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Prevalence and characterization of Listeria monocytogenes, Salmonella and Shiga toxin-producing Escherichia coli isolated from small Mexican retail markets of queso fresco
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Prevalence and characterization of *Listeria monocytogenes*, *Salmonella* and Shiga toxin-producing *Escherichia coli* isolated from small Mexican retail markets of queso fresco

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Queso fresco (QF) is a handmade cheese consumed and produced in Latin America. In Mexico, QF production is associated with a microbiological risk. The aim of the study was to determine the incidence and characterization of *Listeria monocytogenes*, *Salmonella* spp., and Shiga toxin-producing *Escherichia coli* (STEC) in QF from retail markets of the north-western State of Sinaloa, Mexico, and to assess the effect of physicochemical parameters on *Listeria* presence. A total of 75 QF samples were obtained. *L. monocytogenes*, *E. coli*, and coliforms were detected in 9.3, 94, and 100 %, respectively. *Salmonella* was not detected. STEC isolates showed virulence genes. Microbial loads were above the maximum values recommended by the Official Mexican Standards. Physicochemical parameters such as water activity (a_w), moisture content, pH, and salinity played a role in *Listeria* prevalence in QF. Rigorous control in QF made in Culiacan, Mexico is needed to reduce the risk of foodborne pathogens.

**Keywords:** queso fresco; *Listeria monocytogenes*; *Salmonella*; *Escherichia coli*; Shiga toxin; food safety

Introduction

The production, consumption, and diversity of cheese in Mexico, and other Latin American countries, have a long tradition. Queso fresco (QF) is the most popular type of cheese consumed in Mexico, as well as in the Southern and Western United States, where a significant proportion (~53 %) of the population is Hispanic (Clark et al. 2001; Van Hekken & Farkye 2003). QF is a fresh white cheese, typically made with unpasteurized milk, with relatively high moisture content (55–58 %), high pH levels (5–6.3), low salt content (1.4–1.6 %), and no specific starter culture added during production (Torres & Chandan 1981; Van Hekken & Farkye 2003). The typical cheese flavor is influenced by the native microflora present in raw milk (Reny et al. 2008), and could be the primary reason why consumers prefer it over QF made from pasteurized milk. Small farmers generally produce QF on a small scale with very little technology (MacDonald et al. 2005; Cesin et al. 2007), using raw milk and simple equipment such as kitchen utensils. QF is sold to wholesalers or directly to end consumers, usually in
small markets and/or door-to-door, without labels, while transported and stored in ice chests with very few food safety precautions (Vazquez-Salinas et al. 2001; Cesin et al. 2007; Moreno-Enriquez et al. 2007).

The production of QF is commonly obtained by the enzymatic coagulation of raw milk with rennet and coagulation enzyme without any microbiological monitoring program since it is processed in households by artisans. The use of unpasteurized milk in the production of QF is in conflict with the Mexican Official Regulation, which states that cheeses should be manufactured with pasteurized milk to guarantee safety (Secretaria de Salud 1996). However, the Mexican authorities fail to monitor the application of good manufacturing practices by the small producers when making QF, resulting in an unsafe product that can cause health problems to the general population.

In the last decade, QF made in the United States has been involved in a number of foodborne outbreaks associated with the presence of *Listeria monocytogenes* and *Salmonella typhimurium* due to the insufficient pasteurization conditions implemented (CDC 2001; MacDonald et al. 2005; Koustas et al. 2010). Two outbreaks associated with multidrug-resistant *S. typhimurium* DT10 in California, United States, in 1997 were linked to the consumption of raw milk Mexican-style cheese from street vendors (Cody et al. 1999). During 2000, more than 13 cases and 5 deaths of listeriosis attributed to QF were reported in North Carolina (CDC 2001; MacDonald et al. 2005). An outbreak of 12 cases of listeriosis associated with consumption of QF contaminated with *L. monocytogenes* was also reported in Texas in 2003 (Hise et al. 2004). Recently, the US has been involved with two outbreaks of listeriosis linked with domestic and imported cheeses (CDC 2012, 2013). Shiga toxin-producing *Escherichia coli* (STEC) especially *E. coli* O157:H7 has been considered an important foodborne pathogen causing hemorrhagic colitis and hemolytic uremic syndrome (Wang et al. 1997; Paton & Paton 1998). Outbreaks of *E. coli* O157:H7 associated with the consumption of raw milk dairy products have been well documented in Canada and France (Honish et al. 2005; Espie et al. 2006; FSN 2013). In 2010, an outbreak of *E. coli* O157:H7 linked with cheese made from raw milk was reported in the US (CDC 2010), but no reports have been documented due to the consumption of QF produced in Latin American countries contaminated with this serotype. Furthermore, fecal contamination during the manual milking process, coupled with the high moisture content and high pH, is a factor contributing to the presence and growth of enteric pathogens such as *L. monocytogenes*, *Salmonella*, and STEC (Griffin & Tauxe 1991; Wang et al. 1997; Pintado et al. 2005; Brito et al. 2008). The occurrence and levels of microorganisms such as *L. monocytogenes*, *Salmonella* spp., and STEC in QF in Mexico are unclear and more research is needed in this arena. Thus, the aim of the present study was to establish the incidence and characterization of these target pathogens in QF obtained from retail markets of the northwestern State of Sinaloa, Mexico.

**Materials and methods**

**Sample collection**

A total of 75 QF samples, each weighting approximately 500 g each, were obtained from 75 independent retail merchants (small markets selling cutting portions of QF upon purchase) in Culiacan, the capital of the State of Sinaloa, Mexico, over an 8-month period. Samples were placed in a portable cooler for transport to The Centro de Investigacion en
Bacterial analyses were conducted within 24 h upon collection.

**Physicochemical analysis**
Physicochemical analysis such as water activity ($a_w$), pH, moisture, and salinity was performed according to the Association of Official Analytical Chemists (William 1998). A 10 g sample of the QF sample was weighed and mixed with 90 mL of water in a blender and filtered through Whatman paper No.1. Water activity ($a_w$) was measured by Aqualab model X-2 (Aqua Lab, Pulman, WA); pH was measured with an automated titrator (Mettler Toledo Mod. DL-50®) and moisture was determined by drying 3 g of QF in a conventional oven at 95–105 °C for 24 h. For salinity, a 10 g sample of QF was dried at 550 °C for 24 h in a conventional oven, followed by addition of 5 mL HCl and filtering through Whatman paper No.5. Triplicate samples for each parameter were measured.

**Identification, isolation, and characterization of microorganisms in QF**
QF samples were analyzed for *Listeria* spp., *Salmonella* spp., *E. coli*, STEC, and coliforms. *Listeria* spp. and *Salmonella* were recovered from each QF sample using the FDA-Bacteriological Analytical Manual standard enrichment/recovery method with some modification (http://www.cfsan.fda.gov/~ebam/bam-10.html) as shown below. *E. coli* coliforms were recovered by using membrane filtration, and STEC was detected by biochemical and molecular characterization analysis.

**Listeria**
For *Listeria*, briefly, a 25 g portion of QF was diluted in 225 mL of modified *Listeria* enrichment broth (Difco, Lake, NJ, USA), followed by hand homogenization for 2 min. The homogenized samples were incubated at 35 °C for 48 h, and 0.1 mL aliquot from the enrichment broths was transferred to *Listeria* enrichment broth base Fraser (Difco, Lake, NJ, USA) and then incubated at 35 °C for 24 h. Then, 10 μL of media of the broth base Fraser were streaked onto PALCAM (Difco, Lake, NJ, USA) and/or *Listeria* ChromAgar (CHROMagar™ Company, Paris). Plates were incubated at 35 °C for 24 h. From each selective plate, five presumptive *Listeria*-like colonies (gray-green colonies surrounded by dark brown to black halos in the medium) were transferred onto trypt-case soy agar and incubated at 35 °C for 24 h for future analysis. In addition, PCR was run on DNA extract of the presumptive *Listeria* isolates (Furrer et al. 1991) and positive isolates were identified when amplification target of the gene (234 bp) was observed in 1.5 % agarose gels stained with ethidium bromide. Positive *Listeria* isolates were confirmed as *L. monocytogenes* by United States Department of Agriculture-Agricultural Research Service-Eastern Regional Research Center (USDA-ARS-ERRC), through biochemical, pulsed field gel electrophoresis (PFGE), and ribotyping techniques. Genomic DNA collected from confirmed *L. monocytogenes* isolates was digested with *Ascl* and the resulting fragments were separated using the PFGE method as described by Gilbreth et al. (2005). Restriction enzyme digestion profiles (REDP’s) obtained from each isolate were analyzed using the Applied Maths BioNumerics software package (version 4.0, Saint-Martins-Latem, Belgium). Isolates were ribotyped using the automated Riboprinter® characterization system with *EcoRI* as specified by the manufacturer (Dupont/Qualicon, Wilmington, DE).
Salmonella

For *Salmonella*, a 25 g portion of QF was diluted in 225 mL of Buffer Peptone Water (Difco), followed by hand homogenization for 2 min. The homogenized samples were incubated at 35 °C for 24 h. After 24 h of incubation, 0.1 mL aliquots from enrichment broths were transferred to 10 mL of Rappaport-Vassiliadis (RV-Oxoid) followed by incubation at 42 °C for 24 h. The broth was plated onto Hektoen enteric agar and incubated at 35 °C for 24 h. Typical *Salmonella*-like colonies (green colonies with black center) were purified on trypticase soy agar and incubated at 35 °C for 24 h. Isolates were examined and screened by PCR, according to Chiu and Ou (1996), to identify *Salmonella* isolates with a gene target of 244 bp. PCR products were visualized by electrophoresis through 1.5 % agarose gels stained with ethidium bromide.

E. coli and coliforms

*E. coli* and coliforms were recovered using membrane filtration (0.45 μm, Gelman Science, Ann Arbor, MI). A 25 g portion of QF was diluted in 225 mL of lactose broth (Difco) followed by homogenization by hand for 2 min. Ten-fold dilutions were made and plated onto ChromAgarECC (Paris, France), a selective medium for the detection of *E. coli* and coliforms, followed by incubation at 44.5 °C for 24 h. From the selective plate, five *E. coli* colonies were transferred onto trypticase soy agar and incubated at 35 °C for 24 h. The *E. coli* isolates were sent to USDA-ARS-ERRC, (Wyndmoor, PA) for the biochemical and molecular characterization. Each isolate was examined and screened by PCR according to Frataminco et al. (2000) to determine whether any of the isolates possessed O157:H7 or *stx* genes. PCR products were visualized by electrophoresis through 1.5 % agarose gels stained with ethidium bromide.

Statistical analyses

Descriptive statistics were performed to quantify the presence of *L. monocytogenes*, *Salmonella*, *E. coli*, and STEC in QF. Analysis of variance (ANOVA) and the stepwise binary logistic regression analysis (LRA) were conducted to compare physicochemical parameters with *Listeria* risk presence/absence in QF with an alpha of 0.05 significance level. The odds ratio (OR) was also calculated. Data were subjected to Minitab (version 14) system for statistical analyses.

Results and discussion

A total of 75 samples of QF were collected from 75 retail markets over an 8-month period. All samples were analyzed for aw, pH, moisture and salt levels, and for the presence of *L. monocytogenes*, *Salmonella*, *E. coli*, and coliforms. The results showed an average percentage of proximal analysis from the 75 QF samples: aw of 0.980 (SD 0.009), pH value of 5.47 (SD 0.34), moisture 59.86 (SD 4.58), and NaCl 2.53 (SD 3.14). The moisture and salt levels are attributes considered of great importance during QF production. QF from Culiacan samples showed values above the typical levels expected for a QF compared with Saltijeral et al. (1999) which found moisture and salt content levels in QF ranging from 55 to 58 % and from 1.4 to 1.6 %, respectively. It has been shown that high water activity and moisture levels on cheeses made from raw milk favor the survival of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7.
Guraya et al. 1998; Saltijeral et al. 1999; Silva et al. 2003). The average pH of 5.47 was below the range expected for this type of cheese (Tunick & Van Hekken 2010). The low pH value observed in this study may be attributed to unsanitary processing conditions and/or to inadequate storage at abusive temperatures as described by Lin et al. (2006).

*L. monocytogenes* was detected in 7/75 (9.3%) of the samples tested. Coliforms were present in 75/75 (100%), at levels varying from $5 \times 10^2$ to $4.0 \times 10^6$ with an average of $2.1 \times 10^5$ CFU/g. *E. coli* was isolated from 70/75 (94%), at levels ranging from $\leq 1.0$ to $3.4 \times 10^5$ CFU/g with an average of $1.7 \times 10^4$ CFU/g (detection limit 1.0 CFU/g). *Salmonella* was not detected (detection limit 1.0 CFU/g) in any of the analyzed samples. Other studies have documented the presence of microbial contamination in QF produced in Mexico, showing in some cases 100% incidence of *E. coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterobacter* spp., and *Streptococcus durans* (Diaz-Cinco et al. 1998; Araujo et al. 2002). A study conducted by Ansay and Kaspar (1997) showed 58% of *E. coli* in soft and semi-hard cheese; Diaz-Cinco et al. (1998) reported *E. coli* in all QF samples tested; and Öksüz et al. (2004) reported a 60% incidence of *E. coli* in cheese samples made with raw milk, and high total coliform bacteria counts $6.0 \times 10^4$ CFU/g. In addition, Aygun et al. (2005) reported $4.3 \times 10^3$ CFU/g of *E. coli* and $1.0 \times 10^3$ CFU/g of coliforms in Carra cheese made with raw milk, which has also confirmed our results. The present study also showed a clear correlation between the presence of *L. monocytogenes* and *E. coli* in QF; all *L. monocytogenes* positive-QF samples were positive for *E. coli*.

### L. monocytogenes

A total of 35 *L. monocytogenes* isolates, from seven positive samples, were analyzed by pulsed field electrophoresis pulsotypes. Fourteen of the isolates belonged to Pulsotypes I, whereas Pulsotypes II and III contained one isolate each (Table 1). Table 1 shows a comparison of *L. monocytogenes* in QF in soft cheese from Sinaloa and Sonora, Mexico, and Minas Frescal Cheese (MFC) from Brazil (Moreno-Enriquez et al. 2007; Brito et al. 2008).

<table>
<thead>
<tr>
<th>Location</th>
<th>Year</th>
<th>Cheese type</th>
<th>Prevalence (positive sample/total samples)</th>
<th>Number isolates/positive samples</th>
<th>Pulsotype (number isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culiacan, Sinaloa, Mexico</td>
<td>2007</td>
<td>QF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.3 % (7/75)</td>
<td>16/7</td>
<td>6 (14)</td>
</tr>
<tr>
<td>(present study)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8 (1)</td>
</tr>
<tr>
<td>Sonora, Mexico</td>
<td>2004/2005</td>
<td>QF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.4 % (5/149)</td>
<td>18/5</td>
<td>1 (3)</td>
</tr>
<tr>
<td>(Moreno-Enriquez et al. 2007)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3 (6)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9 (88)</td>
</tr>
<tr>
<td>Minas Gerais, Brazil</td>
<td>2005</td>
<td>MFC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11 % (6/55)</td>
<td>88/6&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>(Brito et al. 2008)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Non-pasteurized.

<sup>b</sup>Pasteurized MFC.

<sup>c</sup>All isolates, serotype 1/2a.
The predominance of Pulsotype I in QF samples obtained from Culiacan suggests at least two possibilities: there is a common source of contamination for *L. monocytogenes* Pulsotype I, and/or *L. monocytogenes* Pulsotype I thrives in the raw milk and/or process environment used for this QF preparation. In Brazil, Da Silva et al. (1998) recovered *L. monocytogenes* from 7 of 17 samples (41%) of MFC made from raw milk and in 1 of 33 samples (3%) of MFC made from pasteurized milk. Da Silva et al. (1998) also observed three serotypes (1/2a, 1/2b, and 3b) from the cheese made from pasteurized milk, concluding that improper pasteurization, post-processing contamination, and poor hygiene in the processing plant must be responsible for the high frequency of *Listeria* spp. In Mexico, Moreno-Enriquez et al. (2007) found 5 of 149 (3.4%) QF samples from retail markets positive for *L. monocytogenes*. Furthermore, Rebagliati et al. (2009) established the incidence of *L. monocytogenes* in soft and semi-soft cheese at about 10%. The results of the present study are in agreement with those of the above-mentioned studies.

There is a relatively high incidence of *L. monocytogenes* in QF produced in Culiacan; however, no cases of listeriosis from QF have been reported in this region. One reason might be attributed to the lack of scientific-based knowledge of *L. monocytogenes* and the lack of reporting or recording listeriosis cases in Mexico. The poor hygiene during preparation and the improper refrigeration temperature at which QF is often stored during commercialization may have favored the survival of *L. monocytogenes*. In addition, Brito et al. (2008) demonstrated that storage coolers are also sources of *L. monocytogenes* contamination of cheese made from pasteurized milk.

**STEC**

The presence of virulence attributes (*stx*₁, *stx*₂, *eae*, and H7) was characterized. Eighty-one strains of *E. coli* were isolated from 75 QF samples displaying at least one of the four PCR primer products. None of the isolates were O157 (Table 2).

Even though pasteurization of milk greatly reduces the risk of pathogens, it is often omitted during non-commercial, unregulated production of fresh raw milk cheese, non-aged cheese (MacDonald et al. 2005), making them an important sources of illness. Human infections with *E. coli* O157:H7 have been linked to the consumption of unpasteurized milk. In Canada and the US, cheese made with raw milk was implicated in outbreaks of *E. coli* O157:H7 (Honish et al. 2005; CDC 2010). During the cheese-making process, *E. coli* may contaminate the milk and equipment, and might also multiply or be part of the microflora in the processing room and thus cross-contaminate the cheese. Wang et al. (1997) demonstrated that temperature abuse during shipping and handling can result in significant growth of *E. coli* O157:H7.

According to the Official Mexican Standards (NOM-121-SSA-1994), the fecal coliforms count must be ≤ 100 MPN/g, while *Listeria* and *Salmonella* must be absent in a 25 g portion of cheese. The U.S. FDA since the mid-1980s has also implemented a

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Stx₁</th>
<th>Stx₂</th>
<th>Stx₁ + Stx₂</th>
<th>ae</th>
<th>H7</th>
<th>Stx₁ and aea</th>
</tr>
</thead>
<tbody>
<tr>
<td>81</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>6</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. Isolates of Shiga toxin producing *E. coli* (STEC) from QF.
zero-tolerance policy for *L. monocytogenes* (Knight et al. 2008). However, the present study reports that QF from Culiacan, Mexico, is above the limit set by FDA and Mexican regulations for fecal coliforms and *Listeria*, representing a potential risk to consumers of this region. Despite the fact that coliforms indicate the possible presence of enteropathogenic bacteria, *Salmonella* was not detected in any of the analyzed samples.

**Listeria prediction**

Data analysis to evaluate potential relation between *Listeria* presence/absence in QF and physicochemical parameters (*a_w*, moisture, pH, and salinity) was conducted. Only pH (*p = 0.002*) was significant, while moisture (*p = 0.088*) and *a_w* (*p = 0.922*) were statistically no significant. LRA showed that presence of *Listeria* in QF is 1, 14, and 18 times greater as moisture, *a_w*, and pH increases at 1%. On the other hand, a negative relation (*p = 0.288*) was shown between the presence of *Listeria* in QF and salinity. Thus, the risk of *Listeria* in QF decreases 0.2 times when increasing salinity content in 1%. Some studies have stated that an increase in the salt rate might stimulate the inactivation of pathogenic bacteria, such an *E. coli* O157:H7 (Guraya et al. 1998; Öksüz et al. 2004) and other pathogenic microorganisms. Furthermore, the inactivation of pathogenic bacteria in QF may not be solely based on increasing sodium chloride and/or other physico-chemical parameters; the implementation of preventive strategies should also be enforced.

**Conclusions**

QF is widely consumed in Culiacan, Sinaloa Mexico. The manufacturing is performed essentially by artisans and independent merchants who often do not have environmental monitoring programs in place. The levels of contamination found in QF produced in this region are an indicator of inadequate conditions and sanitization measures during production. Control of pathogenic microorganisms during artisanal production of QF is a challenge in this region; a safety management system, adequate temperatures during processing, storage, and transport to the retail markets, equipment being properly disinfected after contact with QF among other measures should be implemented to avoid contamination of QF and the proliferation of undesirable microbes. In addition, more epidemiological studies need to be conducted to correlate the number of foodborne cases with the presence of pathogenic microorganisms with retail Mexican-style soft cheese.

**References**


