

Full Length Research Paper

Indicator microorganisms, *Salmonella*, *Listeria monocytogenes*, Staphylococcal enterotoxin, and physicochemical parameters in requeson cheese

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Requeson cheese is an artisanal dairy product widely consumed in Latin America. However, no data is available on its microbiological and physicochemical quality. Eighty-four samples were collected. Identification and enumeration of indicator microorganisms and pathogens were done by conventional culture methods, meanwhile staphylococcal enterotoxin by immunoassay. Bromatological parameters were measure collection day. Indicator and pathogenic microorganisms per samples were aerobic mesophilic bacteria (n = 100), coliform bacteria (n = 100), molds-yeasts (n = 70), *Staphylococcus aureus* (n = 74), and *Salmonella* (n = 3). Neither *Listeria monocytogenes* nor Staphylococcal enterotoxin was detected. Temperature ranged from 3 to 19°C, pH from 5.1 to 6.9, acidity from 0.1 to 0.7%, and sodium from 290 to 3970 mg/100 g. Protein content ranged from 2.1 to 13% and lipids from 4.1 to 13%. Starch as adulterant was present in 56 samples.

Key words: Indicator microorganism, *Salmonella*, *Listeria monocytogenes*, Staphylococcal enterotoxin, macronutrients, sodium.

INTRODUCTION

Food contamination is a direct consequence of inadequate hygiene practices during manufacture, handling, transport, storage and marketing.

Microorganisms from other sources are transferred to the surface of foods where they have the nutrients needed to proliferate to levels ranging from 10² to 10⁵ CFU/cm²

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(Quiroz-Santiago et al., 2009). In the dairy industry, for example, both milk and utensils can contribute to microorganism load, including pathogenic microorganisms (Jayarao et al., 2006; Marth and Steeler, 2001; Vázquez-Salinas et al., 2001). Cheeses made from raw milk are especially susceptible, and can contain pathogenic bacteria such as *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Latorre et al., 2009; Schoder et al., 2003; Torres-Vitela et al., 2012; Tunick and Van Hekken, 2010). However, cheeses made from pasteurized milk can also contain microorganisms when handling and marketing conditions are inadequate (Licitra, 2010). In one example, both *Klebsiella* and *Enterobacter* species were identified from fresh cheese made from pasteurized milk (Renyé et al., 2008), demonstrating the importance of proper hygiene measures in cheese production installations. Control of refrigeration temperature is also a critical parameter in ensuring the safety of fresh cheese (Bishop and Smukowski, 2006).

Precise classification of cheese types is difficult due to the diversity of names, country of origin and lack of knowledge of the manufacturing process. In Mexico, a number of fresh cheeses are made, including requesón, ricotta, cottage, *panela*, *adobera*, “feta”, and cream cheese. Some of these cheeses are made from whey, and can be solid, semi-solid or soft. These are made using one of two processes: concentration of the whey followed by molding; or thermal coagulation of whey with or without addition of acid, and finished fresh or aged (CODEX, 2011). Requesón cheese is produced via heat precipitation of whey proteins (mainly β -lactoglobulin and α -lactalbumin) in an acid medium. The final product is bland, soft in texture, salty and granulated, with high moisture content, and requires refrigerated storage (NOM-243-SSA, 2010). It has low macronutrient content, but contains micronutrients such as sodium, iron and calcium (Pérez-Lizaur et al., 2014). Requesón cheese is widely consumed in Latin America and Spain, and is considered highly perishable. Despite its popularity, no reports have been published on its safety or the presence of pathogenic microorganisms in requesón cheese (Pintado and Malcata, 2000). Neither are there reports on its marketing or nutritional quality. Requesón cheese is made from the whey discarded from manufacture of other cheese types, meaning its nutrient and mineral contents can vary widely between different producers. These variations can determine the type of microorganisms present in the final product (Pitado et al., 2005).

The present study objective was to identify and quantify the presence of aerobic mesophilic bacteria, coliform bacteria, molds and yeasts, *Staphylococcus aureus*, *Salmonella*, *L. monocytogenes* and Staphylococcal enterotoxin in requesón cheese acquired from independent retailers. Data were also reported as bromatological parameters, the temperature at the time of purchase, pH, acidity, sodium concentration, and starch,

protein and lipids contents.

MATERIALS AND METHODS

Sample collection

Requesón cheese samples were acquired from fifty-five small, independent dairy retailers located in five distribution centers in the metropolitan area of Guadalajara, Mexico. Over a six month period, a total of 84 samples were taken of requesón cheese sold in bulk (Marques de Cantú, 1991). Each sample (500 g) was immediately placed in a sterile plastic bag, hermetically sealed and transported in a cooler (Igloo, Houston, USA) to the laboratory for analysis within one hour of collection.

Sample preparation

Sub-samples (10 g) from each sample were placed in a sterile plastic bag with 90 ml sterile peptone diluent (SPW; Bioxon Mexico) and pummeled in a stomacher for 1 min. Subsamples were analyzed to quantify (APC), Coliform bacteria (CB), *S. aureus*, and Yeasts and Molds (Y-M). Separate sub-samples (25 g) were taken for *L. monocytogenes* and *Salmonella* identification, placed in sterile plastic bags containing 225 ml sterile *Listeria* enrichment medium (LEM; DIFCO, Sparks, MD) or sterile lactose broth (LB; Bioxon Mexico), and pummeled in a stomacher for 1 min.

Microbiological analyses

Analysis of APC, CB, Y-M, *S. aureus*, *Salmonella* and *L. monocytogenes* were done following methods described in the U.S. Food and Drug Administration (Bacteriological Analytical Manual [BAM], 2013). Briefly, for APC, CB, Y-M and *S. aureus*, serial dilutions in SPW (Bioxon, Mexico) from the sub-sample homogenates were prepared. Aliquots (1 ml) of each dilution were transferred to petri dishes (three petri dishes per dilution). Plate count agar (PCA), violet red bile agar (VRBA) and potato dextrose agar (PDA) containing 50 mg/L ampicillin (Bayer, Leverkusen, Germany) and 0.6% dichloran rose bengal (Sigma-Aldrich, St. Louis, MO) were poured into the inoculated petri dishes.

Determination of *S. aureus* was done by taking 0.1 ml of each dilution and spreading it onto Baird-Parker medium (BPM) using a sterile bent glass streaking rod. The PCA, VRBA and BPM plates were incubated at 35°C/48 h, while PDA plates were incubated at 25°C/5 days.

Sub-samples for *Salmonella* identification were incubated in LB at 35°C for 18 to 24 h. Enrichment was done in soy Rappaport Vassiliadis broth (RV_B) and tetrathionate broth (T_B) for 24 h at 41.5°C. The RV_B and T_B were streaked onto plates containing bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar and incubated for 24 h at 35°C. Three to five typical *Salmonella* colonies were randomly chosen from each selective medium for biochemical identification using urea broth, lysine-iron agar (LIA) and triple sugar iron (TSI). The *Salmonella* spp. genus was identified by serological analysis using somatic antigen polyvalent serum (Antiserum poli A&V1, Bioxon, Mexico).

Subsamples for *L. monocytogenes* quantification were incubated in *Listeria* enrichment medium base containing sodium pyruvate (BD DIFCO, Sparks, MD) at 30°C for 48 h. The medium was then

Table 1. Average, mean, maximum and minimum values for indicator microorganisms and *S. aureus* contents in requeson cheese samples (n=84).

Parameter	Aerobic mesophilic bacteria*	Coliform bacteria*	Molds/Yeasts	<i>S. aureus</i> *
Average	7.7	2.9	4.9	5.8
Maximum	8.9	6	6.5	7.7
Minimum	5.2	0.5	2.6	3.3
Mean	7.6	2.4	4.8	5.7

*Data expressed as log CFU/g.

streaked onto Oxford medium (OXA) and incubated at 35°C for 48 h. Fresh mobility and sheep blood hemolysis tests were done for the typical colonies and the CAMP and biochemical tests (API Listeria, bioMérieux) were done to confirm dubious reactions. Presence of staphylococcal enterotoxin in both cheese types was determined following AOAC procedure 12095 B-ES-2003/09. Briefly, samples (25 g) were mixed with 25 ml extraction buffer, homogenized in a stomacher (Lab-Blender 400) for 2 min and incubated at 18 to 25°C for 15 min. Two milliliters of supernatant were transferred into a 1.5-ml Eppendorf tube and centrifuged for 15 min at 4,000 g. The pH was adjusted to between 7.5 and 8.09 using 1 N NaOH and then 500 ml of solution transferred to a SET2 strip (bioMérieux, Marcy-l'Étoile, France).

Physicochemical parameters

Temperature measurements were taken from the geometric center of each sample using a thermometer with a probe (Cooking Thermo Timer, Cooper Atkins, Middlefield, USA). For each pH measurement, 100 ml deionized water was added to the bag, the contents manually homogenized from outside the bag for 1 min and pH measured with a potentiometer (pH meter 320, Corning, Corning, USA). Acidity was measured as a percentage (%) by manually homogenizing 10 g sample with 25 ml deionized water, adding phenolphthalein and titrating with 0.1 N sodium hydroxide. Sodium was quantified using Mohr titration. Requesón cheese samples (10 g each) were placed in precipitate flasks, 15 ml deionized water at 50°C added and mixed with a glass rod. Another 20 ml hot distilled water were added to disperse the sample, which was transferred to a matrass and completed to 100 ml with deionized water. The sample was run through filter paper, 50 ml of permeate placed in an Erlenmeyer flask and 1 ml potassium chromate added. This solution was titrated with a standard 0.1 M silver nitrate solution until a persistent red-brown color appeared for 30 s (Nielsen, 2010a).

Protein/nitrogen quantification was done following the Kjeldahl method. Digestion was done using a 5 g sample and adding 25 ml concentrated sulphuric acid and a 5 g Missouri tablet as catalyzer (Merck, Darmstadt, Germany). It was then neutralized with sodium thiosulfate and the distillate captured in 50 ml boric acid with methylene blue and methyl red as indicators. This solution was titrated with 0.1 N HCl. Protein content was measured using a 6.38 conversion factor (Nielsen, 2010c, 2010). Lipids were analyzed using the Gerber method. A sample (11 g) was placed in a Gerber bottle, and 10 ml 90% sulfuric acid and 1 ml isoamyl alcohol added. The bottle was closed, shaken and centrifuged for 4 min. It was then placed in a water bath at 60°C for 5 min and lipids content measured (Nielsen, 2010b). Starch was quantified qualitatively by adding an iodine-iodide solution to a 5 g sample; appearance of a blue color indicated the presence of starch.

RESULTS AND DISCUSSION

Microbiological analysis

Levels of APC, CB and Y-M were generally high (Table 1). Of the 84 analyzed samples, 27 had APC levels of 9.0 to 8.1 log CFU/g, 46 had levels between 8.0 and 7.1 log CFU of BMA/g, and eleven had levels <7 log CFU of BMA/g. Overall, CB levels ranged from 0.5 to 6 log CFU/g. Thirty-six samples had levels from 1.1 to 3.0 log CFU/g, while 29 samples had levels between 3.1 and 5.0 log CFU/g. All samples were contaminated with Y-M, with 70 having levels ranging from 4.1 to 6.0 log CFU/g (Table 1). Contamination with *S. aureus* was also ubiquitous, with 74 samples having levels between 5.1 and 7.0 log CFU/g (Table 1). Staphylococcal enterotoxin was not detected in any of the analyzed samples. *Salmonella* was isolated in only three samples and *L. monocytogenes* was not detected in any.

Both in Mexico and developed countries such as the US, identity regulations do not include the composition of the cheeses of Latin America; this makes naming and describing them quite difficult (Van Hekken and Farkye, 2003). Requeson cheese is a fresh cheese consumed widely in Mexico. It consists of a soft granular mass that will not hold a mold, and is produced only from the whey discharged after cheese production. Generally made on a small scale, often using artisanal techniques, it is most frequently marketed in bulk in independent dairy stores, where it can be exposed to temperature abuse.

Requeson cheese manufacture involves heating to 85 to 95°C, which substantially reduces or inactivates most non-sporulated bacteria in the whey. However, it can easily be recontaminated under the poor safety conditions with which it is normally handled and marketed in Mexico. Its high nutrient and moisture contents, and near-neutral pH (Hough et al., 1999; Pintado et al., 2001), allow microorganisms to quickly become active if it is not adequately refrigerated. In response to the lack of requeson cheese safety and microbiology data in Mexico, the present microbiological analysis was limited to identifying indicator microorganisms commonly used to evaluate food safety and microbiological quality. Analyses were also done of the presence of two

pathogenic bacteria frequently reported in fresh cheeses, and Staphylococcal enterotoxin.

The generally high indicator microorganism frequencies observed here may have resulted from both direct and cross-contamination (Table 1). Levels this high can be produced in two ways. The first scenario is that initial contamination level was low but microbial growth became extremely active, quickly reaching maximum concentrations. The second is that contamination level was high during preparation, with no later microorganism growth; which is the most likely scenario in the present case. However, substandard food safety conditions during requeson cheese preparation and marketing can greatly increase the likelihood of both possibilities.

Approximately 50% of the samples had CB counts >3 log CFU/g. This microbial group may contribute to cheese flavor and aroma (Menendez et al., 2001), but in requeson cheese its presence is linked to inadequate post-thermal treatment hygiene practices (Renyé et al., 2008). Although no previous studies have been done of requeson cheese microbiology, the present results can be compared to reports on similar types of cheese. For example, high CB and Y-M levels have been reported for other fresh cheeses (Renyé et al., 2008; Williams and Withers, 2010).

The main pathogenic microorganisms involved in cheese contamination are *L. monocytogenes*, *S. aureus*, *Salmonella* and *E. coli* pathotypes (Williams and Withers, 2010). The present analyses focused on *Salmonella*, *L. monocytogenes* and *S. aureus*, because *Salmonella* (Franco et al., 2010; Heredia et al., 2001; Soto-Beltran et al., 2015), *S. aureus* (Aguilar et al., 1983; Parrilla-Cerrilo et al., 1993) and *L. monocytogenes* (Latorre et al., 2009; Moreno-Enriquez et al., 2007; Rodas-Suárez et al., 2006) have been isolated from different foods in Mexico. From a public health perspective, *Salmonella* infections, and *S. aureus* food poisoning are endemic in Mexico; indeed, from 2009 to 2011, 381,320 salmonellosis cases, 139,000 typhoid fever cases and 122,200 food poisoning cases were reported (Secretaría De Salud, 2011).

Salmonella can be found in cheeses made from raw or pasteurized milk. Of the different cheese types, fresh cheeses are most frequently involved in disease outbreaks traced to cheese consumption (Michanie et al., 2001). In the present data, 3.5% of the requeson cheese samples were *Salmonella*-positive, a low frequency near that reported previously. For example, in a study of Requeijão (a semi-hard Brazilian cheese) none of 25 samples were found to have *Salmonella* (Viana et al., 2009). In a study of fresh cheeses, *Salmonella* was not identified in any of the samples, although *L. monocytogenes* was identified (Menendez et al., 2001). The latter microbe is common in fresh cheeses and frequently implicated in outbreaks from fresh cheese consumption (CDC, 2012; Jackson et al., 2010; MacDonald et al., 2005; Torres-Vitela et al., 2012; Vázquez-Salinas et al., 2001). However, *L. monocytogenes*

was not detected in the present requeson cheese samples.

In contrast, *S. aureus* levels surpassed 5 log CFU/g in 94% of the samples and coagulase-positive strains were detected. Staphylococcal enterotoxin was not detected in the analyzed samples, but the high frequency of *S. aureus* and requeson cheese is near neutral pH and high nutrient content could still provoke staphylococcal enterotoxin synthesis (Williams and Withers, 2010).

Most of the samples (77%) were stored or marketed under conditions of temperature abuse ($>5^{\circ}\text{C}$), which favors growth of microbes, including pathogenic bacteria (FDA, 2011; Food Code, 2009). In addition, the samples near-neutral pH and low acidity can promote bacterial development and survival (Pintado et al., 2001). The *Salmonella*-positive samples were found to have temperature/pH/acidity values of $6^{\circ}\text{C}/\text{pH } 6.1/0.14$ acidity and $15^{\circ}\text{C}/\text{pH } 6.2/0.27$ acidity, values that may have favored this pathogen's survival in the requeson cheese. This is supported by a study of the relation between physicochemical parameters and *Listeria* presence in fresh cheese (Soto-Beltran et al., 2014). In this study, pH was found to be significantly ($P < 0.05$) related to *Listeria* presence and negatively ($P > 0.05$) linked to salinity. Other studies have confirmed that changes in fresh cheese physicochemical parameters can favor microbial activity. In a study of ricotta cheese, storage at 17°C was found to negatively affect organoleptic and microbiological characteristics and lower shelf life to 12.5 days compared to refrigerated storage (Hough et al., 1999).

Physicochemical parameters

Temperature varied widely in the samples at the moment of sampling (Table 2). Nineteen were $<5^{\circ}\text{C}$ and the remaining 65 were $>5^{\circ}\text{C}$; of the latter, eleven were between 17 and 19°C . Values for pH ranged from 5.1 to 6.9 with an average of 6.1 (Table 2). Titratable acid, expressed as lactic acid, averaged 0.3% in the samples with a range of 0.1 to 0.7%. Sodium was >466 mg/100 g in 76 of the samples.

Composition analysis showed most of the samples (61) to have protein content between 5.1 and 7.0 g/100 g, while casein ranged between 2.2 and 7 g/100 g of cheese (Table 3). Lipids content ranged from 4.1 to 13 g/100 g with 44 samples having values between 9.1 and 11 g/100 g. Starch was detected in 56 of the samples.

The significant differences ($P < 0.05$) observed here in sample physicochemical parameters may be due to variations in whey characteristics caused by its origin, type and source process. Requeson cheese nutrient content will also be affected by the composition of the whey used to make it. Whey contains substantial amounts of protein, lipids, lactose and calcium (Miranda-Miranda et al., 2009). Whey protein is essentially albumin

Table 2. Average, mean, maximum and minimum values for sodium content, acidity, pH and temperature in requeson cheese samples (n=84).

Parameter	Sodium (mg/100)	Acidity (%)	pH	Temperature (°C)
Average	1660	0.3	6.10	11
Maximum	3970	0.7	6.90	19
Minimum	290	0.13	5.10	3
Mean	1360	2.80	6.1	9.6

Table 3. Distribution of requeson cheese samples based on total protein, casein and lipids contents*.

Sample	Total protein	Casein	Lipids
1.1 a 2.0	-	-	-
2.1 a 3	3	12	-
3.1 a 4	8	12	-
4.1 a 5	4	52	4
5.1 a 6	29	0	4
6.1 a 7	32	8	4
7.1 a 8	0	-	16
8.1 a 9	4	-	4
9.1 a 10	4	-	28
10.1 a 11	-	-	16
11.1 a 12	-	-	4
12.1 a 13	-	-	4

*Data expressed as g/100 g requeson cheese.

and globulin because any casein is removed during cheese curdling (Franchi, 2010; Vázquez-Puente et al., 2010). Requeson cheese protein content is influenced by whey acidity and processing temperature. In one study, protein content doubled when the whey was acidified to 0.45% and heated to 93°C (Raftari et al., 2009). This contrasts with the present results in which samples with the highest protein content (8.4 and 9.6%) had low acidity (0.1 to 0.2%).

According to the Mexican Food Equivalents System 2014 (Sistema Mexicano de Alimentos Equivalentes - SMAE), requeson cheese is a very low-fat (6.38 g/100 g) animal product (Pérez-Lizaur et al., 1993). However, of all samples in the present study, 76 samples surpassed the very low-fat cut-off level. In the (CODEX, 2014), dairy products are classified as non-fat or skim when they contain ≤10% lipids. In the present study, only 38% of the samples fell within this classification while the remaining 62% had 10 to 13% lipids, which would classify them as low-fat products.

For the sodium content, SMAE indicate 466 mg/100 g as average contents for this element in the requeson cheese. However, in the study, only eight samples presented 290 to 380 mg of sodium/100 g, meanwhile 76 samples were >466 mg of sodium/100 g. Sodium chloride improves cheese flavor and lengthens its shelf life by

reducing water activity, thereby inhibiting bacterial development (Hutton, 2002). The USDA National Nutrient Database for Standard Reference (USDA, 2014), does not include a nutritional description for requeson cheese, but it is generally not recommended to use high sodium levels to decrease pathogen presence in this kind of food (Soto-Beltran et al., 2014).

On the other hand, level of protein in samples was lower than SMAE (11.94 g of protein/100 g of requeson cheese). The objective of determination of starch is because of its use as a stabilizer and anti-agglutinating agent in some fresh cheeses, although its use is not recommended in cheeses produced from whey (CODEX, 2014). This is notable since over half the samples contained starch.

Conclusions

In Mexico, requeson cheese production is essentially artisanal and it is sold largely through independent marketers. This constitutes a serious challenge in the pathogen control and food safety in fresh cheeses in general, and particularly, in requeson cheese. The present results indicate requeson cheese in Mexico to be at high risk of contamination and highlight an acute need for implementing adequate food safety measures throughout the product's trajectory, from production to marketing. Contamination risk was especially high during post-production handling and storage. Sample contamination could have been substantially reduced with use of individual packaging and proper retail storage conditions. As is the case with other highly perishable products, avoiding contamination of requeson cheese requires standardized operating and hygiene procedures during production, storage, transport to retailers and marketing. A good first step towards this goal would be to ensure fulfillment of applicable regulations controlling requeson cheese physicochemical and food safety parameters to ensure product characteristics and hygiene.

Conflict of Interests

The authors have not declared any conflict of interests.

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