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Evaluating the thermoresistance of *Bacillus cereus* strains isolated from wheat flour

Avaliação da resistência térmica de cepas de *Bacillus cereus* isoladas de farinha de trigo

Evaluación de la resistencia térmica de cepas *Bacillus cereus* aisladas de harina de trigo

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Abstract
Wheat flour is often used to prepare confectionery and baked goods, however, it can be contaminated by aporulating microorganisms contaminated during harvest or improper storage. The aim of this study was to isolate *Bacillus cereus* strains from different wheat flour brands and to evaluate their thermoresistance in different confectionery products. It was done in order to investigate the risks posed by food prepared with flour contaminated with *B. cereus* to consumers' health. The investigation of *B.cereus* was realized in five brands of different wheat flours were collected and named A to E. The isolated strains were subjected to boiling tests *in vitro* to evaluate their thermoresistance. In addition, confectionery products were prepared with flour contaminated with *B. cereus* strains. These products were subjected to different cooking and *B. cereus* strain ATCC®30301™ was used as control. Flour brands were contaminated with *B. cereus;* and counts ranged from 0.25 to 1.57 log CFU/g. The strains presented higher thermoresistance in the confectionery products than in the test conducted *in vitro*. Based on our results, it was concluded that *B. cereus* strains are thermoresistant. Moreover, if the flour is contaminated with this bacterium, food products subjected to thermal treatments may remain contaminated. In addition, it is suggested that there is some mechanism (not observed in our study) that could directly influence the thermoresistance of strains found in food.
Keywords: Wheat flour; *Bacillus cereus;* Contamination; Thermoresistance; Food.

Resumo
A farinha de trigo é frequentemente usada para prepare de produtos de confeitaria e panificados, entretanto, pode estar contaminada por microrganismos esporulados principalemnte durante a colheita ou armazenamento inadequado. O objetivo deste estudo foi isolar cepas de *Bacillus cereus* de diferentes marcas de farinha de trigo e avaliar sua termoresistência em diferentes produtos de confeitaria. Isso foi feito com o objetivo de investigar os riscos apresentados por alimentos preparados com farinha contaminada com *B. cereus* para a saúde dos consumidores. A investigação de *B. cereus* foi realizada em cinco marcas de diferentes farinhas de trigo coletadas e denominadas de A a E. As cepas isoladas foram submetidas a testes de fervura in vitro para avaliação de sua termorresistência. Além disso, produtos de confeitaria foram preparados com farinha contaminada com cepas de *B. cereus*. Esses produtos foram submetidos a diferentes cozimentos e a cepa de *B. cereus* ATCC®30301 ™ foi usada como controle. Todas as marcas de farinha que estavam contaminadas com *B. cereus* e as contagens variaram de 0,25 a 1,57 log CFU / g. As cepas apresentaram maior termoresistência nos produtos de confeitaria do que no teste realizado in vitro. Com base em nossos resultados, concluiu-se que as cepas de *B. cereus* são termorresistentes e se a farinha estiver contaminada com essa bactéria, os produtos embora sejam submetidos a tratamentos térmicos podem permanecer contaminados. Além disso, sugere-se que exista algum mecanismo (não observado em nosso estudo) que poderia influenciar diretamente na termorresistência das cepas encontradas nos alimentos.

Palavras-chave: Farinha de trigo; *Bacillus cereus*; Contaminação; Termoresistência; Alimentos.

Resumen
La harina de trigo se usa a menudo para preparar productos de repostería y panadería, sin embargo, puede contaminarse con microorganismos esporulados principalmente durante la cosecha o el almacenamiento inadecuado. El objetivo de este estudio fue aislar cepas de *Bacillus cereus* de diferentes marcas de harina de trigo y evaluar su termorresistencia en diferentes productos de repostería. Esto se hizo con el fin de investigar los riesgos que plantean los alimentos preparados con harina contaminada con *B. cereus* para la salud de los consumidores. La investigación de *B. cereus* se llevó a cabo en cinco marcas de diferentes harinas de trigo recolectadas y nombradas de la A a la E. Las cepas aisladas se sometieron a pruebas de ebullición in vitro para evaluar su termorresistencia. Además, se prepararon productos de confitería con harina contaminada con cepas de B. cereus. Estos productos se sometieron a diferentes cocciones y se utilizó como control la cepa de *B. cereus* ATCC®30301 ™. Todas las marcas de harina que estaban contaminadas con *B. cereus* y los recuentos oscilaron entre 0,25 y 1,57 log UFC / g. Las cepas mostraron una mayor termorresistencia en productos de confitería que en el ensayo realizado in vitro. Con base en nuestros resultados, se concluyó que las cepas de *B. cereus* son termorresistentes y si la harina está contaminada con esta bacteria, los productos, aunque sometidos a tratamientos térmicos, pueden permanecer contaminados. Además, se sugiere que existe un mecanismo (no observado en nuestro estudio) que podría influir directamente en la termorresistencia de las cepas que se encuentran en los alimentos.
Palabras clave: Harina de trigo; *Bacillus cereus*; Contaminación; Termorresistencia; Comida.

1. Introduction

Wheat flour is the main raw material used to make dough-based products; it has high nutritional value and, consequently, is a staple food for the world population (Kucek et al., 2017). Sixty-two percent (62%) of the flour used as raw material in Brazil in 2017 was destined to the bakery sector, 23% was added with flour enhancers, 14% to biscuit flour and 1% was used in other segments (Abitrigo, 2018). It is widely used by the population as the main component in the dough to be added with other ingredients because it makes the food-production process easier, is easy to be handled and presents stability during storage. (Minguita et al., 2015; Katyal et al., 2015).

The flour production process comprises milling and/or grinding the grain (*Triticum aestivum* L.) - or other wheat species belonging to genus *Triticum* - to transform the endosperm into flour; next, the bran is separated from the germ to enable the preparation of higher-quality products (Scheuer et al., 2014). However, the production of farinaceous products has been facing issues such as the contamination with thermoduric and sporulated microorganisms like *Bacillus cereus*, which can deteriorate the products and pose risks to human health (Senesi and Ghelardi, 2010; Chaves et al., 2011).

This microorganism is a Gram-positive, facultative anaerobic, spore-forming bacterium found in different environments such as soil, vegetation, water and animal hair. This bacterium multiplies at temperatures ranging from 25 to 37°C, pH ranging from 5.0 to 9.3 and water activity ranging from 0.92 to 0.95. In addition, it may remain viable for long periods in the environment and in food, because of its ability to form spores resistant to severe conditions (Paiva et al., 2009).

The *B. cereus* is ubiquitous in the environment and can be recovered from food and food materials. It also forms heat resistant spores that are likely to survive the mild heat treatments given to these foods. (Setlow, 2006; Webb et al., 2019). Moreover, spores are extremely dormant and may survive thousands of years in the wet. (Wohlgemuth; Kämpfer, 2014).

Wheat flour can be contaminated with *B. cereus* at all food production stages - from grain harvesting to its processing into dough and ready-to-eat product. Thus, the development of this bacterium in food products can contribute to the incidence of food-borne diseases (Ferreira, 2003).

B.cereus is often found in dry food, it is associated with outbreaks that take place after the intake of food such as sauces, sausages, soups, oven-baked food, rice, doughs, puddings, milk powder, flours, cereals and salads. *B. cereus* is the main cause of food poisoning outbreaks caused by contaminated cereals; this bacterium triggers reactions such as vomiting and diarrhea due to the emetic toxin and enterotoxins produced by it during food processing (Faille et al., 2014; Liu et al., 2018).

Controlling this bacterium in food products is not an easy task due to its ability to produce thermoresistant spores and to adapt to thermally-treated food. This difficulty contributes to their survival and dissemination in the environment, as well as to the contamination of raw materials and post-processing products (Carlin et al., 2010; Abee et al., 2011).

Thus, the aim of the current study was to evaluate the thermoresistance of *B. cereus* strains isolated from wheat flours, by taking into consideration that this raw material is used to prepare several food products that are subjected to different cooking types. The condition in which *B. cereus* recorded the highest thermoresistance was also investigated in this study in order to contribute scientific information that can help to control food contamination by this bacterium.

2. Methodology

2.1 Investigating strains of the *Bacillus cereus* group on wheat flours

Three samples from each of the five brands of flours (named A, B, C, D and E) were collected and subjected to microorganism counting in order to check whether the flour was contaminated with strains of the *B. cereus* group. The counting procedure complied with Normative Instruction 62/2003 of the Ministry of Agriculture, Livestock and Food Supply (MAPA). Twenty-five grams (25g) of each sample were aseptically homogenized in 0.1% peptone water in order to get a 1:10 (and, subsequently, 1: 100) dilution, which was seeded in Petri dishes containing phenol-egg yolk-mannitol-polymyxin B (MYP agar) and incubated at 30 ± 1°C for 48 h.

Colonies surrounded by opaque precipitation halo with rosy background were counted and isolated, since they represented typical colonies of the *B. cereus* group. Next, staining (Gram staining) and biochemical (nitrate motility and reduction, α-hemolysis, tyrosine decomposition, rhizoid growth and presence of crystalline inclusion corpuscles) tests were performed. Results were expressed in colony-forming units per gram (CFU/g).

2.2 Evaluating the thermoresistance of strains of the *Bacillus cereus* group.

To verify the thermoresistance of strains of the *Bacillus cereus* group was used methodology of Johnson;Nelson; Busta (1982) with adaptations.

Three (3) *B. cereus* group strains isolated from the investigated wheat flours were selected to evaluate their thermoresistance; and *B. cereus* ATCC®33019™ strain was used as a control.

The thermoresistance of *B. cereus* was evaluated during the preparation of confectionery products (cake, cream, cupcake and pudding) whose doughs were based on ingredients such as wheat flour, sugar, eggs, milk, margarine and baking powder. All the ingredients were mixed in a plastic vessel; after the doughs showed adequate consistency and growth, they were transferred to a pan in order to be cooked/baked.

The doughs were contaminated with a known number of *B. cereus* strains and/or spores (10^6 CFU/g) and homogenized to assure uniform microorganism distribution before they were cooked. The confectionery products, as well as their cooking method, time and temperature, are described in Table 1.

Table 1. Confectionery product-cooking methods.

Confectionery product	Cooking method	Cooking time/temperature
Cake	Electric oven	50 minutes at 180ºC
Cream	Electric oven	20 minutes at 96ºC
Cupcake	Electric oven	30 minutes at 180ºC
Pudding	Water bath	90 minutes at 80ºC

Source: Authors.

Were analysed portions of the food that were used as a negative control (absence of bacteria from the *B.cereus* group) before being contaminated and as a positive control (presence of bacteria from the *B. cereus* group) portions of the food after contamination with the test strains.

A digital thermometer used for cooking purposes (model TP-101) was adopted to check whether the oven, water bath and direct-heating temperatures were correct. After the heating/baking process was over, the doughs were covered and kept at room temperature (natural cooling). The analysis was performed in triplicate only after the products were fully cooked, i.e., when they were ready for consumption.

Subsequently, *B. cereus* strains (ATCC®33019 ™ and wild strains isolated from the wheat flours singly) were diluted in sterile water at a concentration of 10^6 CFU/ml in a test tube and subjected to test *in vitro*, when they were subjected to baking in a glass Becker. The strains were subjected to approximately 96°C for 0, 5, 10, 15, 20, 25, 30 and 35 minutes. Then, *B. cereus* were counted according to the methodology described.

2.3 Statistical analysis

The Assistat software version 7.7 (2016) was used in association with Excel 2013 to generate a database to enable the analysis of the Tukey test results ($p < 0.05$).

3. Results and Discussion

The microbiological analyses showed that all the investigated flours were contaminated with *B. cereus* group. The recorded amounts ranged from 0.25 log CFU/g to 1.57 log CFU/g; however, they were within the limits (3.48 log CFU/g) recommended by the National Sanitary Surveillance Agency (Table 2). In addition, there were no statistical differences in counts between the herein investigated brands.

Table 2. Comparison of microbiological evaluation data between wheat flour samples from 5 different brands.

Brand	*Bacillus cereus* Group (log CFU*/g)
A	0.25 ± 0.66 [a]
B	0.54 ± 0.93 [a]
C	1.57 ± 0.92 [a]
D	0.86 ± 1.14 [a]
E	0.75 ± 0.97 [a]

*CFU (Colony-Forming Unit). Different letters indicate statistical differences between samples ($p < 0.05$). Source: Authors.

The incidence of this bacterium in the evaluated flours corroborated the study by Prado et al. (2005), who found that 5 (12.5%) out of 40 cassava flour samples were contaminated with *B. cereus*.

Souza et al. (2015) recorded different values (from 2.3 x 10^2 to 1.8 x 10^3) for the incidence of *B. cereus* in cassava flour - unlike our study, which recorded values within the maximum limits recommended by Anvisa (National Health Surveillance Agency), the aforementioned study recorded values above 10^3.

According to Arsenem et al. (2008), values higher than 6 log CFU/g indicate bacterial multiplication; thus, these values represent a risk factor for human health because toxins are produced during *B. cereus* multiplication in the food. Although the amounts of *B. cereus* recorded in our study were lower than 6 log CFU/g, it is worth emphasizing that the contamination is real and that this flour can favor the multiplication of this bacterium at the time it is used and hydrated during food processing.

Other studies, such as the one conducted by Reyes et al. (2007) showed that milk is another food where this bacterium can be found, since 20 out of 63 different *B. cereus* strains found in 260 pasteurized, UHT and powdered milk samples were able to produce toxins.

Based on the thermoresistance analyses, *B. cereus* was able to survive after cooking confectionery products such as cake, cream, cupcake and pudding (Table 3).

Table 3. *B. cereus* counts in confectionery products subjected to thermal treatment.

Strains	**Product**	**Mean**
		***Log CFU/g**
ATCC®33019 ™	Cake	4.53 ±0.91
	Cream	4.69 ±0.38
	Cupcake	4.57 ±0.99
	Pudding	5.77 ±0.40
A	Cake	5.04 ±0.92
	Cream	5.30 ±0.58
	Cupcake	5.73 ±0.33
	Pudding	5.87 ±0.13
B	Cake	4.35 ±0.62
	Cream	4.47 ±0.47
	Cupcake	5.85 ±0.36
	Pudding	4.25 ±0.44
C	Cake	5.63 ±0.08
	Cream	5.52 ±0.06
	Cupcake	5.93 ±0.12
	Pudding	5.21 ±0.09

*CFU (Colony-Forming Unit). Source: Authors.

Based on Table 4, there was no statistical difference in *B. cereus* thermoresistance between the tested food products. Therefore, food composition and cooking temperature did not influence the thermoresistance of the tested strains.

Table 4. Comparison of *B. cereus* count between confectionery products.

Confectionery products	*Bacillus cereus* Group(Log CFU*/g)
Cake	4.76 a
Cream	4.91 a
Cupcake	5.45 a
Pudding	5.18 a

* CFU (Colony-Forming Unit). Different letters indicate statistical differences between samples (p <0.05). Source: Authors.

Our results were similar Fazzoni et al. (2013) and Sánchez et al. (2014), who found incidence of *B. cereus* in several foods made from wheat flour. According to the aforementioned studies, the high count of *B. cereus* may have resulted from the raw material, as well as from poor production, storage and handling conditions.

These food types, besides being able to present the bacterium in its sporulated form, are often stored in unappropriate temperature conditions after their preparation. These conditions favor spore germination and enable the intake of the vegetative form of the bacterium and/or of its toxin (Luu-Thi et al. 2014).

Other studies, such as the one conducted by Rubio and Andres (2015), showed that *B. cereus* can survive in the food after the cooking process is over and that food storage temperature can directly affect the post-cooking multiplication of this bacterium. The aforementioned author compared white rice to vitaminized rice and found that white rice stored at 10°C recorded maximum spore count 2.8 log CFU/g, whereas vitaminized rice recorded 4.8 log CFU/g. On the other hand, white rice and vitaminized rice stored at 25°C recorded maximum spore count 5.4 log CFU/g and 8.4 log CFU/g, respectively.

Our results corroborate with Eijlander et al. (2011) that spores of *Bacillus* species can survive stress conditions, for example, heating food. These insufficient inactivation associated with nutrients found in food can contribute the germination of these spores and results in multiplicationn these bacteria, with great risk of food spoilage and food poisoning after consumption.

Moreover, spore properties and germination efficiency are clearly affected by differences in environmental conditions, as for example, the presence or absence of certain nutrients or chemical compounds in the foods, the pH, temperature and time of exposure at high temperatures (Eijlander et al. 2011).

The table 5 presents the results of thermoresistance comparison between the tested *B. cereus* strains. Wild strains A and C showed higher thermoresistance than the ATCC strain in the tested food products.

Table 5. Comparison of thermoresistance between *B. cereus* strains tested in different confectionery products.

Strains	*Bacillus cereus* Group(Log CFU*/g)
ATCC®33019 ™	4.52 b
A	5.41 a
B	5.04 ab
C	5.33 a

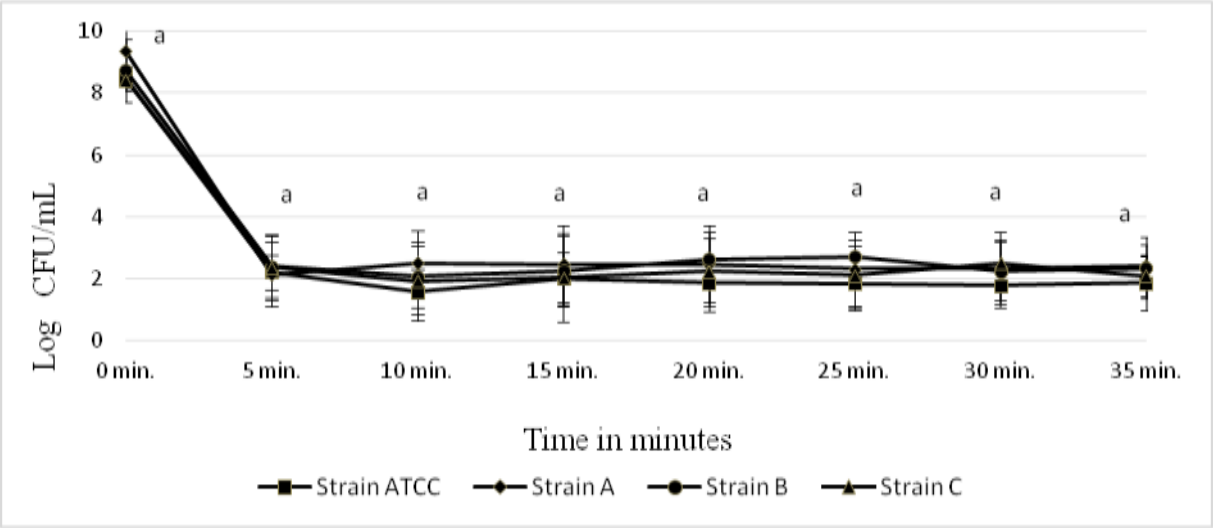
* CFU (Colony-Forming Unit). Different letters indicate statistical differences between samples (p <0.05). Source: authors.

In light of the foregoing, it is suspected that this bacterium has adapted to flour storage media, since it is able to survive in products presenting low water activity for long periods. Consequently, when the flour is used to make food, the dormant spores are hydrated that associated with the new environmental conditions (pH, temperature, osmotic pressure,

nutrients) start forming new cells already adapted to the new ready-to-eat food (Van der Auwera, et al., 2007; Hoon et al. 2010; Mckenney et al. 2013).

On the other hand, results of the test conducted *in vitro* showed that vegetative cells were eliminated during the first 5 minutes of the cooking process in sterile water. However, boiling *in vitro* was unable to destroy the spores, which survived (2.0 log CFU/mL, on average) up to 35 boiling minutes (Figure 1).

Figure 1. Comparison between *B. cereus* cooking times in test conducted *in vitro*.



Different letters indicate statistical differences between strains ($p < 0.05$). Source: authors.

Our results corroborate the study by Rossi et al. (2012), who stated that boiling is a heat treatment capable of destroying only vegetative cells because the water-boiling temperature does not exceed 100°C.

Results of all boiling times were statistically different from those of the initial time (before boiling) in the test conducted *in vitro*. As it was previously mentioned, data showed that vegetative cells were destroyed and only the spores remained after the first five boiling minutes, since they were able to resist up to 35 boiling minutes (Table 6).

Table 6. Mean LogCFU/ml of the test conducted *in vitro* thermoresistance evaluation of *B.cereus* strains (ATCC®33019 ™ and wild strains isolated from the wheat flours) tested separately.

Time	Mean LogCFU/ml
Zero minutes	8.83 a
Five minutes	2.25 b
Ten minutes	1.99 b
Fifteen minutes	2.21 b
Twenty minutes	2.23 b
Twenty-five minutes	2.29 b
Thirty minutes	2.20 b
Thirty-five minutes	2.23 b

Different letters indicate statistical differences between samples ($p < 0.05$). Source: Authors.

Results recorded by Bradshaw et al. (1975) and Dufrenne et al. (1995) were similar to ours. The aforementioned studies evaluated several *B. cereus* spore strains and found that one strain was thermoresistant for 2.90 minutes at 90°C, whereas the other psychrotrophic *B. cereus* strains were thermoresistant from 2.8 to 9.2 minutes at 90°C. Moreover, Wang (1994) analyzed the kinetic parameters of thermal resistance in the main sporulated organisms found in fluid milk samples, among them, *B. cereus*, which recorded thermoresistance for 3.8 minutes at 35.9°C.

Based on figure 1, there was no statistical difference in thermoresistance *in vitro* between the tested *B. cereus* strains.

B. cereus thermoresistance *in vitro* has been investigated by several researchers such as Den Besten et al. (2010), who showed that the boiling efficiency in destroying this bacterium may vary if one takes into consideration the number of vegetative cells and spores in the sample: the larger the number of spores, the higher the thermoresistance. The aforementioned authors concluded that the number of *B. cereus* vegetative cells decreased after 9 minutes at 44.5°C, whereas the number of *B. cereus* in the stationary phase did not decrease during 15 minutes at 44°C; they were the specimens most resistant to heat.

Other studies, such as the one conducted by Valerio et al. (2012), showed that the initial contamination of semolina (durum wheat) used for Algerian couscous production was 20 CFU/g of *B. cereus* spores after heat treatment at 80°C for 10 minutes.

According to data described in Tables 5 and 6, the herein tested *B. cereus* strains recorded higher thermoresistance (mean = 5.0 log CFU/ml) in confectionery products when compared with the results of the test conducted *in vitro* (mean = 2.0 log CFU/ml).

The higher thermoresistance of *B. cereus* in the analyzed food can be explained by the fact that these products enable higher thermoresistance, thus assuring additional heat protection. In addition, food can also work as good nutritional medium for this bacterium, since this medium enables favorable enzyme and spore production conditions (Sánchez, 2016).

It is known that genus *Bacillus* comprises resistant microorganisms capable of adapting to extreme environmental conditions. These microorganisms actively grow at temperatures ranging from 25 to 37°C, besides presenting psychotropic properties depending on their ability to grow in food stored at temperatures ranging from 3 to 75°C (Paiva et al., 2009).

On the other hand, growth conditions also affect their thermoresistance. Lound et al. (2017) believe that such resistance is associated with the low water content inside the cell. These researchers saw that heat induces intense water molecule vibration and, consequently, breaks the disulfide and hydrogen bonds of the intracellular proteins. However, this vibration does not happen in vegetative cells due to limited water availability, which, in its turn, protects cellular proteins from denaturizing at high temperatures. Therefore, incorrect food storage temperature, water activity, pH and food composition were probably the factors that most contributed to *Bacillus cereus* thermoresistance during the cooking process.

However this study may have been limited by its structure because the experiment *in vitro* was conducted only in wet conditions and water conducts heat better. Although the food tested has water, we believe the *in vitro* results may have been better because water directly conducted heat and contributed to cell death.

Thus, our results can contribute to the development of new research to a deeper understanding of the mechanisms involved in spore resistance, adaptation and killing (and heterogeneity there-in) may lead to improved models for spore behavior prediction.

In addition, studies can be developed to verify the spread of *B.cereus* in environments such as bakeries or bakery industries, because according to Garcia et al. (2019) flour has a particular importance in the dissemination of fungal spores because they could disperse in the processing air during food processing.

4. Conclusion

Results of the current study allowed us to conclude that flours may be contaminated with *B. cereus*, and that this bacterium can survive cooking and remains in ready-to-eat food products. Facts that may lead to health issues if the flour is not properly stored. Consequently, if the flour is contaminated, the food to be prepared with this ingredient can also be contaminated by this pathogen, even if it is subjected to different heat treatments.

In addition, food composition can provide better heat protection for *B. cereus*, since this bacterium presented higher thermoresistance in food than in tests conducted *in vitro*. This outcome suggests that there are some mechanisms (not observed in our study) that could directly influence the thermoresistance of the strains found in food.

Thus, we suggest that news research be made to verify the mechanisms of *B.cereus* thermoresistance in foods.

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