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Occurrence and antimicrobial susceptibility of *Bacillus cereus,* isolated from RTE foods in Republic of Kosovo

Driton Sylejmani*, Avni Robaj and Mentor Alishani

University of Prishtina, Faculty of Agriculture and Veterinary, Str. Bill Clinton, 10000, Republic of Kosovo
**Corresponding author:* driton.sylejmani@uni-pr.edu

Abstract

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The aim of this study was to investigate the presence of *B. cereus* and antimicrobial susceptibility isolates from ready-to-eat-foods. A total of 150 samples of ready-to-eat-foods were collected from different restaurants in Kosovo. Samples were examined for the isolation and enumeration of *Bacillus cereus*. Serial dilutions of the samples were carried out, and cultured on Mannitol Egg Yolk Polymyxin Agar (MYP), using the spread plate technique. *B. cereus* was confirmed with standard biochemical methods. Out of the 150 RTE food samples examined, 44 (29.3%) samples were positive for *Bacillus cereus*. Based on food safety criteria and our results, out of 44 positive samples with *B. cereus* of the RTE foods examined, 41% were considered good, 36.3% satisfactory, and 13.3% unsatisfactory. Antibiotic susceptibility was performed by the Kirby-Bauer disc diffusion method. Generally, *Bacillus cereus* isolates expressed high resistance to penicillin, ampicillin and amoxicillin/clavulanic acid, while high sensitivity to amikacin, chloramphenicol, gentamicin, followed by erythromycin, ciprofloxacin, tetracycline, and trimethoprim-sulfamethoxazole. Our study indicates the contamination levels of *B. cereus* isolated from RTE foods, and demonstrates the potential hazards of *B. cereus* in RTE foods in Kosovo.

Keywords: occurrence; *Bacillus cereus*; enumeration; antimicrobial susceptibility

Introduction

Bacillus cereus is a Gram positive facultatively anaerobic, spore forming bacilli, that causes foodborne diseases, and is widespread in nature and foods (Marrollo, 2016). The spores of the *B. cereus* group bacteria show heat resistance (Le Marc et al., 2022). *B. cereus* may produce toxins (Schoeni & Wong, 2005), which cause food poisoning usually when insufficiently chilled food is kept for several hours before consumption. Based on the report of European Food Safety Agency (EFSA, 2005), 1–33% of food-borne poisonings are caused by *B. cereus*. The organisms are common in nature, and due to their resistant endospores they may survive different stresses during food production, e.g. drying and heat treatment. *B. cereus* is frequently found in processed products/prepared food items, according to data on the prevalence of *Bacillus* in food and animals in the European Union (EFSA/ECDC, 2018). Ready-to-eat (RTE) foods, are very popular, as they are intended for direct consumption, while on the other side, have been shown to be frequently contaminated with pathogenic bacteria, such as *Bacillus cereus* (Batchoun et al., 2011; Ji-Yeon & Jong-Hyun, 2015; Yang et al., 2017).

In general, foods such as rice, pasta, and dairy products, are common vehicles for *Bacillus cereus*, which is a common cause of food poisoning (Glasset et al., 2016; Jessberger et al., 2020; Rodrigo et al., 2021). During the cultivation, harvesting, and handling process, rice might be contaminated with vegetative cells and endospores of *B. cereus* (Kindle et al., 2019; Rodrigo et al., 2021). Contamination by *Bacillus* *cereus* in food may occur after heat treatment or food production, during food processing, preparation, transport, storage, and distribution (Bennett et al., 2013). Under improper storage conditions of food after cooking, the spores germinate and the vegetative cells multiply (Logan, 2011; Rouzeau-Szynalski et al., 2020). *B. cereus* has been isolated from a variety of foods, particularly RTE foods, such as cooked rice and mixed salad (Park et al., 2009; Yu et al., 2019), dairy products (Seza et al., 2014), and fresh vegetables (Valero et al., 2002). The major threat to food safety is mainly related with the production of spores and biofilm, which is particularly difficult to eliminate (Huang et al., 2020).

While there are various estimates of the number of *B. cereus* cells required to cause illness, there is a general agreement that foods containing <10^3CFU/g are safe for human consumption (Vilas-Boas et al., 2007). It is reported that *B. cereus* should be 10^5 –10^8 CFU/g in order to cause emetic or diarrheal illness (EFSA, 2005). The European Union (EU) announced in 2016, that 413 foodborne outbreaks resulting from *Bacillus* toxins had affected 6657 individuals, 352 of whom were hospitalized, but did not have a fatal consequence (EFSA, 2016). However, certain severe cases of foodborne illness by *B. cereus* leading to mortality or organ failure have sporadically been reported in several countries (Pósfay-Barbe et al., 2008; Naranjo et al., 2011).

Antibiotic treatment is still the main method for treating bacterial infections, including those caused by *B. cereus*, however, the extensive use of antimicrobials has led to the emergence of antibiotic-resistant strains, including those resistant to multiple antibiotics, which can cause routine treatments to fail (Friedman et al., 2016). Thus, determing the antibiotic resistance profile of *Bacillus cereus* important for informing drug selection for treatment regimens. The aim of the present study was to determine the occurrence of *B. cereus* in ready-to-eat foods in Kosovo, level of contamination and the antimicrobial susceptibility.

Material and Methods

A total of 150 RTE food samples were collected randomly from different restaurants in Kosovo. The samples included 30 cooked rice, added with vegetables, 30 cooked rice without vegetables, 30 pudding, 30 pasta, and 30 salads of raw vegetables. The sample consisted of collecting approximately 100 g of RTE product, which was deposited in sterile bags and transported in a cooler (at 4-8ºC) to the laboratory immediately, and analyzed within 24 h of sample collection.

Sample preparation

Using aseptic technique, 25 g of each sample was weighed and homogenized with 225 ml of phosphate-buffered saline (PBS), using stomacher bags. Quantitative detection of *B. cereus* in each sample was performed by using the direct plating method (Tallent et al., 2012). The homogenized samples were serially diluted in 9 ml of PBS until 10^4. The dilutions were spread on Mannitol-Egg-Yolk Polymyxin (MYP, Oxoid, UK)) agar separately in duplicate. Plates were incubated at 30ºC for 24 h. The suspected colonies on MYP agar plates (pink color surrounded by egg yolk-like precipitate, or cloudy halo due to lecithinase production), were identified microscopically and biochemically according to (Rosenquist et al., 2005; Procop et al., 2017).

Antimicrobial susceptibility testing

Antibiotic susceptibility of all *B. cereus* isolates was tested using the Kirby–Bauer disk diffusion method (Bauer et al., 1966; CLSI, 2010; Gao et al., 2018). All isolates were grown in brain heart infusion broth (Oxoid) for 24 h at 30°C, without agitation, followed by spreading on Mueller-Hinton (Merck, Darmstadt, Germany) agar plates. A total of ten different antibiotic discs (Oxoid, UK) containing the antibiotics amikacin (AMK 30 µg), ampicillin (AMP 10 µg), amoxicillin/clavulanic acid (AMC 20/10 µg), chloramphenicol (CHL 30 µg), ciprofloxacin (CIP 5 µg), erythromycin (ERY 15 µg), gentamicin (GEN 10 µg), penicillin G (PEN 10 µg), tetracycline (TET 30 µg) and trimethoprim-sulfamethoxazole (SXT 25 µg), were used for susceptibility testing on Mueller Hinton Agar. After incubating for 18 h at 36 ± 1^0C, the inhibition zones were measured and interpreted referring to the Clinical and Laboratory Standards Institute (CLSI, 2010) for *S. aureus* (Gao et al., 2018).

Results and Discussion

Out of the 150 RTE food samples examined, 44 (29.3%) samples were positive for *Bacillus cereus* (Figure 1). The rate of contamination observed in this study was lower than has been reported by Yu et al. (2020) and Titilayo et al. (2020). Among the examined RTE foods, *B. cereus* has been most frequently found in cooked rice with vegetables and salads of raw vegetables (Table 1). *B. cereus* was detected in 14/30 of cooked rice with vegetables, followed by salads of raw vegetables 10/30, cooked rice without vegetables 9/30, pasta 6/30 and pudding 5/30 (46.7%, 33.3%, 30%, 20% and 16.7%, respectively). According to data in previously published studies, there are differences regarding the detection rate of *B. cereus* in different types of food samples. The occurrence of *B. cereus* in cooked rice in our study was lower than in the previous study, isolated from cooked rice in Mexico (Alvadore-Zárate et al., 2020), which was reported as 89%, and from dishes with boiled rice in Eastern China we value 71.4% (Chen et al., 2022). Our findings (33.3%) for the presence of *B cereus* in salads of raw vegetable samples are higher, compared to the rate of 19.4%, reported by previous studies by Jonkuvienė et al. (2012), and even compared with only fresh vegetables at a rate of 15%, reported by Lin et al. (2023). According to findings of Choma et al. (2000), *B. cereus* was detected in 30% of raw vegetable samples sold in France, almost similar to our results, while Flores-Urbán et al. (2014) reported that *B. cereus* was identified in 57% of the 100 analyzed samples of different vegetables. The frequency of *B. cereus* contamination of pasta found in our research was low compared with the results reported by Pluta et al. (2019), while it is almost close if compared to the level of spaghetti contamination reported by Titilayo et al. (2020).

Fried, boiled rice, pasta and salads with fresh vegetables, are among the main dishes on the kitchen menu in our country, therefore the research of *B cereus* in these foods is of particular importance, because they can also pose a potential risk to public health from food poisoning. According to Oh et al. (2011), *B. cereus* spores can survive boiling and cooking, and pasteurization can even activate some of the spores (heat activation), which can begin to germinate (Tewari & Abdullah, 2015). If raw rice is contaminated with high level of *B. cereus* spores, the spores will germinate and produce toxin after cooking and during storage at inappropriate temperature. Therefore, cooked food should not be stored at room temperature (Montanhini et al., 2013).

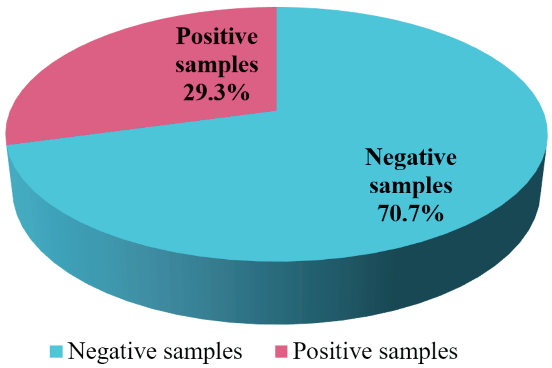

Fig. 1. Occurrence of *Bacillus cereus* in RTE food samples

Table 1. Occurrence of *Bacillus cereus* in RTE food samples

Type of RTE food sample	The No. of examined samples	No. of positive samples, %
Cooked rice with vegetables	30	14 (46.7%)
Cooked rice without vegetables	30	9 (30%)
Pudding	30	5 (16.7%)
Pasta	30	6 (20%)
Salads of raw vegetables	30	10 (33.3%)
Total	150	44 (29.3%)

A number of safety criteria for *B. cereus* in RTE foods have been developed. In present study, the quality of the examined samples regarding the *B. cereus* count was estimated using the guidelines for the microbiological quality of ready-to-eat foods (NSW Food Authority, 2009), which shows four grades of the microbiological quality, related to the *B. cereus* count; $<10^2$ cfu/g is considered good, 10^2-$<10^3$ cfu/g is considered acceptable, 10^3-$<10^4$ cfu/g is considered unsatisfactory, and $\geq 10^4$ cfu/g is considered unacceptable.

The highest contamination was found in samples of cooked rice with vegetables and salad of fresh vegetables. Based on these criteria and results (Table 2), out of 44 positive samples with *B. cereus* of the RTE foods examined, 18 samples (41%) were considered good, while 16 (36.3%) were satisfactory. Our study revealed that the most unsatisfactory samples came from the cooked rice with vegetables 4/30 (13.3%), followed by the salad with fresh vegetables 3/30 (10%), cooked rice without vegetables 2/30 (6.7%) and pasta 1/30 (3.3%). Despite the findings of our study in RTE foods, there was no presence of *B cereus* that reached the unacceptable criterion, the analyzed foods may present concern for public health.

The antimicrobial susceptibility profile of the *B. cereus* isolates is shown in Table 3. Various susceptibility patterns against 10 types of antibiotics were exhibited. All the *B. cereus* isolates (n=58) exhibited 100% resistance to ampicillin and penicillin consistent with published reports, that *B. cereus* isolates from either clinical, food sources and RTE foods were mostly resistant to penicillin and ampicillin (Park et al., 2009; Chon et al., 2012; Merzougui et al., 2014; Zhang et al., 2017; Yu et al., 2020), while 96.55% of isolates were resistant to amoxicillin/clavulanic acid. Penicillin, ampicillin and amoxicillin-clavulanic acid are β-lactam antibiotics and resistance to these drugs, is as a result of synthesis of β-lactamase by *B. cereus* (Park et al., 2009; Bottone, 2010). In the present study, 98.3%, 96.6.0%, 94.8%, 82.8%, 75.9% and 72.4% of the isolates showed susceptibility to chloramphenicol, amikacin, gentamicin, erythromycin, ciprofloxacin, and tetracycline, respectively. Susceptibility to gentamicin in our study was almost similar with the works of other authors (Chaves et al., 2011; Chon et al., 2012; Organji et al., 2015), who reported 100% sensitivity to gentamicin by *B. cereus* strains recovered from food. Also, similar findings in relation to sensitivity to gentamicin (96.47%) and to chloramphenicol (98.3%), were reported and by Yu et al. (2020)

Table 2. Microbial quality* of the examined samples based on their *B. cereus* count

The examined RTE food samples	Positive samples with *B. cereus*	Good $<10^2$	Satisfactory (10^2-$<10^3$cfu/g)	Unsatisfactory (10^3<10^4 cfu/g)	Potentially hazardous ($\geq10^4$ cfu/g)
Cooked rice with vegetables (N = 30)	14/30	8	2	4	–
Cooked rice without vegetables (N = 30)	9/30	3	4	2	–
Puding (N = 30)	5/30	4	1	–	–
Pasta (N = 30)	6/30	2	3	1	–
Salads of raw vegetables (N = 30)	10/30	1	6	3	–
Total (150)	44/150 (29.3%)	18 (41%)	16 (36.3%)	10 (22.7%)	–

*According to the public health guidelines (NSW Food Authority, 2009)

Table 3. Antibiotic susceptibility of *Bacillus cereus* isolates from ready-to-eat foods

No.	Antimicrobial agents	*Bacillus cereus* isolates (n = 58)		
		Resistant	Intermediate	Susceptible
1	Erythromycin ERY (15µg)	4 (6.9%0	6 (10.3%)	48 (82.8%)
2	Ciprofloxacin CIP (5µg)	2 (3.4%)	12 (20.7%)	44 (75.9%)
3	Amikacin AMK (30µg)	0 (0.00%)	2 (3.4%)	56 (96.6%)
4	Penicillin PEN (10µg)	58 (100.00%)	0 (0.00%)	0 (0.00%)
5	Ampicillin AMP (10µg)	58 (100.00%)	0 (0.00%)	0 (0.00%)
6	Gentamicin GEN (10µg)	2 (3.4%)	1 (1.7%)	55 (94.8%)
7	Amoxicillin/clavulanic acid AMC (20/10µg)	56 (96.55%)	0 (0.00%)	2 (3.45%)
8	Chloramphenicol CHL (30µg)	0 (0.00%)	1 (1.72%)	57 (98.30%)
9	Tetracycline TET (30µg)	6 (10.35%)	10 (17.24%)	42 (72.41%)
10	Trimethoprim-sulfamethoxazole SXT (25µg)	16 (27.6%)	6 (10.3%)	36 (62.1%)

and Chen et al. (2022). Most of the isolates were sensitive to tetracycline (72.4%) and our result was in agreement with work of Titilayo et al. (2020) but higher than that reported by Chon et al. (2012). Compared to our findings, Chen et al. (2022) reported that 95.6% of *Bacillus cereus* isolates were sensitive to tetracycline, while Ankolekar et al. (2009) reported that 98% of their *B. cereus* strains were resistant to tetracycline.

Sensitivity to ciprofloxacin was 75.9%, and the result of our study was similar by the work of Titilayo et al. (2020) and Yu et al. (2020), who reported that 70.1% and 78.80% of isolates were sensitive to the drug, respectively. Luna et al. (2007) and Chon et al. (2012) had reported that *B. cereus* isolates were 100% sensitive to ciprofloxacin. 82.8% of the *Bacillus cereus* isolates, displayed susceptibility to erythromycin, and our findings were in line with the findings of other authors (Park et al., 2009; Titilayo et al., 2020). The higher level of sensitivity to erythromycin compared to our result was reported by previous studies (Chon et al., 2012; Seza et al., 2014; Chen et al., 2022).

The isolates demonstrated sensitivity (62.1%) against trimethoprim/sulfamethoxazole, and it was in contradiction with the finding of Chen et al. (2022), who reported that *B. cereus* isolates from ready-to-eat rice products in Eastern China were 84.6% resistance to trimethoprim/sulfamethoxazole.

Conclusion

Although it was a first report, the results of our study shows that *B. cereus* can occur in RTE foods in restaurants in our country. The occurrence of *B. cereus* in RTE foods in Kosovo, indicates possible high risk of foodborne infections, that could occur as a result of the consumption of these products. Therefore, it is recommended to adopt measures to minimize the contamination with *B. cereus* from the very outset, even before processing in order to avoid the growth of the organism to dangerous levels. RTE foods should be consumed immediately as a precaution, if not, they should be stored at a temperature below 7°C or above 63°C for two hours after cooking, to prevent spore germination. Prompt cooling or heating the food for storage can minimise the potential of the spore to germinate during the holding time.

Conflict of interest

Authors declare no conflict of interest.

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