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Full Length Research Paper

Detection of *Staphylococcus* species and staphylococcal enterotoxins by ELISA in ice cream and cheese consumed in Burdur Province

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In this study, 50 ice cream and 50 cheese samples from Burdur Province of Turkey were examined for total mesophilic aerobic bacteria, Micrococcus/Staphylococcus and *Staphylococcus aureus*. In ice cream, total aerobic mesophilic microorganisms, Micrococcus/Staphylococcus and *S. aureus* were determined at levels of 4.29, 3.78 and 3.00 $\log_{10}$cfu/g, respectively. In cheese, these organisms were present at 8.21, 6.21 and 5.80 $\log_{10}$cfu/g, respectively. Enterotoxin analyses were performed with Enzyme Linked Immunosorbent Assay (ELISA). Enterotoxins were detected in 3 cheese samples and were identified as staphylococcal enterotoxins B, C and E. Further attention should be given to the bacteriological standards for milk used in cheese and ice cream production.

Key words: Staphylococcal enterotoxin, ELISA, cheese, ice cream.

INTRODUCTION

Foods commonly associated with staphylococcal food poisoning fall into general categories such as meat and meat products, salads, cream-filled bakery products, and dairy products (Wieneke et al., 1993; Bennett and Monday, 2003). Many of these items are contaminated during preparation in homes or food service establishments and subsequently mishandled prior to consumption. In processed foods, contamination may result from human, animal, or environmental sources. In raw food, especially animal products, the presence of *Staphylococcus aureus* is common and may not be related to human contamination (Bennett and Monday, 2003). Staphylococcal contamination of animal hides, feathers, and skins is common and may or may not result from lesions or bruised tissue. Contamination of dressed animal carcasses by *S. aureus* is common and often unavoidable. Raw milk and unpasteurized dairy products may contain large numbers of *S. aureus*, usually a result of staphylococcal mastitis (Bennet and Monday, 2003). Milk is often described as a complete food because it contains protein, sugar, fat, vitamins, and minerals (Komorowski and Early, 1992). All of the nutritional components that make these types of dairy products an important part of the human diet, also support the growth of pathogenic organisms (Galal Abdel Hameed, 2006).

S. aureus is an important pathogen due to a combination of toxin-mediated virulence, invasiveness, and antibiotic resistance. The spectrum of staphylococcal infections ranges from pimples and furuncles to toxic shock syndrome and sepsis, most of which depend on numerous virulence factors. On the other hand, some infections, such as staphylococcal food poisoning, rely on one single type of virulence factor, the staphylococcal enterotoxins (SEs). Staphylococcal gastroenteritis is caused by the ingestion of food that contains one or more

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enterotoxins, which are produced only by some stapylococcal species and strains. Although enterotoxin production is believed generally to be associated with *S. aureus* strains that produce coagulase and thermonuclease, many species of Staphylococcus that produce neither coagulase nor thermonuclease are known to produce enterotoxins (Jay, 2000). Although enterotoxins are produced mainly by coagulase positive staphylococci (CPS), some coagulase-negative staphylococci (CNS), involved in a variety of human and animal infections, have also raised interest (Cunha et al., 2006). Very little is known about the growth of CNS in foods (Su and Wong, 1993). These strains have rarely been implicated in food poisoning because they do not grow rapidly in foods. Nevertheless, CNS can contaminate foods because humans are common carries of these microorganisms and some may be related to specific human infections (Cunha et al., 2006). There are 14 serologically distinct SEs, designated A, B, C, D, E and G through O (Anon, 2008). However, commercially available test kits for the detection of SE in food or in cultures include only SEs A through E (Anon, 2008). A toxin dose of less than 1.0 µg in contaminated food will produce symptoms of staphylococcal intoxication. This toxin level is reached when *S. aureus* populations exceed 10^6 cfu. However, in highly sensitive people a dose of 100 to 200 ng is sufficient to cause illness (Bennett and Monday, 2003). Staphylococcal toxins destroyed at 121°C for 3 to 8 min by heating, but they cannot be destructed by ambient storage, drying, or freezing (Baird-Parker, 1990; Jay, 2000). Staphylococcal toxins are resistant to proteolytic enzymes such as trypsin, chymotrypsin, rennin, papain (Anon, 2008). Staphylococcal toxins can also be effected by irradiation. The D-values (dose required to inactivate 90% SEB) were 27 kGy with buffer and 97 kGy with milk (Anon, 2008). The symptoms of staphylococcal food poisoning are abdominal cramps, nausea, vomiting, sometimes followed by diarrhea (never diarrhea alone). The onset of symptoms is rapid (from 30 min to 8 h) and usually spontaneous remission is observed after 24 h (Loir et al., 2003).

Several enzyme-linked immunosorbent assay (ELISA) methods have been proposed for the identification of staphylococcal enterotoxins and are currently the most commonly used methods (Bennet and Monday, 2003; Normanno et al., 2005). Of the competitive and noncompetitive ELISA-based methods, the noncompetitive, double antibody sandwich ELISA, appears to be the most popular for routine toxin identification (Carmo et al., 2002; Bennet and Monday, 2003). White cheeses that are offered for consumption in Burdur Province of Turkey are generally produced by conventional methods in enterprises without modern technology. They are produced as using raw milk or lack of heating process in the production, and the cheesemarker has to rely on the natural microflora of milk. Ice cream is commonly made in small-scaled production like open/artisanal shops (pastry shop's) in Burdur. The production processes do not follow safety standards regularly. The purpose of the present study was to investigate the rate of *S. aureus* and the presence of staphylococcal enterotoxins in cheese and ice cream samples by ELISA.

MATERIALS AND METHODS

Samples

In this study, 50 ice cream and 50 white cheese samples (250 g each) were obtained from Burdur Province of Turkey. Ice cream and cheese are generally produced with traditional methods by farmers and openly sold in local bazaar. All of the samples were collected in sterile plastic bags and transported to the laboratory on ice.

Enumeration of total aerobic mesophilic bacteria

The total aerobic mesophilic bacteria (TAMB) were determined using conventional methods (Maturin and Peeler, 1998). 10 g of each sample was suspended in 90 ml sterile buffered peptone water (0.85% NaCl+0.1% peptone) and 0.05 ml of 10^{-1} to 10^{-6} dilutions were drop plated onto the surface of the plate count agar (Merck, Darmstadt, Germany). After 48±2 h incubation at 30±1°C, colony forming units (cfu) were counted and calculated per gram of sample.

Enumeration of *Staphylococcus* spp. and *S. aureus*

10 g of each sample were diluted with 90 ml of sterile buffered peptone water and homogenized in a stomacher (Masticator, IUL Instruments-Spain). Samples of 10^{-1} to 10^{-6} dilutions in 0.1 ml were then spread on the surface of Baird-Parker Agar (BD, Becton Dickinson and Company, France) supplemented with egg yolk and tellurite. The plates were incubated at 37°C for 24 to 48 h.

The colonies were classified as typical for *S. aureus* (jet black to dark gray, smooth, convex, entire margins, with an opaque zone and a clear halo beyond the opaque zone) and counted (Bennett and Lancette, 1998). Ten colonies from each sample were selected and transferred to individual tubes of TSB agar (as stock cultures). A series of tests were then performed on the isolates, including Gram stain, catalase, coagulase, anaerobic fermentation of glucose and mannitol, haemolysis in blood agar, production of acetoin, methyl red, voges-proskauer, urease test, DNase, and TNase activity (Bennett and Lancette, 1998).

Detection of staphylococcal enterotoxins

The presence of staphylococcal enterotoxins (A, B, C, D, E) was determined qualitatively by a sandwich ELISA Staphylococcal Enterotoxin Kit (RIDASCREEN® Set A, B, C, D, E; R-Biopharm AG, Darmstadt, Germany). A detection limit defined by manufacturer for this test kit was approx.0.2 to 0.7 ppb in food. Based on its sensitivity the RIDASCREEN® Set A, B, C, D, E test is consequently clearly superior to the immunodiffusion procedure which has a detection limit of 0.1 mg/ml. The extraction and test steps were performed following the instructions of the kit manufacturer. To prepare the extract, 15 ml of PBS buffer (pH 7.4) were added to 10 g of sample and the mixture was shaken for

Table 1. The bacteria isolated from cheese samples. Number of bacteria (log 10 cfu/g).

Bacteria	N	n	%	Min	Max	Median	SE
Total aerobic mesophilic bacteria	50	50	100	6.30	10.8	8.21	0.19
Micrococcus/Staphylococcus (CPS and CNS)	50	28	56	2.48	8.70	6.21	0.30
S.aureus	50	18	36	3.11	8.18	5.80	0.37

CPS: Coagulase Positive Staphylococcus; CNS: Coagulase negative Staphylococcus; N: Number of cheese samples; n:Number of positive cheese samples; SE: Standart error.

Table 2. The bacteria isolated from ice cream samples. Number of bacteria (log 10 cfu/g).

Bacteria	N	n	%	Min	Max	Median	SE
Total aerobic mesophilic bacteria	50	48	96	2.30	7.64	4.29	0.12
Micrococcus/Staphylococcus (CPS and CNS)	50	19	38	2.00	3.78	3.02	0.12
S.aureus	50	7	14	1.30	5.50	3.00	0.50

CPS: Coagulase Positive Staphylococcus; CNS: Coagulase negative Staphylococcus; N: Number of ice cream samples; n:Number of positive ice cream Samples; SE: Standart error.

Table 3. The count and enterotoxin type of *S. aureus* isolated from cheese samples.

No. of cheese samples	Count of *S. aureus* (cfu/g)	Type of Staphylococcal enterotoxin
28	2.6x105	B
32	5.5x107	C
48	8.0x105	E

15 min.

The mixture was then centrifuged for 10 min at 3500x g and 15°C. The supernatant was sterilized by membrane filtration (0.2 µm, SM 16534, Sartorius, Minisart) to eliminate food particles and 100 µl of the filtrate was added to wells from A to G (the wells A to E in the microtiter strips are coated with specific antibodies against Staphylococcal enterotoxins A to E, the wells F and G serve as controls and are coated with antibodies of non-immunized animals) of one microtiter strip. A 100 µl aliquot of the positive control was added to well H, present toxins was binded to specific capture antibodies. The plate was mixed gently by manual shaking and left at room temperature for 1 h. The wells were then washed three times, each time with 250 µl per well of washing buffer.

A 100 µl aliquot of enzyme conjugate was then added to the wells and the plate was mixed gently by manual shaking and left at room temperature for 1 h. After another 3 washes with 250 µl washing buffer, 50 µl of the substrate and 50 µl of chromogen mixture were added. The plate was mixed gently by manual shaking and the plate again left at room temperature for 30 min. Finally, 50 µl of stop solution were added into each well, followed by gentle manual shaking. The optical densities were measured at 450 nm with a plate reader (ELX800, Universal Microplate Reader, BIO-TEK).

Statistical analysis

The results were analyzed using Minitab 15 statistical software (Minitab version 15.1.0.0, 2006) with the descriptive statistics.

RESULTS

The prevalence of microorganisms isolated from cheese and ice cream samples (log_{10}cfu/g) are given in Tables 1 and 2. Of the 50 cheese samples examined, 3 (6%) were determined with staphylococcal enterotoxins. Distribution of *S. aureus* and its enterotoxin producing strains isolated from cheese samples are given in Table 3. However, none of the ice cream samples was detected with staphylococcal enterotoxins.

DISCUSSION

The TAMB counts of white cheese samples were detected ranged from 4.30 to 10.53 log_{10} cfu/g by many researches (Sagun et al., 2001; Gulmez and Guven, 2001; Gulmez et al., 2001; Tekinsen, 2004; Sancak et al., 2006; Hamid and El Owni, 2007). In our study, the count of TAMB was detected to be 8.21 log_{10} cfu/g, which is higher than the results obtained by Sagun et al. (2001), Gulmez et al. (2001), Gulmez and Guven (2001) and Sancak et al. (2006), but similar to the results of Tekinsen (2004), and Hamid and El Owni (2007). The high count of TAMB found in cheese samples may be attributed to the

number of bacteria in milk materials, production conditions which were neither modern nor hygienic, unsuitable conditions storage, low salt concentration, non-hygienic equipment, and contaminations induced by the environment and personnel.

In the present study, the average Micrococcus/ Staphylococcus (CPS and CNS) count was 6.21 $\log_{10}$ cfu/g levels in 56% of the cheese samples. This result contrasted with that reported by Sagun et al. (2001), who reported an average Micrococcus/ Staphylococcus count of 1.39 $\log_{10}$ cfu/g in white cheese and the average Micrococcus/Staphylococcus count was 3.01 $\log_{10}$cfu/g in herby cheese. Sancak et al. (2006) found an average Micrococcus/Staphylococcus count of 4.93 $\log_{10}$ cfu/g in herby cheese. The average *S. aureus* count was $4.0x10^4$ cfu/g in non-ripened cheese and was $5.5x10^2$ cfu/g in ripened cheese (Gulmez et al., 2001). Gulmez and Guven (2001) reported an average Staphylococcus count of $4.1x10^3$ cfu/g and an average *S. aureus* count of $4.9x10^2$ cfu/g in cheese. Sagun et al. (2001) showed that, the average *S. aureus* count was 0.64 $\log_{10}$cfu/g in white cheese and 0.99 $\log_{10}$cfu/g in herby cheese. Tekinsen (2004) reported an average CPS count of 4.34 $\log_{10}$cfu/g levels in 57.5% of the cheese samples tested. In a study by Hamid and El Owni (2007), the average *S. aureus* count was 1.73 $\log_{10}$cfu/g in cheese samples. In the present study, the average *S. aureus* count was 5.80 $\log_{10}$ cfu/g levels in 36% of the cheese samples. In this study, CPS counts were above the maximum tolerable microbiological limit (10^3 cfu/g) for cheese according to the Turkish Food Codex (Anon, 2010). The high count of *S. aureus* found in cheese samples might be attributed to the high initial numbers of *S. aureus* in milk contamination during processing. The presence of *S. aureus* may be resulted from either insufficient pasteurization of milk, or transmitted from human and animal. The main reservoir of *S. aureus* is skin, nasal cavity, and throat in human and animal. Therefore, *S. aureus* can be transmitted in milk from animal or from human and environmental sources at pre- or post-pasteurization.

In our study, the average TAMB count was 4.29 $\log_{10}$ cfu/g levels in 96% of the ice cream samples. The TAMB counts of ice cream samples were detected ranged from 2.00 to 7.00 $\log_{10}$cfu/g by many researches (Erol et al., 1998; Bostan and Akin, 2002; Patir et al., 2004; Guner et al., 2004; Yaman et al., 2006). In this study, the findings were in line with those reported by Patir et al. (2004), Yaman et al. (2006), whereas they were not supported by the results of Erol et al. (1998), Guner et al. (2004), and Bostan and Akin (2002). The high TAMB count observed in the present study might be due to initial microflora of the milk used in ice cream making and other ingredients and their quality, the environment, insufficient heat treatment and poor personal hygiene.

In the present study, the average Micrococcus/ Staphylococcus (CPS and CNS) count was 3.2 $\log_{10}$ cfu/g levels in 38% of the ice cream samples and the average *S. aureus* count was 3.0 $\log_{10}$ cfu/g levels in 14% of the ice cream samples. According to the Turkish Food Codex, CPS counts were above the maximum tolerable microbiological limit (10^3 cfu/g) for ice cream (Anon, 2010). In other studies, Micrococcus/Staphylococcus, and *S. aureus* counts have been reported in analysed ice cream samples (Erol et al., 1998; Araujo et al., 2002; Guner et al., 2004; Patir et al., 2004; Agaoglu and Alemdar, 2004; Yaman et al., 2006). Patir et al. (2004) found average Micrococcus/Staphylococcus levels of 2.36 $\log_{10}$cfu/g, while for *S. aureus* they were 1.74 $\log_{10}$ cfu/g. Erol et al. (1998) reported that 20 to 30.8% of their samples had levels of 10^2 to 10^4 cfu/g for CPS. Agaoglu and Alemdar (2004) isolated *S. aureus* in 10 (13.3%) out of 75 ice cream samples. Araujo et al. (2002) found *Staphylococcus* spp. in 35 (77.7%) out of 45 ice cream samples. Guner et al. (2004), determined the average *S. aureus* levels at 1.2 to $1.7x10^3$ cfu/g, and Yaman et al. (2006) reported that 24 samples tested positive for *S. aureus* ranging from 10^2 to 10^4 cfu/g in ice cream samples. The high counts of Micrococcus/Staphylococcus and CPS found in some ice cream samples might be attributable to the high initial numbers of these organisms arising from milk contamination during processing. It is obvious from the previous and the present data that ice cream samples are frequently subjected to Staphylococci contamination which may indicate inadequate personel hygiene of workers or sales people.

In the our study, the cheese samples in which staphylococcal enterotoxin B, C and E were detected to contain *S. aureus* count $2.6x10^5$, $5.5x10^7$ and $8.0x10^5$ cfu/g, respectively (Table 3). According to the Turkish Food Codex, staphylococcal enterotoxins should not occur in food (Anon, 2010). Enterotoxigenic staphylococci are generally considered to require levels of at least 10^5 to 10^6 cfu/g or ml to produce detectable amounts of SE (Anon, 2008). The presence of enterotoxigenic staphylococcal strain in milk and cheeses represents a risk for consumers, even in low numbers (Carmo et al., 2002; Normanno et al., 2005). The presence of staphylococcal enterotoxin in cheese and ice cream has been investigated in several studies carried out in Turkey (Demirel and Karapinar, 2004; Bostan et al., 2006; Sancak et al., 2006) and in other countries (Carmo et al., 2002; De Reu et al., 2002; Normanno et al., 2005; Joffe and Baranovics, 2006) by many researchers. For example, Carmo et al. (2002) determined that all 3 cheese samples suspected of causing food poisoning in Brazil contained staphylococci at concentrations of $\geq 2.0x10^8$, $1.0x10^8$, $1.0x10^5$ cfu/g, respectively, and enterotoxins were detected as SEA and SEB; SEA, SEB and SEC; and SEA and SEB, respectively. De Reu et al. (2002) reported that *S. aureus* count $>10^4$ cfu/g was found in 17% (first sampling) and 4% (second sampling) cheeses made with raw milk. All samples with *S. aureus*

counts >10^3 cfu/g were tested for enterotoxins. In one cheese sample, two staphylococcal enterotoxins (SEA and SEC) were detected. Demirel and Karapinar (2004) reported that *S. aureus* was detected in the range of $1.0x10^1$ to $3.0x10^5$ cfu/g in 18.7% of 75 cheese samples. The number of enterotoxin-containing samples was 17 and the distribution of toxins were in the order of SEA, SEB, SEC and SED. Normanno et al. (2005) reported that 375 (23.7%) of the 1578 cheese samples were contaminated with coagulase positive staphylococci. Of the 161 isolates examined, 99 (61.5%) were confirmed to be *S. aureus* and they produced SEA, SEC, SED, SEA+SED, SEC+SED. The number of enterotoxin-containing samples was 17 and the distribution of toxins were in the order of SEA, SEB, SEC and SED. Joffe and Baranovics (2006) isolated *S. aureus* in 4 (4.3%) of the 92 cheese samples and staphylococcal enterotoxin were detected in 1 (25.0%) cheese sample.

In contrast, Bostan et al. (2006) reported that *S. aureus* at <10 to $9.2x10^3$ cfu/g was found in 30 white cheeses, but staphylococcal enterotoxins were not detected by the ELISA in any of the cheese samples tested and the detection limit of this test kit was 0.25 ng/ food. Sancak et al. (2006) reported that *S. aureus* <2 to 4.7 $\log_{10}$ cfu/g was found in cheese samples, but staphylococcal enterotoxins were not detected in any of the 50 herby cheese samples analysed. In our study, the average *S. aureus* count was detected to be 3.00 $\log_{10}$cfu/g, but staphylococcal enterotoxins were not detected in ice cream samples. These results were not in agreement with the results obtained by Normanno et al. (2005), who reported that 23 (6.6%) of the 350 ice cream samples tested were contaminated with CPS. Of the four isolates examined, four were *S. aureus* and 2 were enterotoxigenic and produced SEA+SEB.

In conclusion, cheese and ice cream produced by traditional production methods in enterprises without modern technology in Burdur. In particular, dairy animals with subclinical *S. aureus* mastitis, may shed large numbers of *S. aureus* organisms into the milk. However, contamination of raw milk and raw milk products from human handling or from the environment during manufacture also is possible. These contaminations may cause important public health risks. Therefore, greater attention should be given to bacteriological standards for the milk that is used in cheese and ice cream production. When scaling up food production from household level to industrial level, general hygienic practices need to be integrated into the process. Teaching and training programs, for those working at the dairies, can possibly improve the situation.

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