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Full Length Research Paper

Determination of antibiotic susceptibility and fatty acid methyl ester profiles of *Bacillus cereus* strains isolated from different food sources in Turkey

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In this study, a total of 77 *Bacillus cereus* isolates were obtained from four different food samples (58 raw milks, 8 chickens, 7 cereals and 4 meats) consumed in Turkey by using Chromogenic Bacillus Cereus Agar (Oxoid, CM1036). They were tested for susceptibilities to a total of 10 different antibiotics (penicillin, oxacillin, sulphamethoxazole, rifampicin, apromycin, amikacin, tobramycin, kanamycin, gentamicin and oflaxacin). In addition, they were determined to be in fatty acid methyl esters (FAMEs) group of the strains. All the isolates were identified as *B. cereus* based on colonical, cellular morphology and biochemical characters, including FAME analysis. A total of 25 different fatty acids were detected in 77 strains tested, but 16 of them appeared as minor components, in less than 2%. The strains had 15:0 iso 3OH (30.25%), 16:0 iso (11.23%), 17:0 iso (9.20%),16:0 (9.02%),13:0 iso (8.85%), 14:0 iso (6.79%), 15:0 anteiso (4.83%), 14:0 (4.58%), 16:1 ω6c (3.92%) and 16:1 ω7c (3.92%) as the major fatty acids (FA). Based on FAME analysis, the isolates were clustered into three main groups. Cluster 1, 2 and 3 were composed of the 71, 3 and 3 strains, respectively. All the strains in each cluster showed an extremely high degree of similarity (95 - 100%) to each other. Antibiotic resistant profile showed that all strains were resistance to penicillin and oxacillin, but they were highly susceptible to gentamicin and oflaxacin.

Key words: Antibiotic, *Bacillus cereus*, FAME, fatty acid, MIS, resistance.

INTRODUCTION

Bacillus cereus is a Gram-positive, facultative anaerobic and spore-forming bacterium. It is widely distributed in nature and a common contaminant of foods such as rice, spices, meat, cereal, various vegetables, egg and dairy product (Johnson, 1984; Slaghuis et al., 1997). *B. cereus* is found in both vegetative cell and endospore form (Ankolekar et al., 2009). Spores of various *Bacillus* species are metabolically dormant and can remain in this state for a long period of time (Prestamo et al., 2007). Under certain conditions, strains of this species produce haemolysins, phospholipases C and also emetic toxins and enterotoxins (Rusul and Yaacob, 1995; Andersen-Borge et al., 2001; Agata et al., 2002).

In recent years, there have been an increasing number of reports establishing *B. cereus* as a food-spoilage and food-poisoning organism. It can cause two types of illness: the diarrhoeal form and the emetic form (Blakey and Priest, 1980). Whereas the former type is caused by complex enterotoxins produced during vegetative growth in the small intestine, the latter type is produced by growing cells in the food (Granum and Lund, 1997; Michelet et el., 2006; Roy et al., 2007).

Bacillus species are the most frequently isolated bacilli from natural environments. But accurate identification for these bacterial species is difficult in many cases since they share many important morphological and biochemical properties (Kwon et al., 2009). Recently, whole-cell fatty acid (FA) analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), carbon pattern utilization, genomic profiling and microarray techniques are

Abbreviations: FAMEs, Fatty acid methyl esters; **FA,** fatty acids; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **MIS,** microbial ıdentification system; **NA,** nutrient agar; **NB,** nutrient broth; **TSA,** trypticase soy agar.

commonly used molecular identification techniques. Whole-cell fatty acid methyl esters (FAMEs) profiles have been used in bacterial classification for over 35 years and have become increasingly important in bacterial identification. The first genus-wide FAME analysis of the genus *Bacillus* was done by Kampfer (1994) (Slabbinck et al., 2008). FAMEs analysis using the Sherlock Microbial Identification System (MIDI, Inc., Newark, DE, USA) (MIS) is a reliable, fast, cost-effective and sensitive method for identification of bacteria from various sources (Roy, 1988; Sasser, 1990).

Strains of food-borne bacterial pathogens that are resistant to a variety of antibiotics have become a major health concern (Kiessling et al., 2002; Roy et al., 2007). Many studies have shown that *B. cereus* strains isolated from different food sources have resistant strains against some antibiotics (Willinghan et al., 1996; Ultee et al., 2002; Moon et al., 2006; Antwerpen et al., 2007; Roy et al., 2007; Brown and Jang, 2008). But there is not any study related to antibiotic susceptibilities and FAME profiles of *B. cereus* strains isolated from different food sources including milk, meat, chicken and cereal in Turkey.

The aim of this study was to determine the antibiotic susceptibility of 77 isolates of *B. cereus* isolated from different food sources (raw milk, chicken, cereal and meat) consumed in Turkey and to analyse the whole cell FAMEs of the these bacterial strains. This is the first study combining both the FAME analysis and antibiotic susceptibility of *B. cereus* isolates in Turkey.

MATERIALS AND METHODS

Isolation of the bacterial strains

The bacteria were isolated from the following 4 different food sources: 58 raw milk, 8 chickens, 7 cereals and 4 meats. The meat, chicken and cereal samples were obtained from local markets and the raw milk was collected from a dairy farm located in Erzurum city, Turkey. The samples were brought to the laboratory for the isolation of *B. cereus* under sanitary conditions in sterilized cold bags and processed on the same day. Ten grams (10 g) of each meat, chicken and cereal sample was homogenized in 90 mL sterile distilled water in a stomacher for about 2 min. The homogenates and raw milk were serially diluted and 1 mL of the last dilute were poured plated in Chromogenıc Bacillus Cereus Agar (CM1036) media and incubated in anaerobic jar at 37°C for 48 - 72 h for typical colony formation. The *B. cereus* colonies appeared as a blue-green, dry, rough surface, with a blue-green roundness on CM. Typical colonies of *B. cereus* were then transferred to Nutrient Agar (NA) for determination of biochemical and cultural characteristics of the isolates. Bacteria were grown on NA for routine use and maintained in Nutrient Broth (NB) with 15% glycerol at -80˚C for long-term storage.

Biochemical characterisation

Gram stain, catalase, nitrate reduction, Voges-Proskauer, glucose, starch hydrolysis and anaerobe utilisation of glucose tests were performed by the routine microbiological procedures as suggested by Shinagawa (1990), Pirttijarvi et al. (1998), Nout et al. (1998), and Altayar and Sutherland (2006), and these confirmed the identify of *B. cereus*.

Identification of the bacterial strains by microbial identification system (MIS)

Preparation and analysis of FAMEs from whole cell FA of bacterial strains were performed according to the method described by the manufacturer`s manual (Sherlock Microbial Identification System version 5.5 MIDI, Inc., Newark, DE, USA) (Mıller and Berger, 1985; Roy, 1988). All strains were grown on Difco Trypticase Soy Agar (TSA) for 48 h at 37°C. Approximately 40 mg cells (wet weight) were transferred to 13 x 100 mm glass tubes fitted with Teflon-lined screw caps and fatty acids were extracted using methods described by Miller (1982). FAMEs were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA, USA) with a fused-silica capillary column (25 m x 0.2 mm) with cross-linked 5% phenyl methyl silicone. FAME profiles of each bacterial strain were identified by comparing the commercial databases (TSBA 40) with the MIS software package. The identity of bacterial strains was revealed by computer comparison of FAME profiles of the unknown test strains with those in the library. A dendogram, based on the FAs, patterns of the strains were generated by clustering the euclidian distance of the fatty acids with the unweighted pair group method with arithmetic average algorithim that is provided by the MIDI software package.

Statistical analyses of FAMEs

In order to determine whether there is a statistically significant difference among the obtained FAMEs for 3 clusters, variance analyses were carried out using SPSS 10.0 software package. Differences between means were tested by Duncan test ($\alpha = 0.05$).

Susceptibility to antibiotics

The antibiotic susceptibility was determined by the disc agar diffusion method (Murray et al., 1995). A total of ten antibiotics including penicillin, oxacillin, sulphamethoxazole, rifampicin, apromycin, amikacin, tobramycin, kanamycin, gentamicin and oflaxacin were tested. Three colonies of 24 h-old culture were transferred to about 5 mL Tryptic Soy Broth (TSB) and incubated at 37°C for 24 - 48 h on rotary shakers (130 rpm) until the broth became moderately turbid. Absorbance of bacterial suspension at 600 nm was adjusted to 10^8cfu/mL in sterilized water. Bacterial suspension (100 µL) containing 10^8 cfu/mL of the bacterium was spread by a sterile swab on Tryptic Soy Agar (TSA) medium. After drying for 15 min, various antibiotic susceptibility test discs (6 mm) put aseptically in the middle of the inoculated plates. Bacterial cultures were incubated at 35 ± 2°C for 48 h and then inhibition zones around the discs were measured in diameter (mm). The assay was replicated three times.

RESULTS

A total of 77 *B. cereus* strains isolated from raw milk (58 strains), chicken (8 strains), cereal (7 strains) and meat (4 strains) using CM are listed in Table 1. The strains were identified initially based on their colonial morphology on plates. *B. cereus* colonies appeared as blue-green, in color with dry and rough surface on this agar media. MIS results based on fatty acid methyl esters of whole strains were confirmed as *B. cereus*. Similarity index of all strains were between 0.476 and 0.945.

Table 1. Number of bacterial strains (NBS) and their strain number from different food sources identified as *B. cereus.*

Origin of strains	NBS	Strain no
Raw milk	58	ND4, ND5, ND6, ND7, ND8, ND9, ND10, ND12, ND13, ND14, ND15, ND17, ND18, ND19, ND20, ND21, ND22, ND23, ND24, ND25, ND26, ND27, ND28, ND29, ND30, ND32, ND33, ND34, ND35, ND36, ND37, ND38, ND39, ND42, ND44, ND46, ND47, ND49, ND52, ND53, ND54, ND55, ND56, ND57, ND58, ND60, ND61, ND62, ND63, ND64, ND66, ND67, ND68, ND69, ND70, ND71, ND72 and ND73
Chicken	8	ND82, ND83, ND84, ND85, ND86, ND88, ND91 and ND92
Cereal	7	ND74, ND75, ND76, ND77, ND78, ND79 and ND80
Meat	4	ND1, ND2, ND3 and ND87

Table 2. Biochemical characteristics of *B. cereus* strains.

Biochemical tests	The number of positive strains (%)	The number of negative strains (%)
Voges-Proskauer	77 (100)	0 (0)
Nitrate reduction	77 (100)	0 (0)
Glucose test	77 (100)	0 (0)
Gram stain	77 (100)	0 (0)
Starch hydrolysis test	77 (100)	0 (0)
Catalase test	77 (100)	0 (0)
Anaerob utilisation of glucose	75 (97.41)	2 (2.59)*

*These strains (ND91 and ND92) were isolated from chicken.

Biochemical test results of the strains were given in Table 2. Gram stain, catalase, nitrate reduction, Voges-Proskauer, glucose, and starch hydrolysis test results of all strains were positive. Anaerobe utilisation of glucose test results of 75 strains (97.41%) was positive, but 2 strains (2.59%) isolated from chicken showed negative results.

FAME profiles from *B. cereus* isolates were submitted to cluster analysis and the resulting dendogram showed in Figure 1. Based on FAME analysis, the isolates were clustered into three main groups. Cluster 1, 2 and 3 were composed of the 71, 3 and 3 *B. cereus* strains, respectively. All the isolates in each cluster showed an extremely high degree of similarity (95 - 100%) to each other.

Proportion of the FAMEs in each cluster were given in Table 3. A total of 25 different FAs were detected in 77 strains tested, but 16 of them appeared, as minor components, their amount being less than 2%. The strains had 15:0 iso 3OH (30.25%), 16:0 iso (11.23%), 17:0 iso (9.20%), 16:0 (9.02%), 13:0 iso (8.85%), 14:0 iso (6.79%), 15:0 anteiso (4.83%), 14:0 (4.58%), 16:1 ω6c (3.92%) and 16:1 ω7c (3.92%) as the major FAs. Cluster 1 had higher amounts of 15:0 iso 3OH, 16:0 iso and 17:0 iso and less quantities of 17:0 cyclo than cluster 2 and 3. Cluster 2 had higher amounts of 14:0, 16:0 and 17:0 cyclo and lesser quantities of 13:0 iso and 14:0 iso than cluster 1 and 3. Cluster 3 had higher amounts of 13:0 iso, 13:0 anteiso, 15:0 anteiso A, 18:1 ω9c and 18:2 ω6,9c and 15:0 iso 3OH, 15:0 anteiso, 16:0, 16:0 iso and 17:0 iso than clusters 2 and 3.

The results for susceptibility of 77 *B. cereus* strains to 10 different antibiotics were given in Table 4. Antibiotic resistance profile showed that all strains were resistant to penicillin and oxacillin, but they were higly susceptible to gentamicin and oflaxacin. In addition, most of the strains were resistant to sulphamethoxazole (89.6%) and higly susceptible to amikacin (97.4%). Only 11 strains were resistant to rifampicin (14.3%) and the rest of 65 strains (84.4%) were susceptible. Most of the strains were also susceptible to apromycin (97.4%), tobramycin (88.3%) and kanamycin (67.5%).

DISCUSSION

A total of 77 *B. cereus* strains was isolated from four different food sources and investigated their antibiotic susceptibility and FAME group. It was found that *B. cereus* was the most common pathogen in the raw milk. Giffel et al. (1995) also stated that *B. cereus* is a common contaminant in raw milk. Due to its complex biochemical composition and high water activity, milk serves as an excellent culture medium for the growth of many kinds of microorganisms. In addition, it is known that usually milk is contaminated with different kinds of microorganisms at milk collecting places. The other studies reported that the highest numbers of *B. cereus* spores in raw milk are found during the grazing season (Slaghuis et al., 1997)

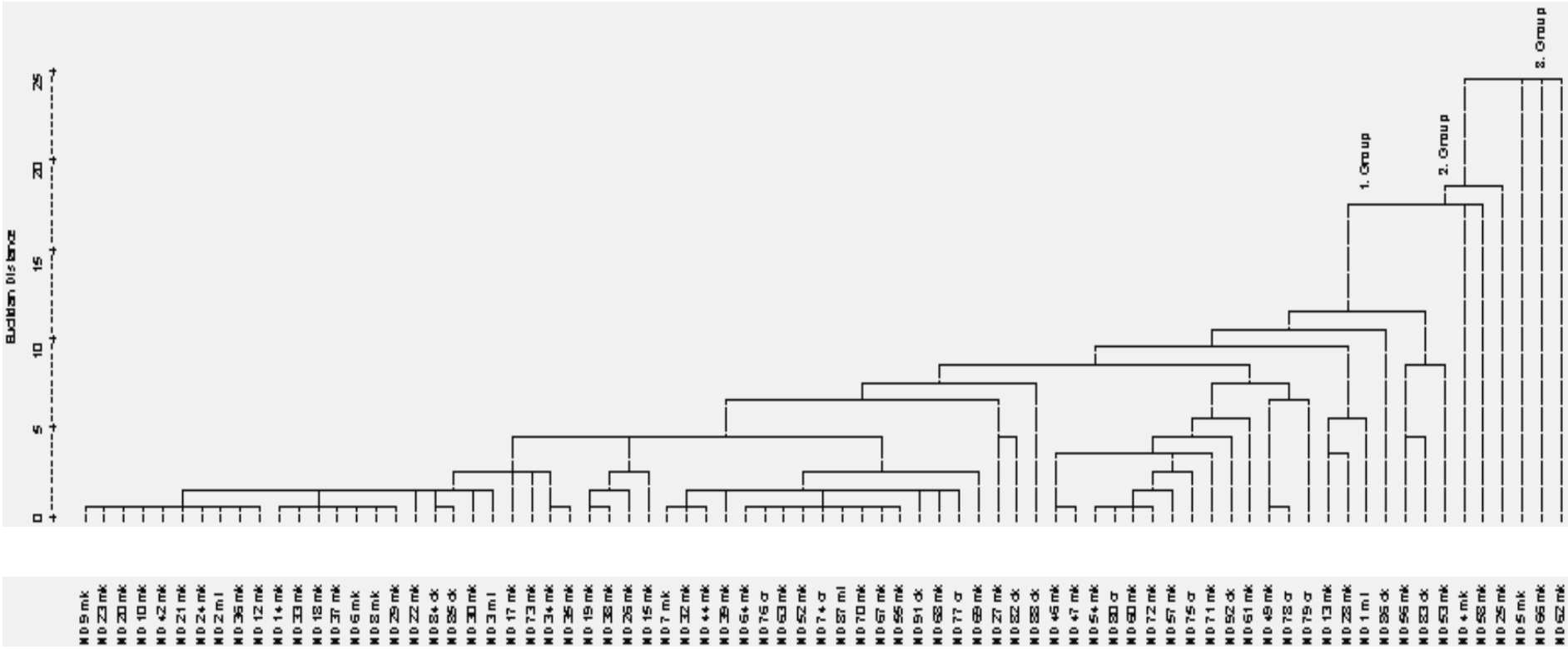

Figure 1. Dendogram of *B. cereus* strains generated by cluster analysis of FAME profiles showing subgroups (mk, mt, ck and cr) were added to strain name of bacteria isolated from milk, meat, chicken and cereal, respectively.

mainly due to contamination of the teats by soil (Christiansson et al., 1999). Food-borne illness resulting from consumption of food contaminated with Gram-positive and Gram-negative bacteria has been of vital concern to public health. In developed countries, good hygiene practices, adequate preservative technique for processed foods and use of antimicrobial substances such as antibiotics are the various ways of control and treatment (Aboaba et al., 2006). But, the development of bacterial resistance to presently available antibiotics has necessitated the search for new antibacterial agents (Davis, 1994).

Biochemical test results were highly similar to all isolates tested. MIS results based on FAMEs of whole strains were confirmed as *B. cereus*. There are many studies stated that FA analysis is a useful tool for identifying bacterial species including *Bacillus* (El-Helow, 2001; Whittaker et al., 2005). In this work, a FAME analysis procedure which was successfully employed for the identification of *B. cereus* strains. According to dendogram of *B. cereus* strains generated by cluster analysis of FAME profiles, the isolates were clustered into three main groups. The difference between groups changed from 2 to 24%. However, there was no relation between the groups and source of the strains. Several different typing methods for *B. cereus* have been used to try to find contamination sites in the dairy plant. Lin et al. (1998) used FAME profiles to differentiate between different strains of *B. cereus.*

One important characteristic of the cluster 1 was

Table 3. Proportion of the fatty acid methyl esters (%) in clusters 1, 2 and 3 of *B. cereus* strains.

Fatty acids	1. Cluster	2. Cluster	3. Cluster	SEM
12:0	0.10[a]	0.50[ab]	1.08[b]	0.15 ± 0.05
12:0 iso 3OH	0.96	0.43	1.39	0.96 ± 0.14
13:0 iso	8.86	7.56	9.91	8.85 ± 0.41
13:0 anteiso	0.66[a]	0.00[a]	2.99[b]	0.72 ± 0.12
14:0	4.54[a]	5.28[b]	4.88[a]	4.58 ± 0.16
14:0 iso	6.85	5.35	6.81	6.79 ± 0.20
15:0 iso 3OH	30.48	28.33	26.33	30.25 ± 0.56
15:0 anteiso A	0.20	0.00	1.56	0.24 ± 0.12
15:0 anteiso	4.88	4.40	3.97	4.83 ± 0.23
16:0	8.82	14.94	8.04	9.02 ± 0.33
16:0 Iso	11.39	9.95	8.43	11.23 ± 0.31
16:1 ω7c alchol	0.00[a]	0.00[a]	0.38[b]	0.01 ± 0.01
iso 17:1 ω5c	0.66	0.00	1.05	0.65 ± 0.13
anteiso 17:1 ω9c	002[a]	1.30[b]	0.00[a]	0.06 ± 0.04
iso 17:1 ω10c	0.09	0.00	0.52	0.10 ± 0.05
17:0 cyclo	0.00[a]	4.05[c]	2.12[b]	0.23 ± 0.13
17:0 iso	9.36	8.53	5.78	9.20 ± 0.38
17:0 anteiso	1.62	0.77	1.63	1.59 ± 0.17
18:1 ω9c	0.00[a]	0.00[a]	1.18[b]	0.04 ± 0.04
16:1 ω6c	3.91	3.81	4.09	3.92 ± 0.16
16:1 ω7c	3.92	3.81	4.09	3.92 ± 0.16
18:2 ω6,9c	0.00[a]	0.00[a]	1.55[b]	0.06 ± 0.05
17:1 isol/anteiso B	0.19	0.00	0.00	0.17 ± 0.07
12:0 aldehyde	1.13	1.18	2.16	1.17 ± 0.16
18:1 ω7c	0.03[a]	1.51[b]	0.00[a]	0.08 ± 0.06

SEM: Means ± standard error in the same line with the same letter are not significantly different according to the test of Duncan (□□= 0.05).

Table 4. Number of resistant (NR), susceptible (NS) and highly susceptible (NHS) *B. cereus* strains to a total of ten different antibiotics and percent of them (%).

Antibiotics (mg/disk)	Resistant (NR)				Total (%)	Susceptible (NS)				Total (%)	Highly susceptible (NHS)				Total (%)
	mk	mt	ck	cr		mk	mt	ck	cr		mk	mt	ck	cr	
Penicillin (10)	58	4	8	7	77 (100)	0	0	0	0	0 (0.0)	0	0	0	0	0 (0.0)
Oxacillin (1)	58	4	8	7	77 (100)	0	0	0	0	0 (0.0)	0	0	0	0	0 (0.0)
Sulphamethoxazole (25)	51	4	8	6	69 (89.6)	7	0	0	1	8 (10.3)	0	0	0	0	0 (0.0)
Rifampicn (5)	7	1	3	0	11 (14.2)	50	3	5	7	65 (84.4)	1	0	0	0	1 (1.2)
Apromycin (15)	0	0	0	0	0 (0.0)	56	4	8	7	75 (97.4)	2	0	0	0	2 (2.6)
Amikacin (30)	0	0	0	0	0 (0.0)	1	0	1	0	2 (2.5)	57	4	7	7	75 (97.4)
Tobramycin (10)	0	0	0	0	0 (0.0)	49	4	8	7	68 (88.3)	9	0	0	0	9 (11.6)
Kanamycin (30)	0	0	0	0	0 (0.0)	39	3	6	4	52 (67.5)	19	1	2	3	25 (32.4)
Gentamicin (120)	0	0	0	0	0 (0.0)	0	0	0	0	0 (0.0)	58	4	8	7	77 (100)
Oflaxacin (5)	0	0	0	0	0 (0.0)	0	0	0	0	0 (0.0)	58	4	8	7	77 (100)

the absence of 17:0 cyclo. Whereas cluster 2 had the highest amounts of 14:0, 16:0, anteiso 17:1 ω9c, 17:0 cyclo and 18:1 ω7c. Furthermore, 14:0 and 16:0 were also detected in the clusters 1 and 3, but their amounts

were detected lesser than that in the cluster 1. The results of this study showed that the strains had 15:0 iso 3OH (30.25%), 16:0 iso (11.23%), 17:0 iso (9.20%), 16:0 (9.02%), 13:0 iso (8.85%), 14:0 iso (6.79%), 15:0 anteiso (4.83%), 14:0 (4.58%), 16:1 ω6c (3.92%) and 16:1 ω7c (3.92%) as the major fatty acids. Güven and Mutlu (2008) also found some results of this study. However, another study showed that FAs iso 17:1 ω10c is present in *B. cereus* vegetative cells and spores and 16:0 2OH and 17:0 iso 3OH are present in spores but not in the vegetative cells (Whittaker et al., 2005). The methyl-hexadecanoic (anteiso C17:0) and 15- methylhexa-decanoic (iso C17:0) were the main fatty acids analysed in *Bacillus* sp. (Mohammad and Alireza, 2007). It is well known that variation in growth conditions can affect lipid composition in bacteria.

Strains of food-borne bacterial pathogens that are resistant to a variety of antibiotics have become a major health concern (Kiessling et al., 2002; Roy et al., 2007). In this study, antibiotic resistance profile showed that all strains were resistant to penicillin and oxacillin, as also been reported in the previous papers (Schlegelova et al., 2003; Antwerpen et al., 2007; Roy et al., 2007; Mérens et al., 2008; Ankolekar et al., 2009; Park et al., 2009). Consequently, , this may be the first report demonstrating resistance of *B. cereus* strains isolated from milk, meat, cereal and chicken to penicillin and oxacillin in Eastern Anatolia Region of Turkey.

This study showed that all *B. cereus* strains isolated from milk, meat, cereal and chicken showed an extremely high degree of similarity (95 - 100%) to each other in each cluster based on FAME analysis. Their antibiotic resistance profile showed that all strains were resistant to penicillin and oxacillin, but they were highly susceptible to gentamicin and oflaxacin. In addition, MIS system based on FAMEs might be used alone as a routine method for the identification of *B. cereus* and this method gave the similar identification results of biochemical tests.

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