

Investigation of Microbiological Quality of Some Dairy Products in Kırklareli: Detection of *Salmonella* spp. and *Listeria monocytogenes* by Real Time PCR

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This study was carried out for the evaluation of the microbiological quality of various dairy products including white cheese, kashar cheese, butter, and milk cream purchased in retail markets in the province Kırklareli. Coliform bacteria, *Escherichia coli* and mold-yeast counts were investigated by the standard cultural method and found to be 59 (74.7 %) and 54 (68.4 %), and 68 (86.1 %) of the total 79 samples, respectively. *Staphylococcus aureus* was detected in 18 (22.8 %) samples. *Listeria monocytogenes* and *Salmonella* spp detected by the Real Time PCR and *Listeria monocytogenes* was detected in only two kashar cheese samples, while *Salmonella* spp. were not detected in any samples. The microbiological contamination level was higher than the permitted level according to the European standard in most of the investigated products in spite of the low level of the pathogen detection. These results also showed poor microbiological quality and inadequate production conditions of these products.

Keywords: Dairy Product, Microbiological quality, Turkey, Real Time PCR

Kırklareli’nde Üretilen Bazı Süt Ürünlerinin Mikrobiyolojik Kalitesinin Araştırılması: *Salmonella* spp. ve *Listeria Monocytogenes*’ in Real Time PCR Kullanarak Teşhis Edilmesi

Bu çalışma Kırklareli piyasasından temin edilen beyaz peynir, kaşar peynir, tereyağı, ve krema gibi çeşitli süt ürünlerinin mikrobiyolojik kalitelerinin belirlenmesi amacıyla yürütülmüştür. 79 örnekten 59 (%74,7), 54 (%68,4) ve 68 (%86,1) örnekte sırasıyla *Koliform* bakteri, *E.coli* ve küf/maya bulunmuştur. *Staphylococcus aureus* ise 18 (% 22, 8) örnekte tespit edilmiştir. *Listeria monocytogenes* yalnızca 1 kaşar örneğinde tespit edilirken, herhangi bir örnekte *Salmonella* spp. bulgusuna rastlanılmamıştır. Yalnızca bir örnekte patojen mikroorganizma bulgusuna rastlanmasına rağmen örneklerin çoğunun mikrobiyolojik yükü Avrupa standartlarına göre izin verilen seviyenin üzerinde çıkmıştır. Bu sonuçlar ürünlerin mikrobiyolojik kalitesinin çok düşük olduğuna ve yetersiz hijyen ve sanitasyona işaret etmektedir.

Anahtar Kelimeler: Süt ürünleri, Mikrobiyolojik kalite, Real time PCR

Introduction

Food safety is a great task that accompanies the food processing flow sheet from raw material to final product. Although pasteurized dairy products are presently considered as safe food products to be consumed, pathogenic bacteria which can be transmitted by post-pasteurization and contaminated to dairy products endanger the public health and thereby the dairy industry. So far, many pathogenic microorganisms, such as *Salmonella* spp., *Listeria monocytogenes*, verotoxin producing *Escherichia coli* (VTEC), and *Staphylococcus aureus* have been reported as the causal agents of food-borne diseases and/or food

spoilage (McCabe-Sellers and Samuel, 2004). Several studies have demonstrated that the sources of contamination were raw milk, inadequately pasteurized milk, or post-pasteurization contamination with organisms originally derived from raw milk or during the manufacturing process and due to inadequate personal hygiene and sanitation (De Buyser et al., 2001).

The production and consumption of dairy products have a long tradition in Turkey and the variety of dairy products is widely known. Dairy

production in Turkey, as a candidate of EU membership, which was on a small scale till 1970s, has expanded at a high rate about 10% per annum over the last years. In recent years, the total milk production reached to 17.4 million tons with an increase over than 2.4 million tons between 2011 and 2012 (Anonymous 2012).

Verification of the microbiological quality of dairy products in Turkey has shown lack of food safety standards and hygiene (Elmalı *et al.*, 2005). Although most of these studies (Kayıoğlu *et al.*, 2003; Öksüz *et al.*, 2004) are related to a few regions of Turkey, still there are limited data about microbiological quality of dairy products. Therefore, the main objective of this study was to assess the microbiological quality of dairy products available at retail sale in the markets in European Side of Turkey. The presence of foodborne pathogens such as *Salmonella* spp., coagulase-positive staphylococci and *L. monocytogenes* was investigated, as suggested by the legislation on national food security, and also the counts of coliform bacteria and mould-yeasts were determined as indicators of milk quality and hygiene during the production process.

Materials and Methods

Collection of the samples

In this study, a total of 79 dairy product samples including white cheese ($n = 40$), kashar cheese ($n = 17$), butter ($n = 13$), and milk cream ($n = 9$) were analyzed. The samples were randomly collected from retail stores in retail markets in the province Kirklareli in European side of Turkey (the Marmara region) from December 2012 till April 2013. The samples were randomly selected and collected from retail markets in the province Kirklareli in European side of Turkey (the Marmara region) from December 2012 to April 2013. The samples were immediately transported into the laboratory in refrigerated containers at 4°C for further preparation and analysis.

Microbiological analysis

Dairy samples were analyzed in terms of the counts of total coliforms, *Escherichia coli*, total mold-yeasts, and *S. aureus* using conventional cultural methods and the presence of *Salmonella* spp. and *L. monocytogenes* was determined using real time PCR assay. For this aim, sample (10 g)

was transferred to 90 ml 0.1% peptone water (Oxoid, Basingstoke, Hampshire, England) and homogenized with Stomacher Lab-Blender 400 (Seward Medical, London, UK). Appropriate 10-fold dilutions of the samples were prepared in sterile peptone water. *Escherichia coli* and total coliforms were determined using most probably number (MPN) method in Lauryl sulphate tryptose broth (Oxoid) at 37°C for 24 hours. Verification of *E. coli* and total coliform was carried out by using EC broth and brilliant green bile broth. Total mould-yeast count was determined in Rose Bengal Chloramphenicol Agar (Oxoid) by using surface plating method and plates were incubated at 25°C for 5–7 days. *S. aureus* was determined by surface plating on Baird–Parker agar (Oxoid) and incubating plates at 37 °C for 30–48 hours. Coagulase test was also applied for verification of typical *S. aureus* colonies. The analyses were performed in triplicate and the results were expressed as cfu g⁻¹.

Determination of foodborne pathogens using real time PCR assay

Salmonella spp. and *L. monocytogenes* were detected using real time PCR assay after pre-enrichment procedure. For pre-enrichment, 25 g of sample were blended in a stomacher (Seward Stomacher 400 Lab System, Norfolk, UK) with 225 ml of half Fraser Broth (Oxoid) for *L. monocytogenes*, and 225 ml of buffered peptone water for *Salmonella* spp. and incubated at 30 °C and 37 °C for 24 h, respectively. A 1.5 ml aliquot of enrichment samples was used for DNA extraction performed according to kit procedure (Food poroff, Bioteccon Diagnostic, Germany). Subsequently, DNA was measured by nano-drop spectrophotometer (Thermo Scientific NanoDrop 2000C, USA). Extracted samples were stored at -20°C until Real Time PCR application. 1.5-100 ng µl⁻¹ DNA concentration was performed in Real Time PCR application. Real-time PCR amplification was performed using the “Light Cycler 480 Fast DNA master hybridization probes” kit (Roche Diagnostics, Mannheim, Germany). Final 25 µl reaction volume (18 µl master mix, 1µl Enzyme solution, 1µl internal control solution and 5 µl DNA solution) was used for Real Time PCR application. Amplifications were carried out by the Light Cycler Nano System (Roche Diagnostics, Germany) using a thermal cycling protocol consisting of 37 °C 4 min and 95 °C for 15 min

followed by 50 cycles at 95 °C for 5 s and at 60 °C for 1 min.

Evaluation of the microbiological quality of dairy products was carried out according to European Union standards on regulation on microbiological criteria (European Commission 2005, 2007) of food products.

Result and Discussion

In the present study, regarding the distribution of microbial populations, 86.1 % of the samples were found to have total yeast-mould counts exceeding 10 cfu g⁻¹ while the results varied from 10¹ to 10⁵ cfu g⁻¹ for dairy products, where as most samples had a count ranging from 10³ to 10⁵ cfu g⁻¹ (Table 1). 65.8 % of the samples did not conform with the criteria for dairy products established in Turkish Food Codex (10³cfu g⁻¹). The levels of mould and yeast counts obtained in this study were comparable to those found by Akyüz *et al.* (1998), Özdemir *et al.* (1998), Aksu *et al.* (1999), Türkoğlu *et al.* (2003) for manufactured dairy products in Turkey. High mould-yeast numbers indicate the poor hygienic conditions along with manufacture, production and marketing of dairy products such as cheese. In general, mould counts are useful for indicating the shelf-life duration and microbial quality of food, because there are not only the main causative for food spoilage, moreover high counts are considered to be a hazard for public health due to mycotoxin production.

In this study, 74.7% (n=59) and 68.4 (n=54) of the samples were contaminated with coliform bacteria and *E. coli* with different levels, respectively while a remarkable number of the samples (38.0 % and 35.4 %, respectively) had coliform bacteria and *E. coli* levels exceeding 103 MPN g⁻¹. However, only one butter sample showed *E. coli* count higher than 3MPN g⁻¹(Table 1).

In several studies, *E. coli* levels in cheese samples were reported as follows; 58% in soft and semi-hard cheeses (Ansay and Kaspar, 1997), 32.8% in Damietta and 20.8% in Kareish cheese (Aman *et al.*, 1998). According to the Turkish food codex regulation on microbiological criteria

(Anonymous, 2011), *E. coli* counts have a maximum value of 10² cfu g⁻¹ in dairy products including white cheese, kashar cheese, butter and milk cream. High levels of *E. coli* may occur due to improper handling or storage conditions, microbiologically poor quality of raw materials and cross-contamination after processing (Beuchat and Ryu, 1997). In several studies about *E. coli* levels in cheese samples were reported as follows; 58% in soft and semi-hard cheeses (Ansay and Kaspar, 1997), 32.8% in Damietta and 20.8% in Kareish cheese (Aman *et al.*, 1998). *E. coli* and coliform bacteria are often considered as indicator microorganisms, and their presence implies a risk that other enteric pathogens may be present in the sample. The presence of coliform bacteria is not necessarily an indicator for a direct fecal contamination of milk, but more precisely an indicator of poor hygiene and sanitary practices during milking and further processing steps (Yücel and Ulusoy, 2006). In addition, the high level of contamination of these products deserves special attention, particularly during manufacturing.

In our study, the moderate occurrence of *S. aureus* was detected in the white cheese (12.7 %), kashar cheese (3.8%) and milk cream (6.3%) samples and at levels ranging from 10¹ to <10⁴ cfu g⁻¹. The coagulase positive staphylococcus counts for nine samples were above the maximum tolerable microbiological limit (10³ cfu g⁻¹ or ml⁻¹) according to the Turkish Food Codex (Anonymous, 2011). Two samples showed *S. aureus* levels exceeding 10⁵ cfu g⁻¹ which is considered as a significant risk due to the enterotoxin production. Although the Turkish Food Regulation analog to the Regulation 2005/2073/EC defines levels exceeding 10⁵ cfu g⁻¹ as unsatisfactory in raw milk cheeses, in unripened soft cheeses made from milk that has undergone pasteurisation levels exceeding 10² cfu g⁻¹ also demand improvements in production hygiene. The occurrence of *S. aureus* in cheese samples (15 %) detected in this study is in accordance with the data reported by other studies from Turkey. Tekinsen and Özdemir (2006) detected *S. aureus* level between 10² and x10⁷ cfu g⁻¹ in Van otlu (Herb) cheese which processed from raw milk. Günsen and Büyükyörük (2003) found that 3.2% of 125 kashar cheese samples were contaminated with *S. aureus*.

Table 1. Microbiological results on most probable number of coliform bacteria and *Escherichia coli* and microbial counts of *Staphylococcus aureus* and mould-yeasts, in 79 dairy product samples.

Count interval	Coliforms bacteria ^{a, b}				<i>Escherichia coli</i> ^{a, b}				<i>Staphylococcus aureus</i> ^{c, d}					mould-yeasts ^{c, d}				
	<3	3 – 10 ²	>10 ² –10 ³	>10 ³	<3	3-10	>10 ² –10 ³	>10 ³	<10 ¹	10 ¹ -<10 ²	10 ² -<10 ³	10 ³ -<10 ⁴	>10 ⁴	<10 ¹	10 ¹ - <10 ²	10 ² - <10 ³	10 ³ - <10 ⁴	>10 ⁴
White Cheese (n=40)	7	7	6	20	10	6	6	18	30	1	4	4	1	10	2	9	10	9
Kashar Cheese (n=17)	7	6	2	2	8	5	2	2	14	n.d.	2	1	n.d.	nd	nd	4	4	9
Milk cream (n=9)	1	n.d.	2	6	1	n.d.	2	6	4	n.d.	2	2	1	nd	nd	nd	2	7
Butter (n=13)	5	6	n.d.	2	6	5	n.d.	2	n.d.	n.d.	n.d.	n.d.	n.d.	1	nd	1	3	8

^aRange in MPN g⁻¹; ^bdetection limit at >3 MPN g⁻¹; ^crange in cfu g⁻¹; ^ddetection limit at >10 cfu g⁻¹; n.d.: not detected

Can and Çelik (2012) found that 5% of a total 100 cheese samples from Ankara were contaminated with *S. aureus*. In the present study, we did not find *S. aureus* neither in kashar cheese nor in butter samples. Similarly, Gülmez et al. (2004) did not detect any *S. aureus* contamination in 50 kashar cheese samples analyzed in Kars, Turkey. The differences in these results may be due to differences in cheese production techniques, and whether the processed milk was raw or heat treated. Compared to our results, contamination rates of different types of cheeses with *S. aureus* were reported as 25% De Luca et al. (1997), 20% Araujo et al. (2002), 15.3% Akineden et al. (2008), 3.8% El-Sharoud and Spano (2008) from Italy, Brazil, Germany and Egypt, respectively. In Italy, 22 (16.3%) samples were positive for *S. aureus* in 135 cheese samples (De Luca et al. 1997). In France, the prevalence of enterotoxigenic *S. aureus* in dairy products was determined to be 7.3% Lamprell et al. (2004). It has been shown in some studies that raw milk is a potential source of contamination of cheese (Tondo et al. 2000; Andre et al. 2008). A high contamination level of *S. aureus* is necessary for the production of toxin sufficient to be considered as threat for public health (Le Loir et al. 2003).

While *Listeria monocytogenes* was detected using real time PCR in only two kashar cheese samples, *Salmonella* spp. was not detected in any of the samples. Some researchers have reported positive results for presence of *Listeria monocytogenes* in processing fields and equipments (Arıcı et al. 1999; Gülmez and Güven 2001; Güner and Telli 2011). In the present study, the occurrence of *L. monocytogenes* in kashar cheese samples showed that production of kashar cheese in two manufacturers were carried out lack of hygienic condition. Besides non-hygienic production, insufficient heat treatment plays an important role in *L. monocytogenes* contamination. Some researchers reported wide range of *L. monocytogenes* contamination sources and environmental effects. Scaack and Marth (1988) reported that contamination of *L. monocytogenes* can take place in post pasteurization stage from contaminated equipment during processing, storage and distribution. Some researchers reported positive result for detection of *Listeria monocytogenes* from processing field and equipments (Walker et al. 1991; Menendez et al. 1997; Mehmetoğlu et al. 2011). In this study, although most of the dairy products didn't show contamination with *Salmonella* spp. and *L.*

monocytogenes, other microbiological quality parameters were not in a desirable level. These parameters indicate the efficiency of the compliance with hygienic condition during production and post-production stages of processed food.

Conclusion

In this study, Real time PCR method was used for detection of *Salmonella* spp. and *L. monocytogenes*. *Salmonella* spp. was not found in any samples while *L. monocytogenes* was detected in only two of the total 79 samples. This result suggested that dairy products in retail market in European side of the Turkey (Kırklareli) would generally be considered as acceptable in terms of food safety according to European standards. But two cheese samples was found to be *L. monocytogenes* positive. In order to prevent pathogen microorganisms' contamination, some hygienic procedures such as cleaning, disinfection, and good post processing techniques and procedures should be applied. In addition to Real time PCR method, standard cultural methods were also applied for determination of microbiological quality and hygienic conditions of the dairy products. These results about dairy products indicated that processing of dairy product was not carried out in compliance with hygiene standardization. The results of the present study also indicated that it would be met European standard on the Microbiological criteria of the dairy products in European side of the Turkey in the case of the applying hygienic standards.

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