Recurrent and Sporadic *Listeria monocytogenes* Contamination in Alheiras Represents Considerable Diversity, Including Virulence-Attenuated Isolates[▽]†

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Microbiological characterization of alheiras, traditional smoked meat sausages produced in northern Portugal, had previously shown that more than 60% of the lots analyzed were contaminated with *Listeria monocytogenes* at levels higher than 100 CFU/g. In order to better understand *L. monocytogenes* contamination patterns in alheiras, we characterized 128 *L. monocytogenes* isolates from alheiras using a variety of subtyping techniques (i.e., molecular serotyping; arsenic, cadmium, and tetracycline resistance typing; and pulsed-field gel electrophoresis [PFGE]). Subtyping of isolates from products collected on two separate dates provided evidence for the persistence of specific *L. monocytogenes* PFGE types in the production and distribution chains of alheiras from four different processors. A subset of 21 isolates was further characterized using ribotyping and Caco-2 cell invasion assays to evaluate the pathogenic potential of *L. monocytogenes* present in alheiras. Caco-2 invasion assays revealed seven isolates with invasion efficiencies that were less than 20% of that of the control strain 10403S. All seven isolates had premature stop codons in *inlA* that represented three distinct mutations, which had previously been observed in isolates from the United States or France. Our findings indicate the need for a comprehensive approach to control *L. monocytogenes* in alheiras, including strategies to reduce persistence. The presence of considerable diversity in invasion phenotypes among *L. monocytogenes* strains present in alheiras, including the presence of subtypes likely to be virulence attenuated, may provide an opportunity to initially focus control strategies on the subtypes most likely to cause human disease.

Listeriosis, a food-borne disease caused by the ubiquitous bacterium *Listeria monocytogenes*, represents an important public health problem. Listeriosis mainly occurs in pregnant women and newborns, the elderly, or immunocompromised individuals. In 1999, Mead et al. (31) reported that *L. monocytogenes* causes an estimated 2,500 human cases of listeriosis, including 500 deaths, in the United States on an annual basis. While there is no surveillance for *L. monocytogenes* cases in Portugal, recent evidence indicates that human listeriosis occurs in Portugal at levels similar to those encountered in other developed countries (1). Worldwide, *L. monocytogenes* has been detected in dry and semidry fermented sausages as well as in delicatessen meats and other ready-to-eat (RTE) meat and poultry products, which are generally considered to be important vehicles for food-borne listeriosis (45). Listeriosis outbreaks linked to meat products have also been reported worldwide (for examples, see references 11, 12, 19, 30, 39, and 42).

A recent preliminary microbiological characterization of alheiras, traditional smoked meat sausages produced in northern Portugal (5), showed that more than 60% of the lots analyzed were contaminated with *L. monocytogenes* at levels higher than 100 CFU/g (5). These smoked meat products represent an important part of the diet in rural areas of Portugal and are also becoming increasingly popular in urban areas. For example, annual production of alheiras in the Mirandela region ("alheiras de Mirandela"), the main alheira-producing region, has been increasing since 1998 and reached an annual production of 136 tons in 2003 (37, 38). While alheiras are generally not considered RTE foods, they are often consumed without heating that is sufficient to kill *L. monocytogenes.* Thus, the presence of *L. monocytogenes* in these products represents a potential food safety issue.

Locally produced and artisanal foods may represent potentially important sources of human listeriosis, as supported by a large listeriosis outbreak in France (279 cases) linked to the consumption of pork tongue in jelly (19). We thus conducted this study to better understand *L. monocytogenes* contamination patterns in alheiras through the application of modern molecular and tissue culture methods. Specifically, we characterized a collection of 128 *L. monocytogenes* isolates from alheiras using subtyping techniques. A subset of isolates was further characterized using tissue culture invasion assays to evaluate the pathogenic potential of *L. monocytogenes* present in alheiras. The goal of this study was not only to specifically study *L. monocytogenes* contamination patterns in alheiras but also to more broadly demonstrate how modern microbiological approaches can be used to characterize *L. monocytogenes* contamination patterns in traditional and often locally produced foods. Improved understanding of *L. monocytogenes* contamination in artisanal foods will also be critical for better attribution of food-borne listeriosis cases to specific specialty food

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TABLE 1. PFGE type diversity of *L. monocytogenes* isolated from alheiras samples from different processing plants[a]

Processor[b]	Sampling date	Product type[c]	PFGE pattern type and MPN for[d]:			
			Composite sample 1		Composite sample 2	
			PFGE type(s) (no. of isolates)	MPN	PFGE type(s) (no. of isolates)	MPN
1	03/18/2004	MAP	341 (1), 342 (1)	0.47	NA	<0.2
2	03/18/2004	MAP	343 (3)	0.18	343 (2)	0.40
3	11/12/2003	MAP	NA	<0.2	NA	<0.2
	04/24/2004	MAP	197 (4), 342 (3), 345 (3)	0.8	345 (15)	1.7
4	09/24/2003	B	344 (3)	2.3	NA	<0.2
	04/23/2004	B	NA	<0.2	NA	<0.2
5	11/12/2003	B	347 (2), 348 (1), 349 (7)	>240	347 (7), 348 (1)	>240
	06/30/2004	B	NA	<0.2	NA	<0.2
6	09/29/2003	VP	351 (1), 352 (1), 353 (1), 354 (1), 355 (1)	6.9	352 (1), 355 (1)	0.9
7	05/18/2004	B	**356** (2), 357 (1), 358 (1)	>240	**356** (1)	>240
	07/06/2004	VP	**356** (1)	>240	**356** (1)	>240
8	05/18/2004	VP	**130** (3), 359 (2)	>240	**130** (2)	>240
	06/30/2004	VP	**130** (1)	3.3	NA	<0.2
9	05/25/2004	VP	360 (4)	>240	360 (3)	>240
	07/06/2004	VP	NA	<0.2	NA	<0.2
10	05/18/2004	MAP	**361** (7)	>240	**361** (7)	161
	06/29/2004	MAP	**361** (1)	2.7	**361** (1)	5.0
11	05/25/2004	B	**358** (5), 362 (1), 363 (2)	>240	362 (2), 363 (4), 366 (1)	>240
	07/06/2004	B	346 (1), **358** (1)	2.7	**358** (1)	2.2
12	10/15/2003	B	NA	<0.2	344 (1), 353 (9), 365 (2)	0.7
	04/22/2004	B	NA	<0.2	NA	<0.2

[a] See Table S3 in the supplemental material for an extended version of this table, which also indicates the specific *L. monocytogenes* testing results obtained with different methods (MPN, VIDAS, and ISO) for each sample.
[b] All four samples collected from processor 13 were negative, and processor 13 is thus not listed in this table.
[c] Samples were classified according to packaging typing, including vacuum packaging (VP), modified atmosphere packaging (MAP), and bulk (B).
[d] PFGE types found on both sampling dates in a given plant (indicating persistence) are shown in bold. MPN values are shown as *L. monocytogenes* cells/g. NA, not applicable (indicates that the analyzed composite sample was negative for *L. monocytogenes*).

groups that may not currently be considered in risk assessments.

MATERIALS AND METHODS

Sampling. Samples of alheiras were collected from retail establishments, and the products sampled represented either (i) bulk product, (ii) vacuum-packed product, or (iii) product with modified atmosphere packaging (MAP) (Table 1). The samples collected represented products from 13 different processors located in the northern region of Portugal. For 10 processors, samples were collected on two separate dates; for the remaining 3 processors, samples were collected on only a single date. On each sample collection date, six different product samples were collected for a given processor. All samples were transported to the laboratory in chilled, insulated boxes and then stored at 4°C until analysis, which was performed within 48 h of collection. As some *L. monocytogenes* growth may occur during this storage period (9), even though products with pH values similar to that of alheiras (i.e., summer sausage) showed the least increase in *L. monocytogenes* numbers during storage (9), quantitative data on *L. monocytogenes* contamination in the sampled product may not reflect bacterial numbers at the retail stage. The *L. monocytogenes* numbers determined are deemed to be relevant though, as they likely reflect numbers that may be present in alheiras at the time of preparation by consumers.

***L. monocytogenes* isolation and enumeration.** For testing, six alheiras samples collected from a given processor on a given date were used to generate two composite 25-g samples (each composite sample contained material from all six alheiras samples). Composite samples were tested using the five-tube most-probable-number (MPN) technique for enumerating bacterial pathogens in foods as described by the U.S. FDA (6). The media used for the MPN technique were those detailed in the International Organization for Standardization (ISO) standard procedure ISO 11290-1 for *L. monocytogenes* detection (17), except that only a single selective medium, i.e., polymyxin-acriflavine-LiCl-ceftazidime-esculin-mannitol (PALCAM) agar, was used for plating. All media were obtained from Merck (Darmstadt, Germany). Briefly, 25 g of composite alheiras samples was homogenized in 225 ml of half Fraser selective broth for 2 min using a Stomacher laboratory blender (Seward, Norfolk, United Kingdom). A 10-ml aliquot of this homogenate was inoculated into each of five tubes with 10 ml of half Fraser selective broth. Two additional five-tube sets of half Fraser selective broth were inoculated with 1 ml and 0.1 ml of the initial homogenate. After the tubes were incubated at 30°C for 24 h, 0.1 ml was transferred from each tube of half Fraser selective broth to a tube containing 10 ml of Fraser selective broth with subsequent incubation at 37°C for 48 h. Each tube of Fraser selective broth was streaked onto PALCAM agar, which was incubated at 37°C for 48 h. Five *Listeria*-like colonies were subcultured on tryptone soy agar plates supplemented with 0.6% yeast extract and confirmed as *L. monocytogenes* by standard procedures, including selected sugar fermentation tests and the CAMP test. The MPN index was calculated using the FDA MPN table available online (http://www.cfsan.fda.gov/~ebam/bam-a2.html#tables).

Due to logistical constraints (i.e., the availability of appropriate supplies), only a subset of samples was also tested by two other methods. Specifically, for 36 out of 46 composite samples, *L. monocytogenes* detection was also performed using the VIDAS automated immunoassay system (bioMérieux, Marcy l'Etoile, France). Briefly, the initial homogenate (25 g of composite alheiras sample in 225 ml of half Fraser selective broth) that was used for MPN determinations was incubated at 30°C for 24 h. Subsequently, 0.1 ml of this broth was transferred to 10 ml Fraser selective broth. After an incubation at 37°C for 24 h, 0.5 ml of the Fraser selective broth was inoculated into VIDAS strips. For all samples positive by VIDAS, one loop of the Fraser selective broth was streaked onto PALCAM agar and five presumptive *L. monocytogenes* colonies were confirmed as described above. In addition, for 26 of the 46 composite samples, *L. monocytogenes* enumeration was also performed according to ISO 11290-2 (18). The initial homogenate was incubated at 20°C for 1 h for resuscitation, and subsequently, the homogenates and serial tenfold dilutions were surface plated in duplicate onto PALCAM plates, which were incubated at 37°C for 48 h. Presumptive *L. monocytogenes* colonies were confirmed as described above. These methods are detailed here, as some of the isolates obtained by these methods were used for subtyping (see Tables S1 and S3 in the supplemental material).

Isolate information. All isolates were stored frozen at −80°C. Isolate information and subtyping data from this study are archived and freely available through the Pathogen Tracker 2.0 database (http://www.pathogentracker.net).

Resistance typing and molecular serotyping. Overall, 128 *L. monocytogenes* isolates were characterized by subtyping (see Table S1 in the supplemental material). For composite samples that yielded only a few confirmed *L. monocy-*

TABLE 2. Characteristics of *L. monocytogenes* isolates from alheiras that show attenuated invasion in Caco-2 cells

Isolate no.[a]	Molecular serotype[b]	Ribotype	PFGE type	Processor no.	Avg no. of intracellular bacteria (%)[c]	PMSC mutation type[d]	Nucleotide location of PMSC mutation	Type of mutation	Length of predicted protein (aa)[e]	Reference or source for PMSC
F7-001	1/2c or 3c	DUP-1039C	341	1	6.4	8	1637	Deletion of A	576	21
F7-002	1/2a or 3a	DUP-1062D	342	1	11.5	6	1474	C → T substitution	491	35
F7-010	1/2a or 3a	DUP-1062D	342	3	15.2	6	1474	C → T substitution	491	35
F7-061	1/2a or 3a	DUP-1039C	356	7	13.0	4	12	Deletion of A	8	R. Orsi and M. Wiedmann
F7-089	1/2c or 3c	DUP-1039C	361	10	18.3	8	1637	Deletion of A	576	21
F7-094	1/2c or 3c	DUP-1039C	361	10	17.4	8	1637	Deletion of A	576	21
F7-127	1/2a or 3a	DUP-1053E	365	12	18.1	6	1474	C → T substitution	491	35

[a] Full isolate numbers carry the prefix FSL (Food Safety Laboratory), e.g., FSL F7-001.
[b] Molecular serotypes were determined as reported by Doumith et al. (4).
[c] Average intracellular bacterial numbers for a given isolate are relative to average intracellular bacterial numbers for the laboratory control strain 10403S.
[d] The assignment of numbers to PMSC mutation types is not sequential for consistency with a previous study (32).
[e] aa, amino acids.

togenes colonies, all isolates obtained by the different detection methods (MPN method, VIDAS, or ISO) were characterized by subtyping. For all other positive samples, approximately 10% of confirmed *L. monocytogenes* isolates from a given sample (i.e., 1 to 15 isolates per composite sample, yielding 2 to 25 isolates per processor) were selected for subtyping.

Molecular serotyping was performed using a previously described multiplex PCR assay (4) that detects serotype-specific marker genes. This assay differentiates five *L. monocytogenes* subtypes and classifies the four most common disease-associated serotypes (i.e., 1/2a, 1/2b, 1/2c, and 4b) into unique subgroups. Each multiplex PCR profile (A to E) contains *L. monocytogenes* isolates belonging to more than one serotype, including profiles A (serotypes 1/2a and 3a), B (serotypes 1/2b, 3b, and 7), C (serotypes 1/2c and 3c), D (serotypes 4b, 4d, and 4e), and E (serotypes 4a and 4c) as noted previously (4). All isolates were also characterized for resistance to cadmium and arsenic (29) and to tetracycline (46) using previously reported methods (29, 46).

PFGE. Pulsed-field gel electrophoresis (PFGE) of all 128 isolates characterized by resistance typing and molecular serotyping was performed according to the standard PulseNet protocol of the CDC (13) using a CHEF Mapper XA (Bio-Rad Laboratories, Hercules, CA). XbaI-digested *Salmonella enterica* serovar Braenderup (CDCH9812) DNA was used as a reference size standard (16). PFGE pattern images were acquired using a Bio-Rad Gel Doc with Multi-Analyst software (version 1.1) (Bio-Rad Laboratories) and compared using the Applied Maths BioNumerics version 3.5 software package (Applied Maths, Sint-Martens-Latem, Belgium). Similarity clustering analyses were performed with BioNumerics using the unweighted pairs group matching algorithm and the Dice correlation coefficient with a tolerance of 1.5% and an optimization of 1.5%. Combined PFGE types (based on both ApaI and AscI patterns) were given a four-digit sequential number (e.g., 0366).

Simpson's index of discrimination. Molecular diversity and the suitability of typing methods for the differentiation of strains within independent populations were determined using Simpson's numerical index (*D*) as described by Hunter and Gaston (15). Values for *D* range between 0 and 1, with a value of 1 indicating the most diverse population and thus the most discriminatory subtyping method.

Caco-2 invasion assays. For each processor, one isolate representing a given typing profile (i.e., a combination of molecular serotypes and resistance types) was selected for characterization using a Caco-2 invasion assay, yielding a total of 21 isolates (as the same typing profiles were sometimes found in products from two to five processors) that were tested for invasion. Caco-2 invasion assays were performed as previously described (32) with minor modifications. The tumor-derived human colorectal epithelial cell line Caco-2 (ATCC HTB-37) was grown using Dulbecco's minimal essential medium (DMEM) with Earle's salts (Gibco, Gaithersburg, MD) containing 20% fetal bovine serum (HyClone, Salt Lake City, UT), 1% nonessential amino acids, 1% sodium pyruvate, 1.5 g/liter sodium carbonate (Gibco), penicillin G (100 U/ml), and streptomycin (100 μg/ml). For invasion assays, 5.0×10^4 Caco-2 cells were seeded into 24-well plates (Costar, Corning, NY) in DMEM with fetal bovine serum, but without antibiotics. After Caco-2 cells were subsequently grown for 48 h at 37°C, Caco-2 monolayers were inoculated with approximately 2×10^7 *L. monocytogenes* CFU/well. *L. monocytogenes* isolates were grown in brain heart infusion at 30°C for 18 h without shaking prior to inoculation. Inoculated Caco-2 monolayers were incubated at 37°C for 30 min, followed by three washes with 1 ml sterile phosphate-buffered saline to remove any unattached, extracellular *L. monocytogenes*. Subsequently, 1 ml of prewarmed fresh DMEM (prepared as described above, but without antibiotics) was added. At 45 min postinoculation, the medium was aspirated and 1 ml of fresh Caco-2 medium containing 150 μg/ml gentamicin (Gibco) was added to kill remaining extracellular bacteria. At 90 min postinoculation, the medium was aspirated and Caco-2 cells were lysed by the addition of 500 μl of ice-cold sterile distilled water and vigorous pipetting. After cell lysis, intracellular *L. monocytogenes* cells were enumerated by plating (with an automated spiral plater [Spiral Biotech, Norwood, MA]) on brain heart infusion agar plates, which were subsequently incubated at 37°C for 24 h.

A standard laboratory control strain (10403S) was included as a control in each invasion assay. Four independent invasion assays were performed for each *L. monocytogenes* isolate tested. For the first assay, *L. monocytogenes* populations used for the inoculation of Caco-2 cells were enumerated. Because the bacterial numbers in the cultures were very consistent between strains, the inoculum in the subsequent experiments was verified only by using readings of the optical density at 600 nm. For each experiment, invasion efficiencies for different isolates were reported as intracellular bacterial numbers recovered from each well (at 90 min postinfection) relative to recovered numbers for 10403S.

Automated ribotyping. *L. monocytogenes* isolates that had reduced Caco-2 invasion efficiencies were characterized by automated EcoRI ribotyping using the RiboPrinter microbial characterization system (DuPont Qualicon, Wilmington, DE) as previously described (43). Ribotype patterns were analyzed using the RiboPrinter software, which normalizes fragment pattern data for band intensity and relative band position compared to those of the molecular weight marker. The RiboPrinter automatically assigns to each ribotype pattern a DuPont identification number (e.g., DUP-1039), which was confirmed by visual inspection. If visual inspection revealed that a given DuPont identification number included more than one distinct ribotype pattern, which generally differed by the position of only one weak band, then each pattern was designated with an additional alphabetized letter (e.g., DUP-1039A and DUP-1039B) (43).

***inlA* sequencing.** For seven *L. monocytogenes* isolates that showed reduced invasion efficiencies in Caco-2 cells (a less than 20% average invasion efficiency relative to 10403S [Table 2; see Table S1 in the supplemental material]), we initially sequenced approximately 800 nucleotides (nt) at the 5′ end of *inlA* using PCR amplification and sequencing with primers *inlA pro F* and *inlA pro R* (see Table S2 in the supplemental material; R. Orsi and M. Wiedmann, unpublished data). As no premature stop codons were found in the 5′ end of *inlA* in six *L. monocytogenes* isolates, the entire *inlA* open reading frame (except for 2 to 26 nt at the 3′ end for four isolates) was sequenced for these isolates by using additional sets of primers (see Table S2 in the supplemental material) (32). Nucleotide sequences were proofread and aligned with SeqMan and MegAlign (part of the DNAStar software package; Lasergene, Madison, WI), respectively.

Nucleotide sequence accession numbers. *inlA* sequences have been deposited in GenBank under accession numbers EF195259 to EF195265.

RESULTS

***L. monocytogenes* prevalence and levels.** *L. monocytogenes* was isolated from composite samples from 12 processors (Ta-

TABLE 3. *L. monocytogenes* prevalence by alheiras packaging type

Packaging type	No. of samples tested	No. of samples (%)	
		Positive for *L. monocytogenes*	With MPN of >100 *L. monocytogenes* cells/g
Bulk	20	10 (50)	6 (30)
Vacuum	14	9 (64)	6 (43)
MAP	12	9 (75)	2 (17)

ble 1); all four composite samples for processor 13 were negative for *L. monocytogenes*. The number of composite samples contaminated with *L. monocytogenes* ranged from one to four per processor; for processors 7, 10, and 11, all four composite samples were positive. *L. monocytogenes* numbers (as determined by the MPN method) ranged from 0.18 to >240 cells/g. Overall, 14 of 46 composite samples (30%) were found to have an MPN of >100 *L. monocytogenes* cells/g. For processor 7, all four composite samples showed MPNs of >240 *L. monocytogenes* cells/g. While MAP and vacuum-packaged products showed the highest prevalence of *L. monocytogenes*-positive samples, with vacuum-packaged samples showing the highest prevalence of samples with MPNs of >100 *L. monocytogenes* cells/g (Table 3), the prevalence of positive samples and samples with MPNs of >100 *L. monocytogenes* cells/g did not differ significantly ($P > 0.05$ by chi-square test) between categories.

***L. monocytogenes* subtype diversity and discriminatory power of resistance typing, molecular serotyping, and PFGE.** A total of 128 *L. monocytogenes* isolates from 12 processors were characterized by molecular subtyping. Molecular serotyping by multiplex PCR and combined cadmium, arsenic, and tetracycline resistance typing differentiated these 128 isolates into four and six distinct groups, respectively. Simpson's index (D) values for molecular serotyping and for resistance typing were 0.74 and 0.66, respectively. The combination of molecular serotyping and resistance typing data yielded 12 typing profiles (representing unique combinations of molecular serotypes and arsenic, cadmium, and tetracycline resistance profiles) with increased discriminatory power ($D = 0.87$) over either method alone.

PFGE differentiated the 128 isolates into 21 ApaI and 18 AscI restriction patterns that yielded 26 combined ApaI and AscI PFGE types (Fig. 1). Simpson's index further confirmed that PFGE was considerably more discriminatory ($D = 0.94$) compared to molecular serotyping and resistance typing. ApaI PFGE showed higher discriminatory power ($D = 0.93$) than did AscI PFGE ($D = 0.89$). The combination of molecular serotyping, resistance typing, and PFGE typing data yielded 28 types ($D = 0.94$) compared to 26 PFGE types ($D = 0.94$), indicating that the use of these other methods in addition to PFGE provides for a limited increase in discriminatory power. The number of PFGE types found among isolates from one composite sample ranged from one to five (Table 1).

Despite the large number of PFGE types (26) encountered among the alheiras isolates, seven PFGE types (345, 347, 349, 353, 358, 360, and 361) represented the majority ($n = 75$; 58.6%) of the *L. monocytogenes* isolates characterized. PFGE type 345 was the most common (18 isolates; 14.1%), followed by types 361 (16 isolates; 12.5%) and 353 (10 isolates; 7.8%). Most of the PFGE types (20 out of 26) were represented by multiple isolates (2 to 18); 16 PFGE types were found in multiple composite samples. Four PFGE types (342, 344, 353, and 358) were found in composite samples from two processors; no given PFGE types were found among composite samples from more than two processors. Among the composite samples from the three processors that were collected at the same retail establishment (processors 1, 2, and 3), composite samples from two processors yielded the same PFGE type (PFGE type 342, processors 1 and 3), but the positive alheiras samples with this PFGE type were MAP packaged, thus making cross-contamination at retail unlikely.

A total of 24 PFGE types were unique among the isolates characterized in this study compared to PFGE patterns for 539 *L. monocytogenes* isolates that represent 495 isolates from the northeastern United States (predominantly New York) (8) and 44 isolates in a diversity collection, which includes isolates from selected outbreaks (7). PFGE type 130, which was represented by six isolates characterized in this study, had previously been assigned to one human clinical isolate as well as to one isolate from an urban environment, both collected in New York State (8). PFGE type 197, which was represented by four isolates characterized in this study, had previously been assigned to one isolate obtained from an RTE meat product (ham) in New York State.

Subtype patterns in composite samples from different processors. A total of 11 composite samples contained multiple PFGE types (i.e., between two and five PFGE types per composite sample) (Table 1). In contrast, for nine composite samples that had multiple isolates characterized by PFGE, all isolates showed the same PFGE type (Table 1).

When considering all composite samples tested from a given processor, products from a total of eight processors yielded multiple PFGE types with two to five different PFGE types per processor. While multiple PFGE patterns found in products from a given processor were usually clearly distinct (differences of more than three bands), products from four processors included isolates with closely related PFGE patterns (differences of three bands or less). For example, composite samples from processor 11 yielded isolates exhibiting two closely related restriction patterns (358 and 362); the ApaI patterns of these isolates differ by only two bands (i.e., the presence of only a 400-kb band in type 358 and the presence of only a 375-kb band in type 362). For products from four processors (2, 4, 9, and 10), all isolates had the same PFGE pattern.

For four processors (7, 8, 10, and 11), isolates with the same PFGE type were found in composite samples obtained on both sampling dates; the time between the two sampling dates ranged from 6 to 7 weeks (Table 1); for two of these processors, all samples collected were vacuum or MAP packaged, suggesting that contamination did not occur at the retail stage or during distribution (Table 1). These findings indicate the persistence of a given PFGE type in the production chain of alheiras produced by these four processors. Processor 7 (persistent PFGE type 356) showed high levels of *L. monocytogenes* (MPN of >240 *L. monocytogenes* cells/g) in all four composite samples. The other three processors with evidence for persistence showed high levels of *L. monocytogenes* (MPN > 240 *L. monocytogenes* cells/g) in composite samples collected on sampling date 1, while levels in composite samples from sampling date 2 were lower (MPN ≤ 5 *L. monocytogenes* cells/g), pos-

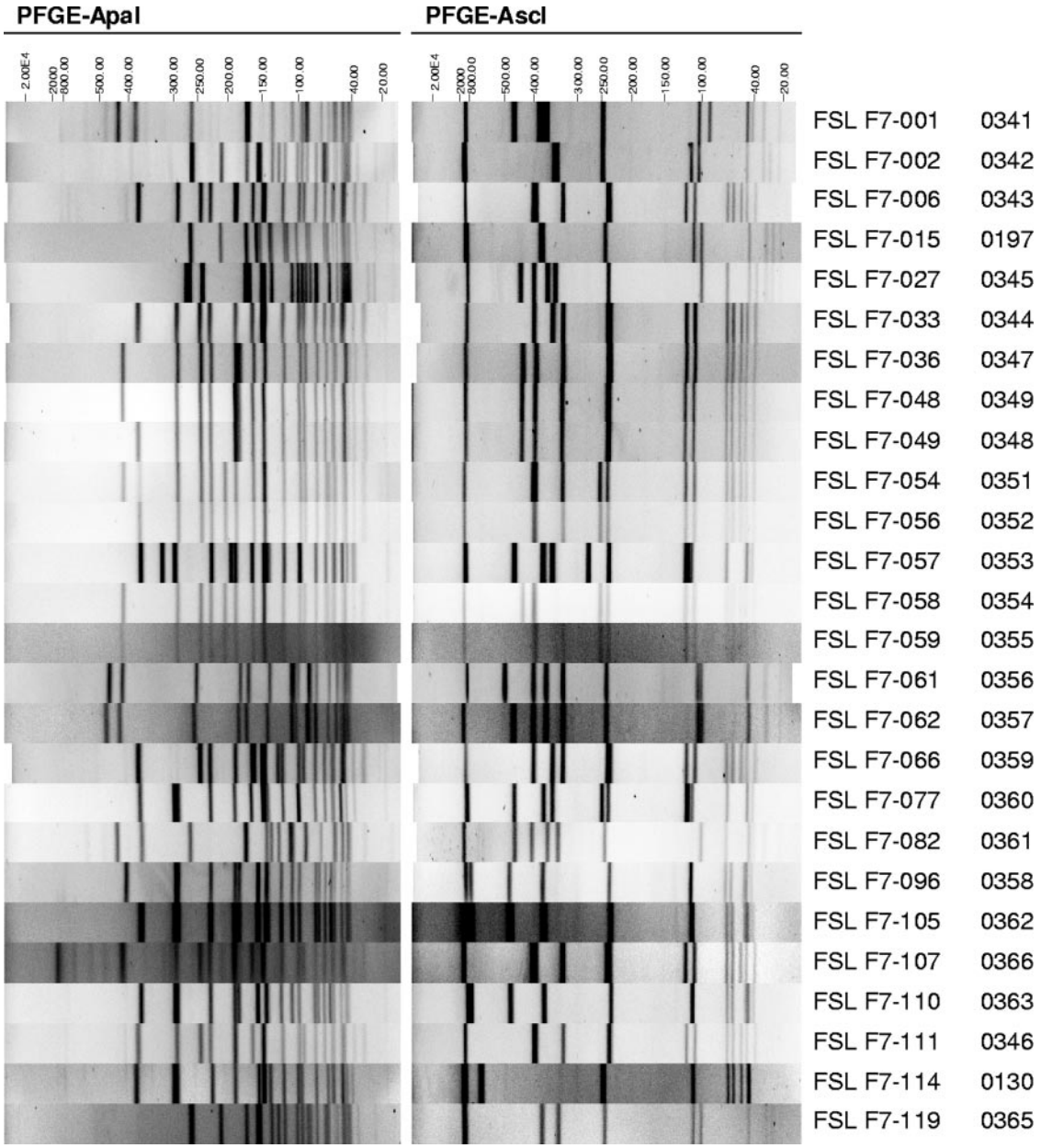

FIG. 1. ApaI and AscI PFGE restriction patterns for all 26 combined PFGE types found among *L. monocytogenes* isolates from alheiras. ApaI and AscI restriction patterns are shown in the left and right columns, respectively. Numbers on the right side are isolate numbers (e.g., FSL F7-001) and combined PFGE pattern designations (e.g., 0341).

sibly because processors took corrective actions after being informed of the results from sampling date 1.

Invasion capability of selected *L. monocytogenes* subtypes, ribotyping, and *inlA* DNA sequencing. We compared the abilities to invade Caco-2 cells of 21 *L. monocytogenes* isolates from alheiras representing each typing profile observed per processor; isolates were considered to have the same typing profiles if they had the same molecular serotype and cadmium/arsenic/tetracycline resistance type. The 21 isolates showed average intracellular bacterial numbers (i.e., invasion efficiencies) relative to the control strain 10403S ranging from 6.4 to 393.5% (Fig. 2). Natural food isolates with reduced invasion efficiency in Caco-2 cells have previously been shown to carry mutations in *inlA* that lead to premature stop codons (PMSCs), which appear responsible for reduced invasiveness (20, 21, 32, 33, 35, 40). Therefore, we screened for *inlA* PMSC mutations in the seven *L. monocytogenes* isolates from alheiras showing relative invasion efficiencies of <20%, indicating a reduced invasion phenotype in vitro (Table 2). These seven *L. monocytogenes* isolates belonged to five different PFGE types (341, 342, 356, 361, and 365) and three ribotypes (DUP-1039C, DUP-1053E, and DUP-1062D).

Partial sequencing of the 5′ end of *inlA*, where a PMSC mutation had previously been reported for some DUP-1039C

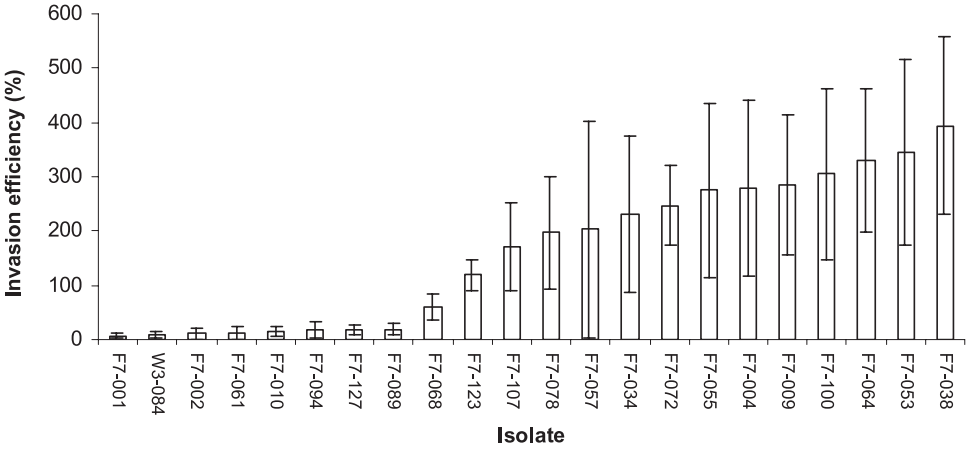

FIG. 2. Invasion efficiency in Caco-2 cells of 21 selected isolates from alheiras. In addition to the 21 alheiras isolates, strain FSL W3-084 (a Δ*inlA* mutant strain) is included as an invasion-attenuated control. Average intracellular bacterial numbers at 90 min postinoculation of Caco-2 cells (relative to standard laboratory control strain 10403S) are shown. Values shown are averages of four independent replicates; the error bars represent standard deviations.

isolates, revealed the presence of a previously described PMSC mutation (PMSC type 4 [Table 2]) (R. Orsi and M. Wiedmann, unpublished data) in one DUP-1039C isolate (FSL F7-061); PMSC type 4 represents the deletion of an adenine at nt 12, yielding a predicted truncated InlA protein of eight amino acids. Sequencing of the full-length *inlA* open reading frame for the remaining six isolates (except for 2 to 26 nt at the 3′ end of four isolates) revealed *inlA* PMSCs in all isolates (Table 2). PMSC type 6 (found in three isolates belonging to ribotypes DUP-1053E and DUP-1062D) represents the replacement of a cytosine by a thymine at nt 1474. This mutation was first described for isolates from France (35). PMSC type 8 (observed in three DUP-1039C isolates) represents the deletion of an adenine at nt 1637; this mutation has also previously been described for isolates from France (21). Interestingly, two PFGE types associated with PMSCs, type 356 (PMSC type 4) and type 361 (PMSC 8), showed evidence for persistence in the production/distribution chain of processors 7 and 10, respectively.

DISCUSSION

L. monocytogenes testing of alheiras, a traditional Portuguese meat product, including the subtype characterization of 128 *L. monocytogenes* isolates and tissue culture characterization of a subset of these isolates, showed (i) high *L. monocytogenes* prevalence, levels, and subtype diversity in alheiras; (ii) persistence of *L. monocytogenes* in the alheiras production and/or distribution chain, providing a particular challenge for *L. monocytogenes* control; and (iii) a subpopulation of *L. monocytogenes* isolates from alheiras exhibiting reduced invasion efficiency in Caco-2 cells, most likely due to different premature stop codon mutations in *inlA*. These findings indicate the need for a comprehensive approach to control *L. monocytogenes* in alheiras, including strategies to reduce *L. monocytogenes* contamination in general as well as to control *L. monocytogenes* persistence.

***L. monocytogenes* in alheiras is found at considerable incidence and levels and shows considerable PFGE diversity.** The observation that 28 of 46 composite alheiras samples were positive for *L. monocytogenes*, including 14 samples with *L. monocytogenes* MPNs of >100/g, indicates that increased efforts to control *L. monocytogenes* in this product are critical. Overall PFGE type diversity for the *L. monocytogenes* isolates from alheiras was higher than subtype diversity detected by other methods, consistent with previous reports that showed a high discriminatory power of PFGE for subtype characterization of *L. monocytogenes* (8, 43, 47). The use of PFGE or another highly discriminatory subtyping method rather than potentially cheaper less discriminatory methods is thus advisable when performing initial evaluations of *L. monocytogenes* contamination patterns in specific food products.

Interestingly, we observed considerable subtype diversity within a given composite sample (up to five PFGE types/composite sample). These findings are consistent with previous studies that also describe the presence of multiple *L. monocytogenes* subtypes within a single composite sample (22, 41). These findings indicate the importance of subtyping multiple isolates in certain situations, particularly when testing composite samples. For example, for samples collected from processing plants or sites within a plant that show a high prevalence and history of multiple subtypes, subtype characterization of multiple isolates may be valuable, while for plants or sites that rarely show *L. monocytogenes* contamination, the subtyping of a single isolate may generally be sufficient (as contamination in these cases is likely to represent a single incident and/or source).

PFGE typing indicates sporadic and persistent *L. monocytogenes* contamination in alheiras. Overall, PFGE data provided evidence that alheiras from some plants are characterized by sporadic (transient) *L. monocytogenes* contamination, as indicated by the isolation of multiple PFGE types and different PFGE types from composite samples collected on different dates. The presence of up to five different *L. monocytogenes* PFGE types in composite samples collected on a single date (e.g., processor 6) may suggest serious hygiene or sanitation problems, possibly including frequent cross-contamina-

tion. While raw meat used for alheira production is boiled for extended time periods, most likely representing an effective kill step for *L. monocytogenes*, some ingredients with presumably lower risks for the introduction of *L. monocytogenes* (e.g., bread and spices), are added after boiling and thus could be a source of *L. monocytogenes*. The most likely source of *L. monocytogenes* contamination in alheiras, though, is the processing plant environment, as a number of studies indicate that post-processing contamination from environmental sources in processing plants represents the main source of *L. monocytogenes* contamination of finished products, including that of foods that do not undergo a listericidal heat treatment (e.g., cold smoked salmon) (24).

PFGE also provided evidence for the persistence of specific *L. monocytogenes* subtypes in alheiras from four processors. For each of these processors, samples collected on two different dates yielded identical PFGE types, most likely indicating that these PFGE types survived in a given plant over time and regularly contaminated products. These findings are consistent with a considerable number of studies that have shown the persistence of specific *L. monocytogenes* subtypes in meat, poultry, smoked fish, and dairy processing plants (2, 24, 25, 27, 48), including persistence in one plant for at least 10 years (23). As we were not able to gain access to processing plants for the study reported here, it was not possible to further define the specific site(s) of persistence or the specific source of contamination. The persistence of specific subtypes associated with products from a given processing plant could thus have occurred at any point in the production chain, including the processing plant, the raw material supply, or, for bulk products, the retail stage. As a number of studies that have found persistent *L. monocytogenes* contamination in foods have been able to identify definitive or likely sites of persistence in the environment of the processing plant (24, 27, 28), these plants represent the most likely site of persistence. As all samples tested here had to be collected from retail operations, it is theoretically possible that at least some of the *L. monocytogenes* isolated from bulk alheira samples originated from the retail environment, particularly since *L. monocytogenes* persistence in the retail environments has been reported previously (44). In general, the contamination of foods at the retail stage is most likely to occur in products that are further processed (e.g., sliced or mixed) or frequently handled (10), which does not apply to alheiras, which are sold whole (i.e., not sliced) and exposed to limited handling. Thus, contamination at the retail stage is likely to have had limited, if any, contributions to *L. monocytogenes* contamination found in the alheiras sampled in this study, particularly since 26 of 46 samples were vacuum or MAP packaged.

A considerable number of *L. monocytogenes* isolates from alheiras show attenuated invasiveness in Caco-2 cells. A number of previous studies have suggested that *L. monocytogenes* strains may differ in virulence potential (14, 20) and that at least some *L. monocytogenes* strains isolated from foods may have a reduced likelihood to cause human disease. In particular, there is evidence that selected *L. monocytogenes* subtypes that are more common in foods than among human clinical cases show reduced abilities to invade the human intestinal epithelial Caco-2 cell line and thus may be virulence attenuated (20). Interestingly, some studies have identified specific mutations in the *L. monocytogenes* virulence gene *inlA* that appear to be at least partially responsible for the reduced virulence potential of these strains (34, 36, 40). The surface protein InlA, encoded by *inlA*, is critical in the pathogenesis of human listeriosis (20, 26) as it facilitates the invasion of the intestinal epithelial cells, a critical step preceding systemic infection. Previous studies have identified at least 12 different naturally occurring *inlA* mutations that lead to the production of secreted and truncated InlA molecules in *L. monocytogenes* (21, 32, 34–36, 40). In this study, we observed that a subpopulation of *L. monocytogenes* isolated from alheiras also showed reduced ability to invade Caco-2 cells and possessed PMSC mutations in *inlA*, which are predicted to lead to the production of secreted and truncated InlA. Interestingly, alheiras from two plants showed persistence as well as a high number of *L. monocytogenes* isolates with PMSC mutations, consistent with a previous report which indicated that *L. monocytogenes* subtypes with PMSCs are generally found at higher numbers in foods compared to other *L. monocytogenes* subtypes without these mutations (3).

While *L. monocytogenes* isolates with *inlA* PMSC mutations have so far been reported only for isolates collected in France (21, 34, 35, 40) and the United States (32), the isolation of *L. monocytogenes* with *inlA* PMSC mutations in an indigenous product produced in Portugal further suggests that these types of virulence-attenuated *L. monocytogenes* strains are widely distributed. The identification and characterization of these virulence-attenuated *L. monocytogenes* and their ecology and distribution will not only be critical for an accurate assessment of the human health hazard associated with *L. monocytogenes*-contaminated foods but may also provide an opportunity to focus control strategies and efforts on products found to contain those strains most likely to cause human disease, even though policies based on the precautionary principle may consider all *L. monocytogenes* strains an equal human health risk.

Conclusions. While *L. monocytogenes* has been found in a variety of foods, including RTE deli meats, dairy products, and smoked and other RTE fish products, efforts to control *L. monocytogenes* are often focused on a few products that are most commonly linked to human listeriosis. For example, a risk assessment conducted in the United States by the FDA, USDA, and CDC (45) identified RTE deli meat products as the most common cause of human listeriosis cases in the United States (estimated to cause 1,599 human cases/year), followed by pasteurized milk; however, risk assessments may underestimate the public health hazards associated with regional foods, which are often not included in these assessments, as limited or no data on *L. monocytogenes* prevalence or levels are usually available for regional foods. Increased efforts for understanding *L. monocytogenes* contamination patterns in previously understudied foods are thus needed to reduce levels of human listeriosis and improve food attribution for this important food-borne disease. Our study of alheiras, a traditional Portuguese smoked meat product, highlights the challenges associated with studies of regional foods and may serve as a road map for future studies of other regional and locally distributed traditional and artisanal products, which often develop from homemade products into industrially produced foods with, at first, limited food safety controls. In our study, six of the nine processing plants for which samples were col-

lected twice showed *L. monocytogenes* MPNs of >240/g in their products in the first sampling. Only one of these plants showed MPNs of >240/g in the second sampling (after plants had been notified of the results of the first sampling), indicating improvements which may already have a positive public health impact. Even though samples could not be collected inside the plants, the use of molecular subtyping methods provided important initial information about *L. monocytogenes* contamination patterns. As the alheira processing plants included in this study have started to permit environmental sampling of the facilities, likely as a consequence of this study, continued application of subtyping methods to characterize *L. monocytogenes* from different samples (e.g., raw materials, partially processed sausages, and ingredients) will facilitate the identification of contamination sources.

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