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METHODS FOR DETECTING *Bacillus cereus* CONTAMINATION IN DAIRY PRODUCTS - A FOOD SAFETY PERSPECTIVE

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Abstract

Every microbial environment starts with an initial contamination from microorganisms present in soil, water, and atmospheric dust. The unique characteristics of each environment ultimately dictate which species or types of associations become dominant. Consequently, microorganisms with high resistance to inhibitory or lethal factors often thrive in food products, on various surfaces, and on machinery and equipment. Bacillus cereus serves as a prime example, widely distributed in nature and considered an opportunistic pathogenic species. Research conducted between 2021 and 2023 focused on several categories of milk powder samples collected from sales networks in Călăraşi County, Ilfov County, and Argeş County. These categories included skimmed milk powder with 1% fat, milk powder with 26% fat, organic milk powder with 26% fat, milk powder for children aged 4-5 years, and of milk powder for infants aged 9-12 months. The analysis of these five products involved two categories of methods: confirmation on MYP agar, blood agar, and real-time PCR testing for the amplification and detection of specific Bacillus cereus DNA. Identification of species within the B. cereus group was conducted using specific tests.

Key words*: Bacillus cereus, microbial niche, milk powder, pathogen.*

INTRODUCTION

A well-balanced diet plays a vital role in the normal growth and development of individuals. Adequate nutrition not only fosters improved health and reduced vulnerability to illnesses but also aids cognitive development and intellectual achievement (Posan et al., 2022). Ensuring the microbiological quality of both raw materials, such as milk sourced from animals raised in suitable microclimates, free from mammary gland inflammatory diseases, under optimal hygienic conditions, and the final product, while considering the intended consumer categories, is of utmost importance. The quality of the finished products depends on the raw materials quality, the ingredients used and the processing technologies (Ianitchi et al., 2023).

Milk holds significant importance as a food product because of its intricate chemical makeup, nutritional value, and excellent digestibility. It stands as a product of immense socio-economic significance, contributing to the overall health and well-being of the population. (Mihai et al., 2022).

To prevent the occurrence of consumer illnesses, all food products need to adhere to bacteriological standards. This entails ensuring that they are free from pathogenic germs and that the levels of saprophytic organisms remain within the maximum permissible limits. (Suler et al., 2021).

There are some bacteria which can contaminate milk through various means. *Bacillus cereus* is one of them. *Bacillus cereus* belongs to the *Bacillus* genus, which falls within the *Bacillaceae* family alongside the *Clostridium* genus. It is characterized as Gram-positive, sporogenous, noncapsulogenous, aerobic, facultative anaerobic, and peritrichous, meaning it has flagella distributed around its cell. It thrives optimally at moderate temperatures (although its spores can withstand high temperatures) and neutral pH levels, with the ability to grow in environments with high moisture content.

In the food processing industry, it serves as a crucial microbiological parameter that must be considered to ensure food safety.

The pathogenicity of *Bacillus cereus* relies on a broad spectrum of extracellular metabolites produced primarily during its logarithmic growth phase and stationary phase. Some of these metabolites are recognized as toxins or virulence factors, identified based on their behaviour in vivo and their epidemiological associations.

Among the main toxins produced by *Bacillus cereus* are diarrheagenic enterotoxin, vomiting toxin, haemolysins, phospholipases, and cytolysins. Both the diarrheagenic enterotoxin and the vomiting toxin are responsible for inducing clinical symptoms associated with two types of gastrointestinal illnesses resembling food poisoning: a syndrome typified by gastrointestinal disturbances, with an incubation period of 8-16 hours (Zhou et al., 2008; Lund et al., 2000; Sastalla et al., 2013).

Foods commonly implicated in these cases include dairy products such as milk, minced meat, portioned meat, vegetables, and cereal preparations containing corn or corn starch. (Alecu & Ţogoe, 2008; Organji et al., 2007)

The number estimated to induce the diarrheal type of food poisoning ranges from approx. 5 to 8 log CFU of vegetative cells or spores of *Bacillus cereus* (Sastalla et al., 2013) and an emetic syndrome, often associated with the consumption of rice and rice-based preparations.

The strains involved in this case develop optimally at temperatures between 35-45°C. The other toxins are virulence factors in wound, eye, or systemic infections produced by *B. cereus*. Diarrheic enterotoxin (ED) is an antigenic, thermolabile protein, composed of several subunits and inactivated by proteolytic enzymes. Emetic toxin (ET) is very resistant to heat (90 minutes at 126°C) and to proteolytic enzymes. *B cereus* strains usually produce one, very rarely, both types of exemplified toxins.

The objective of this study was to assess the prevalence of *Bacillus cereus* in various types of milk powder available in the Romanian market. Subsequently, the isolated strains were examined to determine their classification within the group.

MATERIALS AND METHODS

Research conducted between 2021 and 2023 focused on several categories of milk powder samples collected from sales networks in Călăraşi County, Ilfov County, and Argeș County. These categories included skimmed milk powder with 1% fat, milk powder with 26% fat, organic milk powder with 26% fat, milk powder for children aged 4-5 years, and of milk powder for infants aged 9-12 months.

The varieties of milk powder were purchased from stores across Romania, transported under optimal conditions, and subjected to examination before the expiration date. In total, 100 samples were examined. This number included 63 samples of milk powder with 26% fat (32 in 2021 and 3 in 2022), 10 samples of skimmed milk powder with 1% fat (3 in 2021 and 7 in 2022), 10 samples of organic milk powder with 26% fat (6 in 2021 and 4 in 2022), 10 samples of milk powder for infants aged 9-12 months (3 in 2021 and 7 in 2022), and 10 samples of milk powder for children aged 4-5 years (3 in 2021 and 4 in 2022).

The 5 varieties were analysed using 2 categories of methods: confirmation on MYP agar, blood agar, and real-time PCR testing for amplification and detection of *Bacillus cereus*-specific DNA. Identification of species belonging to the *B. cereus* group was performed through specific tests.

The laboratory protocol consisted of the following steps:

- Sample collection was conducted under aseptic conditions; a quantity of 10 g was placed into sterile plastic bags containing 90 ml of diluent. The samples were homogenized for 10 minutes using a STOMACHER 80 and serial dilutions were subsequently performed (ISO, 2004, 2017);
- The samples were inoculated into tubes containing tryptic soy broth, and the tubes were then incubated for 48 hours at 30°C. Subsequently, they were checked for dense growth, typical of *B. cereus*;
- Passages were performed onto Petri dishes by streaking onto the surface of MYP isolation media (mannitol with egg yolk emulsion and polymyxin B) and blood agar, followed by incubation for 24-48 hours at 30°C;

- Subcultures were then conducted on blood agar from colonies that produced lecithinase to confirm *B. cereus*;
- Confirmation of *B. cereus* was achieved by checking for the presence of lecithinase and the absence of mannitol fermentation on MYP medium. *B. cereus* colonies appeared large, with a dark pink colour on a violet background, surrounded by a zone of egg yolk precipitation. On blood agar, *B. cereus* colonies appeared large, surrounded by a distinct halo of haemolyses, which did not intensify upon refrigeration (specific to *B. cereus*) (Alecu & Ţogoe, 2008);
- The enumeration of *Bacillus cereus* (total number of vegetative cells and spores) in the examined products was conducted according to the colony counting technique outlined in ISO 7932:2004 (ISO 7932:2004; Ehling-Schulzet al., 2015).

From each MYP agar plate, 5 lecithinase-positive colonies were selected and transferred onto nutrient agar, from which microscopic preparations were made. *B. cereus* appears as a large, Gram-positive bacillus with rounded ends, arranged in longer or shorter chains, and with ellipsoidal endospores located centrally or sub-terminally, which usually do not deform the vegetative cell.

Following the suspension culture, the following confirmation tests were conducted (Table 1).

Table 1. Confirmation tests for *Bacillus cereus* (ISO 7932:2004)

Confirmation tests	Results
Gram stain	Gram-positive bacillus
Lecithinase (MYP) production	negative
Mannitol fermentation on agar (MYP)	negative
Reduction of nitrates in nitrites	positive
Production of acetyl-methyl-carbinol	positive
Tyrosine decomposition	positive
Growth in the presence of 0.001% lysozyme	positive

These fundamental characteristics are shared with other members of the *B. cereus* group, including the rhizoid strains of *B. cereus* var. *mycoides*, pathogenic bacteria of the crystalliferous insect, *B. thuringiensis*, and those pathogenic to mammals, *B. anthracis*.

Subsequently, the varieties of infant milk powder were subjected to ensure food safety and real-time PCR verification using the *Bacillus cereus* Taqman PCR Detection Kits. The tests were performed according to the manufacturer's instructions.

The enumeration of *Bacillus cereus* (the total number of vegetative cells and spores) in the examined products was conducted according to the colony counting technique outlined in ISO 7932:2004 (ISO, 2004)

For the differentiation of members of the *B. cereus* group, the isolates were then subjected to the following laboratory protocol:

1. Mobility test

Inoculation into BC medium for mobility testing by stabbing with a straight inoculating needle (3 mm suspension of 24-hour culture), incubation for 18-24 hours at 30°C (if bacterial growth diffuses throughout the culture medium, the bacteria are mobile; if growth occurs only along the inoculation line, the bacteria are non-motile). Alternatively, inoculation of a bacterial suspension onto the surface of an agar slope, incubation for 6-8 hours at 30°C, and mobility testing by examining between slide and cover slip (most strains of *B. cereus* and *B. thuringiensis* are mobile, possessing peritrichous flagella. *B. anthracis* and almost all strains of *B. cereus* var. *mycoides* are non-motile).

2. Rhizoid growth

Inoculation onto nutrient agar plates, incubation for 48-72 hours at 30°C. We observed ropelike growth characterized by the production of colonies resembling root structures that can extend several centimetres from the point of inoculation.

B. cereus typically forms rough colonies in a galaxy-like pattern, which should not be confused with the ropelike colonies typical of *B. cereus var. mycoides*. Most strains of this variety are non-motile.

3. Haemolytic activity test

Inoculation of a 24-hour bacterial culture suspension onto a sheep blood agar plate and a tryptic soy agar plate, incubation for 24 hours at 35°C (*B. cereus* cultures typically exhibit strong haemolytic activity, producing a zone of complete beta haemolysis measuring 2-4 mm around the bacterial colony; most strains of

B. thuringiensis and *B. cereus* var. *mycoides* also exhibit beta haemolysis, while those of *B. anthracis* are usually non-haemolytic after a 24-hour incubation).

4. Test for protein toxin crystals

Inoculation of a 24-hour bacterial suspension onto nutrient agar, followed by incubation at 30°C for 24 hours, and then allowing it to stand at room temperature for 2-3 days. Preparation of smears and covering with methanol (for 30 seconds), removal, drying over a flame, and staining with 0.5% basic fuchsin. After gently heating over a flame until vapors appear and allowing to stand for 2 minutes, the process is repeated. After 30 seconds, the dye is removed and the slide is rinsed. After drying (without wiping), examination under immersion microscopy for the presence of endospores and dark tetragonal crystals (diamond-shaped). The crystals are usually smaller than the spores. These crystals are abundant in a 3-4 day old culture of *B. thuringiensis* but may not be detected by staining techniques until after sporangium lysis. Therefore, if free spores cannot be observed, cultures are kept at room temperature for several days and re-examined. Typically, protein toxin crystals are produced by *B. thuringiensis* (ISO, 2004).

RESULTS AND DISCUSSIONS

Various types of powdered milk (a product primarily intended for sensitive consumer categories such as infants and young children) are known to be contaminated with *B. cereus*, particularly its spores. (Organji et al., 2015; ISO, 2007)

The obtained results are presented in Table 2 and Figure 1.

Table 2. The prevalence of *Bacillus cereus* species in the analyzed samples

Sample type	No. of analysed samples	Positive reactions	%	Negative reactions	%
Milk powder 26% G	63	5	7.93	58	92
Skimmed milk powder 1%G	10	2	20	8	80
Organic milk powder 26% G	10	1	10	9	100
Milk powder for children 9-12 months	10	-	-	10	100
Milk powder for children 4-5 years old	7	-	-	7	100
Total	**100**	**8**	**8**	**92**	**92**

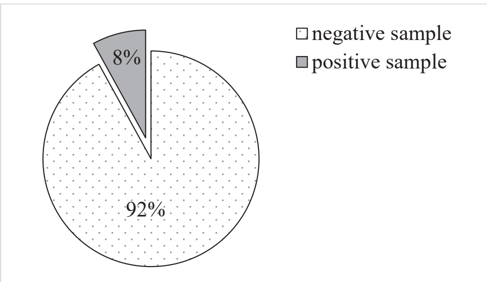

Figure 1. Incidence of Bacillus cereus in the analysed samples

Among the examined products, *Bacillus cereus* was most frequently detected in powdered milk with 26% fat, with a prevalence rate of 7.93%. The bacterial count in these positive samples varied from 1.0 to approximately 3.0 log CFU g-1. For Skimmed Milk Powder with 1% fat, the count of *Bacillus cereus* ranged from 1.0 to about 2.0 log CFU g-1, while for Bio Milk Powder with 26% fat, it was 1.0 log CFU g-1 (Table 3). None of the samples intended for infants and young children tested positive for *Bacillus cereus*.

Table 3. Inventory of *Bacillus cereus* in positive samples

Sample type	***Bacillus cereus* isolates**	***Bacillus cereus* count (log CFU g-1)**
Milk powder, 26% fat	5	1-3
Skimmed milk powder, 1% fat	2	1-2
Organic milk powder, 26% fat	1	1

The identification of species from the *Bacillus cereus* group in the period 2021-2023, for the Milk powder with 26% fat, Skimmed milk powder with 1% fat, and Organic milk powder with 26% fat category is shown in Tables 4-6.

Table 4. Identification of species from the *Bacillus cereus* group in the period 2021-2023, for the category Milk powder with 26% fat

Year	Number of samples	***Bacillus cereus* group**	Number of isolated strains	%
2021	32	*Bacillus cereus* *Bacillus cereus* var. *mycoides*	2 -	6.25 -
2022	31	*Bacillus cereus* *Bacillus cereus* var. *mycoides*	2 1	6,45 3,22
2023	35	*Bacillus cereus* *Bacillus cereus* var. *mycoides*	2 1	5.71 2.85

In 2021, for the Powdered Milk with 26% fat category, 2 strains of *Bacillus cereus* were identified, which represents 6.25% of the total of 32 analysed samples. In 2022, for the Powdered Milk with 26% fat category, 2 strains of *Bacillus cereus* were identified, which represents 6.45% of the total of 31 samples analysed, and one strain of *Bacillus cereus* var. *mycoides* which represents 3.22% of the total of 31 analysed samples. In 2023, 35 samples were analysed, in which 2 samples with *Bacillus cereus* and 1 sample with *Bacillus cereus* var. *mycoides* were identified.

Table 5. Identification of species from the *Bacillus cereus* group in the period 2021-2023, for the category Skimmed milk powder with 1% fat

Year	Number of samples	***Bacillus cereus* group**	Number of isolated strains	%
2021	3	*Bacillus cereus* *Bacillus cereus* var. *mycoides*	1 -	33.3 -
2022	7	*Bacillus cereus* *Bacillus cereus* var. *mycoides*	1 -	14.28 -
2023	7	*Bacillus cereus* *Bacillus cereus* var. *mycoides*	1 -	14.28 -

In 2021, for the Skimmed Milk Powder with 1% fat category, a single strain of *Bacillus cereus* was isolated, which represents 33.3% of the total samples analysed.

In 2022, for the Skimmed milk powder with 1% fat category, a single strain of *Bacillus cereus* was isolated, which represents 14.28% of the total of 7 samples. The same situation occurred in 2023.

Table 6. Identification of species from the *Bacillus cereus* group in the period 2021-2023, for the category Organic milk powder with 26% fat

Year	Number of samples	***Bacillus cereus* group**	Number of isolated strains	%
2021	6	*Bacillus cereus* *Bacillus cereus* var. *mycoides*	1 -	16.66 -
2022	4	*Bacillus cereus* *Bacillus cereus* var. *mycoides*	- -	- -
2023	6	*Bacillus cereus* *Bacillus cereus* var. *mycoides*	1 -	16.66 -

Both in 2021 and 2023, for the Bio Powdered Milk with 26% fat category, a single strain of *Bacillus cereus* was isolated, which represents 16.66% of the total of 6 analysed samples, in 2022 no strains of *B. cereus* were detected.

The incidence of species from the *Bacillus cereus* group from the total strains identified in the samples analysed is shown in Table 7 and Figure 2.

Out of the 131 samples analyzed across all categories of milk powder, 11 samples were found to contain *Bacillus cereus*, and 2 samples contained *Bacillus cereus* var. *mycoides*. Thus, a total of 13 samples, or 9.91% of the samples, were considered contaminated.

Table 7. Frequency of species from the *Bacillus cereus* group in all the analysed samples

Species	Number of samples	Number of isolated strains	%
Bacillus cereus	131	11	8.39
Bacillus cereus var. *mycoides*		2	1.52
Total		**13**	**9.91**

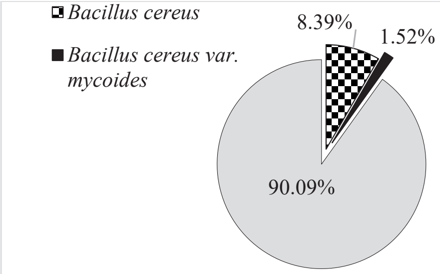

Figure 2. Incidence of species belonging to the *Bacillus cereus* group across all examined samples

CONCLUSIONS

The results of the performed analyses indicate a high level of hygiene and food safety in the processes of obtaining, handling and processing milk powder.

The prevalence of *B. cereus* in powdered milk marketed in Romania demonstrated that there are no significant differences depending on the product, and the contamination level of all analysed products did not exceed 3.0 log. CFU g^{-1}.

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