

ASSESSING EFFICACY OF NANOCERAM® FILTERS FOR VIRUS
CONCENTRATION FROM WATER: RISK ASSESSMENT FOR *LISTERIA* AND
SALMONELLA IN FOOD

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TABLE OF CONTENTS

ABSTRACT.....	10
INTRODUCTION.....	12
Problem Definition.....	12
1. Concentration of Enteric Viruses in Water.....	12
2. Foodborne Pathogens in Mexican-style Soft Cheese.....	13
Literature Review.....	16
1. Concentration and Detection of Enteric Viruses from Water and Wastewater.....	16
2. Foodborne Pathogens in Queso Fresco (QF).....	25
2.1. <i>Listeria monocytogenes</i>	28
2.2. <i>Salmonella</i>	31
2.3. <i>Escherichia coli</i>	33
3. Quantitative Microbial Risk Assessment.....	34
3.1. Hazard Identification.....	35
3.2. Exposure Assessment.....	35
3.3. Dose-Response Assessment.....	36
3.4. Risk Characterization.....	36
Dissertation Format.....	38
PRESENT STUDY	39

TABLE OF CONTENTS – *Continued*

REFERENCES.....	42
APPENDIX A. COMPARISON OF POLIOVIRUS CONCENTRATION FROM WASTEWATER BY TWO DIFFERENT ELECTROPOSITIVE FILTERS.....	60
ABSTRACT.....	61
1. INTRODUCTION.....	62
2. MATERIALS AND METHODS.....	64
2.1.Virus propagation and assay.....	64
2.2. Poliovirus 1 concentration using NanoCeram® and 1MDSfilters.....	65
2.3.Virus elution and secondary concentration.....	67
2.4.Plaque assay.....	68
2.5.Polymerase chain reaction detection of poliovirus.....	68
3. DATA ANALYSIS AND STATISTICS.....	68
4. RESULTS AND DISCUSSION.....	69
5. CONCLUSIONS.....	72
REFERENCES	74
TABLES AND FIGURES.....	77
APPENDIX B. PREVALENCE AND CHARACTERIZATION OF FOODBORNE PATHOGENS ISOLATED FROM RETAIL MEXICAN-STYLE SOFT CHEESE IN SINALOA, MEXICO.....	80

TABLE OF CONTENTS - Continued

ABSTRACT.....	81
1. INTRODUCTION.....	83
2. MATERIALS AND METHODS.....	85
2.1. Sample collection.....	85
2.2. Physicochemical analysis.....	86
2.3. Identification, isolation, and characterization of microorganisms in QF.....	86
2.4. <i>Listeria</i>	86
2.5. <i>Salmonella</i>	88
2.6. <i>Escherichia coli</i> and fecal coliforms.....	88
3. STATISTICAL ANALYSIS.....	89
4. RESULTS AND DISCUSSION.....	89
4.1. Shiga toxin production <i>E. coli</i> (STEC)	91
5. CONCLUSIONS.....	94
ACKNOWLEDGMENTS.....	95
REFERENCES.....	96
TABLES.....	103
APPENDIX C. RISK ASSESSMENT OF LISTERIA MONOCYTOGENES IN QUESO FRESCO IN CULIACAN, MEXICO.....	105

TABLE OF CONTENTS - *Continued*

ABSTRACT.....	106
1. INTRODUCTION.....	108
2. MATERIALS AND METHODS.....	111
2.1. Sample collection and laboratory analysis.....	111
2.2. Dose-response assessment.....	111
2.3. Exposure assessment.....	113
2.3.1. Number of servings of QF and serving size.....	114
2.3.1.1. Healthy and compromised population....	114
2.3.1.2. Elderly population.....	115
3. RESULTS AND DISCUSSIONS.....	115
4. CONCLUSIONS.....	118
ACKNOWLEDGMENTS.....	119
REFERENCES.....	120
TABLES.....	125

ABSTRACT

Water quality, and therefore human health, may be significantly affected by the presence of pathogenic enteric microorganisms derived from improper disposal of wastewater to aquatic environments. Detection of waterborne viruses is complex due to the difficulties in concentrating the sample and then in detecting the virus by cell culture or molecular techniques. Methods used to concentrate enteric viruses from water have remained largely unchanged for nearly 30 years. The U.S. Environmental Protection Agency requires the use of 1MDS electropositive filters for concentrating enteric viruses from water; however, these filters are expensive for routine viral monitoring. The NanoCeram® filter, an electropositive cartridge filter, has been proposed as a new alternative for large volumes of water. The objective of the study was: to evaluate the effectiveness of NanoCeram® filters for the concentration of poliovirus-1 from wastewater samples and compare to 1MDS cartridge filters. This study suggested that NanoCeram® filters are a viable alternative to the use of 1MDS filters for viral monitoring in surface waters and wastewaters.

L. monocytogenes outbreaks with Latin-style soft cheese have been well-documented; however, more information to characterize the human health risk associated with the consumption of queso fresco (QF) using unpasteurized milk is needed. The objectives of the study were: i) to evaluate the prevalence of *Listeria*, *Escherichia coli*, *Salmonella* and fecal coliforms in QF obtained from markets in the northwestern state of Sinaloa,

Mexico, and ii) to address the human health impact associated with the consumption of QF contaminated with *L. monocytogenes* using quantitative microbial risk assessment (QMRA). The study suggested that QF produced in Culiacan, Sinaloa, Mexico have microbial loads above the maximum values recommended by the Official Mexican Regulations; and QMRA can be used to interpret microbial contamination data for impacts on public health.

INTRODUCTION

Problem definition:

1. Concentration of Enteric Viruses in Water

Enteric viruses may be present naturally in aquatic environments or, more commonly, are introduced through human activities such as leaking sewage and septic systems, urban runoff, agricultural runoff, and wastewater discharge. Transmission to humans is possible because of several factors. Viruses may remain viable in water for extended periods of time, they, sometimes, are able to survive conventional drinking water and wastewater treatments, and they can be reintroduced into water from the effluent of treatment facilities. Several studies have indicated that only a fraction of cases of waterborne disease are ever reported. Craun suggested in 1991 that fewer than half of waterborne outbreaks occurring in the United States are reported and investigated. In recent years, significant steps have been taken to develop rapid, sensitive and efficient methods for the monitoring of waterborne viruses. Because the levels of enteric viruses are often low in water, large volumes (up to thousands of liters) are frequently processed before analysis via inoculation on cultured host cells or by molecular methods. Different types of filters and filtration methods have been used to collect and concentrate viral particles from water. The only U.S. Environmental Protection Agency (USEPA) approved method for collecting viruses from large volumes of drinking water (required by the Information Collection Rule) is based on the use of electropositive Virosorb® 1MDS filter (CUNO, Meriden, CT, USA) with elution using 1.5% beef extract with 0.05

M glycine at a pH of 9.5 (EPA, 1996). This method has been extensively used in field studies and outbreak investigations. However, this method has several disadvantages: 1) an additional concentration step is required following elution, resulting in viral loss, 2) the filters cost \$150-180 and higher per unit, 3) these filters cannot be used to effectively concentrate viruses from waters with a high salinity (e.g., seawater), and 4) this beef extract used as an eluting buffer contains some substances which can interfere with molecular assays such as PCR. This study describes a new electropositive filter (NanoCeram® Virus Sampler) which cost approximately \$40 per unit for concentrating enteric viruses from large volumes of water. Additionally, the NanoCeram® and the 1MDS filters were evaluated in a side-by-side comparison for their effectiveness in concentrating viruses from wastewaters.

2. Foodborne Pathogens in Mexican-style Soft Cheese

Foodborne diseases have a major public health impact and are responsible for approximately 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths in the United States each year (Scallan et al. 2011). It is estimated that 5.5 million (59%) foodborne illnesses are caused by viruses, 3.6 million (39%) by bacteria, and 0.2 million (2%) by parasites. The pathogens responsible for the largest numbers of illnesses are norovirus (58%), non-typhoidal *Salmonella* spp. (11%), *Clostridium perfringens* (10%), and *Campylobacter* spp. (9%). The leading causes of death are non-typhoidal *Salmonella* spp. (28%), *Toxoplasma gondii* (24%), *Listeria monocytogenes* (19%), and norovirus (11%) (Scallan et al. 2011).

Dairy products are an important vehicle of gastrointestinal infections worldwide. From 1998 through 2008, 86 outbreaks due to the consumption of raw milk or raw milk products were reported to the Centers for Disease Control and Prevention (CDC). These outbreaks resulted in 1,676 illnesses, 191 hospitalizations, and 2 deaths. Most of these cases were caused by *Escherichia coli* O157:H7, *Campylobacter* spp., and *Salmonella* spp. (CDC, 2011a).

Cheese production and markets have emerged as important elements of the dairy industry over the past three decades. In Mexico, a small number of companies process dairy products with a few large companies dominating the market. In 2008, Mexican cheese exports accounted for \$19,747,936 (SAGARPA-SIAP, 2008). Mexican national cheese production increased from 116,409 tons in 1997 to 149,988 tons in 2008, with 27% of the total as queso fresco (QF) production (SARGA-SIAP, 2008). QF is a Mexican-style soft cheese found throughout Mexico and Central and South America, and is very popular in the southern and western United States where a significant portion of the populations are Hispanic (Clark et al. 2001; Van Hekken and Farkye, 2003). QF is often made from raw milk with minimal processing. Therefore, if contaminated with enteric pathogenic microorganisms, QF may represent a health hazard to the consumer. Nevertheless, the use of raw milk during its production gives QF its distinctive flavor, texture, and cooking properties that consumers prefer over pasteurized cheeses.

The Mexican market for QF is often served by artisanal companies which are exclusively family affairs located in premises attached to their homes (Cesin et al. 2007).

Furthermore, producers sell the cheeses to wholesalers or directly to consumers at small markets and/or door-to-door with no labels, transporting their products in ice chests with minimal food safety precautions (Vazquez-Salinas et al. 2001; Cesin et al. 2007; Moreno-Enriquez et al. 2007). This, along with the high moisture content and high pH of QF, contributes to the growth of enteric pathogens such as *L. monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 (Pintado et al. 2005; Brito et al. 2008). Outbreaks related with the consumption of raw milk Mexican-style soft cheeses contaminated with *Listeria* and *Salmonella* have been reported in the United States. Milk safety regulations in Mexico are as stringent as (and sometimes more than) comparable U.S. codes; however, U.S. regulations are enforced while those in Mexico are not (Outlaw and Nicholson, 1994). The number of disease outbreaks related to the consumption of QF in Mexico is unknown. More information is needed to identify contaminating pathogenic microorganisms during QF production in Mexico. In addition, there is also a need for interpreting such data on human health. The major goals of the present research were to establish the prevalence and occurrence of foodborne pathogens isolated in retail Mexican-style cheeses from the northwestern state of Sinaloa, Mexico. Quantitative microbial risk assessment (QMRA) was also used to determine the potential human health impact associated with the consumption of QF contaminated with *L. monocytogenes* in this region.

Literature review:**1. Concentration and Detection of Enteric Viruses from Water and Wastewater**

Viruses are an important cause of waterborne diseases, which are responsible for 14% of outbreaks and 38% of illnesses associated with drinking water in the United States (Lambertini et al. 2008). Between 1971 and 2004, 748 drinking water outbreaks and 575,207 cases of illness were reported in the U.S., and 8% were enteric viruses (Karim et al. 2009; Barwick et al. 2000). To prevent or reduce the public health threat from waterborne outbreaks, it is necessary to develop sensitive methods for monitoring human viruses in various environments (Li et al. 2010), yet monitoring and identifying etiologic agents from water samples remains difficult (Morales-Morales et al. 2010).

There are four basic steps in virus analysis: sample collection, elution, secondary concentration, and virus detection (Pepper et al. 2009). For sample collection, it is often necessary to pass large volumes of water or wastewater (100 to 1,000 liters) through a filter because of the low numbers of viruses presence (Farrah and Preston, 1985; Goyal et al. 1980; Li et al. 2010). The viruses are concentrated from the water by adsorption onto the filter. Recovery of viruses involves elution of the virus from the filter as well as a secondary concentration step to further reduce the sample volume before assay (Pepper et al. 2009; Karim et al. 2009). Viruses can be detected using cell culture or molecular methods such as polymerase chain reaction (PCR) (Sobsey et al. 1982; Toze, 1999). The major factors that influence the quality of enteric virus detection methods are: (a) the variability in the types, amounts, and conditions of viruses present in environmental

samples, (b) the characteristics, quality, and size of the samples, and (c) the characteristics and conditions of the virus recovery and assay procedure (Sobsey, 1982). Several concentration methods have been developed and successfully applied in the past for concentrating enteric viruses from large volumes of water and wastewater, including the use of glass powder (Wyn-Jones and Sellwood, 2001), ultrafiltration (Sobsey, 1982; Olszewski et al. 2005; Winona et al. 2001), polyelectrolyte adsorption (Wallis et al. 1970; Hill et al. 1971), polyethylene glycol hydroextraction (Shuval et al. 1967), and filter adsorption-elution (Wallis, 1967; Haramoto et al. 2007).

Glass powder is composed of borosilicate glass beads of 100-200 μm diameters that form a good adsorbent for viruses in a fluidized matrix bed. These have the advantage that the filter matrix cannot become clogged as with glass fiber systems. Glass powder is used to concentrate tap water and sewage (Wyn-Jones and Sellwood, 2001). For low samples volumes (< 100 L), the method produces a low eluate volume which does not need secondary concentration prior to inoculation onto cell cultures. However, the performance for the recovery varies widely with the type of sample, from 60% with potable water to 20% with wastewater. Also, there is a necessity for acidification of the sample which may inactivate some of the virions (Hugues, et al. 1993).

Polyelectrolyte PE60 lends itself to the concentration of viruses present in water. Wallis et al. (1969) reported that viruses added to sewage or naturally found in sewage were preferentially adsorbed to insoluble polyelectrolyte PE60 with a high recovery efficiency. When the polyelectrolyte-reactive sites are occupied by a variety of organic

compounds, the virus is not efficiently adsorbed. Because of its unstable nature and lot-to-lot variation in efficiency for the concentration of viruses, this method has not been used in recent years (Pepper et al. 2009).

Positively charged glass wool, held together by a binding agent and coated with mineral oil, possesses both hydrophobic and electropositive sites on its surface which adsorb and concentrate viruses from water (Wyn-Jones and Sellwood, 2001; Lambertini et al. 2008). It has been used in virus monitoring involving wastewater, drinking water, groundwater, river water, and reservoirs (Ehler et al. 2005). Glass wool has also been demonstrated to be significantly superior in concentrating viruses in wastewater over glass powder (94% versus 46%) (Hugues et al. 1993). The principal limitation of this material is the high variability of the virus recovery, ranging from 24% to 81%, and the inhibition of molecular techniques due to the enhanced adsorption of organic matter (Wyn-Jones and Sellwood, 2001; Pepper et al. 2009).

Concentration of viruses by ultrafiltration offers important advantages over other filtration systems by simultaneously concentrating parasites, viruses, and bacteria with efficiencies ranging from 60% to 108% (Winona et al. 2001; Morales-Morales et al. 2003; Hill et al. 2007). Ultrafiltration uses a size-exclusion-based mode of concentration, where molecules smaller than the pore size of the filter pass through the membrane and out of the system and larger particles are concentrated in the filter retentate (Wyn-Jones and Sellwood, 2001; Morales-Morales et al. 2003). The cross-flow circulation pattern with recirculation of the retentate reduces fouling of the membrane and makes it possible

to filter large volumes of turbid water while maintaining the organisms in suspension. The pH, salinity and humic acids do not have a significant impact on ultrafiltration performance (Hernandez-Morga et al. 2009). Ultrafiltration has been used for tap water, lake water, seawater (Pepper et al. 2009), and estuarine water (Hernandez-Morga et al. 2009). Although ultrafiltration is still a promising alternative, it suffers from a lack of portability and high equipment costs.

The class of filters most commonly used for virus collection from large volumes of water is microporous filters that are used for virus adsorption and elution, commonly known as the VIRADEL (for virus adsorption-elution) method (APHA, 2005; Maier et al. 2009). VIRADEL involves passing the water through a filter to which the virus (that is negatively charged) adsorbs. The pore size of the filters is much larger than the viruses. Adsorption takes place by a combination of electrostatic and hydrophobic interactions (Gerba, 1984). Two general types of filters are available: electronegative filters (with a negative surface charge) and electropositive filters (with a positive surface charge). VIRADEL filter methods suffer from a number of limitations. For instance, suspended matter in water tends to clog the filters, thereby limiting the volume that can be processed and interfering with the elution process (Gerba et al. 1978; Pepper et al. 2009). Dissolved and colloidal organic matter in some waters can also interfere with virus adsorption to filters (Gerba et al. 1978; Gerba, 1984; Sobsey et al. 1984) due to the virus being associated with solids and competition for adsorption sites. Finally, the concentration efficiency varies depending on the type of virus, presumably because of differences in

their isoelectric point (pH at which the net charge on a particle of interest is zero), which influence the net charge of the virus at various pH's (Hsu et al. 2007; Pepper et al. 2009). Viruses display a wider range of isoelectric points, making their net surface charge dependent on changes in pH. Viruses will be positively charged below their isoelectric point and negatively charged above this pH (Gerba, 1984), thus playing a significant role in controlling the viral adsorption to charged filters.

Electronegative filters are composed of either cellulose esters or fiberglass with organic resin binders (Pepper et al. 2009). Because the filter is negatively charged, the water sample has to be conditioned to an acidic pH (pH 3.5) to ensure that the viruses are positively charged by using either divalent (e.g., $MgCl_2$) (Victoria et al. 2009) or trivalent ($AlCl_3$) salts (Haromoto et al. 2007). High salt concentrations and a pH below the virus isoelectric point will promote both electrostatic (ionic) and hydrophobic interactions between the viruses and filters (Gibbons et al. 2010). This will reduce the net negative charge usually associated with viruses, maximizing their adsorption. The most commonly used electronegative filter is the Filterite. Generally, it is used as a 10-inch (25.4 cm) pleated cartridge with either a 0.22 μm or 0.45 μm nominal pore size rating (Maier et al. 2009). Filterite filters are ideal for use when concentrating viruses from seawater and waters with high amounts of organic matter and turbidity (Gerba et al. 1978); however, due to the need for conditioning the water prior to filtering and the need for additional materials and equipment such as pH meter, electronegative filters are difficult to use for field sampling and for large volume sampling.

The electropositive charged filters may be composed of fiberglass or cellulose with or without polymeric resin, which creates a net positive surface charge that enhances adsorption of the negatively charged viruses. The Virosorb® 1MDS filter is recommended by the United States Environmental Protection Agency (USEPA) as the standard method for collecting enteric viruses from large volumes of drinking water (EPA, 2001). These filters adsorb viruses efficiently over a wide pH range normally found in waters without the need for addition of polyvalent salts (Maier et al. 2009); nevertheless, they do not perform well for raw wastewaters and waters with high organic matter and cannot be used with waters with a pH above 8.5-9.0 (Sobsey and Glass, 1980). Also, a pre-filter may be necessary to increase the capacity of the virus filters (Pepper and Gerba, 2004).

The methods used to concentrate viruses also concentrate a variety of other solutes such as humic acids, organic matter, and proteins which may interfere with conventional as well as molecular detection techniques (Guttman-Bass et al. 1985; Sobsey and Hickey, 1985; Wyn-Jones and Sellwood, 2001; Hill et al. 2007). These inhibitors might be already present in highly polluted water or might be introduced during the concentration step and remain in the final sample concentrates (Abbaszadegan et al. 1993; Schwab et al. 1995). In particular, many naturally occurring inorganic and organic solutes inhibit the polymerases used for polymerase chain reaction (PCR) amplification of target genomes (e.g., reverse transcriptase and *Taq* DNA polymerase) (Tsai and Olson, 1992). Nucleases and proteases may also degrade virus genomes before

they can be amplified by PCR (Schwab et al. 1995) or organic compounds may bind magnesium and calcium ions, and the nucleotides required by nucleic acid polymerases, causing them to precipitate and preventing them from being used as cofactors for the enzymes involved in PCR (Kopecka et al. 1993; Abbaszadegan et al. 1993). In addition, these solutes found in water can cause cytotoxicity in some cell lines (Pepper et al. 2009).

Beef extract is the most widely used solution to elute adsorbed viruses from filters and other surfaces. It contains a high concentration of poorly characterized components which can interfere with molecular detection methods (Schwab et al. 1995; Toze, 1999). Some methods, such as sephadex columns, ion exchanges resins, and cesium chloride density centrifugation are efficient at removing inhibitory solutes such as organic, inorganic and salt compounds (Schwab et al. 1995). These methods remove or neutralize other interfering substances found in environmental samples to achieve higher detection efficiencies. However, a number of these methods are complex or require expensive sophisticated machinery, making them unsuitable for routine use (Toze, 1999).

Ikner et al. (2011) found that the positively charged NanoCeram® filters could rapidly adsorb a variety of viruses (MS2 coliphage, poliovirus 1, Coxsackievirus B5, and echovirus 1) and the viruses could successfully be eluted with a sodium polyphosphate based buffer followed by secondary concentration by ultrafiltration. This method did not cause inhibition in PCR amplifications. Polyphosphates are highly negatively charged and work as dispersants by altering the surface charge of microbes, particles, and filter surfaces. They reduce the zeta potentials of all surfaces in the system, thus imparting a

more negative surface charge and increasing electrostatic repulsion. Long chain polyphosphates may also increase particle-particle (or particle-surface) repulsion due to steric effects of the polyphosphate molecules attached to the respective surfaces (Polaczyk et al. 2007).

The traditional methods used to detect viruses from water rely on cell culture followed by observation of the cells for cytopathogenic effects or by enumeration of clear zones or plaque forming units (PFU) in cell monolayers stained with vital dyes (Pepper et al. 2009). These methods have the disadvantages of being time consuming, expensive, and requiring highly trained personnel (Wyn-Jones and Sellwood, 2001); however, this is still the best method to determine the infectivity of viruses concentrated from environmental samples (Fong and Lipp, 2005). Despite this, some enteric viruses such as caliciviruses or hepatitis A virus cannot be detected, or can be detected only with great difficulty (Cho et al. 2000) because of their inability to grow in cell culture. Buffalo Green Monkey (BGM) cells are commonly used for enterovirus assays, but not all human enteroviruses grow in this cell line. Thus, more than one cell line is required to be able to identify enteroviruses in a sample (Abbaszadegan et al. 1993). Due to these limitations, a lot of recent research has been directed to the detection of viral nucleic acid using molecular techniques such as polymerase chain reaction, semi-nested PCR, nested PCR, and real time PCR (for quantification) (Fong and Lipp, 2005).

The use of PCR to detect viruses in water has several benefits over conventional techniques. PCR has the potential of specificity, sensitivity, rapidity, and the capacity to

detect small amounts of target nucleic acid in a sample. In particular, reverse transcriptase PCR (RT-PCR) can detect as little as 0.001 viral PFU in a sample (Shwab et al. 1995) and has emerged as an effective method to detect enteric viruses in water (Abbaszadegan et al. 1999). Integrated cell culture PCR (ICC-PCR) has aided the specific detection of infectious enteric viruses (Reynolds et al. 2001; Fong and Lipp, 2005).

The need for a simple, reliable, and efficient method to quantitatively concentrate enteric viruses from large volumes of water continues to be a challenge. Although Virosorb® 1 MDS filters have demonstrated efficient virus adsorption from a range of water quality types for both small and large volumes, it is costly (~\$180 in 2010). It is also relatively ineffective for the concentration of viruses from seawater, most likely due to the higher salt concentration and pH levels relative to freshwater. Recently, a promising and cost effective (~\$40 per unit in 2010) alternative, the NanoCeram® Virus Sampler, has been described for the concentration of enteric viruses from tap water, river water, and seawater (Karim et al. 2009; Bennet et al. 2010; Gibbons et al. 2010; Ikner et al. 2011; Li et al. 2010) with high efficiencies.

NanoCeram® filters are electropositive filters composed of a non-woven matrix of microglass fibers (0.6 μm in length) coated with boehmite-derived nanoalumina fibers (~2 nm in diameter by ~250 nm in length). The nanoalumina coating confers an extensive surface area to the NanoCeram® (~500 m^2/g), resulting in an effective pore size of ~2 μm (Gibbons et al. 2010). Karim et al. (2009) reported retention efficiency of poliovirus 1 (10^5 PFU) from tap water and river water (100 L) using the NanoCeram® filter of 51%

and 38%, respectively. Karim et al. (2009) also found no difference in virus recovery from tap water with a pH range of 6 to 9.5. Ikner et al. (2011) obtained high virus recovery efficiencies with a number of viruses (66% for poliovirus 1, 83% for echovirus 1, 77% for Coxsackievirus B5, and 56% for MS2 coliphage) using these filters. Bennett et al. (2010) also found that these filters were much better for the recovery of MS2 from artificial seawater than 1MDS filters (63% versus 30%). In addition, the recovery of poliovirus from seawater (44%) was higher than that reported for 1MDS filters. Gibbons et al. (2010) also observed high recoveries of viruses from seawater using these filters.

2. Foodborne Pathogens in Queso Fresco (QF)

During the past 20 years, knowledge of foodborne pathogens has increased at an unprecedented rate. There is an increasing demand by the consumer for high quality natural food, free from artificial preservatives and contaminating microorganisms. An estimated 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths in the United States each year are caused by foodborne diseases (Scallan et al. 2011). The leading causes of death are non-typhoidal *Salmonella* spp. (28%), *Toxoplasma gondii* (24%), *Listeria monocytogenes* (19%), and norovirus (11%) (Scallan et al. 2011).

Cheeses are ready-to-eat (RTE) food products that do not undergo any further treatment to ensure their safety before consumption (Kousta et al. 2010). Various type of cheeses have occasionally been implicated in foodborne outbreaks associated with severe symptoms and high fatality rates. The pathogenic microorganisms implicated are usually *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, and

Salmonella (Cody et al. 1999; Linnan et al. 1988; Kousta et al. 2010). The farm environment constitutes a source of raw milk contamination with *Salmonella*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* (Kousta et al. 2010). Moreover, the presence of these pathogenic bacteria in raw milk cheeses pose a threat to human health due to the increased number of cases and the severity of symptoms (Kousta et al. 2010).

One of the most popular varieties of cheese consumed in Mexico and other Latin American countries is queso fresco (QF). It is also the most popular Hispanic-style cheese in the southern and western United States, where a significant portion of the population is Hispanic (Clark et al. 2001; Van Hekken and Farkye, 2003). QF is known for having a mild milky flavor, a soft and creamy texture, a short shelf-life. It is therefore usually eaten fresh. It is characterized by being high in moisture content (55% to 58%), having a low pH (pH 5 to 6.3), and having a high salt content (1.4% to 1.6%) (Van Hekken and Farkye, 2003). QF is generally made by farmers on a small scale with unregulated, noncommercial processors using raw milk and traditional techniques (Cesin et al., 2007; McDonald et al., 2005). These techniques include acidification by the indigenous lactic acid bacteria present in milk and the action of rennet (Torres-Llanez et al. 2006).

The traditional use of raw milk in the production of QF gives the cheese its distinctive flavor, texture, and cooking properties. Cheese made from pasteurized milk results in a more uniform product of better sanitary quality; however pasteurization

adversely affects its flavor quality, since it eliminates some of the indigenous microflora in the milk, which are partly responsible for the development of the typical cheese flavor. Producers and consumers usually prefer the organoleptic (sensory) qualities of QF produced using raw milk over pasteurized milk (Renye et al. 2008). One of the main disadvantages of QF is the lack of sanitary practices in processing. Microbial contamination during cheese production may originate from various sources: starter culture, brine, floor and packaging material, cheese vat, cheese cloth, and curd cutting knife, and cold room and production room air (Kousta et al. 2010). Post-pasteurization contamination, manufacturing and handling processes, equipment, and temperature abuse during transport and storage conditions might result in high levels of pathogenic microorganisms in the cheese (Araujo et al. 2002). Brito et al. (2008) demonstrated that storage coolers are source of *L. monocytogenes* contamination of cheese made from pasteurized milk. Wang et al. (1997) also demonstrated that temperature abuse during shipping and handling can result in significant growth of *E. coli* O157:H7.

QF is sold both door-to-door and in small local markets, where the cheese may not be stored at adequate refrigeration temperature (Moreno-Enriquez et al. 2007). QF is sometimes processed illegally or under insufficient pasteurization conditions in the United States (McDonald et al. 2005). The absence of any rigorous control of temperature and relative humidity during storage makes the final quality of the product quite variable (Torres-Llanez et al. 2006). The use of unpasteurized milk in the production of QF violates the Mexican Official Regulation that states cheeses should be

manufactured with pasteurized milk to guarantee their safety (Secretaria de Salud, 1996); however, the regulation has not been enforced. These conditions represent a significant source of contamination by pathogenic microorganisms when it is not inspected and raw milk is used as the main ingredient (MacDonald et al. 2005).

As a result, QF has been involved in a number of foodborne outbreaks in the last 20 years in states such as California and North Carolina in episodes involving mainly the presence of *L. monocytogenes* and *Salmonella typhimurium* (Linnan et al. 1988; Cody et al. 1999; MacDonald et al. 2005; Kousta et al. 2010). Other bacteria reported in outbreaks related to cheeses to a lesser degree are *Staphylococcus aureus* and *Escherichia coli* (Kousta et al. 2010). The presence of these pathogenic microorganisms can lead to serious health problems which can potentially be fatal to consumers.

2.1. *Listeria monocytogenes*

Listeria monocytogenes is a ubiquitous Gram-positive, facultative, intracellular foodborne pathogen that causes listeriosis, a severe infectious foodborne disease. Each year in the United States, 2,500 persons become seriously ill as a result of *L. monocytogenes* infection, and 500 of these persons die. Listeriosis has a 30% case fatality rate (Mead et al. 1999). The Centers for Disease Control and Prevention's (CDC) FoodNet active surveillance program indicated that there were 158 cases per million people in 2009 (CDC, 2010). Particularly at risk are individuals with lowered immune function including pregnant women, newborns, the elderly, and the immunocompromised (FAO, 2004). Infections that occur during pregnancy can precipitate stillbirth,

miscarriage, premature birth, and infection in newborns. Sixty percent of cases of listeriosis among person 10-40 years of age occur among pregnant women, and 27% of cases of listeriosis occur among persons in all age groups (Lorber, 1997). Of the 13 known serotypes of *L. monocytogenes*, serotype 4b strains causes 50% of the listeriosis cases worldwide, whereas most of the *L. monocytogenes* strains found in foods are serotypes 1/2a and 1/2b (Gilbreth et al. 2005).

Dairy products such as soft cheeses contaminated with *L. monocytogenes* have been implicated in foodborne illness outbreaks in the United States. In Los Angeles County, California in 1985, 63 cases of invasive disease, predominantly in pregnant Hispanic women and their offspring and in immunocompromised adults, were traced to the consumption of a Mexican-style soft cheese that was contaminated with *L. monocytogenes* of the same serotype (4b) (CDC, 1985; Linnan et al. 1988). The analysis of this outbreak showed that the most frequent cause for cheese contamination was using raw milk for its production (Linnan et al. 1988). During 2000, more than 13 cases and 5 deaths of listeriosis were reported in North Carolina. The outbreak was attributed to the consumption of a noncommercial, homemade Mexican-style cheese which was made of raw milk contaminated with *L. monocytogenes* and sold to unlicensed cheese makers by a local dairy or by street vendors (CDC, 2001; MacDonald et al. 2005). *Listeria* isolation in patients, raw milk and cheese was analyzed using pulsed field gel electrophoresis, which proved the genome identity. Of the 13 identified cases, 12 were women with an average age of 21 years (range 18-38 years) and one was an immunocompromised 70 year old

male. Ten of the women were pregnant and *L. monocytogenes* infection caused deaths of five fetuses, three premature babies, and two infected newborns. The 11th woman was 5 months postpartum when she checked into a local hospital with meningitis caused by *L. monocytogenes* and no previous medical condition (CDC, 2001; MacDonald et al. 2005). An outbreak of listeriosis associated with QF was also reported in Texas in 2003 (Hise et al. 2004), again highlighting the risk posed by the use of raw milk in the manufacture of soft cheeses and unripened cheeses. These outbreaks have caused public health officials to recommend that raw milk and dairy products prepared from raw milk not be consumed by susceptible populations, particularly pregnant women.

Although the exact route of contamination of QF with *L. monocytogenes* is not known, several sources have been proposed, including the use of contaminated raw milk, contaminated processing surfaces, water and air, and contamination by workers (Moreno-Enriquez et al. 2007). Because of the public health significance of *L. monocytogenes*, U.S. regulatory agencies established a policy whereby ready-to-eat (RTE) foods contaminated with the organism at a detectable level are deemed adulterated. Since the establishment of this zero tolerance policy in the 1980's, the food industry has made major changes in an effort to eradicate the organism from RTE products and processing environments (Chen et al. 2003; Knight et al. 2008). According to the Official Mexican Standards NOM-121-SSA-1994 (Secretaria de Salud, 1996), *Listeria* and *Salmonella* must be completely absent in 25 g of cheese, while the fecal coliform count has to be less than 100 MPN/g.

2.2. *Salmonella*

Salmonella is a very large group of rod-shaped, Gram-negative bacteria comprising more than 2,000 known serotypes that are members of the Enterobacteriaceae. All of these serotypes are pathogenic to humans and can cause a range of symptoms from mild gastroenteritis to severe illness or death (Gerba et al. 2009). All age groups are susceptible, but symptoms are most severe in the elderly, infants, and the immunocompromised.

In the United States, an estimated 1.4 million cases of non-typhoidal *Salmonella* cases are reported annually, resulting in more than 16,000 hospitalizations and 580 deaths (Mead et al. 1999). Approximately 25% of all salmonellosis cases reported to the Centers for Disease Control and Prevention (CDC) are caused by a single serotype, Typhimurium (CDC, 1996). The CDC's FoodNet active surveillance program indicated that there were 7,039 cases of salmonellosis per million people in 2009 (CDC, 2010). In 2010, CDC FoodNet sites, which cover about 15 percent of the American population, reported nearly 20,000 illnesses, 4,200 hospitalizations, and 68 deaths from nine foodborne infections. Of these, *Salmonella* caused more than 8,200 infections, nearly 2,300 hospitalizations, and 29 deaths. The CDC also estimates that there are 29 infections for every lab-confirmed *Salmonella* infection (CDC, 2011b).

Salmonella is one of the most important pathogenic genera implicated in foodborne bacterial outbreaks and is mostly associated with poultry; however, they have been also linked to outbreaks associated with the consumption of various types of cheeses

(Bryan and Doyle, 1995). Several studies have shown that *Salmonella* species have been linked with the consumption of soft cheeses made from unpasteurized milk in the United States, England and Wales, France, Canada, and Switzerland (Maguire et al. 1992; Desenclos, et al. 1996; Ellis et al. 1998; Villar et al., 1999; Pastore et al. 2008). It has been reported that salmonellosis has been caused by fewer than 10 cells of *Salmonella* bacteria in cheddar cheese (Ratna and March, 1986).

Salmonella spp. has also been detected in various retail cheeses such as tulum cheese, a semi-hard cheese made from raw milk. In a study conducted in Turkey, six out of 250 (2.4%) samples collected from various markets were positive for *Salmonella* spp. (Colak et al. 2007). In a case controlled study, the infections with *Salmonella typhimurium* were primarily associated with eating raw milk, Mexican-style soft cheese purchased from unknown street vendors, friends, or relatives (Villar et al. 1999). Two outbreaks in California in 1997 were linked to the consumption of raw milk, Mexican-style cheese contaminated with multidrug-resistant *Salmonella* Typhimurium DT104 (Cody et al. 1999). *Salmonella* was not detected in any of the 4,437 samples of fresh, ripened, and semi-hard cheeses made from raw, or pasteurized milk that were analyzed in two studies in the UK during 2004 and 2005 (Little et al. 2008). No positive samples of *Salmonella* were detected in any type of cheese collected from retail stores in a study conducted in Greece (Angelidis et al. 2006).

2.3. *Escherichia coli*

Escherichia coli is a Gram-negative, rod shaped bacterium used as an indicator of direct or indirect fecal contamination of foods, and therefore, the possible presence of enteric pathogens. The greatest application of testing for *E. coli* is in the assessment of the overall quality of the food and the hygienic conditions during food processing. In cheese, *E. coli* is used as an indicator to assess post-pasteurization contamination and its presence may indicate inadequate pasteurization, poor hygiene conditions during processing, or post-processing contamination (Kornaki and Johnson, 2001).

Shiga toxin-producing *Escherichia coli* (STEC), especially *E. coli* O157:H7, has been considered an emerging foodborne pathogen which causes hemorrhagic colitis, abdominal pain, and occasionally fever; along with hemolytic uremic syndrome (Wang et al. 1997; Paton and Paton, 1998) which develops in 5-8% of cases (Honish et al. 2005). The Centers for Disease Control and Prevention's (CDC) FoodNet active surveillance program indicated that there were 459 and 264 cases of STEC O157 and STEC non-O157 per million people in 2009 (CDC, 2010), respectively.

The main natural reservoir of STEC is ruminants such as cattle (Kasrazadeh and Genigerogis, 1995; Espie et al. 2006); however, fecal contamination during manual milking, along with poor hygiene practices during cheese preparation, allows for the presence of STEC in raw milk (Griffin and Tauxe, 1991; Wang et al. 1997). Since dairy cattle are asymptomatic carriers of *E. coli* O157:H7, milk and milk products are thought to be risky foods from the standpoint of infection risk (Oksuz et al. 2004). The infective

dose of *E. coli* O157:H7 is 10-100 organisms, or even lower in the case of susceptible groups (Armstrong et al. 1996).

E. coli O157:H7 has caused more foodborne outbreaks related to the consumption of raw and pasteurized milk (Upon and Coia, 1994) than cheese. Nevertheless, outbreaks associated with the consumption of raw milk hard cheese and with fresh unpasteurized goats' cheese have been well documented in Canada and France (Honish et al. 2005; Espie et al. 2006). On the other hand, *E. coli* O157:H7 were not detected in any of the cheeses and retail samples in a survey conducted in the US (Ansay and Kaspar, 1997). Similarly, there have been no reported cases due to the consumption of Hispanic style soft cheese contaminated with *E. coli* O157:H7 (Kasrazadeh and Genigerogis, 1995).

3. Quantitative Microbial Risk Assessment

Microbiological risk assessment can be used to provide an estimate of the probability of illness from a specific pathogen in a given population from an exposure (Swaminathan and Gerner-Smidt, 2007). It includes the identification and quantification of factors contributing to the frequency of occurrence of adverse effects. Microbiological risk assessment includes four basic steps: hazard identification, hazard characterization, exposure assessment, and risk characterization (Rocourt et al. 2003). Risk assessment, risk management, and risk communication together constitute risk analysis (Maier et al. 2009).

Microbial risk assessment is a unique scientific approach that is able to link microbial data from food and the various data on human disease to provide a clear

estimation of the impact of contaminated food on human public health. It is also a powerful tool to clearly assess the efficacy of each possible mitigation strategy. The development and use of this tool encourages international collaboration and provides a transparent system for comparison of foodborne risk and mitigation potential. Microbiological risk assessment therefore provides risk managers with clear information to develop programs to reduce the risk associated with foodborne pathogens (Rocourt et al. 2003).

3.1. Hazard Identification

Hazard identification is the identification of biological, chemical and physical agents capable of causing adverse health effects and which may be present in a particular food or group of foods (Rocourt et al. 2003). In the case of pathogens, this is complicated because several outcomes ranging from asymptomatic infection to death are possible. These outcomes depend upon the complex interaction between the pathogenic agent and the host. This interaction, in turn, depends on the characteristics of the host (including pre-existing immunity, age, nutrition, ability to mount an effective immune response) as well as the nature of the pathogen (type and strain of the organism and its capacity to elicit an immune response) (Gerba et al. 2009).

3.2. Exposure Assessment

Exposure assessment is the process of measuring or estimating the intensity, frequency, and duration of human exposures to an agent. Exposure can occur via inhalation, ingestion of water or food, or through the skin (Rocourt et al. 2003). The

ultimate goal of exposure assessment is to evaluate the level of pathogens at the time of exposure. It may also include an assessment of actual or anticipated human exposure and/or consumption (Notermans and Hoornstra, 2000). Contaminant sources, release mechanisms, transport, and transformation characteristics are all factors of exposure assessment, as are the nature, location, and activity patterns of the exposed population (Gerba et al. 2009). The quantification of exposure involves models with three set of variables: concentration of microbes in the media, exposure rates (magnitude, frequency, duration), and quantification of biological characteristics of receptors (e.g., body weight, immunity to microbial pathogens) (Gerba et al. 2009).

3.3. Dose Response Assessment

Dose response is the process of obtaining quantitative information on the probability of human illness following exposure to a hazard; it is the translation of exposure into harm (Rocourt et al. 2003). This assessment is usually expressed mathematically as a plot showing the response in living organisms to increasing doses of the agent (Gerba et al. 2009). Animal models have also been used to establish dose-response curves. Although the dose-response established by using animal models provides data which can explain certain aspects of the mechanism of infection, translating animal dose-response data to humans is questionable.

3.4. Risk Characterization

Risk characterization is the qualitative and/or quantitative estimation, including attendant uncertainties and variability, of the probability of occurrence and the severity of

the adverse health effects in a given population based on hazard identification, hazard characterization, and exposure assessment (Notermans and Hoornstra, 2000; Rocourt et al. 2003).

Dissertation Format

This dissertation is comprised of three appendices. Appendix A is a research based manuscript entitled “Comparison of poliovirus concentration efficiencies of electropositive filters from wastewater” that will be submitted for publication. This manuscript compares the efficiency of a newly developed method based on the VIRADEL (virus adsorption-elution) technique for the concentration, recovery and secondary concentration of viruses from wastewater to the currently used method. Appendix B is a second research based manuscript entitled “Prevalence and characterization of foodborne pathogens isolated from retail Mexican-style soft cheese in Sinaloa, Mexico” which will also be submitted for publication. This research establishes the incidence and characterization of *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7, and fecal coliforms in queso fresco obtained from retail markets of the northwestern state of Sinaloa, Mexico. Appendix C is a third research based manuscript entitled “Risk assessment of *Listeria monocytogenes* in queso fresco in Mexico” which performs a quantitative microbial risk assessment to address the human health impact associated with the consumption of queso fresco contaminated with *Listeria monocytogenes*. This paper will also be submitted for publication.

PRESENT STUDY

This dissertation contains three appendices: The manuscript in Appendix A provides the evaluation of the effectiveness of NanoCeram® filters for the concentration of poliovirus-1 from wastewater samples and a comparison of virus recovery efficiency to that of 1MDS cartridge filters, currently the only USEPA approved method for collecting viruses from large volumes of drinking water. Wastewater secondary treatment samples were spiked with poliovirus 1 and passed through NanoCeram® VS2.5”-5 and Virosorb® 1MDS filters. A sodium polyphosphate based buffer (pH 9.3) was used for the elution from the NanoCeram® filters followed by secondary concentration using Centricon Plus-70 centrifugal filters. Virosorb® 1MDS cartridge filter elution was performed using 3% beef extract at pH 9.0 with secondary concentration by organic flocculation. Statistical analyses were performed to compare the filter and overall method efficiencies. The overall method efficiency found for the NanoCeram® and 1MDS filters were 54% and 22%, respectively. The study demonstrated that NanoCeram® filters have the ability to efficiently recover poliovirus from large volumes of wastewater and can be used as an inexpensive alternative to 1MDS filters for such applications.

Appendix B is a second manuscript describing the incidence and characterization of *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7, and fecal coliforms in queso fresco obtained from retail markets of the northwestern state of Sinaloa, Mexico, and also an assessment of the effect of physicochemical parameters of queso fresco in the viability of *Listeria* spp. occurrence. A total of 75 queso fresco samples were obtained

from independent retail markets in Culiacan, Sinaloa, Mexico. The FDA-Bacteriological Analytical Manual standard enrichment/recovery and membrane filtration method were used to detect the microorganism of interest. Descriptive statistics and analysis of variance (ANOVA), along with stepwise binary logistic regression analysis (LRA), were conducted. *L. monocytogenes*, *E. coli*, and fecal coliforms were recovered in 9.3%, 94%, and 100% of the 75 queso fresco samples tested, respectively. *Salmonella* were not recovered from any of the samples. *E. coli* isolates possessed virulence genes for *stx*₁, *stx*₂, *eae*, and H7. The occurrence of *Listeria* spp. increased by 1% in queso fresco as water activity, moisture, and pH increased. The levels of contamination found in this study are an indicator of an inadequate sanitization measures and may cause a health problem if manufacturing conditions are not improved.

Appendix C is a third, research based manuscript which evaluated the prevalence and types of *Listeria* that may be present in queso fresco obtained from markets in the northwestern state of Sinaloa, Mexico, followed by the use of quantitative microbial risk assessment to address the human health impact associated with the consumption of QF contaminated with *Listeria monocytogenes*. *Listeria* isolates were confirmed by API-*Listeria* biochemical assay, pulsed field gel electrophoresis, and ribotyping. *L. monocytogenes* was found in 9.3% of queso fresco samples. Risk of illness was estimated assuming a serving size of 4.5 g and either 1 or 21.4 servings in a given year for healthy and compromised individuals. In addition, 16.4 servings per person per year was assumed for persons over 65 years of age (elderly). The data are presented to illustrate the number

of expected cases of listeriosis in Culiacan, Mexico per serving per year with doses of 10^2 , and 10^5 CFU/25 g of queso fresco. The average number was from negligible to 0.01 for healthy individuals, 1.84×10^{-5} to 48 for the immunocompromised, and 1.69×10^{-6} to 4 for the elderly. On the other hand, the cases of listeriosis assuming 16.4 and 21.4 servings at the same doses ranged from negligible to 0.23 for the healthy individual population, 3.93×10^{-5} to 102 for the immunocompromised, and 2.77×10^{-5} to 72 for the elderly. The QMRA conducted in this study demonstrated that the consumption of contaminated QF represents a risk of *L. monocytogenes* illness in Culiacan's population. Therefore, greater insight on prevalence, levels, and types of *L. monocytogenes* in QF will augment efforts to better manage the threat of listeriosis.

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APPENDIX A

**COMPARISON OF POLIOVIRUS CONCENTRATION FROM WASTEWATER
BY TWO DIFFERENT ELECTROPOSITIVE FILTERS**

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ABSTRACT

Enteric viruses enter aquatic environments through natural or human activities and have been shown to play an important role in the contamination of surface waters, groundwater, and drinking water. The levels are often low, and as such, typical virus sampling involves a primary concentration of viruses using electronegative or electropositive filters. The U.S. Environmental Protection Agency requires the use of Virosorb® 1MDS electropositive filters for concentrating enteric viruses from water; however, these filters are expensive for routine viral monitoring. An inexpensive electropositive cartridge filter, the NanoCeram® Virus Sampler has been proposed as a new alternative. The goal of this study was to compare the relative efficiencies of NanoCeram® and 1MDS electropositive filters for poliovirus 1 concentration from treated wastewaters. The wastewater samples were spiked with poliovirus 1 and concentrated in side-by-side comparisons using these two filter types. The captured virus was then eluted from the filters using a sodium polyphosphate based buffer or a solution of 3% beef extract for the NanoCeram® and 1MDS filters, respectively. This was in turn followed by a secondary concentration step using either centrifugal ultrafiltration (NanoCeram® filter eluates) or organic flocculation (1MDS filter eluates). Although the virus capture and elution efficiencies did not differ significantly, the difference between the overall method efficiencies was found to be statistically significant with 57% (\pm 15%) and 23% (\pm 7%) poliovirus-1 recovery for the NanoCeram® and the 1MDS methods, respectively.

1. INTRODUCTION

Wastewater discharges are the most common source of enteric viral contamination of surface waters. Over the last three decades, a number of different techniques have been developed for the recovery and concentration of human enteric viruses from water including glass beads, glass wool and ultrafiltration (Winona et al. 2001; Wyn and Sellwood, 2001; Olszewski et al. 2005; Lambertini et al. 2008). The VIRADEL (viral adsorption and elution) method has been the most commonly used method for concentrating viruses from water (Sobsey and Glass 1984; American Public Health Association 2005). This method involves the filtration of water samples through a negatively or positively charged microporous membrane filter (0.2-0.45 μm pore size) with adsorption of the viruses to the membrane.

The positively charged Virosorb® 1MDS (CUNO Inc., Meriden, CT) is the most commonly used filter for virus concentration from fresh waters (EPA 2001), river water (Karim et al. 2009) and wastewater (Sobsey and Glass 1984; Rose et al. 1984); nevertheless, these filters are costly and cannot be used with marine waters (Bennet et al. 2010). Recently, the NanoCeram® Virus Sampler, a lower cost electropositive filter composed of nanoalumina fibers infused into a microglass fiber/cellulose matrix (NanoCeram® Argonide Corporation, Sanford, FL, USA) has been described for the concentration of enteric viruses from various waters (dechlorinated tap water, source waters, and seawater) (Bennet et al. 2010; Gibbons et al. 2010; Ikner et al. 2011; Karim et al. 2009) with good recovery efficiencies.

Different methods have been described to elute viruses from charged filters, including the use of glycine-NaOH and/or beef extract (1.5% to 3%) at an alkaline pH (pH 9.0 to 9.5) (American Public Health Association 2005; Ikner et al. 2011; Karim et al. 2009; Sobsey and Glass 1984). Additionally, primary eluates often require a secondary concentration step to further reduce the volume for subsequent assay by cultural and/or molecular methods. The use of beef extract for virus elution from the filters allows for secondary concentration via organic flocculation; however, beef extract is known to contain substances that can interfere with molecular assays such as reverse transcriptase polymerase chain reaction (RT-PCR) (Abbaszadegan et al. 1993). Recently, Ikner et al. (2011) developed a new method using a sodium polyphosphate buffer to elute viruses from the NanoCeram® filters followed by a secondary ultrafiltration concentration step that appears to be compatible with both cell culture and PCR assays. This method yielded virus recovery efficiencies comparable to the established method using 1MDS filters followed by organic flocculation. The objective of the current study was to compare the relative efficiencies of this new method using the NanoCeram® filters with the standard method using 1MDS filters for the concentration of poliovirus 1 from treated (activated sludge) wastewater.

2. MATERIALS AND METHODS

2.1. Virus propagation and assay

Poliovirus 1 (PV-1) (strain LSc-2ab) was obtained from the Department of Virology and Epidemiology at the Baylor College of Medicine (Houston, TX). PV-1 was maintained on BGM (Buffalo green monkey kidney; obtained from Don Dahling at the United States Environmental Protection Agency, Cincinnati, OH) cell line monolayers with MEM (minimum essential media) containing 5% calf serum (CS; HyClone Laboratories, Logan, UT) at an incubation temperature of 37°C with 5% CO₂. Following the observation of $\geq 90\%$ destruction of the monolayer, the cell culture flasks were frozen (at -20°C) and thawed (at 37°C) three successive times to release the viruses from the host cells. The suspension was centrifuged (1000 x g for 10 min) to remove cell debris, followed by precipitation with polyethylene glycol (PEG; 9% w/v) and sodium chloride (5.8% w/v) performed overnight at 4°C (Black et al. 2009). The viruses were then centrifuged (15,300 x g for 30 min at 4°C). After re-suspension of the virus pellet in phosphate-buffered saline (pH 7.4; Sigma-Aldrich, St. Louis, MO), a Vertrel XF extraction was performed at a 1:1 ratio to promote monodispersion of the virus and the removal of lipids (centrifugation at 1,900 x g for 15 min at 4°C) according to Black et al. (2009). The top aqueous layer containing the PV-1 was carefully removed using a pipette and then filtered using a syringe filter (pre-wetted with 2 ml of 1.5% beef extract) with a pore size of 0.22 μm (Millex; Millipore, Bedford, MA). The viruses passing through the filter were collected in sterile tubes and stored at -80°C until use. Viral titrations for PV-1

were performed using 10-fold serial dilution plaque-forming assays described by Bidawid et al. (2003). Host cell monolayers in 6-well tissue culture plates (Corning Inc., Corning, NY) were rinsed twice with 0.025M TRIS buffered saline [0.32L TBS-1 (31.6 g/L Trizma base, 81.8 g/L NaCl, 3.73 g/L KCl, 0.57 g/L Na₂HPO₄- anhydrous) in 3.68 L ultrapure H₂O] and then inoculated with 0.1 ml volumes of 10-fold serial dilutions (in duplicate) of the virus stock and incubated at 37°C for 30 min. Following this incubation period, 3 ml of a molten solution of MEM containing 1.5% Bacto-agar (Becton, Dickinson and Co., Sparks, MD), 2% FBS (fetal bovine sera) (HyClone Laboratories, Logan, UT), 1 M HEPES buffer (Mediatech Inc., Manassas, VA), 7.5% sodium bicarbonate (Fisher Scientific, Fair Lawn, NJ), 10 mg/ml kanamycin (HyClone Laboratories, Logan, UT), 100X antimycotic (HyClone Laboratories, Logan, UT), and 200 mM glutamine (Glutamax; HyClone Laboratories, Logan, UT) was added as an overlay to each well and allowed to solidify. The plates were then incubated at 37°C with 5% CO₂ for two days. Following incubation, the agar overlays were removed and the cell monolayers were stained with 0.5% (w/v) crystal violet (Sigma-Aldrich, St. Louis, MO) dissolved in ultrapure water and mixed 1:1 with 95% ethanol and the plaques were counted to enumerate the PV-1.

2.2. Poliovirus 1 concentration using NanoCeram® and 1MDS filters

On three separate occasions, two 40 L volumes of activated sludge effluent were obtained from the Regional Wastewater Treatment Reclamation Department in Pima County, Arizona prior to chlorination. The samples were collected in sterile 20 gallon

carboys (Nalgene, International Corporation, Rochester, NY) and transported immediately to the University of Arizona. Each sample was analyzed within 3 hours following collection for pH, temperature, total suspended solids, turbidity, and total chlorine using standard methods (American Public Health Association 2005). Following these analyses, 10 mg/L of sodium thiosulfate was added to remove any residual chlorine.

Pre-sterilized electropositive NanoCeram® filters (VS2.5"-5") and ZetaPlus® Virosorb® 1MDS filters were used to concentrate PV-1 from the two 40 L wastewater samples in separate experiments in the following manner: A one-liter volume was removed and spiked with approximately 10^5 plaque forming units (PFU) of PV-1 in a polypropylene beaker and the solution was thoroughly mixed using a stir plate and stir bar for 15 minutes. The one-liter virus suspension was then re-added to the wastewater sample to bring the volume back to 40 L and the sample was mixed thoroughly for an additional 15 minutes. To determine the initial titer of PV-1, three 10-ml samples were collected from the 40 L wastewater volume for assay. A 20 L volume of the sample was then added to stainless steel pressure vessel (Alloy Products, Waukesha, WI) and positive pressure was applied to the vessel using N_2 gas to obtain a flow rate of approximately 3.5 L/min through the NanoCeram® filter. Following this, the remaining 20 L of the sample was added to the vessel and concentrated through the same filter. Filter effluents (10 ml volumes) were collected after the passage of 10, 20, and 30 L to determine the amount of virus retained by each filter.

2.3. Virus elution and secondary concentration

PV-1 elution and secondary concentration for the NanoCeram® Cartridge was performed using the method described by Ikner et al. (2011) which uses a sodium polyphosphate based elution buffer [1.0% (w/v) sodium polyphosphate, 0.01 M phosphate, 0.05 M glycine buffer solution; pH 9.3] followed by a secondary concentration step of 70 ml of the filter eluate using Centricon Plus-70 centrifugal ultrafilters (30 kDa cut-off; Millipore, Billerica, MA). The average volume of the secondary concentrates using this method was 1.4 ± 0.3 ml. The remaining 350 ml volume remaining of the primary eluate was not concentrated further.

Elution and secondary concentration of PV-1 from wastewater for the 1MDS filters are described in “Standard Methods for the Examination of Water and Wastewater” (American Public Health Association 2005) using 3% beef extract (Becton, Dickinson and Co., Sparks, MD) at pH 9.0 and secondary concentration via organic flocculation. This resulted in a final volume of 30 ml. The remaining pellet was resuspended in a small volume (30 ml) of 0.45 N (0.15 M) Na_2HPO_4 and adjusted, if necessary, to a final pH of 7.4.

All non-viral contaminants were removed using a pre-blocked (with 2 ml of 1.5% beef extract) Millex syringe filter with a pore size of 0.22 μm (Millipore, Bedford, MA). Finally, 1 ml of kanamycin sulfate (10 mg/ml) (HyClone Laboratories, Logan, UT), gentamicin (5 mg/ml) (HyClone Laboratories, Logan, UT) and antibiotic/antimycotic (100 X) (HyClone Laboratories, Logan, UT) were added. The samples were then

aliquoted into 1.5-ml volumes in cryogenic vials and stored at -80°C until quantitative infectivity assays were performed.

2.4. Plaque assay

PV-1 was quantified using the ten-fold serial dilution plaque-forming assay on BGM cells as described previously (Bidawid et al. 2003).

2.5. Polymerase chain reaction detection of poliovirus

One-step reverse transcriptase PCR (RT-PCR) followed by nested PCR as described by Rodriguez et al. (2008) was used to determine if the virus concentrates contained any PCR-inhibiting substances. The PCR products (bands of 195 and 105 bp) were visualized via gel electrophoresis (Rodriguez et al. 2008). The 195 bp RT-PCR product is not always observed with PV-1 positive samples; therefore, the more sensitive nested PCR assay (105 bp product) was performed following the RT-PCR.

3. DATA ANALYSIS AND STATISTICS

The ability of the NanoCeram® and 1MDS filters to effectively concentrate (the filter retention efficiency) PV-1 was calculated by comparing the total number of PV-1 measured in the effluent samples by the total number present in the influent samples [i.e., $100 * (1 - (\# \text{ in effluent} / \# \text{ in influent}))$]. The elution (virus recovery) efficiency from each filter was determined by comparing the number of PV-1 recovered from the filter (the primary concentrate) to the number of PV-1 originally adsorbed (the influent titer less the effluent titer) to the filter [i.e., $100 * (\# \text{ in eluate} / \# \text{ adsorbed to the filter})$]. The secondary concentration efficiency was determined by comparing the total number of

PV-1 in the secondary concentrate to the number of PV-1 found in the filter eluate [i.e., $100 * (\# \text{ in secondary concentrate} / \# \text{ in primary concentrate})$].

In order to determine the overall method efficiency for each of the filters tested, the number of PV-1 in the secondary concentrate was compared with an equivalent volume from the original 40 L influent sample. For example, the number of PV-1 found in the secondary concentrate from the experiments using the NanoCeram® filters is equivalent to the number of PV-1 found in approximately 6.6 L of the original 40 L wastewater sample. The number of PV-1 in the secondary concentrate was divided by the number of PV-1 from the equivalent volume of the influent and then multiplied by 100. The standard deviations for all of the efficiencies were also calculated for the three wastewater samples tested with each type of filter.

A two-tailed Student's *t* test was used to compare the PV-1 recovery efficiencies between experiments conducted with the NanoCeram® and the 1MDS filters. Differences were considered significant if the resultant *P* value was ≤ 0.05 .

4. RESULTS AND DISCUSSION

In the present study, the NanoCeram® filter was evaluated for its ability to effectively concentrate PV-1 from wastewaters using a new VIRADEL approach (Ikner et al. 2011). This was compared to the established standard method using 1MDS filters and organic flocculation. The physical and chemical characteristics of the wastewater used in this study are shown in Table 1. The results of PV-1 recovery and elution efficiencies using these two filters for the three separate wastewater samples collected on different days are

shown in Table 2. The PV-1 capture efficiencies were high for both filter types, with the NanoCeram® outperforming the 1MDS filters (Table 2). This difference was not statistically significant, however. The NanoCeram® filter efficiency of $>97\%$ ($\pm 1\%$) for the recovery of PV-1 in wastewater was similar to the efficiencies reported for PV-1 in tapwater by Ikner et al. (2011) ($>99.92 \pm 0.01\%$) and in deionized water by Karim et al. (2009) ($84\% \pm 9\%$).

Virus recovery efficiencies are influenced by water quality, including temperature, pH, turbidity, organic matter, and salt ions with virus adsorption sites on filters (Sobsey 1982; Victoria et al. 2009). The wastewater samples in the present study had low turbidity and low suspended solids (Table 1). These characteristics may reduce the interferences with virus adsorption to the NanoCeram® filters, thereby increasing adsorption efficiency. The high retention efficiency of the NanoCeram® filters can also be related to a high isoelectric point (pI of ~ 9.0) which maintains a constant electropositive charge in the filter. In comparison, 1MDS filters become more negatively charged as the pH of the water increases (Sobsey and Glass 1980).

Beef extract has been successfully used for the last 30 years as part of the VIRADEL elution procedure from a spectrum of water quality matrices (American Public Health Association 2005; Ikner et al. 2011; Gibsson et al. 2010); nevertheless, beef extract contains substances which potentially interfere with virus detection by molecular assays. Table 2 shows the PV-1 elution efficiencies from both filter types. The sodium polyphosphate based buffer used with the NanoCeram® filters yielded a modestly greater

virus recovery ($63\% \pm 22\%$) than the 3% Beef extract (pH 9) with the 1MDS filters ($55\% \pm 17\%$). Ikner et al. (2011) reported a recovery efficiency of $69\% (\pm 8\%)$ of PV-1 in tapwater from the NanoCeram® filters using this sodium polyphosphate buffer. In contrast, Karim et al. (2009) reported $51\% (\pm 26\%)$ and $67\% (\pm 6\%)$ elution efficiencies for PV-1 (from 100 L of tapwater) from NanoCeram® and 1MDS filters, respectively, using 1.5% beef extract buffered with 0.05 M glycine (pH 9) for both filter types. Rose et al. (1984) showed 92% elution efficiency for coliphages (sewage) from 1MDS filter using 3% beef extract. Likewise, Bennett et al. (2010) reported elution efficiencies of $37\% (\pm 12\%)$ and $51\% (\pm 6\%)$ for PV-1 (from deionized water) from 1MDS filters, respectively, using a similar elution buffer (1.5% beef extract, 0.05 M glycine, 0.01% Tween 80, pH 9.5). The sodium polyphosphate based elution buffer was also more effective for the elution of MS2 coliphage from NanoCeram® filters than beef extract solutions (up to $86\% \pm 9\%$ versus $34\% \pm 18\%$ for 3% beef extract) in a previous study (Ikner et al. 2011).

The secondary concentration step for PV-1 from the NanoCeram® filters using the Centricon Plus-70 was more efficient ($98\% \pm 35\%$) when compared to organic flocculation from the 1MDS eluates ($45\% \pm 4\%$). In addition, this new method resulted in a secondary concentrate with a smaller volume 1.4 ml (equivalent to 8.4 ml for the entire eluate) versus 30 ml. A similarly high secondary concentration efficiency ($95\% \pm 5\%$) was observed using this method by Ikner et al. (2011) to concentrate PV-1 from tapwater.

The efficiency of recovery of PV-1 using organic flocculation was lower than the 70% (\pm 8%) reported by Guttman-Bass and Nasser (1984) from wastewater.

Due to the disparity between the efficiencies of the secondary concentration steps, the difference in the overall efficiencies for the NanoCeram® (57% \pm 15%) and 1MDS filter (23% \pm 7%) methods was found to be statistically significant ($P = 0.037$). The use of organic flocculation simultaneously collects inhibitory organic materials (e.g., humic and fulvic acid) naturally present in the environment that have to be removed prior to molecular analyses such as PCR (Sobsey and Glass 1984; Abbaszadegan et al. 1993). The semi-nested RT-PCR amplification of the wastewater concentrates are shown in Figures 1 and 2. Amplification of the NanoCeram® eluate / Centricon secondary concentrates exhibited similar results to a previous study conducted by Ikner et al. (2011) in which the sodium polyphosphate based buffer does inhibit the PCR, yet this inhibition was eliminated during the secondary concentration step without the need for additional inhibitor removal steps (Figure 1). PCR inhibition was also observed with the 1MDS beef extract eluate; however, it is difficult to ascertain whether or not this inhibition was the reason for the lack of amplification of viral nucleic acid following the organic flocculation step or if the lower virus recovery was responsible (Figure 2).

5. CONCLUSIONS

The recovery of poliovirus-1 from wastewater using the NanoCeram® filters was significantly greater than the recovery using 1MDS filters. In addition, the final virus concentrate volume was lower for the NanoCeram® method and there appeared to be less

interference with viral detection via PCR. Therefore, the NanoCeram® filters have the ability to efficiently recover poliovirus from large volumes of wastewater and can be used as an inexpensive alternative to 1MDS filters for such applications.

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TABLES AND FIGURES

Table 1. Physico-chemical wastewater characteristics.

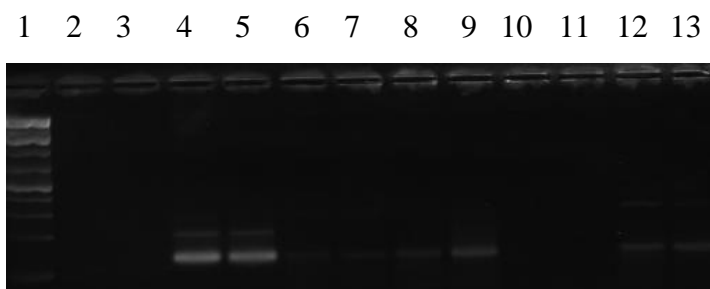
Characteristic	Range	Mean
pH	6.74 - 6.89	6.83
Temperature (°C)	24.4 - 25.7	24.0
Total Suspended Solids (mg/L)	0.44 - 0.45	0.45
Turbidity (NTU)	2 - 32	2
Total chlorine (mg/L)	0.5 - 0.6	0.5

Table 2. The concentration and recovery of poliovirus 1 from wastewaters using NanoCeram® Virus Samplers and Virosorb® 1MDS filters.

Filter	Influent titer (Log ₁₀ /40L ± SD)	Virus retention (% ± SD)	Elution efficiency (% ± SD)	Secondary Concentration efficiency (% ± SD)	Method efficiency (% ± SD)
NanoCeram®	5.3 ± 0.1	> 97 ± 1	63 ± 22	98 ± 35	57 ± 15
1MDS	5.3 ± 0.2	> 89 ± 7	55 ± 17	45 ± 4	23 ± 7

SD = Standard Deviation

Figure 1. Semi-nested reverse transcription PCR amplification of poliovirus1 from wastewater and wastewater concentrates (recovered via the NanoCeram® / Centricon centrifugal ultrafiltration method).



Lane 1 – 1-kb Ladder

Lanes 2 & 3 – Negative control (no virus)

Lanes 4 & 5 – Poliovirus-positive control (seeded with 10^8 PFU/ml)

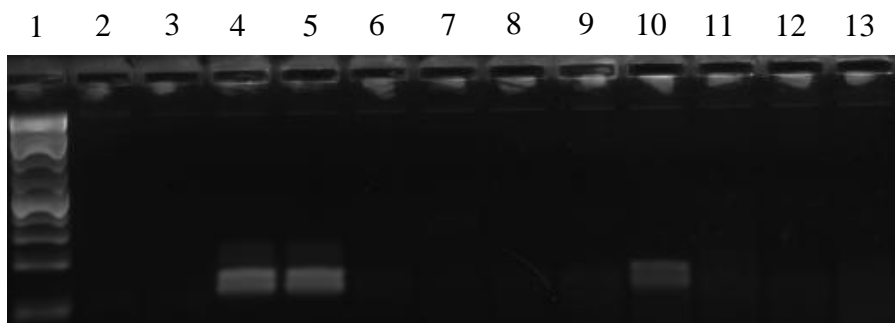
Lanes 6 & 7 – Wastewater control (seeded with 10^5 PFU/ml of PV-1)

Lanes 8 & 9 – Wastewater control 2 (diluted 1:10 with nuclease-free water and seeded with 10^5 PFU/ml of PV-1)

Lanes 10 & 11 – Primary filter eluate from NanoCeram® filters (wastewater originally seeded with 10^5 PFU/40 L)

Lanes 12 & 13 – Secondary concentrate from Centricon Plus-70 ultrafilter (concentrated from filter eluate in Lanes 10 & 11)

Figure 2. Semi-nested reverse transcriptase PCR amplification of poliovirus 1 from wastewater and wastewater concentrates (recovered via the 1MDS / organic flocculation method).



Lane 1 – 1-kb Ladder

Lanes 2 & 3 – Negative control (no virus)

Lanes 4 & 5 – Poliovirus-positive control (seeded with 10^8 PFU/ml)

Lanes 6 & 7 – Wastewater control 1 (seeded with 10^5 PFU/ml of PV-1)

Lanes 8 & 9 – Wastewater control 2 (diluted 1:10 with nuclease-free water and seeded with 10^5 PFU/ml of PV-1)

Lanes 10 & 11 – Primary filter eluate from 1MDS filters (wastewater originally seeded with 10^5 PFU/40 L)

Lanes 12 & 13 – Secondary concentrate from organic flocculation (concentrated from filter eluate in Lanes 10 & 11)

APPENDIX B**PREVALENCE AND CHARACTERIZATION OF FOODBORNE PATHOGENS
ISOLATED FROM RETAIL MEXICAN-STYLE SOFT CHEESE IN SINALOA,
MEXICO**

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ABSTRACT

Queso fresco (QF) is a handmade cheese consumed in Latin American and some regions of the United States. The objectives of this study were: i) establish the incidence and characterization of *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* and fecal coliforms in QF obtained from retail markets of the Northwestern state of Sinaloa, Mexico, and ii) assess the effect of physicochemical parameters of QF in the viability of *Listeria* spp. by Logistic Regression Analysis. A total of 75 samples of QF were obtained from independent retail merchants over an 8-month period in 2007. *L. monocytogenes* and *Salmonella* were recovered from each QF sample using the United States Food and Drug Administration-Bacteriological Analytical Manual standard enrichment/recovery method whereas *E. coli* and coliforms were recovered by use of membrane filtration. *L. monocytogenes*, *E. coli* and fecal coliforms were recovered in 9.3% (16 isolates), 94% (419 isolates) and 100% of the 75 QF samples tested, respectively, whereas *Salmonella* cells were not recovered. Of the 81 isolates of *E. coli*, 4 (5%) isolates showed virulence genes for *stx*₁, 4 (5%) for *stx*₂, 56 (69%) for *eae*, 9 (11%) for H7, 6 (7%) were positive for both *stx*₁ and *stx*₂, and 2 (3%) were positive for *stx*₁ and *aea*. *Listeria* spp. occurrence related to water activity, moisture, and pH. Results showed that microbial loads in all 75 QF samples tested were higher than the acceptable levels recommended by the Official Mexican Standards. Thus, 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*, physicochemical parameters, food safety.

1. INTRODUCTION

The production, consumption, and diversity of cheeses in Mexico and other Latin American countries have a long tradition. Queso Fresco (QF) is the most popular type of cheese consumed both in Mexico and in the southern and western United States, where a significant portion of the population is Hispanic (Clark et al. 2001; Van Hekken and Farkye, 2003). QF is a fresh, white cheese, made with unpasteurized milk, with a relatively high moisture content (55% to 58%), low pH (5.0 to 6.3), and low salt content (1.4%-1.6%). No specific starter culture is added during its production (Torres and Chandan, 1981; Van Hekken and Farkye, 2003). The typical QF flavor is influenced by the native microflora present in raw milk (Renyé et al., 2007). This is the reason that consumers prefer QF over pasteurized milk. Raw milk cheese may only be sold in the U.S. if it is ripened for 60 days (Code of Federal Regulations 2007, *sec.* 1240.61); however, the QF flavor is adversely affected after two to three weeks (Paht, 1991).

The production of QF is generally by small farmers with little knowledge of the potential microbial risks (Cesin et al., 2007; McDonald et al., 2005). In general, QF is sold to wholesalers or directly to end consumers in small markets and/or door-to-door by very small producers. The product is sold without labels and is transported in ice chests with few food safety precautions (Vazquez-Salinas et al., 2001; Cesin et al., 2007; Moreno-Enriquez et al., 2007). In addition, the hand manipulation during processing, the high moisture content, and the high pH of QF are all factors that may contribute to the viability of foodborne pathogens such as *Listeria monocytogenes*, *Salmonella*, and

Escherichia coli (Pintado et al., 2005; Brito et al., 2008). Consumers still prefer QF because of social customs and the notion that fresh is better than processed cheese. The Mexican Official Regulation (Norma Oficial Mexicana NOM-143-SSA1-1994) states that cheeses should be manufactured with pasteurized milk to guarantee their safety (Secretaria de Salud, 1996); however, this regulation is not enforced.

In the last decade, Mexican-style soft cheeses produced in the United States have been involved in a number of foodborne outbreaks associated with *L. monocytogenes* and *Salmonella Typhimurium* (CDC, 2000; CDC, 2001; MacDonald et al., 2005; Kousta et al., 2010). In 2000, more than 13 cases and five deaths of listeriosis were reported in North Carolina (CDC, 2001; MacDonald et al., 2005). In addition, an outbreak of listeriosis was associated with QF in Texas in 2003 (Hise et al., 2004). In 1997, two outbreaks associated with multidrug-resistant *S. Typhimurium* DT104 in California were linked to the consumption of raw milk Mexican-style cheese from street vendors (Villar et al., 1999; Cody et al., 1999).

L. monocytogenes is the causative agent of listeriosis, a severe foodborne disease that is characterized by a 30% mortality rate in certain sensitive populations. Particularly at risk are the young, the elderly, the immunocompromised, and pregnant women (FAO, 2004). The typical symptoms include nausea, vomiting, and diarrhea while complications such as abortion have also been reported.

Outbreaks of *E. coli* O157:H7 associated with the consumption of raw milk dairy products have been documented in Canada and France (Honish et al., 2005; Espie et al.,

2006), but there have been no reported cases in Latin American countries from the consumption of QF. Nevertheless, fecal contamination during manual milking processes, along with poor hygiene practices during cheese preparation, allows for potential exposure to shiga-toxin producing *E. coli* (STEC), *L. monocytogenes*, *Salmonella*, and other pathogenic microorganism found in raw milk and in the processing environment (Griffin and Tauxe, 1991; Wang et al., 1997).

Culiacan is the capital of the Sinaloa state, with summertime temperatures approaching 42°C and a humidity ranging from 40–60%, resulting in a heat index between 50°C to 55°C. Thus, the final quality of the QF products is quite variable. The occurrence of microorganisms such as *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 in QF in this particular region is unclear. The goals of the present study were to characterize and establish the incidence of *L. monocytogenes*, *Salmonella*, *E. coli*, and fecal coliforms in QF obtained from retail markets in the northwestern state of Sinaloa, Mexico and to assess the effect of physicochemical parameters of QF on the presence of *Listeria*.

2. MATERIALS AND METHODS

2.1. Sample collection. A total of 75 QF samples (each weighing approximately 500 g) were obtained from 75 independent retail merchants in Culiacan, the capital of the state of Sinaloa, Mexico. The selection was made using logistic regression analysis (LRA) over an 8-month period (January to August 2007). Samples were placed in a portable freezer for transport to the Centro de Investigacion en Alimentacion y Desarrollo

(CIAD) in Culiacan, Sinaloa. Bacterial analyses were conducted within 24 hours following collection.

2.2. Physicochemical analyses. Physicochemical analyses such as water activity, pH, moisture, and salinity were carried out according to the Association of Official Analytical Chemists (AOAC, 1998). A 10 g sample was weighed and mixed with 95 mL of water in a blender then filtered through a Whatman no.1 paper. The water activity (a_w) was measured using an electronic water activity meter (Aqualab Model CX2; Pulman, Washington, USA) following the manufacturer instructions. For pH measurements, a 10-g portion of the QF sample was blended with 95 ml of water and then the pH was measured using a potentiometer (Orion, Boston, MA, USA). The moisture content was determined by drying the QF (3 g) in an oven at 95°C to 105°C for 24 hours. Lastly, for the salt content determination, the QF samples were dried in an oven at 550°C for 24 hours, followed by the addition of 5 mL HCl and filtration through a Whatman no. 5 filter.

2.3. Identification, isolation, and characterization of microorganisms in QF. QF samples were analyzed for *Listeria* spp., *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli*, and fecal coliforms. *Listeria* spp. and *Salmonella* were recovered from each QF sample using the FDA Bacteriological Analytical Manual standard enrichment/recovery method (BAM, 1998).

2.4. *Listeria*. For *Listeria*, a 25 g portion of the QF was added to 225 ml of *Listeria* enrichment demi-fraser broth base (Difco, Sparks, MD) followed by hand

homogenization for 2 minutes. After incubation of the homogenized samples at 35°C for 48 hours, a 0.1-mL aliquot of the enriched demi-fraser broth was transferred to *Listeria* enrichment broth (Difco, Sparks, MD), and then incubated at 35°C for an additional 24 hours. Following incubation, 10 µL of the enrichment broth was streaked onto *Listeria* agar base PALCAM (Becton Dickinson, Sparks, MD) and/or *Listeria* ChromAgar (Becton Dickinson, Sparks, MD). These are differential and selective media for the detection of *Listeria* spp. for the detection of *L. monocytogenes* in food matrices. These plates were incubated at 35°C for 48 hours. From each PALCAM and/or *Listeria* ChromAgar plate, five presumptive *Listeria* colonies were subcultured on trypticase soy agar (TSA; Becton Dickinson, Mexico) plates and incubated at 35°C for 24 hours.. Additionally, polymerase chain reaction (PCR) was run on the DNA extract of the presumptive *Listeria* isolates (Furrer et al., 1991) and positive isolates were identified via amplification of the target gene (234 bp) was observed when the PCR product was visualized using 1.5% agarose gel electrophoresis after staining with ethidium bromide.

Positive *Listeria* isolates were sent to the United States Department of Agriculture's Agricultural Research Service Eastern Regional Research Center (USDA-ARS-ERRC located in Wyndmoor Pennsylvania) for the biochemical and molecular characterization of *L. monocytogenes* isolates. Genomic DNA was digested with *AscI* restriction endonuclease and the resulting fragments were separated using the pulsed field gel electrophoresis (PFGE) method 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). The DNA from confirmed *L. monocytogenes* isolates was ribotyped using the automated Riboprinter® (Dupont/Qualicon, Wilmington, DE) characterization system with *EcoRI* endonuclease as specified by the manufacturer.

2.5. *Salmonella*. For *Salmonella*, a 25 g portion of QF was added to 225 ml of buffered peptone water (Difco, Sparks, MD), following by hand homogenization for 2 minutes. The homogenized samples were incubated at 35°C for 24 hours. After incubation, a 0.1-ml aliquot was transferred to 10 ml of Rappaport-Vassiliadis R10 broth (Difco, Sparks, MD) following by incubation at 35°C for 24 hours. This enrichment broth was then used to inoculate Hektoen enteric (BD Bioxon, Mexico) agar and incubated at 35°C for 24 hours. Typical *Salmonella*-like colonies were subculture on TSA plates and incubated at 35°C for 24 hours. Multiple isolates were examined and screened by PCR according to Chiu and Ou (1996) to identify *Salmonella* isolates with a gene target of 244 bp. The PCR products were visualized by electrophoresis as before.

2.6. *Escherichia coli* and fecal coliforms. *E. coli* and fecal coliforms were recovered using the membrane filtration method (0.45 µm pore size, Gelman Science, Ann Arbor, MI). A 25 g QF sample was added to 225 ml of lactose broth (Difco, Sparks, MD) and homogenized by hand for 2 minutes. Ten-fold serial dilutions were performed and inoculated onto CHROMagar™ ECC (CHROMagar, Paris, France), a selective medium for the detection of *E. coli* and fecal coliforms, followed by incubation at 44.5°C

for 24 hours. From the selective CHROMagar ECC plate, five putative *E. coli* colonies were subcultured on TSA plates and incubated at 35°C for 24 hours.

The *E. coli* isolates were sent to USDA-ARS-ERRC for biochemical and molecular characterization. Each *E. coli* isolate was transferred to Sorbitol-MacConkey (Difco, Sparks, MD) agar and incubated at 37°C for 18 to 24 hours, followed by transfer to brain heart infusion (BHI) agar plates. Each isolate was examined and screened by PCR according to Fratamico et al. (2000), using primers specific for shiga toxin (*stx1* and *stx2*), hemolysin (*hly*), chromosomal flagella of the H7 serogroup (H7), and attaching and effacing genes (*eae*). The PCR products were visualized by agarose electrophoresis as described previously.

3. STATISTICAL ANALYSIS

Descriptive statistics were performed to determine the presence of *Listeria* spp., *L. monocytogenes*, *Salmonella*, *E. coli*, and fecal coliforms. In addition, physicochemical parameters were compared to predict the risk of *Listeria* presence in the QF samples. Analysis of variance (ANOVA) and stepwise binary logistic regression analysis (LRA) were conducted. The odds ratios (OR) were also calculated. Minitab (version 14) was used for the statistical analysis with a significance level of 5%.

4. RESULTS AND DISCUSSION

A total of 75 samples of QF were collected from 75 retail markets over an 8-month period in 2007. Samples were analyzed for physicochemical parameters, *L. monocytogenes*, *Salmonella*, *E. coli*, and fecal coliforms. These results are shown in

Tables 1 and 2. The average composition of the QF samples was the following: moisture 59.9 (\pm 4.6%), salt 2.9 (\pm 3.6%), water activity value of 0.980 aw (\pm 0.009), and pH of 5.5 (\pm 0.34) (Table 3). The moisture and salt levels of QF were above the typical levels expected for a fresh soft cheese. These attributes are of great importance for QF production (Saltijeral et al., 1999). These levels were also higher than pasteurized cheeses, which range from 47% to 57% moisture content with a 2% to 3% salt concentration (Secretaria de Salud, 1996; Saltijeral et al., 1999; Tunick and Van Hekken, 2010). The average pH of 5.5 was well below the range expected for this type of cheese (pH 6.2) (Tunick and Van Hekken, 2010). This may be attributed to the poor processing conditions and/or inadequate storage at abuse temperatures (Lin et al., 2006). Silva et al. (2003) showed that pH (4.9-5.3), high water activity, and high moisture levels (55%-58%) favor the survival of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 (Guraya et al., 1998; Saltijeral et al., 1999).

Listeria spp. was found in 26 of 75 samples (34.6%) and *L. monocytogenes* was detected in 7 of 75 samples (9.3%). *Salmonella* was not detected in any of the analyzed samples (Table 1); fecal coliforms were present in 100% of the samples (Table 2), ranging from 5×10^2 to 4×10^6 CFU/g with an average of 2.1×10^5 CFU/g of QF. *E. coli* was isolated from 70 of 75 samples (94%) (Table 2), ranging from <1 to 3.4×10^5 CFU/g with an average of 1.7×10^4 CFU/g of QF. Previous studies have demonstrated similar poor sanitary quality of QF produced in Mexico based on the common occurrence of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterobacter* spp., and

Streptococcus durans in the cheeses (Diaz-Cinco *et al.*, 1998; Araujo *et al.*, 2002). It would follow then that QF containing high levels of fecal contamination is also at risk for being contaminated with pathogenic *E. coli*. Our study showed a clear correlation between the presence of *E. coli* and *Listeria* in QF. All *Listeria* positive QF samples were also positive for *E. coli*. Our results agree with the study conducted by Ansay and Kaspar (1997) in which *E. coli* was present in 58% of soft and semi-hard cheese samples; Diaz-Cinco *et al.* (1998) reported *E. coli* in 100% of QF samples; Oksuz *et al.* (2004) reported a 60% incidence of *E. coli* in cheese samples made with raw milk, and high total coliform bacteria counts (6.0×10^4 CFU/g). In addition, Aygun *et al.* (2005) reported 4.3×10^3 CFU/g of *E. coli* and 1.0×10^4 CFU/g of coliforms in Carra cheese made with raw milk.

The reason for high numbers of coliforms in QF is due to the use of raw milk and insufficient hygienic conditions during its manufacturing, storage, and selling periods. In addition, the absence of any antimicrobial barriers in the QF composition (e.g., no starter culture, high moisture content) might allow for the growth of pathogenic microorganisms. Kasrazadeh and Genigeorgis (1995), showed that soft Hispanic-style cheese with a pH value of 6.6, a moisture content of 60%, a low brine concentration of 1.61%, and manufactured without the use of a starter culture is an excellent substrate for the growth of enterohemorrhagic *E. coli* if the storage temperature is $\geq 10^\circ\text{C}$.

4.1. Shiga Toxin producing *E. coli* (STEC). Human infections with *E. coli* O157:H7 are linked to the consumption of unpasteurized milk. 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. Even though pasteurization of milk greatly reduces the risk of pathogens, it is often omitted during non-commercial, unregulated production of fresh, non-aged cheeses (McDonald et al. 2005), making them an important source of illness. In an outbreak of *E. coli* O157:H7 in Canada, raw milk cheddar cheese was implicated (Honish et al. 2005). On the other hand, *E. coli* O157:H7 was not detected in any of the cheese and retail samples in a survey conducted in the United States (Ansary and Kaspar, 1997). Similarly, there have not been any reported cases caused by the consumption of Hispanic style soft cheese contaminated with *E. coli* O157:H7 (Kasrazadeh and Genigerogis, 1995).

To determine the *E. coli* isolates' potential as human pathogens, the presence of virulence genes (*stx*₁, *stx*₂, *eae*, and H7) and *Stx* expression were characterized. Eighty-one strains of *E. coli* were isolated from the 75 QF samples that possessed at least one of these genes. None of the isolates were found to be *E. coli* O157 (Table 4).

According to the Official Mexican Standards (NOM-121-SSA-1994), fecal coliform counts have to be less than 100 MPN/g, while *L. monocytogenes* and *Salmonella* must be absent in 25 g of cheese. Since the mid-1980's, the U.S. Food and Drug Administration (FDA) has had a zero tolerance policy regarding *L. monocytogenes* in cheeses (Knight et al. 2008). However, our results indicate that QF from Culiacan, Mexico has bacterial levels above these limits, representing a potential risk to consumers. The presence of fecal coliforms indicates that enteropathogenic bacteria may also be present. Regardless, *Salmonella* was not detected in any of the analyzed samples (Table 2).

A total of 16 *L. monocytogenes* isolates from 7 positive samples were analyzed using PFGE, exhibiting three different pulsotypes. The majority of the isolates belonged to pulsotype I, whereas pulsotypes II and III contained one isolate each. The predominance of pulsotype I in the 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 QF preparation. Delgado da Silva et al. (1998) observed three serotypes (1/2a, 1/2b, and 3b) in 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 this type of cheese. *L. monocytogenes* was found in 7 of 17 samples (41%) of Minas Frescal Cheese (MFC) made from raw milk and in 1 of 33 samples (3%) of MFC made from pasteurized milk in Brazil (Delgado da Silva et al., 1998). In Mexico, Moreno-Enriquez et al. (2007) found 5 of 149 (3.4%) QF samples from retail markets positive for *L. monocytogenes*. Rebagliati et al. (2009) established the incidence of *L. monocytogenes* in soft and semi-soft cheese to be up to 10%. These results are in agreement with those of the present study (Table 2); however, no cases of listeriosis from QF have been reported in Mexico. One reason might be related to the lack of knowledge and therefore cases that go unrecognized and unreported. The poor hygiene during preparation and the improper refrigeration temperature at which QF is stored may favor the survival and growth of *L.*

monocytogenes, increasing the potential risk to consumers of QF. This highlights the need for vigilance in maintaining hygienic conditions in the processing environment.

Logistic regression analysis showed that the incidence of *Listeria* increases as water activity, moisture content and pH increases. However, the incidence of *Listeria* in QF decreased with increasing salinity content, though this was not significant ($P = 0.288$). Risk values for *Listeria* spp. in QF from Culiacan, Mexico are shown in Table 5. Some studies have stated that increasing the salt concentration might enhance the inactivation of pathogenic bacteria such as *E. coli* O157:H7 (Guraya et al. 1998; Oksuz et al. 2004).

5. CONCLUSIONS

QF is widely consumed in Mexico and is manufactured primarily by independent merchants without any environmental monitoring programs. The presence of bacterial pathogens and fecal coliforms in significant percentages of QF samples indicates poor processing conditions and inadequate sanitization measures and suggests an important role of QF as a vehicle of transmission of pathogenic bacteria. The consumption of QF contaminated with *Listeria* might constitute a high risk for consumers when water activity, moisture, and pH are high. The control of foodborne diseases related to the consumption of QF continues to be a challenge; a safety management system consisting of good manufacturing practices, adequate temperatures during processing and storage, properly disinfecting equipment after contact with QF need to be implemented to avoid such contamination.

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TABLESTable 1. Presence of *Listeria* spp., *L. monocytogenes* and *Salmonella* in QF.

Pathogenic Bacteria	Presence/Absence	Number of positive samples/ total sample
<i>Listeria</i> spp.	Presence	26/75
<i>L. monocytogenes</i>	Presence	7/75
<i>Salmonella</i>	Presence	0/75

Table 2. Occurrence of *Escherichia coli* and Fecal coliforms in QF.

Pathogenic Bacteria	Log CFU/g (mean)	Number of positive samples/ total sample
Fecal coliforms	2.13×10^5	100/75
<i>Escherichia coli</i>	1.7×10^4	70/75

Table 3. Proximal composition analysis of the 75 QF samples.

Sample	Moisture ^a	Salt ^a	Water Activity ^b	pH
	Mean (Range)			
Queso fresco	59.86±4.58	2.93 ± 3.57	0.980±0.009	5.47 ± 0.34
	(47.62-71.21)	(0.71-16.59)	(0.940-1.0)	(4.47-6.03)

^a %^b aw

Table 4. Isolates of Shiga toxin producing *Escherichia coli* (STEC) from QF.

Total isolates/positive isolates	Virulence profile					
	No. isolates / (%)					
	<i>Stx₁</i>	<i>Stx₂</i>	<i>Stx₁ + Stx₂</i>	<i>eae</i>	H7	<i>Stx₁</i> and <i>aea</i>
446/81	4(5)	4(5)	6(3)	56(69)	9(11)	2(3)

Table 5. Risk values for *Listeria* spp. in QF from Culiacan, Mexico.

Physicochemical parameters	Risk ^c
Moisture ^a	1.109
Salt ^a	0.288
Water Activity ^b	13.915
pH	18.265

^a %^b aw^c Risk based on presence/absence when parameters increase in 1%

APPENDIX C**RISK ASSESSMENT OF LISTERIA MONOCYTOGENES IN QUESO FRESCO
IN CULIACAN, MEXICO**

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ABSTRACT

The presence of *Listeria monocytogenes* in queso fresco (QF) is a major public health concern due to the popularity of this cheese. *L. monocytogenes* outbreaks with Latin-style soft cheese have been well-documented; however, more information to characterize the human health risk associated with the consumption of QF using unpasteurized milk is needed. The aims of this study were to evaluate the prevalence and types of *Listeria* that may be present in QF obtained from markets in the northwestern state of Sinaloa, Mexico, followed by the use of quantitative microbial risk assessment (QMRA) to address the human health impact associated with the consumption of QF contaminated with *L. monocytogenes*. *Listeria* isolates were confirmed by API-*Listeria* biochemical assay, pulsed field gel electrophoresis, and ribotyping. *L. monocytogenes* was found in 7/75 (9.3%) QF samples collected showing three different pulsotypes (pulsotype I, II and III). Risk of illness was estimated assuming a serving size of 4.5 g and either 1 or 21.4 servings in a given year for healthy and compromised individuals. In addition, 16.4 servings/person/year was assumed for persons over 65 years of age (elderly). The probability of illness associated with the consumption of one serving with a dose of 10^2 , and 10^5 CFU/25 g of QF in healthy individuals ranged from negligible to 1.75×10^{-8} and from 1.27×10^{-10} to 3.31×10^{-4} for the compromised and the elderly. In contrast, the probability of illness for 16.4 and 21.4 servings at the same doses ranged from negligible to 3.74×10^{-7} for healthy individuals, 2.71×10^{-10} to 7.08×10^{-4} for the compromised and 2.08×10^{-9} to 5.42×10^{-3} for the elderly. The average number of expected cases of listeriosis

in Culiacan, Mexico, per serving per year with doses of 10^2 , and 10^5 CFU/25 g of QF was from negligible to 0.01 for healthy individuals, 1.84×10^{-5} to 48 for the compromised, and 1.69×10^{-6} to 4 for the elderly. The cases of listeriosis assuming 16.4 and 21.4 servings at the same doses ranged from negligible to 0.23 for the healthy individual population, 3.93×10^{-5} to 102 for the compromised, and 2.77×10^{-5} to 72 for the elderly. This study shows how QMRA can be used to interpret microbial contamination data for impacts on public health.

Keywords: Quantitative Microbial Risk Assessment, *Listeria monocytogenes*, Queso Fresco, cheeses, listeriosis.

1. INTRODUCTION

Listeriosis outbreaks associated with the consumption of Latin-style soft cheese, such as queso fresco (QF), has increased in recent years raising concerns regarding its quality as they are processed with raw milk by artisanal techniques (McDonald et al. 2005). QF is an essential ingredient in Mexican cuisine characterized by its snow white color, crumbly texture, lightly salty flavor (Van Hekken and Farkye, 2003), and consumed without the holding requirement for a raw milk cheese sold in the United States (Code of Federal Regulations, 2004). In the United States, outbreaks associated with *L. monocytogenes* and QF have been well documented due to the increasing Hispanic population. In Los Angeles County, California, 142 human cases of listeriosis affecting pregnant Hispanic woman and their neonates and fetuses were associated with one brand of Mexican-style soft cheese produced in the U.S. that was contaminated with unpasteurized milk (Linnan et al. 1988). During 2000, more than 13 cases and 5 deaths of listeriosis were attributed to the consumption of a noncommercial, fresh, Mexican-style cheese. The QF contaminated with *L. monocytogenes* was made of contaminated raw milk traced from one dairy farm in North Carolina (CDC, 2001; MacDonald et al. 2005). An outbreak of listeriosis associated with QF was also reported in Texas in 2003 (Hise et al. 2004). QF from Latin-style soft cheese from Central and South America has been associated with *L. monocytogenes* (Brito et al. 2008). In Brazil, Delgado da Silva et al. (1998) recovered *L. monocytogenes* from 7 of 17 (41%) samples of homemade minas frescal cheese made from raw milk, 3 of 53 (5.6%) of ripened cheeses and 1 of 33 (3%)

of pasteurized cheeses. Brito et al. (2008) recovered *L. monocytogenes* in 6 of 55 samples (11%) of minas frescal cheese made with pasteurized milk believed to be contaminated during cold storage. In Mexico, Moreno-Enriquez et al. (2007) collected samples of QF from farms, cheese - processing plants, and retail markets and found 5 of 149 (3.4%) positive for *L. monocytogenes*. No detectable difference in the effectiveness of either the Mexican Official Regulation NOM-143-SSA1-1995/Food and Drug Administration (FDA) Bacteriological Analytical Manual methods and U.S. Department of Agriculture Food Safety and Inspection Service method (USDA-FSIS) to recover *Listeria* from cheese or environmental samples was observed. Also, in Mexico, Chaidez et al. (2007) found a 9.3% prevalence of *L. monocytogenes* in QF from independent merchants within Culiacan, the capital of Sinaloa. All of those outbreaks highlight the risk posed when raw milk is used for cheese production, and the ability of *L. monocytogenes* to survive in food processing environments.

Although pasteurization of milk greatly reduces the risk of *L. monocytogenes*, it is often omitted during non-commercial, unregulated production of fresh, non-aged cheese (McDonald et al. 2005). Producers and consumers of QF usually prefer the organoleptic qualities of cheeses produced using raw milk over pasteurized milk. However, the relatively high moisture content (55 to 58%), low pH levels (pH 5 to 6.3), and low salt content (1.4-1.6%) (Van Hekken and Farkye, 2003) as well as hand manipulation during processing, distribution and selling increase the likelihood of *L. monocytogenes* contamination. In addition, the uses of traditional methods by small processors are

contributing factors favoring contamination and survival of *L. monocytogenes* and other pathogens in QF (Pintado et al. 2005; Brito et al. 2008).

Culiacan is located in the northwestern part of Mexico. This particular region has a very hot and humid summer with an average temperature of 42 °C. The production of QF is made using raw (unpasteurized) milk by unregulated processors and commonly sold in small markets and/or door-to-door where the cheese may or may not be stored at adequate refrigeration temperature. The production of QF is commonly obtained by the enzymatic coagulation of raw milk with rennet and coagulation enzyme without any monitoring program for *L. monocytogenes* since it is processed in households by artisanal techniques increasing the risk to those people who consume QF. A commonly used method for recovering *L. monocytogenes* from food sources in Mexico is the “Mexican official regulation” including NOM-143-SSA1-1995 (Secretaria de Salud, 1995) which is implemented by the national food processors. However, it is not enforced as good manufacturing practices by the small producers.

Quantitative Microbial Risk Assessment (QMRA) can be used to address the human health impact associated with the consumption of QF contaminated with *L. monocytogenes*. The QMRA is a four-tiered approach that includes hazard identification, exposure assessment, dose-response modeling, and risk characterization (Bemrah et al. 1998; Hass et al. 1999) and interprets monitoring data for public health impact. Due to the lack of information related to cases of human listeriosis in Mexico, the aim of this study was to evaluate the prevalence and types of *Listeria* that may be present in QF

obtained from markets in the northwestern state of Sinaloa, Mexico, and apply QMRA to estimate the risk of illnesses from *L. monocytogenes*.

2. MATERIALS AND METHODS

2.1. Sample Collection and Laboratory Analysis

QF was obtained from 75 independent merchants within Culiacan city limits, using the stepwise binary logistic regression analysis (LRA) between January and August of 2007. Samples were placed in a portable freezer for transport to the Centro de Investigacion en Alimentacion y Desarrollo (CIAD), Culiacan, Sinaloa, and analyzed within 24 h of collection. *L. monocytogenes* was recovered using the FDA-Bacteriological Analytical Manual Standard enrichment/recovery (presence and absence) method (FDA, 2003). A single 25 g portion was obtained and diluted in 225 ml of *Listeria* pre-enrichment broth (DIFCO, Sparks, MD), followed by hand homogenization for two min. Each sample was plated onto PALCAM and *Listeria* Chrom Agar and incubated for 48 h at 35 °C. When possible, multiple isolates were selected from each plate for further characterization. Biochemical and molecular characterization of *L. monocytogenes* were carried out using the API-*Listeria* biochemical assay and the pulsed-field gel electrophoresis (PFGE) method.

2.2. Dose-Response Assessment

To address a single exposure to *L. monocytogenes* in QF, the generated data were fitted in a dose-response model by using the Weibull-Gamma equation suggested by Farber et al. (1996):

$$P_i = 1 - [1 + (N)^b / \beta]^{-\alpha}$$

where P_i is the probability of illness for an individual exposed to N dose of *L. monocytogenes* cells ingested, and α , β and b are parameters associated with dose-response. In establishing this relationship, factors such as the pathogenicity of the strain and the vulnerability of the host are considered. Normal healthy individuals rarely develop clinical listeriosis symptoms after consuming contaminated food. The compromised and elderly populations are the most vulnerable to developing disease. For all populations, the parameters $\alpha = 0.25$ and $b = 2.14$ were used. β of $10^{15.26}$ and β of $10^{10.98}$ were used for the healthy population, and for the elderly population, respectively (Bemrah et al. 1998). The FDA-Bacteriological Analytical Manual Standard enrichment/recovery method was used to estimate the presence of *L. monocytogenes* in QF with a detection limit of ≤ 1 CFU per sample. Because this is only a presence and absence method to detect *L. monocytogenes*, the concentrations of *L. monocytogenes* (lower and upper) used in this QMRA were assumed using the study of Pini and Gilbert (1988). They calculated a lower concentration of $<10^2$ CFU/g and higher of 10^5 CFU/g of *L. monocytogenes* in soft cheeses by enrichment and by a modification of the U.S. Food and Drug Administration (FDA) methodology (Table 1) (Pini and Gilbert, 1988). Nevertheless, *L. monocytogenes* in Spanish unripened soft cheese (queso fresco) of pH 6.5 at 7 °C can reach a concentration around 10^7 CFU/g after 10 days in the absence of a starter culture. These levels have been found in some naturally contaminated cheeses (Farber and Peterkin, 1991; Lake et al. 2005).

According to Bemrah et al. (1998), for a typical cheese serving of 31 g, the estimated probabilities of consuming a dose of *L. monocytogenes* greater than 10^2 , 10^3 and 10^5 were 41%, 8.3% and 0.08%, respectively. Notermans and Hoornstra (2000) found that the average yearly exposures to 10^3 , 10^5 and $>10^6$ *L. monocytogenes* occur 19.3, 3.8, and 0.8 times, respectively. The results of this study showed that 7 of 75 samples were positive for *L. monocytogenes*, thus these seven positive QF samples were considered in the dose-response assessment.

The cumulative risk (CR) of listeriosis associated with consumption of raw-milk cheese is a combination of the probability of illness linked to the consumption of one cheese serving (R_i) and the number of servings per capita/year (C):

$$CR = 1 - \prod_{i=1}^C (1 - R_i) = 1 - \prod_{i=1}^C (1 - V \times P_i)$$

where V is the proportion of virulent *Listeria* strains, P_i is the probability of illness for an individual exposed to a dose (N) of *L. monocytogenes* cells. The probability of illness linked to the consumption of one cheese serving (R_i) was calculated using P_i times the probability that the consumed strains of *L. monocytogenes* are virulent. The probability of virulent strains was assumed to be $V=0.1$ (Bemrah et al. 1998).

$$R_i = V \times P_i$$

2.3. Exposure Assessment

Exposure assessment addresses the likelihood that an exposure to a hazard will occur; it also addresses the quantity of the hazardous substance in the exposure. The

prevalence of *L. monocytogenes* in cheese has been found to range from 0.5 to 10%, depending on survey design, type of cheese, and isolation methods (Bemrah et al. 1998).

The amount of *L. monocytogenes* in a QF serving was assumed to be a random variable following a Poisson distribution, and the ingested dose was dependent on the amount of *L. monocytogenes* present in 25 g of QF and how much of it was ingested. According to the GEMS/Food regional diet (regional per capita consumption of raw and semi-processed agricultural commodities) (2003), the typical serving for the Latin American population is approximated 4.5 g/person/day (WHO, 2003). The amount of QF ingested was assumed to be 4.5 g/person/day. Table 1 summarizes the assumptions in this exposure assessment.

The number and size of QF servings in Culiacan, Sinaloa, Mexico, were determined using the data from the National Nutrition Survey (NNS) which uses the data from a study conducted in the Netherlands, where 279 individual dietary records were deemed to represent consumption of a serving of soft cheese from a total survey population of 4,636 (Lake et al. 2005). The total Culiacan population obtained from the Mexican Census (2005) is 793,730 (<http://www.inegi.org.mx/inegi>).

2.3.1. Number of servings of QF and serving size

2.3.1.1 Healthy and compromised population

$$\begin{aligned} \text{Annual number of servings (healthy and compromised population)} &= 279 \times \\ &793,730/4,636 \times 365 \\ &= 1.7 \times 10^7 \text{ servings} \end{aligned}$$

Based on a total population of 793,730, the number of servings per person per year is 21.4, which is remarkably similar to the results from the USA and New Zealand, in which the number of servings per person per year was 21.7, and 21.9, respectively (Lake et al. 2005).

2.3.1.2 Elderly population

From the NNS, 50 individual dietary records out of the 279 dietary records were deemed to represent consumption of a serving of soft cheese for an individual aged 60 years or more. A total of 1,087 people aged 60 years or more completed dietary recall questionnaires as part of the NNS (Lake et al. 2005).

According to the Mexican Census (2005), 13,359 individuals in Culiacan were aged 65 or older. The annual number of servings (elderly population) = $50 \times 13,359 / 1,087 \times 365$
 $= 2.2 \times 10^5$ servings

The number of servings per person per year in the elderly population is 16.4. Assessment of how the number of consumed servings per capita and per year influenced the incidence of human listeriosis was made using 21.4 serving for healthy/compromised population and 16.4 serving for the elderly (Table 1).

3. RESULTS AND DISCUSSION

Listeria monocytogenes was found in 7 of 75 (9.3%) samples collected in Culiacan, Sinaloa, Mexico (Chaidez et al. 2007). A total of 16 isolates from the 7 positive samples were analyzed by pulsed field gel electrophoresis (PFGE) showing three different

pulsotypes. The majority of the isolates belonged to pulsotype I; whereas, pulsotypes II and III contained one isolate each (data not shown) (Chaidez et al. 2007). More than 90% of QF samples were not contaminated with *L. monocytogenes*, which is in accordance with the data shown by Bemrah et al. (1998) in that the prevalence of *L. monocytogenes* in cheeses ranged from 0.5-10% and Moreno-Enriquez et al. (2007) which found 3.4% of QF from retail markets positive for *L. monocytogenes*. Table 1 shows the parameters and assumptions for exposure of *L. monocytogenes* in QF. The simulated concentration of *L. monocytogenes* (CFU/25 g) of QF used in this assessment ranged from 10^2 to 10^5 CFU/25 g with an average of 10^3 CFU/25 g. According to the most recent Mexican population Census (2005), Culiacan has a population of 793,730, from this amount 634,984 correspond to the normal healthy individuals, 145,387 are considered compromised, and 13,359 individuals are over 65 years old (elderly). All of the assumptions to estimate the risk of illness in this QMRA were made assuming a serving size of 4.5 g and either a 1 or 21.4 servings in a given year for healthy and compromised individuals. In addition, a serving size of 16.4 was assumed for persons over 65 years of age. Table 2 shows the estimated probability of illness of *L. monocytogenes* in healthy, compromised and elderly populations that consumed QF contaminated with *L. monocytogenes* at levels of 10^2 , 10^3 , and 10^5 CFU/25 g.

The probability of illness associated with the consumption of one serving with a dose of 10^2 , and 10^5 CFU/25 g of QF in healthy individuals ranged from negligible to 1.75×10^{-8} and from 1.27×10^{-10} to 3.31×10^{-4} with a mean of 1.10×10^{-4} for the compromised and

elderly. On the other hand, the risk of illness assuming 16.4 and 21.4 servings at the same doses ranged from negligible to 3.74×10^{-7} for the healthy population, 2.7×10^{-10} to 7.08×10^{-4} with a mean of 2.36×10^{-4} for the compromised and 2.08×10^{-9} to 5.42×10^{-3} with a mean of 1.80×10^{-3} for the elderly (Table 3).

Assuming that QF was contaminated at the levels determined in this assessment and assuming that all individuals in Culiacan's population consumed 4.5 g of contaminated QF as reported by the GEMS/Food regional diet (WHO, 2003), table 4 provides the number of estimated cases of *L. monocytogenes* illness in this region. Using the proportion of the overall Culiacan population, the average number of expected cases of listeriosis in Culiacan, Mexico, assuming one serving with a dose of 10^2 , and 10^5 CFU/25 g of QF per year was from negligible to 0.01 for the healthy population, 1.84×10^{-5} to 48 with a mean of 16 for the compromised, and 1.69×10^{-6} to 4 with a mean of 1 for the elderly. The number of expected cases of listeriosis assuming 16.4 and 21.4 servings per year at the same doses ranged from negligible to 0.23 for the healthy population, 3.93×10^{-5} to 102 with a mean of 34 for the compromised, and 2.77×10^{-5} to 72 with a mean of 24 for the elderly (Table 5).

Risks may be overestimated by assuming that the entire population of Culiacan consumed QF and that each consumer eats at least 4.5 g/serving/day. This QMRA also assumes that the contamination during cheese preparation is similar when cheese is made from pasteurized milk. It is also assumed that temperature at retail did not affect produce quality and that *L. monocytogenes* growth is constant. One other potential influence of

QF quality includes cross-contamination of the product from transporting between retailer and consumer. In addition, the time between production and consumption was not considered in this QMRA.

There is a need for standardization protocols and better reproducibility among brands/batches of QF from independent merchants in Culiacan which will allow them to enhance the quality and safety of the QF. This is important because listeriosis is more likely to occur in pregnant women, neonates, individuals who are immunocompromised, those undergoing immunosuppressive therapies, and the elderly (Swaminathan and Gerner-Smidt, 2007). Greater insight on prevalence, levels, and types of *L. monocytogenes* in soft cheese will augment efforts to better manage the threat of listeriosis, especially since the data of cases of listeriosis in Culiacan, Sinaloa, are unknown.

4. CONCLUSIONS

The QMRA conducted in this study demonstrated that the consumption of contaminated QF represents a risk of *L. monocytogenes* illness in Culiacan's population. The number of cases of listeriosis is predicted to be greater among compromised and elderly populations. Control of *L. monocytogenes* in cheeses is difficult; however, improved sanitation practices, identification and elimination of probable contaminants, and modification of processing practices and equipment design in areas of high risk should minimize the likelihood of product contamination.

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TABLES

Table 1. Parameters and assumptions for exposure of *Listeria monocytogenes* in queso fresco

Parameter	Assumption
<i>Listeria monocytogenes</i> /25 g of Queso Fresco	$<10^2, 10^3, 10^5$ CFU ^{ab}
Limit of Detection	≤ 1 CFU per 25 g
Amount of Queso (g) consumed per serving	4.5 g ^c
Number of serving per healthy and compromised population	21.4
Number of servings per elderly population	16.4

^a Pini and Gilbert, 1988

^b Colony forming units

^c Lake et al. 2005

The amount of *L. monocytogenes* in QF servings was assumed to be random, and the dose was dependent on the amount of *L. monocytogenes* present in 25 g of QF.

Table 2. Estimated probability of *Listeria monocytogenes* illness for one serving of queso fresco

Population	10^2 ^c	10^3 ^c	10^5 ^c
Healthy ^b	NR ^a	NR ^a	1.75×10^{-8}
Compromised/Elderly ^b	1.27×10^{-10}	1.75×10^{-8}	3.31×10^{-4}

^a Negligible risk

^b Assuming one serving of 4.5 g/person/day

^c Concentration of *L. monocytogenes* (CFU/25 g) in queso fresco

Table 3. Risk of illness associated with the consumption of multiple servings of queso fresco

Population	10 ^{2e}	10 ^{3e}	10 ^{5e}
Healthy ^b	NR ^a	NR ^a	3.74 x 10 ⁻⁷
Compromised ^c	2.71X10 ⁻¹⁰	3.74X10 ⁻⁸	7.08X10 ⁻⁴
Elderly ^d	2.08X10 ⁻⁹	2.87X10 ⁻⁷	5.42X10 ⁻³

^a Negligible risk

^{b, c} Assuming 21.4 servings/person/year

^d Assuming 16.4 servings/person/year

^e Concentration of *L. monocytogenes* (CFU/25 g) in queso fresco

Table 4. Estimated incidence of listeriosis in Culiacan's population for a single exposure of queso fresco

Population	10 ^{2d}	10 ^{3d}	10 ^{5d}
Healthy ^a	NR ^e	NR ^e	0.01
Compromised ^b	1.84X10 ⁻⁵	2.54X10 ⁻³	48
Elderly ^c	1.69X10 ⁻⁶	2.33X10 ⁻⁴	4

^a 634,984 inhabitants

^b 145,387 inhabitants

^c 13,359 inhabitants

^d Concentration of *L. monocytogenes* (CFU/25 g) in queso fresco

^e Negligible risk

Table 5. Estimated incidence of *Listeria monocytogenes* illness in Culiacan's population for multiples exposures of queso fresco

Population	10^{2c}	10^{3c}	10^{5c}
Healthy ^a	NR ^d	NR ^d	0.23
Compromised ^a	3.93×10^{-5}	5.43×10^{-3}	102
Elderly ^b	2.77×10^{-5}	3.83×10^{-3}	72

^a Assuming 21.4 servings/person/year

^b Assuming 16.4 servings/person/year

^c Concentration of *L. monocytogenes* (CFU/25 g) in queso fresco

^d Negligible risk