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Original Article

# Evaluation of multidrug-resistant *Bacillus* strains causing public health risks in powdered infant milk formulas

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ABSTRACT

*Background:* Antibiotic-resistant bacteria are one of the major global health issues that can affect humans, animals, and the environment. Antibiotic-resistant bacteria have emerged as opportunistic pathogenic bacteria that are frequently isolated from both clinical patients and healthy individuals. The aim of this study was to characterize the antibiotic-resistant bacteria isolated from powdered infant formulas marketed in Riyadh, Saudi Arabia.

*Methods:* Infant powdered milk formulas were purchased from different pharmacies located within Riyadh, and ten products of powdered milk formulas designed for children of various ages were then transferred to the laboratory in the Department of Botany and Microbiology at King Saud University, Riyadh. Isolation and purification of *Bacillus* species were both performed according to standard protocols. The identification test was performed using the automated Vitek 2 system (BioMerieux, France), and antibiotic sensitivity tests were performed using the disk-diffusion method incorporating standard antibiotic disks foramikacin (30 μg/disk), gentamicin (10 μg/disk), imipenem (10 μg/disk), moxifloxacin (5 μg/disk), cefoperazone (75 μg/disk), cefpodoxime (10 μg/disk), ceftazidime (30 μg/disk), and cefepime (30 μg/disk). Statistical analysis was performed using Ward's method to obtain antibiotic resistance of the isolates.

*Results:* The results obtained from the milk samples indicated that all isolates were sensitive to amikacin, gentamicin, and moxifloxacin. A group of isolates obtained from milk was resistant to cefoperazone by 6.49%, cefpodoxime by 25.9%, ceftazidime by 14.28%, and cefepime by 19.48%.

*Conclusions:* Based on these findings, we concluded that the powdered infant formula marketed in Riyadh City may act as a source of bacterial isolates that are resistant to several standard antibiotics.



## Introduction

Infant formulas are the primary source of nutrition for many infants and are fed to them during a sensitive period of development, likely inducing short- and long-term effects on infant health. In terms of food safety, infants and children are considered as a high-risk group of individuals, as their immune systems may not be fully developed. Infants are at a high hazard of gain microbial infections caused by pathogenic antibiotic-resistant strains [1]. Thus, it is reasonable that the safety of products used for infants should be scrutinized more than that of foods designed for adults, who have developed several mechanisms to cope with nutrient inadequacies and excesses [2]. The fact that antimicrobial resistance presents a significant and worldwide clinical problem attests to the success and speed of bacteria to develop resistance to standard antibiotics [3]. Mechanisms of antibiotic resistance in bacteria vary and include target protection, target substitution, detoxification, and inhibition of intracellular antibiotic accumulation [4]. Bacteria possess three mechanisms by which DNA may be transferred from one cell to another, including transformation, transduction, and conjugation [5,6]. The development of resistance to multiple antibiotics provides a particularly striking example of how bacterial strains are able to resist different categories of standard antibiotics [7].

Not all resistant bacteria cause diseases and, therefore, in many cases they do not harm their carriers. Under suitable conditions, however, such bacterial can either induce the onset of a disease or transfer the gene that provides antibiotic resistance to another bacterial pathogen [8]. It has been reported that the infections caused by resistant bacteria can be fatal to infants, and globally, more than

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200.000 newborns die annually of infections caused by antibiotic-resistant bacteria [9]. In infants, antibiotic resistance of gut bacteria can be natural or can be acquired by genetic alterations within the microbial genome or by horizontal transfer of resistance genes located on various types of mobile DNA elements [10,11].

The U.S. Food and Drug Administration (FDA, 2018) defined infant formula as "a food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk" [12].

Although many studies discuss the microbial and chemical safety of powdered infant formula milk, there are few investigations regarding antibiotic-resistant *Bacillus* spp. that may be spread through this very important product. For example, an opportunistic pathogenic *Enterobacter sakazakii*, *Pantoea* (*Enterobacter*) *agglomerans*, and *Bacillus cereus* strains have all been detected in powdered infant formula milk where their antibiotic resistance patterns have been studied [13,14,15,16]. The recent investigation reported that *C. sakazakii* strains are the most foodborne bacteria that could be isolated from powdered infant formula throughout the world [17].

A local research group in Iraq isolated and studied the susceptibility patterns of *Bacillus* spp. and non-*Bacillus* spp. obtained from powdered infant formula (PIF) and dried infant cereals [18]. *Bacillus* spp. are widely applied as food microbial supplements. In general, scientific information concerning antibiotic-resistant *Bacillus* strains is limited, and concerns regarding the ability of these strains to transfer antibiotic resistance genes are increasing [19].

The aim of our present study was, therefore, to evaluate the prevalence of antibiotic-resistant *Bacillus* spp. in powdered infant formula milk marketed in Riyadh city, Saudi Arabia.

## Material and methods

### *Specimens collection*

Sample collection was performed over the course of two academic semesters (2017–2018). Infant powder milk samples were collected from pharmacies in Riyadh City. Ten powdered milk products were selected from the most consumed products based on advice from pharmacies. All products used in the study are produced by international companies, and all samples were taken based on the various age ranges of children. Milk samples for three different age ranges were tested, including range 1, which was specific to the period of birth (from the first day of birth to six months of the child's age), range2, which included the end of the first six months until the child finishes the first year, and range3 that begins at the end of the first year until the child reaches three years of age.

All samples were coded (C1 to C10) in an effort to not identify the companies that manufactured the products used for this study, as this study did not obtain permission from government agencies to publish results that included the name of these companies. The commercial infant formula cans were transported to the Microbiology Laboratory, Department Botany and Microbiology, College of Science, King Saud University and stored at 4 °C.

### *Isolation of microorganisms*

For the infant formula samples, 10 g of each were dissolved into 90 mL of sterile normal saline solution (0.89% NaCl), and then ten-fold serial dilutions were performed to obtain $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, and $10^{-6}$ dilutions. Of these dilutions, 1 mL of each was cultivated on 20 mL of sterile nutrient agar (Oxoid, UK) prepared in a sterile plastic petri dish, and then incubation was performed at 37 °C for mesophilic bacteria and 50 °C for thermophilic and thermoduric bacteria. Bacterial growth was observed daily, and every individual colony formed on the surface of the nutrient medium was picked and sub-cultivated on new medium to obtain a single culture. To obtain pure bacterial cultures, the sub-cultivation on the nutrient medium was performed at least three times, and the purification then was investigated by examining macroscopic and microscopic characteristics. The macroscopic and microscopic characteristics were studied using mannitol salt agar (Oxoid, UK) and light microscopy (Motic, Taiwan). The bacterial cultures that possessed similar macroscopic and microscopic features were considered as the same bacterial isolates. Seventy-five single bacterial cultures purified from the previous stage were preserved in a sterile skim milk medium containing glycerol solution (30%) at −80 °C until needed.

### *Identification*

All bacterial isolates were Gram-positive aerobic endospore-forming bacteria, and for that reason, the identification was performed using the automated Vitek 2 system (BioMerieux, Marcy-1'Eotile, France) incorporating a Vitek BCL card that can reliably identify *Bacillus* spp. [20]. The optical density of bacterial suspensions in sterile saline solution (0.89% NaCl) was adjusted to a McFarland score of 2 ± 0.2 using Vitek 2 DensiChek (bioMerieux). The BCL card was filled automatically, sealed, and incubated at 35 °C for 14 h with readings every 15 min.

### *Antibacterial drug susceptibility tests*

The susceptibility testing for B. licheniformis, B. cereus, B. thurinigiensis, B. mycoides, B. alicyclobacillus, B. acidotesreslris, B. acidocadasius, B. subtilis, B. amyloliguefaciens,and B. megaterium was performed using an assay determined by the National Committee for Clinical Laboratory Standards (NCCLS) [21]. The test was performed using standard antibiotic disks (Himedia, India) and Mueller–Hinton medium plates (Oxoid, UK). The standard antibiotic disks used in this work consisted of amikacin (30 μg), gentamicin (10 μg), imipenem (10 μg), moxifloxacin (5 μg), cefoperazone (75 μg), cefpodoxime (10 μg), ceftazidime (30 μg), and cefepime (30 μg). The bacterial isolates were sub-cultivated at 35 °C for 24 h on nutrient broth at least three times prior to the susceptibility testing. The bacterial suspension was prepared from a single colony to obtain an optical density (O.D) of 0.65 at 670 nm using a sterile saline solution (0.89% NaCl). Mueller-Hinton agar was prepared according to the manufacturer's instructions and poured onto sterile plastic plates. Bacterial suspensions (100 μL) were spread onto the surface of Mueller-Hinton agar, and then the standard antibiotic disks were gently placed onto the surface of the medium. The plates were incubated at 8 °C for 30 minutes and then incubated aerobically at 35 °C for 18 h.The diameter of inhibition zone was then manually measured. *E. coli* ATCC 25922 and Staphylococcus aureus ATCC 25923 were used as controls. Susceptibility breakpoints for the standard antibiotics used in this assay are listed in Table 1.

### *Statistical analysis*

The Hierarchical cluster of data recorded during the course of this investigation were statistically analyzed using Ward's method in IBM® SPSS® statistical version 25 [22].

## Results and discussion

### *Isolation and identification*

In the present study, the macroscopic and microscopic characteristics of bacterial isolates indicated that all isolates obtained

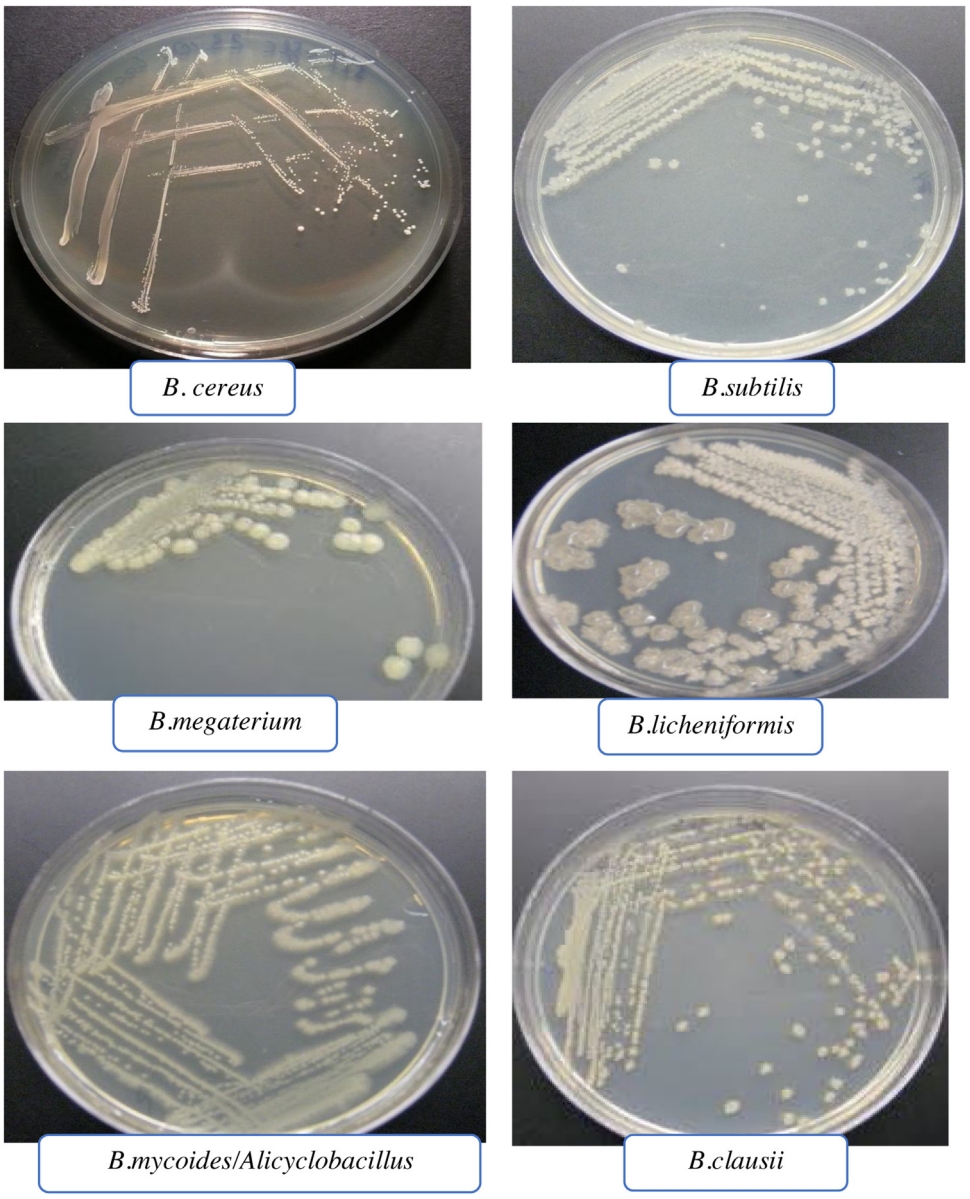


**Fig. 1.** Macroscopic characteristics of the bacteria isolated from powder infant formula on nutrient agar incubated at 35 °C for 24 h.

**Table 1**
Susceptibility breakpoints (mm diameter) for the standard antibiotics used in this study.

| Antibiotic (μg/disk) | Susceptible | Intermediate | Resistant |
|---|---|---|---|
| Amikacin (30 μg) | ≥17 | 15 to 16 | ≤14 |
| Gentamin (10 μg) | ≥15 | 13 to 14 | ≤12 |
| Imipenem (10 μg) | ≥16 | 14 to 15 | ≤13 |
| Moxifloxacin (5 μg) | ≥24 | 21 to 23 | ≤20 |
| Cefoperazone (75 μg) | ≥21 | 16 to 20 | ≤15 |
| Cefpodoxime (10 μg) | ≥21 | 18 to 20 | ≤17 |
| Ceftazidime (30 μg) | ≥18 | 15 to 17 | ≤14 |
| Cefepime (30 μg) | ≥18 | 15to 17 | ≤14 |

*The breakpoints used for the above antibiotics were for *Staphylococcus* spp. according to zone diameters of antimicrobial agents in accordance with CLSI guidelines 2011. It has been reported that breakpoints of antibiotics for *Staphylococcus* spp. can be used to test *Bacillus spp (non-Bacillus anthracis)* [34].

from powdered infant formula were *Bacillus* spp. and *Alicyclobacillus* sp. Fig. 1 shows the bacterial isolates and their features on nutrient agar. Of the bacterial isolates obtained from powdered infant formula milk, 77 have been identified using automated VITEK 2 system. These isolates included *B. subtilis, B. cereus, B. megaterium, B. clausii, B. licheniformis*, and *Alicyclobacillus* sp. The results indicated that out of 77 bacterial isolates, 22.07% were isolated from C6, followed by 20.77% from C2, 12.98% from C3, 11.68% from C7, 9.09% from C8 and C10, 5.19% from C5 and C9, 2.59% from C1, and 1.29% from C4 (Fig. 2).

In this study, thermophilic rod-shaped endospore forming bacteria have been isolated from all tested powdered infant formula products. Standardized organization for the Cooperation Council for the Arab States of the Gulf (GSO/FDA 1016/2014) acknowledged the possibility of isolation of *Bacillus* spp. from powdered infant formula. Given this, the isolation of related spore-forming bacteria in this investigation is not surprising. The frequency of isolation of *Bacillus* species from powdered infant formula varies by product according to quality manufacturing practices. Several studies have been performed that isolated and identified *Bacillus* spp. from several infant foods, including powder milk [23,24,25].

The vast majority of bacteria isolates (80.51%) were identified as *B. licheniformis*, followed by *B. subtilis* (10.3%), *B. cereus* (5.19%), *B.*

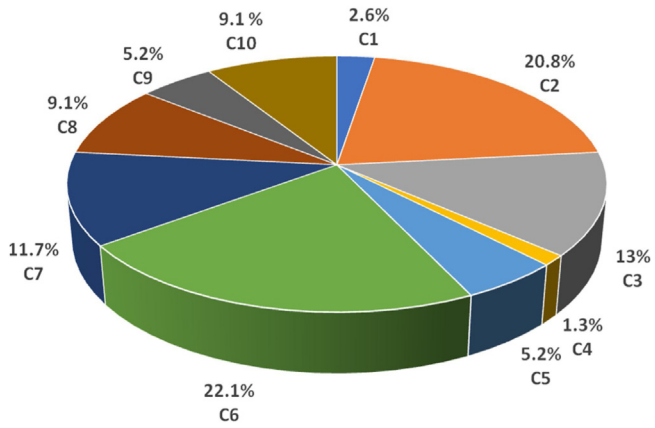


**Fig. 2.** Percentage (%) of the bacterial isolates derived from the powder infant formula marketed in Riyadh. (C1 to C10 are the products studied in this work). (N = 77).

*megaterium* (1.2 %), *B. clausii* (1.2%), and *Alicayclobacillus* sp. (1.2%), according to the results obtained from this study.

*B. lichenifromis* is one of subtilis group bacteria that was identified as the major isolate in this work. *B. lichenifromis* is associated with numerous food spoilage cases and a number of food-borne infections. In general, *B. licheniformis* is one of the most common bacterial contaminants in dairy products [26,27].

The data in Table 2 indicate that *B. licheniformis* was isolated from all tested products, with the exception of product C4. Of the *B. licheniformis* isolates, 24.1% were isolated from product C6, while 19.3% were isolated from product C2, 14.5% from product C7, 12.9% from product C3, 11.2% from product C8, 8.06% from product C10, and 3.2% from product C3. All isolates of *B. subtilis* were isolated from products C1, C2, C3 C6, and C10, and they were not all isolated from products C4, C5, C7, C8, and C9. For the *B. cereus* isolates, 50% were isolated from product C2, while no isolates were obtained from products C1, C3, C5, C6, C7, C8, and C10. Product C6 was the only source of *B. megaterium* and *B. clausii*, and product C5 was the only source of *Alicayclobacillus sp.* In this study, one isolate of *B. cereus* was isolated from product C4. This product was the only source of *B. cereus*, and it completely was free of all other bacterial isolates.

The microbial biodiversity of the powdered infant formula products was limited to few species of *Bacillus* and *Alicayclobacillus sp.* This is due to the nature of the production processes for those products where they are subjected to thermal treatments during spray drying that allows only some thermophilic endospore-forming bacteria to survive. Isolation of mesophilic bacteria at 37 °C) from the powdered infant formula milk products is indicative of post-production contamination or bad manufacturing practices.

It should be noted powdered infant formula is not considered a sterile product. There are many scientific reports that confirm that through the use of current industrial techniques, it is not possible to remove all microbes from powdered infant formula. In some investigations, certain microbes were identified in powdered infant formula in unopened and opened containers of powdered infant formula [27,28,29].

*Antibiotic susceptibility pattern of bacterial isolates*

In the present study, the susceptibility testing of 77 bacterial strains of *B. subtilis, B. cereus, B. megaterium, B. clausii, B. licheniformis, and Alicyclobacillus* isolated from powdered infant formula was performed using eight of the standard antibiotics. The results arranged in Table 3 indicate that 8.1%, 21%, 14.5%, and 19.4% of *B. licheniformis* strains were resistant to cefoperazon cefpodoxim, ceftazidime, and cefepime, respectively. Of the isolates of *B. subtilis*, 12.5%were resistant to cefpodoxim and to cefepime, and 50%, 25%, and 12.5% of *B. cereus* isolateswere resistant to cefpodoxim, ceftazidime, and cefepime, respectively. The only isolate of *B. megaterium* was resistant to cefpodoxime and cefepime, and the only isolate of *B. clausii* was resistant to ceftazidime. The *Alicyclobacillus* sp. Isolate was resistant to ceftazidime and cefepime.

In general, all the bacterial isolates evaluated in the present work were susceptible to amikacin, gentamicin, imipenem, and moxifloxacin, while 6.5%, 22.1 %, 5.6%, and 19.5% of the isolates were resistant to cefoperazon, cefpodoxime, ceftazidime, and cefepime, respectively.

Our data indicate that there are six categories of antibiotic-resistant bacteria that can be isolated from powdered infant formula. Specifically, the first is the cefpodoxime-resistant strains, the second is the ceftazidime-resistant strains, the third is the cefepime-resistant strains, the fourth is the cefoperazon-resistant strains, the fifth is the cefpodoxim- and cefepime-resistant strains, and the sixth is the cefpodoxim-, ceftazidime-, and cefepime-resistant strains.

The National Committee for Clinical Laboratory Standards recommended using cefpodoxime to detect for extended-spectrum β-lactamase (ESBL) production [30]. Given this, the present results confirm that extended-spectrum beta-lactamase (ESBL)-producing strains are prevalent in powdered infant formula. Of note, β-Lactamase gene expression has been determined in some *Bacillus*strains [31], and this finding is in agreement with our present study. Cefepime-resistant *Bacillus* spp. isolated in the present study (cefepime is one of β-lactam antibiotics) have been isolated from humans [32] and dairy products [33]. In the present investigation, several strains that possess resistance to multi-antibiotic treatments have been isolated, and these results are in agreement with numerous studies that reported the occurrence of *Bacillus* spp. that are resistant to multi-antibiotic treatments in several sources, including food [34,35]. These data indicated an incidence of *Bacillus* strains resistance to the third and fourth generation of cephalosporins (ceftazidime and cefepime, respectively).

Our results show that 17 of the 54 cefoperazone-susceptible bacterial isolates were isolated from product C6, and 15 of these isolates were identified as *B. licheniformis* strains. Additionally, 10 of the 54 cefoperazone- susceptible bacterial isolates were isolated from product C2, and six of these were identified as *B. licheniformis* strains. In product C3, eight isolates were identified as cefoperazone- susceptible, and seven of these were identified as cefoperazone- susceptible *B. licheniformis* strains. From all products, seven of the 77 isolates were identified as cefoperazone-resistant bacteria, and four of these were identified as *B. licheniformis* that were isolated from product C2.

With regard tocefopodoxime-susceptible bacterial strains isolated from several products examined in this study, 34 bacterial siolates were cefopodoxime-susceptible strains isolated from all tested products; 9 of these 34 strains were isolated from product C6, and 8 strains were isolated from product C2. In product C6, all cefopodoxime-susceptible strains were identified as *B. licheniformis*, while in product C2, six isolates were identified as cefopodoxime-susceptible *B. licheniformis* strains. Additionally, our data indicated that cefopodoxime-susceptible *B. licheniformis* strains were isolated from product C7 and product C8. Of these *Bacillus* strains, 19 of 77 isolates were identified as cefopodoxime-resistant.

Ceftazidime-susceptible isolates were identified in all tested products, with the exception of product C4. Additionally, ceftazidime-resistant isolates (11 out of 77 isolates) have been isolated from all products, except products C1, C2, C7, and C9. *B. licheniformis* strains accounted for 8 of the ceftazidime-resistant isolates (11 total isolates). Cefepime-resistant bacterial isolates were isolated from products C1, C2, C5, C6, C7, C8, and C10,

**Table 2**
Total number of bacterial isolates derived from the powder infant formula products.

| Bacterial Isolates | Total no. of isolates | (No.) % of bacterial isolates in each product | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 |
| *BI*[a] | 62 | (1) 1.6% | (12) 19.3% | (8) 12.9% | (0) | (2) 3.2% | (15) 24.1% | (9) 14.5% | (7) 11.2% | (3) 4.83% | (5) 8.06% |
| *BS* | 8 | (1) 12.5% | (2) 25% | (2) 25% | (0) | (0) | (1) 12.5% | (0) | (0) | (0) | (2) 25% |
| *BC* | 4 | (0) | (2) 50 | (0) | (1) 25% | (0) | (0) | (0) | (0) | (1) 25% | (0) |
| *BM* | 1 | (0) | (0) | (0) | (0) | (0) | (1) 100% | (0) | (0) | (0) | (0) |
| *BI* | 1 | (0) | (0) | (0) | (0) | (0) | (1) 100% | (0) | (0) | (0) | (0) |
| *Al* | 1 | (0) | (0) | (0) | (0) | (1) 100% | (0) | (0) | (0) | (0) | (0) |

[a] *B. licheniformis (BL), B. subtilis (BS), B. cereus (BC), Bacillus megaterium (BM), Bacillus clausii (BI), and Alicyclobacillus sp. (AL).* C1-C10 are the products.

**Table 3**
Antibiotic susceptibility pattern of the different bacteria isolated from powder infant formula marketed in Riyadh.

| B1(N = 62)[a] | | B2(N = 8) | B3(N = 4) | B4(N = 1) | B5(N = 1) | B6(N = 1) | Total (N = 77) |
|---|---|---|---|---|---|---|---|
| A1[b] | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) | S (100%)[c] |
| | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) |
| | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) |
| A2 | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) |
| | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) |
| | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) |
| A3 | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) |
| | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) |
| | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) |
| A4 | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) | S (100%) |
| | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) |
| | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) |
| A5 | S (58.1%) | S (87.5%) | S (75%) | S (100%) | S (100%) | S (100%) | S (63.6%) |
| | I (33.8%) | I (12.5%) | I (25%) | I (0%) | I (0%) | I (0%) | I (29.9%) |
| | R (8.1%) | R (0%) | R (0%) | R (0%) | R (0%) | R (0%) | R (6.5%) |
| A6 | S (51.6%) | S (62.5%) | S (50%) | S (0%) | S (100%) | S (0%) | S (51.9%) |
| | I (27.4%) | I (25%) | I (0%) | I (0%) | I (0%) | I (100%) | I (26%) |
| | R(21 %) | R(12.5%) | R (50%) | R (100%) | R (0%) | R (0%) | R (22.1) |
| A7 | S (71%) | S (100%) | S (75%) | S (100%) | S (0%) | S (0%) | S (70.1%) |
| | I (17.7%) | I (0%) | I (0%) | I (0%) | I (0%) | I (0%) | I (14.3%) |
| | R (14.5%) | R (0%) | R (25%) | R (0%) | R (100%) | R (100%) | R (15.6%) |
| A8 | S (61.3%) | S (50%) | S (75%) | S (0%) | S (100%) | S (0%) | S (57.1%) |
| | I (19.4%) | I (37.5%) | I (12.5%) | I (0%) | I (0%) | I (0%) | I (23.4%) |
| | R(19.4%) | R (12.5%) | R (12.5%) | R (100%) | R (0%) | R (100%) | R (19.5%) |

[a] B1=*B. licheniformis*, B2=*B. subtilis*, B3=*B. cereus*, B4=*B. megaterium*, B5=*B. clausii,* and B6= *Alicyclobacillus* sp.
[b] A1=Amikacin, A2=Gentamicin, A3=Imipenem, A4=Moxifloxacin, A5=Cefoperazone, A6=Cefpodoxime, A7=Ceftazidime and A8=Cefepime.
[c] S = susceptible, I = intermediate and R = resistant.

and these strains were not isolated from any of the other tested products. Our results indicate that 11 of the77 isolates were cefepime-resistant strains, and nine of these strains were identified as *B. licheniformis* strains. *B. licheniformis* accounts for 4 of the cefepime-resistant strains isolated from product C2.

In the present study, we tested a number of different powdered infant formula milk products, and we found that they all contained varied amounts of *Bacillus* strains that were resistant to antibiotics. Our data confirmed the presence of antibiotic-resistant *B. licheniformis* strains as the predominant bacterial strain in all tested products. The importance of this work is highlighted by previous studies that have confirmed that the *Bacillus* spp. possess the ability to transfer of antibiotic resistance genes. Several strains of *Bacillus* are widely applied in food science as probiotics for humans and animals. Although the use using of *Bacillus* strains in food is increasing, there is limited data concerning their resistance to standard antibiotics and their ability to transfer antibiotic resistance genes [19,36]. There are several bacterial strains such *Acinetobacter* strains have been detected in milk powder, these strains have no ability to resist almost standard antibiotics such as ciprofloxacin, cefepime, and meropenem, and tobramycin [37].

A dendrogram (Fig. 3) derived from Ward Linkage and Euclidean Distance analyses indicates that all bacterial isolates from this study can be grouped into 5 clusters based on their susceptibility to the eight standard antibiotics used in this study. The isolates in the first group were sensitive to all of the antibiotics, with the exception of ceftazidime and cefepime. In the second group, the isolates were sensitive to all of the antibiotics, while in the third group, the isolates were sensitive to all the antibiotics, with the exception of cefepime. The isolates of the fourth group were sensitive to all the antibiotics, with the exception of cefpodoxime to which theywere resistant. In the fifth group, the isolates were sensitive to imipenem, gentamicin, amikacin, and cefoperazone, and they were resistant to cefpodoxime, ceftazidime, and cefepime.

## Conclusions

The data derived from this study indicated that the powdered infant formula marketed in Riyadh may serve as a source of numerous *Bacillus* species; 80% of the isolates were identified as *B. Licheniformis*. These bacteria are considered as one of the most important contaminants in powdered infant formula.

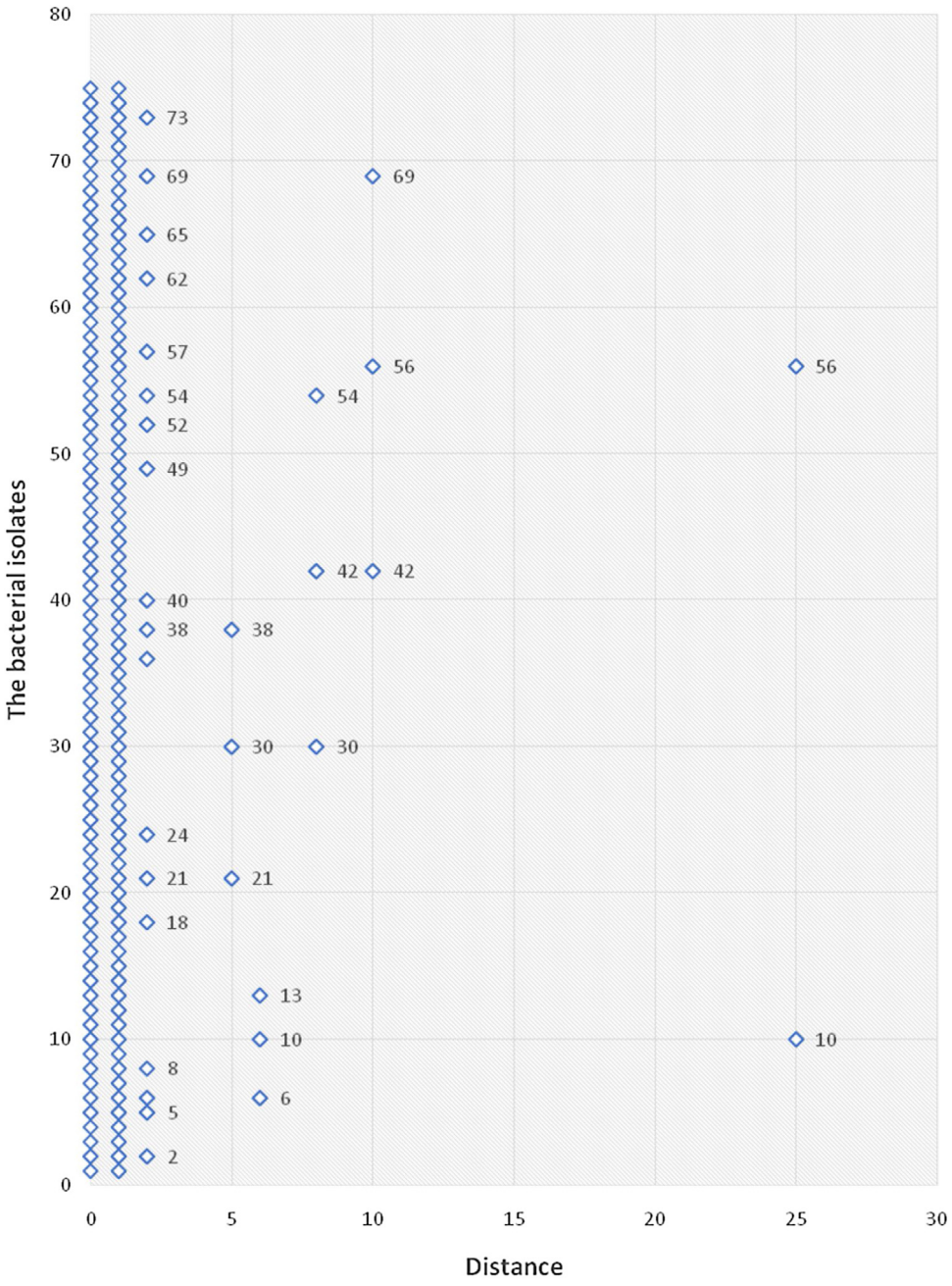


**Fig. 3.** Dendrogram of bacterial isolates with Ward Linkage and Euclidean distance using SPSS. (N = 75).

Of the isolated *B. licheniformis* strains, 24.1% and 19.3% were found in products C6 and C2, respectively. All tested powdered infant formula products contained *Bacillus* spp. with variations in the frequency of isolation. Additionally, we concluded that powdered infant formula may serve as an important source of *Bacillus* spp. resistant to mono-antibiotics and multi-antibiotics. The antibiotic-resistant strains from powdered infant formula were identified as cefpodoxime-resistant strains; ceftazidime-resistant strains; cefepime-resistant strains; cefoperazon-resistant strains; cefpodoxim and cefepime-resistant strains; and cefpodoxim, ceftazidime, and cefepime-resistant strains. Extended-spectrum beta-lactamase (ESBL)-producing strains and *Bacillus* strains that possess the ability to resist third and fourth generations of cephalosporins (ceftazidime and cefepime, respectively) have also been identified. The results of this study highlight the need to apply susceptibility testing using standard antibiotics to routine microbiological analyses of powdered infant formulas in Saudi Arabia.

## Competing interests

None declared.

## Ethical approval

Not required.

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## References


[1] Pärnänen K, Hultman J, Satokari R, Rautava S, Lamendella R, Wright J, et al. Formula alters preterm infant gut microbiota and increases its antibiotic resistance load. bioRxiv 2019:1–22, http://dx.doi.org/10.1101/782441v1. Article ID 782441.

[2] Diego P. Microbiological assessment of some powdered infant formulas: from quality to antibiotic resistance evaluation. J Food Process Technol 2013;4(12):284.

[3] Bennett P. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. Br J Pharmacol 2008;153(S1).

[4] Benveniste R, Davies J. Mechanisms of antibiotic resistance in bacteria. Annu Rev Biochem 1973;42:471–506.

[5] O'connel MP. Genetic transfer in prokaryotes: transformation, transduction, and conjugation. In: Puhler A, Timmis KN, editors. Advanced molecular genetics. Berlin Heidelberg: Springer-Velage; 2012. p. 2–12.

[6] Percival SL, Bowler P, Russell D. Bacterial resistance to silver in wound care. J Hosp Infect 2005;60:1–7.

[7] David MZ, Robert SD. Community-associated methicillin-resistant *Staphylococcusaureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev 2010;23(3):616–87.

[8] Byington CL, Enriquez FR, Hoff C, Tuohy R, Taggart EW, Hillyard DR, Carroll KC, Christenson JC. Serious bacterial infections in febrile infants 1 to 90 days old with and without viral infections. Pediatrics 2004;113(6):1662–6.

[9] Groer MW, Gregory KE, Louis-Jacques A, Thibeau S, Walker WA. The very low birth weight infant microbiome and childhood health. Birth Defects Research Part C: Embryo Today. Reviews 2015;105(4):252–64.

[10] Normark BH, Normark S. Evolution and spread of antibiotic resistance. J Intern Med 2002;252:91–106.

[11] Courvalin P. Transfer of antibiotic resistance genes between gram-positive and gram-negative bacteria. Antimicrob Agents Chemother 1994;38(7):1447–51.

[12] FDA (2018). https://www.fda.gov/food/people-risk-foodborne-illness/questions-answers-consumers-concerning-infant-formula#1. (03/07/2018).

[13] Iversen C, Forsythe S. Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. Trends Food Sci Technol 2003;14(11):443–54.

[14] Mardaneh J, Dallal MMS. Isolation, identification and antimicrobial susceptibility of *Pantoea* (*Enterobacter*) *agglomerans* isolated from consumed powdered infant formula milk (PIF) in NICU ward: First report from Iran. Iran J Microbiol 2013;5:263.

[15] Izadi Z, Mahmoud S, Ali E, Soleimanian-Zad S. Fabrication of an electrochemical DNA-based biosensor for *Bacillus cereus* detection in milk and infant formula. Biosens Bioelectron 2016;80:582–9.

[16] Zhou X, Jianxin G, Yaojian H, Songzhe F, Chen H. Antibiotic resistance pattern of *Klebsiella pneumoniae* and *Enterobacter sakazakii* isolates from powdered infant formula. Afr J Microbiol Res 2011;5(19):3073–7.

[17] Chen Y, Zhang Y, Wang X, Ling J, He G, Shen L. Antibacterial activity and its mechanisms of a recombinant Funme peptide against *Cronobacter sakazakii* in powdered infant formula. Food Res Int 2019;116:258–65.

[18] Adimpong DB, Sørensen K, Thorsen L, Stuer-Lauridsen B, Abdelgadir WS, Nielsen DS, et al. Antimicrobial susceptibility of *Bacillus* strains isolated from primary starters for African traditional bread production and characterization of the bacitracin operon and bacitracin biosynthesis. Appl Environ Microbiol 2012;78:7903–14.

[19] Halket G, Dinsdale AE, Logan NA. Evaluation of the VITEK2 BCL card for identification of *Bacillus* species and other aerobic endospore-formers. Lett Appl Microbiol 2010;50:120–6.

[20] Clinical, L.S. Institute. Performance standards for antimicrobial susceptibility testing. PA: Clinical and Laboratory Standards Institute Wayne; 2012.

[21] Fisher RA. Contributions to mathematical statistics. Oxford, England: Wiley; 1950.

[22] Rahimi E, Fahimeh A, Hassan M, Zienab TB, Jalali M. *Bacillus cereus* in infant foods: prevalence study and distribution of enterotoxigenic virulence factors in Isfahan Province, Iran. Sci World J 2013:1–6. Article ID 292571.

[23] Pei X, Shuran Y, Li Z, Jianghui Z, Xiaoyu S, Xiaoning H, et al. Prevalence of *Bacillus cereus* in powdered infant and powdered follow-up formula in China. Food Control 2018;93:101–5.

[24] Sadek ZI, Abdel-Rahman M, Azab MS, Darwesh OM, Hassan MS. Microbiological evaluation of infant foods quality and molecular detection of *Bacillus cereus* toxins relating genes. Toxicol Rep 2018;5:871–7.

[25] Turnbull P. The role of *Bacillus* genus in infection. Culture 1997;18:5–8.

[26] Frank JF. Milk and dairy products. In: Doyle MP, Beuchat LR, editors. Food microbiology: fundamentals and frontiers. ASM Press, American Society of Microbiology; 2007. p. 141–55.

[27] FAO, WHO. *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered formulae: external microbiological risk assessment series 15; 2008. ISBN 978-92-5-106119-0.

[28] Himmelright I, Harris E, Lorch V, Anderson M. *Enterobacter sakazakii* infections associated with the use of powdered infant formula—Tennessee. External JAMA 2001;287:2204–5.

[29] Baumbach J, Rooney K, Smelser C, Torres P, Bowen A, Nichols M. *Cronobacter* species isolation in two infants—New Mexico, 2008. MMWR Morb Mortal Wkly Rep 2009;58:1179–83.

[30] Oliver A, Weigel LM, Rasheed JK, McGowan JE, Raney P, Tenover FC. Mechanisms of decreased susceptibility to cefpodoxime in Escherichia coli. Antimicrob Agents Chemother 2002;12:3829–36.

[31] Chen Y, Tenover FC, Koehler TM. β-Lactamase gene expression in a penicillin-resistant *Bacillus anthracis* strain. Antimicrob Agents Chemother 2004;12:4873–7.

[32] Kıvanç SA, Kıvanç M, Güllülü G. Automated ribotyping and antibiotic resistance determining of *Bacillus* spp. from conjunctiva of diabetic patients. Iran J Basic Med Sci 2014;17:138.

[33] Owusu-Kwarteng J, Wuni A, Akabanda F, Tano-Debrah K, Jespersen L. Prevalence, virulence factor genes and antibiotic resistance of *Bacillus cereus* sensu lato isolated from dairy farms and traditional dairy products. BMC Microbiol 2017;17:65.

[34] Meena BS, Kapoor KN, Agarwal RK. Occurrence of multi-drug resistant *Bacillus cereus* in foods. J Food Sci Technol 2000;37:289–91.

[35] Khasnabis J, Adhikari P, Chowdhury D, Rai C, Roy A. Incidence of multiple drug resistant *Bacillus cereus* in some popular snacks and sweets sold in Kolkata city, India. Indian J Microbiol Res 2017;4:14–9.

[36] Luna VA, King DS, Gulledge J, Cannons AC, Amuso PT, Cattani J. Susceptibility of *Bacillus anthracis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus thuringiensis* to 24 antimicrobials using Sensititre® automated microbroth dilution and Etest® agar gradient diffusion methods. J Antimicrob Chemother 2007;60:555–67.

[37] Cho GS, Li B, Rostalsky A, Fiedler G, Rösch N, Igbinosa E, et al. Diversity and antibiotic susceptibility of *Acinetobacter* strains from milk powder produced in Germany. Front Microbiol 2018;27(9):536.