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## Detection and Characterization of *Listeria monocytogenes* in São Jorge (Portugal) Cheese Production

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### ABSTRACT

*Listeria monocytogenes* is a foodborne pathogen that can cause serious invasive disease in humans. Because human listeriosis cases have previously been linked to consumption of contaminated cheese, control of this pathogen throughout the cheese production chain is of particular concern. To understand the potential for *L. monocytogenes* transmission via São Jorge cheese, a Portuguese artisanal cheese variety that bears a Protected Denomination of Origin classification, 357 raw milk, curd, natural whey starter, and cheese samples representative of the production chain of this cheese were collected over one year and tested for the presence of *L. monocytogenes* and selected physicochemical parameters. Although neither *L. monocytogenes* nor other *Listeria* spp. were detected in whey, curd, or cheese samples, 2 of the 105 raw milk samples analyzed were positive for *L. monocytogenes*. These 2 raw milk isolates represented a ribotype that has previously been linked to multiple human listeriosis outbreaks and cases elsewhere, indicating the potential of these isolates to cause human listeriosis. On average, physicochemical parameters of São Jorge cheese ripened for 4 mo presented values that likely minimize the risk of *L. monocytogenes* outgrowth during ripening and storage (mean pH = 5.48; mean moisture = 37.79%; mean NaCl concentration = 4.73%). However, some cheese samples evaluated in this study were characterized by physicochemical parameters that may allow growth and survival of *L. monocytogenes*. Even though our results indicate that raw milk used for São Jorge cheese manufacture as well as finished products is rarely contaminated with *L. monocytogenes*, continued efforts to control the presence of this pathogen in the São Jorge cheese production chain are urged and are critical to ensure the safety of this product.

**Key words:** *Listeria monocytogenes*, Azores, cheese, food safety

### INTRODUCTION

*Listeria monocytogenes* is a gram-positive opportunistic pathogen that has been isolated from many natural and human-created environments. Human listeriosis is a primarily foodborne infection that occurs predominantly in elderly and immunocompromised people, as well as in pregnant women (Low and Donachie, 1997; Mead et al., 1999). Clinical symptoms of invasive listeriosis include septicemia, encephalitis, and meningitis, as well as miscarriage in pregnant woman. In addition, listerial gastroenteritis in immunocompetent hosts has been reported (Dalton et al., 1997; Fsihi et al., 2001). Even though listeriosis is a rare disease, invasive human foodborne listeriosis represents a considerable public health concern because of its high case mortality rate of about 20% (Mead et al., 1999). *Listeria monocytogenes* also causes disease in dairy cattle, including abortions and central nervous system infections, and is commonly isolated from dairy farm environments and fecal material from dairy cattle (Husu, 1990; Nightingale et al., 2004).

Most human listeriosis cases appear to be linked to consumption of ready-to-eat (RTE) food products that have undergone listeriocidal heat treatments but have been contaminated postprocessing from environmental sources. However, some cases and outbreaks have been linked to consumption of food products that did not undergo heat treatment and appear to have been contaminated by on-farm sources (Lundén et al., 2004). In particular, *L. monocytogenes* contamination of fermented dairy products made from raw milk may be due either to use of contaminated raw milk or to postprocessing contamination from environmental sources not directly linked to raw milk (Broseta et al., 2003; Kells and Gilmour, 2004). For example, a human listeriosis outbreak linked to consumption of contaminated Hispanic-style cheese may have been caused by the use of *L. monocytogenes*-contaminated raw milk in cheese making (Linnan et al., 1988; Ryser, 1999).

Measures are taken and regulations enforced in most countries to reduce contamination of RTE foods with *L. monocytogenes*. Italy and the United States, for ex-

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ample, have a so-called “zero tolerance” policy for the presence of *L. monocytogenes* in RTE foods, meaning that this species must be absent from a 25-g food sample. Other countries, which consider such a goal of “zero tolerance” to be difficult to comply with, have standards that limit the presence of *L. monocytogenes* up to a maximum of 100 cfu/g in certain foods (Pagotto et al., 2002). *Listeria monocytogenes* control measures recommended by the Health and Consumer Protection Directorate of the European Commission include, among others, hygiene practices, risk communication, and consumer education (European Commission, 1999). However, this group considers the product profile to be a critical point for preventive measures against *L. monocytogenes*. Specifically, stringent control measures are required for RTE foods that potentially permit growth of *L. monocytogenes*, such as foods with high pH or high water activity ( $a_w$ ), particularly if these RTE foods did not receive any listeriocidal (e.g., pasteurization) treatment (European Commission, 1999). The requirements of these control measures are consistent with various studies (Adams and Nicolaidis, 1997; European Commission, 1999) showing that *L. monocytogenes* can grow to high levels in foods stored at refrigerating temperatures, even though the pathogen is generally a poor competitor that can easily be overgrown by other psychrotolerant or psychrophilic bacteria present in foods.

As stressed above, consumption of *L. monocytogenes*-contaminated cheeses has been linked to a number of human listeriosis outbreaks (Linnan et al., 1988; Adams and Moss, 1995b; Ryser, 1999; Boggs et al., 2001). Artisanal cheeses produced with raw milk in small dairy farms or small processing operations may be at particular risk for *L. monocytogenes* contamination because of the lack of heat treatment of the raw materials, as well as because of the potentially higher risk of product contamination from environmental sources (e.g., soil, manure) if production sites lack sophisticated approaches to control contamination (Guerra et al., 2001). In addition, manufacture on farms and in small plants often lacks reproducibility, thus leading to products that may suffer from a lack of consistency for physicochemical parameters (pH, moisture, and salt), which may act as a hurdle to prevent undesirable bacterial growth (Cole et al., 1990; Adams and Moss, 1995a; Bolton and Frank, 1999; European Commission, 1999; Tienungoon et al., 2000).

São Jorge cheese is a Portuguese cheese that has been produced on the island of the same name in Azores for more than 300 yr. This product received a Protected Denomination of Origin, or Appellation d'Origine Contrôlée, classification in 1984 and is a main source of income for residents of the island. São Jorge cheese is

manufactured in small processing plants from raw cow's milk and in the absence of any commercial starter; hence, production does not include a processing step that kills *L. monocytogenes* that may be present in the raw milk. Manufacture includes coagulation at 30 to 31°C of whole raw milk following acidification with whey from the previous day's production. Cutting, cooking, and whey draining are followed by hand salting, pressing, and subsequent ripening at 12 to 14°C for 4 to 12 mo. Emerging European market rules enforcing stricter quality and safety requirements for rural and artisanal food products, and increasing acceptance of this cheese by consumers and its economic importance for São Jorge inhabitants, make it critical to better understand potential food safety hazards, such as *L. monocytogenes* contamination, that may be associated with the production of São Jorge cheese. The main objective of this study was thus 1) to investigate the prevalence of *L. monocytogenes* in raw milk and cheese randomly collected on São Jorge Island, and 2) to characterize the physicochemical parameters of São Jorge cheese that may affect the growth and survival of *L. monocytogenes*.

## MATERIALS AND METHODS

### Sample Collection

Between July 2002 and May 2003, a total of 105 raw milk samples, 90 curd samples, 96 natural whey starter samples, and 66 cheese samples (obtained after 4 mo of ripening) were collected. All samples were obtained, once a month, from 7 small dairy-processing plants (A through G) that produce São Jorge cheese. Combined, these dairies receive approximately 52,000 L of raw milk per day in the summer from 273 farmers who deliver milk twice a day (once in the morning and once in the afternoon) in stainless-steel cans without refrigeration. On arrival, the milk is routinely stirred in the can with a milk stirrer and each farmer subsequently transfers the milk into the receiving bulk tank, filtering the raw milk through a white cloth as it is transferred; this white cloth is regularly replaced with a sanitized clean cloth. When the receiving bulk tank is full, all milk is pumped from this tank into the coagulation vat.

At each sampling visit, 200 mL of raw milk, 200 mL of whey starter (sampled immediately before addition to the open vat), 100 g of salted curd, and 200 g of 4-mo-old cheese were aseptically collected and transferred into individual sterile bags or flasks. Samples were transported to Centro de Investigação de Recursos Naturais (Universidade dos Açores, Ponta Delgada, Portugal) in insulated coolers containing cold packs and were analyzed within 10 h of collection.

**Table 1.** Incidence of *Listeria monocytogenes* in all raw milk, cheese, and curd samples

Type of product	Number of samples analyzed	Number of positive samples collected in a given month/number of samples collected										
		Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Raw milk	105	1/14	0/7	0/7	0/7	0/10	0/10	0/10	0/10	0/10	1/10	0/10
Whey starter	96	0/14	0/7	0/7	0/7	0/12	0/12	0/10	0/7	0/7	0/7	0/6
Curd	90	0/12	0/7	0/7	0/7	0/10	0/10	0/10	0/7	0/7	0/7	0/6
Cheese	66	0/7	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/5

### Chemical Analysis

Subsamples were prepared from all samples to determine pH using a pH meter (Hanna Instruments, Padova, Italy); curd and cheese subsamples were used to determine moisture and NaCl content, as described by James (1999).

### Microbiological Analyses

Sample preparation and *L. monocytogenes* detection were performed as described by Struijk et al. (2001). Briefly, milk, whey, curd, and cheese samples (25 mL or g) were aseptically transferred into flasks containing 225 mL of *Listeria* enrichment broth base (Oxoid, Basingstoke, Hampshire, UK). Flasks were then placed in a Certomat H incubator (B. Braun International, Melsungen, Germany), and incubated at 30°C with 100 rpm agitation. After 24 and 48 h, 0.1-mL aliquots of the enrichments were surface-plated on Palcam agar (Oxoid). After incubation of the plates for 48 h at 30°C, 5 *Listeria*-like colonies (if present) were sub-streaked on brain heart infusion agar and were presumptively identified by morphology, gram staining, and catalase production.

### PCR Analysis

An *L. monocytogenes*-specific PCR assay targeting the hemolysin gene (*hlyA*) was used, as described by Kabuki et al. (2004), to screen presumptive *Listeria* colonies to identify *L. monocytogenes* isolates. Briefly, overnight cultures in brain heart infusion broth of putative *Listeria* isolates were lysed using lysozyme and proteinase K, and lysates were then used to perform the *L. monocytogenes*-specific PCR targeting *hlyA*. Polymerase chain reaction products were separated by gel electrophoresis using a 1.5% agarose gel; a positive result (i.e., the presence of a PCR product of approximately 860 bp) indicated that the isolate tested represented *L. monocytogenes*.

### Ribotyping

Isolates identified as *L. monocytogenes* by PCR were further characterized by automated *EcoRI* ribotyping,

performed using the RiboPrinter Microbial Characterization System (DuPont-Qualicon, Wilmington, DE). Automated ribotyping involved *EcoRI* digestion of chromosomal DNA, followed by Southern hybridization with an *Escherichia coli rrnB* rRNA operon probe, as described elsewhere (Bruce, 1996). Images were acquired with a charge-coupled device camera and processed using the RiboPrinter custom software. This software normalizes fragment pattern data for band intensity and relative band position compared with the molecular weight marker, thus providing a high level of reproducibility and standardization. Ribotype patterns were automatically assigned a DuPont ID (e.g., DUP-1042) by the RiboPrinter, which was confirmed by visual inspection. If visual inspection indicated that a given DuPont ID included more than one distinct ribotype pattern, each pattern was designated by an alphabetically assigned letter suffix (e.g., DUP-1042A and DUP-1042B represent 2 distinct ribotype patterns within DUP-1042; Gray et al., 2004). Distinct ribotype patterns within a given DuPont ID generally differed by only a position of a single weak band.

## RESULTS AND DISCUSSION

Collection and testing of 357 raw milk, curd, natural whey starter, and cheese samples representing the production chain of São Jorge cheese 1) revealed an overall low prevalence of *L. monocytogenes*, and 2) indicated that, on average, the physicochemical parameters of sampled São Jorge cheese after 4 mo of ripening were likely to minimize the risk of *L. monocytogenes* outgrowth in this cheese.

### Prevalence of *L. monocytogenes* in Cheese and Ingredients

The overall prevalence of *L. monocytogenes* among 357 raw milk, curd, natural whey starter, and cheese samples, randomly taken and representative of the production chain of São Jorge cheese, was very low. Although neither *L. monocytogenes* nor other *Listeria* spp. were detected in whey, curd, or cheese samples, 2 of the 105 raw milk samples analyzed tested positive for *L. monocytogenes* (Table 1), corresponding to a raw milk

FSL ID#	Source	Location	Period	Ribotype	Ribotype image
H4-601	Milk	Azores	2004	DUP-1042B	
H4-602	Milk	Azores	2004	DUP-1042B	
J1-116	Human epidemic	UK	1988–1990	DUP-1042B	
J1-225	Human epidemic	USA (MA)	1983	DUP-1042B	

**Figure 1.** *Eco*RI ribotypes for *Listeria monocytogenes* raw milk isolates and isolates with a matching DUP-1042B ribotype obtained from human patients associated with the listeriosis outbreaks in Massachusetts, United States (1983), and in the United Kingdom (1988 to 1990; Jeffers et al., 2001). FSL = Food Safety Laboratory; DUP = DuPont.

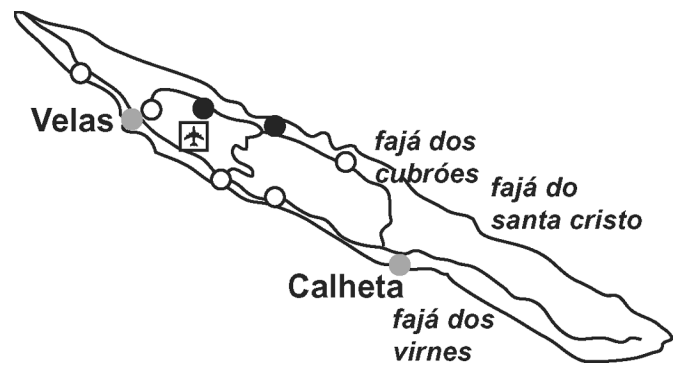
prevalence of 1.9%. No other *Listeria* species were isolated from the raw milk samples tested. Overall, a total of 40 presumptive *Listeria* isolates were tested by the *L. monocytogenes*-specific PCR assay.

Surveys of raw milk collected from farms in different countries found a wide prevalence of *L. monocytogenes* contamination (Adams and Moss, 1995b), ranging from 0.32% in Germany, to 4.2 and 4.4% in France and Holland, respectively, up to 7% in Mexico (Carlos et al., 2001). Kells and Gilmour (2004) reported a 22.2% prevalence of *L. monocytogenes* in raw milk samples collected in Ireland. Sanchez et al. (1998) reported an incidence of 3.6 and 2.71% of *L. monocytogenes* and *Listeria innocua*, respectively, in raw milk produced in Spain, whereas Guerra et al. (2001) reported a 5% incidence of *L. monocytogenes* in milk and dairy products sold in mainland Portugal. Therefore, the prevalence levels in São Jorge raw milk and cheese appear to be comparatively low.

In a seroepidemiological study of listeriosis in cows, Boerlin et al. (2002) reported that 11% of healthy dairy cows in Switzerland simultaneously presented antibodies toward *L. monocytogenes* listeriolysin O and internalin A antigens, which are indicative of *L. monocytogenes* infection of the tested cattle. In that study, feeding silage (corn silage, but not grass silage) was found to be a significant risk factor for positive *Listeria* serology. Cattle breed and hygiene on the farm have also been reported as significant factors associated with a *Listeria*-positive serological status of animals on a given farm. In addition, a large number of studies have indicated that clinical listeriosis in ruminants is often associated with feeding poor-quality silage (Adams and Moss, 1995b; Driehuis and Oude-Elferink, 2000; Boerlin et al., 2002). Hence, differences in *L. monocytogenes* incidence for raw milk samples collected in different parts of the world may be related to distinct cattle-feeding practices. The low incidence of *L. monocytogenes* found in our study encompassing raw milk samples

collected on São Jorge Island may thus be explained by the fact that cattle in the Azores graze all year round because of the mild climatic conditions (winter temperatures range from 12 to 15°C) and that very little (if any) grass silage is fed to cows on this island. Consistent with this hypothesis, Husu (1990) and Fenlon (1996) also found that it is uncommon for grazing animals to shed detectable levels of *L. monocytogenes*. Broseta et al. (2003) also reported that contamination of raw milk with *Listeria* is usually more common in the winter, most likely because silage feeding in many parts of the world is more common in that season. In this context, it is important to emphasize that our study included all 4 seasons.

Interestingly, the 2 *L. monocytogenes* isolated from raw milk shared the same ribotype pattern (DUP-1042B, Figure 1), even though they were obtained from raw milk collected at 2 different dairies (Figure 2) and in 2 different months; isolate CM1 was obtained from milk collected in dairy C in July 2002, whereas isolate GM1 was obtained from milk collected in dairy G in April 2003. It should be emphasized that ribotype DUP-



**Figure 2.** Map of the São Jorge Island showing the locations of the 7 dairy-processing operations included in this study; operations where *Listeria monocytogenes* was detected in raw milk samples (●); operations without *L. monocytogenes*-positive samples (○).

**Table 2.** Physicochemical parameters (mean value  $\pm$  SD) for all raw milk, cheese, and curd samples

Parameter measured	Raw milk	Whey starter	Curd	Cheese
pH	6.80 $\pm$ 0.15	5.69 $\pm$ 0.51	5.77 $\pm$ 0.19	5.48 $\pm$ 0.20
NaCl content, %	ND <sup>1</sup>	ND	2.55 $\pm$ 0.94	4.73 $\pm$ 0.66
Moisture, %	ND	ND	52.25 $\pm$ 5.15	37.79 $\pm$ 3.70

<sup>1</sup>ND = not determined.

1042B not only represents a *L. monocytogenes* subtype commonly associated with human listeriosis infections in the United States (Gray et al., 2004), but also has been implicated as the causative subtype of multiple human listeriosis outbreaks, including outbreaks in the city of Boston in 1979 and in the state of Massachusetts in 1983 in the United States, and in the United Kingdom from 1988 to 1990 (Jeffers et al., 2001). Hence, the *L. monocytogenes* subtypes found likely have the potential to cause human disease, highlighting the need for continued efforts to control *L. monocytogenes* contamination of São Jorge cheeses.

#### **Physicochemical Parameters of São Jorge Cheese Limit the Likelihood of *L. monocytogenes* Outgrowth During Cheese Ripening and Storage**

The ability of *L. monocytogenes* to grow or survive in a food product depends on a variety of physicochemical parameters, including pH,  $a_w$ , and NaCl content (Conner et al., 1986; Bolton and Frank, 1999; Learson et al., 1999). The average physicochemical parameters associated with São Jorge cheese ripened for 4 mo (Table 2) are such that they should limit (if not prevent) the growth of *L. monocytogenes*; the pH values ranged from a maximum of 5.96 to a minimum of 5.10, with a mean of 5.48; the salt concentrations ranged from a maximum of 6.30% to a minimum of 3.20%, with a mean of 4.73%; and the moisture levels averaged 37.79%. Previous studies have specifically shown that *L. monocytogenes* does not grow at a pH below 5.3 when the  $a_w$  is lower than 0.93 (Tienungoon et al., 2000), or at a pH below 4.46 regardless of the  $a_w$  (Cole et al., 1990; Farber et al., 1996). In addition, an  $a_w$  range between 0.85 and 0.6 (typical for intermediate-moisture foods), which corresponds roughly to a moisture content of 15 to 50%, prohibits the growth of gram-negative bacteria as well as a large number of gram-positive bacteria (Adams and Moss, 1995a).

Although the combination of the average values found for pH, moisture, and salt content should essentially be able to prevent *L. monocytogenes* growth in most São Jorge cheeses, some products representing extreme values (e.g., a pH of 5.96) may permit growth of this pathogen to some extent. In addition, there is a

potential risk for *L. monocytogenes* initially present in nonrefrigerated raw milk or curd to grow to high numbers when the  $a_w$  is still very high (i.e., at the beginning of ripening), thus allowing survival of at least some *L. monocytogenes* cells until the end of the natural ripening period.

## **CONCLUSIONS**

Even though our results indicate that raw milk used for São Jorge cheese production as well as ripened São Jorge cheeses may rarely be contaminated with *L. monocytogenes*, continued efforts to control and monitor the presence of this pathogen in the São Jorge cheese production chain are critical, particularly because physicochemical parameters for some cheeses might permit growth of *L. monocytogenes*. Lack of refrigeration of raw milk in particular represents a potential risk factor that may allow growth of *L. monocytogenes* to high numbers, hence facilitating subsequent survival and persistence in the final cheese. Continued *L. monocytogenes* surveillance, along with the development of risk management strategies and application of the general principles of food hygiene and Hazard Analysis and Critical Control Point guidelines (European Union, 1999; Guerra et al., 2001) should allow successful control of *L. monocytogenes* in São Jorge cheese manufacture, particularly because the baseline prevalence of this pathogen appears to be low in the cheese production continuum.

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