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Analysis of spore-forming bacterial contaminants in herbs and spices and evaluation of their heat resistance

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Abstract

The antimicrobial activities of certain spices and herbs are well-documented. Herbs and spices are often exposed to bacterial contamination, spore-forming bacteria in particular. The spores introduced during the production process result in a contamination that causes food alteration during storage. The aim of this study was to isolate spore-forming bacteria from commercially available spices and herbs, such as ginger, cinnamon, black pepper, curcumin, saffron, and clove. The API 50 CHB/E medium was used to identify different genera of spore-forming bacteria isolated from samples. We found that these belonged to the genus *Bacillus*, such as *Bacillus cereus*, which was isolated from cinnamon, and *Bacillus subtilis*, and isolated from curcumin and black pepper. The third species belonged to the genus *Brevibacillus*, *Brevibacillus laterosporus*, which was isolated from ginger. The D-values (decimal reduction) of each bacterium were determined at 80, 85, 90, 95, and 100 °C. The Z-value was determined for each bacterium. The whole-cell protein profiles of the identified bacteria were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Since spore-forming bacterial contamination of herbs and spices can occur during their harvest, exsiccation, or through cross-contamination during packaging, we propose different methods for spore elimination.

Keywords: spices; spore-forming bacteria; D-values; Z-values; SDS-page.

Practical Application: Herbs and spices contained a sporeforming and heat resistant bacteria belonged to the genus of Bacillus and Brevibacillus.

1 Introduction

Spices and herbs are aromatic substances used in food as coloring and flavoring agents. Spice production is increasing and these ingredients are now widely used in the development of new food products. Spices are divided according to their flavor intensity in strong spices, such as pepper, chili, and ginger; mild spices, such as paprika and coriander; aromatic spices, such as cinnamon, curcumin, clove, cumin, and aniseed; dried herbs, such as basil, bay, dill, marjoram, tarragon, and thyme; and aromatic vegetables, such as onion and garlic (Peter, 2001). The mixture of herbs and spices has also been added as a last category. Spices and herbs are generally harvested under poor sanitary conditions, and most drying processes are done directly on the soil. Therefore, they become contaminated with a high load of microorganisms, such as mycotoxins produced by molds, pathogenic bacteria such as salmonella, and also bacterial spores, as mentioned in numerous articles and reviews (Carlin, 2011; McKee, 1995). In all heat-treated foods, bacterial spores are a threat since they can survive the processes used in the food industry, such as cooking, pasteurization, sterilization, and disinfection.

Herbs and spices come in contact with the soil during exsiccation. Drying methods differ among countries and depend on the type of spice, region of production, and regional economic development. The traditional method of harvesting and drying spices on the ground is still common in the developing countries, hence the direct contact with the soil. There are several techniques that could be used for drying spices, such as solar, hot air, and microwave exsiccation (Jin et al., 2018). Other methods, such as boiling, can also be critical in the eliminating bacterial spores contamination of spices.

Microbial load is increased during food and food product manufacturing (Postollec et al., 2012), and the addition of spices is part of this industry (Hampikyan et al., 2009). Although the number of bacterial spores is low, their diversity is quite high (McKee, 1995). Bacterial spores are resistant to heat, so they can germinate and sporulate during the multi-step manufacturing process. Certain species of spore-forming bacteria cause food poisoning (Hariram & Labbé, 2015; Van Doren et al., 2013), such as *Bacillus cereus*, *Clostridium perfringens*, and *Clostridium botulinum*, while others cause product alterations that result in food degradation and significant economic loss. Instability

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of canned foods may occur because of the presence of spore-forming bacteria in the herbs and spices (André et al., 2013; Witkowska et al., 2011). Better knowledge of the amount of spores present could be helpful for evaluating the risks associated with the use of these ingredients (Van Doren et al., 2013).

Several decontaminating processes to remove bacterial spores, such as ionizing treatments, are very effective, but are still poorly accepted by consumers and require marketing authorizations (Mathot et al., 2021). Steam-heating treatments alter the sensory and physicochemical properties of spices. Fumigation with ethylene oxide can produce carcinogenic or mutagenic compounds. However, alternative methods have been developed (Mathot et al., 2021).

Knowledge of spice and herb bacterial spore contamination is, therefore, of great interest to food manufacturers. A similar thermal death time is key in the study of spore-forming bacteria, especially those present in canned foods. Therefore, our first objective was to determine the levels of bacterial spores found in herbs and spices and identify these bacteria. Second, we evaluated their the heat resistance by determining the thermal death time (D-value) and Z-values. The sources of contamination should be limited to avoid or reduce the number of these bacteria in the future.

2 Materials and methods

2.1 Spice and herb samples

Five samples of ginger, cinnamon, curcumin, black pepper, saffron, and clove, for a total of 25 samples, were collected from markets in Riyadh City, Saudi Arabia.

2.2 Isolation and identification of Bacillus sp. in herbs and spices

Ten grams of herb and spice samples were collected under sterile conditions and placed in sterile plastic bags containing 90 mL of diluent (0.85% sterilized saline solution). The mixtures were homogenized for 10 min in a STOMACHER 80 homogenizer (BA 7020 type, Seward Ltd., Worthing, UK). Thus, dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were obtained. Spore-forming bacteria were enumerated on *Bacillus cereus* agar base (HiMedia, M8333) supplemented with *B. cereus* selective supplement (Oxoid SR99) and 25 mL of sterile egg yolk emulsion (Oxoid SR47). A suspected colony of *Bacillus sp.* or related genera was detected when it appeared bluish in color, because of the bromothymol blue indicator, with a surrounding precipitate of the same color due to lecithinase production (from the egg yolk). The colonies were then isolated for confirmation of their affiliation to this species. The total count of *Bacillus sp.* and related genera was done on potato dextrose agar supplemented with polymyxin.

2.3 Biochemical identification of Bacillus sp., isolates

API 50 CHB/E medium (BioMérieux, SA, F-69280 Marcy l'Etoile, France) was used to identify *Bacillus* and related genera, as well as gram-negative rods belonging to the Enterobacteriaceae and Vibrionaceae families. It is a ready-to-use medium that allows fermentation of 49 carbohydrates on the API 50 CH strip (Ref. 50 300). The API 20 E strip (Ref. Twenty 100) may be used in association with the API 50 CH strip to provide supplementary tests (essential for Enterobacteriaceae and Vibrionaceae). The API 20 E reagent kit (Ref. Twenty 120) was used to complete the tests. API 50 CHB/E was applied in accordance with the manufacturer's instructions for the biochemical identification of *Bacillus*. The identified isolates were stored at −80 °C until further analyses. *Bacillus sp*. were further identified by extracting the total protein of cells and performing polyacrylamide gel electrophoresis (PAGE).

2.4 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Isolates were prepared for SDS-PAGE analysis, in accordance with the method described by Scarcelli et al. (2001). Electrophoresis was performed with a 12% polyacrylamide running gel and a 4% stacking gel, with a 0.025 M Tris 0.19 M glycine buffer, pH 8.3, and 100 µL of sucrose buffer (50 mM Tris–HCl, pH 8; 40 mM EDTA, pH 8; 0.75 M sucrose).

2.5 Preparation of cell extract

One hundred milliliters of overnight culture of *B. cereus* ATCC 14579 and the isolates identified by API 50 CHB/E (100 µL) were inoculated into 10 mL of fresh brain heart infusion (BHI) medium (Oxid, CM1135) and grown to a 620-nm optical density (OD) of 0.6. The cells were collected and weighed, and 250 mg of cells was suspended in 100 µL of TES buffer (50 mM Tris HCl, pH 8, 1 mM EDTA, 25% sucrose). Twenty microliters of lysozyme (50 mg/mL) and 5 µL mutanolysin (5000 u/mL) were added to the suspended cells in the TES buffer and incubated at 37 °C for 30 min. Five to ten microliters of 20% SDS was added, and the contents were mixed until they became clearly visible. These were stored at −20 °C for 1 to 2 d (Yehia & Al-Dagal, 2014). Thirty microliters of cell extracts (standard and isolated bacteria) was loaded onto the SDS-PAGE. Electrophoresis was performed at 25 °C in a vertical tank using a constant-voltage power supply until the bromophenol blue tracking dye reached the bottom of the gel. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Bio-Rad, Marnes-la-Coquette, France) in water:methanol:acetic acid (6.5:2.5:1) for 18 h at room temperature. Gel destaining was performed by continuous agitation in methanol:acetic acid:water (20:10:70 v/v/v) until obvious protein bands were obtained. The whole-cell protein profiles of presumptive *Bacillus* isolates were compared with those of the standard strain ATCC 14579 of *B. cereus* using SDS-PAGE.

2.6 D-value

The D-value, or decimal reduction value, is known as the inactivation, at a given temperature, of 90% of the bacterial population or 1 log of the initial population . Active cultures of each identified bacterium were obtained by inoculating one colony of each bacterium into BHI broth (Oxoid, CM1135) and allowing it to grow at 37 °C for 24 h. One milliliter of active 24 h culture was diluted in screw-top test tubes containing 9 mL of BHI medium. The tubes with diluted solutions (in duplicate)

were exposed to heat in a water bath at 80, 85, 90, and 95 °C, each temperature for 1, 2, 3, 4, and 5 min. After each heat treatment, the tube was quickly cooled in an ice water bath for 20 s. To count the surviving cells, serial dilutions were performed, and 1 mL of each sample was poured onto BHI agar and incubated at 37 °C for 24-48 h. Colony-forming units per mL (CFU/mL) were recorded for every treatment. The optical density (OD) of the dilutions was recorded after a 24-h incubation at 37 °C. D-values were determined from the linear sections of the survivor plots using linear regression analysis. D-values are reported in seconds and defined as the time required to achieve a 1 log reduction in the bacterial population at a designated temperature. Before treatment, the number of cells in the inoculated medium ranged from 10^6 to 10^7 cells/mL.

2.7 Z-value

The Z-value is the increase in temperature required to decrease the number of organisms by 1 log at a specified D-value. The Z-values were determined at different temperatures (80, 85, 90, and 95 °C) and compared with the D-values, which were calculated using the formula Z = slope − 1 (the temperature change necessary to induce a 10-fold change in the D-value) (Holdsworth, 1997).

3 Results and discussion

The total count of bacteria isolated from curcumin was 5.3 log10 CFU/g followed by ginger, 4.6 log10 CFU/g, black pepper, 4.2 log10 CFU/g, and cinnamon, 3.3 log10 CFU/g. No bacteria were found in cloves or saffron (Table 1).

The common bacterial contamination of curcumin and black pepper was identified as *Bacillus subtilis*, according to the identification using API 50 CHB/E kits, databases (V4.1), and the analytical profile index. *Brevibacillus laterosporus* and *Bacillus cereus* were identified in ginger and cinnamon, respectively.

Kumar et al. (2016) isolated nine bacterial strains from the rhizospheric soil of curcumin (*Curcuma longa*), namely *Bacillus subtilis* CL1 and *Bacillus sp.* CL3, *Burkholderia thailandensis* CL4, *Agrobacterium tumifaciens* CL5, *Klebsiella sp.* CL6, *Bacillus cereus* CL7, *Pseudomonas putida* CL9, *Pseudomonas fluorescens* CL12, and *Azotobacter chroococcum* CL13. Plants were grown in the Botanical Garden of Banaras Hindu University, India.

Table 1. Total count of bacteria on potato dextrose agar supplemented with polymyxin.

Herbs/spices	Sample No.	Scientific name	log10 CFU/mL
Clove	5	*Syzygium aromaticum*	0
Cinnamon	5	*Cinnamomum zeylanicum*	3.3
Black pepper	5	*Piper nigrum*	4.2
Curcumin	5	*Curcuma domestica*	5.3
Ginger	5	*Zingiber officinale*	4.6
Saffron	5	*Crocus sativus*	0

Therefore, it becomes evident that the contamination of curcumin by *Bacillus subtilis* vegetative cells or its spores occurs through exsiccation and ground processes, allowing soil bacteria to come in contact with these spices.

The endospore-forming bacteria *Brevibacillus laterosporus* Laubach (formerly *Bacillus laterosporus*) appears to be rod-shaped and morphologically characterized by the production of a typical canoe-shaped parasporal body firmly attached to one side of the spore, which determines its lateral position in the sporangium (Ruiu, 2013). This bacterium has been isolated from a wide variety of substances or materials as well as from the soil (Oliveira et al., 2004). *B. laterosporus* was also detected in rice samples. Cottyn et al. (2001) reported the presence of this species in raw rice. *B. laterosporus* produces vegetative cells, spores, and sometimes parasporal bodies.

Bacillus cereus is a rod-shaped, gram-positive, motile, aerobic-to-facultative anaerobic bacterium that forms endospores (Tewari & Abdullah, 2015). Because Bacillus bacteria form endospores, they can distribute in many habitats, such as soil, water, and food. They can also survive in extreme environments, such as geothermal pools, where the temperature exceeds 60 °C. *B. cereus* is ubiquitous and usually contaminates foods and commercial products along with other spore-forming bacteria responsible for food spoilage and poisoning (Okanlawon et al., 2010; Berthold-Pluta et al., 2019). Thanh & Tram (2018) isolated a variety of *Bacillus spp*. from the rhizosphere of pepper, including 11 strains identified as *B. cereus*, *B. weihenstephanensis*, *B. megaterium*, *B. subtilis*, and *B. pumilus*. The total count of *B. cereus* in seven cinnamon samples ranged from 1.10 to 2.65 log10 CFU/g (Fogele et al., 2018). They also found that *B. cereus* was present in nine (18%) blackground pepper samples at 2.08-3.09 log10 CFU/g, whereas the total count in curry samples ranged from 2.03 to 2.76 log10 CFU/g. Mustard had the lowest *B. cereus* concentration (1 log10 CFU/g). The authors also indicated the concentration present in onion powder, garlic powder, red bell pepper, and cayenne pepper, namely < 10 log10 CFU/g, 2.08 log10 CFU/g, 1.91 log10 CFU/g, and 2.43 log10 CFU/g, respectively. The amount of *B. cereus* in thyme, basil, dill, rosemary, and parsley varied from 1.20 to 2.98 log10 CFU/g. Supermarket basil had the highest concentration of *B. cereus* (2.98 log10 CFU/g), while supermarket parsley had the lowest (1.20 log10 CFU/g).

3.1 Identification of bacterial isolates

The API 50 CHB system biochemically identified three genera (*Bacillus* and *Brevibacillus)*, and three species in our samples. *B. laterosporus* was a common spore-forming bacterium in ginger, *B. cereus* was identified in cinnamon, and *B. subtilis* in curcumin and black pepper (Table 2).

The whole-cell protein profiles obtained from bacteria identified by API 50 CHL/E were compared with reference isolates for species identification. By comparing the protein bands of each microbe on SDS-PAGE (Figure 1), it can be concluded that lanes 2 and 3 are similar to lane 1 of *B. cereus* ATCC 14579 in terms of number, shape, and number of bands. Lane 4 is similar to lane 3 of the bacteria identified by API 50 CHL/E, *B. subtilis*. Lanes 6 and 7 represent *B. laterosporus*. Lanes 8 and 9 are similar to

Table 2. Identification of herb and spice isolates with API 50 CHB/E kits.

Isolates	API 50 CHB/E V3.0 profile	Identification	Similarity % (Nb.)
B. cereus ATCC 14579	- + - - - + - - - - - + + + - - - - - - - - + + + + + + + - - + + - - - - + + - - - - - - - - + - - + + - - + - - - - + + +	***Bacillus cereus 1***	**99.7**
Isolate 1 (Cinnamon)	- + - - - + - - - - - + + + - - - - - - - - + + + + + + + - - + + - - - - + + - - - - - - - - + - - + + - - + - - - - + + -	***Bacillus cereus 1***	**99.7**
Isolate 2 (Curcumin)	- + - - + + + - - - - + + + - - - + + + - + - - - + + + + + + - + - - - + + - - - - - - - - - - - - - - - - + + - - - + + -	***Bacillus subtilis* A**	-
Isolate 3 (Ginger)	- + - - - + - - - - - + + + - + - + + + - - - - - + - + + - - + + - - - - - - - - - - - - + - - - - - - - - + + +	***Brevibacillus laterosporus***	**83.3**
Isolate 4 (Black pepper)	- + - - - + + + - - - + + + - - - + + + - + - - + + + + + + + + + - - + + + - - - - - - - - - - - - - + - - + - - - - + + +	***Bacillus subtilis* B**	**95.8**

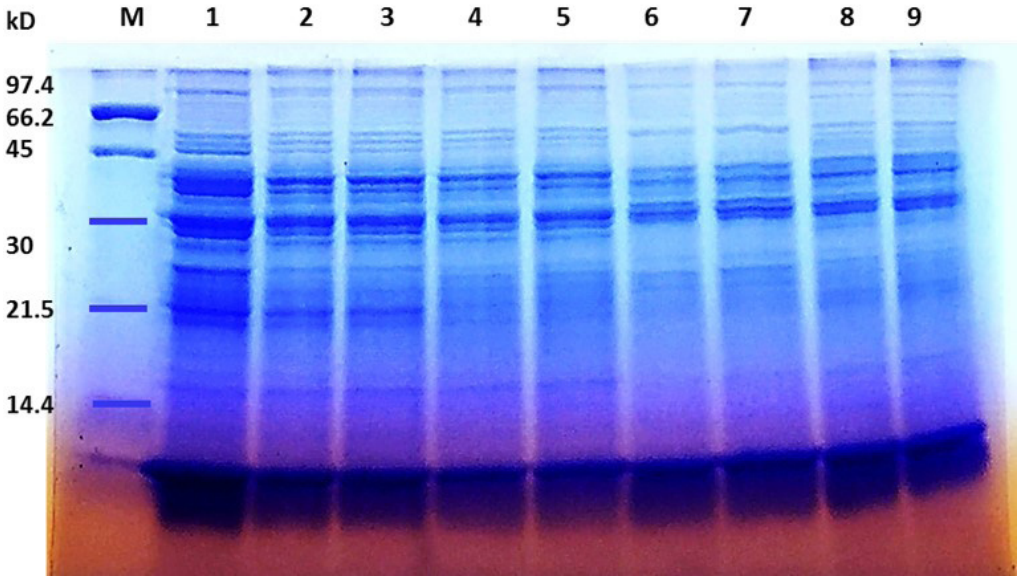

Figure 1. Total protein profiles of *Bacillus cereus* ATCC 14579 and isolates of spices identified by API 50CHB/E on SDS/PAGE. Lane M: molecular weight standard (kD); Lane 1: *B. cereus* ATCC 14579: total protein (positive control); Lanes 2 and 3: *B. cereus* (cinnamon); Lanes 4 and 5: *Bacillus subtilis* A (curcumin); Lanes 6 and 7: *Brevibacillus laterosporus* (ginger); Lanes 8 and 9: *B. subtilis* B (black pepper).

lanes 3 and 4 of *B. subtilis* in terms of the shape and number of bands. SDS-PAGE is an important molecular technique used for the identification of whole-cell proteins at the species level and has the advantage of being simple and rapid to perform. However, this technique requires extensive data to cover all the known target species (Leisner et al., 1994).

3.2 Spore heat resistance

Survivor curves for *Brevibacillus laterosporus* spores (ginger) were determined at 80, 85, 90, 95, and 100 °C in BHI broth. The D-values were 4.0, 3.8, 3.2 and 3.1 min, respectively (Figure 2A-D). The Z-value of *B. laterosporus*, determined from D-value plots (decimal reduction curves), was 7.5 °C (Figure 2E). In previous studies, the D-values for *B. laterosporus* at 85, 90, 92.5, and 95 °C were found to be 20.5, 6.4, 3.9, and 2.1, respectively, with a Z-value 10.1 °C (Shehata & Collins, 1972).

Survivor curves for *B. cereus* spores (ginger) were determined at 80, 85, 90, 95, and 100 °C in BHI broth. The D-values were 5.0, 4.8, 4.6, 4.4, and 3.4 min, respectively (Figure 3A-E). The Z-value of *B. cereus* was 12 °C (Figure 3F). Shehata & Collins (1972) had previously determined the Z-value of *B. cereus* to be 9.4 °C. In whole-milk medium, the D-values for the *B. cereus* ATCC 7004 strain were reported to be 18.6 and 3.18 s at 100 and 106 °C, respectively (Mazas et al., 1998), with Z-values ranging from 7.7 to 9.3 °C (Mazas et al., 1999). Xu et al. (2006) reported that a Z-value for the *B. cereus* D17 strain of 9.5 °C.

The D-values of *Bacillus subtilis* A were determined for both isolates in BHI broth at 80, 85, 90, and 95 °C; these were 2.4, 2.25, 2.1, and 1.95 min, respectively (Figure 4A-D), with a Z-value of 9.5 °C (Figure 4E). While, the D-values of *Bacillus subtilis* B were determined for both isolates in BHI broth at 80, 85, 90, and 95 °C; these were 3.0, 2.3, 2.0, and 1.85 min, respectively (Figure 5A-D), with a Z-value of 15 °C (Figure 5E). Put & Aalbebsberg (1967), determined the heat resistance of spores of two strains of *B. subtilis,* Bac 1–11 and Bac 1–12, which were isolated from processed fried rice and processed

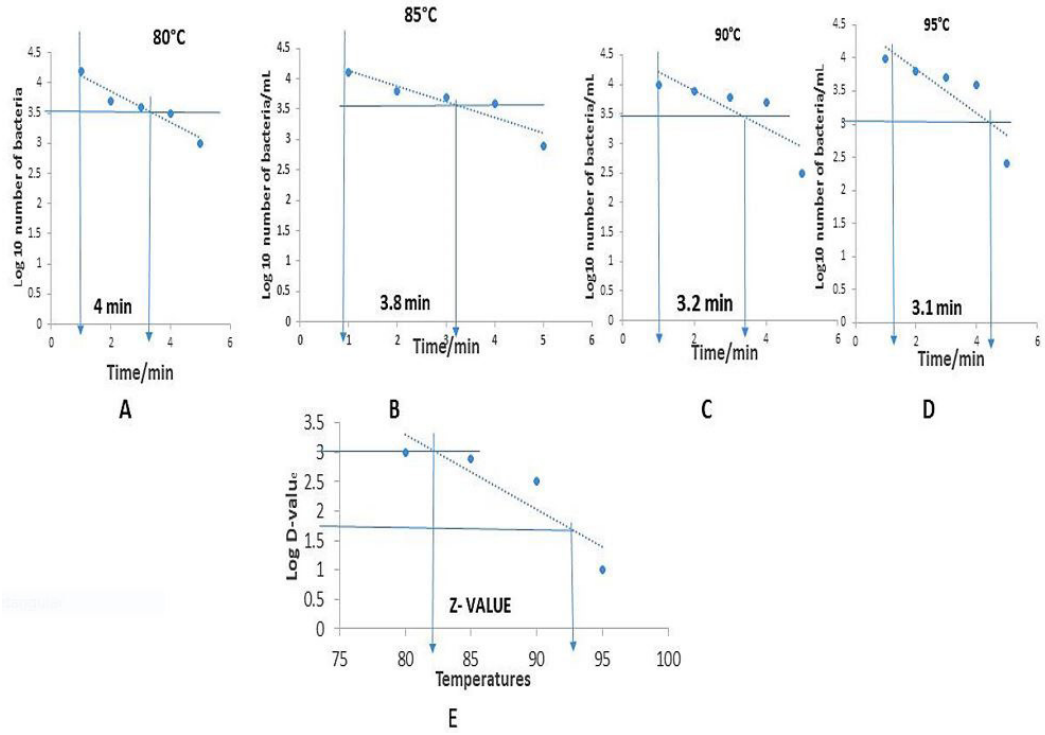

Figure 2. The D-values of *Brevibacillus laterosporus* at of 80, 85, 90 and 95 °C (A-D), and the calculated Z-value of 7.5 °C (E).

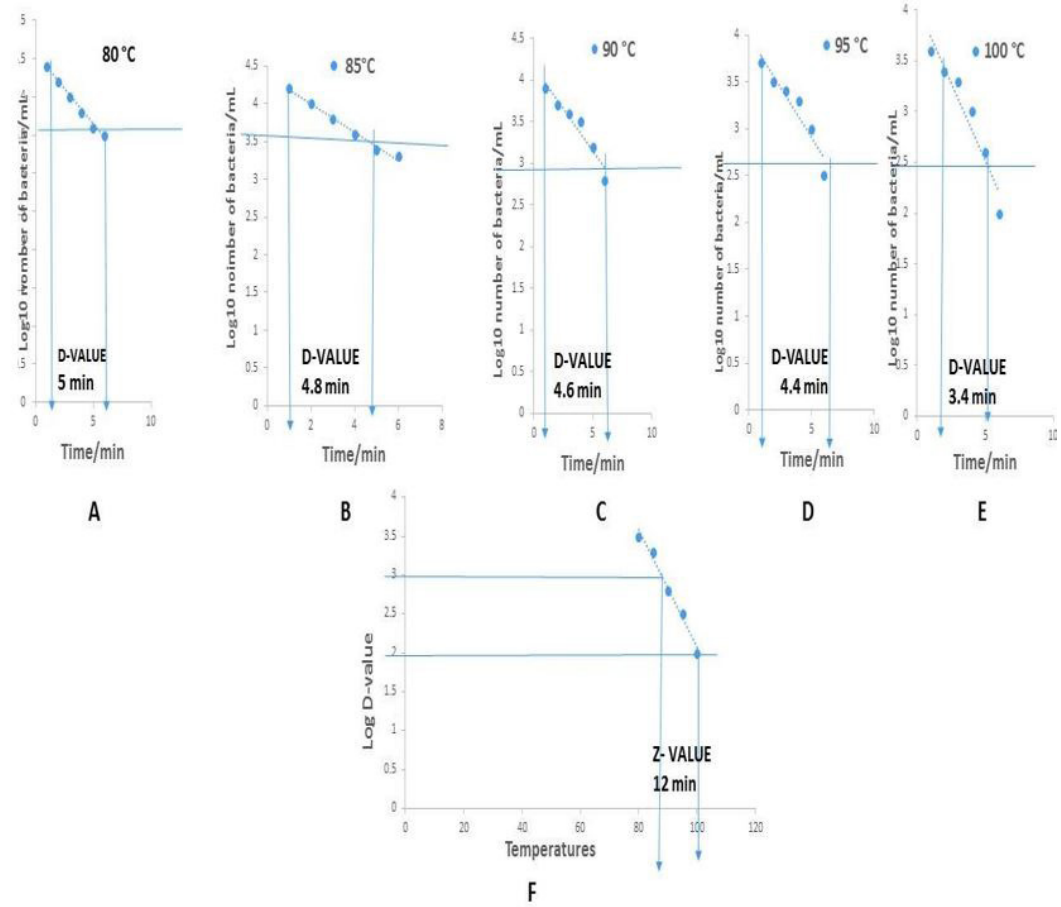

Figure 3. D-values of *Bacillus cereus 1* at 80, 85, 90, 95, and 100 °C (A-E), and the calculated Z-value of 12 °C (F).

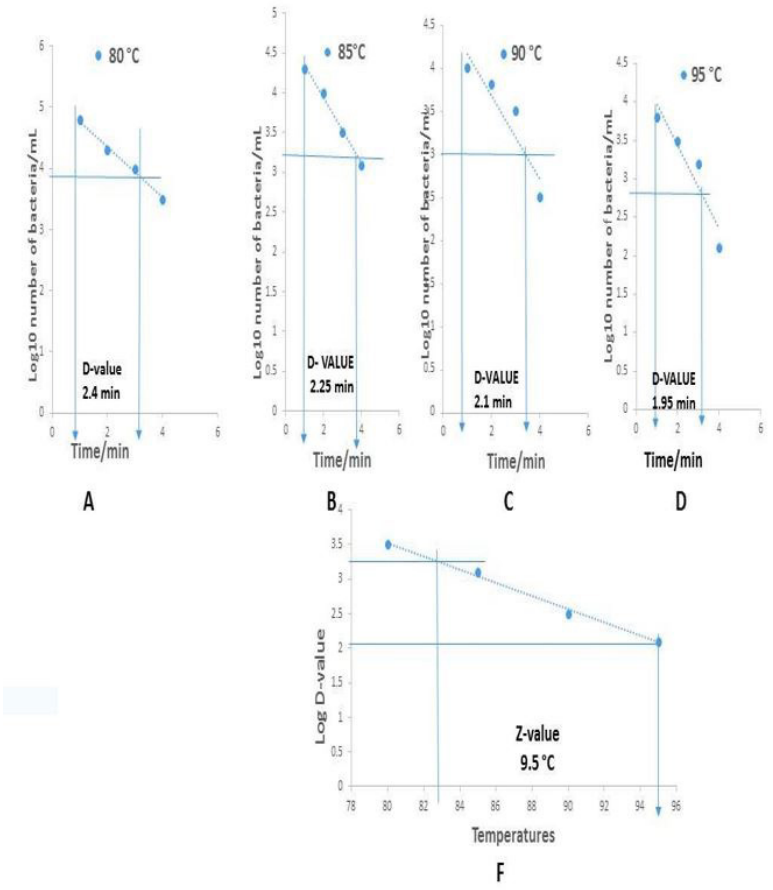

Figure 4. D-values of *Bacillus subtilis* A at 80, 85, 90, and 95 °C (A-D), and the calculated Z-value of 9.5 °C (E).

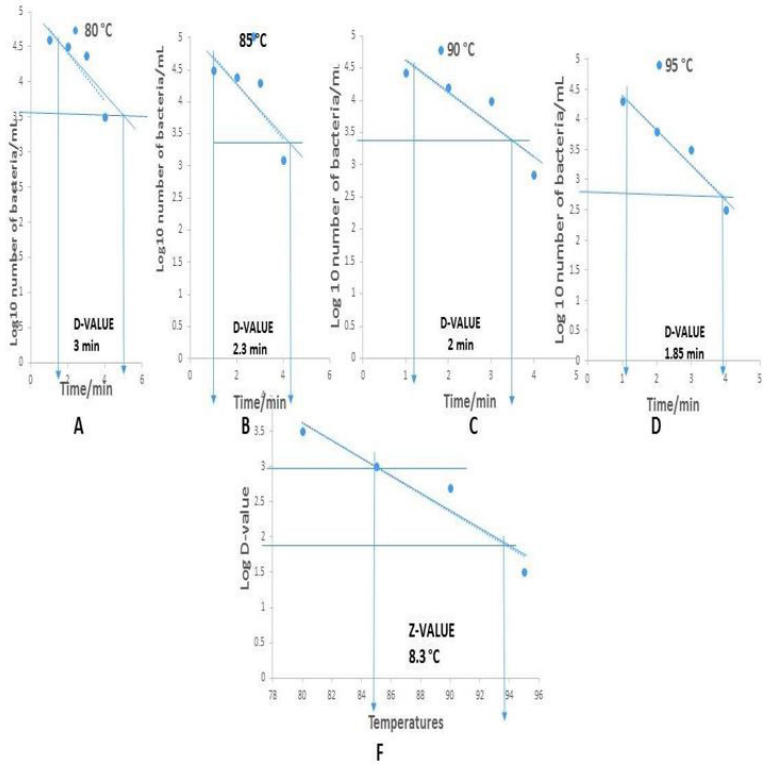

Figure 5. D-value of *Bacillus subtilis* B at 80, 85, 90, and 95 °C (A-D), and the calculated Z-value of 15 °C (E).

evaporated milk, respectively. The calculated D-values ($D_{112.5}$) f Bac 1–11 spores in M/40 phosphate buffer (pH 6.8) and fried rice (pH 6.4) were 4.2 and 4.5 min (Z = 18 and 20 resp.), respectively. The calculated $D_{112.5}$ values of Bac 1–12 spores in M/40 phosphate buffer (pH 6.8) and evaporated milk (pH 6.4) were 5.0 and 6.7 min (Z = 18 and 23 resp.), respectively. The extrapolated D_{121} values were 0.57, 0.76, 0.71, and 1.45 min, respectively. Evelyn et al. (2021) determined that the D-values for *B. subtilis* in 11 °Brix pineapple juice were 2.1 ± 1.7 min at 100 °C, 6.8 ± 0.9 min at 95 °C, and 13.2 ± 0.5 min at 90 °C ($p < 0.05$). Moreover, Rodriguez et al. (1993) reported the D-values for *B. subtilis* 075-T-0 in tomato juice were 5.7 min at 100 °C and 15.8 min at 95 °C.

4 Conclusions

The spore-formers found in the herb and spice samples mainly belonged to the genera *Bacillus* and *Brevibacillus*. Such contamination is very common and sometimes reaches high levels, as in curcumin, ginger, black pepper, and cinnamon. *Bacillus cereus* and *Bacillus subtilis* were the most frequently detected species, followed by *Brevibacillus*. Bacteria contaminate spices because these are mostly produced in developing countries on small farms using traditional production methods. Spices become contaminated by bacterial spores in two main ways: by direct contact with the soil during their harvest or exsiccation (e.g., black pepper), or by cross-contamination when cooked in water (e.g., curcumin). Based on these observations, we proposed different methods that can be used to eliminate bacterial spores from spices, while indicating their efficiency and limitations. In our study, *Bacillus cereus* was found to be the most heat resistant bacterium, capable of tolerating 100 °C for 3.4 min, followed by *Brevibacillus laterosporus*, with 3.1 min at 95 °C, and *Bacillus subtilis*, with 1.95 and 1.85 min at 95 °C.

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