PCR DETECTION OF PATHOGENIC BACTERIA IN MILK AND SOFT CHEESE UNDER DIFFERENT COW'S PRODUCTION SYSTEMS IN MEXICO

Ameer E. ELFARASH [a,*], Francisco J. SOLORIO-SÁNCHEZ [b], Rafael A. ROJAS-HERRERA [c], Victor M. TOLEDO-LOPEZ [d,] and Yaser M. A. EL-DERWY [b,e]

[a] Department of Genetics, Faculty of Agriculture, Assiut University, Assiut (71526), Egypt.
[b] Faculty of Veterinary Medicine and Animal Science (CCBA), Autonomous University of Yucatan (UADY), Merida, Yucatan (97100), Mexico.
[c] Faculty of Chemical Engineering, Autonomous University of Yucatan (UADY), Merida, Yucatan (97203), Mexico.
[d] Merida Institute of Technology, Merida, Yucatan (97118.), Mexico.
[e] Department of Dairy science, Faculty of Agriculture, Assiut University, Assiut (71526), Egypt.

***Corresponding author:** Ameer E. ELFARASH
Department of Genetics, Faculty of Agriculture, Assiut University, Assiut (71526), Egypt.
Tel: (+2) 01005392548 E-mail: aelfarash@aun.edu.eg

ABSTRACT

Thirty six samples of raw milk were collected from three different farms located in Merida, Yucatan, Mexico and seventy two samples of soft cheese were manufactured from thirty six samples of raw milk and thirty six samples of pasteurized milk from the previous collected raw milk during May 2013 to April 2014. The first and second farm located at Faculty of Veterinary Medicine and Animal Science, Autonomous University of Yucatan (FMVZ/UADY) used two different production regimes; intensive silvopastoral system (ISS) and traditional system (TS). The third farm in Kampepen under (ISS). The collected raw milk samples were divided into three portions, one portion of raw milk and two portions for soft cheese manufacture from (raw and pasteurized milk) for PCR screening. PCR screening was performed for one hundred and eight samples to detect four types of the most dangerous foodborne pathogens (*Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*). PCR results showed that almost samples were negative for all tested pathogens except six samples (5.56%); 3 samples of raw milk and other 3 samples of soft cheese manufactured from this raw milk under traditional system which were positive for *Staphylococcus aureus*.

Key words: PCR, Pathogenic bacteria, Raw milk, Soft cheese, Production systems.

INTRODUCTION

Cheese is the generic name for a group of fermented milk-based food products. There are more than 1,000 varieties of cheese (Fox *et al.* 2004). Most of the Mexican cheeses are soft cheese and made locally from raw milk (Olson *et al.* 2011; Renye *et al.* 2011; Aldrete-Tapia *et al.* 2014; Gould *et al.* 2014; Pereira-Suarez *et al.* 2014). The quality and safety of the cheese depends on raw milk contamination by microorganisms, efficiency of processing and the hygienic practice applied in dairy farms and dairy factors, transportation from dairy farms to dairy factors, handling and manipulation of milk during

manufacture. All together can impair its utility and renders the product unsafe for human consumption (Leuschner and Boughtflower 2002). Due to the high microbial contamination present in raw milk, pasteurization is commonly used to eliminate all pathogenic and most of nonpathogenic organisms before its further processing into cheese (Silvestre *et al.* 2008; Elizondo-Salazar *et al.* 2010; Silva *et al.* 2010; Morales-Celaya *et al.* 2012; Van Gysel *et al.* 2012).

In Mexico, consumers prefer soft cheese manufactured from cow's raw milk due to the presence of microorganisms which enhances the flavor of the soft cheese, but this raised the concern about the safety of the cheese (Solano-López and Hernández-Sánchez 2000; Morales-Celaya *et al.* 2012). There are no enough data on cheese contaminated with species, especially; *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes* present in Mexican and American markets (Torres-Llanez *et al.* 2006; Omiccioli *et al.* 2009). These pathogens are the most dangerous foodborne bacterial in terms of human health and disease, and their detection through current microbiological analyses methods involves considerable spending of time and effort (Omiccioli *et al.* 2009). Pathogenic bacteria can produce numerous toxins and leading cause of gastroenteritis and vomiting resulting from consumption of contaminated dairy product (Nedelkov 2000; U.S. FDA 2001). Contaminated milk and soft cheese with pathogenic bacteria often are involved in outbreaks (Bergdoll 1997).

PCR is a rapid and sensitive diagnostic method for detecting pathogenic bacteria. Therefore, it was used for detecting the pathogenic bacteria in this research according to (Holland *et al.* 2000; Gouws and Liedemann 2005; Omiccioli *et al.* 2009; Wu *et al.* 2012).

In Mexico, intensive silvopastoral systems based on tropical grasses and *Leucaena leucocephala* are gradually expanding in the feeding of milking cows. Incorporation of tree legumes such as *Leucaena leucocephala* in silvopastoral systems represents an alternative in tropical ruminant production as it plays an important role in providing a forage rich in nutrients, especially protein, vitamins, and minerals (Barros-Rodríguez *et al.* 2012).

In this study, we focused on PCR detection of four types of pathogens (*Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes*) present in raw milk and soft cheese manufactured from raw and pasteurized milk under intensive silvopastoral and traditional systems in Yucatan, Mexico. The ultimate goal of this study was to help dairy industry to produce healthy and safe milk as well as soft cheese to the consumers in Mexico. Also, appropriate action can be taken to minimize healthy risk.

MATERIALS AND METHODS

Milk samples:

Three raw milk samples (11 L/sample) were collected monthly in a total of thirty six samples from three different farms located in Merida, Yucatan, Mexico during May 2013 to April 2014. The milking cows of the first and second farm located at Faculty of Veterinary Medicine and Animal Science, Autonomous University of Yucatan (FMVZ/UADY) were reared under two different production regimes; intensive silvopastoral system (ISS) and traditional system (TS). The milking cows of the third farm in Kampepen, Merida, Yucatan, Mexico were reared under intensive silvopastoral system (ISS). The differences in the nutrient ingredients between production systems are shown in (Table 1).

A bulk of fifteen milking cows from each production system was used for getting raw milk samples.

Table 1. The nutrient ingredients of the experimental production systems during May 2013 to April 2014 in Merida, Yucatan, Mexico.

Production system	Ingredients	Percentage
Intensive Silvopastoral System (Kampepen farm)	➢ *Leucaena leucocephala*	7%
	➢ Grass	76.9%
	➢ Supplements:	
	• 29.1% Soy hulls	
	• 29.1% Wheat bran	16.1%
	• 38.8% Ground corn	
	• 3% Minerals salts	
Intensive Silvopastoral System (UADY farm)	➢ *Leucaena leucocephala*	28.72%
	➢ Grass	47.30%
	➢ Sorghum	23.98%
Traditional System (UADY farm)	➢ Grass	48.02%
	➢ Supplements:	
	• 60% Maize	
	• 30% Soy hulls	51.98%
	• 5% Soya milled	
	• 5% Minerals and Calcium	

Production systems:

In the first and second farms (FMVZ/UADY), 24 multiparous (≥3 calves) *Bos indicus* x *Bos taurus* cows (Holstein and Brown Swiss x Zebu were used; with proportion of European genes ranging from 50-75%) at calving. The mean body weight of the cows was 509 ± 74 kg (mean ± SD) with a body condition score (BC) of 6 ± 0.3 points. All the cows were familiarized to the consumption of Leucaena forage as part of the routine diet. The cows were divided in two homogenous groups according to the previous milk yield and calving interval, and were allocated at calving to the following treatments: (ISS) (n=12), a Intensive Silvopastoral System, consisting of an association of stargrass and Leucaena (*Cynodon nlemfuensis* - *Leucaena leucocephala*); (TS) (n=12), a Traditional System (control) group where the cows grazed in a monoculture of stargrass. The grazing area was 4.3 hectare for (ISS) and 3.0 ha for (TS), with stocking rates of 2.4 AU/hectare (1 animal unit [AU] = 450 kg live weight) and 2 AU/ha for (ISS) and (TS), respectively. The stocking rates were fixed based on previous works for (ISS) (Mayo 2013) and (TS) taking into account the forage availability. To keep the stocking rate in both treatments during the experiment, there were incorporated external animals as necessary, using the "put and take" method. Both groups of cows were grazed from (5:00 pm to 5:00 am) and from (8:00 am to 1:00 pm). In order to avoid weight losses which could affect the response variables, all the cows were supplemented during the milking in the morning and evening (am and pm) to fulfill their protein and energy requirements for milk yield. Because of the composition of the forages, it was used ground sorghum as an energy supplement for (ISS) and a commercial concentrate to supply energy and protein for (TS) as shown in (Table 1). All the cows had access to a mineral mixture and water *ad libitum*.

In the third farm (Kampepen), 24 multiparous (≥3 calves) *Bos indicus* x *Bos taurus* cows (Holstein x Zebu were used; with proportion of European genes ranging from 50-75%) at calving. The mean body weight of the cows was 537 ± 53 kg (mean ± SD) with a body condition score (BC) of 6 ± 0.8 points. All the cows were familiarized to the consumption of Leucaena forage as part of the routine diet. All the cows were reared under Intensive Silvopastoral System (ISS), consisting of an association of guinea grass and Leucaena (*Pannicum maximum* - *Leucaena leucocephala*). The grazing area was 7 hectare for (ISS) with stocking rates of 3.5 AU/hectare (1 animal unit [AU] = 450 kg live weight). The cows were milked dally at 8:00 am, all the cows were supplemented during the milking in the morning as shown in (Table 1). To keep the stocking rate in the summer and winter, during the experiment in the summer (3.5 kg dry matter additional citrus peel) to supplement the animals during the time of milking. The cows grazed from 12:00 pm to 8:00 am the next day.

Cheese manufacture:

Six soft cheese samples were manufactured monthly in a total of 72 samples from the previous collected milk. The soft cheeses in each one of the three production system were manufactured from raw and pasteurized milk in the dairy laboratory at (FMVZ/UADY).

One portion of raw milk (5 L) was used for soft cheese manufacturing, while the other (5 L) was pasteurized to 73°C for 15 sec. followed by sudden cooling at 5°C then warmed to 40°C, 3% of salt was added to raw and pasteurized milk. 100 L of each milk was coagulated by adding 10-15 ml of liquid rennet (Cuamix) in 30-40 min. After that, the curd was cut and the pieces of curd were powered into a fine cotton cloth in aluminum molds and pressed by light weight for 24 hours to excrete the whey. Then, the obtained soft cheese were weighted to determine the percentage of yield.

Microbial enrichment:

To improve the sensitivity of detection by PCR, an

enrichment step was done in the laboratory of Microbial genetics, department of genetics at the Faculty of Agriculture, Assiut University, Assiut, Egypt. Milk and soft cheese samples were added to an enrichment media and then incubated at 36±1°C for 24 h for the further investigation. To identify the pathogen, different enrichment media were used as follow: Trypticase Soy Broth (TSB) for *Escherichia coli* O157:H7 (Lye *et al.* 2013); Baird-Parker Agar Base for *Staphylococcus aureus* (Rahimi and Alian 2013); Listeria Enrichment Broth Base for *Listeria monocytogenes* (Amagliani *et al.* 2006); and selenite brilliant green sulfa enrichment (SBG) broth for *Salmonella enterica* serovar Typhimurium (Chang *et al.* 1999).

DNA extraction:

DNA extraction by boiling method was carried out as previously described with some modifications (Peng *et al.* 2013). In brief, 1 ml of overnight cultivated cultures were centrifuged at 13,000×g. at 4 °C, the pellet was dissolved in 100 µl of sterilized ddH_2O, followed by vigorous homogenization by vortexing for 30s. The suspensions were incubated at 100 °C in a boiling water-bath for 15 min and immediately frozen at 0 °C on ice. Subsequently, the suspensions were centrifuged at 13,000×g. at 4 °C for 15 min. The supernatants were transferred to a clean 1.5 ml tube and stored at -20 °C until PCR analysis.

PCR amplification:

In this study four types of pathogens were screened; *Escherichia coli* O157:H7 as previously described (Holland *et al.* 2000); *Salmonella*

enterica serovar Typhimurium as previously described (Park *et al.* 2009); *Staphylococcus aureus* as previously described (Wu *et al.* 2012); and *Listeria monocytogenes* as previously described (Gouws and Liedemann 2005). All the one hundred and eight different milk and soft cheese samples were PCR screened with specific pair of primers presented in (

Table 2) to detect the presence of each separated pathogenic strain.

The polymerase chain reaction was carried out in a cycler Sensoquest Gradient (Germany). The reaction was conducted in a final volume of 25 µl containing 1× concentrated PCR buffer (containing 20mM Tris-HCl, pH 8.4, 1.5 mM MgCl2, and 50mM KCl), 100 nM primers, 0.2mM dNTP mix, 1 U *Taq* polymerase (Thermo Scientific, Fisher Scientific - USA) and 3 µl of DNA solution. Negative and positive controls were included in all experiments.

The optimized temperature and time profile of the PCR reaction was as follows: Initial denaturation: 94°C, 5 min; 30 cycles with the following step cycle profile: denaturation 94°C, 30 s; annealing (54-68°C), 30 s; extension 72°C, 1 min; final extension 72°C, 5 min.

PCR samples were separated by electrophoresis in 1% agarose gel in TAE buffer 1× concentrated and stained with Ethidium Bromide (0.5 µg/ml). The PCR samples were calibrated using Smart Ladder (Eurogentec) with DNA fragments ranging between 200 bp and 10,000 bp to determine the molecular weights of the amplicons. Gels were visualized on a Transilluminator (Ultra-Violet Product, Upland, CA, USA,) and photographed using an Orange filtered Olympus digital camera (Olympus model No. C-7070, wide zoom, Japan).

Table 2. The primer sequences and codes which used for detecting of pathogenic bacteria in raw milk and soft cheese manufactured from raw and pasteurized milk under (ISS) and (TS) during May 2013 to April 2014 in Merida, Yucatan, Mexico.

Strain	Primer	Primer sequence (5'→ 3')	Tm	Size (bp)	Reference
S. aureus	St.a-Fw	AACTCTGTTATTAGGGAAGAACA	55.3	756	(Wu *et al.* 2012)
	St.a-Rv	CCACCTTCCTCCCCGTTGTCACC	67.8		
S. Typhimurium	Sa.t-Fw	AACAACGGCTCCGGTAATGAGATTG	63.0	310	(Park *et al.* 2009)
	Sa.t-Rv	ATGACAAACTCTTGATTCTGAAGATCG	60.4		
E.coli O157:H7	Ec-Fw	ACACTGGATGATCTAGTGG	54.5	614	(Holland *et al.* 2000)
	Ec-Rv	CTGAATCCCCCTCCATTATG	57.3		
L. monocytogenes	Lm-Fw	CATTAGTGGAAAGATGGAATG	54.0	730	(Gouws and Liedemann 2005)
	Lm-Rv	GTATCCTCCAGAGTGATCGA	57.3		

RESULTS AND DISCUSSION

Data concerning the presence of bacterial species like; *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus,* and *Listeria monocytogenes* in soft cheese are scarcely available (Torres-Llanez *et al.* 2006; Renye *et al.* 2008; Omiccioli *et al.* 2009).

The obtained results indicated that, all of the one hundred and eight samples were negative to; *Escherichia coli* O157:H7*, Salmonella enterica* serovar Typhimurium*,* and *Listeria monocytogenes*. The results in agreement with those found by EC (2004a; 2004b). While, for *Staphylococcus aureus* screening, 6 out of 108 examined samples were positive (5.56 %) 3 samples of raw milk and other 3 samples of soft cheese manufactured from this raw milk under traditional system (Table 3& Fig. 1).

Interestingly, all of the contaminated raw milk and soft cheese manufactured from this raw milk samples by *Staphylococcus aureus* were detected only in the traditional system, while it was not detected in the rest of raw milk and soft cheese samples under intensive silvopastoral system in both FMVZ and Kampepen farms (Table 3& Fig. 1).

On the other hand, no detection of pathogenic bacteria was found in all soft cheese samples manufactured from pasteurized milk under the all production systems, these result in agreement with (Silvestre et al. 2008; Elizondo-Salazar et al. 2010; Silva et al. 2010; Van Gysel et al. 2012). In this regard, raw milk and its products are considered a big reason of food risk because they are an ideal medium for bacterial growth. In addition, Goto *et al.* (2007) found that Pasteurization affected the quantity of DNA of *Staphylococcus aureus* found in milk when tes

by Real-Time PCR. The results presented in (Table 3) indicated that the *Staphylococcus aureus* which detected in soft cheese manufactured from raw milk proved the elimination role of pasteurization for all *Staphylococcus aureus* pathogens in soft cheese manufactured from pasteurized milk.

Soft cheeses manufactured from raw milk are highly susceptible to contain the same pathogens which found in raw milk. On the other hand, some other factors capable of eliminating pathogenic bacteria in soft cheese manufactured from raw milk, i.e., ripening of cheese in a period not less than few weeks or months which reduce cheese pH and eliminate any pathogenic bacteria in soft cheese manufactured from raw milk (Millet *et al.* 2006).

As it known that, *Staphylococcus aureus* is the most dangerous foodborne disease in terms of human health and it must not be found in dairy products (EC 2004a; 2004b). It is clear that raw milk and soft cheese manufactured from raw milk contains *Staphylococcus aureus* only during three sequential months (Table 3) from Feb. to Apr. 2014, it might be due to the probability of infected cows with mastitis in one or more of the selected cows in this study. In agreement with the finding of Hebert *et al.* (2000); Barkema *et al.* (2006) whom stated that the infection with mastitis is common in cows. *Staphylococcus aureus* may occur in the milk of cows with clinical or sub-clinical mastitis or as the result of contamination by handlers (Hudson 2010). Therefore, more

attention must be given to animal health for producing safety dairy products. Additionally, more attention must be given to the hygiene of workers in dairy plants.

Table 3. PCR detection of *Staphylococcus aureus* in raw milk and soft cheese manufactured from raw and pasteurized milk under (ISS) and (TS) during May 2013 to April 2014 in Merida, Yucatan, Mexico.

112

production systems	Traditional system (FMVZ/UADY)			Intensive silvopastoral system (FMVZ/UADY)			Intensive silvopastoral system (Kampepen)		
Samples	Raw milk	Cheese from raw milk	Cheese from pasteurized milk	Raw milk	Cheese from raw milk	Cheese from pasteurized milk	Raw milk	Cheese from raw milk	Cheese from pasteurized milk
May 2013	-	-	-	-	-	-	-	-	-
Jun. 2013	-	-	-	-	-	-	-	-	-
Jul. 2013	-	-	-	-	-	-	-	-	-
Aug. 2013	-	-	-	-	-	-	-	-	-
Sept. 2013	-	-	-	-	-	-	-	-	-
Oct. 2013	-	-	-	-	-	-	-	-	-
Nov. 2013	-	-	-	-	-	-	-	-	-
Dec. 2013	-	-	-	-	-	-	-	-	-
Jan. 2014	-	-	-	-	-	-	-	-	-
Feb. 2014	+	+	-	-	-	-	-	-	-
Mar. 2014	+	+	-	-	-	-	-	-	-
Apr. 2014	+	+	-	-	-	-	-	-	-

FMVZ/UADY: Farm of Faculty of Veterinary Medicine and Animal Science, Autonomous University of Yucatan.

+ : Contaminated samples. - : Non contaminated samples.

Fig. 1. Agarose electrophoresis separation of one of the agarose gels used in the detection of *Staphylococcus aureus*, which is presented. Lane L: Molecular weight Ladder. Lane C: Positive control. Empty lanes mean that these samples were not contaminated with *Staphylococcus aureus*, while lanes with bands (+) mean that these samples are contaminated with *Staphylococcus aureus*.

PCR detection showed that all samples were negative for almost tested pathogens (*Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes*) except six samples (5.56%); 3 samples of raw milk and other 3 samples of soft cheese manufactured from this raw milk under traditional system which were positive for *Staphylococcus aureus*. Once milk has been contaminated with *Staphylococcus aureus*, they can subsequently grow in dairy products manufactured from this contaminated milk. The milk pasteurization is recommended with animal health care and hygiene of workers in dairy plants. The results obtained for detected the pathogenic bacteria in the present study were compared with European Commission (EC) and it clearly indicate that the consumption of raw milk and soft cheese manufactured from raw milk under traditional system in (FMVZ/UADY) farm is not recommended due to the presence of *Staphylococcus aureus*.

REFERENCES

Aldrete-Tapia A, Escobar-Ramirez MC, Tamplin ML and Hernandez-Iturriaga M (2014) High-throughput sequencing of microbial communities in Poro cheese, an artisanal Mexican cheese. *Food Microbiol* **44** 136-141.

Amagliani G, Omiccioli E, Campo A, Bruce I, Brandi G and Magnani M (2006) Development of a magnetic capture hybridization-PCR assay for *Listeria monocytogenes* direct detection in milk samples. *Journal of applied microbiology* **100** 375-383.

Barkema HW, Schukken YH and Zadoks RN (2006) Invited Review: The role of cow, pathogen, and treatment systemn in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J Dairy Sci* **89** 1877-1895.

Barros-Rodríguez M, Solorio-Sánchez J, Ku-Vera J, Ayala-Burgos A, Sandoval-Castro C and Solís-Pérez G (2012) Productive performance and urinary excretion of mimosine metabolites by hair sheep grazing in a silvopastoral system with high densities of *Leucaena leucocephala*. *Tropical animal health and production* **44** 1873-1878.

Bergdoll MS (1997) Toxic shock syndrome. *Rev J Venom Anim Toxins* **1** 6–21.

Chang CT, Yuo CY, Shen HC, Li AM, Chen CY, Chou JI and Huang SP (1999) Recovery of Salmonella by using selenite brilliant green sulfa enrichment broth. *Journal of clinical microbiology* **37** 4120-4123.

Elizondo-Salazar JA, Jones CM and Heinrichs AJ (2010) Evaluation of calf milk pasteurization systems on 6 Pennsylvania dairy farms. *J Dairy Sci* **93** 5509-5513.

European Commission (2004a) Regulation (EC) No. 852⁄2004 of the European Parliament and of the Council of 29 April 2004 on hygiene of foodstuffs. Official Journal of the European Community L139 1-54.

European Commission (2004b) Regulation (EC) No. 853⁄2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Official Journal of the European Community L139 55-205.

Fox PF, mcsweeney PL, Cogan TM and Guinee TP (2004) Cheese: Chemistry, Physics and Microbiology: General Aspects. 3 ed. Academic Press

Goto M, Takahashi H, Segawa Y, Hayashidani H, Takatori K and Hara-Kudo Y (2007) Real-time PCR method for quantification of

Staphylococcus aureus in milk. *J Food Prot* **70** 90-96.

Gould LH, Mungai E and Behravesh CB (2014) Outbreaks attributed to cheese: differences between outbreaks caused by unpasteurized and pasteurized dairy products, United States, 1998-2011. *Foodborne Pathog Dis* **11** 545-551.

Gouws PA and Liedemann I (2005) Evaluation of diagnostic PCR for the detection of *Listeria monocytogenes* in food products. *Food Technology and Biotechnology* **43** 201-20

Hebert A, Sayasith K, Senechal S, Dubreuil P and Lagace J (2000) Demonstration of intracellular *Staphylococcus aureus* in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. *FEMS Microbiol Lett* **193** 57-62.

Holland J, Louie L, Simor A and Louie M (2000) PCR detection of *Escherichia coli* O157: H7 directly from stools: evaluation of commercial extraction methods for purifying fecal DNA. *Journal of clinical microbiology* **38** 4108-4113.

Hudson JA (2010) Evaluation of methods for detection of coagulase positive *staphylococcus* and staphylococcal toxin in milk and cheese. Prepared for New Zealand Food Safety Authority under project MFS/09/09 – Evaluation of methods for detection of coagulase positive *Staphylococcus aureus* and staphylococcal toxin in milk and cheese, as part of overall contract for scientific services pp 1-30.

Leuschner RG and Boughtflower MP (2002) Laboratory-scale preparation of soft cheese artificially contaminated with low levels of *Escherichia coli* O157, *Listeria monocytogenes*, and *Salmonella enterica* serovars Typhimurium, Enteritidis, and Dublin. *J Food Prot* **65** 508-514.

Lye Y, Afsah-Hejri L, Chang W, Loo Y, Puspanadan S, Kuan C, Goh S, Shahril N, Rukayadi Y and Khatib A (2013) Risk of *Escherichia coli* O157: H7 transmission linked to the consumption of raw milk. *International Food Research Journal* **20** 1001-1005.

Millet L, Saubusse M, Didienne R, Tessier L and Montel MC (2006) Control of *Listeria monocytogenes* in raw-milk cheeses. *Int J Food Microbiol* **108** 105-114.

Morales-Celaya M, Lobato-Calleros C, Alvarez-Ramirez J and Vernon-Carter E (2012) Effect of milk pasteurization and acidification method on the chemical composition and microstructure of a Mexican pasta filata cheese. *LWT-Food Science and Technology* **45** 132-141.

Nedelkov D, Rasooly A and Nelson RW (2000) Multitoxin biosensor-mass spectrometry analysis: a new approach for rapid, realtime, sensitive analysis of staphylococcal toxins in food. *Int J Food Microbiol* **60** 1–13.

Olson DW, Van Hekken DL, Tunick MH, Tomasula PM, Molina-Corral FJ and Gardea AA (2011) Mexican Queso Chihuahua: functional properties of aging cheese. *J Dairy Sci* **94** 4292-4299.

Omiccioli E, Amagliani G, Brandi G, Bruce IJ and Magnani M (2009) Simultaneous direct detection of *Salmonella spp.*, *Listeria monocytogenes* and *Escherichia coli* O157 in milk samples by magnetic extraction and multiplex PCR. *Journal of Rapid Methods & Automation in Microbiology* **17** 195-213.

Park SH, Kim HJ, Cho WH, Kim JH, Oh MH, Kim SH, Lee BK, Ricke SC and Kim HY (2009) Identification of *Salmonella enterica* subspecies I, *Salmonella enterica* serovars Typhimurium, Enteritidis and Typhi using multiplex PCR. *FEMS microbiology letters* **301** 137-146.

Peng X, Yu KQ, Deng GH, Jiang YX, Wang Y, Zhang GX and Zhou HW (2013) Comparison of direct boiling method with commercial kits for extracting fecal microbiome DNA by Illumina sequencing of 16S rrna tags. *Journal of microbiological methods* **95** 455-462.

115

Pereira-Suarez AL, Estrada-Chavez Y, Zuniga-Estrada A, Lopez-Rincon G, Hernandez DU, Padilla-Ramirez FJ and Estrada-Chavez C (2014) Detection of Mycobacterium tuberculosis complex by PCR in fresh cheese from local markets in Hidalgo, Mexico. *J Food Prot* **77** 849-852.

Rahimi E and Alian F (2013) Presence of enterotoxigenic *Staphylococcus aureus* in cow, camel, sheep, goat, and buffalo bulk tank milk. *Veterinarski Arhiv* **83** 23-30.

Renye J, Somkuti G, Vallejo-Cordoba B, Van Hekken D and Gonzalez-Cordova A (2008) Characterization of the microflora isolated from queso fresco made from raw and pasteurized milk. *Journal of Food Safety* **28** 59-75.

Renye JAJr, Somkuti GA, Van Hekken DL and Guerrero Prieto VM (2011) Short communication: characterization of microflora in Mexican Chihuahua cheese. *J Dairy Sci* **94** 3311-3315.

Silva R, Cruz AG, Faria JA, Moura MM, Carvalho LM, Water EH and Sant'Ana AS (2010) Pasteurized milk: efficiency of pasteurization and its microbiological conditions in Brazil. *Foodborne Pathog Dis* **7** 217-219.

Silvestre D, Ruiz P, Martinez-Costa C, Plaza A and Lopez MC (2008) Effect of pasteurization on the bactericidal capacity of human milk. *J Hum Lact* **24** 371-376.

Solano-López C and Hernández-Sánchez H (2000) Behaviour of *Listeria monocytogenes* during the manufacture and ripening of Manchego and Chihuahua Mexican cheeses. *International journal of food microbiology* **62** 149-153.

Torres-Llanez M, Vallejo-Cordoba B, Díaz-Cinco M, Mazorra-Manzano M and González-Córdova A (2006) Characterization of the natural microflora of artisanal Mexican Fresco cheese. *Food control* **17** 683-690.

U.S. Food and Drug Administration (2001) Foodborne pathogenic microorganisms and natural toxins. Available at:

http://www.fda.gov/Food/FoodSafety/FoodborneIllnes/FoodborneIllnessFoodborne PathogensNaturalToxins/BadBugBook/ucm070015.htm. Accessed 2 October 2009.

Van Gysel M, Cossey V, Fieuws S and Schuermans A (2012) Impact of pasteurization on the antibacterial properties of human milk. *Eur J Pediatr* **171** 1231-1237.

Wu Q, Li Y, Hu H, Wang M, Wu Z and Xu W (2012) Rapid Identification of *Staphylococcus aureus*: FISH Versus PCR Methods. *Lab Medicine* **43** 276-280.