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Extracellular enzyme production and enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from cheese in Turkey

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ABSTRACT

The aim of the present study was to investigate the biochemical characteristics, extracellular enzyme production and enterotoxigenic genes contents of 6 *Bacillus cereus* and 22 *Bacillus thuringiensis* strains, isolated from 100 cheese samples in Turkey. Crystal morphologies of *B. thuringiensis* strains were found either spherical ($n = 12$) or spherical and irregular-shaped ($n = 10$) by phase contrast microscopy. *B. cereus* and *B. thuringiensis* strains were found to produce extracellular enzymes, respectively: gelatinase (83% and 91%), DNase (83% and 77%), lecithinase (83% and 95%), protease on skim milk agar (100% and 100%), protease on milk agar (100% and 91%), protease on casein agar (83% and 77%), xylanase (100% and 45%), and cellulase (0% and 41%), and amylase (83% and 27%). All of the strains, except for Bt-D1, hydrolyzed Tween 20 (96%), but not Tween 80 or tributyrin. Pectinolytic activity was obtained to be the least frequent (4%). PCR analysis showed that all strains contained *nheA*, *nheB*, *nheC* and *hblD* genes. The *hblA* and *hblC* genes were present in 2 and 4 of *B. thuringiensis* strains, respectively. The *bceT* gene was detected in 1 *B. cereus* and 9 *B. thuringiensis* strains. The *entFM* gene was detected more frequently in *B. thuringiensis* (82%) than in *B. cereus* strains (50%). To our knowledge, this is the first report about the isolation and identification of enterotoxigenic *B. cereus* and *B. thuringiensis* strains from cheese samples in Turkey.

1. Introduction

Bacillus cereus is considered to be a common food pathogen and *Bacillus thuringiensis* is distinguishable from this organism by the production of insecticidal crystal proteins (δ-endotoxins) inside the cell during sporulation (Höfte & Whiteley, 1989). These two species are genetically similar and plasmids encoding for insecticidal crystals can be transferred to *B. cereus*, making the *B. cereus* a crystal-producing *B. thuringiensis* (Gonzáles, Brown, & Calton, 1982). Therefore, they can be considered as one species on the basis of genetic evidence (Chen & Tsen, 2002).

In dairy products, the presence of *Bacillus* spp. is inevitable and the spore-formation of this organism allows it to easily survive after pasteurization (Christiansson, Bertilsson, & Svensson, 1999). The growth of *B. cereus* spoils the quality of dairy products due to the production of protease, lipase and phospholipase, and these in turn, causes some defects such as off-flavours, sweet curdling and bitty cream (Davies & Wilkinson, 1973; Meer, Baker, Bodyfelt, & Griffiths, 1991). Therefore, in milk and dairy products, it decomposes casein into peptides and amino acids, and milk fat into free fatty acids, thus degrading the quality of milk products and shortening their shelf life (Torkar & Matijašić, 2003). The occurrence of *B. cereus* as a contaminant of cheese was previously reported (Helmy, El-Bakey, & Mohamed, 1984; Iurlina, Saiz, Fuselli, & Fritz, 2006; Tham, Hadju, & Danielsson-Tham, 1990). Although *B. thuringiensis* has been isolated from creamery silo milk (Phillips & Griffiths, 1986), pasta, bread, milk (Damgaard, Larsen, Hansen, Bresciani, & Jørgensen, 1996), and ready-to-eat food (Rosenquist, Smidt, Andersen, Jensen, & Wilcks, 2005), no information about the prevalence of *B. thuringiensis* in cheese has been previously published.

B. cereus produces several toxins, including emetic toxin and diarrhoeal enterotoxins. Hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe) and cytotoxin K (CytK) proteins are considered as the primary virulence factors in *B. cereus* diarrhea (Granum, O'Sullivan & Lund, 1999; Lund, Debuyser, & Granum, 2000) and the other toxins enterotoxin FM (EntFM), enterotoxin T (BceT) or haemolysin II (HlyII) are regarded as three possible enterotoxins (Dohmae et al., 2008). HBL complex is composed of three proteins, B, L_1 and L_2 (Beecher & Wong, 1994; Beecher & Wong, 1997) transcribed from the genes *hblC* (encoding L_2), *hblD* (encoding L_1), and *hblA* (encoding B) (Heinrichs, Beecher, MacMillan, & Zilinskas, 1993; Ryan, Macmillan, & Zilinskas, 1997). NHE complex is also composed of three proteins, NheA, NheB and NheC encoded by the three genes *nheA*, *nheB* and *nheC*, respectively (Granum, O'Sullivan, & Lund,

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1999). Also, *B. thuringiensis* strains, including the strains used in commercial insecticides are reported to encode and produce diarrhoeal enterotoxins similar to those produced by *B. cereus* (Damgaard et al., 1996; Damgaard et al., 1997; Jensen et al., 2002a; Perani, Bishop, & Vaid, 1998).

This is the first study to determine the presence of *B. cereus/B. thuringiensis* strains in Turkish cheese and their enterotoxin gene content. Our purpose was (i) to isolate *B. cereus/B. thuringiensis* from cheese samples collected from different regions of Turkey, (ii) to identify the isolates using biochemical tests (iii) to determine the extracellular enzyme productions and (iv) to search the presence of *hblA*, *hblC* and *hblD* genes of the HBL complex; the *nheA*, *nheB* and *nheC* genes of the NHE complex; *bceT* and *entFM* genes.

2. Materials and methods

2.1. Isolation of the strains

A total of 100 cheese samples were collected from different regions of Turkey (Table 1). First, ten-fold 10 g of each sample was added into 90 ml 0.1% (wv^{-1}) peptone water. The samples were mixed well, homogenized by vigorous vortexing at room temperature for 3 min. Ten-fold dilution was prepared in 20% (vv^{-1}) glycerol–peptone water. A 50 μl aliquot from this dilution was inoculated into 5 ml Nutrient Broth (NB) (Applichem) and incubated at 37 °C for 18 h with shaking at 150 rpm. The tubes were pasteurized at 80 °C for 10 min to eliminate non-sporulating bacteria. The suspension was streaked onto chromogenic *B. cereus* agar (BCA) supplemented with chromogenic *B. cereus* selective supplement (Oxoid). The plates were incubated at 37 °C overnight and blue/green colonies were subcultured on chromogenic BCA until obtaining a pure culture. After identification by biochemical tests (Gram staining and catalase test), the isolated strains were stored in sterile NB containing 20% (vv^{-1}) glycerol at −80 °C.

2.2. Phase contrast microscopy

The strains belonging to *B. cereus/B. thuringiensis* group were streaked on T3 medium (tryptone 3 g l^{-1}, tryptose 2 g l^{-1}, yeast extract 1.5 g l^{-1}, sodium phosphate 0.05 M [pH 6.8], MnCl 0.005 g l^{-1} and agar 15 g l^{-1}) (Travers, Martin, & Reichelderfer, 1987) and examined by a phase contrast microscope (Olympus CX31) and image analyzing system (Olympus DP-2 BSW) in order to investigate the presence of crystal proteins after 24- and 48-h incubations at 37 °C. Those producing parasporal inclusions were assigned to *B. thuringiensis*, while the parasporal inclusion-lacking isolates were classified as *B. cereus* (Ohba & Aratake, 1994).

Table 1
Distribution of *B. cereus* (Bc) and *B. thuringiensis* (Bt) strains and the sampling regions in Turkey.

Sampling region	No. of samples	No. of samples containing Bc/Bt	No. of isolated Bc strains	No. of isolated Bt strains
Nevsehir	35	1	–	3
Edirne	16	1	–	2
Izmir	14	4	1	8
Manisa	14	1	–	2
Tekirdag	11	3	4	5
Ardahan	4	–	–	–
Erzincan	2	1	1	1
Konya	1	–	–	–
Tunceli	1	–	–	–
Mugla	1	1	–	1
Kutahya	1	–	–	–
Total	100	12	6	22

2.3. Biochemical characteristics of the strains

Hemolysis and motility tests were performed according to the methods by Ehling-Schulz et al. (2005) and Guttmann and Ellar (2000), respectively. Utilization of 19 carbohydrates and growth of the strains in 2.5%, 5%, 7.5% and 10% (wv^{-1}) NaCl in NB was analyzed in 96-well plates.

2.4. Extracellular enzyme production

The strains were screened for extracellular enzyme productions at 37 °C according to the protocols described by the following studies: gelatin liquefaction (Harrigan, 1998), lecithinase activity (Guttmann & Ellar, 2000), xylanase, amylase and cellulase production (Bragger, Daniel, Coolber, & Morgan, 1989), and pectinolytic activity on pectinase I medium (yeast extract 1 g l^{-1}, $(NH_4)_2SO_4$ 2 g l^{-1}, Na_2HPO_4 6 g l^{-1} K_2PO_4 3 g l^{-1}, polygalactronic acid 5 g l^{-1}, agar 15 g l^{-1}) and pectinase II medium (yeast extract 1 g l^{-1}, $(NH_4)_2SO_4$ 2 g l^{-1}, Na_2HPO_4 6 g l^{-1}, KH_2PO_4 3 g l^{-1}, pectin from citrus peel 5 g l^{-1}, agar 15 g l^{-1}) (Kobayashi et al., 1999). Lipolytic activity of the strains was determined using Tween 20, Tween 80 (Haba et al., 2000) and tributyrin agar medium (Merck). Protease screening was performed on skim milk agar (Priest, Goodfellow, & Todd, 1988), milk agar (Nutrient Agar (NA) containing 10% (vv^{-1}) UHT milk) and casein agar (casein 10 g l^{-1}, yeast extract 3 g l^{-1}, NaCl 5 g l^{-1} and agar 20 g l^{-1}, pH 7.4). DNase production was examined on DNase agar media (Oxoid).

2.5. Genomic DNA isolation

Genomic DNA was extracted by first freezing and then boiling the cells (Bravo et al., 1998). Strains were grown at 37 °C for 16 h. A loopful of cells was scraped off from NA plate and resuspended in 150 μl sterile water. After 20 min freezing at −80 °C, the samples were boiled for 10 min to lyse the cells completely. Cell debris was removed by centrifugation (11000 g, 10 s). The supernatant containing genomic DNA was stored at −20 °C.

2.6. Examination of enterotoxin genes

The strains were tested for the presence of enterotoxin genes (*nheA*, *nheB*, *nheC*, *hblA*, *hblC*, *hblD*, *bceT* and *entFM*). The primer sets used in this study are presented in Table 2. Each amplification process was performed in a 50 μl reaction mixture containing 100 ng

Table 2
The primer sequences and amplicon sizes used in PCR analysis.

Gene	Primer sequences (5′→3′) F: forward; R: reverse	Amplicon size (bp)	Reference
bceT	F-TTACATTACCAGGACGTGCTT R-TGTTTGTGATTGTAATTCAGG	428	Agata et al. (1995)
entFM	F-ATGAAAAAAGTAATTTGCAGG R-TTAGTATGCTTTTGTGTAACC	1269	Asano, Nukumuzi, Bando, Iizuka and Yamamoto (1997)
hblA	F-GCTAATGTAGTTTCACCTGTAGCAAC R-AATCATGCCACTGCGTGGACATATAA	874	Mäntynen and Lindström (1998)
hblC	F-CGAAAATTAGGTGCGCAATC R-TAATATGCCTTGCGCAGTTG	411	Moravek et al. (2004)
hblD	F-AGGTCAACAGGCAACGATTC R-CGAGAGTCCACCAACAACAG	206	Moravek et al. (2004)
nheA	F-ATTAAGGTAAATGCGATGAG R-GCTTCAGTTTGTGATAACTT	671	Jensen et al. (2002b)
nheB	F-CTATCAGCACTTATGGCAG R-ACTCCTAGCGGTGTTCC	769	Granum et al. (1999)
nheC	F-TGGATTCCAAGATGTAACG R-ATTACGACTTCTGCTTGTGC	683	Guinebretiére et al. (2002)

of genomic DNA as the template, 5 μl of 10× reaction buffer (100 mM Tris–HCl (pH 8.8), 500 mM KCl, 0.8% (vv^{-1}) Nonident P-40, and 1.5 mM $MgCl_2$), 10 μM of each of the primers, 0.2 mM each of the four dNTPs (Fermentas) and 2 U *Taq* DNA polymerase (Fermentas). The diarrhoeagenic strain of *B. cereus* (ATCC 14579) was used as a positive control. PCR products were analyzed in 1.5% (wv^{-1}) TAE agarose gels and all PCR experiments were performed twice for each strain.

3. Results

3.1. Identification and phase contrast microscopy

The prevalence of *B. cereus*/*B. thuringiensis* strains in the cheese samples is shown in Table 1. In total, 100 cheese samples were analyzed and 12 of them were found to contain *B. cereus*/*B. thuringiensis* strains. Twenty-eight isolates with the typical phenotypic characteristics of *B. cereus*/*B. thuringiensis* on chromogenic BCA were isolated. All *B. cereus*/*B. thuringiensis* strains were found to produce spores after 24 h incubation and 22/28 (79%) strains of *B. cereus*/*B. thuringiensis* were assigned to *B. thuringiensis* due to the formation of crystal proteins by phase contrast microscopy (Fig. 1). Spherical crystals were found in 12 strains, while spherical and irregular-shaped crystals were found in the remaining 10 *B. thuringiensis* strains.

3.2. Biochemical characteristics

In this study, 5 *B. cereus* and 21 *B. thuringiensis* strains exhibited β-hemolysis, confirming the presence of a hemolytic enterotoxin. All *B. cereus* and 21 *B. thuringiensis* strains were actively-motile after 72 h incubation. All strains produced acid from glucose, ribose and maltose; however, arabinose, mannitol, xylose, rhamnose, melebiose, raffinose, and sorbitol were not utilized by the strains. Acid production from other carbohydrates was variable (Table 3). In addition, all strains were able to grow in the presence of 2.5% and 5% (wv^{-1}) NaCl.

3.3. Extracellular enzyme production

The most frequent extracellular enzyme activities were as follows for *B. cereus*/*B. thuringiensis* strains, respectively: gelatin hydrolysis (83% and 91%), DNase (83% and 77%) and lecithinase production (83% and 95%), protease on skim milk agar (100% and 91%), milk agar (83% and 77%), and casein agar (83% and 77%), amylase (83% and 27%) and xylanase (100% and 45%). All of the strains except Bt-D1 hydrolyzed Tween 20 (96%), but not Tween 80 and tributyrin. Cellulase was only produced by 41% of *B. thuringiensis* strains. Pectinolytic activity was obtained to be the least frequent activity; which was only produced by the strain Bt-L11C on pectinase II medium (Table 3).

According to the production of extracellular enzymes, 13 different patterns were observed among 6 *B. cereus* and 22 *B. thuringiensis*. *B. cereus*/*B. thuringiensis* strains could be divided into two and twelve groups, respectively (Table 3). Among *B. cereus* strains, most strains ($n = 5$) belonged to group I, and one strain to group II. According to the presence of extracellular enzymes in *B. thuringiensis*, identical strains were obtained among groups XIII ($n = 4$), IV ($n = 4$), XII ($n = 3$), III ($n = 2$) and VI ($n = 2$).

3.4. Examination of enterotoxin genes

The distribution of enterotoxin genes among strains is shown in Fig. 2. It was found that all *B. cereus*/*B. thuringiensis* strains contained *nheA*, *nheB*, *nheC* and *hblD* genes. *B. cereus* strains were devoid of *hblA* and *hblC* genes. However, 2 *B. thuringiensis* strains contained *hblA* and 4 *B. thuringiensis* strains had *hblC*. The *bceT* gene was detected only in 1 *B. cereus* and 9 *B. thuringiensis* strains. Interestingly, the *entFM* gene was detected more frequently in *B. thuringiensis* (82%) than in *B. cereus* (50%). Based on the results of the PCR, *B. cereus*/*B. thuringiensis* strains were divided into three and seven groups. Also, the strains which were identical in enzyme patterns were not identical at the level of enterotoxin genes (Table 3).

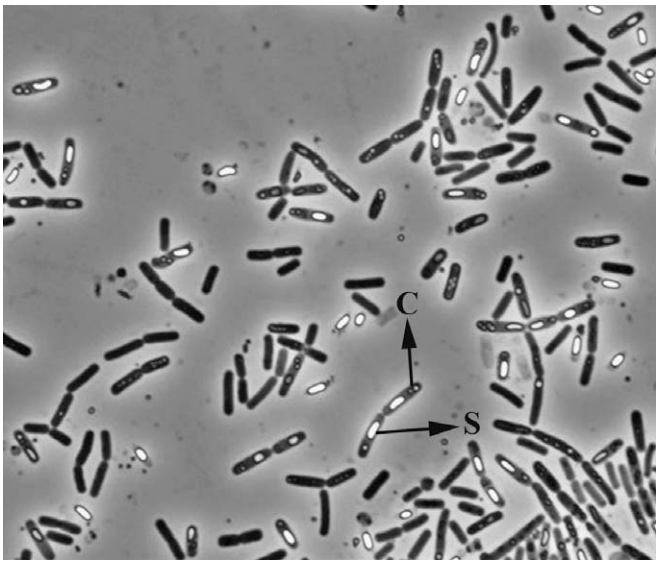

Fig. 1. Phase contrast microscope image of a 48-h sporulated culture of *B. thuringiensis* strain Bt-L11B. The arrowheads indicate S, spore and C, crystal.

4. Discussion

B. cereus/*B. thuringiensis* strains, isolated from different Turkish cheese samples, were well characterized in this study on the basis of a number of biochemical and genotypic characteristics. *B. cereus*/*B. thuringiensis* strains were detected in 12% of the samples. The fact that cheese is a good source for the isolation of *B. cereus* (Cosentino, Mulargia, Pisano, Tuveri, & Palmas, 1997; Iurlina et al., 2006; Rukure & Bester, 2001), but there has been no information about the isolation of *B. thuringiensis* strains from cheese. Rukure and Bester (2001) reported that *B. cereus* spores can survive, germinate and grow into vegetative cells during the early stages of cheese manufacturing process, but not during the final stages of the process or ripening. Ahmed, Moustafa, and Marth (1983) examined the presence of *B. cereus* in 400 milk and dairy products and they isolated *B. cereus* strains from 9%, 14%, 35%, and 48% of raw milk, cheese, pasteurized milk and ice cream samples, respectively. The low incidence of *B. cereus*/*B. thuringiensis* strains can be explained due to the textural characteristics of Turkish white cheese. It has a soft texture when fresh, after ripening it becomes semi-hard or semi-soft (Öner, Karahan, & Aloğlu, 2006). In fact, the type of cheese samples affects the potential growth of *B. cereus*. In soft cheeses (refrigerated below 8 °C), the incidence of *B. cereus* was 4% (Tham et al., 1990), while this ratio was 50% in hard cheeses (Helmy et al., 1984).

Most of the isolated *B. cereus*/*B. thuringiensis* strains were found to produce extracellular enzymes: lecithinase, gelatinase, lipase and protease. These enzymatic activities are very important for dairy industry. Lipolytic activity was shown as the reason for both rancid and fruity off-flavours in milk products (Downey, 1980; Johnston & Bruce, 1982). In addition, proteolytic activity is related to spoilage of UHT milk, sweet curdling of milk, bitterness, other off-flavours in cheese, and textural or body defects in cultured dairy products (Cousin, 1982; Dairy Research Foundation, 1986).

Table 3
Biochemical characteristics, extracellular enzyme production and enterotoxin gene contents of *B. cereus* (Bc) and *B. thuringiensis* (Bt) strains.

	Biochemical characteristics											Extracellular enzyme production												Enterotoxin gene contents								
	Fermentation of carbohydrates																															
Strain code	Motility	Hemolysis	Mannose	Fructose	Salicin	Sucrose	Galactose	Lactose	Melesitose	Trehalose	Glycine	Amylase	Gelatinase	Lecithinase	DNase	Protease on Milk Agar	Protease on Casein Agar	Lipase on Tween 20	Xylanase	Cellulase	Pectinase	Group	*hblA*	*hblC*	*hblD*	*nheA*	*nheB*	*nheC*	*entFM*	*bceT*	Group
Bc-T9A	+	+	–	+	+	–	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	I	–	–	+	+	+	+	–	–	A
Bc-T9A1	+	+	–	+	+	+	+	+	–	–	–	+	+	+	+	+	+	+	+	–	–	I	–	–	+	+	+	+	–	–	A
Bc-T9B	+	+	–	+	+	–	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	I	–	–	+	+	+	+	+	–	B
Bc-T11A1	+	+	–	+	+	–	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	I	–	–	+	+	+	+	+	–	B
Bc-MN6A	+	+	–	+	+	–	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	I	–	–	+	+	+	+	–	–	A
Bc-E2	+	–[b]	–	+	–	–	–	–	–	+	–	–	–	–	–	+	–	+	+	–	–	II	–	–	+	+	+	+	+	+	C
Bt-T4A	+[a]	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	III	+	+	+	+	+	+	+	+	D
Bt-T4A1	+	+	–	+	+	–	+	+	–	+	+	+	+	+	–	+	+	+	+	+	–	III	+	+	+	+	+	+	–	+	E
Bt-T9B1	+	+	–	+	+	–	+	+	–	–	–	+	+	+	+	+	+	+	+	–	–	I	–	–	+	+	+	+	–	–	A
Bt-T9B2	+	+	–	+	+	–	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	I	–	+	+	+	+	+	+	–	F
Bt-T11A	+	+	–	+	+	–	+	+	–	–	–	+	+	+	+	+	+	+	+	–	–	I	–	+	+	+	+	+	–	–	G
Bt-MN6	+	+	–	+	+	–	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	I	–	–	+	+	+	+	–	–	A
Bt-T16B	+	+	–	+	–	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	–	IV	–	–	+	+	+	+	+	–	B
Bt-T16A1	+	+	+	+	+	+	+	+	–	+	+	–	+	+	+	+	+	+	+	+	–	V	–	–	+	+	+	+	+	–	B
Bt-MN13	+	+	–	+	–	+	+	+	–	+	–	–	+	+	+	+	–	+	+	–	–	VI	–	–	+	+	+	+	+	–	B
Bt-MN13A	+	+	–	+	–	–	+	+	–	+	–	–	+	+	+	+	–	+	+	–	–	VI	–	–	+	+	+	+	+	+	C
Bt-E3	+	–	–	+	–	–	–	–	–	+	–	–	–	–	–	+	–	+	–	–	–	VII	–	–	+	+	+	+	+	+	C
Bt-D1	+	+	–	+	–	+	–	–	–	+	+	–	+	+	+	+	+	–	–	–	–	VIII	–	–	+	+	+	+	+	–	B
Bt-L3A	+	+	–	+	–	+	–	+	–	+	+	–	+	+	–	–	–	+	+	–	–	IX	–	–	+	+	+	+	+	–	B
Bt-L3B	+	+	–	+	–	+	+	+	–	+	+	–	+	+	–	–	–	+	–	+	–	X	–	–	+	+	+	+	+	–	B
Bt-L11C	–	+	–	–	–	–	–	+	–	+	+	–	+	+	+	+	+	+	–	+	+	XI	–	–	+	+	+	+	+	+	C
Bt-L11B	+	+	+	+	+	–	–	+	–	+	+	–	+	+	+	+	+	+	–	+	–	XII	–	–	+	+	+	+	+	+	C
Bt-L10	+	+	+	+	+	–	+	+	–	+	+	–	+	+	+	+	+	+	–	+	–	XII	–	–	+	+	+	+	+	+	C
Bt-L10A	+	+	–	+	+	–	+	+	–	+	+	–	+	+	+	+	+	+	–	+	–	XII	–	–	+	+	+	+	+	+	C
Bt-L10B	+	+	–	+	+	–	+	+	–	+	+	–	+	+	+	+	+	+	–	–	–	XIII	–	–	+	+	+	+	+	–	B
Bt-N15A	+	+	–	+	–	+	–	–	–	+	+	–	+	+	+	+	+	+	–	–	–	XIII	–	–	+	+	+	+	+	+	C
Bt-N15A1	+	+	–	+	–	+	+	+	–	+	+	–	+	+	+	+	+	+	–	–	–	XIII	–	–	+	+	+	+	+	–	B
Bt-N15B	+	+	–	–	–	+	–	+	–	+	+	–	+	+	+	+	+	+	–	–	–	XIII	–	–	+	+	+	+	+	–	B
ATCC 14579	+	+	–	+	+	+	–	+	–	+	–	+	+	+	–	+	–	–	–	+	–	XIV	+	+	+	+	+	+	+	+	D

[a] Positive, [b] Negative

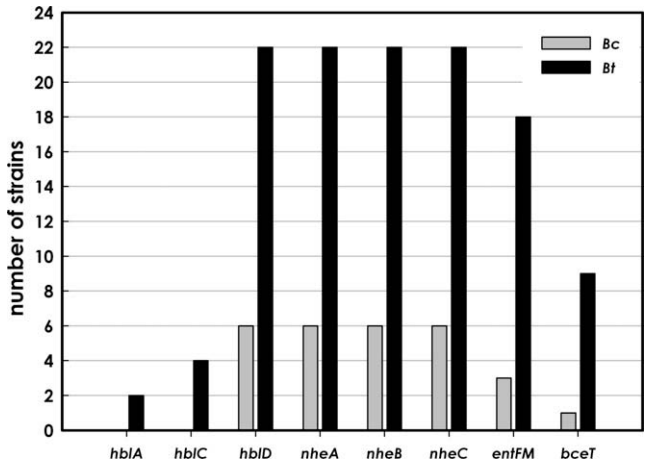

Fig. 2. Distribution of enterotoxin genes among *B. cereus* and *B. thuringiensis* strains.

It is known that *B. cereus* strains isolated from dairy products adapt to environmental conditions, especially for the fermentation of lactose which is an uncommon carbon source. In this study, 83% *B. cereus* and 86% *B. thuringiensis* strains were able to ferment lactose. Similarly, te Giffel, Beumer, Granum, and Rombouts (1997) observed that 20% of 134 *B. cereus* strains isolated from pasteurized milk samples could ferment lactose, whereas this ratio was less than 1% in the strains isolated from non-dairy products (te Giffel, Beumer, Leijendekkers, & Rombouts, 1996).

The presence of diarrhoeal enterotoxin genes in the isolated strains was screened by PCR (Gaviria Rivera, Granum, & Priest, 2000; Hansen & Hendriksen, 2001; Tsen, Chen, Hsieh, Sheu, & Chen, 2000; Zahner et al., 2005). In the detection of enterotoxin genes, all strains were found to carry the three *nhe* genes. Recently, it has been shown that almost all *B. cereus/B. thuringiensis* strains contain *nhe* genes (Beecher, 2001; Gaviria Rivera et al., 2000; Guinebretiére, Broussolle & Nguyen-The, 2002; Hansen & Hendriksen, 2001). In our study, all strains were also found to contain *hblD* gene. In another study, Hansen and Hendriksen (2001) reported that 95% *B. thuringiensis* strains and 64% *B. cereus* strains contained this gene. *B. cereus* strains did not contain the *hblA* and *hblC* genes, while the prevalence of these two *hbl* genes was low in *B. thuringiensis* strains, only 2 (9%) and 4 (18%) strains were positive for *hblA* and *hblC* genes, respectively. Mäntynen and Lindström (1998) detected *hblA* gene in 52% of their *B. cereus* strains and only in 1/41 of their *B. thuringiensis* strains. On the other hand, Hansen and Hendriksen (2001) reported the high prevalence of the *hblA* gene in 31/41 *B. thuringiensis* and in 13/22 *B. cereus* strains. They also showed that 40 *B. thuringiensis* (98%) and 16 *B. cereus* (73%) strains contained *hblC*.

The presence of *bceT* gene was relatively rare in our *B. cereus* (17%) and *B. thuringiensis* (41%) strains and this situation was also observed in the other studies (Agata, Ohta, Arakawa, & Mori, 1995; Granum et al., 1996; te Giffel et al., 1997; Hsieh, Sheu, Chen, & Tsen, 1999). On the other hand, Zahner et al. (2005) reported that *bceT* was found in 86% of *B. thuringiensis* strains and in 78% of *B. cereus* strains. The *entFM* gene was detected at a significantly higher proportion among *B. thuringiensis* strains (82%) than detected in the *B. cereus* strains (50%). In a previous study, Hsieh et al. (1999) found the *entFM* gene in 93% of *B. cereus* strains and in 78% of *B. thuringiensis* strains.

PCR analysis is used as a routine detection method for the enterotoxin genes in a large variety of strains (Svensson et al., 2007). Lower sensitivity levels are observed due to high degree of molecular heterogeneity in genes of Hbl and Nhe components leading to false negative PCR results (Schoeni & Wong, 1999; Guinebretiére et al., 2002). PCR is a specific and accurate method, but it only identifies potential HBL producing bacteria that may not always correlate to the expression of toxin components (Kumar, Muralli, & Batra, 2008). Therefore, the use of combined methods such as immunology and PCR may be necessary to detect the presence of toxin genes (Svensson et al., 2007; Ouoba, Thorsen, & Varnam, 2008).

The majority of our strains exhibited hemolysis and these results also confirmed the presence of a hemolytic enterotoxin. It is interesting to note that 26 strains that were negative for *hblA* gene also showed hemolytic activity on blood agar. A similar result was reported by Gray, Banada, O'Neal and Bhunia (2005). They could not find the *hblA* gene in three strains which also showed hemolytic activity. They suggested that the PCR primers were targeted only toward the B component of the hemolytic gene. Although this component was not present in these strains, two L components of HBL might be found to exhibit hemolysis (Beecher, Schoeni, & Wong, 1995; Beecher & Wong, 1997). We could not find any correlation between the *hbl* genes and hemolysis. Ouoba et al. (2008) indicated that the production of toxins is not necessarily related to hemolytic activity. Only two strains were found to contain the three genes for the HBL complex. The other strains showing hemolysis contained one or two of *hbl* genes. Two strains which did not show hemolysis were found to contain the *hblD* gene by PCR.

In conclusion, *B. cereus/B. thuringiensis* strains isolated from cheese samples should be regarded as potential enterotoxin producers according to PCR results. Therefore, much more detailed research on the phenotypic expression of enterotoxins should be performed. In the light of this study, it is important to prevent the contamination of cheese by *B. cereus/B. thuringiensis* strains. Moreover, new attempts should be undertaken to eliminate these organisms during cheese manufacturing for food safety and quality.

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