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# Comprehensive risk assessment of infant exposure to *Bacillus cereus* bacteria in commercially baby flour marketed in Bechar, South western Algeria

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**Abstract:**
Infants represent the foundation of society, therefore, safeguarding their health is an essential responsibility. This research focuses on the biological factors that may lead to organic diseases in infants due to the consumption of weaning foods contaminated with the sporulated bacterium *Bacillus cereus* in Bechar region of southwest Algeria. In recent years, the consumption of infant flour has increased. Although this product is designed for children, it is inherently non-sterile and produced at temperatures below 50 degrees Celsius, making it susceptible to bacterial proliferation. *Bacillus cereus* was detected in 78% of the samples, with concentrations ranging from 2 to 3 log. Resistance to temperature was assessed at 90°C, 95°C, and 98°C, demonstrating its resilience, certain strategies were shown to be effective in eradicating the bacterium.
**Key words**: Infants, Weaning foods, Bacillus cereus, Resistance



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## 1. Introduction

Infant and young child nutrition is an important topic, and the majority of healthcare professionals agree on the superiority of breastfeeding (Jackson and Nazar, 2006). Indeed, breast milk, this crucial liquid during the first three months of an infant's life, is sufficient to cover their nutritional needs; for this reason, the World Health Organization (WHO) recommend that mothers breastfeed their children until they are 2 years old (WHO, 1995).

However, from six months onward, the needs for energy and micronutrients, particularly iron, exceed what breast milk can provide, making it necessary to introduce complementary foods such as infant cereals (Brown and *al*., 1998; Genève, 2001). These food preparations are formulated to provide essential additional energy and nutrients, but they can be contaminated by microorganisms, notably *Bacillus cereus* (Delmas and *al*., 2010). This bacterium produces toxins that can cause vomiting or diarrhea and is widespread in the environment, which explains its presence in ingredients such as milk, cereals (Ziane and *al*., 2014), and flour (Valero and *al*., 2003). Its resistant spores facilitate its survival and proliferation under certain environmental conditions, particularly temperature (Difallah ans *al*., 2021). It is in this context that our work takes place, with the choice of *B.cereus* as a study model in the different brands of infant flour consumed in the town of Bechar, southwest Algeria in order to assess the probability of exposure of children to the biological contaminant of the group *B. cereus* exceeds the doses tolerable by the organism which we call risk and evaluate their thermoresistance following the development of the bacteria which will attract our attention, and in the last part of the study we tried to estimate the growth and the concentration of *B .cereus* at different storage temperatures and consumption. Since 2017, Algeria has included the detection of spore-forming bacteria, including *Bacillus cereus*, in its microbiological criteria. Despite the low water activity of infant cereals, spores can persist, germinate, and produce toxins depending on storage conditions, making these foods a potentially risky product. This study therefore contributes to the prevention and protection of infant health.

## 2. Materials and methods:

The sample was taken at different points of sale (pharmacies). Thus, the sampling method was used to locate a random point of sale on the map of the city of Béchar as described by Grawitz (2001). The preparation schedule was collected from

the manufacturer's instructions on the container of each brand and the behaviors of nurses, only for families with a baby. Each preparation template defines a scenario.

In total, 70 samples of infant flour of 10 different brands and tastes were collected from 20 selected points of sale, then transferred to the laboratory in their sales conditions for microbiological analysis. The exterior surface of the infant flour bag was disinfected with alcohol (99.8%) and then opened under aseptic conditions. From each sample unit, 10 g was taken and diluted in 90 mL of peptone water.

The mixture is then put in a water bath at 80°C for 10 min at temperatures ranging from 98 to 100°C and then develops into the infant flour meal. At this level, different questions could be asked and would require further investigation to obtain precise answers such as:

Does the manufacturer's recommended temperature eliminate B. cereus in the meal? Do mothers follow the manufacturer's instructions? Is the mother safer than recommended by the manufacturer? Is it necessary to improve a preparation plan? Thus, this article aims to evaluate (1) the methods of preparing meals based on infant flour according to different actors (manufacturers' instructions and childminders), (2) their resistance to heat, (3) the decimal reduction ratio at each preparation temperature, and (4) the concentration of *B. cereus sensu lato* in the infant's flour meal.

In this context, it should be important to note that *B. cereus sensu lato* is a group of bacteria belonging to the genus *Bacillus* and presenting close phylogenetic relationships but affiliated to different phylogenetic subgroups (I to VII) with high ecological diversity (Guinebretière et al., 2008). This group includes *B. pseudomycoides, B. weihenstephanensis, B. mycoides, B. anthracis, B. cereus sensu stricto, B. cytotoxicus and B. thuringiensis* (Guinebretière et al., 2008).

Next, decimal dilutions in series were carried out in peptone water from previously heated mixtures. A volume of 0.1 mL of each dilution was spread onto the surface of Mossel Agar plates (Tryptone (10.0 g); Meat extract (1.0 g); D-mannitol (10.0 g); Sodium chloride (10.0 g); Phenol red (25.0 g. mg); sterile egg yolk emulsion (100.0 mL); bacteriological agar (13.5 g); pH 7.2 ± 0.2) (Biokar diagnostics, France), then incubated at 30°C for 24 h for isolation and enumeration. *B. cereus sensu lato* is Gram positive, rod shaped, catalase positive and forms endospores.

For the production of spores, a volume of 0.5 mL of the culture was spread on the surface of a nutrient agar medium (Biokar diagnostics, Beauvais, France) supplemented with 40 mg/L of MnSO4 and 100 mg/L of CaCl2, then incubated at 30 °C for the time necessary for the sporulation of the bacterial population seeds. Sporulation was monitored by observation under a phase contrast microscope during 5 days of incubation.

The spores of *B. cereus sensu lato* spores were collected using a sterile spatula by scraping the surface of the agar. The collected spores were suspended in 20 ml of sterile distilled water. The prepared spore suspension was centrifuged at 500 × g for 30 min. The pellet was collected and resuspended in 20 ml of sterile distilled water

This operation was repeated twice. The pellet obtained was taken up in a water/ethanol mixture (v/v) and placed at 4°C for 12 h to eliminate the rest of the vegetative forms. The mixture was then centrifuged at 500 x g for 30 min. The treated strains underwent three washing cycles with sterile distilled water under the same centrifugation conditions.

The pellets previously collected were then resuspended in a minimum volume greater than 200 µL of sterile distilled water to thus have a high concentration of spores. The stock of B. cereus sensu lato spores obtained was stored for a minimum of one month at 4°C in sterile distilled water before the heat treatment step.

A volume of 100 µL of spore stock was suspended in 10 mL of BHI (Brain Heart Infusion Broth) (Biokar diagnostics, Beauvais, France). Then, a volume of 1 mL was dispensed into sterile tubes. After heating at different temperatures between 90°C and 98°C, the contents of each tube were quickly poured into 9 ml of sterile physiological water.

The enumeration of survival spores was carried out by inclusion of 0.5 ml of the contents of the heated tubes in 15 ml of nutrient agar, then incubated at 37°C for 48 h.

For the determination of the thermal resistance parameters, the thermal resistance parameters were estimated by first-order kinetics (equation 1). Otherwise, the influence of temperature (T °C) on bacterial sensitivity to heat was quantified by the classical sensitivity parameter zT °C, using Equation 2.

When preparing meals, warm boiled water (or milk) was added to the infant's flour and then mixed. As a dry product, infant flour quickly absorbs boiled (heating) water (or milk) added to reach the maximum temperature (Tmax) in the meal, then decreases as it cools. The method of preparation was collected from 100 families.

Mothers were asked the following questions: (1) Do you follow the meal preparation instructions labeled by the brand sold? (2) Do you know exactly the temperature used for such a preparation? (3) If so, what is it? (4) If no, what is the temperature range used: [60-80°C] or [80-100°C]? As for the temperature recommended by the manufacturer, it was taken from the label instructions (45°C and 50°C).

the meal was prepared as follows: a volume of 150 mL of heated water (at the temperature studied for each scenario and data collected) was gradually added to the

30 g of infant flour in the bottle and mixed well to record the profile of temperature every 1 minute.

When the temperature profile was recorded, the decimal reduction ratio "n" was estimated at each temperature kinetic (T°C). The Bigelow method was used, based on the calculation of the partial biological destruction value (L(T)) for each temperature kinetic recorded every minute. Then, the decimal reduction ratio (nT °C) was calculated according to equation 4.

The L(T) were calculated throughout the time spent for the temperature to reach 45°C. The parameters of thermal resistance (DT °C and z values), biological destruction L(T) and decimal reduction ratio (n) were estimated using Excel in Microsoft office. The probabilistic distribution, adjustment and Monte Carlo simulation were carried out by @Risk software version 5 (Palisade). As for the ANOVA analysis, version 22 of SPSS software was used.

## 3. Results and Discussion:

As for the level of contamination in this study, the median concentration varied between 2.4 and 3.9 log CFU/g. To estimate the thermal resistance parameters (DT °C), linear regression was adopted for each isolate using all the curves. The results show heterogeneity in the heat resistance of the isolates studied. DT °C values range from 3.24 to 5.52, e.g. 95°C. As for thermal sensitivity, thermal treatments were carried out and varied from 11.56°C to 89.74°C

Concerning the method of preparation, most manufacturers recommend heating the water then letting the temperature drop to 45°C, while no heat treatment is required when using milk for meal preparation. 16% of meal preparations used fresh or room temperature milk and 70% reheated it. Others add spices (1.1%) as well as fruits and vegetables (9.8%).

The manufacturer does not recommend the use of heated milk, perhaps, to preserve its components and because it has undergone prior sterilization during its production process. According to the objectives of this study, frequency of use is not necessary. According to a survey conducted as part of this study, only 41.5% of mothers follow the infant meal preparation recommendations established by manufacturers, while 49% of them do not follow the label instructions. Indeed, these mothers have no information on the safe preparation schedule and prefer to use hot water (more than 60°C) to ensure the death of the microbes probably present (as they called) in the infant's flour without taking into account guaranteeing the nutritional quality of prepared dishes. Indeed, 37.5% and 5.8% of mothers prepared the infant's

meal with water heated to 60-80°C and 80-100°C respectively, The results are summarized in Table 1.

Concerning the exposure time, it depends on the initial temperature. This is the time spent (min) necessary to reach the consumption temperature above 45°C. Exposure times were calculated for each temperature (50°C, 60°C, 70°C, 80°C, 90°C, 100°C) The results are summarized in Figure 1. They were used to calculate the biological destruction value (L(T)). Whatever the temperature tested, the temperature of the meal decreases. For a temperature < 50°C, the temperature directly reaches 45°C in 3 min.

The biological killing value (L(T)) was calculated for each kinetic temperature and tested isolate. L(T) was proportional to temperature. The results showed that the high values were observed at 100°C (median = 5.40; mean = 7.36; 95e = 19.92). Then, these values were used to calculate the decimal reduction ratio "n" at each temperature. The "n" values depended on the preparation temperature and L(T). However, the lowest temperature effect was estimated for temperature <50°C, high values were reported at 100°C (median = 2.22; mean = 3.16; 95e = 12.35) , while low values were reported at 50°C (median = 0.02 = mean; 95e = 0.35) The results are summarized in Table 2.

It should be noted that the prevalence (74%) of *B. cereus sensu lato* seemed similar to the prevalence (78%) reported by Heini (2018) and higher than 3.5% reported by Zhang et al. (2017). As for their concentrations in the samples analyzed, they agreed with those reported (3 to 4 log CFU/g) by Zhang et al. (2017). The presence of *B. cereus sensu lato* in the analyzed samples is evident because (1) infant flour is not a sterile product, (2) and is produced with various ingredients generally contaminated with the *B. cereus* group. Indeed, several ingredients of infant flour are contaminated by *B. cereus sensu lato*, mainly rice (Sarrias et al. 2002), corn (Byaruhanga et al. 1999), wheat (Valerio et al. 2012) or oats and other cereals (Daczkowska-Kozon et al. 2009).

The *B. cereus sensu lato* group is mainly present in raw materials, which increases its probability and confirms its presence in infant flour. The presence of *B. cereus sensu lato* in infant flour could be due to several factors: (1) the contaminated raw material, (2) the ability of *B. cereus sensu lato* to produce a spore form to survive under conditions hostile, particularly at low aw of this product (infant flour). Fortunately, at this stage, *B. cereus sensu lato* does not exceed a critical concentration (5 log CFU/g) and could decrease after preparation with lukewarm boiling water depending on the preparation methods.

The problem is not only related to the existence of *B. cereus sensu lato* in the product, but also to (1) its resistance to heat and its ability to grow and reach a critical concentration (5 log cells per g) and (2) the low preparation temperature for the two patterns (manufacturing instructions and mother preparation). Thus, the level of *B. cereus* could increase particularly during their survival and growth during preparation and storage respectively. In fact, Sadek et al. (2018) consider infant formula to be an excellent medium for bacterial growth. Buss da Silva et al. (2017) showed the growth of three tested strains of *B. cereus* in reconstituted infant formula. Otherwise, Messelhäusser et al. (2014) reported that cereal-based infant formula, rich in vitamins and trace elements, is known to promote cereulide synthesis.

The concentration of *B. cereus sensu lato* in different preparations was estimated by equation 5 developed by Nauta (2001) and Membré and Valdramidis (2016). The probabilistic approach was used to simulate the concentration of *B. cereus sensu lato* using the Poisson distribution: [*B. cereus* sl]s = Riskfish([*B. cereus* sl] × m0 ×10 ) (5) where [B. cereus sl]s: survival concentration (CFU per quantity prepared) of *B. cereus sensu lato* in the meal after heating; [*B. cereus* sl]0: Distribution of the initial concentration (CFU per gram of infant flour analyzed) of B. cereus sensu lato. It was adjusted by RiskDuniform (C1, C2, C3,…, Ci) × RiskBeta (number of contaminated samples + 1, no contaminated samples + 1). C1 to Ci are the concentrations of *B. cereus sensu lato* in each positive sample (1 to i respectively). As for the probability of appearance of toxigenic strains among these concentrations, the RiskUniform distribution (0; 1) was used. In the absence of information on the distribution of toxigenic bacteria among the *B. cereus* group, it was assumed that toxigenic B. cereus could represent any percentage (ranging from 0 to 100%) of all B. cereus groups. . cereus which was therefore described by a RiskUniform(0;1) as shown by Ziane et al. (2019).

Monte Carlo simulations of initial concentrations of toxigenic *B. cereus* in infant flour before preparation were assumed to be (median = 2.489; mean = 2.615; 99e = 3.75) log CFU/g. After preparation, the possible concentrations of toxigenic B. cereus and according to equation 5 the low values (CFU per g of prepared meal) were estimated for a temperature of 100 °C. °C (min=0, median = 0.004; mean = 0.135; 99e = 1.24 log CFU/g) followed by the 90 °C scenario (min=0, median = 0.036; mean = 0.142, 99e = 1.226 log CFU/ g). Both of these temperatures are safe for infants, while heating to the temperature recommended by manufacturers is unsafe and no significant effect (ANOVA $P < 0.001$) on initial concentration was observed.

## 3. **Tables & Figures:**

**Table (1):Impact of mothers' educational level on infant feeding.**

| N° | Questions pour les pratiques de préparations de repas | % de ménage pour chaque réponse | | |
|---|---|---|---|---|
| | | **Oui** | **Non** | **ND** |
| **01** | Education nutritionnelle | **20** | **80** | / |
| **02** | Conscience de danger mcirobiologique dans la FI | **80** | **15** | **5** |
| **03** | Source d'information | **Télévision, réseaux sociaux** | | |
| **04** | Lave main avant manipulation de FI | **92,4** | **7,6** | / |
| **05** | Re-lavage des ustensiles utilisés | **90** | **6** | **4** |
| **06** | Eau de préparation Minéral<br>Robinet | **86**<br>**14** | / | / |

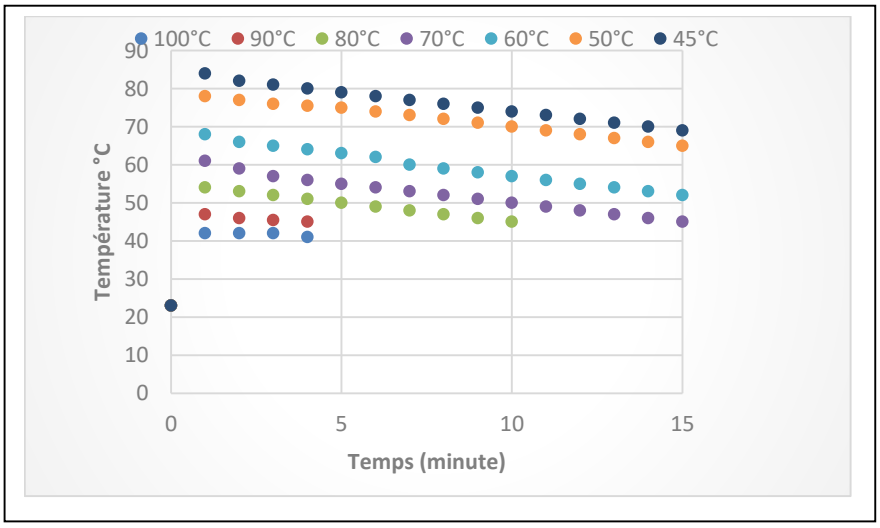
**Table (2): Results of the evaluation of critical concentrations during the consumption of infant flour meals. The concentrations are simulated from the concentrations of spores resulting from each heat treatment.**

| Etape | Symbole | Unité | Distribution |
|---|---|---|---|
| Contamination initiale | | | |
| Concentration de *B. cereus* dans les échantillons analysés | $[B.\ cereus\ sl]_0$ | Ufc/g | RiskUniform($N_1$ à $N_{72}$) |
| Probabilité de *B. cereus* toxinogène | Prt | | RiskUniform(0 :1) |
| Concentration de *B. cereus* dans les échantillons analysés | $[B.\ cereus]_0$ | Ufc/g | $[B.\ cereus\ sl]_0 \times$ Prt |
| Nombre des échantillons analysés | Pt | | // |
| Nombre des échantillons non contaminé | Pn | | // |
| Nombre des échantillons contaminé | Pp | | Pt-Pn |
| Prévalence de *B. cereus sensu lato* dans les échantillons analysé | P | | ((Pt-Pn)/Pt)×100 |
| Distribution de *B. cereus sensu lato* dans la farine infantiles commercialisé dans la région de Béchar | $[B.\ cereus]_{FI}$ | Ufc/g | RiskBéta(Pt-Pn+1 ;Pn+1) × $[B.\ cereus]_0$ |
| | | | |
| Temps de réduction décimale | $D_{T°C}$ | min | Eq (1) |

| | | | |
|---|---|---|---|
| Sensibilité au traitement thermique | $z_{T°C}$ | °C | Eq (2) |
| Cinétique de la température | | °C | Riskpert() |
| Destruction biologique | L(T) | °C | Eq (3) |
| Nombre de réduction décimale | n | | Eq (4) |
| Concentration de *B. cereus* toxinogène survivante | $[B.\ cereus]_s$ | Ufc/g | Eq (5) |
| Croissance de *B. cereus* toxinogène | | h | Eq (6) |
| Température cardinale : minimale/maximale/optimale | $T_{min}/T_{max}/T_{opt}$ | °C | Littérature (Tableau 22) |
| Taux de croissance optimum | $\mu_{opt}$ | $h^{-1}$ | Eq (8) |
| Modèle gamma γ | $\gamma_{T°C}$ | °C | Eq (7) |
| Taux edc roissance maximum dans la farine infantile | $\mu_{T°C}$ | $h^{-1}$ | Eq (9) |
| Temps de latence dans la farine infantile | $\lambda_{T°C}$ | h | Eq (10) |
| Concentration de *B. cereus* toxinogène dans le repas au moment de la consommation | $[B.\ cereus]_{repas}$ | Ufc/g | Eq (11) |
| Evaluation de l'exposition | P≥5 log ufc | | 1-Risktarget($[B.\ cereus]_{repas}$ ;5) |
| Fréquence de chaque scenario temps/température | Ps | | Questionnaire d'enquête |
| Nombre de repas contient ≥ 5 log ufc pour 100 enfants | | | P × Ps |

**Source:** Difallah et *al*, .2021

**Fig.1. Concentration (log UFC) of B.cereus during meal preparation $10^2$**



## 5. Conclusion

Great attention should therefore be paid to the consumption of infant flour in terms of the emergence of potential microbial hazards such as spores of the bacteria group *B. cereus sensu lato*, known for their resistance to heat. Depending on the preparation patterns, *B. cereus sensu lato* could be involved in food poisoning. Our results showed that 75% of infant flour samples were contaminated with *Bacillus*. Furthermore, the results of this article highlight the effectiveness in reducing the rate of *B. cereus sensu lato* compared to the manufacturer's instructions, only the modes of use need to be improved. Thus, some recommendations could be established to avoid problems linked to this type of bacteria: - Choose a relevant raw material; - Continuously improve the process and control it; - Review the preparation instructions and improve the solubility of the product at high temperatures; - Reduce the quantity of flour from 200 g to 100 g per package; - Add the storage condition to the packaging. Finally, as perspectives for this study, additional experiments will have to be carried out in the future, on: (1) determining the affiliation of *B. cereus* to different groups; (2) study their growth in the infant's flour meal; (3) conduct a microbial risk assessment to obtain sufficiently in-depth details about this type of bacteria, which will undoubtedly help industries during the production process and stores during storage.

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