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Research Article

Investigation of Toxin-Producing and Antibiotic-Resistant *Bacillus cereus* in Spices Used in the Production of Sucuk

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Abstract

In this study, a total of 100 samples of seven types of spices were investigated to determine the presence of toxin-producing and multiple antibiotic-resistant *Bacillus cereus* and other food-borne microorganisms. The spices tested included 18 samples of ground black pepper (*Piper nigrum*), 17 samples of red pepper (*Capsicum frutescens*), 12 samples of ground red pepper (*Capsicum frutescens*), 20 samples of cummin (*Cuminum cyminum*), 16 samples of pimento (*Pimenta dioica*), nine samples of garlic powder (*Allium sativum*) and eight samples of sucuk spice mixes that were obtained from various retail shops and sucuk production units in the Bursa province between January and December 2014. These spices are used to prepare sucuk because of their flavouring and seasoning properties. The samples were analysed for *Bacillus cereus*, *Escherichia coli*, coagulase-positive *Staphylococcus aureus*, *Salmonella* spp., yeast, moulds and coliforms. For the identification of *B. cereus*, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS, Germany) was used. The analysis showed that all samples had coliform, yeast and mould and *B. cereus* maximum and minimum counts between <10 and $1.1x10^5$ cfu/g, $1x10^2$ and $9.3x10^4$ cfu/g, and $1x10^2$ and $4.8x10^4$ cfu/g, respectively. Furthermore, coagulase-positive *S. aureus* was identified in five samples, *E. coli* was identified in two samples, and *Salmonella* spp. was identified in none. A total of 39 (92.8%) *B. cereus* isolates were resistant to two or more antibiotic agents. In addition, 31 (73.8%) *B. cereus* isolates tested positive for production of both non-haemolytic enterotoxin (NHE) and haemolysin BL (HBL) enterotoxins. These results show that some spices contain harmful microorganisms. To reduce microbial counts and to improve poor microbiological quality in spices, hygiene programmes must be corrected in the pre- and post-harvesting period.

Keywords: Spices, Sucuk, *Bacillus cereus*, Multiple antibiotic resistance, Toxin producing, MALDI-TOF.

Sucuk Üretiminde Kullanılan Baharatlarda Toksin Üreten ve Antibiyotiğe Dirençli *Bacillus cereus*'un Araştırılması

Öz

Bu çalışmada, toksin üreten ve çoklu antibiyotiklere dirençli *Bacillus cereus* ve diğer gıda kaynaklı mikroorganizmaların varlığını belirlemek için yedi çeşit baharattan toplam 100 numune araştırılmıştır. Çalışmanın amacı doğrultusunda; Ocak ve Aralık 2014 tarihleri arasında Bursa ilinde, çeşitli perakende satış mağazaları ve sucuk üretim ünitelerinden elde edilen 18 kara biber örneği (Piper nigrum), 17 kırmızı pul biber örneği (Capsicum frutescens), 12 toz kırmızı biber örneği (Capsicum frutescens), 20 kimyon numunesi (Cuminum cyminum), 16 yenibahar örneği (Pimenta dioica), dokuz sarımsak tozu (Allium sativum) ve sekiz sucuk baharatı karışımları örnekleri analiz edilmiştir. Bu baharatlar, aromaları ve baharat özelliklerinden dolayı sucuk hazırlamak için kullanılmaktadır. Örnekler, *Bacillus cereus*, *Escherichia coli*, koagülaz pozitif *Staphylococcus aureus*, *Salmonella* spp., Maya-küf ve koliformlar yönünden analiz edilmiştir. *Bacillus cereus*'un tanımlanmasında Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS, Germany) kullanıldı. Analizler, tüm örneklerin koliform, maya ve küf ve *B. cereus* en yüksek ve en düşük sayılarının sırasıyla <10 and $1.1x10^5$ cfu/g, $1x10^2$ and $9.3x10^4$ cfu/g, and $1x10^2$ and $4.8x10^4$ cfu/g arasında olduğunu gösterdi. Ayrıca, beş örnekte koagülaz pozitif *S. aureus*, iki örnekte *E. coli* belirlenirken, hiçbir örnekte *Salmonella* spp.

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varlığı tespit edilemedi. Toplam 39 (% 92.8) B. cereus izolatının iki veya daha fazla antibiyotik ajana dirençli olduğu belirlendi. Ek olarak, *B. cereus* izolatlarının 31 (% 73.8) adeti non-hemolitik enterotoksin (NHE) ve hemolizin BL (HBL) enterotoksinlerinin üretimi için pozitif test edildi. Bu sonuçlar, bazı baharatların zararlı mikroorganizmaları içerdiğini göstermektedir. Mikrobiyal sayımı azaltmak ve baharatlarda zayıf mikrobiyolojik kaliteyi arttırmak için hijyen programları hasat öncesi ve hasat sonrası düzeltilmelidir.

Anahtar Kelimeler: Baharat, Sucuk, *Bacillus cereus*, Çoklu antibiyotik direnci, Toksin üretimi, MALDI-TOF.

1. Introduction

Sucuk is a popular traditional meat product that is produced from minced beef meat, hard beef fat tissue, salt, nitrite and/or nitrate and various spices including cumin, garlic, black pepper, pimento, and red pepper (Pehlivanoglu et al., 2015). However, sucuk may contain microorganisms that first come in contact with the meat through exposure to air and water, through employee handling, or through the spices used in its production. These microorganisms can seriously affect the health of anyone who consume these products.

Spices are valuable products that grow in a natural environment and are commonly used in a variety of ways, including in the preparation of meat products. Spices are a key ingredient throughout the world because of the preservation, flavouring, colouring and aromatic properties they confer to meat products, particularly sucuk, salami, pastrami and meatballs (Hampikyan et al., 2009; Verluyten et al., 2004). However, the use of contaminated spices in meat products can add to the existing microflora and thus impair the quality of these products and cause serious foodborne illness (Koohy-Kamaly-Dehkordy et al., 2013; Shinagawa et al., 1988). Yeasts and moulds, coliforms, *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, and the toxin-producing and spore-forming bacterium *Bacillus cereus* are the known possible contaminants of spices.

The spore-forming, toxin-producing bacterium *B. cereus* is one of the possible contaminants of spices (Eglezos, 2010). This bacterium can be found in soil and is widely distributed in the environment. *B. cereus* causes severe food poisoning, producing a diarrhoeal syndrome induced by haemolysin BL (HBL) and non-haemolytic enterotoxin (NHE) (Hansen and Hendriksen, 2001; Lund and Granum, 1996). *B. cereus* spores are heat-resistant and can germinate when cooled improperly, and the nutrients within meat support the growth of the resulting vegetative cells (Eglezos, 2010). Spices may also contain the enterotoxigenic *B. cereus*, usually at counts below 10^3 cfu/g but sometimes at higher levels (10^5-10^6 cfu/g), and food to which it is added may cause food poisoning if the product is inappropriately handled or stored (Cameron, 1998; Little et al., 2003). In the study conducted by Aksu et al. (2000), 63.44% of the spices were found to be contaminated with *B. cereus* ranging between 10^2 and $3.2x10^3$ cfu/g. A survey performed by Tulu et al. (2013) showed coliform counts ranging from $3.8 x10^5$ to $1.0 x 10^6$ cfu/g in red chilli spice samples.

Although B. cereus has been isolated from various spices at rates ranging from 28% to 100% (Hampikyan et al., 2009; Aksu et al., 2000; Erol et al., 1999; Agaoglu et al., 1999) there have not been any studies that have investigated the enterotoxin-producing capabilities or the antibiotic resistance of *B. cereus* in spices in Turkey.

Yeast and moulds may also contaminate spices. Mould contamination is dangerous due to their production of mycotoxins. These microorganisms have previously been isolated from various spices (Moss and Baker, 2000; Banerjee and Sarkar, 2003). Spices may be contaminated by coliforms associated with improper and inadequate hygienic practices and faecal contamination, *E. coli* in particular. The presence of *E. coli* in red pepper spice samples was reported by Parveen et al. (2014). *Salmonella* spp. is a leading cause of acute bacterial gastroenteritis, and *S. aureus* is another important foodborne pathogen. Several studies have reported the contamination of spices by both microorganisms. For instance, Banarjee and Sarker (2003) reported *Salmonella* spp. in 2.6% of spice samples. In the U.S., Julseth and Deibel (1974) found *S. aureus* in two of 12 black pepper samples.

In 2011, the Republic of Turkey Ministry of Food, Agriculture and Livestock established a maximum limit of 10^4 cfu/g spice of both coagulase-positive *S. aureus* and *B. cereus*. *Salmonella* spp. count should be zero in a 25 g sample.

The purpose of this study was to establish the microbiological quality of spices used in sucuk production in the Bursa province in relation to the presence of toxin-producing and antibiotic-resistant *B. cereus*. In addition, yeast and moulds, coliforms, *E. coli*, coagulase-positive *S. aureus* and *Salmonella* spp. were investigated. To our knowledge, this is the first report on the incidence of toxigenic and multiple antibiotic-resistant *B. cereus* in spices in Turkey.

2. Material and Method

2.1. Sample Collection

Between January and December 2014, a total of 100 samples of seven types of spices, including 18 samples of ground black pepper, 17 samples of red pepper, 12 samples of ground red pepper, 20 samples of cumin, 16 samples of pimento, nine samples of garlic powder and eight samples of sucuk spice mixes, were purchased from various markets, retail shops and sucuk production premises located in the Bursa province of Turkey. The samples were transported to the laboratory and analysed as soon as possible at <4°C.

2.2. Isolation and Identification of *Salmonella* spp.

Salmonella spp. isolation was performed according to the ISO 6579 standard (ISO, 2002). Briefly, the sample pre-enrichment was combined with 225 ml Buffered Peptone Water (Merck, Belgium), homogenized for two min with a Seward Stomacher 80 Lab System (Seward, London, UK) and incubated at 37°C for 24 h. After pre-enrichment and incubation, 1 ml of the sample was transferred to Muller-Kauffmann Tetrathionate-Novobiocin Broth (Oxoid, UK), and 0.1 ml of the sample was transferred to Rappaport-Vassiliadis Medium with Soya Broth (Oxoid, UK). The inoculated broths were incubated for primary enrichment for 24 h at 37°C and 41.5°C. After incubation, the cultures were inoculated using a loop onto both Xylose Lysine Deoxycholate (Merck, Belgium) agar and Xylose Lysine Tergitol (Merck; Belgium) agar. After selective plating at 37°C for 24 h, potential *Salmonella* colonies were subjected to biochemical identification by API 20E (bioMerieux, France).

2.3. Isolation and Identification of Coliforms and *E. coli*

For coliform and *E. coli* isolation and enumeration, serial 10-fold dilutions of samples were made in saline peptone water and plated onto the relevant selective media. Total coliform was grown on Violet Red Bile (Bio life, Italy) agar using the "pour" plate technique, and plates with 30-300 colonies were used for enumeration after 24-48 hours of incubation at 37ºC. After the incubation, typical colonies (red colonies with halos) were inoculated into lactose broth (Oxoid, UK) in a Durham tube for 44°C 24 h. After incubation, acid and gas formation-positive colonies were confirmed to be *E. coli* using the IMVC series of tests (indole, methyl red, Voges-Proskauer and citrate). Indol (+), methyl red (+), Voges-Proskauer (-) and citrate (-) indicated the presence of *E. coli* type-1.

2.4. Isolation and Identification of Coagulase-positive *S. aureus*

All samples were serially diluted in 9 ml of saline peptone water, and each dilution was spread on Baird Parker Agar (Oxoid, UK) plates. The plates were incubated at 37°C for 48 h. All colonies on Baird Parker with opaque halos surrounded by a clear zone were accepted as coagulase-positive *S. aureus*. From each Baird Parker Agar plate, 2 typical colonies were chosen, and a coagulase test was conducted for confirmation.

2.5. Isolation and Identification of Yeast and Moulds

Saline peptone water was used as a diluent for samples, and each dilution was spread on Potato Dextrose Agar (Oxoid, UK) using the spread plate method. Plates were incubated at 22°C for 3-5 days.

2.6. Isolation of *B. cereus*

All samples were serially diluted in 9 ml of saline peptone water, and each dilution was spread on Bacara Agar (bioMerieux, France) using the spread-plate method. Plates were incubated at 30°C for 48 h. Following incubation, plates were examined for typical colonies (pink/orange with halos), and at least one colony of each typical colony type was picked from each of the plates for identification using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS, Germany).

2.7. Identification of Presumptive *B. cereus* Colonies Using MALDI-TOF-MS

Identification of the isolates was performed using MALDI-TOF MS by the Duzen Laboratories Group in Istanbul. For this purpose, the colonies of presumptive *B. cereus* were subcultured to Tryptic Soy Agar (Oxoid, UK) and were confirmed using MALDI-TOF-MS. This technique is a promising platform for quick, flexible, and reliable identification of isolates originating from food. A portion or the whole colony on Tryptic Soy Agar (Oxoid, UK) agar was directly transferred onto a spot on the 96-spotted polished steel target plate. The spot was then covered with 1 µl of CHCA matrix solution. After air drying completely, the plate was loaded into the Bruker Microflex LT (Bruker Daltonics, Germany) instrument. The spectra were generated in linear positive ion mode with delayed extraction in a mass range of 4 to 10 kDa using a 337 nm nitrogen laser with a frequency of 60 Hz. The automated data analysis was processed with MALDI Biotyper Realtime Classification and Biotyper software version 3.1 (Bruker Daltonics). The obtained spectra were analysed using a standard pattern-matching algorithm, which compared the raw spectra with the spectra of the Bruker library using the standard settings. The results were listed in a ranking table, expressed as log (score) values ranging from 0 to 3 as recommended by the manufacturer. Score values of >1.7 generally indicate relationships at the genus level, and values of >2.0 generally indicate relationships at the species level. The highest score was used for species identification. The Bruker library contains >80,000 spectra covering 2048 species and 385 genera. If the result was below 1.7, the colony was transferred into screw cap tubes and mixed thoroughly in 0.3 ml of double-distilled ultrapure water. Next, 0.9 ml of pure ethanol was added to the tubes, and after vortexing, they were centrifuged at 13,000 × g for 2 min. The supernatant was discarded, and the pellet was mixed thoroughly with 50 µl of 70% aqueous formic acid. After the addition of 50 µl of acetonitrile, the mixture was centrifuged at 13,000 × g for 2 min. One microliter of the microorganism extract supernatant was placed onto the polished steel and covered with 1 µl of CHCA matrix solution and loaded to the instrument. Data analysis was processed as previously described.

2.8. Screening of Enterotoxic *B. cereus*

Haemolytic enterotoxin (HBL) and non-haemolytic enterotoxin (NHE) production were assessed using the Duopath® Cereus Enterotoxins Test kit (Merck, Belgium) according to the manufacturer's instructions. Briefly, *B. cereus* isolates were suspended in 1 ml Caseinhydrolysate Glucose Yeast extract Broth (Base) (Merck, Belgium) (with 1% glucose) and incubated at 37°C for 4 h. The Caseinhydrolysate Glucose Yeast extract Broth (Base) (Merck, Belgium) preparations were cooled to room temperature; then, 150 µl was transferred to the circular sample port on the test. The results were read after 30 minutes.

2.9. Antibiotic Resistance Testing

Antibiotic resistance of *B. cereus* isolates was tested using Mueller-Hinton Agar (Oxoid, UK) by the Kirby-Bauer disc diffusion method (Bauer et al., 1966). The disks used (Oxoid) and antibiotic concentrations were as follows: ampicillin (AMP, 10 µg), penicillin G (10 U), tetracycline (30 µg), erythromycin (15 µg), kanamycin (30 µg), chloramphenicol (30 µg), neomycin (30 µg), oleandomycin (15 µg), cephalothin (30 µg), streptomycin (10 µg), polymyxin B (300 U), and vancomycin (30 µg). *B. cereus* ATCC 10876 was used as control strain. According to the inhibition zone measured, the isolates were classified as resistant or susceptible as recommended by Bauer et al. (1966).

3. Results and Discussion

In total, 100 samples consisting of seven types of spices, including 18 samples of ground black pepper, 17 samples of red pepper, 12 samples of ground red pepper, 20 samples of cumin, 16 samples of pimento, nine samples of garlic powder and eight samples of sucuk spice mixes were analysed to determine their microbiological quality. The results obtained in this study are summarized in Table 1. The results of testing for the identification and toxin-producing capability of *B. cereus* isolates are shown in Table 2. The number of resistant and susceptible *B. cereus* isolates are indicated in Table 3. In addition, multiple antibiotic resistance patterns observed among *B. cereus* isolates are given in Table 4.

This analysis revealed that the samples had coliform, yeast and mould, and *B. cereus* counts between <10 and $1.1x10^5$ cfu/g, $1x10^2$ and $4.4x10^5$ cfu/g and $1x10^2$ and $4.8x10^4$ cfu/g, respectively. The average coliform counts of ground black pepper, red pepper, pimento, cumin, ground red pepper, garlic

powder and sucuk spice mixes were $2.7x10^3$ cfu/g (61.11%), $9x10^1$ cfu/g (5.88%), $4.2x10^3$ cfu/g (50%), $1.7x10^4$ cfu/g (55%), $2.3x10^4$ cfu/g (33.33%), $3.7x10^2$ cfu/g (22.22%) and $2.3x10^4$ cfu/g (100%), respectively. *E. coli* was identified in two sucuk spice mixes (25% of those tested). Coagulase-positive *S. aureus* was isolated in five samples (5% of those tested), and *Salmonella* spp. were found in none of them.

In the present study, yeast and mould were counted in samples of ground black pepper, red pepper, pimento, cumin, ground red pepper, garlic powder and sucuk spice mixes at the average levels of $7.5x10^4$ cfu/g (33.33%), $3.4x10^3$ cfu/g (29.41%), $1.3x10^4$ cfu/g (56.25%), $2.5x10^3$ cfu/g (35%), $4.0x10^4$ cfu/g (66.66%), $9.5x10^2$ cfu/g (22.22%), $1.3x10^4$ cfu/g (62.5%), respectively. Additionally, the highest contamination levels of yeast and moulds ($4.4x10^5$ cfu/g) were obtained from ground black pepper.

In the current study, 100% (n=42) of *B. cereus* isolates produced NHE enterotoxin. Further, 31 (73.8%) of the *B. cereus* isolates tested positive for both NHE and HBL enterotoxins.

The resistance of *B. cereus* isolates against penicillin G was found to be 92.8% (n=39), followed by ampicillin (83.3%; n=35), cephalotin (80.9%; n=34) and polimixin B (61.9%; n=26). Resistance to tetracycline (14.2%), kanamycin (11.9%), oleondamycin (2.3%), erythromycin (2.3%) and vancomycin (2.3%) were also observed in our study. No isolates were resistant to chloramphenicol, streptomycin or neomycin (Table 3).

In 2011, the Republic of Turkey Ministry of Food, Agriculture and Livestock established a maximum limit of 10^4 cfu/g spice of both coagulase-positive *S. aureus* and *B. cereus*. *Salmonella* spp. count should be zero in a 25 g sample. Varying counts of coliform bacteria have been reported in different spices. Elmali and Yaman (2005) indicated that coliform microorganism counts were $1.1x10^4$, $1.3x10^4$, $4.2x10^2$, $4.5x10^3$ and $1.0x10^3$ cfu/ g for black pepper, powdered red pepper, granulated red pepper, cumin and sumac, respectively. In another work (Vural, 2004), the average coliform counts of ground black pepper, red pepper, pimento, cumin and ground red pepper were $5.9x10^3$, $1.6x10^4$, $1.9x10^4$, $2.5x10^4$ and $9.9x10^4$ cfu/g, respectively.

These results are in accordance with the values found in the current study. On the other hand, the average coliform count of red pepper in this study ($9x10^1$ cfu/ g) was closer to those reported by other groups. A survey performed by Parveen et al. (2014) showed a coliform count of $7.20x10^1$ MPN/g in red pepper. In the present study, we didn't find any contamination with *E.coli* in the samples. However, a survey performed by Vural (2004) indicated that the average counts of *E. coli* were $4.8x10^3$, $1.6x10^4$, $4.2x10^2$, $5.4x10^2$ and $6.5x10^2$ cfu/g in ground black pepper, red pepper, pimento, cumin and ground red pepper, respectively. *E. coli* was found in 26.6% of the samples investigated by Elmali and Yaman (2005). In another work (Debs-Louka et al., 2013), *E. coli* counts ranged from <10 to $9x10^3$ cfu/g, <10 to $3x10^4$ cfu/g and <10 to $7x10^3$ cfu/g in black pepper powder, seven spices mixture and cumin powder, respectively.

Elmali and Yaman (2005) reported yeast and mould counts in black pepper, powdered red pepper, granulated red pepper and cumin samples at the average numbers of $3.8x10^5$, $1.2x10^5$, $3.4x10^6$, $4.2x10^4$ and $1x10^6$ cfu/g, respectively. These results are close to the bacterial counts obtained in our study. On the other hand, Parveen et al. (2014) counted yeast and mould at the average number of $1x10^2$ cfu/g in red pepper alone. Filiz (2001) reported the presence of yeast and mould at the average number of $5x10^2$ cfu/g. Vural (2004) reported $4x10^3$, $1.3x10^4$, $1.9x10^3$ and $1.5x10^4$ cfu/g of yeast and mould in ground black pepper, red pepper, pimento, cumin and ground red pepper, respectively, which is in agreement with the results of this study.

In the current study, *Salmonella* spp. was found in none of the spice samples. Similarly, Debs-Louka et al. (2013) and García et al. (2001) found no *Salmonella* spp. in a variety of spice samples. On the other hand, in the study of Sagoo et al. (2009), 2833 dried spices and herbs of different varieties were sampled across the United Kingdom in 2009, and thirty-one (1.1%) herb samples were found to have been contaminated with different *Salmonella* serovars at retail premises.

Konuma et al. (1988) reported that *B. cereus* was present in 39.7% of tested spices at levels between 10^2-10^4 cfu/g. Similar results were reported by Deambrosis and Da Silva (1992), who found that the rate of isolation of *B. cereus* from spice samples was 41%. Our results are consistent with these observations. On the other hand, Kneifel and Berger (1994) and Rosenberger and Weber (1993) reported the absence of *B. cereus* in spices.

In our study, the highest contamination levels (unsatisfactory) ($\geq 10^4$ cfu/g) were obtained from cumin ($4.8x10^4$ and $1x10^4$ cfu/g), ground black pepper (4.6×10^4, $1.1x10^4$ and $1x10^4$ cfu/g) and pimento (1.9×10^4 cfu/g) for *B. cereus*. A similar result has been observed by Aksu et al. (2000) who found the maximum *B. cereus* count was in cumin. Moreover, 8% (8 out of 100) of spices tested did not comply with (unsatisfactory or unacceptable quality) both the Turkish Food Codex criteria (2011) and the Commission Recommendation 2004/24/EC for B. cereus counts (*B. cereus* $\geq 10^4$ cfu/g). These findings are similar to data reported by several researchers (Powers et al., 1976; De Boer et al., 1985; Pafumi, 1986; Kovács-Domján, 1988; Giaccone et al., 1996; Te Giffel et al., 1997). In comparison to our results, higher (5.72 log10 cfu/g) and lower *B. cereus* levels (3.93 log10 cfu/g) were observed by Hampikyan et al. (2009) and Elmali and Yaman (2005), respectively. In another previous study, *B. cereus* was detected at levels $\geq 10^4$ cfu/g in 0.3% (2/647) of single spice samples (3rd Trimester National Microbiological Survey, 2005).

In a few extreme cases *B. cereus* at levels reaching 10^5-10^8 cfu/g have been reported in various spices (Banerjee and Sarkar; 2003; Antai, 1988). However, most reports indicate the presence of this pathogen at levels which are in agreement with our findings ($<3x10^3$ cfu/g). In other studies of the microbiological status of herbs and spices, *B. cereus* was detected at $\geq 10^4$ cfu/g in up to 49% of tested samples (Hampikyan et al., 2009; Little et al., 2003; Sagoo et al., 2009; Kneifel and Berger, 1994; Pafumi, 1986; Temelli and Anar, 2002).

In previous studies, Hariram and Labbe (2015) identified the production of both enterotoxins in 52% of *B. cereus* isolates from spice samples. The high prevalence of the NHE enterotoxin among *B. cereus* isolates has been demonstrated in many studies (Hariram et al., 2015; Guinebretiere et al., 2002; Moravek et al., 2006; Schoenı et al., 2005; Wehrle et al., 2009; Yang et al., 2005). We also obtained positive results for NHE in 100% (n=34) of our samples. Hariram and Labbe (2015) identified HBL enterotoxin production in 87% of *B. cereus* isolates.

Table 1. Presence of yeast & mold, coagulase positive S. aureus, *coliforms &* E. coli *in spice samples (n= 100)*

Sample type (st)	Microorganism							
	Yeast & mold		**Coagulase positive *S. aureus***		**Coliform & *E. coli***			
	No positive / no st (%)	**Mean (cfu/g)**	**No positive / no st (%)**	**Mean (cfu/g)**	**Coliform positive / no st (%)**	**Mean (cfu/g)**	***E. coli* positive / Coliform positive (%)**	**Mean (cfu/g)**
Red pepper	5/17 (29)	$3.4x10^{3}$	2/17 (12)	$1.6x10^{3}$	1/17 (6)	$9X10^{1}$	1/1 (100)	$9X10^{1}$
Ground red pepper	8/12 (67)	$4.0x10^{4}$	0/12 (0)	-	4/12 (33)	$2.3x10^{4}$	0/4 (0)	-
Ground black pepper	6/18 (33)	$7.5x10^{4}$	1/18 (6)	-	11/18 (61)	$2.7x10^{3}$	0/11 (0)	-
Pimento	9/16 (56)	$1.3x10^{4}$	1/16 (6)	-	8/16 (50)	$4.2x10^{3}$	0/8 (0)	-
Cummin	7/20 (35)	$2.5x10^{3}$	0/20 (0)	-	11/20 (55)	$1.7x10^{4}$	0/11 (0)	-
Garlic powder	2/9 (22)	$9.5x10^{2}$	0/9 (0)	-	2/9 (22)	$3.7x10^{2}$	0/2 (0)	-
Sucuk spice mix	5/8 (63)	$1.3x10^{4}$	1/8 (13)	-	8/8 (100)	$2.3x10^{4}$	2/8 (25)	$2.7x10^{3}$
Total	**42/100**		**5/100**		**45/100**		**3/100**	

Table 2. The results of testing for isolation, identification and toxin-producing capability of B. cereus *isolated from spices (n= 49)*

Sample	**Isolation**	**Identification**		Toxin production	
	***B. cereus* count (cfu/g)**	**MALDI-TOF-MS**		NHE	HBL
		Positive	**Log (score)**		
Red pepper (1)	$2x10^2$	+	2.053	+	+
Red pepper (15)	$4x10^2$	+	2.082	+	+
Red pepper (44)	$2x10^2$	+	1.842	+	+
Red pepper (75)	$1x10^2$	+	2.014	+	+
Red pepper (82)	$2x10^2$	+	1.958	+	-
Ground red pepper (6)	$1x10^3$	+	1.780	+	+
Ground red pepper (11)	$1.2x10^2$	-	-	-	-
Ground red pepper (49)	$2x10^3$	+	2.087	+	+
Ground red pepper (59)	$1x10^3$	+	2.234	+	+
Ground red pepper (68)	$3x10^3$	-	-	-	-
Ground red pepper (78)	$3x10^2$	+	2.294	+	-
Ground black pepper (3)	$3.4x10^3$	+	1.938	+	+
Ground black pepper (8)	$1x10^3$	+	1.834	+	+
Ground black pepper (13)	$4.6x10^4$	+	1.860	+	+
Ground black pepper (34)	$1x10^4$	+	2.114	+	+
Ground black pepper (40)	$9.8x10^3$	+	2.024	+	-
Ground black pepper (47)	$1x10^3$	+	1.998	+	-
Ground black pepper (57)	$2.2x10^3$	+	2.098	+	+
Ground black pepper (61)	$2x10^2$	+	2.136	+	+
Ground black pepper (62)	$2x10^3$	+	2.227	+	+
Ground black pepper (71)	$1x10^2$	+	2.153	+	+
Ground black pepper (79)	$1x10^3$	+	2.02	+	+
Ground black pepper (83)	$5x10^2$	+	2.138	+	-
Ground black pepper (84)	$1.1x10^4$	+	1.782	+	+
Pimento (2)	$1.9x10^4$	+	2.183	+	+
Pimento (7)	$1x10^3$	+	1.931	+	+
Pimento (12)	$7x10^3$	+	2.110	+	-
Pimento (18)	$2x10^3$	+	1.764	+	+
Pimento (33)	$1.4x10^3$	+	1.748	+	-
Pimento (43)	$2x10^2$	+	1.833	+	+
Pimento (50)	$3x10^2$	-	-	-	-
Pimento (58)	$2x10^2$	+	2.153	+	-
Cummin (4)	$4.8x10^4$	+	1.948	+	+
Cummin (9)	$1.1x10^3$	+	1.764	+	+
Cummin (16)	$1x10^4$	+	2.233	+	-
Cummin (46)	$2x10^2$	+	1.981	+	+
Cummin (72)	$1x10^2$	-	-	-	-
Cummin (76)	$2x10^2$	+	1.898	+	+
Garlic powder (10)	$2x10^4$	+	1.838	+	-
Garlic powder (17)	$3x10^3$	+	1.953	+	-
Garlic powder (51)	$1x10^3$	-	-	-	-
Garlic powder (55)	$3x10^2$	+	2.228	+	+
Garlic powder (70)	$1.5x10^4$	-	-	-	-
Garlic powder (95)	$4x10^2$	+	1.551	+	+
Sucuk spice mixes (19)	$7x10^2$	+	1.864	+	+
Sucuk spice mixes (45)	$1x10^2$	-	-	-	-
Sucuk spice mixes (74)	$7x10^3$	+	1.848	+	+
Sucuk spice mixes (89)	$2x10^2$	+	2.237	+	+
Sucuk spice mixes (100)	$1x10^2$	+	1.976	+	+

NHE: nonhaemolytic enterotoxin, HBL: haemolysin BL, + positive, - negative

Table 3. Number of resistant and susceptible B. cereus *isolates to 12 different antibiotics*

Antibiotics	No. of *B. cereus* isolates (n=42)	
	No. of resistant isolates	**No. of susceptible isolates**
Oleondamycin (15 µg)	1	39
Tetracycline (30 µg)	6	26
Polymixin B (300 U)	26	-
Chloramphenicol (30 µg)	-	38
Erythromycin (15 µg)	1	37
Penicillin G (10 U)	39	-
Cephalothin (30 µg)	34	7
Ampicillin (10 µg)	35	7
Kanamycin (30 µg)	5	26
Vancomycin (30 µg)	1	40
Streptomycin (10 µg)	-	42
Neomycin (30 µg)	-	42

Table 4. Multidrug resistance patterns observed among B.cereus *isolates (n= 39)*

Source	No. of multiple resistant isolates	Resistance pattern
Red pepper	1	P, PB
Ground red pepper	1	
Garlic powder	1	P, AMP
Cummin	1	P, PB, AMP
Sucuk spice mixes	2	P, KF, AMP
Pimento	2	
Ground black pepper	2	
Garlic powder	1	
Cummin	3	
Garlic powder	1	P, PB, KF
Cummin	1	
Ground red pepper	1	P, PB, KF, AMP
Pimento	3	
Red pepper	3	
Ground black pepper	5	
Sucuk spice mixes	1	
Ground red pepper	1	P, KF, AMP, TE
Garlic powder	1	
Cummin	1	P, PB, KF, AMP, TE
Ground black pepper	1	
Ground black pepper	4	P, PB, KF, AMP, K
Ground black pepper	1	P, PB, KF, AMP, TE, K
Pimento	1	P, KF, AMP, VA, E, OL

Ol, oleandomycin; Te, tetracycline; Pb, polymixin B; C, chloramphenicol; E, erythromycin; P, penicillin G; Kf, cephalothin; Amp, ampicillin; K, kanamycin; Va, vancomycin; S, streptomycin; N, neomycin

The broad application of antimicrobials in agriculture and veterinary medicine may lead to the emergence of resistant pathogens. These pathogens may enter the food chain through various foods, including meat and meat products, and may potentially affect human health (Khachatourians, 1998; Phillips et al., 2004). A remarkable variation to a wide range of antimicrobial agents has been described in the resistance of B. cereus isolated from spices in studies worldwide. Multiple antibiotic-resistant B. cereus has been reported to be associated with some food samples (Tewari et al., 2012; Meena et al., 2000).

A total of 41 (97.6%) B. cereus isolates were resistant to at least one antibiotic agent (Table 3) and 39 (92.8%) were resistant to two or more antibiotic agents (Table 4). This is in agreement with previous data that found high resistance rates in bacterial populations from spices (Hassan and Altalhi, 2013; Brown and Jiang, 2008). In only one isolate from red pepper (3% of total isolates from this source), no resistance was found (data not shown). In addition, 11 different patterns of multiple resistance were detected among the B. cereus isolates ranging from resistance to 2 drugs to resistance to 6 drugs. The most common multiple antibiotic resistance pattern was penicillin G, polymyxin B, cephalothin and ampicillin, which was observed in 33.3% (n=13) of isolates.

Previous work has shown that B. cereus isolates are highly susceptible to chloramphenicol and tetracycline and less sensitive to penicillin (Whong and Kwaga, 2007). In respect to chloramphenicol, penicillin and tetracycline resistance, our results align with those of Whong and Kwaga (2007), who determined chloramphenicol, penicillin and tetracycline resistance percentages of 0%, 82% and 6.7%, respectively, for B. cereus isolates from various foods, including spice samples.

4. Conclusions and Recommendations

To the authors' best knowledge, this is the first report on the incidence of toxigenic and multidrug resistant B. cereus in spices in Turkey.

As shown in this study, a significant subset of the spices studied contained high numbers of B. cereus. Further, multidrug resistance and toxin production were observed in a majority of the isolates. The multidrug resistance patterns observed in this study suggest that these antibiotic agents are highly abused and can be found at sublethal doses in the environment. Occurrence of multidrug resistance and diarrhoeal toxin-producing B. cereus in high proportions of spices used in meat products may pose a significant public health hazard (Tewari, 2012).

Manufacturers should understand the microbiological risks involved in production of meat products and should apply a farm-to-table approach to food safety. Manufacturers may also use sterilized spices to reduce the risk of contamination of meat products with these microorganisms. As a recommendation, we can say that manufacturers should source spices from certified suppliers complying with systems such as QA, GMP and ISO systems. They should also apply GMP rules, and HACCP systems should be applied in their premises.

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