying risk factors for eggshell contamination by Bacillus cereus group bacteria in French laying farms. British Poultry Science 2013;54(3):298–305.

Kramer J, Gilbert R, Doyle M. Foodborne bacterial pathogens. New York: M. Dekker; 1989. p.22–70.

Liang R, Tian J, She R, Meng H, Xiao P, Chang L. Airborne microbial composition in a high-throughput poultry slaughtering facility. Journal of Food Protection 2013;76(3):413–419.

López AC, Minnaard J, Pérez PF, Alippi AM. A case of intoxication due to a highly cytotoxic *Bacillus cereus* strain isolated from cooked chicken. Food Microbiology 2015;46:195–199.

Lues JFR, Theron MM, Venter P, Rasephei MHR. Microbial composition in bioaerosols of a high-throughput chicken-slaughtering facility. Poultry Science 2007;86(1):142–149.

Lund T, De Buyser ML, Granum PE. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Molecular Microbiology 2000;38(2):254–261.

Lund T, Granum PE. Characterisation of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. FEMS Microbiology Letters 1996;141(2–3):151–156.

Mahami T, Togby-Tetteh W, Kottoh DI, Amoakoah-Twum L, Gasu E, Annan SNY, *et al.* Microbial food safety risk to humans associated with poultry feed: the role of irradiation. International Journal of Food Science 2019;6915736.

Majed R, Faille C, Kallassy M, Gohar M. *Bacillus cereus* biofilms—same, only different. Frontiers in Microbiology 2016;7.

Marchand S, Block JD, Jonghe VD, Coorevits A, Heyndrickx M, Herman L. Biofilm formation in milk production and processing environments; influence on milk quality and safety. Comprehensive Reviews in Food Science and Food Safety 2012;11(2):133–147.

Mehta DS, Metzger LE, Hassan AN, Nelson BK, Patel HA. The ability of spore-formers to degrade milk proteins, fat, phospholipids, common stabilizers, and exopolysaccharides. Journal of Dairy Science 2019;102:10799–10813.

Niu C, Gilbert ES. Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. Applied and Environmental Microbiology 2004;70(12):6951–6956.

Oltuszak-Walczak E, Walczak P. PCR detection of *cytK* gene in *Bacillus cereus* group strains isolated from food samples. Journal of Microbiological Methods 2013;95(2):295–301.

Osman KM, Kappell AD, Orabi A, Al-Maary KS, Mubarak AS, Dawoud TM, *et al.* Poultry and beef meat as potential seedbeds for antimicrobial resistant enterotoxigenic *Bacillus* species: A materializing epidemiological and potential severe health hazard. Scientific Reports 2018;8(1):11600.

Ouoba LII, Thorsen L, Varnam AH. Enterotoxins and emetic toxins production by *Bacillus cereus* and other species of *Bacillus* isolated from Soumbala and Bikalga, African alkaline fermented food condiments. International Journal of Food Microbiology 2008;124(3):224–230.

Pande VV, Devon RL, Sharma P, McWhorter AR, Chousalkar KK. Study of *Salmonella typhimurium* infection in laying hens. Frontiers in microbiology, 2016;7(203).

Park YB, Kim JB, Shin SW, Kim JC, Cho SH, Lee BK, *et al.* Prevalence, genetic diversity, and antibiotic susceptibility of *Bacillus cereus* strains isolated from rice and cereals collected in Korea. Journal of Food Protection 2009;72(3):612–617.

Pretorius C, Buys EM. Extended shelf life milk processing: Effect of simulated cleaning in place on the germination and attachment of *Bacillus cereus* spores. International Journal of Dairy Technology 2021;74: 75-83.

Quarles CL, Gentry RF, Bressler GO. Bacterial contamination in poultry houses and its relationship to egg hatchability. Poultry Science 1970;49(1):60–66.

Rajkovic A, Uyttendaele M, Dierick K, Samapundo S, Botteldoorn N, Mahillon J, *et al.* Risk profile of the *Bacillus cereus* group implicated in food poisoning. Brussels: Superior Health Council Belgium; 2008. p.80. Available from: https://www.health.belgium.be/sites/default/files/uploads/fields/fpshealth_theme_file/19060475/Risicoprofiel%20voor%20Bacillus%20cereus%20Groep%20in%20voedsel%20toxi-infecties%3A%20situatie%20in%20België%20en%20aanbevelingen%20%5BBijlage%5D%20%28januari%202010%29%20%28HGR%208316%29.pdf.

Romero D, Vlamakis H, Losick R, Kolter R. An accessory protein required for anchoring and assembly of amyloid fibres in *B. subtilis* biofilms. Molecular Microbiology 2011;80(5):1155–1168.

SADER - Secretaria de Agricultura y Desarrollo Rural. Prevé Sader aumento en producción de carne de pollo y huevo para el 2019 [cited 2019 Jun 17]. Available from: https://www.gob.mx/agricultura/prensa/preve-sader-aumento-en-produccion-de-carne-de-pollo-y-huevo-para-el-2019-205085

Salfinger Y, Tortorello ML. Compendium of methods for the microbiological examination of foods. Washington: American Public Health Association; 2015. Available from: https://doi.org/10.2105/MBEF.0222

Samiullah Roberts JR, Chousalkar KK. Effect of production system and flock age on egg quality and total bacterial load in commercial laying hens. Journal of Applied Poultry Research 2014;23(1):59–70.

SIAP - Servicio de Información Agroalimentaria y Pesquera. Huevo para plato. Producción, precio y valor. Anuario Estadístico de la Producción Ganadera; 2019 [cited 2020 May 10].). Available from: https://nube.siap.gob.mx/cierre_pecuario/.

Siriporn Chaemsanit AA. Isolation of total aerobic and pathogenic bacteria from table eggs and its contents. Food and Applied Bioscience 2015;3:19.

Smith A, Rose SP, Wells RG, Pirgozliev V. The effect of changing the excreta moisture of caged laying hens on the excreta and microbial contamination of their egg shells. British Poultry Science 2000;41(2):168–173.

Smith DP, Berrang ME, Feldner PW, Phillips RW, Meinersmann RJ. Detection of *Bacillus cereus* on selected retail chicken products. Journal of Food Protection 2004;67(8):1770–1773.

Stenfors Arnesen LP, Fagerlund A, Granum PE. From soil to gut: *Bacillus cereus* and its food poisoning toxins. FEMS Microbiology Reviews 2008;32(4):579–606.

Storgårds E, Tapani K, Hartwall P, Saleva R, Suihko ML. microbial attachment and biofilm formation in brewery bottling plants. Journal of the American Society of Brewing Chemists 2006;64(1):8–15.

Trudeau S, Thibodeau A, Côté JC, Gaucher ML, Fravalo P. Contribution of the broiler breeders' fecal microbiota to the establishment of the eggshell microbiota. Frontiers in Microbiology 2020;11:666.

Vilain S, Pretorius JM, Theron J, Brozel VS. DNA as an Adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms. Applied and Environmental Microbiology 2009;75(9):2861–2868.

Wei S, Chelliah R, Park BJ, Park JH, Forghani F, Park YS, *et al.* Molecular discrimination of *Bacillus cereus* group species in foods (lettuce, spinach, and kimbap) using quantitative real-time PCR targeting *groEL* and *gyrB*. Microbial Pathogenesis 2018;115:312–320.

Wijman JGE, de Leeuw PPLA, Moezelaar R, Zwietering MH, Abee T. Air-liquid interface biofilms of *bacillus cereus*: formation, sporulation, and dispersion. Applied and Environmental Microbiology 2007;73(5):1481–1488.