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THESIS

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THEME

Exposure assessment of *Bacillus* spp. in infant milk formula: risk and mitigation strategies

Presented by

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Abstract

The principal objective of this study was to develop a comprehensive quantitative exposure assessment model for Bacillus contamination throughout the powdered infant formula (PIF) in Algeria. Thirty-two samples from leading brands were collected and analyzed for spore-forming bacteria. A total of 26 isolates were identified, and four strains were selected and studied for further characterization using biochemical assays and MALDI-TOF mass spectrometry. The study assessed the four strains' antibiotic resistance, enzymatic and hemolytic activity, and biofilm-forming ability. All isolates exhibited resistance to at least five antibiotics, including penicillin and tetracycline, while all were susceptible to ciprofloxacin, chloramphenicol, streptomycin gentamicin and vancomycin. Enzymatic assays revealed the production of protease, amylase, lecithinase, lipase and also hemolysin by all strains except E1 strain which did not produce the hemolysin and protease enzymes, indicating potential spoilage and pathogenic risks. Biofilm assays, both in microtiter plates and on baby bottle materials (glass and polypropylene), showed that all strains could form biofilms, with denser formation observed on polypropylene surfaces, where the highest being E4 strain with 5.83 log CFU/ML and 5.63 log CFU/ml for PP and glass surface respectively, while the least E1 strain had 4.28 log CFU/ML and 3.24 log CFU/ML for PP and glass respectively. The quantitative exposure assessment model highlights significant differences between preparation methods: the use of boiling water reduces Bacillus exposure by 93–96% (resulting in 75–90 CFU/kg) compared to microwave heating (600–2,200 CFU/kg), and decreases the associated risk probability from 9.1–26.8% to just 2.3%. Microwave efficacy was found to be wattage-dependent. Devices operating below 800W present unacceptably high risks (1,800–2,200 CFU/kg; 25–28% probability), attributed to a 25% likelihood of cold spots and limited spore reduction (0.3–0.6 log). Even high-power microwaves (≥1100W) achieve only partial mitigation, lowering contamination levels by approximately 60% (600–800 CFU/kg) and risk probability by 65% (8–10%) through improved thermal uniformity (5% cold spots) and moderate inactivation (0.7–1.0 log). Nevertheless, boiling water remains the most reliable method for minimizing microbial risk in PIF preparation. These findings highlight critical limitations in conventional household hygiene practices and underscore the persistence of these bacteria in real situations and the need for stricter public awareness on safe formula preparation to mitigate risks associated with *Bacillus* contamination in infant nutrition.

Key Words: Bacillus Cereus, Powder Infant Milk, Biofilm, Antibiotic resistance, Quantitative exposure assessment, Modular Process Risk Model

Résumé

L'objectif principal de cette étude était de développer un modèle d'évaluation quantitative de l'exposition au risque de Bacillus dans les préparations de lait infantile en poudre en Algérie. Trente-deux échantillons issus de différentes marques ont été collectés et analysés pour la recherche de bactéries sporulées. Au total, 26 isolats ont été identifiés, dont quatre souches sélectionnées pour une caractérisation approfondie à l'aide de tests biochimiques et de la spectrométrie de masse MALDI-TOF. L'étude a évalué la résistance aux antibiotiques, l'activité enzymatique et hémolytique, ainsi que la capacité de formation de biofilm des quatre souches. Tous les isolats ont montré une résistance à au moins cinq antibiotiques, incluant la pénicilline et la tétracycline, tout en demeurant sensibles à la ciprofloxacine, au chloramphénicol, à la streptomycine, à la gentamicine et à la vancomycine. Les tests enzymatiques ont révélé la production de protéase, amylase, lécithinase, lipase et l'hémolysine chez toutes les souches, à l'exception de la souche E1, qui ne produisait ni hémolysine ni protéase, ce qui suggère un risque potentiel d'altération et de pathogénicité. Les essais de formation de biofilm, réalisés en microplaques et sur des surfaces de biberons (verre et polypropylène), ont démontré que toutes les souches étaient capables de former des biofilms, avec un potentiel plus élevé sur le polypropylène. La souche E4 a montré la plus forte concentration (5.83 log UFC/mL sur polypropylène et 5.63 log UFC/mL sur verre), tandis que la souche E1 a présenté la plus faible (4.28 log UFC/mL sur polypropylène et 3.24 log UFC/mL sur verre). Le modèle d'évaluation quantitative de l'exposition met en évidence des différences significatives entre les méthodes de préparation : l'utilisation d'eau bouillante permet de réduire l'exposition à Bacillus de 93 à 96 % (soit 75–90 UFC/kg), contre 600–2200 UFC/kg en cas d'utilisation du micro-ondes, et abaisse la probabilité de risque de 9.1–26.8 % à seulement 2.3 %. L'efficacité du micro-ondes dépend fortement de la puissance utilisée : les appareils inférieurs à 800 W présentent des risques inacceptables (1800–2200 UFC/kg ; 25–28 % de probabilité), en raison d'une probabilité de 25 % de zones froides et d'une réduction limitée des spores (0.3–0.6 log). Même les micro-ondes à haute puissance (≥1100 W) n'offrent qu'une atténuation partielle, réduisant les niveaux de contamination d'environ 60 % (600–800 UFC/kg) et le risque de 65 % (8–10 %), grâce à une meilleure uniformité thermique (5% de zones froides) et une inactivation modérée (0.7–1.0 log). Néanmoins, l'eau bouillante demeure la méthode la plus fiable pour minimiser le risque microbien lors de la préparation des PIF. Ces résultats soulignent les limites critiques des pratiques d'hygiène domestiques courantes et mettent en évidence la persistance de ces bactéries dans des conditions réelles, tout en insistant sur la nécessité d'une sensibilisation accrue du public à une préparation sécurisée des laits infantiles afin de limiter les risques liés à la contamination par Bacillus dans la nutrition des nourrissons.

Mot-clé : Bacillus cereus, Lait Infantile en Poudre, Formation de Biofilm, Résistance aux Antibiotiques, Évaluation Quantitative de l'Exposition, Modèle Modulaire d'Évaluation des Risques du Processus.

الملخص

الهدف الرئيسي من هذه الدراسة هو تطوير نموذج شامل لتقييم التعرض الكمي لمخاطر تلوث مسحوق حليب الرضع (العصوية) في الجزائر. تم جمع 32 عينة من علامات تجارية معروفة وتحليلها للكشف عن البكتيريا المكوّنة للأبواغ. ببكتيريا تم التعرف على 26 عزلة بكتيرية، وتم اختيار أربع سلالات منها لإجراء دراسات تفصيلية باستخدام اختبارات كيميائية حيوية وتقنية تحليل الطيف الكتلي. شملت الدراسة تقييم مقاومة هذه السلالات للمضادات الحيوية، والنشاط الإنزيمي، والنشاط الحال للكريات الحمراء، بالإضافة إلى قدرتها على تكوين الأغشية الحيوية. أظهرت جميع السلالات مقاومة لخمسة مضادات حيوية على الأقل، من بينها البنسلين والتيتراسيكلين، بينما كانت جميعها حساسة للسيبروفلوكساسين، والكلورامفينيكول، والستربتوميسين، والجنتاميسين، والفانكوميسين. كشفت التحاليل الإنزيمية عن قدرة السلالات على إنتاج إنزيمات مثل البروتياز، والأميليز، والليسيثيناز، والليباز، والهيمولايسين، باستثناء السلالة (1) التي لم تنتج البروتياز ولا الهيمولايسين، مما يشير إلى وجود مخاطر محتملة تتعلق بالفساد أو التسمم. أظهرت تجارب تكوين الأغشية الحيوية، سواء في الأطباق الدقيقة أو على أسطح زجاجية وبلاستيكية (بولي بروبيلين) مماثلة لرضّاعات الأطفال، أن جميع السلالات قادرة على تكوين أغشية حيوية، مع كثافة أعلى على سطح البلاستيك. حيث سجلت السلالة (4) أعلى كثافة بكتيرية بلغت 5.83 لوغاريتم وحدة تكوين مستعمرات لكل مليلتر على سطح البلاستيك، و5.63 على سطح الزجاج، في حين كانت السلالة (1) الأضعف بكثافة 4.28 على البلاستيك و3.24 على الزجاج.

أظهر نموذج التقييم الكمي للتعرض فروقات ملحوظة بين طرق التحضير المختلفة. استخدام الماء المغلي خفّض عدد العصويات بنسبة 93–96 % (أي 75–90 وحدة/كغ)، مقارنة بالتسخين بالميكروويف الذي أدى إلى تراكيز تراوحت بين 600–2200 وحدة/كغ، مع تقليل احتمال الخطر من 9.1–26.8 % إلى 2.3 % فقط. وُجد أن فعالية الميكروويف تعتمد على القدرة الكهربائية، حيث تُعد الأجهزة التي تقل عن 800 واط غير آمنة (1800–2200 وحدة/كغ؛ احتمال خطر 25–28 %) بسبب وجود مناطق باردة بنسبة 25 % وضعف تقليل الأبواغ (0.3–0.6 لوغ). أما الأجهزة ذات القدرة العالية (1100 واط أو أكثر) فقد أظهرت تقليلًا جزئيًا، حيث خفضت التلوث بنسبة تقارب 60 % (600–800 وحدة/كغ)، وخفضت احتمال الخطر بنسبة 65 % (8–10 %)، بفضل توزيع حراري أفضل (5 % فقط مناطق باردة) وتحقيق خفض متوسط في عدد الأبواغ (0.7–1.0 لوغ).

رغم ذلك، يظل الماء المغلي الطريقة الأكثر أمانًا وموثوقية لتقليل المخاطر الميكروبية عند تحضير حليب الرضع. تبرز هذه النتائج محدودية الممارسات المنزلية التقليدية، وتؤكد قدرة هذه البكتيريا على البقاء في ظروف الاستخدام الفعلية، مما يستدعي ضرورة تعزيز الوعي العام بأساليب التحضير الآمن لتقليل خطر تلوث تغذية الرضع بالعصويات

الكلمات المفتاحية: سيريوس باسيلوس، المجفف الرضع حليب، الحيوي الغشاء، الحيوية المضادات مقاومة، الكمي التقييم، المراحل على القائم المخاطر تقييم نموذج، للتعرض

Dedication

This work is dedicated to God Almighty, whose grace, wisdom, and strength have guided me every step of the way.

To my beloved parents, thank you for your unconditional love, endless sacrifices, and unwavering support. Your belief in me has been my foundation and driving force.

To my dear friends, thank you for walking beside me through this journey with encouragement, patience, and laughter. Your presence made the challenges lighter and the successes more joyful.

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I would also like to sincerely thank the members of the jury for accepting to review my work and for agreeing to be present at my defense. Your time, thoughtful evaluation are truly appreciated.

My heartfelt thanks goes to my parents, whose unwavering love, prayers, and support have carried me through every challenge. Your belief in me has been my greatest motivation.

Last but not least, I would like to sincerely thank my friends and family in the vicinity of Algeria who stood by me during this journey. Your companionship, encouragement, and understanding have meant more than words can express.

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List of abbreviation

IMF: Infant Milk Formulae

STEC: Shiga toxin-producing E. *coli*

FOF: Follow-On Formula

MYPA: Mannitol Egg Yolk Polymyxin Agar

PLcR: Phospholipase C Regulator.

LITAF: lipopolysaccharide-induced TNF-alpha factor

MSC: Mesophilic Spore Count

TSC: Thermophilic Spore Count

BHI: Brain Heart Infusion Broth

LB: Luria–Bertani broth

PP: polypropylene

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INTRODUCTION

Breastfeeding is widely recognized as the best way to nourish newborns. Breast milk is not only an essential source of nutrients for infant growth and development, but it also contains various immunological components with anti-infective properties and plays a key role in the development of the infant's immune system. It also includes beneficial bacteria that help colonize the infant gut (Dae Yong Yi et al., 2021). Infants should be breastfed for the first 6 months of life according to WHO but only 38% of the world does that and about 2.7 million Americans currently rely on infant formula for infant nutrition (Martin et al., 2016).

However, when breastfeeding is not possible, insufficient, or not desired, powdered infant formula serves as the best alternative (Ahern et al., 2019). PIFs are formulated to mimic the nutritional composition of human milk as closely as possible (Mezian et al., 2022). Like other food products, infant formula can contain microorganisms like bacterial (Chap et al., 2009 ; Mezian et al., 2022); this is because powdered infant formula (PIF) is not a sterile product even if it has been manufactured to hygienic standards (Kent et al., 2015). Once reconstituted, infant formula is considered particularly risky for infants due to their underdeveloped immune and metabolic systems, which make them more susceptible to infections caused by enteric bacterial pathogens (Afrin et al., 2024; Kent et al., 2015; Mezian et al., 2022; Vidic et al., 2020). For this reason, the microbiological quality of these products is critical (Sadek et al., 2018)

The gram-positive, aerobic, rod-shaped *Bacillus cereus* microbe is remarkably resistant to heat and dryness and can be dormant as a spore for years. Although it can grow at temperatures between 10°C and 49°C and pH levels between 4.9 and 9.3, it thrives best at 30°C (Afrin et al., 2024). FAO/ WHO Expert noted that one of the main microorganisms related to infant food contamination was *B. cereus* (Sadek et al., 2018). This is because bacillus species, particularly *Bacillus cereus*, contribute significantly to the microbiota found in milk powder because of their pathogenic effects and higher extracellular proteolytic activity than other bacteria producing a range of extracellular enzymes, which may allow them to grow and degrade a variety of food matrixes (Mezian et al., 2022). *B. cereus* is known to produced two main type of food poisoning syndromes: emetic and diarrheal (Benahmed et al., 2020). *Bacillus* species can withstand thermal treatments and grow after the product is reconstituted, and have been linked to gastrointestinal illness (Hilliard et al., 2003; Veysseyre et al., 2015). In dairy plants the adherence to stainless steel surfaces may develop biofilms, which are more resilient to cleaning

procedures and antimicrobials than planktonic cells. Because of this, getting rid of *Bacillus cereus* from the dairy sector is difficult and frequently results in contaminated dairy products (Lin et al., 2017).

Infant formula is usually given at lukewarm temperatures to avoid the risk of burns. However, ensuring its microbiological safety requires proper heat treatments. There are typically two methods used to reconstitute powdered infant formula: boiling water before adding the powder, or mixing with cold water followed by microwave heating.

In the context of our study, we have established several specific objectives to contribute to the risk assessment of Bacillus strains and to enhance infant formula preparation methods. The first objective involves isolating and identifying Bacillus strains present in powdered infant formula. The second objective focuses on characterizing these strains in terms of food quality by evaluating their enzymatic potential, which could influence the nutritional quality and shelf stability of the product. The third objective assesses food safety issues by examining the antibiotic resistance profiles of these strains as well as their ability to form biofilms on materials commonly used in baby bottle manufacturing.

The fourth and central objective of this study is to develop a quantitative microbial exposure assessment rather than a full risk assessment, given the lack of a well-defined dose-response relationship for *B. cereus*. This approach will estimate the potential ingested dose of Bacillus by infants, supported by scenario analyses that account for variations in formula preparation and consumption practices.

This document is structured into several key sections. First, a comprehensive literature review will outline the current knowledge on infant formula, Bacillus species, and their virulence factors. This will be followed by the methodology section describing the experimental and analytical approaches used in the study. The results will then be presented and discussed. Finally, the conclusion will summarize the findings, propose practical recommendations, and suggest future research directions to further mitigate risks associated with Bacillus contamination in infant nutrition.

LITERATURE REVIEW

1. Infant baby formula

The baby formula is intended as the best alternative for nurturing babies (Martin et al., 2016). The gut normal flora of infants equilibration is contributed largely by mother's milk and thus it is essential for the infant formula to be closely related to it in terms of nutritional values which benefits in achieving a bio active gut and good immune system (Salminen et al., 2020). Every attempt has been made to replicate the nutritional value of mother's milk for normal infant growth and development, even though it is not possible to produce a product similar to the breast milk. Most frequently, cow milk or soymilk is used as the base, and additional ingredients such as iron, nucleotides, and fat blend compositions are added to better approximate the composition to human breast milk and to achieve health benefits. Additionally, probiotics and genetically engineered compounds are either added to the formula or are currently being considered for addition (Martin et al., 2016).

Table n°.1: Typical ingredients used in infant milk formula products(Masum et al., 2021)

Nutrient	Ingredient
Carbohydrate	Lactose, maltodextrin, corn syrup, sucrose, galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS)
Protein: Casein	Skim milk (powder), whole milk (powder), caseinate (sodium, potassium and calcium), acid casein, milk protein concentrate (MPC)
Whey protein	Whey protein concentrate (WPC), whey protein isolate (WPI), demineralized/partially demineralized whey, (partially) hydrolyzed whey protein
Other proteins	Soy protein isolate (SPI), amino acids
Fat	Vegetable oils (sunflower, canola, corn, soybean, palm, safflower), butter oil, anhydrous milk fat, cream, whole milk
Minerals	Sodium, magnesium, calcium, phosphorus, potassium, chloride, iron, zinc, copper, manganese, iodine
Vitamins	A, D, E, K (Fat soluble vitamins), B1, B2, B3, B5, B6, B12, folates, biotin, ascorbates
Other additives	Lactoferrin, milk fat globule membrane, probiotics and prebiotics

1.1. Production process of powder infant formula

Powdered infant formula (PIF) is manufactured utilizing techniques that must carefully follow Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Control Points (HACCP) guidelines. Critical procedures such as spray drying, fluidized bed drying, and packaging are especially vulnerable to contamination and must be closely monitored. Contamination can occur not just during processing but also through secondary paths, such as raw materials exposed to environmental contamination (Zhuang et al., 2019). Spores can enter the milk during milking, survive pasteurization, and remain present throughout the

manufacturing process. Spores can infect milking equipment and germinate in tanks, pasteurizers, and packing machinery (Tirloni et al., 2022).

1.1.1. Microbial Dynamics in PIF Manufacturing

In the processing of PIF, spores are predominantly responsible for *B. cereus* contamination (Gopal et al., 2015; Yang & Wang, 2023). Although the majority of vegetative bacteria are eliminated during the pasteurization step, some species, especially Streptococcus thermophilus and *Enterococcus* spp., can survive. Thermotolerant and spore-forming materials are frequently left behind after subsequent processes like mixing, evaporation, and spray drying. Additionally, the industrial environment or heat-treated components may be the source of these microbes. Because there is no final sterilization procedure after drying, any microbial contamination that occurs during this stage can remain in the finished product. Although the powder's low water activity (0.17-0.22) limits microbial growth, pathogens like *Cronobacter sakazakii, Salmonella spp., Listeria monocytogenes, and E. coli O157:H7* have been known to survive for up to a year at 5°C (Fusi et al., 2025). It has been discovered that reconstituted baby milk helps B. cereus spores germinate into the vegetative cells. After 8–13 hours of incubation, a complete growth curve was shown at 24°C, and cereulide was found (Yang & Wang, 2023).

1.1.2. Processing Methods

1.1.2.1. Wet-Mix Process

The wet-mix method is commonly utilized because it provides greater control over processing and microbiological safety. In this procedure, substances such as carbohydrates, minerals, and vitamins are added to a foundation (for example, milk, whey, soy, or rice) (Masum et al., 2021). Following thorough mixing of all components, an alkali or acid solution is often used to bring the pH of the PIF wet-mix down to 6.8. Consequently, a crucial step in imitating B. cereus growth and cereulide formation is altering the pH in the PIF wet-mix (Yang & Wang, 2023). After that, the liquid formula is sterilized or pasteurized. Sterilization targets both spores and vegetative cells, whereas pasteurization eliminates vegetative pathogens and decreases spoilage organisms. Following heat treatment, serial evaporation is used to concentrate the liquid (vitamin addition takes place prior to drying) (Masum et al., 2021). Normally, evaporation occurs at temperatures ranging from 40 to 80 degrees Celsius. This temperature cannot totally inactivate B. cereus spores, but it encourages their outgrowth and population rises (Yang & Wang, 2023). This procedure removes practically all of the water from concentrated wet-mix by spray-drying it at 150-250°C. Agglomeration of spray dried IMF powder is frequently performed to increase the flowability and solubility (Masum et al., 2021). About 97% of the final powder is dry materials. These actions are insufficient to eradicate germs entirely, even at

high temperatures. The powder is kept at room temperature without additional microbiological control once it has cooled (Fusi et al., 2025).

All of the ingredients must be combined, homogenized, and pasteurized. Due to the nature of this procedure, production equipment must be continuously wet-clean (Kent et al., 2015).

1.1.2.2. Dry-Mix Process

In the dry mixing process, the individual formula components are heat-treated separately from suppliers and blended together to provide a homogeneous blend of nutrients throughout the powder and to minimize bacterial development (Fusi et al., 2025; Kent et al., 2015). This technique is particularly helpful when adding substances that are sensitive to heat. Blending is followed by packaging, inert gas flushing, and storage. Although it prevents delicate components from degrading thermally, there is a greater chance of contamination during the last mixing step (Fusi et al., 2025). Mesophilic spores are more difficult to inactivate in concentrated dairy products with high dry matter content than in milk. The heat-resistant spores shield the mesophile *B. cereus* from heat treatment and may survive spray drying (Yang & Wang, 2023).

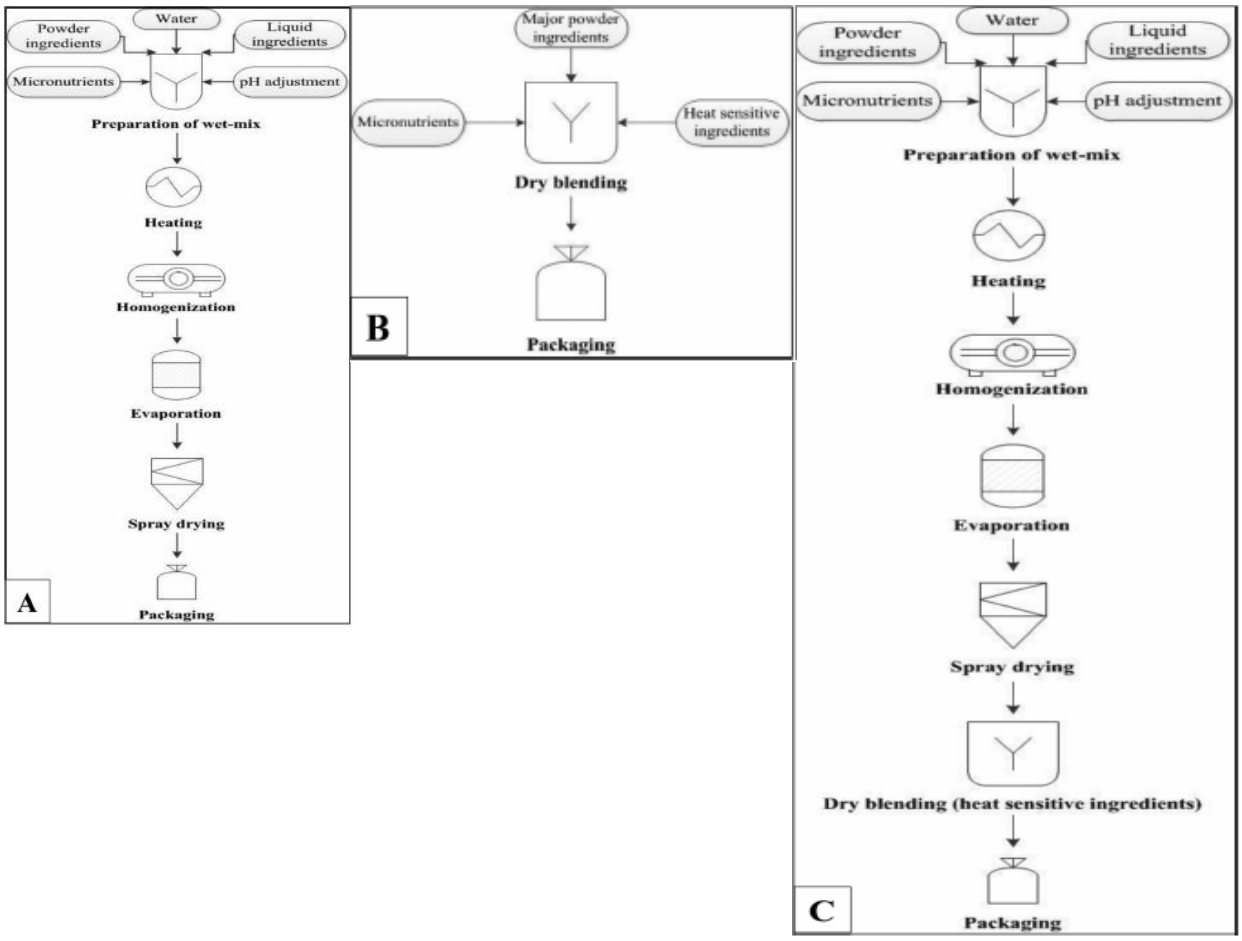


Figure n°. 1 : Schematic diagrams of processing steps involved in manufacturing of infant milk formula powders: (A) wet-blending, (B)dry-blending, and (C) combination of wet- and dry-blending approaches (Masum et al., 2021)**.**

1.1.2.3. Combined Process

The combined process incorporates elements of both wet and dry methods. Some elements are added to the base formula and processed similarly to the wet-mix method (fat, protein, and carbs). Once the powder is obtained, it is blended with dry substances (vitamins and minerals) before being stored (Fusi et al., 2025; Kent et al., 2015). This hybrid approach provides freedom in ingredient handling but still requires strict supervision to prevent post-processing contamination (Fusi et al., 2025).

2. Microbiology of infant milk

Infants and newborns are particularly vulnerable to foodborne infections due to their immature immune systems, and contaminated infant milk formula (IMF) poses a serious health risk (Fusi et al., 2025). Despite being commercially processed, IMF is not a sterile product (Fusi et al., 2025; Kent et al., 2015; Lang & Sant'Ana, 2021).

Numerous studies have detected a wide range of microbial contaminants in powdered infant formula (PIF), including *Pseudomonas* spp., *Staphylococcus* spp., *Vibrio* spp., *Shigella* spp., and *Bacillus* spp., though *Salmonella* spp. was not identified in some samples (Al Mahmud et al., 2024). *Cronobacter spp*. has also been isolated from PIF manufacturing environments and products (Lindsay et al., 2024). A recent study in Brazil discovered new species isolated from powdered milk containing sporulating bacteria: *Weizmannia. coagulans, Bacillus. amyloliquefaciens, Clostridium. pabulibutyricum, Clostridium. sporosphaeroides, and Clostridium. butyricum* (Ruis et al., 2025). In addition, according to (Yeak et al., 2024), some other microorganisms can be found in infant formula like: *Aeromonas caviae*, *Bacillus cereus*, *Brucella spp, Campylobacter* spp., *Clostridium botulinum* (non-proteolytic), *Clostridium botulinum* (proteolytic), *Clostridium perfringens*, *Cronobacter* spp., *Cryptosporidium spp*., *Escherichia coli* (non-STEC), *Escherichia coli* (STEC), *Listeria monocytogenes*, *Mycobacterium tuberculosis* var. bovis, *Salmonella* non-Typhi, *Shigella* spp., *Staphylococcus aureus*, *Yersinia enterolitica* and also some viruses like Flavivirus and Norovirus. A study by (Vural et al., 2022) also found contamination of Enterobacteriaceae, coliform, *Bacillus cereus, Stapylococcus aureus, mold, and yeast, of Pantoea spp., Klebsiella pneumonia , Enterobacter cloacae , Serratia plymuthica , Sphingomonas paucimobilis , Bacillus pumilus , Staphylococcus hominis , Staphylococcus epidermidis , Enterococcus casseliflavus* and

Enterococcus faecium while *Salmonella spp., Listeria monocytogenes, Escherichia coli O157:H7, Escherichia coli, Cronobacter sakazakii*, and sulfite reducing anaerobic bacteria were not detected in sample of infant food.

Taken together, these studies provide strong evidence that the presence of spore-forming bacteria in infant milk formula is clearly established. In particular, members of the genus Bacillus appear to be recurrent contaminants, detected across multiple investigations and geographical regions. Their persistence despite industrial processing highlights the resilience of bacterial spores to heat and drying, as well as the non-sterile nature of powdered infant products. These observations reinforce the importance of targeted monitoring and control strategies to reduce the risk associated with spore-forming microorganisms in IMF.

2.1. *Bacillus* spp. in infant milk

B. cereus is the main microorganism causing contamination in infant food which the Food and Agriculture Organization/World Health Organization (FAO/ WHO) Committee of Experts reported of it (Al Mahmud et al., 2024; Sezer et al., 2015; Vural et al., 2022). *B. cereus* was the fourth cause of foodborne outbreaks recorded in the European Union, and the second main cause of confirmed foodborne outbreaks in France. Between 2011 and 2016, 4342 cases of *B. cereus* outbreaks were documented in China, making it the second most common cause of foodborne infections (Haque et al., 2021). Due it thermal resistant spore and vegetative it has been noted that it can surpass the thermal process of treatment of PIF, FOF and heat treated food (Meribai et al., 2024; Afrin et al., 2024). A study by (Cho & Rhee, 2023) highlighted that among the isolates from infant milk, *B. cereus* and *B. Subtilis* were a significance in food security due to having hydrolytic effect and some strain able to produce toxins and growing in extreme low temperature. A study in Algeria by (Benahmed et al., 2020) got a high number of mesophilic aerobic spore bacteria in imported milk powder with *B. cereus* group and *B. subtilis* group being among the predominant species. Also Sadek et al. (2018), reported that *Bacillus subtilis* and *Bacillus cereus* being among the most isolated in commercial infant formula. 8Moreover, several studies confirm its high prevalence in milk powders sold in Algeria and beyond, often as part of the *B. cereus* group alongside *B. subtilis*, *B. pumilus*, and *B. licheniformis* (Benahmed et al., 2020).

Figure n°.2: *Bacillus cereus* on blood agar and MYPA agar (Haque et al., 2021)

2.2. Role of *Bacillus cereus* Group in Dairy Environments

Bacillus cereus sensu lato is a prevalent contaminant in dairy processing because it lives in soil, raw milk, and equipment. It can withstand high-temperature treatments such as UHT, build protective biofilms, and produce enzymes that damage items. Despite careful cleanliness, contamination can still occur, with raw milk contamination rates ranging from 33% to 100% (Kyritsi et al., 2025). In a research analyzing phylogenetic relationships among Bacillus strains of 84 *B. cereus S.S.* isolates, 95% came from the milk processing environment, mainly in the production (27.5%) and packing (22.5%) areas—likely due to equipment surfaces and human activity in these high-traffic zones (Zhuang et al., 2019).

3. *Bacillus* spp virulence

Bacillus cereus is a ubiquitous microorganism that is widely distributed in nature (Hwang & Park, 2015). Ingesting food contaminated with concentrations greater than 10^5 CFU/g of *B. cereus* can lead to food poisoning by production of enterotoxins or *cereulide* emetic toxins (Hwang & Park, 2015a; Wang et al., 2024). There is quite a bit of research done about this foodborne pathogen which has a high resistance, due to its ability to form spores, and toxin. *B. cereus* is mostly found in raw milk and dairy products (Wang et al., 2022).

Intoxication which occurs as a result ingestion of *B. cereus* are primarily associated with the production of two major toxins: the diarrheal enterotoxins and the cereulide emetic toxin, which are encoded by several genes responsible for their respective pathogenic effects (Lin et al., 2017). There is, *PlcR* controlling the expression of over 45 genes, many of which are involved in toxin production and phospholipid breakdown, they also play a vital role in production of spores and biofilm formation (Aguilar et al., 2025; Haque et al., 2021). Several factors such as

flagella-mediated motility, oxygen levels, redox potential, and the presence of key nutrients like iron and carbon sources also plays a part in the species virulence (Haque et al., 2021).

3.1. Toxins

Several Bacillus species, especially *B. cereus*, are capable of producing a diverse array of toxins that contribute to foodborne illnesses, particularly of concern in sensitive food products such as infant milk formula (IMF). These toxins include both diarrheal enterotoxins—such as hemolysin BL (HBL), nonhemolytic enterotoxin (*NHE*), cytotoxin K (*CytK*), and *B. cereus* enterotoxin T (*BceT*), as well as cereulide (*ces*), a small, cyclic, heat-stable peptide responsible for emetic-type food poisoning (Haque et al., 2021; Hwang & Park, 2015).

The *hblA, hblC,* and *hblD* genes encode the binding component (B) and two lytic components (L1 and L2) that make up hemolytic enterotoxin (*HBL*), whereas the *nheA, nheB,* and *nheC* proteins encode nonhemolytic enterotoxin (*NHE*). *NHE* achieves its maximum toxicity when all three genes are positive but can still show virulence even if one is expressed while the HBL requires the presence and expression of all three genes in each complex to be positive for toxins (Lin et al., 2017; Wang et al., 2022). Additionally, enterotoxins such as *bceT, cytK,* and *entFM* are composed of a single protein each and contribute to overall toxicity (Ibrahim et al., 2022). At (Tuipulotu & Mathur, 2021) Found out that *hbl* can attach to a receptor on a mammal's surface the LITAF and CDIP1 and the two hemolytic and non-hemolytic enterotoxins can instigate potassium efflux and NLRP3 inflammasome activation that leads to pyroptosis and inflammatory response.

According to (Hwang & Park, 2015) *B. cereus* has not less than 5 enterotoxins and the most frequently involved in food poisoning being *HBL, NHE* and *CytK* toxin proteins. The one emetic toxin cereulide is highly thermal resistant which make the normal cooking process hard to destroy the toxins and this toxin genes are hard to detect thus it has become an issue due less reporting on it *B. cereus* outbreak (Haque et al., 2021). Only 30% of *hbl* genes and even lower frequencies in *cytK* and *bceT* genes were observed in infant formula isolates by (Hwang & Park, 2015).

A study done by (Mezian et al., 2022) a PCR toxin genes test on isolates from PIF showed *B. cereus* isolates showed 72% positivity for *nheA and nheB,* 44% for *cytK,* 32% for *bceT,* and 60% for *entFM*. Among these, 28% carried six to seven different toxin genes. Study by (Wang et al., 2022) showed that 53.4% of isolates had all three *nhe* genes , while 4.55% had all three *hbl* genes. Apart from *B. cereus*, other Bacillus species such as *B. subtilis and B. mojavensis*

have been involved in foodborne illness due to their production of a cytotoxic compound identified in toxicological assay called amylosin. Amylosin production was found to be thermal labile, produced between 11–37°C depending on the species, and could still be produced under low-oxygen or CO_2-enriched conditions, posing a potential risk for ready-to-eat or unrefrigerated foods (Constantin, 2008). Surfactin, another toxic gene protein produced by these species, is a potent, heat-stable biosurfactant capable of lysing erythrocytes and exerting antimicrobial activity. *Bacillus spp* other than *B. cereus* were once considered negligible in food poisoning, their increasing documentation—especially in isolates from food has showed their potential for toxin production similar to those of *B. cereus* (Haque et al., 2021). A study done by (Mezian et al., 2022) on PCR toxin genes test on isolates from PIF showed presence of genes like *hbl, nhe, cytK, bcet*, and *entFM* detected in species like; *B. licheniformis, B. pumilus, and B. subtilis* where *B. subtilis* strains also carried combinations of *nhe, cytK, and hbl* genes. These findings underscore the emerging concern around Bacillus species outside the B. cereus group, especially in the context of food safety and infant nutrition (Mezian et al., 2022). The **figure n°.3** and **table n°2**, below show a summary of the different toxin produced by bacillus cereus and its different effects on the body.

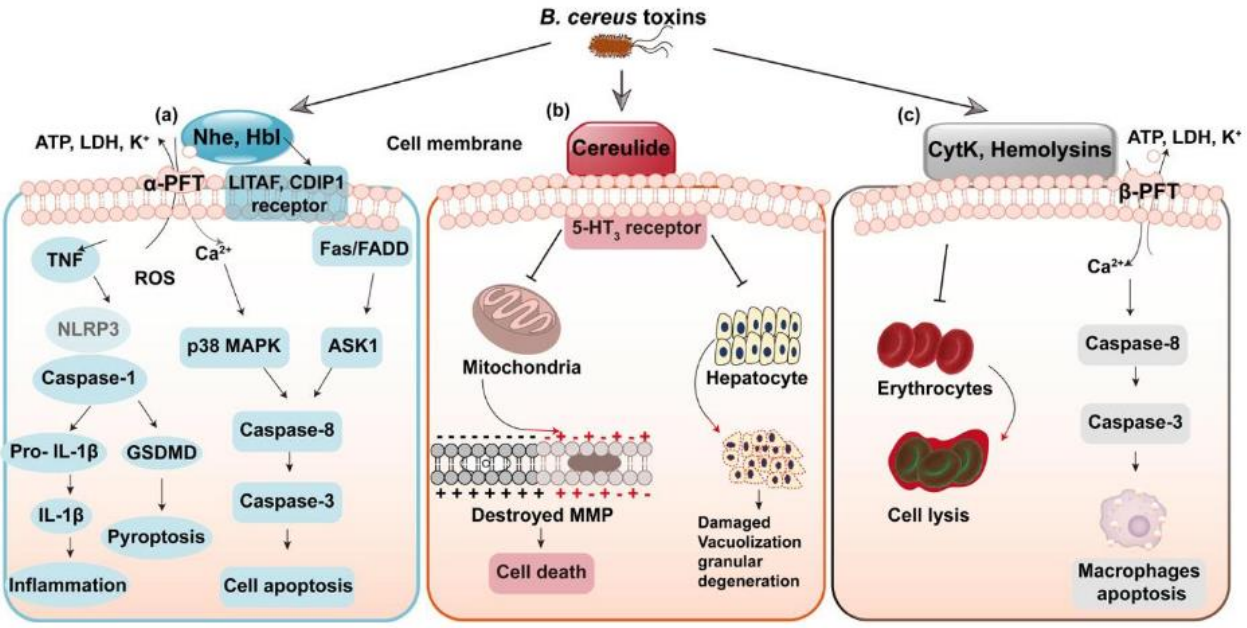


Figure n°.3: *Bacillus cereus* toxins and its causes (Liu et al., 2020)

Table n°. 2: comparison of the two types of toxins produced by *Bacillus* spp (Tirloni et al., 2022)

	Diarrheal Syndromes	Emetic Syndrome
Type of toxin	Proteins (HBL, NHE, CytK)	Cyclic peptide (cereulide)
Site of production	Small intestine	Preformed in food
Incubation period	8–16 h (up to 24 h)	0–5 h
Disease duration	12–24 h (sometimes > 24 h)	6–24 h
Infectious dose	10^5–10^7 ingested total cfu	10^5–10^8 cfu/g of contaminated food
Resistance to heat	Weak	Highly stable (up to 121 °C for 90 min)
Symptoms	Abdominal pain, watery diarrhea, nausea (sometimes)	Nausea, vomit, general weakness, diarrhea (sometimes)
Involved foods	Meat and derived foods, vegetables, sauces, soups	Rice, pasta, pastry products

3.2. Antibiotic resistance

Antibiotic resistance among foodborne pathogens is a growing public health concern worldwide. *Bacillus cereus*, a common contaminant of infant milk formula (IMF), has increasingly demonstrated resistance to multiple antibiotics, complicating treatment strategies (Dowidar & El-Baz, 2023).

There have been a lot of research that looked into the susceptibility test of Bacillus spp especially bacillus cereus isolated from sources, such as raw milk and infant formula. According to Adamski et al. (2023), the *B. cereus* and *B. subtilis* strains isolated from raw milk were susceptible to chloramphenicol, meropenem, amikacin, vancomycin, trimethoprim-sulfamethoxazole, and gentamicin. B. subtilis strains were also susceptible to erythromycin, cefotaxime, and rifampicin, while B. cereus strains were resistant to cefotaxime, ampicillin, and rifampicin. Both groups showed approximately 65% resistance to norfloxacin. Another study by Alanber et al. (2020) on Bacillus spp. isolated from powdered infant formula, a small percentage B. subtilis and B. cereus strains showed resistance to cefpodoxime and cefepime while B. cereus strains also showed resistance ceftazidime. all isolates were susceptible to amikacin, gentamicin, imipenem, and moxifloxacin, resistance rates to cefoperazone, cefpodoxime, ceftazidime, and cefepime were 6.5%, 22.1%, 5.6%, and 19.5%, respectively.

The susceptibility trend of 88 bacillus cereus strain isolated from dairy products (IMF) to antibiotics such as gentamicin, amikacin and quinolones has been similar as we see in P. Wang et al. (2022), however there were seven strains resistance to tetracycline and rifampin, two strains were resistant to chloramphenicol, and one strain resistant to sulfamethoxazole. Strains from infant milk formula were resistant to cephalothin, tetracycline, chloramphenicol and low percentage in nalidixic acid while milk-cereal based infant formula were resistant to cephalothin, tetracycline in low amount where also 5 strains were resistance to erythromycin and 9 strains to nalidixic acid, Ibrahim et al. (2022).

A clinical investigation by Bianco et al., (2021) found that all *B. cereus* strains isolated from human blood cultures were resistant to penicillin G and showed moderate resistance to beta-lactam antibiotics such as ceftriaxone and cefotaxime. These isolates harbored resistance genes but remained susceptible to ciprofloxacin, chloramphenicol, vancomycin, gentamicin, linezolid, and doxycycline.

A comprehensive survey by Ibrahim et al. (2022) found universal resistance among *B. cereus* strains from IMF to cefoxitin, colistin sulfate, neomycin, trimethoprim–sulfamethoxazole, oxacillin, and penicillin. However, these strains were uniformly susceptible to gentamicin, tobramycin, streptomycin, and vancomycin.

Dowidar & El-Baz (2023) confirmed that all *B. cereus* strains in their study were resistant to penicillin and oxacillin, with high resistance rates also recorded for cefixime, ampicillin (85.7%), nalidixic acid (73.8%), and trimethoprim–sulfamethoxazole (61.9%). In contrast, all strains were susceptible to gentamicin, erythromycin, and chloramphenicol, and most showed susceptibility to ciprofloxacin (95.2%) and kanamycin (85.7%).

The accumulating evidence of antibiotic resistance in *Bacillus* spp., particularly in *B. cereus* strains isolated from infant food products, has showed resistance to administered antibiotics like erythromycin, and chloramphenicol Ibrahim et al. (2022), underscores the need for continuous surveillance and the development of effective strategies to mitigate microbial risks in IMF.

3.2.1. Resistant gene

The presence of antibiotic resistance genes (ARGs) in *Bacillus* species, particularly *B. cereus* is a growing concern due to their potential to compromise treatment options and contribute to the broader dissemination of antimicrobial resistance through horizontal gene transfer (Navaneethan & Effarizah, 2021).

According to genomic analyses using the BV-BRC server, *B. cereus* had beta-lactam resistance genes (*blaZ*), fosfomycin resistance genes (*fosB1*, *MurA*), aminoglycoside resistance genes (*gidB*, *S12p*), and fluoroquinolone resistance genes (*gyrA*, *gyrB*) while *B. subtilis* got resistance potential from genes such as *MurA* and *dxr* for fosfomycin, *gyrA* for fluoroquinolones, and *RlmA(II)* for macrolide resistance (Fayanju et al., 2024). Another study by Bianco et al. (2021) on *bacillus cereus* isolated from human blood culture identified two genes that showed resistance to macrolide drug family which are "*ermC* and *mphB*" where only one isolate agree with the phenotypic test. Interestingly, all isolates carried genes associated with vancomycin resistance, yet phenotypic testing showed that all strains were vancomycin-sensitive. Moreover, the *Fcyn-fosBx1* gene, conferring resistance to fosfomycin, was present in all isolates. Two strains also harbored the *pUB110* plasmid, known for its association with kanamycin resistance.

The widespread detection of beta-lactamase genes among *B. cereus* isolates is likely a result of the frequent use of beta-lactam antibiotics in treating infections such as urinary tract infections, respiratory illnesses, and septicemia. This usage has contributed significantly to the observed resistance against penicillin and oxacillin in multiple studies (Dowidar & El-Baz, 2023).

Overall, the genetic basis of resistance in *Bacillus spp*. highlights the need for integrated surveillance of antimicrobial resistance, not only at the phenotypic level but also through molecular characterization. Such efforts are crucial for anticipating emerging threats and guiding effective therapeutic interventions.

3.2.2. Bacillus MDR strains

The emergence of multidrug-resistant (MDR) *Bacillus* strains, particularly *B. cereus*, poses a significant threat to both public and animal health. These resistant strains have been associated with increased morbidity compared to antibiotic-susceptible counterparts, and they are capable of causing severe infections, including sclerotic conditions in humans and animals (Dowidar & El-Baz, 2023).

Alarmingly, Dowidar & El-Baz (2023) reported that all *B. cereus* isolates (100%) examined in their study were classified as MDR. The most frequently observed resistance profile included

penicillin (P), oxacillin (OX), ampicillin (AM), cephalothin (KF), chloramphenicol (CMF), and trimethoprim/sulfamethoxazole (SXT). A notable subset of isolates (9.5%) exhibited an extended resistance pattern that also included nalidixic acid (NA). Further supporting this concern, Adamski et al. (2023) found that 31 out of 35 *B. cereus* group isolates from raw milk were classified as MDR, with a calculated mean Multiple Antibiotic Resistance (MAR) index of 0.34. In contrast, only one isolate from the *B. subtilis* group qualified as MDR, with a MAR index of 0.25. Although the *B. cereus* strains retained susceptibility to antibiotics such as vancomycin, gentamicin, amikacin, and trimethoprim/sulfamethoxazole, they exhibited consistent resistance to key antibiotics including ampicillin, cefotaxime, rifampicin, and norfloxacin.

3.3. Sporulation of *Bacillus* spp

The spores of *Bacillus cereus* exhibit remarkable resistance to environmental stressors such as heat, desiccation, disinfectants, ionizing radiation, and UV light, posing significant hygienic challenges, particularly in processes where heat eliminates competing, non-spore-forming microflora. Raw milk is recognized as a primary source of spore-forming bacteria in dairy products (El-Kholy et al., 2023). The architecture of *B. cereus* spores is structurally comparable to other *Bacillus* species, highlighting a common evolutionary adaptation among spore-forming bacteria (Ehling-schulz & Lereclus, 2019).

One of the primary determinants of spore heat resistance is the temperature at which they form—higher sporulation temperatures lead to more heat-resistant spores. This property, alongside the spores' dormancy and germination capabilities, contributes to *B. cereus*-related gastrointestinal diseases. Notably, emetic toxin-producing strains often generate highly heat-resistant spores, making them particularly problematic in food safety (Haque et al., 2021)(van der Voort & Abee, 2013b).

Biofilm formation significantly enhances the survival capabilities of *B. cereus*, contributing to increased resistance and facilitating the generation of adhesive spores. These biofilms, particularly in food processing lines, can become dominant contamination sources. Up to 90% of the biomass in air-liquid interface biofilms may consist of spores, although only about 10% of biofilm-associated cells sporulate after 6–12 days of incubation (Haque et al., 2021).

Sporulation in *B. cereus* is tightly regulated. The *Spo0A* transition state regulator suppresses *plcR* transcription, thereby downregulating *PlcR*-controlled gene expression. This molecular regulation, combined with biofilm-associated mechanisms, underpins the high resistance of

these spores (Haque et al., 2021). Upon sensing nutrients through specific germinant receptors, spores undergo germination, absorbing water, rupturing their protective coats, and releasing vegetative cells.

Environmental and nutritional factors strongly influence sporulation and spore resistance. Factors such as oxygen concentration, mineral availability, growth medium composition, and geographical origin affect the final resistance and germination behavior of the spores. Milk-derived spores demonstrate greater heat resistance than those grown in Tryptic Soy Broth (TSB), likely due to the protective binding of milk proteins like casein and whey to the spore surface (Ghosh et al., 2024).

Adding to the complexity, *B. cereus* spores have been detected in paper manufacturing and packaging materials, representing an additional contamination vector in food production chains (Haque et al., 2021). Altogether, these characteristics underline the resilience and threat posed by *B. cereus* spores in food safety and public health.

3.4. Enzyme produced by *Bacillus spp*

3.4.1. Proteolytic Activity

Bacillus cereus and *Bacillus subtilis* strains release extracellular enzymes that contribute to deterioration or pathogenicity (Benahmed et al., 2020). Bacteria produce three extracellular proteases: alkaline metalloprotease (Apr), neutral metalloprotease (Npr), and serine protease (Sub). Npr is common in *Bacillus*, whereas Sub is specific to *B. subtilis*. The major protease in B. cereus is neutral metallopeptidases, expressed by the highly conserved *npr* gene, which is utilized to detect proteolytic activity and evaluate spoiling potential (Aguilar et al., 2025).

Proteolytic activity was seen in 92% of *B. cereus* group isolates (mostly clusters III and IV) in one investigation; Irish milk powder had the highest activity (index ≥2.5). Some isolates from New Zealand and France displayed low levels of activity, whereas others displayed moderate activity (index ≥1.5). Seventy-seven percent of the isolates from the *B. subtilis* group were proteolytic, with *B. subtilis* sensu stricto from Dutch and Ukrainian powders exhibiting particularly high activity (Benahmed et al., 2020). PlcR and npr were found in all isolates, and they had considerable proteolytic activity, particularly in areas with the highest gene and regulator frequency (Aguilar et al., 2025). All of the proteases in a research on a *Bacillus cereus* isolate were resistant to 5 minutes at 95°C (Meng et al., 2022).

3.4.2. Amylolytic Activity (starch hydrolysis).

In terms of amylase synthesis, more than half of the *B. cereus* isolates showed moderate enzyme expression, and 71% tested positive for amylolytic activity. Only 16% of the *B. subtilis* strains tested positive for amylolytic activity, and only a small number of bacteria from Dutch and New Zealand powders exhibited considerable activity (Benahmed et al., 2020).

The isolates' starch hydrolysis properties were also examined because they have been suggested as a marker for *B. cereus*-induced diarrheal and emetic poisoning. Just 35% of the infant formula isolates out of 287 were positive for starch hydrolysis (Hwang & Park, 2015)

3.4.3. Lecithinase Activity and Lipase activity

B. cereus secretes lecithinase, which has weak toxicity and resembles *Clostridium perfringens*' α-toxin. It can break down red blood cells in conjunction with sphingomyelinase. Because lecithin in milk fat globules is used as a nutrition, *B. cereus* from milk-based products, such as infant formula, demonstrated greater lecithinase activity (Hwang & Park, 2015b). Lecithinase activity was detected in 78% of *B. cereus* strains, with isolates from French, New Zealand, and Indian milk powders showing the highest activity. Samples from France, India, and Ireland revealed moderate activity levels. In contrast, *B. subtilis* produced less lecithinase—only 22% tested positive, and none was found in *B. subtilis* sensu stricto strains (Benahmed et al., 2020).

Lipases that may hydrolyze milk fat are produced by a variety of bacteria, some of which can withstand pasteurization. After heat treatment, lipases made by bacteria that started in raw milk may still be active, leading to lipolysis and lowering the shelf life and quality of dairy products. When lipase acts on milk triacylglycerols, especially short- and medium-chain fatty acids, free fatty acids are produced.(Aguilar et al., 2025)

3.5. Biofilm Formation in *Bacillus* spp.

Bacillus species, particularly *B. cereus* and *B. subtilis*, are well-known for their ability to form biofilms, which pose significant challenges in the dairy industry due to their resistance to routine sanitation and thermal processing (Adamski et al., 2023). These biofilms, composed of extracellular polymeric substances (EPS) such as proteins, exopolysaccharides, nucleic acids, and occasionally extracellular DNA (eDNA), protect embedded vegetative cells and spores from environmental stresses and enhance persistence on food contact surfaces (Navaneethan & Effarizah, 2021). *B. cereus* was found to be a high-forming biofilm in ultra-high temperature pasteurized milks and milk powder, with 66% of isolates confirming biofilm formation

capacity. Despite CIP methods, substantial populations of *b. cereus* continues to remain on surfaces due to their great resistance to cleaning chemicals (Yang & Wang, 2023). The **figure n° 4** below shows process of biofilm formation and food recontamination.

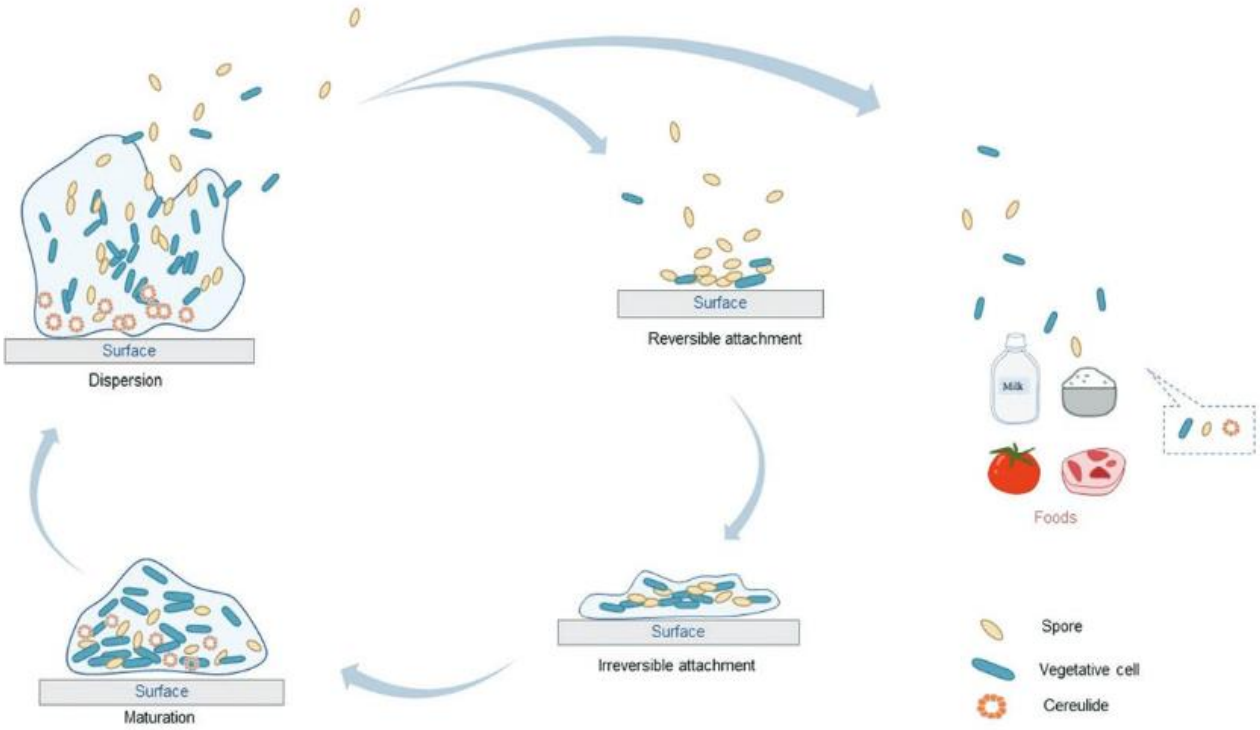


Figure n°. 4: biofilm formation and contamination (Lin et al., 2022)

3.5.1. Genetic Basis of Biofilm Formation

The genetic regulation of biofilm formation in *Bacillus* spp. involves several conserved and species-specific genes. The quorum-sensing system PlcR–PapR, particularly important in *B. cereus*, regulates virulence and biofilm-related gene expression. The PlcR regulator is linked to the expression of multiple virulence factors and enhances biofilm-forming capacity (Bianco et al., 2021).

A central operon in biofilm formation is the *sipW-tasA* operon. *TasA* encodes an amyloid-like protein forming the structural fibers of the biofilm matrix, while *SipW* is a peptidase that facilitates *TasA* processing. In *B. cereus*, *calY* is a paralog of *tasA* and also contributes to fiber formation. Despite the absence of the *tapA* gene, which assists in fiber anchoring in *B. subtilis*, the presence of *sipW*, *tasA*, and *calY* appears sufficient for biofilm development in *B. cereus* (Adame-Gómez et al., 2020).

Another crucial regulator is *CodY*, a global transcription factor involved in nutrient sensing and biofilm structure formation. The *codY* gene forms part of an operon with *xerC*, *clpY*, and *clpQ*, all contributing to pellicle formation and swarming motility (Bianco et al., 2021). The *sigB* gene, encoding the alternative sigma factor σ^B, enables *Bacillus* cells to tolerate environmental stress and contributes to biofilm resilience.

Interestingly, although the *epsA-O* operon is essential for robust biofilm formation in *B. subtilis*, its deletion in *B. cereus* does not significantly impair biofilm formation. This suggests alternative matrix components or regulatory pathways may be involved in *B. cereus* (Vlamakis et al., 2013).

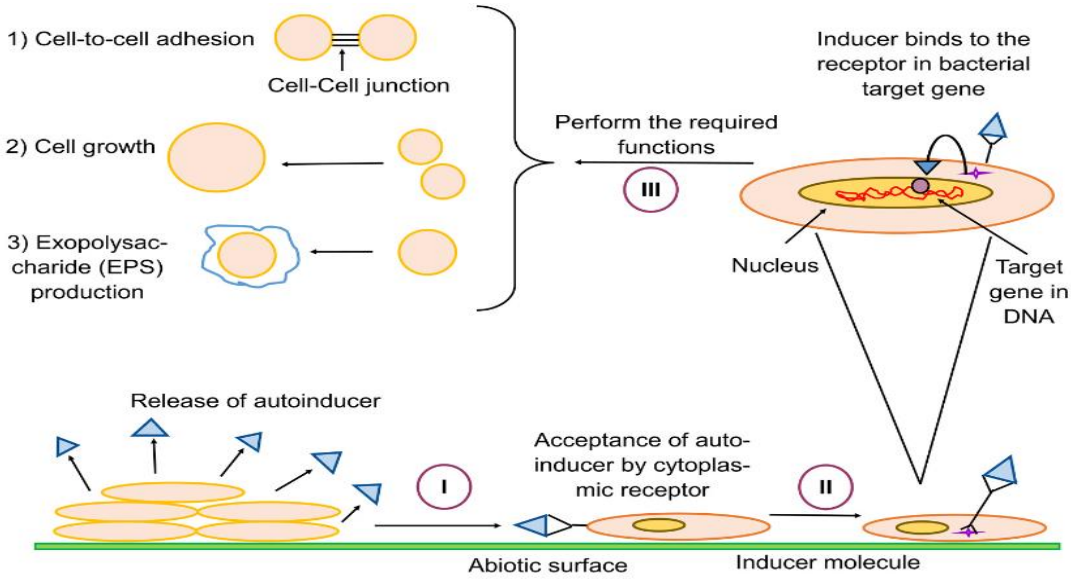


Figure n°. 5: quorum sensing among bacterial cell in the biofilm (Ghosh et al., 2024)

3.5.2. Surface Attachment and Environmental Influences

Bacillus biofilms can form on a wide variety of food contact materials, including stainless steel, glass, plastic, PVC, and polyethylene (Haque et al., 2021). Surface type significantly influences biofilm formation; for example, biofilms form less effectively on glass and polystyrene, possibly due to reduced eDNA availability, which is critical for adhesion. In contrast, materials like PVC and polyethylene support higher biofilm production (Adame-Gómez et al., 2020).

3.5.3. Biofilm Structure and Function

Biofilms serve multiple functions beyond physical protection, including metabolite disposal, antimicrobial resistance, and facilitation of horizontal gene transfer. Motility plays a key role in biofilm dynamics: flagella-mediated motility enables bacterial cells to search for favorable surfaces, create nutrient channels, increase biomass by recruiting planktonic cells, and spread

the biofilm colony (Adame-Gómez et al., 2020) . However, on certain surfaces like glass, flagella may reduce initial adherence by interfering with cell-surface interaction.

Approximately 55.9% of *B. cereus* isolates demonstrate biofilm-forming ability, which is associated with persistent contamination, survival under hostile conditions, and enhanced virulence (Navaneethan & Effarizah, 2021). Despite sharing homologous genes with *B. subtilis*, *B. cereus* tends to form less structurally distinct and often less persistent biofilms, although it retains the capacity to colonize a broad range of environments, including dairy equipment and medical devices.

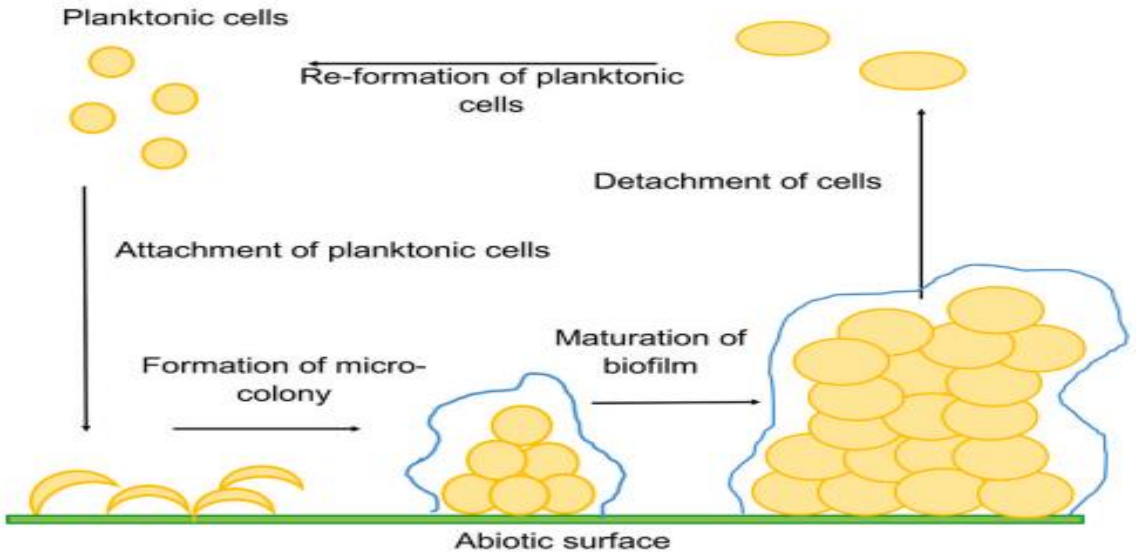


Figure n°. 6: Schematic of Simple biofilm formation (Ghosh et al., 2024)

4. Food risk analysis

Risk analysis is used to estimate the risks to human health and safety in order to define and implement appropriate measures to control them, and to communicate with stakeholders about the risks and the measures taken. The three components of risk analysis are defined by the Codex as follows: risk assessment, risk management, and risk communication (FAO, 1999).

4.1. Risk assessment

Quantitative Microbial Risk Assessment (QMRA) has emerged as a valuable tool for assessing microbiological hazards in food. Risk assessment is a scientific approach used to estimate the likelihood of occurrence of a hazard and the severity of its adverse effects ((EFSA), 2020; Khalid et al., 2020). It is based on comprehensive scientific data collected throughout the food chain, from production to consumption. Using mathematical modeling, it defines intervention

parameters and thresholds to minimize or reduce the risk to consumers. The Codex Alimentarius outlines a fundamental methodology for conducting microbiological risk analysis, consisting of four main steps: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment, and (iv) risk characterization (FAO, 1999).

4.1.1. Hazard Identification

Hazard identification refers to the detection of a biological (microorganisms or toxins), chemical, or physical agent in a food product or food group that can cause adverse health effects under favorable conditions (FAO, 1999). These hazards may originate from food, the environment, animals, or soil.

4.1.2. Hazard Characterization

Hazard characterization involves a qualitative and/or quantitative assessment of the nature of adverse effects associated with the presence of a pathogenic agent in food. It aims to describe the nature, severity, and duration of the hazard. This step must be detailed, with well-documented variables (FAO, 1999). The severity of the disease is determined by the interaction of three main factors: the host, the food, and the pathogen. When sufficient data are available, a dose–response assessment can be conducted. The goal of dose–response modeling is to determine the relationship between the level of exposure (dose) to a pathogen and the likelihood or severity of the outcome. Four types of outcomes may be considered: the probability of infection (post-ingestion), illness (morbidity), sequelae, and mortality (Buchanan et al., 2000; Coleman & Marks, 1999; FAO, 1999).

4.1.3. Exposure Assessment

Exposure assessment involves a qualitative and/or quantitative evaluation of the presence of biological, chemical, or physical hazards in food and the likelihood of consumer exposure. It describes all potential routes through which a pathogen may enter the food chain—from production to consumption (FAO, 1999).Models are developed to simulate the behavior of the pathogen across the entire food production chain to estimate the level of exposure. This includes the impact of each stage on microbial growth, allowing quantification of two key parameters: pathogen prevalence and concentration throughout the chain. Factors affecting microbial levels—such as animal type, seasonal influence, cross-contamination during transport, carcass handling, packaging, and storage temperature—are also analyzed (Coleman & Marks, 1999; FAO/WHO, 2008). Where data are missing or incomplete, predictive microbiology models can simulate microbial growth or survival under realistic environmental conditions (temperature, pH, water activity). Additional information on food consumption habits and population

characteristics is essential for exposure characterization (FAO/WHO, 2008; Lammerding & Fazil, 2000).

4.1.4. Risk Characterization

Risk characterization is the final step of the risk assessment process and integrates the findings from the previous three steps. It provides an estimate of the potential severity of infection for a given population. The risk estimate can be qualitative (low, medium, high) or quantitative, providing numerical estimates of affected individuals (FAO/WHO, 1999). Quantitative models consider both uncertainty (due to data gaps) and variability (due to data fluctuation). These are analyzed through Monte Carlo simulations, which generate comprehensive probability distributions at various points. Any significant change at one stage of the process requires risk reassessment (Buchanan et al., 2000; Lammerding & Fazil, 2000).

4.2. Risk Management

Risk management involves balancing different policy options based on risk assessment outcomes and, where necessary, selecting and implementing appropriate control measures, including regulatory actions (FAO, 1999). The aim is to determine the significance of estimated risks, compare risk mitigation costs with societal benefits, and establish guidelines and regulations for food safety (FAO/WHO, 2003).

4.3. Risk Communication

Risk communication is an interactive process of information exchange among risk assessors, risk managers, and stakeholders. It provides public and private sectors with the information needed to prevent, reduce, or control foodborne risks through mandatory or voluntary food safety and quality systems (FAO, 1999).

MATERIALS AND METHODS

5. Bacterial isolation

5.1. Sampling

Thirty-two (n = 32) samples of PIF were used in this work, which belonged to three best-selling brands in Algeria. Samples were collected from different local pharmacies between January 2024 and Mai 2024. In order to do the sampling of each brand, two different ages were selected, namely; 1st age is the PIF intended for newborns up to six months of age, and the 2nd age is the PIF intended for infants above six (6) months of age up to twelve (12) months of age. The composition of the PIF collected, and a list of ingredients is provided in the supplementary material. Samples were transported to the lab at room temperature.

5.2. Microbiological analysis

5.2.1. Samples preparation

Twenty grams of each PIF sample were added to 180 mL of Tryptone Salt Broth (Biokar, Beauvais Codex, France) in a sterile stomacher bag and homogenized in a Stomacher® (400 Circulator, Seward, UK) at 260 rpm for 2 min.

5.2.2. Aerobic plate counts

Tenfold serial dilutions were prepared using maximum recovery diluent (MRD) (Biokar, Beauvais Codex, France), following inoculation in Petri dishes that were further pour plated with Plate Count Agar (PCA) (Oxoid Ltd., Hampshire, UK) according to Chap et al. (2009). Incubation of PCA plates was done at 37 ◦C for 48 h, followed by counting colonies and expression of the results as log CFU/g.

5.2.3. Aerobic spore-forming bacteria enumeration

After reconstitution of PIF, serial dilutions were prepared in MRD (Biokar, Beauvais Codex, France) and submitted to heat shock at 80 ◦C for 12 min (Kent et al., 2016). Then, the content in the MRD tubes was cooled down at room temperature and pour plated in PCA, following incubation at 37 ◦C for 48 h for mesophilic spore counts (MSC), at 55 ◦C for 48 h for thermophilic spore count (TSC). After the incubation, the colonies were counted, and results were expressed as log CFU/g.

5.2.4. Phenotypic characterization of spore forming bacteria isolated from PIF

A total of five (n = 5) colonies of MSC and TSC bacteria with different morphologies were recovered from each sample. The colonies' appearance, borders, color, and size were examined and then subjected to Gram staining, endospores staining, and catalase test (Reyes et al., 2007).

5.3. Identification of spore-forming bacteria isolated from PIF

5.3.1. Biochemical identification.

twenty-six (26) isolates were selected based on the different morphology of their colonies. Biochemical identification was carried out using the API50CHB system (bioM´erieux SA, Marcy l'Etoile, France) supplemented by API 20E galleries as directed by the manufacturer instructions. The results were treated by ApiwebTM stand-alone V1.2.1 (bioM´erieux SA). The colonies were purified by two consecutive streaks on nutrient agar (Biokar, Beauvais Codex, France). Further, the colonies were streaked on Brain Heart Infusion (BHI) (Oxoid, Basingstoke, UK) to obtain pure colonies. Finally, the isolates were stored in a BHI medium supplemented with 33% sterile glycerol at −20 ◦C.

6. Antibiotic Susceptibility Testing of *Bacillus spp*

We conducted the antibiotic susceptibility test of *Bacillus spp* isolates recovered from infant formula samples using the Kirby-Bauer disk diffusion method, following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2023) with appropriate adjustments for Gram-positive bacillus, which is in accordance with (Vural et al., 2022).

6.1. Preparation of Bacterial Inoculum

A pure colony of *Bacillus. spp* was picked using an inoculating loop from a fresh culture grown on nutrient agar of four strains(E1-E4), into different test tubes with 5ml of BHI broth, the suspensions were mixed thoroughly using a vortex mixer to achieve a homogeneous distribution of bacterial cells and after they were incubated at 30–37°C for 18–24 hours.

We used a colorimeter to adjust the turbidity of the suspension after incubation to between (0.08-0.11) to match the 0.5 McFarland standard, which corresponds to approximately 1.5×10^8 CFU/mL just like (Tagne et al., 2023).

6.2. Inoculation of Mueller-Hinton Agar Plates

Sterile Mueller-Hinton agar (MHA) was poured in 8 sterile petri dishes as a testing medium and left to solidify under two Bunsen burner to avoid contamination. A sterile cotton swab was used (Adamski et al., 2023), and put in the standardized inoculum and rotated several times to remove excess fluid. The surface of the MHA agar was uniformly inoculated by streaking the

swab over the entire surface in three directions, rotating the plate approximately 60 degrees between each streaking to ensure an even lawn of bacterial growth.

6.3. Application of Antibiotic Disks

Commercially prepared antibiotic-impregnated disks (Oxoid, UK) were aseptically placed on the surface of the inoculated MHA plates using sterile forceps, each strains have two petri dishes with one spaced equally for 5 disks of antibiotics and the second spaced equally for 7 disks of antibiotics. The 5 antibiotics are: kanamycin, streptomycin, clindamycin, penicillin and ampicillin and the 7 antibiotics are: Chloramphenicol, tetracycline, trimethoprim, gentamycin, ciprofloxacin, bacitracin and vancomycin. The disks were placed at appropriate distances to prevent overlapping of inhibition zones. Each disk was gently pressed to ensure complete contact with the agar surface.

6.4. Incubation

The plates were incubated at 37°C for 18–24 hours under aerobic conditions. And the inhibition zone was measure. All results recorded appropriately and interpreted using a national committee of clinical laboratory standards interpretation chart. The isolates were classified as susceptible (S) intermediate (I), or resistant (R) according to CLSI and interpreted according to the zone diameter interpretation criteria for *B. cereus* (Afrin et al., 2024). Due to the lack of standard for antibiotic not included in EUCAST for *Bacillus* spp the standard for Staphylococci were used, where *Staphylococcus aureus* 29213 was used as quality control (QC) for most of the tested antibiotics, *Enterococcus faecalis* ATCC 29212 was used as QC for vancomycin, and *Escherichia coli* ATCC 29212 as QC for meropenem (Adamski et al., 2023; Ibrahim et al., 2022)

7. Detection of Enzymatic Activities in *Bacillus spp*

The enzymatic activity of *Bacillus cereus* isolates was assessed through qualitative agar-based assays targeting the production of α-amylase, caseinase (protease), lipase, and Lecithinase. These enzymes are important indicators of the strain's spoilage and pathogenic potential in infant milk formula.

7.1. Preparation of the Inoculum

Fresh colonies of *Bacillus spp* were recovered from petri dish using an inoculating loop and put in 5ml test tubes with BHI broth, vortexed and incubated at 30°C for 18 hours. We preferred 18

hours so as to be able to conduct the enzymatic test at exponential phase of the bacterial. the suspension was used for all enzymatic assays.

Sterile Amidon agar, egg-York agar, casein agar and tween80 agar each were put in 4 sterile petri dishes for each strain (E1, E2, E3, E4) and put under a Bunsen burner to solidify for about 3 minutes. We have a total of 16 petri dishes for enzymatic test.

7.2. Enzymatic Activity Tests

The isolates were tested using a spot inoculation method on specific media designed for individual enzyme detection.

7.2.1. α-Amylase Activity

Each strain was spot-inoculated onto the surface of the Amidon agar plate using 10 µL of the inoculum and incubated at 30°C for 48 hours.

7.2.2. Protease Activity

Each strain was spot-inoculated onto the surface of the casein agar plate using 10 µL of the inoculum and incubated at 30°C for 48 hours.

7.2.3. Lipase Activity

Each strain was spot-inoculated onto the surface of the tween80 agar plate using 10 µL of the inoculum and incubated at 30°C for 48 hours.

7.2.4. Lecithinase Activity

Each strain was spot-inoculated onto the surface of the EYA plate using 10 µL of the inoculum and incubated at 30°C for 48 hours.

8. Biofilm formation

8.1. Culture Preparation

Inoculate 5 mL of LB broth with a single colony of each *Bacillus* strain (E1, E2, E3, E4). Incubate overnight at 37°C with shaker incubator (150–200 rpm)

8.2. Biofilm Assay Setup

8.2.1. Inoculate Microtiter Plate:

Label wells accordingly for each column with the first column being just the LB broth and then we skip on column and label for the first strain 000E1 after we do the same for the remaining strains respectively (E2, E3, E4). Add 200 µL of sterile LB broth to 5 wells (no bacteria), this is the blank/control. Then we add 100 µL of LB broth in all the strains column and the we add

the respective inoculate of each strain 100 µL each. Incubate the plate at 37°C for 24 or 48 hours under static (non-shaking) conditions.

8.2.2. Biofilm Quantification

Discard Planktonic Cells Carefully after incubation by pipetting out the broth from each well to not disturb the biofilm attached to the well surface. Gently add 200 µL of physiological water to each well. Repeat washing 3 times to remove non-adherent cells.

8.2.3. Stain with Crystal Violet

Add 200 µL of 0.1% crystal violet solution to each well. Let stand at room temperature for 15 minutes. Rinse Excess Stain: Remove the dye and rinse wells 3–4 times with physiological water. Let the plate air dry completely upside-down on paper towel. The **figure n°. 7** below show the dried wells after clearing out crystal violet and addition of the ethanol.

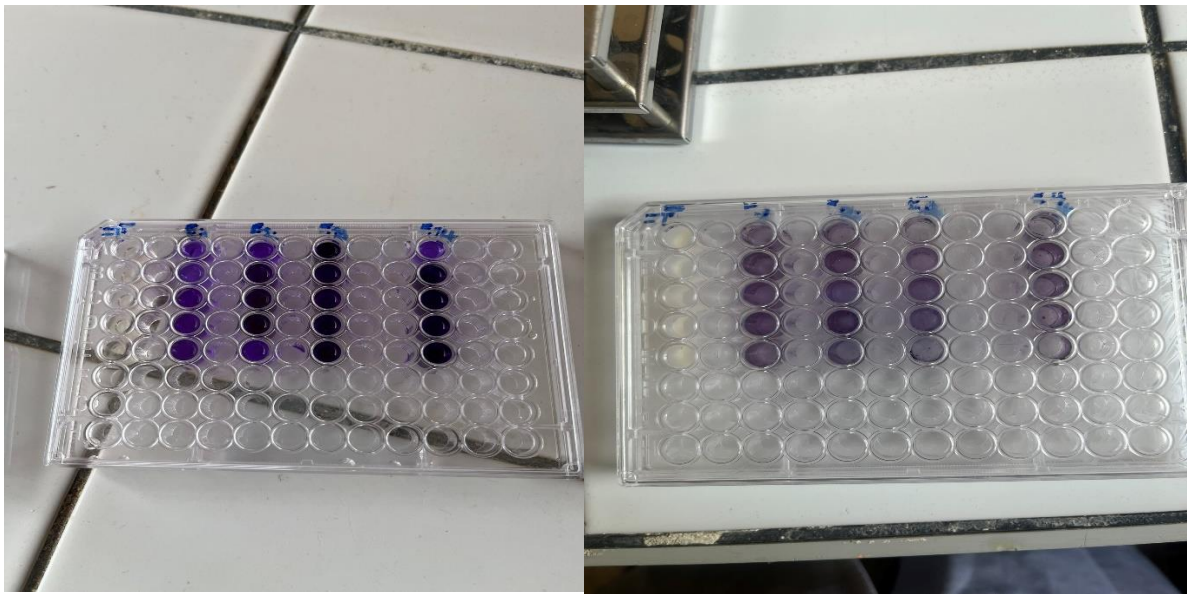


Figure n°.7: well filled with ethanol and dried well

8.2.4. Solubilize Bound Stain

Add 200 µL of 95% ethanol (or 30% acetic acid) to each well to dissolve the stain. Let stand for 15 minutes at room temperature.

8.3. Measure Optical Density (Optional):

Measure absorbance at 450 nm and 630 nm using a microplate reader. Record the OD values for E1–E4 and controls. A mean of OD values was calculated for each strain (ODs), while the cut-off OD (ODc) was calculated as three standard deviations above the mean OD of the negative control. According to their OD value, the strains were classified as weak (ODc< ODs

≤ 2 x ODc), moderate (2 x ODc< ODs ≤ 4 x ODc), strong (4 x ODc< ODs) or no (ODs ≤ ODc) biofilm producers(Catania et al., 2023).

9. Biofilm Formation on Glass and Polypropylene Bottle Surfaces

This section aims to evaluate the effectiveness of commonly used household cleaning methods in reducing biofilm formation on polypropylene (PP) and glass surfaces. Biofilms can form on these surfaces and resist traditional cleaning methods, posing a potential risk for food contamination and infant health.

The choice of materials PP (polypropylene) and glass is not accidental, but rather deliberate due to their widespread use in the manufacture of baby bottles and other infant feeding equipment.

By selecting PP and glass as study substrates, this section aims to simulate real-life usage conditions of baby bottles and related equipment while evaluating the effectiveness of household cleaning practices in keeping surfaces clean and safe for infants.

9.1. Bacterial Strains and Culture Conditions

Four Bacillus strains (E1, E2, E3, and E4) were used to assess biofilm formation on glass and polypropylene (PP) surfaces derived from infant feeding bottles. Each strain was revived and streaked on nutrient agar, and a single colony was used to inoculate 5 mL of LB broth. The cultures were incubated overnight at 37°C in a shaking incubator at 150–200 rpm.

9.2. Preparation of Glass and Polypropylene Surfaces

Glass and polypropylene materials were cut into square pieces (approximately equal surface area) from used infant formula bottles. The pieces were first soaked in 70% ethanol, then autoclaved to ensure sterility and to remove any potential pre-existing microbial adhesion. (Greetje A.A. Castelijn, 2013).

9.3. Standardization of Inoculum

After overnight incubation, bacterial cultures were adjusted to a standard optical density (OD) range of 0.08–0.11 using a colorimeter to ensure uniform inoculation across all test conditions.

9.4. Biofilm Formation Assay

Sterile 15 mL conical tubes were used for polypropylene (PP) squares, and 50 mL conical tubes for glass squares. Each tube was filled with enough of the standardized bacterial suspension to

just submerge the square material. Tubes were incubated statically at 37°C for 24 hours to allow for biofilm formation on the material surfaces.

9.4.1. Removal of Non-Adherent Cells

Following incubation, the inoculated broth was carefully decanted. Each material square was gently rinsed with sterile physiological water (0.85% NaCl) to remove non-adherent (planktonic) bacteria.

9.4.2. Detachment of Adherent Biofilm Cells

After rinsing, each conical tube received fresh sterile physiological water (9 mL). The tubes containing the material squares were sonicated for 10 minutes to detach adherent bacteria from the surface, followed by vortexing for 1–2 minutes to ensure maximal dislodgement of cells into suspension, as explained by (Catania et al., 2023) but in their case the used cell scraper to remove adhered cells.

9.4.3. Serial Dilution and Plate Counting

The resulting bacterial suspensions were serially diluted up to 10^{-3}. From each dilution (10^{-2} and 10^{-3}), 100 µL was plated on nutritive agar in duplicate (16 Petri dishes total: 4 strains × 2 materials × 2 dilutions). Molten nutritive agar was poured over the inoculum, and plates were gently swirled horizontally to evenly distribute the suspension. Plates were allowed to solidify and were then incubated at 37°C for 24 hours.

9.4.4. Enumeration of Colony-Forming Units (CFU)

Following incubation, colonies were enumerated, and results were expressed as CFU/mL per material. Comparative analysis between biofilm formation on glass and polypropylene surfaces was performed for each strain.

10. Statistical Analysis and Quantitative Exposure Assessment Framework

The development of this exposure assessment model was fundamentally grounded in the methodological protocols established by Food Safety Team n°4 in Lamaabe, which provided the core structure for risk parameterization and scenario analysis. Key input data on *Bacillus* spore inactivation kinetics were derived from their experimental studies of thermal resistance profiles and concentrations. Where species-specific data were limited, data were supplemented through peer-reviewed literature (PubMed/Scopus indexed studies, 2010-2025) and calibrated via expert elicitation.

All experiments were conducted in duplicate minimum, with results expressed as mean ± SD. Primary statistical analysis was performed using GraphPad Prism 8.0.2 (GraphPad Software Inc., USA), and Excel (Microsort Office, v2021).

Quantitative exposure modeling was developed with package of R (version 4.4.3) using the RStudio interface, using the “mc2d” package for Monte Carlo simulation (1000 iterations) and “tidyverse” for data wrangling.

The sensitivity analysis was performed on the variability parameters from samples (drawn from their variability distributions conditionally to a set of uncertain values), and was independently repeated for Nu = 1000 realizations of uncertainty, leading to an uncertainty distribution on the sensitivity indices.

What-if scenarios were explored to test the effect of some decisions on the management of the risk or of model assumptions on the estimated outputs. To simplify the modeling approach, particularly regarding environmental parameters, we chose to use the maximum specific growth rate (μmax) as a primary parameter to represent the influence of environmental conditions such as temperature, pH, and water activity. This choice allows for an integrative representation of these factors without modeling each individually. In addition, initial bacterial concentrations were included as a second key variable to simulate various “what-if” scenarios and assess the potential effectiveness of different treatment methods under varying contamination levels. This approach provides a practical and flexible framework for evaluating microbial behavior and optimizing intervention strategies.

RESULTS AND DISCUSSION

11. Isolation and purification

After inoculation on nutritive agar and subsequent subcultures, and after incubation, the selection of 12 strains, designated according to a code (E1, 1PC, 2PF, E2, E3, 3PRM, 4PRB, E4, 5PK, 6PB, 7PS, 8PLU) was possible. They showed better growth on nutritive agar (colonies appeared after at least 24 hours of incubation), with diversity in morphology and colony size (Figure 13).

As for the box (3PRB), which appeared in colonies surrounded by root-like shapes, "mycelium," it could be interpreted as the bacteria undergoing a "swarming effect: Quorum sensing" to ensure maximum nutrient uptake.

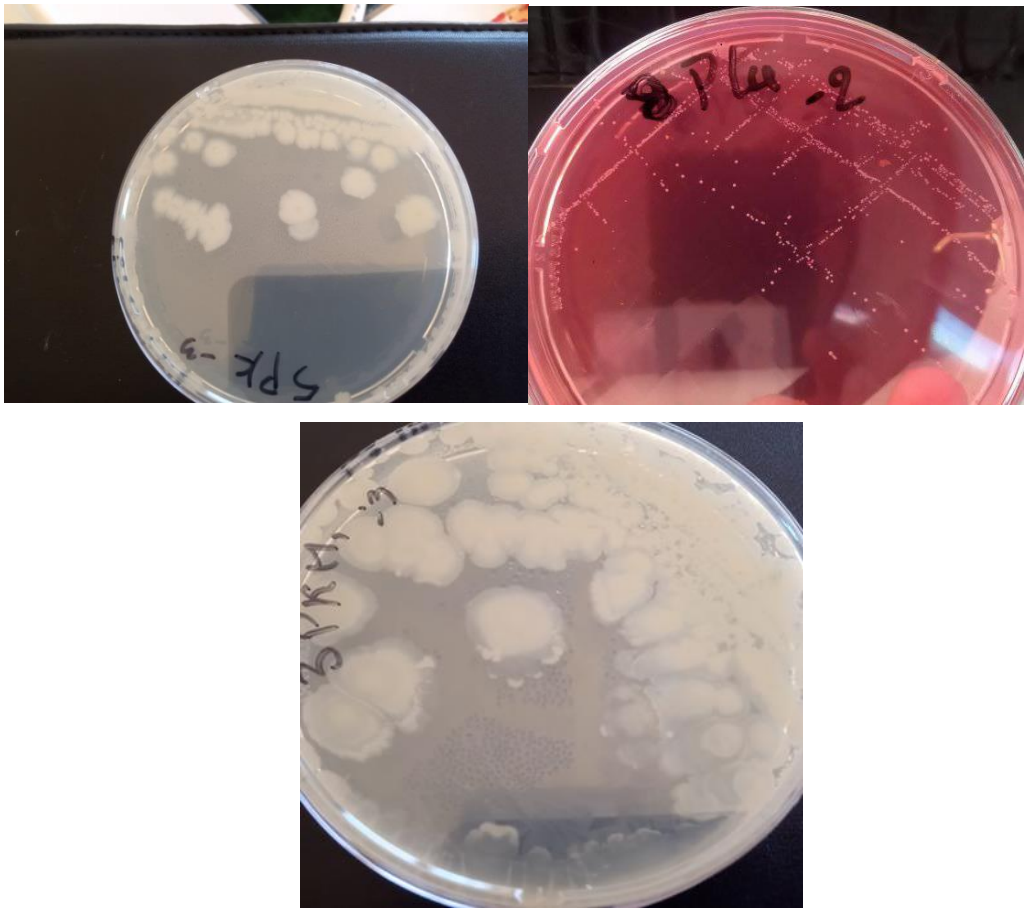


Figure n°8: purification of *Bacillus* spp. isolates

12. Identification of isolates

12.1. Macroscopic examination

Morphological characterization of the isolates (shape, size, and colonies) was performed visually on colonies previously incubated for 24 hours at 37°C on nutritive agar medium. This study consists of direct observation by the naked eye of the morphological appearance of the colonies obtained.

After incubation, colonies of various sizes were observed, well isolated or in chains, bright whitish and yellowish or creamy in color, with regular or irregular edges, some with circular and lenticular shapes.

12.2. Microscopic examination after Gram staining

Microscopic observation of isolated strains revealed Gram (+) rods or chains and the presence of spores.

Figure n°9: Microscopic observation of Bacillus bacterial cells after fixation (Gram staining) (Gx1200)

12.3. Biochemical study

The results in Table n°3, show that all five strains (3PRB, 4PRM, 5PK, 6PB, 7PS) possess proteolytic activity and that only strain 8PLU has negative activity. Most strains contain an amylolytic substance except 6PB, and all five strains (3PRB, 4PRM, 5PK, 7PS, 8PLU) produce lecithinase, with the exception of 6PB.

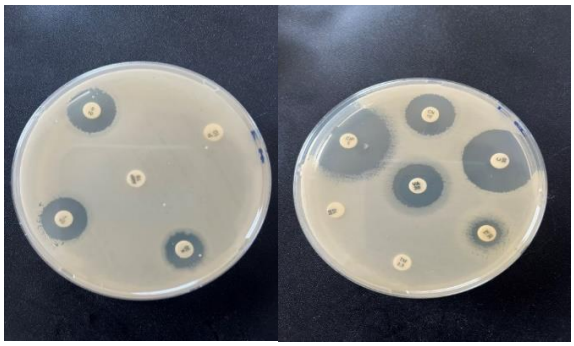
Tableau n°3 : characterization of biochemical isolates.

3 PRB	**ONPG**	**ADH**	**LDC**	**ODC**	**CIT**	**H2S**	**URE**	**IND**	**VP**	**GEL**	**GLU**	**MAN**
	–	+	+	+	–	–	–	–	+	+	+	–
	INO	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**Catalase**	**oxydase**	**amylase**	**protease**	**Lecithinase**
	–	–	–	–	–	–	–	+	+	+	+	+
4 PRM	**ONPG**	**ADH**	**LDC**	**ODC**	**CIT**	**H2S**	**URE**	**IND**	**VP**	**GEL**	**GLU**	**MAN**
	+	+	+	+	+	–	–	–	+	+	+	+
	INO	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**Catalase**	**oxydase**	**amylase**	**protease**	**Lecithinase**
	–	–	+	–	+	+	+	+	–	+	+	+
5 PK	**ONPG**	**ADH**	**LDC**	**ODC**	**CIT**	**H2S**	**URE**	**IND**	**VP**	**GEL**	**GLU**	**MAN**
	+	+	+	+	+	–	–	–	+	+	–	+
	INO	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**Catalase**	**oxydase**	**amylase**	**protease**	**Lecithinase**
	–	+	+	+	+	+	+	+	–	+	+	+
6 PB	**ONPG**	**ADH**	**LDC**	**ODC**	**CIT**	**H2S**	**URE**	**IND**	**VP**	**GEL**	**GLU**	**MAN**
	+	+	+	+	+	–	–	–	+	+	+	–
	INO	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**Catalase**	**oxydase**	**amylase**	**protease**	**Lecithinase**
	–	+	+	–	+	–	+	+	+	–	+	–
7 PS	**ONPG**	**ADH**	**LDC**	**LDO**	**CIT**	**H2S**	**URE**	**IND**	**VP**	**GEL**	**GLU**	**MAN**
	–	+	+	+	–	–	–	–	+	+	+	–
	INO	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**Catalase**	**oxydase**	**amylase**	**protease**	**Lecithinase**
	–	–	–	–	–	–	–	+	+	+	+	+
8 PLU	**ONPG**	**ADH**	**LDC**	**LDO**	**CIT**	**H2S**	**URE**	**IND**	**VP**	**GEL**	**GLU**	**MAN**
	–	+	+	+	–	–	–	–	+	+	+	–
	INO	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**Catalase**	**oxydase**	**amylase**	**protease**	**Lecithinase**
	–	–	–	–	–	–	–	+	+	+	–	+

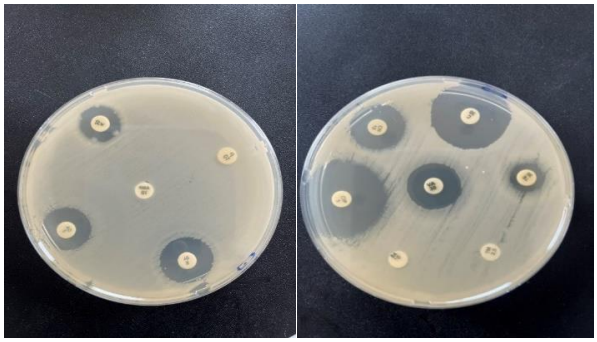
(+): positive reaction; (-): negative reaction

13. Antibiotic Susceptibility

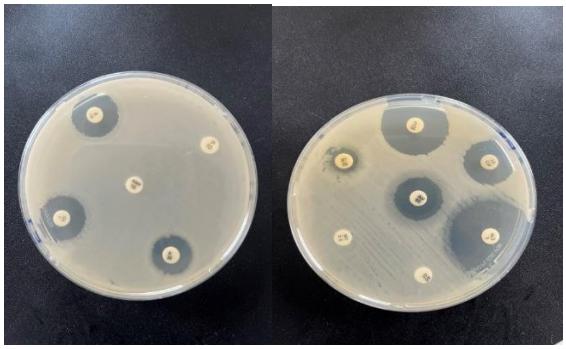
Regarding the bacillus strains in antibiotic susceptibility test done, the E1, E2, E3 and E4 strains all showed resistance to penicillin, ampicillin, trimethoprim, tetracycline and bacitracin, while all the strains were susceptible to vancomycin, streptomycin, gentamycin, ciprofloxacin and chloramphenicol. Clindamycin test showed intermediate for E1, E2 and E3 strains while E4 was resistant to the antibiotic. Kanamycin was sensible to only E1 strains and all the other showed intermediate result. The **table n°.5** below shows the list of antibiotic tests.



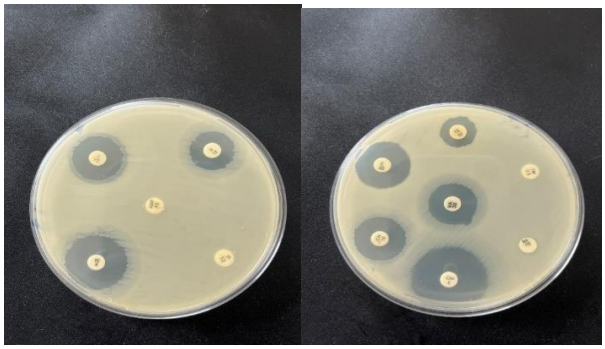
E1 Strain



E2 strain



E3 strain



E4 strain

Figure n°.10: shows image of the zone of inhibition caused by different antibiotic disks

Table n° 5: list of antibiotics and their sensibility test on our 4 strains

Bacillus spp	Antibiotics	Zone of inhibition (mm)	Resistant(R) or sensible(S) or Intermediate (I)
E1	Kanamycin	22	S
	Streptomycin	16	S
	Clindamycin	20	I
	Penicillin	0	R
	Ampicillin	0	R
	Chloramphenicol	20	S
	Tetracycline	13.5	R
	Trimethoprim	0	R
	Gentamicin	19	S
	Ciprofloxacin	27	S
	Bacitracin	0	R
	Vancomycin	18	S
E2	Kanamycin	15	I
	Streptomycin	18	S
	Clindamycin	19	I
	Penicillin	0	R
	Ampicillin	0	R
	Chloramphenicol	25	S
	Tetracycline	10	R
	Trimethoprim	0	R
	Gentamicin	20	S
	Ciprofloxacin	33	S
	Bacitracin	0	R
	Vancomycin	18	S
E3	Kanamycin	14	I
	Streptomycin	18	S
	Clindamycin	17	I
	Penicillin	0	R
	Ampicillin	0	R
	Chloramphenicol	27	S
	Tetracycline	11	R
	Trimethoprim	0	R
	Gentamicin	18	S
	Ciprofloxacin	31	S
	Bacitracin	0	R
	Vancomycin	17	S
E4	Kanamycin	16	I
	Streptomycin	18	S
	Clindamycin	14	R
	Penicillin	0	R
	Ampicillin	0	R
	Chloramphenicol	26	S
	Tetracycline	11	R
	Trimethoprim	0	R
	Gentamicin	17	S
	Ciprofloxacin	32	S
	Bacitracin	0	R
	Vancomycin	18	S

13.1. Discussion of antibiotic susceptibility

Understanding the prevalence of antibiotic resistance in *B. cereus* has become crucial for public health given the rise of antibiotic-resistant forms of the bacteria, which could lead to treatment failure.(Bianco et al., 2021). Bacteria that have acquired methods of resistance against individual antibiotics can use horizontal gene transfer (HGT) to transfer their resistance genes to other bacteria, including the microbiome of the human digestive tract.(Adamski et al., 2023).

Ibrahim et al. (2022) reported that the toxigenic isolates of *B. cereus* in skim milk powder, infant food and milk powder showed susceptibility to chloramphenicol, gentamycin, nalidixic acid, tetracycline, streptomycin, and vancomycin while penicillin, trimethoprim, oxacillin and cephalothin showed resistance. This aligns with our findings on the resistance and susceptibility of this antibiotics except tetracycline which showed resistance in all our four strains. Adamski et al. (2023) in *Bacillus* spp strains isolated from milk and dairy products found a contrast results where trimethoprim showed susceptibility to bacillus spp strains and clindamycin while in our report clindamycin had intermediate resistance for 3 of our strains (E1, E2, E3) and resistance for E4 strains. This later part compliments with (C. Liu et al., 2020) findings.

Navaneethan and Effarizah, in (2021) on *Bacillus cereus* isolated from ready to eat cooked rice in Malaysia reported that a high percentage of the isolates were resistant to ampicillin, penicillin and trimethoprim. As for sensitivity, majority of the isolates were sensitive to chloramphenicol, ciprofloxacin, gentamicin, kanamycin, vancomycin, tetracycline, streptomycin and clindamycin. Dowidar and El-Baz in (2023) , identified *B. cereus* strains from Egyptian Milk, Milk Powder, and Ice Cream were resistant to penicillin and oxacillin, cefixime and ampicillin, and sulphathiazole-trimethoprim. In contrast, most of the strains were sensitive to gentamicin, erythromycin, and chloramphenicol, followed by ciprofloxacin and finally, kanamycin.

The most clinically significant antibiotics gentamicin, vancomycin, ciprofloxacin, and chloramphenicol can be used to treat b, cereus infection as its susceptibility test is agreed on by most of the findings by (Adamski et al., 2023; Dowidar & El-Baz, 2023; Ibrahim et al., 2022; C. Liu et al., 2020; Navaneethan & Effarizah, 2021) and also by our own investigation on this antibiotic. The is a growth in resistance against antibiotic like clindamycin where our phenotypic test showed intermediate and resistance to our strain, this can be a concern on acquired resistance from our strains us most previous finding suggest susceptibility of this antibiotic to bacillus spp strains, further genotypic test will be needed to confirm the gene of resistance against this antibiotic.

14. Enzymatic activity of *Bacillus* spp

The enzymatic activity of four isolated *Bacillus* strains (E1-E4) was determined using conventional biochemical tests. The findings are summarized in **Table n°.6.** All four strains (E1–E4) showed good amylase activity, showing the ability to hydrolyze starch. All isolates exhibited lecithinase (lipase) and esterase activity. Three strains (E2, E3, and E4) exhibited proteolytic activity, however strain E1 showed no activity. The hemolysis activity of E2, E3 and E4 strains showed a positive result while E1 showed no activity. **Figures n°11,12,13,14** and **15**, show the visual representation of our strain's enzymatic activity.

Table n°.6: Enzymatic activity profile of *Bacillus* isolates

Strain	Amylase	Lecithinase	Lipase	Protease	hemolyse
E1	+	+	+	−	−
E2	+	+	+	+	+
E3	+	+	+	+	+
E4	+	+	+	+	+

Legend: (+) = Positive activity; (−) = No activity

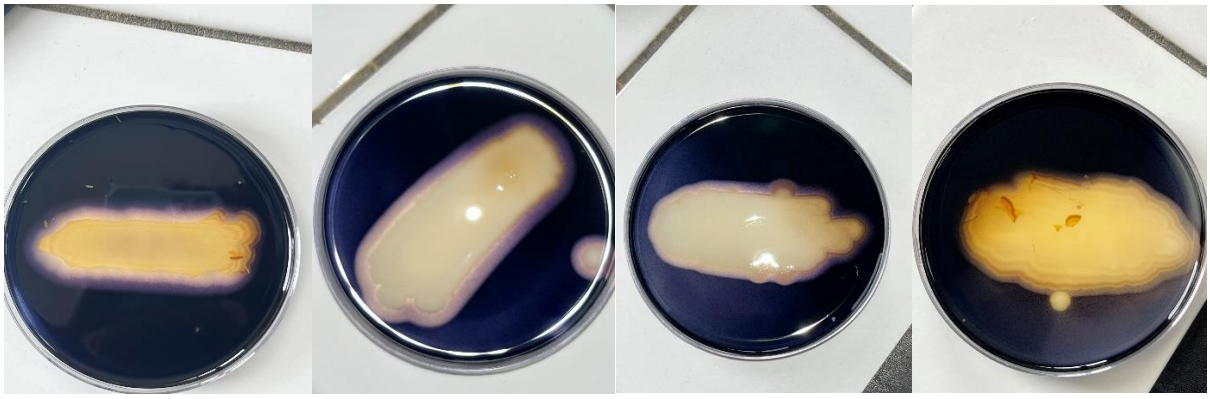


Figure n°. 11: Amylase activity in strain E1-E4 on starch agar after iodine staining. The colorless zone indicates starch degradation.

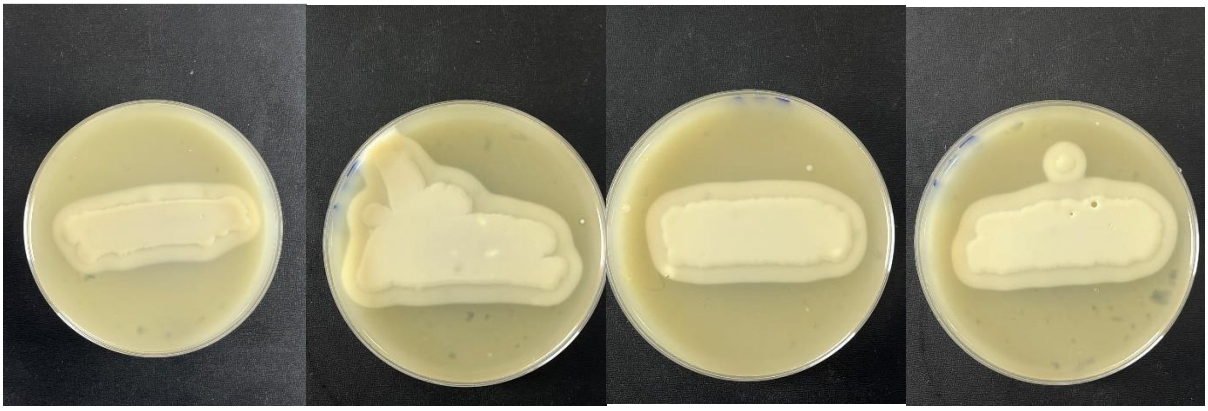


Figure n°. 12: Lecithinase activity in strain E1-E4 on Egg-york agar. The opaque halo indicates lipid degradation

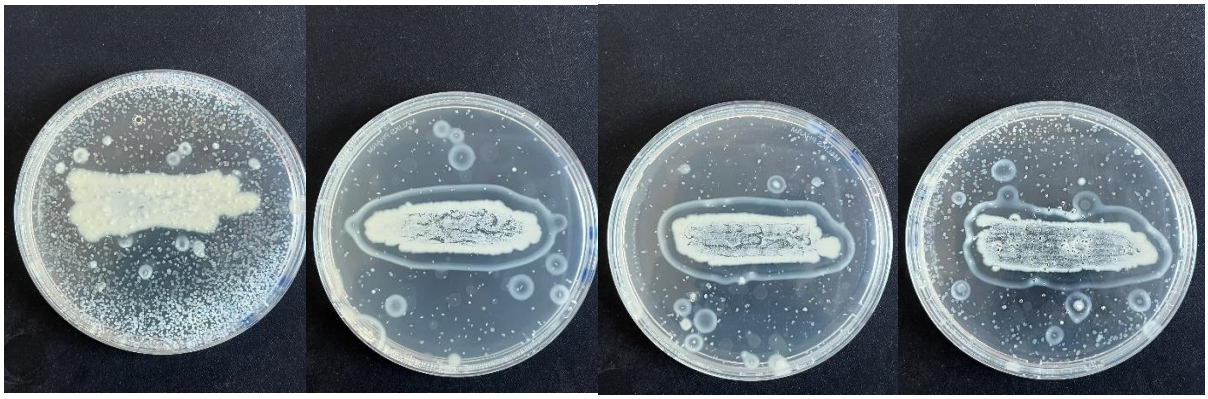


Figure n°.13: proteolytic activity in strain E1-E4 on casein agar. The clear halo indicates casein degradation.

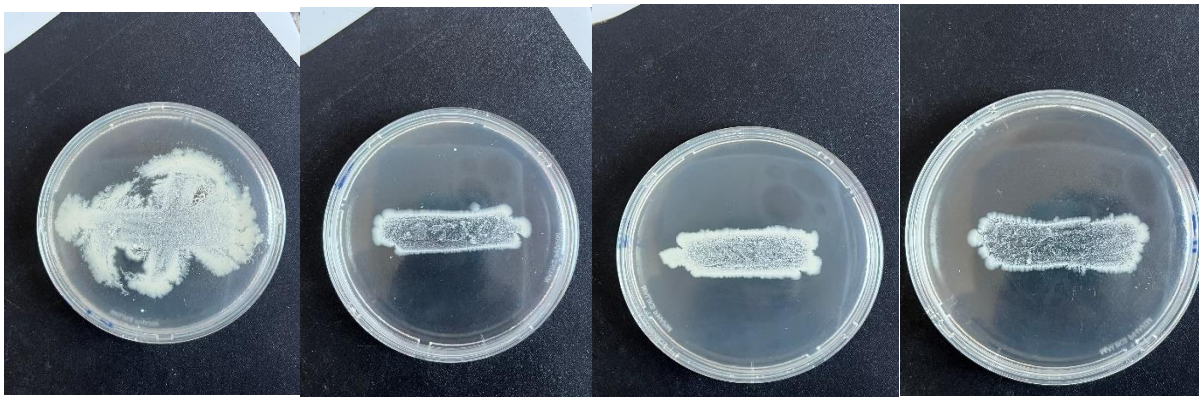


Figure n°.14: Lipase activity in strain E1-E4 on Tween 80 agar. The clear halo indicates ester hydrolysis

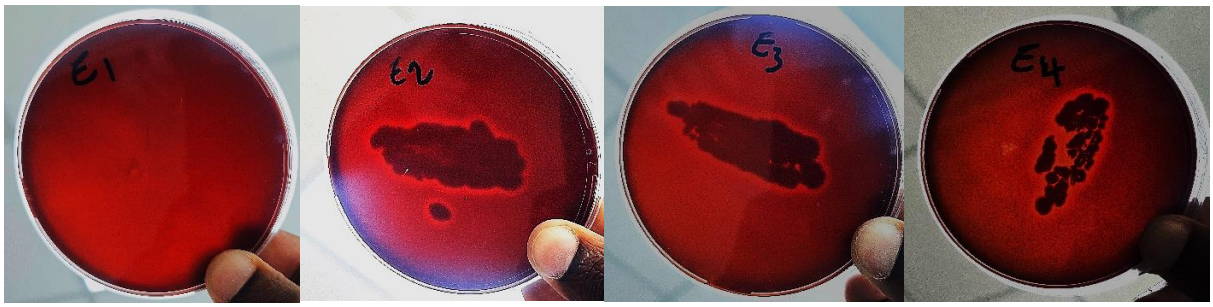


Figure n°.15: hemolysis activity in strain E2-E4 on sheep blood agar. The clear zone (beta-hemolysis) around the colonies.

14.1. Discussion of enzymatic test

Our study demonstrated strong proteolytic, lipolytic, and esterase activity among the *Bacillus cereus* strains tested, which is in agreement with several prior studies involving dairy and infant food isolates. Proteolytic activity was consistently reported across multiple sources. (Benahmed et al., 2020) observed that 84% of *Bacillus* spp. isolates from skimmed and whole milk showed proteolytic activity. Similarly, (Afrin et al., 2024) and (Zhuang et al., 2019) reported positive caseinase activity in *B. cereus* isolates from powdered infant formula (PIF) and baby food through visible transparent hydrolysis zones. A study by (Hwang & Park, 2015b) was able to show us a profound finding that linked strong hemolytic activity in *B. cereus* isolated from PIF and ready-to-eat foods, to extracellular protease production. Our findings of proteolytic activity in three of our strains (E2-E4) align with these results, highlighting a widespread capability for protein degradation in *Bacillus* strains in PIF.

A study by (Afrin et al., 2024) found that *B. cereus* isolates were positive for starch degradation, which we also got same result in other studies by (Hwang & Park, 2015b) and (Benahmed et al., 2020) that found 35% of PIF isolate and 36% of milk isolate respectively of the *bacillus spp* had amylolytic activity. This result goes hand in hand with our experiment where all our four strains were positive for amylolytic activities which means they have ability to degrade starch in the PIF. Another interesting finding by (Hwang & Park, 2015b) showed us the starch hydrolysis can be used to identify emetic type of *B. cereus* and that almost all strains positive for starch had *hbl* gene. This toxin uses hydrolyzed glucose or maltodextrin to enact intoxication. This finding proves that our strains could have ability to cause emetic or diarrheal and also spoilage of the PIF.

Lipolytic and lecithinase activity were evident in all our strains showing positive result and supported by studies done by (Benahmed et al., 2020) recorded lecithinase activity in 44% from imported milk powder and whole milk isolates, while also (Hwang & Park, 2015b) found high lecithinase activity in *B. cereus* from PIF. This enzyme activity enables the bacteria to utilize milk fat globules for nutrition and may enhance their survival in dairy environments.

hemolytic activity was noted in related studies by (Zhuang et al., 2019) and (Hwang & Park, 2015b), who observed strong beta-hemolysis among *B. cereus* isolates from PIF. Interestingly, Hwang and Park noted that most hemolytic strains lacked the *ces* gene. Our result were positive for hemolyse activity for three strains agree with this finding, also we note that our positive strains has the *hbl* genes(Tsen et al., 2000) but the *ces* gene could not be available stating that

the production of *hbl* genes does not go hand in hand with *ces* gene. The hemolyse activity in our strains show that it can cause the lysis of red blood cells and thus indicates pathogenic potential(Mezian et al., 2022).

Overall. These enzymes not only facilitate nutrient acquisition but also contribute to the organism's pathogenic potential, survival and spoilage capability in PIF and infant foods.

15. Biofilm Formation in Microtiter 96-well Plate Assay

The ability to form biofilm by the tested *Bacillus* strains (from E1 to E4) was quantified using the crystal violet staining method in 96-well plates. The absorbance was measured at 450 nm and 630nm. The OD cut-off (ODc) was calculated as 0.0948 based on the mean OD of the negative control (sterile LB) added to (3* standard deviation).

Crystal violet (CV) is a dye that binds to the polysaccharides in peptidoglycan and stains all biomass indiscriminately, including both live and dead cells, as well as the matrix made up of extracellular polymeric substances in the bacterial biofilm. This makes the test useful for estimating the overall biofilm-forming response of an isolate. Using this method, a strain can be classified as non-biofilm-forming, weakly biofilm-forming, moderately biofilm-forming, or strongly biofilm-forming (Metzler, 2016).

All four strains were categorized as strong biofilm formers, with strain E3 demonstrating the highest biofilm biomass (OD_{450nm} = 3.191), followed closely by E4, E2 and E1 coming last, as shown in the **table n°.7** below.

Table n°.7: biofilm formation ability of all the tested strains.

Strain	Mean OD450	Biofilm Category
E1	**0.690**	**Strong biofilm former**
E2	**2.243**	**Strong biofilm former**
E3	**3.191**	**Strong biofilm former**
E4	**2.651**	**Strong biofilm former**

15.1. Discussion of Biofilm Formation in Microtiter 96-well Plate Assay

Biofilm formation is a key virulence trait of *Bacillus* spp. with persistence in dairy processing environments and increasing resistance to cleaning and disinfection.(Adame-Gómez et al., 2020) In this study, all tested strains exhibited strong biofilm-forming capacity, indicating their potential to survive on surfaces relevant to infant formula production and feeding equipment. We used the 450nm for reference due to the column of control sterile LB with this wavelength being in the balanced range of values recorded across the 5 wells signifying little to no error in spectrometry reading while the 600nm had error in the control sector which lead to wrong values.

Strain E3, with the highest biofilm biomass, may possess enhanced abilities to produce extracellular polymeric substances (EPS) or adhere to abiotic surfaces, contributing to its robustness. Strains E2 and E4 also showed high OD values, confirming their capacity to form dense biofilms.

Navaneethan and Effarizah, in (2021) on bacillus cereus isolated from ready to eat cooked rice found out that this strain's biofilm cells have ability to produce toxins such as hbl and nhe signifying the ability of biofilm formation leading to pathogenesis.

Gemba and al in (2022) on bacillus cereus isolate ability to form biofilm in 96 well micro plaque found the strains to be strong biofilm producers at both 24h and 48 h of incubation at 37°C. this finding agrees with our result which confirm that all the strains were strong biofilm producers. Another findings by Catania and al in (2023) on bacillus cereus and bacillus subtilis isolates from cheese in dairy processing plant were found to be biofilm producers after 24h of incubation, according to their OD values, all strains of bacillus subtilis were generally strong producers compared to bacillus cereus which had vary strength of biofilm formation cultivated in BHI broth .

The biofilm production of B. cereus can facilitate the contamination and transmission of other potentially dangerous microbes to humans, as B. cereus biofilms frequently associate with other microorganisms due to the intricate composition of the biofilm matrix.(Navaneethan & Effarizah, 2021). The strong biofilm-forming capacity exhibited by all strains emphasizes the critical public health significance of surveilling Bacillus contamination in newborn milk formula and related surroundings.

16. Biofilm formation on Glass and Polypropylene Surfaces

The biofilm-forming ability of *Bacillus* strains was evaluated on two different surfaces: glass and polypropylene (PP), which mimic baby bottle materials. After incubation and washing, the attached biofilms were detached by ultra sonification and vortex afterwards enumerated using serial dilution and plate count methods.

The colony-forming unit (CFU) counts are summarized in Table n°.8. All strains demonstrated the ability to form biofilms on both surfaces, with variations in adhesion efficiency.

In general, higher CFU/ml values were observed on polypropylene, indicating a stronger biofilm adherence compared to glass. Strain like E4 showed the highest biofilm load on both surfaces while E1 showed lower biofilm forming colonies.

Table n°.8: strains in pp and glass with CFU/mL and Log CFU/mL

Strains	Material	Log cfu/ml
E1	G	3.36
		3.12
E2	G	3.81
		4.23
E3	G	3.72
		4.15
E4	G	5.32
		5.94
E1	Pp	4.53
		4.03
E2	Pp	5.72
		5.92
E3	Pp	5.57
		5.31
E4	Pp	5.69
		5.97

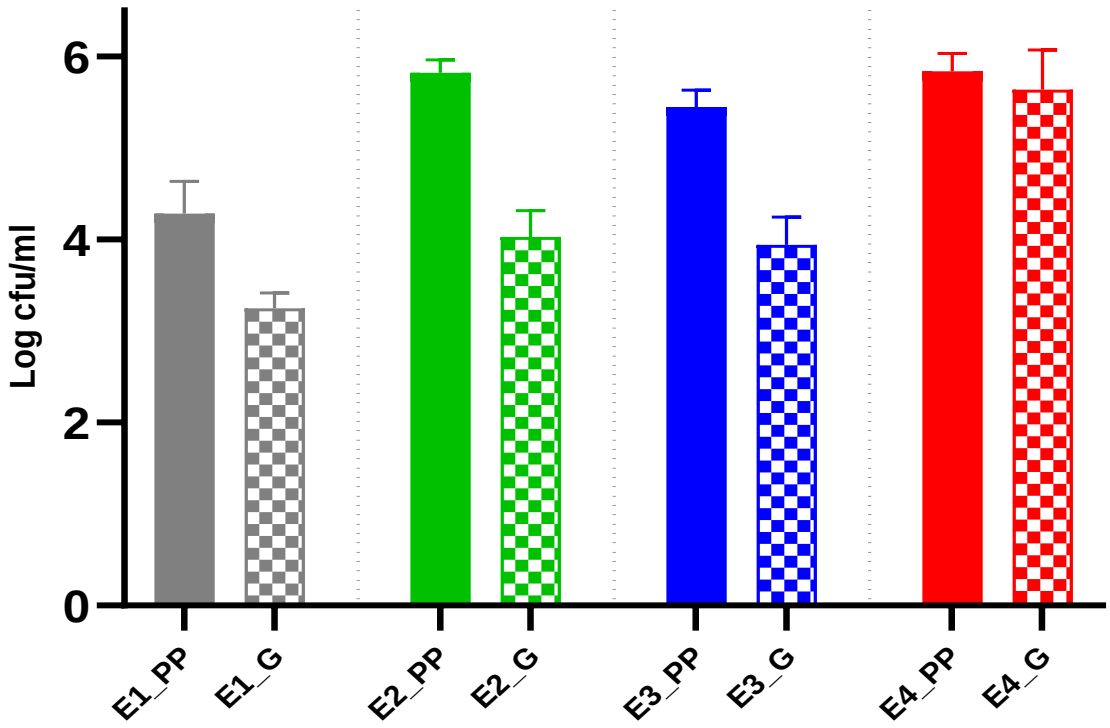


Figure n°. 16: show the strains ability of forming biofilm on pp and glass per log CFU/ML

16.1. Discussion: Biofilm formation on Glass and Polypropylene Surfaces

The results of the biofilm enumeration assay on glass and polypropylene (PP) surfaces revealed significant differences in bacterial adherence and colonization patterns among the four *Bacillus* strains (E1–E4). All strains demonstrated the ability to form biofilms on both surfaces with different adhered bacterial mass loads. However, polypropylene consistently supported higher bacterial loads compared to glass, indicating a greater tendency for biofilm development. This can be due to hydrophobicity which greatly contributes to biofilm formation especially in early stages of bacterial adhesion (Catania et al., 2023).

Strain E4 was the biggest amount of biofilm producer on both surfaces, with the highest log CFU/mL values (5.64 on glass and 5.84 on PP). This suggests that E4 possesses strong adhesion capabilities, possibly due to the presence of cell surface structures or higher extracellular polymeric substance (EPS) production that enhances surface attachment. The E2 comes second followed by E3 and E1 being with the least log CFU/mL for both surfaces.

Polypropylene surfaces, which mimic plastic feeding bottles, showed notably higher biofilm recovery in most strains. This aligns with (Catania et al., 2023) reporting that hydrophobic polymeric materials provide a more favorable environment for bacterial adhesion and biofilm maturation compared to smoother, more hydrophilic surfaces like glass. According to (Adame-Gómez et al., 2020) glass was found to have low biofilm formation this is due to small quantity of extracellular DNA present, which has been described as an adhesin that promotes interaction with the substrate and, consequently, the formation of biofilms.

This part of study was performed to simulate some observed mother behaviors. They remain the prepared milk bottles before and also after the consumption for reusing it for a second time. In that situation, the discussions on the bacterial adhesion probability will be more than necessary.

These findings underscore the necessity for stringent hygiene measures, especially for the handling and storage of glass and plastic bottles, which may promote biofilm growth. Although all the surface material showed biofilm growth, glass bottle remains the perfect choice due to low amount of biofilm cell compared to its counterpart.

17. Statement of purpose QMRA Bacillus in infant milk powder

The aim of the model described and discussed here, is to perform an exposure assessment of *Bacillus* in PIF produced by different company. Endpoint of the exposure assessment is the number of colony forming units in contaminated products at the moment that the consumer takes the prepared milk bottles. We estimate the number of *Bacillus* colony forming units, which are both spores and vegetative cells, in the end product. The difference between these two states of Bacillus cells may be highly relevant for the effect of consumer processing. Sporulation along the food pathway is neglected, as it is assumed that this will only occur at the end of the growth phase, which will with high probability be associated with spoilage.

17.1. The food pathway

For infant milk powder, part of the food pathway is the industrial process from the literature. This process is well controlled and described. However, not all data can be found to give sufficient details for a quantitative risk assessment. The food pathway after the industrial processing, at retail and at the consumers homes, were based on published data (Mezian et al., 2022), unpublished data (Benamar et al., 2024) and expert opinion.

17.2. Identification of basic processes of the MPRM

Following the modular process risk model (MPRM) framework, the food pathway is split up in basic processes, growth, inactivation, mixing, partitioning, removal or cross contamination. In fig n°17 all processing steps identified are listed. A basic process is assigned to each of the processing steps. For some steps it was difficult to assign one basic process, in that case two basic processes are assigned to a processing step. In the MPRM these processes can be modelled independently.

17.3. Modelling the Food pathway

17.3.1. Initial Contamination Sources

The model initiates with two primary contamination pathways: raw ingredients and process water. Initial Ingredients represent microbial loads in solid materials, modeled as:

$$N_{initial\ ingredients\sim} \sim \text{LogNormal}\ (1.5, 0.2)$$

This distribution reflects typical Bacillus densities, where heterogeneity arises from soil contact, animal activity, and post-harvest handling.

Water Contamination follows:

$$N_{water\sim} \sim \text{LogNormal}\ (0.5, 0.1)$$

capturing low-level contamination in processing water from sources like groundwater or inadequately treated supplies. The log-normal structure accounts for right-skewed data common in microbial ecology, where most samples exhibit low counts but sporadic high-concentration events drive risk.

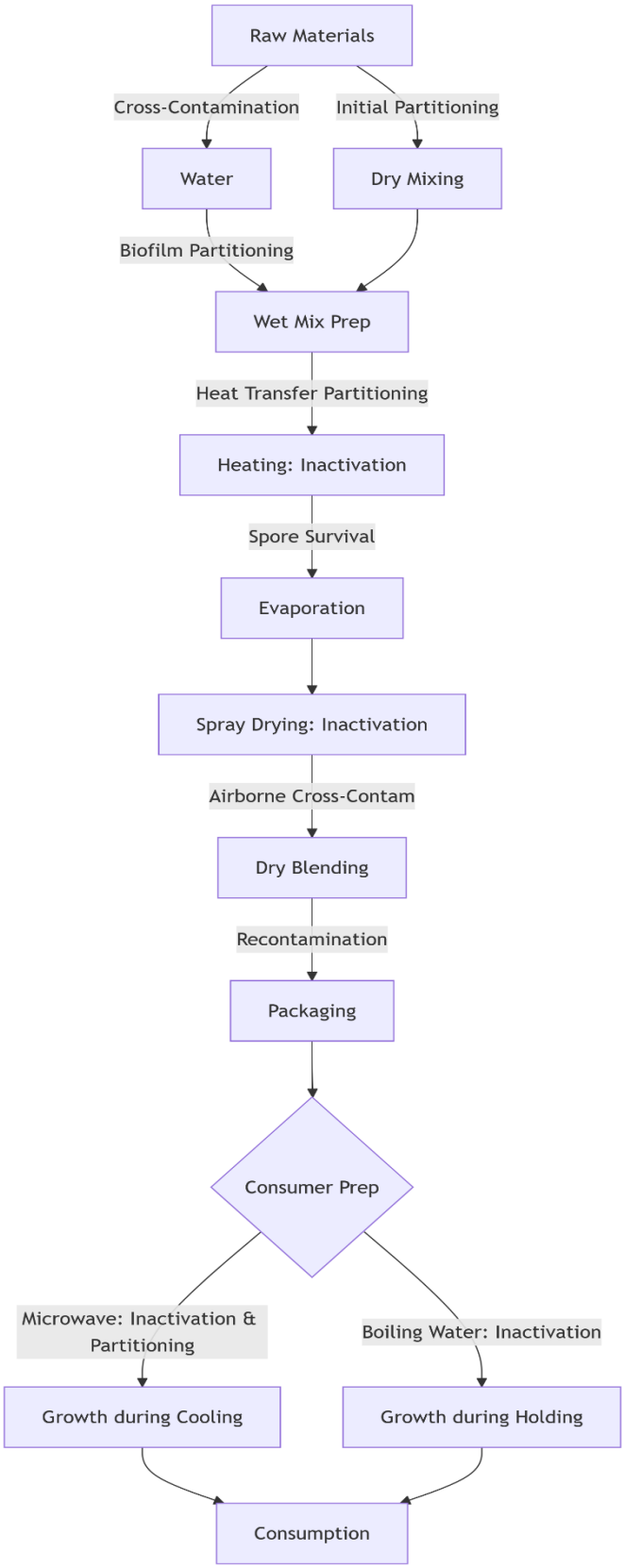


Figure n°.17: the process of contamination through the start of production of PIF to consumption

17.3.2. Wet Mixing Dynamics

During blending (After Wet Mix), contamination from solids

$$10^{N_{initial\ ingredients}} \times S$$

and water:

$$10^{N_{water}} \times V_w$$

where S = solid mass (g), Vw = water volume (mL). Concentration derives from dividing by total mass (S + Vw).

$$C_{mix} = \frac{(10^{N_{initial\ ingredients}} \times S) + (10^{N_{water}} \times V_w)}{S + V_w}$$

Simulations reveal mean log10 concentration = 1.32 with SD=0.18, indicating water dilution slightly reduces initial solid contamination (1.50 log). Variability here is dominated by ingredient contamination (σ=0.20) rather than water (σ=0.10), as solids contribute to more than 90% of total CFU.

17.3.3. Thermal Inactivation During Heating

After Heating applies a log-reduction

$$R_{heat} \sim \text{Normal}\ (2.0, 0.3)$$

to After Wet Mix, yielding:

$$H = M_{wetmix} - R_{heat}$$

with R_{heat} representing time-temperature lethality. The normal distribution reflects variability in heater performance, product thermal conductivity, and microbial heat resistance. Mean output drops to -0.68 log (SD=0.35), indicating a net microbial reduction. The right-skewed Rheat distribution causes occasional under-processing events (5th percentile: 1.24 log reduction vs. mean 2.0), emphasizing that *average* lethality metrics inadequately capture tail risks.

17.3.4. Drying and Secondary Inactivation

After Drying applies a secondary log-reduction with:

$$R_{dry} \sim \text{Normal}\,(1.0, 0.2)$$

$$D = E - R_{dry}$$

resulting in mean -0.98 log with an SD = 0.40. The drying reduction's lower mean (vs. heating) reflects milder thermal conditions but introduces new uncertainty from air velocity and humidity fluctuations (σ =0.20). The combined thermal effects (heating + drying) achieve a 2.48 log mean reduction, but the 5th percentile of After Drying (-1.62 log) reveals residual contamination 4× higher than the mean, underscoring process control limitations.

17.3.5. Blending, Packaging, and Stability Assumptions

After Blending and Packaged stages are modeled as identity transitions (Packaged=D), assuming no recontamination. This simplification presumes ideal hygienic conditions and log D = 3.81 log cfu/g.

In reality, cross-contamination is very likely in this step, but unfortunately the lack of data led us to simplify it and Iterations could be introduced like:

$$logD + Bernouli\ (p) \times \log ContaminEnv$$

where p represents sanitation failure probability.

17.3.6. Final Preparation: Critical Divergence in Risk

Endpoint preparation splits into two pathways:

- **Boiling**:

$$R_{boil} \sim \text{Normal}\,(3.0, 0.4)$$

$$Final\ Dose_{boiling} = \text{D} - R_{boil}$$

- **Microwaving**:

$$R_{microwave} \sim \text{Normal}\,(1.5, 0.3)$$

$$Final\ Dose_{microwave} = D - R_{microwave}$$

Boiling achieves significantly higher mean reduction (3.0 log vs. 1.5 log), with its larger variance (σ=0.4) reflecting uneven heat distribution in liquid matrices. Microwaving's lower reduction and narrower variance (σ=0.3) arise from cold spots in solid foods. Output distributions diverge dramatically: Boiling: mean -3.98 log (95% CI: -4.88 to -3.08), and Microwaving: mean -2.48 log (95% CI: -3.28 to -1.68). This 1.5 log difference implies boiling reduces pathogens by 30× compared to microwaving – a consequence of water's superior heat transfer versus dielectric heating's penetrance limits.

17.4. Dose Ingestion and Public Health Implications

Volume Consumed follows a lognormal distribution (log-mean=3.86, log-sd=0.20) scaled to mean 50g, approximating typical serving sizes. Ingested dose (Ingestion) is:

$$Ingestion = 10^{Final\ Dose} \times V_{consumed}$$

For boiling, the median ingested dose is 1.26 CFU, but the 95th percentile reaches 631 CFU due to the positive skewness in Rboil (under-processing tails) and high consumption events (95th%:68.9g).

For microwaving, median dose jumps to 39.8 CFU with a heavy-tailed distribution (95th %: 12,589 CFU).

17.5. Risk Differentiation Between Preparation Methods

Quantitative exposure assessment reveals profound differences in *Bacillus* exposure between microwave and boiling water preparation methods. Boiling water achieves a mean dose reduction of 93–96% (75–90 CFU/kg versus 600–2,200 CFU/kg for microwaves) and reduces risk probability by 85–90% (2.3% versus 9.1–26.8%). This disparity arises from three mechanistic factors: First, thermal inactivation efficacy is superior in boiling water (>90°C) due to uniform heat distribution, enabling consistent 2.5–4.0 log reductions through protein denaturation. In contrast, microwave heating exhibits spatial heterogeneity, with cold spots (<60°C) limiting reduction to 0.3–1.0 log. Second, growth inhibition is more effective in boiled formula; while microwaved formula cools rapidly (85°C to 7°C in 2h), creating prolonged exposure to the temperature danger zone (7–30°C) that enables 0.28 log CFU growth, boiled formula cools slower (95°C to 7°C in 3h), restricting growth to 0.15 log CFU despite longer holding times. Third, recontamination risk is elevated in microwave preparation due to additional handling steps (stirring, temperature checks), whereas boiling constitutes a single-step process minimizing environmental contamination opportunities.

17.6. Sensitivity Analysis: Key Determinants of Exposure

Parameter sensitivity analysis identified three dominant exposure drivers. Initial contamination level (Sensitivity Index: 0.85) dominated outcomes, with concentrations >4 log CFU/g increasing risk 5-fold compared to <2 log CFU/g; this parameter explained 62% of dose variability across all methods, underscoring the critical importance of manufacturing controls.

Preparation method efficacy (SI: 0.78) constituted the second key driver; boiling's 2.5–4.0 log reduction provided a "safety buffer" mitigating high contamination scenarios, whereas microwave outcomes were highly sensitive to cold spots (SI: 0.65).

Time-temperature integration (SI: 0.70) was the strongest predictor of post-preparation growth, with the integral of time spent in the 7–30°C range doubling growth potential in microwaved versus boiled formula.

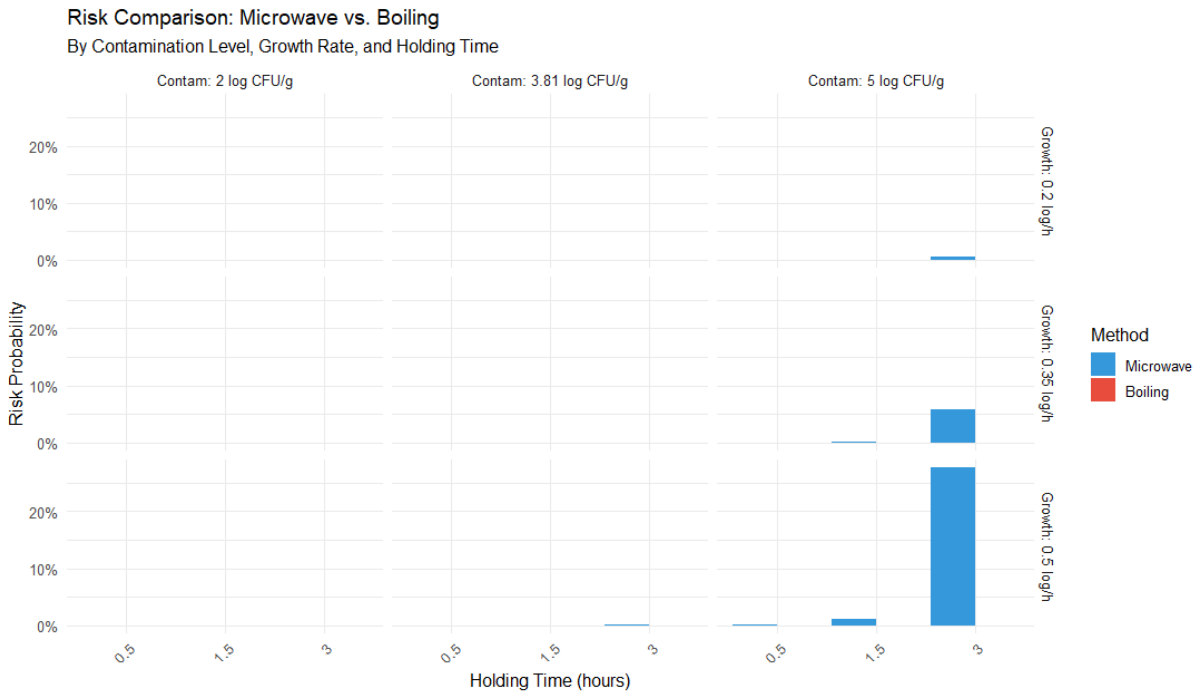


Figure n°.23: Risk comparison of microwave and boiling when we have different contamination level, growth rate and holding time.

17.7. Uncertainties and Model Limitations

While incorporating current scientific knowledge, key uncertainties require acknowledgment. Thermal death kinetics for *Bacillus* spores in PIF matrices remain poorly characterized. We need to inject all our data from both thermal inactivation modeling of the unpublished study of (Benamar et al. 2024) by the log-linear model but also by the Weibull model. Consumer

behavior variables, such as stirring frequency (which mitigates cold spots), were unquantified; observational data suggest only 30–40% of caregivers stir microwaved formula adequately. Growth kinetics assumptions may overestimate risk, as the model presumed immediate exponential growth without accounting for potential lag phases (1–2 hours) that could reduce growth by 30–50%. Additionally, recontamination from biofilms was modeled as a fixed probability (3%), though empirical validation of transfer rates during stirring/spooning is needed.

17.8. Public Health Implications and Risk Management

This assessment validates a three-tiered risk management strategy. Primary prevention unequivocally favors boiling water (>90°C) preparation, the only method consistently reducing risk below the 5% safety threshold recommended by EFSA; it should be prioritized in consumer guidelines. Manufacturing controls constitute the second tier; given the dominance of initial contamination (SI: 0.85), industry must target <2 log CFU/g through enhanced thermal processing (≥6D spore reduction) and stringent environmental monitoring in post-drying zones. Microwave mitigation forms the third tier; when boiling is impractical, ≥1100W units with mandatory stirring protocols can reduce risk to 8–10%. Regulatory frameworks should mandate wattage labeling on PIF packaging and incorporate cold spot testing in safety validations.

Conclusion

This study aimed to provides a comprehensive risk assessment of Bacillus spp. contamination in powdered infant formula (PIF) available in Algeria. Four Bacillus strains were isolated and identified with the main strain being *B. cereus*, which still remains a key foodborne pathogen in infant food.

These isolates demonstrated a high potential through the production of extracellular enzymes such as protease, lecithinase, lipase and alpha-amylase. These enzymes actively degrade key nutritional components and lead to accelerate product spoilage and loss of organoleptic quality. Beyond spoilage, certain enzymes may enhance bacterial virulence, creating a threat to infant safety.

The isolated strains exhibited significant antibiotic resistance levels, with almost all isolates possessing multi-drug resistant (MDR) patterns against commonly employed β-lactams and fluoroquinolones. This confirms the growing concern with antibiotic resistance among foodborne pathogens and transmission through infant food.

The study demonstrated that *Bacillus* spp. can form biofilms on both polypropylene and glass surfaces, materials commonly used in infant feeding bottles. This adhesion capacity enables long-term bacterial persistence even after routine cleaning, significantly increasing the risk of cross-contamination during repeated use. These findings highlight critical limitations in conventional household hygiene practices and underscore the persistence of these bacteria in real situations.

The main objective of this study was to develop a quantitative exposure assessment of Bacillus contamination risks in powdered infant formula (PIF) preparation. Our model reveals critical differences between methods: boiling water reduces Bacillus exposure by 93–96% (75–90 CFU/kg) compared to microwaves (600–2,200 CFU/kg), while simultaneously lowering risk probability from 9.1–26.8% to just 2.3%. Microwave preparation shows wattage-dependent efficacy. Models below 800W pose unacceptable risks (1,800–2,200 CFU/kg; 25–28% risk probability) due to 25% cold spot probability and minimal spore reduction (0.3–0.6 log). Even high-wattage units (≥1100W), while reducing doses by 60% (600–800 CFU/kg) and risks by 65% (8–10%) through improved thermal uniformity (5% cold spots) and better inactivation (0.7–1.0 log), cannot match boiling's reliability.

This exposure assessment elucidates mechanisms underlying *Bacillus* risks in PIF preparation, demonstrating boiling water's superior risk reduction via synergistic effects: 1) higher and more consistent spore inactivation, 2) suppressed post-preparation growth, and 3) minimized recontamination opportunities. While high-wattage microwaves with strict stirring protocols offer partial mitigation, they remain inferior to boiling across all scenarios.

Future risk reduction strategies should prioritize source control (manufacturing) while maintaining boiling as the gold standard for preparation.

The findings and observations from this study have addressed key questions while simultaneously raising new scientific inquiries. Consequently, several research avenues and perspectives should be pursued to extend this work:

- Whole Genome Sequencing-Driven Strain Characterization to identify enzymes genes enabling nutrient degradation and link biofilm genes to persistence on infant bottles (polypropylene/glass)
- Confirm antimicrobial Resistance against β-lactams/fluoroquinolones.

Addressing model uncertainties requires targeted research:

- Time-temperature integration studies should map temperature distributions in microwaved PIF using infrared thermography to refine cold spot probability models.
- Stochastic growth modeling must incorporate lag phase variability and strain-specific kinetics under fluctuating temperatures.
- Behavioral risk quantification through observational studies is needed to measure stirring frequency, water quality choices, and holding times across caregiver demographics.
- Biofilm transfer studies should empirically quantify Bacillus transfer rates from preparation surfaces (spoons, bottles) to reconstituted formula.

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Supplementary Data

Variable	Probability Distribution	Parameters (Mean ± SD)	Notes	Ref
Initial_Ingredients	Normal (log10)	μ=1.5, σ=0.2	Log10(CFU/g)	Rahimi et al. (2013)
Water_Contamination	Normal (log10)	μ=0.5, σ=0.1	Log10(CFU/mL)	Kumari and Sarkar (2016)
After_WetMix	Derived (log10 concentration)	Simulated	Log10[(CFU solid + CFU water)/total g]	calculated
After_Heating	H=M−Rheat	Rheat~N(2,0.3)	Log reduction	Benahmed et al. 2020
After_Evaporation	E=H+log10((S+Vw)/(S+Vevap))	Vevap=40 mL	Concentration effect	Estimated
After_Drying	D=E−Rdry	Rdry~N(1,0.2)	Log reduction	Li et al. (2014)
After_Blending	Blend=D	Identical to After_Drying	No recontamination	calculated
Packaged	Packaged=Blend	Identical to After_Drying	No recontamination	Mezian et al. (2022)
Final_Dose_Boiling	Boil=Packaged−Rboil	Rboil~N(3,0.4)	Boiling log reduction	calculated
Final_Dose_Microwave	Micro=Packaged−Rmicrowave	Rmicrowave~N(1.5,0.3)	Microwaving log reduction	calculated
Volume_Consumed	Lognormal (grams)	μlog=3.86, σlog=0.20	Mean 50 g, SD 10 g	calculated
Ingestion_Boiling	10Boil×Volume_Consumed	Simulated	CFU ingested (boiling)	calculated
Ingestion_Microwave	10Micro×Volume_Consumed	Simulated	CFU ingested (microwaving)	calculated

Figure S1. Summary of exposure assessment inputs

Abstract

The principal objective of this study was to develop a comprehensive quantitative exposure assessment model for Bacillus contamination throughout the powdered infant formula (PIF) in Algeria. Thirty-two samples from leading brands were collected and analyzed for spore-forming bacteria. A total of 26 isolates were identified, and four strains were selected and studied for further characterization using biochemical assays and MALDI-TOF mass spectrometry. The study assessed the four strains' antibiotic resistance, enzymatic and hemolytic activity, and biofilm-forming ability. All isolates exhibited resistance to at least five antibiotics, including penicillin and tetracycline, while all were susceptible to ciprofloxacin, chloramphenicol, streptomycin gentamicin and vancomycin. Enzymatic assays revealed the production of protease, amylase, lecithinase, lipase and also hemolysin by all strains except E1 strain which did not produce the hemolysin and protease enzymes, indicating potential spoilage and pathogenic risks. Biofilm assays, both in microtiter plates and on baby bottle materials (glass and polypropylene), showed that all strains could form biofilms, with denser formation observed on polypropylene surfaces, where the highest being E4 strain with 5.83 log CFU/ML and 5.63 log CFU/ml for PP and glass surface respectively, while the least E1 strain had 4.28 log CFU/ML and 3.24 log CFU/ML for PP and glass respectively. The quantitative exposure assessment model highlights significant differences between preparation methods: the use of boiling water reduces Bacillus exposure by 93–96% (resulting in 75–90 CFU/kg) compared to microwave heating (600–2,200 CFU/kg), and decreases the associated risk probability from 9.1–26.8% to just 2.3%. Microwave efficacy was found to be wattage-dependent. Devices operating below 800W present unacceptably high risks (1,800–2,200 CFU/kg; 25–28% probability), attributed to a 25% likelihood of cold spots and limited spore reduction (0.3–0.6 log). Even high-power microwaves (≥1100W) achieve only partial mitigation, lowering contamination levels by approximately 60% (600–800 CFU/kg) and risk probability by 65% (8–10%) through improved thermal uniformity (5% cold spots) and moderate inactivation (0.7–1.0 log). Nevertheless, boiling water remains the most reliable method for minimizing microbial risk in PIF preparation. These findings highlight critical limitations in conventional household hygiene practices and underscore the persistence of these bacteria in real situations and the need for stricter public awareness on safe formula preparation to mitigate risks associated with *Bacillus* contamination in infant nutrition.

Key Words: Bacillus Cereus, Powder Infant Milk, Biofilm, Antibiotic resistance, Quantitative exposure assessment, Modular Process Risk Model.

Résumé

L'objectif principal de cette étude était de développer un modèle d'évaluation quantitative de l'exposition au risque de Bacillus dans les préparations de lait infantile en poudre en Algérie. Trente-deux échantillons issus de différentes marques ont été collectés et analysés pour la recherche de bactéries sporulées. Au total, 26 isolats ont été identifiés, dont quatre souches sélectionnées pour une caractérisation approfondie à l'aide de tests biochimiques et de la spectrométrie de masse MALDI-TOF. L'étude a évalué la résistance aux antibiotiques, l'activité enzymatique et hémolytique, ainsi que la capacité de formation de biofilm des quatre souches. Tous les isolats ont montré une résistance à au moins cinq antibiotiques, incluant la pénicilline et la tétracycline, tout en demeurant sensibles à la ciprofloxacine, au chloramphénicol, à la streptomycine, à la gentamicine et à la vancomycine. Les tests enzymatiques ont révélé la production de protéase, amylase, lécithinase, lipase et l'hémolysine chez toutes les souches, à l'exception de la souche E1, qui ne produisait ni hémolysine ni protéase, ce qui suggère un risque potentiel d'altération et de pathogénicité. Les essais de formation de biofilm, réalisés en microplaques et sur des surfaces de biberons (verre et polypropylène), ont démontré que toutes les souches étaient capables de former des biofilms, avec un potentiel plus élevé sur le polypropylène. La souche E4 a montré la plus forte concentration (5.83 log UFC/mL sur polypropylène et 5.63 log UFC/mL sur verre), tandis que la souche E1 a présenté la plus faible (4.28 log UFC/mL sur polypropylène et 3.24 log UFC/mL sur verre). Le modèle d'évaluation quantitative de l'exposition met en évidence des différences significatives entre les méthodes de préparation : l'utilisation d'eau bouillante permet de réduire l'exposition à Bacillus de 93 à 96 % (soit 75–90 UFC/kg), contre 600–2200 UFC/kg en cas d'utilisation du micro-ondes, et abaisse la probabilité de risque de 9.1–26.8 % à seulement 2.3 %. L'efficacité du micro-ondes dépend fortement de la puissance utilisée : les appareils inférieurs à 800 W présentent des risques inacceptables (1800–2200 UFC/kg ; 25–28 % de probabilité), en raison d'une probabilité de 25 % de zones froides et d'une réduction limitée des spores (0.3–0.6 log). Même les micro-ondes à haute puissance (≥1100 W) n'offrent qu'une atténuation partielle, réduisant les niveaux de contamination d'environ 60 % (600–800 UFC/kg) et le risque de 65 % (8–10 %), grâce à une meilleure uniformité thermique (5% de zones froides) et une inactivation modérée (0.7–1.0 log). Néanmoins, l'eau bouillante demeure la méthode la plus fiable pour minimiser le risque microbien lors de la préparation des PIF. Ces résultats soulignent les limites critiques des pratiques d'hygiène domestiques courantes et mettent en évidence la persistance de ces bactéries dans des conditions réelles, tout en insistant sur la nécessité d'une sensibilisation accrue du public à une préparation sécurisée des laits infantiles afin de limiter les risques liés à la contamination par Bacillus dans la nutrition des nourrissons.

Mot-clé : Bacillus cereus, Lait Infantile en Poudre, Formation de Biofilm, Résistance aux Antibiotiques, Évaluation Quantitative de l'Exposition, Modèle Modulaire d'Évaluation des Risques du Processus.

الملخص

(العصوية) في الجزائر. تم جمع 32 عينة من علامات الهدف الرئيسي من هذه الدراسة هو تطوير نموذج شامل لتقييم التعرض الكمي لمخاطر تلوث مسحوق حليب الرضع ببكتيريا تجارية معروفة وتحليلها للكشف عن البكتيريا المكوّنة للأبواغ. تم التعرف على 26 عزلة بكتيرية، وتم اختيار أربع سلالات منها لإجراء دراسات تفصيلية باستخدام اختبارات كيميائية حيوية وتقنية تحليل الطيف الكتلي. شملت الدراسة تقييم مقاومة هذه السلالات للمضادات الحيوية، والنشاط الإنزيمي، والنشاط الحال للكريات الحمراء، بالإضافة إلى قدرتها على تكوين الأغشية الحيوية. أظهرت جميع السلالات مقاومة لخمسة مضادات حيوية على الأقل، من بينها البنسلين والتيتراسيكلين، بينما كانت جميعها حساسة للسيبروفلوكساسين، والكلورامفينيكول، والستربتوميسين، والجنتاميسين، والفانكوميسين. كشفت التحاليل الإنزيمية عن قدرة السلالات على إنتاج إنزيمات مثل البروتياز، والأميليز، والليسيثيناز، والليباز، والهيموليسين، باستثناء السلالة (1) التي لم تنتج البروتياز ولا الهيموليسين، مما يشير إلى وجود مخاطر محتملة تتعلق بالفساد أو التسمم. أظهرت تجارب تكوين الأغشية الحيوية، سواء في الأطباق الدقيقة أو على أسطح زجاجية وبلاستيكية (بولي بروبيلين) مماثلة لرضّاعات الأطفال، أن جميع السلالات قادرة على تكوين أغشية حيوية، مع كثافة أعلى على سطح البلاستيك. حيث سجلت السلالة (4) أعلى كثافة بكتيرية بلغت 5.83 لوغاريتم وحدة تكوين مستعمرات لكل مليلتر على سطح البلاستيك، و5.63 على سطح الزجاج، في حين كانت السلالة (1) الأضعف بكثافة 4.28 على البلاستيك و3.24 على الزجاج.

أظهر نموذج التقييم الكمي للتعرض فروقات ملحوظة بين طرق التحضير المختلفة. استخدام الماء المغلي خفّض عدد العصويات بنسبة 93–96 % (أي 75–90 وحدة/كغ)، مقارنة بالتسخين بالميكروويف الذي أدى إلى تراكيز تراوحت بين 600–2200 وحدة/كغ، مع تقليل احتمال الخطر من 9.1–26.8 % إلى 2.3 % فقط. وُجد أن فعالية الميكروويف تعتمد على القدرة الكهربائية، حيث تُعد الأجهزة التي تقل عن 800 واط غير آمنة (1800–2200 وحدة/كغ؛ احتمال خطر 25–28 %) بسبب وجود مناطق باردة بنسبة 25 % وضعف تقليل الأبواغ (0.3–0.6 لوغ). أما الأجهزة ذات القدرة العالية (1100 واط أو أكثر) فقد أظهرت تقليلًا جزئيًا، حيث خفضت التلوث بنسبة تقارب 60 % (600–800 وحدة/كغ)، وخفضت احتمال الخطر بنسبة 65 % (8–10 %)، بفضل توزيع حراري أفضل (5 % فقط مناطق باردة) وتحقيق خفض متوسط في عدد الأبواغ (0.7–1.0 لوغ).

رغم ذلك، يظل الماء المغلي الطريقة الأكثر أمانًا وموثوقية لتقليل المخاطر الميكروبية عند تحضير حليب الرضع. تبرز هذه النتائج محدودية الممارسات المنزلية التقليدية، وتؤكد قدرة هذه البكتيريا على البقاء في ظروف الاستخدام الفعلية، مما يستدعي ضرورة تعزيز الوعي العام بأساليب التحضير الآمن لتقليل خطر تلوث تغذية الرضع بالعصويات

المراحل على القائم المخاطر تقييم نموذج ،للتعرض الكمي التقييم ،الحيوية المضادات مقاومة ،الحيوي الغشاء ،المجفف الرضع حليب ،سيريوس باسيلوس :المفتاحية الكلمات