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Incidence of *Salmonella, Listeria monocytogenes, Escherichia coli* O157:H7, and Staphylococcal Enterotoxin in Two Types of Mexican Fresh Cheeses

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ABSTRACT

Handcrafted fresh cheeses are popular among consumers in Mexico. However, unsafe raw materials and inadequate food safety practices during cheese manufacture and preservation make them a potential public health risk. The incidence of *Salmonella, Listeria, Escherichia coli* O157:H7, and staphylococcal enterotoxin was analyzed in two types of fresh cheese (panela and adobera) commonly marketed in Mexico. A total of 200 samples, 100 panela and 100 adobera, were acquired from 100 wholesale milk product distributors who supply small retailers in the Guadalajara metropolitan area, Jalisco State, Mexico. Pathogens were identified using culture and immunoassay (miniVidas) methods. The presence of staphylococcal enterotoxin was determined by an immunoassay method. Of the 200 analyzed samples, 92 were positive for at least one of the pathogens. The incidence in the panela samples was 56%: 34% *Salmonella,* 16% *E. coli* O157:H7, and 6% *L. monocytogenes*. In the adobera samples, incidence was 36%: 20% *Salmonella,* 4% *E. coli* O157:H7, and 12% *L. monocytogenes*. Staphylococcal enterotoxin was not detected in any of the 200 samples. Choice of technique had no effect on detection of pathogen incidence, although the immunoassay method identified more *Salmonella* serotypes than the culture method. Handcrafted panela and adobera fresh cheeses in Mexico frequently contain pathogenic bacteria and therefore pose a public health risk.

Fresh cheeses such as panela and adobera are made from whole or low-fat cow's milk curd via casein coagulation with rennet, in many cases without thermal treatment *(19)*. These cheeses have high moisture content, a light flavor, no rind, and a short shelf-life, and they require refrigeration *(28)*. The fresh cheeses are produced in coagulation vats using enzymes and/or acids. Once curdled, the whey is drained off; the large cheese clumps are removed, milled into curds, and salted; and the finished cheese is packed in plastic bags for sale *(12)*. A number of cheeses are made in Mexico, including panela, fresco, ranchero, Oaxaca, asadero, mozzarella, morral, adobera, cottage, and crema *(26)*. Panela and adobera are the types most widely sold in the Mexican market.

Many fresh cheese producers in Mexico use unpasteurized (raw) milk because they believe the native microbiota of raw milk confer pleasant aromas and flavors to the final product. A large proportion of cheese consumers also prefer cheeses made with raw milk. However, raw milk products are known to pose a public health risk due to their pathogenic bacteria content *(22)*. Indeed, consumption of raw milk has been associated with campylobacteriosis, salmonellosis, yersiniosis, listeriosis, tuberculosis, brucellosis, cryptosporidiosis, and staphylococcal enterotoxin poisoning *(25)*. A number of disease outbreaks have been traced to consumption of Mexican-style cheeses made with raw milk *(10, 15, 16, 33)*. Most of these outbreaks have been associated with *L. monocytogenes* and have occurred in Hispanic populations accustomed to eating handcrafted cheeses. Listeriosis occurs mainly (65 to 83%) in pregnant women and can lead to death in those susceptible to infection *(7, 14–16)*.

Other microorganisms in contaminated cheeses that have caused outbreaks *(6)* include *Salmonella,* recovered from cheeses made from raw milk *(10, 11),* and *Brucella,* a genus often found in raw milk *(2)*. *Escherichia coli* O157:H7 is uncommon in milk products but has been responsible for disease outbreaks from ingestion of raw cheese *(12)*.

Very limited research has been done on the presence of the abovementioned pathogens in fresh cheeses in Mexico. *Salmonella* was reported in 2.5% of fresh cheese samples from a street market in Mexico City *(1),* and *L. monocytogenes* was identified from fresh cheese samples acquired in small retail stores in Sonora State, Mexico *(19)*.

Fresh cheeses are a basic food in Mexico; 217,000 tons were consumed nationwide in 2005, 60.8% of which was

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produced domestically *(8)*. Of total domestic cheese sales, panela cheese represents 52.3%, highlighting a clear preference among Mexican consumers for fresh cheeses *(32)*. Market demand is supplied by national and transnational companies as well as by family or artisanal companies. The main concern with artisanal cheese production is the health risk arising from the use of raw milk, inadequate food safety practices during production and preservation, and contamination from other raw materials used in the process. These factors, in conjunction with improper storage practices and informal sale in markets, favor pathogen contamination and development *(24)*. The objective of this study was to determine the incidence of *Salmonella, Listeria, E. coli* O157:H7, and staphylococcal enterotoxin in handcrafted panela and adobera cheeses, to better understand the public health risk they may pose.

MATERIALS AND METHODS

Sample collection and preparation. Over a 12-month period (every 2 weeks), fresh cheeses were acquired from 100 randomly selected *(17)* suppliers of milk products; these businesses, located in four wholesale distribution centers in Guadalajara, Jalisco State, Mexico, supply small markets and stores. A sample of each type of fresh cheese was purchased at each supplier, for a total of 100 panela and 100 adobera samples, each weighing approximately 1 kg. Upon purchase and while still in their original packaging, they were placed in stomacher bags, sealed with a rubber band, placed in a refrigerated container (25 by 15 cm; Igloo, Houston, TX), and transported to the laboratory for analysis within 1 h of purchase. Samples totaling 500 g were taken from each cheese, that is, 100-g portions from each side and the center. These were placed in a single stomacher bag and homogenized manually for 1 min.

Microbiological analysis. *Salmonella, E. coli* O157:H7, *L. monocytogenes,* and staphylococcal enterotoxin were identified following the procedures described below. For each positive sample, three to five colonies were selected of each of the pathogens.

Salmonella was identified using, simultaneously, procedures described in the *Bacteriological Analytical Manual (3)* and immunodetection (miniVIDAS, bioMérieux Vitek, Hazelwood, MO). Briefly, 25 g of each subsample was placed in a stomacher bag containing 225 ml of buffered peptone water; the bags were homogenized for 1 min in a stomacher (Lab-Blender 400, Tekmar Co., Cincinnati, OH) and were incubated at 35°C for 18 to 24 h. Enrichment was done in soy Rappaport-Vassiliadis (RV) broth, tetrathionate broth, and Müller-Kauffmann tetrathionate-novobiocin (MKTTn) broth (bioMérieux, Marcy-l'Étoile, France) for 24 h at 41.5°C (RV and tetrathionate broths) or 6 to 8 h at 37°C (MKTTn broth). The RV and tetrathionate broths were streaked on plates containing brilliant green agar, bismuth sulfite agar, or xylose lysine deoxycholate agar and were incubated for 24 to 48 h at 35°C. Three to five typical *Salmonella* colonies were randomly chosen from each selective medium for biochemical identification using urea broth, lysine-iron agar, and triple sugar iron. *Salmonella* was identified by serological analysis using somatic antigen polyvalent serum (antiserum poly A-I & Vi, BD Bioxon, Mexico State, Mexico). The strains identified as *Salmonella* were sent to the National Diagnostic and Epidemiologic Reference Institute (InDRE) in Mexico City for serotype identification.

The immunodetection analysis was done 8 h after incubation. Briefly, 1 ml each of the RV and MKTTn broths were transferred to separate M broth tubes and incubated at 41.5°C for 16 to 24 h. The tubes were then homogenized on a vortex, and 1-ml samples from each tube were mixed together in a sterile tube. The mixture was heated in a water bath at 90°C for 15 min and cooled to room temperature; then 500 μl was transferred to a VIDAS SLM strip (bioMérieux). Samples presumed to be positive were confirmed by culture following the *Bacteriological Analytical Manual* protocol using RV and MKTTn tubes as described above.

To detect *E. coli* O157:H7, the samples were analyzed using, simultaneously, the culture procedure described by Feng and Weagant *(13)* and immunodetection (miniVIDAS, bioMérieux Vitek). Subsamples (25 g) were taken from each cheese type, enriched with modified Trypticase soy broth (225 ml), cefixime (0.0125 mg/liter), cefsulodin (10.00 mg/liter), and vancomycin (8.00 mg/liter) (all from Sigma-Aldrich, St. Louis, MO) and incubated at 35°C for 24 h. Microorganism isolation was done on sorbitol MacConkey medium containing cefixime and tellurite (BD, Franklin Lakes, NJ) or BBL CHROMagar O157 (BD, Mexico State), and both media were incubated at 35 to 37°C for 18 to 24 h. Typical colonies were identified by API 20E and serologically with O157 and H7 antiserums (RIM *E. coli* O157:H7 latex test kit, Remel, Lenexa, KS).

Detection of *E. coli* O157:H7 by immunoassay was done by first preenriching 25 g of each cheese type with 225 ml of modified Trypticase soy broth containing casamino acids (10 g/liter; bioMérieux) and 1.1 ml of acriflavine (Sigma-Aldrich). This was incubated at 41.5°C for 7 h, and 1 ml of the resulting culture was inoculated into 9 ml of MacConkey broth with cefexime and tellurite (180 μl/9 ml; bioMeriéux) and then incubated for 18 h at 35 ± 2°C. Finally, 1 ml of MacConkey broth with cefexime and tellurite was heated at 100°C for 15 min and cooled at room temperature; 500 μl was placed on a VIDAS ECO strip (bioMérieux) and positive samples were confirmed from the tube containing MacConkey broth with cefexime and tellurite, following the procedure described above.

The presence of *L. monocytogenes* was analyzed by enriching 25-g samples of each cheese in 225 ml of *Listeria* enrichment medium (Difco, BD, Sparks, MD), followed by incubation at 30°C for 48 h. Enriched samples were then streaked onto Oxford base medium and incubated at 35°C for 48 h. Fresh mobility and sheep blood hemolysis tests were done for the typical colonies, and the CAMP test and biochemical tests (API Listeria identification test kit, bioMérieux) were done to confirm dubious reactions.

Immunoassay analysis of *L. monocytogenes* was done following the Association française de Normalisation AFNOR BIO-12/11-03/04 method *(5)*. Preenrichment was done by placing 25-g samples of each cheese type in 225 ml of Fraser broth and incubating at 30°C for 24 to 26 h. Each sample type was then homogenized; 1 ml was transferred into a tube containing 10 ml of Fraser broth and was incubated at 30°C for 24 to 26 h. Finally, 500 μl of each was transferred onto a LMO2 strip (bioMérieux). Confirmation of positive results was done from the tube containing Fraser broth, as described above.

Presence of staphylococcal enterotoxin in both cheese types was determined following AOAC procedure 12095 B-ES-2003/09 *(4)*. Briefly, 25-g samples of each cheese type were mixed individually with 25 ml of extraction buffer, homogenized in a Stomacher 400 (Lab-Blender Tekmar Co.) for 2 min, and incubated at 18 to 25°C for 15 min. Of the supernatant, 2 ml was transferred into a 1.5-ml Eppendorf tube and centrifuged for 15 min at 4,000 × *g*. The pH was adjusted to between 7.5 and 8.09 using 1 N NaOH, and then 500 μl was transferred to a SET2 strip (bioMérieux).

TABLE 1. Salmonella, E. coli *O157:H7,* L. monocytogenes, *and staphylococcal toxin incidence in panela and adobera fresh cheeses*[a]

Cheese type	Pathogen	Positive samples by method		
		Total	Culture and immunoassay	Immunoassay alone
Adobera	*Salmonella*	20	16	4
	E. coli O157:H7	4	2	2
	Listeria monocytogenes	12	10	2
	Staphylococcus toxin	0	0	0
Panela	*Salmonella*	34	22	12
	E. coli O157:H7	16	14	2
	Listeria monocytogenes	6	6	0
	Staphylococcus toxin	0	0	0
Total		92	70	22

[a] There were 100 samples of each type of cheese, panela and adobera.

pH, acidity, alkaline phosphatase, and foreign matter determination. To each subsample (10 g) of each cheese type, 10 ml of deionized water was added; after the mixture was manually homogenized, the pH level was measured with a potentiometer (Corning 320 pH meter, Corning, NY). To measure percent acidity, a 10-g subsample of each type of cheese was placed in 25 ml of CO_2-free distilled water and manually homogenized; the mixture was titrated with 0.1 N NaOH, using phenolphthalein as an indicator. Alkaline phosphatase was quantified with a commercial kit (Hycel, Jalisco, Mexico). Foreign matter was identified by close visual inspection of the interior and exterior of each cheese sample, using a knife, fork, and sterile tweezers.

Statistical analysis. An analysis of variance (ANOVA) was run to identify differences between the methods. Analyses were run with the Statgraphics ver. Centurion XV.II program (StatPoint, Inc., Herndon, VA).

RESULTS

Of the 200 analyzed samples, 92 (46%) were positive for at least one of the studied pathogens. Of the 92 positive samples, 60% were acquired between March and May in two of the four wholesale distribution centers in Guadalajara. Seventy samples were found to be positive for pathogen presence by both the immunoassay and culture techniques; the remaining 22 were initially identified as positive only by the immunoassay but were later confirmed by culture. All samples that were found positive by culture were also positive by the immunoassay method (Table 1). The pH levels of 64% of the positive samples ranged from 5.0 to 5.5, whereas all samples with pH levels lower than 4.9 were negative for all pathogens.

Panela cheese had the highest incidence of the studied pathogens, with 56 samples positive for at least one pathogen. In this cheese, *Salmonella* was isolated from 34% of the samples, *E. coli* O157:H7 from 16%, and *L. monocytogenes* from 6% (Table 1). For adobera cheese, 36% of samples were positive for at least one of the three pathogens; *Salmonella* was isolated from 20% of the samples, *E. coli* O157:H7 from 4%, and *L. monocytogenes* from 12% (Table 1). All three pathogens were isolated in 2 of the 100 adobera cheese samples. Staphylococcal enterotoxin was not detected in any of the 200 analyzed cheese samples.

A total of 142 *Salmonella* strains were isolated from 54 panela and adobera cheese samples. Panela cheese had the highest serotype diversity. Although thirteen serotypes were identified, the culture procedure identified only seven; the immunoassay procedure detected a larger number of serotypes (Fig. 1). The *Salmonella* serotypes most frequently recovered were Amsterdam (22.5%), Montevideo

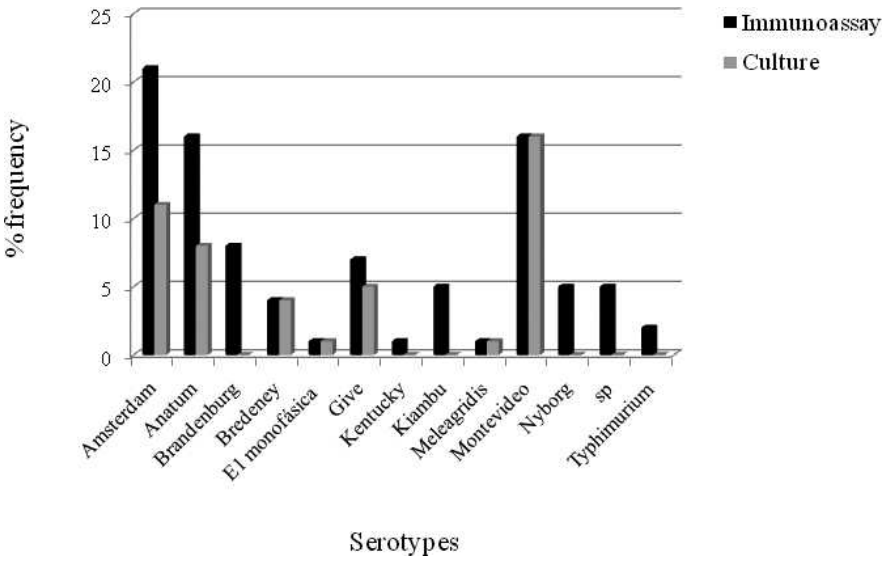

FIGURE 1. *Frequency percentage of* Salmonella *serotypes from fresh cheese samples found to be* Salmonella *positive by immunoassay and culture.*

TABLE 2. *pH and acidity values in panela and adobera fresh cheeses*[a]

Cheese type	pH			Acidity (% lactic acid)		
	Minimum	Maximum	Avg	Minimum	Maximum	Avg
Adobera	4.98	7.3	5.694	0.477	0.675	0.532
Panela	4.92	6.5	5.349	0.378	0.612	0.513

[a] There were 100 samples of each type of cheese, panela and adobera.

(22.5%), and Anatum (16.9%) (Fig. 1). Two strains (1.5%) identified as *Salmonella* Typhimurium were detected by immunoassay from separate samples.

A total of 66 *Listeria* strains were isolated, of which 60.6% corresponded to *L. monocytogenes* and the remaining 39.4% to the species *L. innocua, L. grayi, L. ivannovi,* and *L. seeligeri*. Adobera cheese samples contained no *L. monocytogenes,* and the panela cheese samples contained only *L. monocytogenes* and *L. innocua*. The culture procedure allowed recovery of different *Listeria* species; the immunoassay analysis used *L. monocytogenes*–specific strips and thus detected only this species.

Thirty strains were identified among the 20 samples positive for *E. coli* O157:H7; Shiga toxin production was not analyzed. No differences ($P > 0.05$) in *E. coli* O157:H7 detection was observed between procedures (i.e., culture and ELFA) for either of the two types of cheese studied.

Values for pH and acidity did not differ ($P > 0.05$) between the two types of cheese (Table 2). Most samples (95%) had pH values <5.7, although the range was from 4.92 to 7.0. Acidity in 60% of the samples was between 0.30 and 0.40% (Table 2), and all the cheese samples had alkaline phosphatase values >12 UF/g cheese. Foreign matter was identified in 25% of the samples; the materials found most frequently were human hair, earth, and insect remains, although fragments and splinters of basket were found in the samples of panela cheese.

DISCUSSION

In Mexico, different types of cheese pose potential health risks for consumers. Pathogen presence is affected by such factors as the use of raw milk and lack of aging, as well as by improper conditions during production, packaging, and handling during sale *(21, 28)*. Disease outbreaks due to cheese consumption have most frequently been linked to the use of raw milk or a mixture of raw and pasteurized milk in the production process *(18)*. Fresh cheeses have been the main vehicles for pathogenic agents in these outbreaks; the pathogens involved have included *L. monocytogenes, Salmonella* spp., *E. coli* O157:H7, *Staphylococcus aureus, Brucella melitensis, Clostridium botulinum,* and *Streptococcus equi*.

The results of our study highlight the health risk of consuming fresh cheeses in Mexico as well as the inadequate conditions throughout the chain of production and sale. A number of substandard food safety practices were observed during sample collection at stores, including use of raw milk in cheese fabrication, lack of temperature control at the sale locations, and improper handling by sales staff (data not shown). Use of raw, or incorrectly pasteurized milk, or of mixtures of raw and pasteurized milk in cheese production is indicated by the high residual alkaline phosphatase values (>12 UF/g) observed in the present study; this enzyme is thermolabile and is inactivated during pasteurization *(27)*. The use of extremely inadequate food safety measures during the handcrafting of the sampled cheeses is obvious in the presence of human hair, earth, and insect remains in 25% of the samples. In the panela cheese, the basket fragments and splinters found are artifacts of the circular baskets used to shape and impress basket marks on the final product. An especially egregious violation of good food safety practices was storage of fresh cheeses at room temperature for over 4 h, which promotes pathogen multiplication in cheeses. This violation was observed incidentally during sample acquisition.

Staphylococcal food poisoning is linked to consumption of milk products *(23, 29)*. Absence of these toxins in the analyzed samples was probably due to inhibition of their synthesis by the acidic pH in both types of cheese. Pathogens could also have been inhibited by the acidic pH (most samples had pH levels above 5.0). Our results show a possible protective effect of pH in fresh cheeses made with raw milk, although more studies are needed to confirm that pH levels below 5.0 contribute to inhibiting pathogens in fresh cheeses.

Salmonella is commonly associated with gastrointestinal disorders in developing countries such as Mexico. From 2004 to 2009, 709,278 salmonellosis cases and 228,206 typhoid fever cases were reported in Mexico *(30)*. Data on disease outbreaks in Mexico caused by milk product consumption are scarce, although there are reports of salmonellosis outbreaks linked to fresh cheeses. Between 1980 and 1989, 31.5% of reported outbreaks were due to contaminated cheese; six of the outbreaks were caused by *Salmonella (24)*. A total of 46 outbreaks were reported from 1993 to 2002, three of which were associated with salmonellosis *(31)*. *Salmonella* contamination in cheeses is caused by the use of contaminated milk and by direct contamination from humans *(9)*.

No *L. monocytogenes* or *E. coli* O157:H7 outbreaks linked to cheese consumption have been reported in Mexico to date. This does not mean, however, that these pathogens have not been present in cheese because gastrointestinal disease cases and possible associations with food consumption are significantly underreported in Mexico *(1)*. Data published in the United States show a close relationship between outbreaks and incidence of *L. monocytogenes* and *E. coli* O157:H7; indeed, it is recommended that pregnant women not consume fresh, Mexican-style cheeses to avoid

the risk of spontaneous abortion due to the possible presence of *L. monocytogenes* in these cheeses *(7, 14, 16)*.

Although the present study found no differences between the culture and the immunoassay procedures in the detection of pathogens in cheese samples, each procedure had advantages and limitations. Immunoassay is limited in its use for *Listeria* detection because it detects only *L. monocytogenes,* while the culture procedure can identify various species. In contrast, immunoassay detected more *Salmonella* serotypes, including *Salmonella* Typhimurium, in positive samples than did the culture technique. And immunoassay is able to produce results in a short time, a clear advantage for panela and adobera cheese producers, who need rapid results to ensure product safety.

The present results clearly demonstrate the health risks due to consumption of artisanal panela and adobera cheeses. Nonetheless, because a high proportion of the population prefers handcrafted cheeses, these cheeses maintain their popularity in the marketplace. This ongoing public health risk has led health authorities to propose food safety regulations to meet current demands. The data generated from this study have been used by the federal Secretariat of Health of Mexico to create a regulation, which is still in the development stage, specifically for panela fresh cheese *(20)*.

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