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Putative Cross-Contamination Routes of *Listeria monocytogenes* in a Meat Processing Facility in Romania

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ABSTRACT

Putative routes of *Listeria monocytogenes* contamination, based on the workflow of the employees, were studied in a meat processing facility by investigating 226 samples collected from food contact surfaces, non–food contact surfaces, raw materials, and ready-to-eat meat products on four occasions over a 1-year period. In total, 19.7% of non–food contact surfaces, 22.9% of food contact surfaces, 45% of raw materials, and 20% of ready-to-eat meat products were positive for *L. monocytogenes* (analyzed by the International Organization for Standardization standard method ISO 11290). Pulsed-field gel electrophoresis (PFGE) profiles were determined for a representative subset of these isolates, and 11 distinct pulsotypes were identified, two of which were frequently isolated (T4 and T8) and considered persistent. Strains from the various pulsotypes were screened for the presence of *bcrABC* and *qacH,* the genes responsible for tolerance responses to quaternary ammonium compounds. Two strains harbored *bcrABC*, and these strains had a higher benzalkonium chloride tolerance; however, they were not considered persistent strains. The frequently isolated PFGE pulsotype T8 strains were highly adhesive to abiotic surfaces at 10 and 20°C; however, the pulsotype T6 strain, which was isolated only at the last sampling time, had the highest adhesion ability, and the pulsotype T4 strain (the second most persistent pulsotype) had only modest adhesion. Four putative cross-contamination routes were confirmed by mapping the persistent and other isolates. This information could allow a food safety manager to adjust the work flow to improve the hygienic conditions in a meat processing facility. This study revealed the prevalence and persistence of *L. monocytogenes* strains in a meat processing facility and established the importance of developing strategies to avoid cross-contamination, recalls, and outbreaks of listeriosis.

The foodborne pathogen *Listeria monocytogenes* may causes listeriosis, an infrequent but problematic disease characterized by a mortality rate of 20 to 30% *(29)* and a hospitalization rate greater than 90% *(25, 41)*. Recently, an increase (10.5%) of recorded listeriosis cases was observed in the European Union (EU), with a case fatality rate of 17.8% *(16)*. To protect public health, regulations have been developed related to the occurrence of *L. monocytogenes* in food. In Europe, for foods that do not support growth of *L. monocytogenes* these regulations allow less than 100 CFU/g during the shelf life of the food. For food that supports growth of *L. monocytogenes,* no amount of this pathogen is allowed in five samples of 25 g each unless the food business operator can demonstrate that the *L. monocytogenes* level will not exceed 100 CFU/g during the shelf life of the food *(15)*. Absence of *L. monocytogenes* in 10 samples of 25 g each is required for food for infants and special diets. These regulations are particularly relevant to producers of ready-to-eat (RTE) food products, because additional heat treatment is not required before consumption. According to article 5 of the EU regulation 2073/2005 *(15),* testing of the food processing environment for the presence of *L. monocytogenes* is required wherever RTE food products are produced. However, the regulations do not stipulate the sampling sites, the frequency and the timing of sampling, or the analysis method. Many national food safety authorities via the Rapid Alert System for Food and Feed of the European Commission have reported recalls of RTE meat products such as salami, ham, sausages, pastrami, RTE chicken products, and even RTE meat offal due to *L. monocytogenes* contamination.

The heat treatment applied for preservation of meat products is normally sufficient to eliminate *L. monocytogenes,* but contamination with this pathogen often occurs after the heat treatment, mainly during storage, handling, and slicing *(21)*. In various studies, the main source of contamination has been cross-contamination between the food product and the industrial environments, in which established *L. monocytogenes* strains are difficult to eradicate *(27, 37)*. The biological status of the bacteria,

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the nature and intensity of the contact, the structure of the surface, and the type of product are factors directly responsible for transfer dynamics *(23)*.

L. monocytogenes may persist for months or even years in food processing environments, which increases the risk of (re)contamination of food products and is a major challenge for food safety *(18, 28)*. Persistence has many definitions *(6)* but is loosely defined as repeated isolation through space and time of strains from the same facility that are indistinguishable by molecular methods. The underlying mechanism(s) of persistence are still unknown *(6),* but persistence is important in cross-contamination and recontamination in the processing environment. The challenge lies in determining whether persistence is the result of strain adaptation (due to poor cleaning and disinfection or the residues of incorrectly diluted disinfectants) or is genetically determined (e.g., due to the presence of biocide resistance determinants). Recently, the *bcrABC* cassette and the transporter *qacH,* harbored by the transposon Tn*6188,* have been linked to higher tolerance of *L. monocytogenes* to benzalkonium chloride (BC) and other quaternary ammonium compounds *(13, 31)*. Genes linked to stress responses, such as the stress survival islet (SSI-1), could also influence survival in a food processing environment *(22, 39)*.

L. monocytogenes has frequently been isolated from conveyor belts, floor gutters, and storage tanks, particularly in areas such as dead ends, corner cracks, and crevices where bacteria can adhere and form biofilms *(3, 40)*. *L. monocytogenes* cells in a biofilm are more resistant to sanitizers and disinfectants than are planktonic cells *(3, 34)*. Increased tolerance of *L. monocytogenes* strains to disinfectants such as quaternary ammonium compounds has recently been reported too *(8)*. Recommended disinfectant concentrations will inactivate these strains, but in areas where there could be dilution of the disinfectant or in cracks and crevices that are difficult to clean adequately tolerance may be relevant.

Considering the risk of contamination in the meat processing industry, possible routes of cross-contamination allowing *Listeria* to spread in food processing facilities should be investigated carefully *(11, 27)*. Putative cross-contamination from the processing environment is an important source of *L. monocytogenes* in dairy products *(30)*.

The aim of the present study was to determine putative routes of cross-contamination of *L. monocytogenes* in a meat processing facility and the factors that contribute to prevalence and persistence. This study was performed in a Romanian medium-size meat product company producing both RTE food and products requiring cooking.

MATERIALS AND METHODS

Sampling strategy. The meat processing facility in Romania had a production rate of 20 tons per day. The company had approximately 6 years of experience with the production of raw meat products, such as sausages and meat balls, and RTE food, including sausages, salami, and delicatessen meats. Its products, either vacuum packed or not vacuum packed, were made from pork, beef, chicken, and sheep meat. The production system of this facility was certified according to EN ISO standard 22000/2005 (International Organization for Standardization, Geneva). The samples for the official control of *L. monocytogenes* at the facility were collected by a representative of the facility and analyzed according to ISO 11290 *(24)* in an official laboratory of the Romanian Food Safety Authority. In the last 3 years before this study, about 1,000 samples of RTE products had been tested for the presence of *L. monocytogenes,* and none were positive. However, neither raw material samples nor food processing environment samples were tested for *L. monocytogenes* by the food business operator. This study was undertaken because, contrary to EU regulations, no processing environment samples had been analyzed and the company wished to address this gap in knowledge. Processing and RTE food samples were taken to determine putative transmission routes of *L. monocytogenes* if detected.

Samples were collected on four occasions between May 2012 and March 2013, with 2 to 3 months between each sampling occasion. Sampling covered the entire food processing environment, including the area for processing raw materials, delivery of unheated meat products, and portioning, packaging, and distribution of RTE meat products, targeting the sampling in areas where *L. monocytogenes* occurrence was likely. At each visit, 55 to 58 samples were collected. The main focus of the sampling was the processing environment; 20 or 21 samples were collected from non–food contact surfaces (NFCS; floors, walls, drains, hand washing devices, and door gaskets), 20 to 22 samples were collected from food contact surfaces (FCS; conveyors, belts, tables, slicers, grinders, and knives), 5 samples were collected from raw materials (raw meat and brine), and 10 samples were collected from RTE meat products (vacuum packed and not vacuum packed) (Table 1). For environmental samples, where possible, an area of 900 cm^2 was swabbed with sterile sponges (3M, St. Paul, MN) using a meandering motion; when a 900-cm^2 could not be collected (e.g., knives and slicers), the maximum area possible was swabbed. For liquid samples (brine), 300 ml was collected aseptically and transported to the laboratory in sterile bottles. For raw materials and RTE meat products, 300 to 400 g of each was aseptically collected in sterile bags using sterilized tools. All samples were collected during or at the end of the production day, before cleaning and sanitization of the processing environment or equipment. Gloves and protective clothes were worn during sampling to prevent cross-contamination. Samples were individually packaged, transported to the laboratory in cooled containers (4°C), and analyzed within 12 h.

***L. monocytogenes* isolation by ISO 11290.** The ISO 11290-1:1996/Amd 1:2004 method *(24)* was used for detection and isolation of *L. monocytogenes*. Two enrichment steps were performed (in half-Fraser and full-Fraser broth, Merck KgA, Darmstadt, Germany). After each enrichment step, 20 μl of enriched culture was spread on ALOA (Merck) and PALCAM (Biolife Italiana, Milano, Italy) agar plates, which were then incubated for 48 h at 37°C. Five characteristic colonies (i.e., blue-green colonies with a halo on ALOA or gray-green colonies with a black halo on PALCAM) were confirmed as *L. monocytogenes* by real-time PCR (rt-PCR) *(33)*.

***L. monocytogenes* PCR confirmation.** For the rt-PCR, the DNA extraction procedure was based on a protocol adapted from Oravcová et al. *(33)* and Amagliani et al. *(2)*. A single colony was suspended in 1 ml of phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Merck) and incubated at 95°C for 25 min and then held for 15 min at −20°C. After centrifugation at 18,600 × *g* for 15 min, 5 μl of the supernatant was used as template for the

TABLE 1. *Prevalence of* L. monocytogenes *on food contact surfaces (FCS), on non–food contact surfaces (NFCS), in raw materials, and in ready-to-eat (RTE) meat products determined at four sampling events*

Sample type	Event I		Event II		Event III		Event IV	
	Total no. of samples (no. positive)	Strain(s) tested	Total no. of samples (no. positive)	Strain(s) tested	Total no. of samples (no. positive)	Strain(s) tested	Total no. of samples (no. positive)	Strain(s) tested
FCS								
Knives	1 (0)		1 (1)	AB56	1 (0)		1 (1)	AB124, AB129
Mincing machine	1 (0)		1 (1)	AB65	1 (0)		1 (0)	
Mixing machine	1 (0)		1 (0)		1 (0)		1 (0)	
Deriding machine	1 (1)	AB9	ND[a]		1 (1)		1 (1)	AB180
Bowl cutter	1 (0)		1 (1)	AB61	1 (1)	AB109	1 (0)	
Vacuum packaging machine	1 (1)	AB14	1 (1)	AB49	1 (0)		1 (1)	
Filling machine	2 (0)		1 (0)		1 (0)		2 (0)	
Slicing machine	1 (1)		1 (1)	AB50	1 (1)	AB107	1 (1)	AB167
Scales	ND		2 (1)		2 (1)	AB113	1 (0)	
Plastic boxes	1 (0)		1 (0)		1 (0)		1 (0)	
Stainless steel tables	5 (1)		6 (1)	AB40	6 (2)	AB120, AB116	6 (2)	AB156
Sticks for hanging the products	1 (0)		1 (1)	AB67	1 (0)		1 (0)	
Conveyor belts	1 (0)		1 (0)		1 (0)		1 (0)	
Cutting boards	1 (1)	AB9	1 (1)		1 (0)		1 (1)	AB160
Stainless steel trolley	2 (0)		2 (0)		2 (1)	AB126	2 (0)	
Total no.	20 (5)		21 (9)		22 (7)		22 (7)	
Prevalence (%)	25		42.8		31.8		31.8	
NFCS								
Walls	8 (1)	AB30	8 (0)		8 (1)		8 (1)	AB191
Floors	8 (2)	AB24, AB27	8 (3)	AB63, AB75, AB76	8 (3)		8 (3)	AB194, AB188
Drains	2 (0)		2 (0)		2 (1)	AB140	2 (1)	AB199
Hands washing device	2 (0)		1 (0)		1 (0)		2 (1)	AB204
Door gasket	ND		1 (0)		1 (0)		1 (0)	
Total no.	20 (3)		20 (3)		20 (4)		21 (6)	
Prevalence (%)	15		15		20		28.5	
Raw materials								
Beef	1 (0)		ND		1 (1)	AB152	1 (1)	
Pork	ND		1 (1)		1 (0)		1 (1)	AB220
Chicken	1 (1)	AB31	1 (0)		1 (1)		1 (1)	

TABLE 1. *Continued*

Sample type	Event I		Event II		Event III		Event IV	
	Total no. of samples (no. positive)	Strain(s) tested	Total no. of samples (no. positive)	Strain(s) tested	Total no. of samples (no. positive)	Strain(s) tested	Total no. of samples (no. positive)	Strain(s) tested
Sheep	ND		1 (1)	AB80	ND		ND	
Brine	3 (0)		2 (0)		2 (0)		2 (1)	AB207
Total no.	5 (1)		5 (2)		5 (2)		5 (4)	
Prevalence (%)	20		40		40		80	
RTE products								
Vacuum packed	2 (2)	AB39	4 (4)	AB90, AB92	4 (0)		4 (0)	
Not vacuum packed	8 (0)		6 (2)	AB100	6 (0)		6 (0)	
Total no.	10 (2)		10 (6)		10 (0)		10 (0)	
Prevalence (%)	20		60		0		0	
Grand total no.	55 (8)		56 (20)		57 (13)		58 (17)	
Grand prevalence (%)	14.5		35.7		22.8		29.3	

[a] ND, not determined.

rt-PCR. For all other PCRs, the DNA was isolated using the NucleoSpin Tissue Kit (Macherey-Nagel/VWR International GmbH, Vienna, Austria).

For rt-PCR, the FAM-Tamra probe was replaced with a FAM-BHQ1 probe *(33)*. The PCR was performed with a final volume of 20 μl containing 10 μl of NoRox Kit master mix (Bioline Reagents, London, UK), 0.8 μl of each primer at 400 nM (LMrt3F: 5′-3′; LMrt3Rbis: 5′-TAATTTCCGCTGCGCTATCCG-3′), 0.2 μl of probe at 100 nM (5′-FAM-CCTGGATGACGACGCTCCACTTG-BHQ1-3′), and 5 μl of DNA, made up to volume with distilled water. PCRs were performed in duplicate, with an internal amplification control system to avoid false-negative results, in an Opticon2 rt-PCR system (BioRad, Hercules, CA) using a program consisting of initial denaturation for 1 min at 95°C and 40 cycles of denaturation for 10 s at 94°C, annealing for 1 min at 60°C, and extension for 30 s at 72°C.

Identification of the putative cross-contamination routes. Putative cross-contamination routes were identified by observations of staff work flow during the working day when samples were collected. The observations were equally focused on potential inadequacies in the technological flow and worker movements. Before sampling and observing the process, the facility layout and technological process was presented in detail to the members of the sampling team.

Subtyping of *L. monocytogenes*. A multiplex PCR targeting the *Imo0737, Imo1118, ORF2819, ORF2110,* and *prs* genes was used to differentiate *L. monocytogenes* isolates into the five major subtypes: 1/2a and 3a; 1/2b, 3b, and 7a; 1/2c and 3c; 4b, 4d, and 4e; and 4a and 4c *(12)*. Forty-two of the strains were selected for further analysis (Table 1) based on representation of each sampling occasion, from FCS and NFCS, from different raw materials, and from RTE products. Pulsed-field gel electrophoresis (PFGE) of *L. monocytogenes* isolates was carried out using the International PulseNet protocol *(7)* with two restriction enzymes, *Asc*I and *Apa*I. Isolate similarity dendrograms were generated using Bionumerics version 5.10 software (Applied Maths, Sint-Martens-Latem, Belgium), using the unweighted pair group method with arithmetic mean and the Dice coefficient with tolerance and optimization settings of 1%, as previously described by Fox et al. *(19)*.

Screening for stress resistance genes in *L. monocytogenes*. The 42 *L. monocytogenes* isolates were screened for the presence of *qacH* and *bcrABC* according to Müller et al. *(31)* and Elhanafi et al. *(14)* and for the presence of the five-gene SSI-1 or other related inserts according to Hein et al. *(22)* using appropriate positive and negative controls.

Characterization of adhesion abilities. The adhesion ability of a representative isolate from each of the 11 pulsotypes was determined according to a combination of the methods of Djordjevic et al. *(10)* and Galvão et al. *(20)*. A 200-μl aliquot of an overnight culture of the diluted *L. monocytogenes* strains in brain heart infusion broth with yeast extract (BHIYE; optical density at 600 nm [OD_{600}] of 0.2; Oxoid, Basingstoke, UK) was transferred to a 96-well polystyrene microtiter plate with a U-shaped bottom (Corning, Sigma-Aldrich, St. Louis, MO) and incubated at 4, 10, and 20°C for 24 h.

The culture medium was discarded, and the wells were washed three times with 200 μl of PBS (pH 7.2, Merck) using a multichannel pipette to remove the unattached cells. The PBS was then removed from the wells with the multichannel pipette. Adhered cells were fixed for 15 min with methanol (Merck), air dried for 25 min, stained with

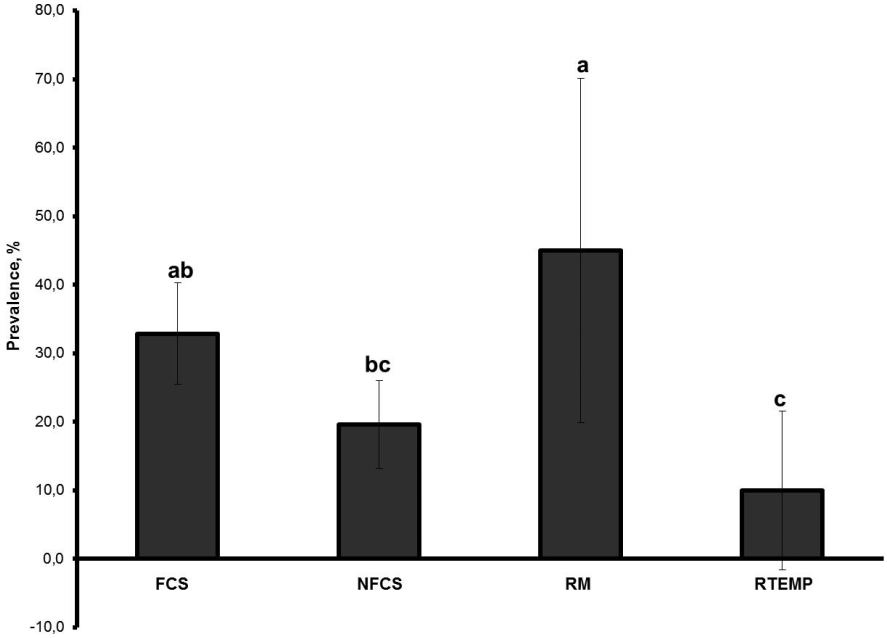

FIGURE 1. L. monocytogenes *prevalence in different categories of samples collected from the meat processing facility. Bars with different lowercase letters are significantly different (*$P < 0.05$*). Sample categories: FCS, food contact surface; NFCS, non–food contact surface; RM, raw materials; RTEMP, ready-to-eat meat products.*

crystal violet solution (1%, wt/vol) (Merck) for 15 min, washed with tap water, and dried again. After drying, 200 μl of 95% ethanol (Merck) was added to each well, and after 30 min the absorbance was measured at 595 nm (OD_{595}). Wells with BHIYE alone were used as negative controls. Each experiment was performed independently in duplicate with six replicates each. The mean OD was calculated (OD test) and compared with the mean OD of the negative controls (OD blank), which was adjusted (OD blank adj.) according to the following equation *(20)*: OD blank adj. = mean OD blank + 3(standard deviation of OD blank).

Each isolate was categorized according to the criteria of Galvão et al. *(20)* as follows: absence of adhesion, OD test ≤ OD blank adj.; weak adhesion, OD blank adj. < OD test ≤ 2 OD blank adj.; moderate adhesion, 2 OD blank adj. < OD test ≤ 4 OD blank adj.; strong adhesion, OD test > 4 OD blank adj.

Determination of MICs. MICs of BC (Sigma-Aldrich), peracetic acid (PA; Sigma-Aldrich), and hydrogen peroxide (H_2O_2; Sigma-Aldrich) were determined for 11 strains (1 strain from each pulsotype). *L. monocytogenes* cultures were grown on Muller-Hinton agar plates supplemented with 2.5% defibrinated sheep blood (Oxoid) as previously described by Müller et al. *(31)* with sanitizer concentrations of 0.0005, 0.001, 0.0015, 0.002, 0.003, and 0.004% (wt/vol) BC, 0.01, 0.02, 0.03, 0.04, and 0.05% (vol/vol) PA, and 0.01, 0.012, 0.014, 0.016, 0.018, 0.02, 0.022, 0.024, and 0.026% (vol/vol) H_2O_2. To define the MIC of PA more closely, 0.028% PA was also used. A 5-μl aliquot of overnight culture diluted in Muller-Hinton broth (Oxoid) (OD_{600} of 0.1, corresponding to 10^8 CFU/ml) was spotted in triplicate onto the agar plates. The MIC was defined as the lowest assessed BC, PA, or H_2O_2 concentration that prevented growth. Experiments were performed as three independent biological replicates.

Statistical analysis. For the prevalence and adhesion studies, means and standard deviations were calculated with Excel 2007 (Microsoft, Redmond, WA). To compare the means for prevalence and adhesion, a posthoc test (Fisher's least significant difference test) was performed using the statistical software SPSS.20 (IBM Corporation, Armonk, NY). Statistical significance was set at $P \leq 0.05$. For the MIC studies, the results were expressed as the midpoint of the interval tested ± half the range tested.

RESULTS

Prevalence of *L. monocytogenes* in the food processing environment, raw materials, and RTE meat products. During the four sampling events, a total of 226 samples were collected: 166 samples from the processing environment (FCS and NFCS), 20 from raw materials, and 40 from RTE meat products (Table 1). The highest prevalence of *L. monocytogenes*–positive samples was observed for raw materials (45%) followed by FCS (32.9%), RTE meat products (20%), and NFCS (19.7%). There was a significant difference ($P < 0.05$) in prevalence between the FCS and NFCS samples and between the raw material and RTE food samples (Fig. 1). Over the four sampling events, the prevalence in FCS samples varied, the prevalence in NFCS samples increased from 15 to 28.5%, the prevalence in raw material samples (including beef, pork, chicken, and sheep meat) increased from 20 to 80%, and the prevalence in RTE food samples decreased. The prevalence of *L. monocytogenes* in RTE products increased from 20 to 60% between sampling events I and II, but on the last two sampling occasions no positive samples were detected.

Subtyping of *L. monocytogenes* isolates. From the 61 *L. monocytogenes* isolates recovered, 42 were selected for further analyses; this group included isolates from different sources and from each sampling occasion. Multiplex PCR for serovar determination revealed that 83.3% of the isolates (35) belonged to serovar 1/2a, 3a and 16.7% (7) belonged to serovar 1/2c, 3c (Fig. 2). PFGE genotyping with the restriction enzymes *Asc*I and *Apa*I revealed 11 pulsotypes (T1 to T11; Fig. 2). Isolates of two pulsotypes, T4 and T8, were found on all sampling occasions, and isolates of pulsotypes T1 and T10 were present on three and two sampling occasions, respectively. In contrast, isolates of pulsotypes T2, T3, T5, T6, T7, T9, and T11 were found on only one sampling occasion. Because of the temporal distribution (over 12 months) and spatial distribution around

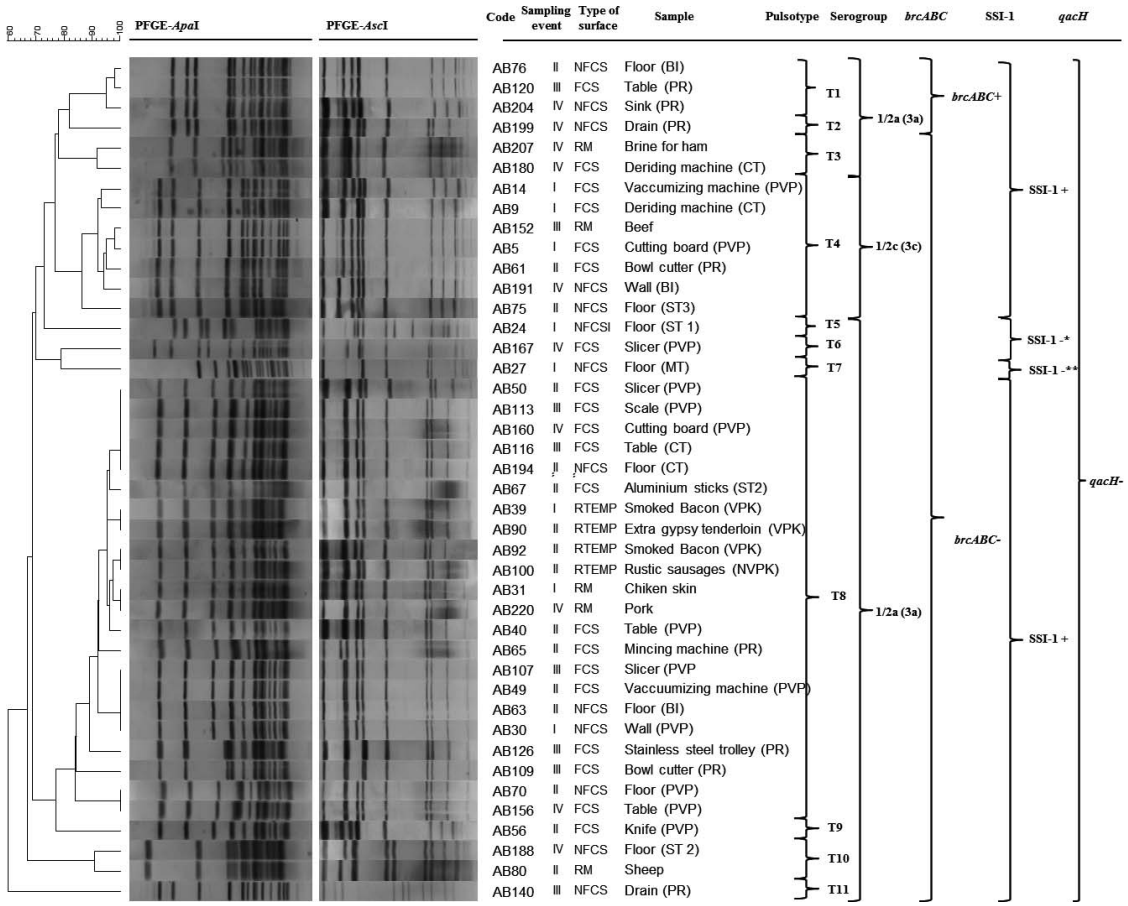

FIGURE 2. *Combined PFGE cluster analysis based on results from the restriction enzymes* Asc*I and* Apa*I, serogrouping, and the presence of resistance genes in 42* L. monocytogenes *isolates. The resistance genes were* bcrABC *(benzalkonium chloride resistance cassette),* qacH *(on the transposon Tn6188), and the SSI-1 (stress survival islet 1) SSI-1−* indicates that SSI-1 was absent but the homologue* LMO2365-481 *was present; SSI-1−** indicates that SSI-1 was absent but homologue genes* lin0464/lin0465 *were present. BI, brine injection area; PR, production area; CT, cutting area; PVP, portioning–vacuum packaging area; ST1, RTE meat product storage area 1; ST2, RTE meat product storage area 2; ST3, poultry meat storage; MT, maturation area; VPK, vacuum-packed RTE meat product.*

the facility, isolates of pulsotypes T4 and T8 were considered persistent.

Screening for the presence of genes related to tolerance to disinfectants revealed that isolates of pulsotypes T1 and T2 harbored the *bcrABC* resistance cassette, but none of the isolates harbored the transporter *qacH* gene. The SSI-1 was detected in isolates of eight pulsotypes, but isolates representing three pulsotypes (T5, T6, and T7) were SSI-1 negative. Isolates of pulsotypes T5 and T6 harbored the *LMO2365_481* homologue gene, and isolates of pulsotype T7 harbored the *lin0464*/*lin0465* homologue genes *(22)* (Fig. 2).

Adhesion to abiotic surfaces. The adhesion ability of 11 isolates representing the 11 pulsotypes increased at higher temperatures (Fig. 3), with the exception of isolates from pulsotypes T5 and T10, which had nearly constant adhesion ability at all tested temperatures. At 4°C, the adhesion ability was moderate for isolates of pulsotypes T1, T6, and T8 and weak for isolates of the other pulsotypes. At 10°C, isolates of pulsotypes T6 and T8 had relatively strong adhesion, and at 20°C strong adhesion was detected for isolates of pulsotypes T1, T6, and T8, but isolates from the other pulsotypes were only moderately adherent. The pulsotype T6 isolate had the strongest adhesion at all temperatures. The frequently isolated pulsotype T8 had strong adhesion at 10 and 20°C. In contrast, the second most frequently isolated pulsotype (T4) had only moderate adhesion, comparable to that of the sporadically isolated pulsotypes T3 and T7. The pulsotype T1 isolate recovered on three sampling occasions had strong adhesion at 20°C, comparable to that of the pulsotype T6 isolate, whereas the pulsotype T10 isolate (also recovered at three time points) had weak adhesion at all temperatures.

Disinfectant susceptibility. Susceptibility testing of isolates of each pulsotype to three disinfectants revealed a higher tolerance to BC for isolates of pulsotypes T1 and T2 (0.0025% ± 0.0005% BC), which harbored the *bcrABC* cassette, compared with the other isolates (BC MIC of 0.00075% ± 0.00025% to 0.00175% ± 0.00025%). No difference was observed in the PA MIC for strains of all 11 pulsotypes. All strains grew at 0.027% ± 0.01% PA and were inhibited at 0.029% ± 0.01%. However, the MIC varied for H_2O_2; isolates from pulsotypes T4 and T7 had slightly higher

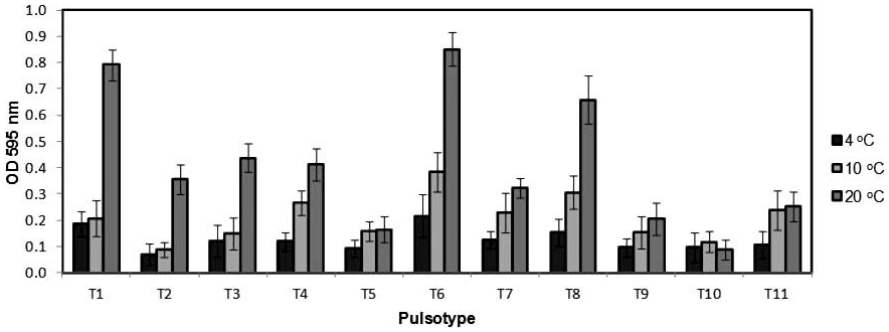

FIGURE 3. *Adhesion ability (OD_{595}) of 11 isolates (one representative of each pulsotype) was determined at 4°C (solid bars), 10°C (light shaded bars), and 20°C (dark shaded bars). Values are mean ± SD of two independent experiments performed in six replicates.*

tolerance to H_2O_2 (Fig. 4). For each disinfectant test, the same result was obtained on each of the three testing occasions.

Putative contamination routes. Observations on the activity within the premises revealed that the same employees were working in both ''clean'' and ''dirty'' areas of the facility and that they circulated between these areas without cleaning and disinfecting their equipment. The vacuum-packed RTE meat products were frequently returned from the delivery area to the packaging room, for example when the vacuum seal was not properly made, presenting another opportunity for cross-contamination. From these observations, four putative routes of contamination were identified (Fig. 5), where the staff or product work flows were inadequate.

DISCUSSION

In this study, *L. monocytogenes* was isolated from 8 of 40 RTE meat products tested. However, the results of the official monitoring plan at the facility indicated that no *L. monocytogenes*–positive samples had been detected during a 3-year sampling period. During the second two sampling events for the present study, no positive isolates

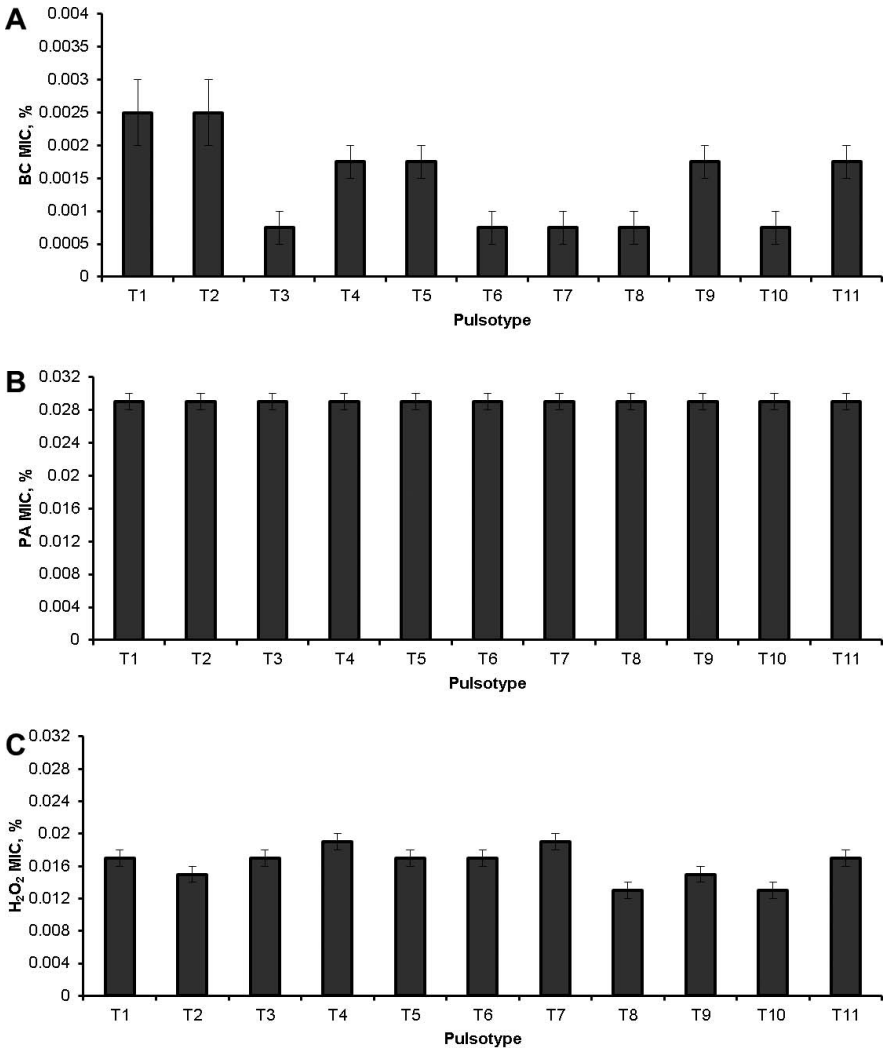

FIGURE 4. *MICs of (A) benzalkonium chloride (BC), (B) peracetic acid (PA), and (C) hydrogen peroxide (H_2O_2) determined for 11 isolates (one representative of each pulsotype). Values are the midpoint of the interval tested ± half the range tested.*

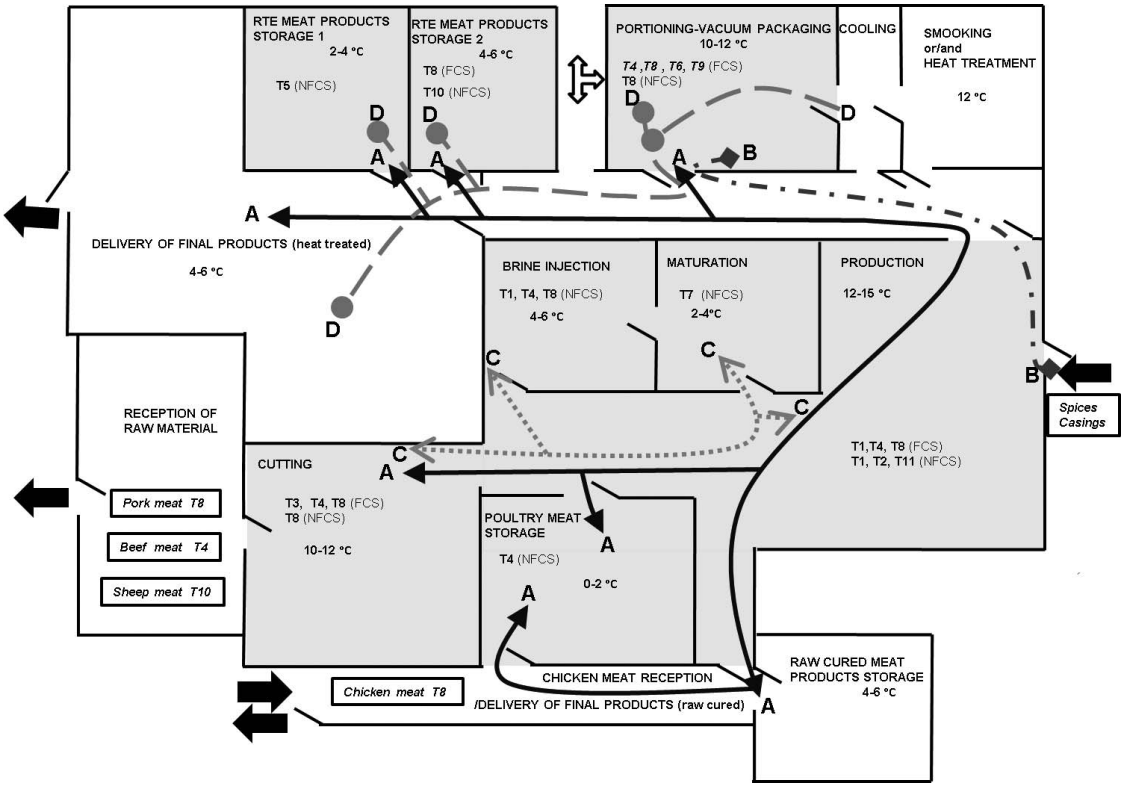

FIGURE 5. *Putative* L. monocytogenes *contamination routes in the processing facility based on distribution of* L. monocytogenes *PFGE pulsotypes (T1 through T11) in the building compartments of the meat processing facility. NFCS, non–food contact surfaces; FCS, food contact surfaces; RM, raw materials; RTE, ready-to-eat meat products. A through D indicate putative contamination routes that could lead to pulsotype distribution as a result of the work flow in the facility. In route A (solid line), the same employees handled both raw materials and finished RTE meat products. No hygiene barriers were in place between these two processes, leading to a high risk of cross-contamination from the raw materials production area to the RTE foods area either directly from the raw materials or indirectly from NFCS or FCS (e.g., transfer of T4 isolates). In route B (dash-dot line), the employees from the portioning–vacuum packaging area crossed the production area to obtain new packages for packaging (e.g., transfer of T8 isolates). In route C (short-dash line), the same workers transported the raw meat from the cutting area to the production area and crossed the brine injection and maturation area (in random order) (e.g., transfer of T1 isolates). In route D (long-dash line), after cooling the RTE meat products were weighted in the delivery area and returned to the portioning–vacuum packaging area. The devacuumed RTE products from the delivery area were then returned to the portioning–vacuum packaging area. Before portioning–vacuum packaging, some products were deposited in storage room 2, and after portioning–vacuum packaging, products were deposited in storage room 1 (e.g., transfer of T8 isolates).*

were detected in the food samples. The detection of positive food samples at the first two sample events may have been an anomaly, but this explanation is unlikely. Other explanations include implementation of the advice on corrective action (given after the first two sampling events) and through processing of a contaminated batch of raw materials (an indistinguishable *L. monocytogenes* pulsotype was found in raw materials, processing environment, and food) at the beginning of this study. Overall, 26.5% of samples from the food processing environment, including both FCS and NFCS (32.9 and 19.7% prevalence, respectively), were positive for *L. monocytogenes*. Raw materials (45% prevalence) used in this factory originated from four animal species (pig, cow, chicken, and sheep), and isolates of a different pulsotype were obtained from each species (Table 1). *L. monocytogenes* prevalence in food samples decreased over time, while the prevalence in raw materials increased, but the prevalence on FCS and NFCS remained similar. After each sampling event, the results were discussed with the facility owner. The lower prevalence in food samples at the last two sampling events may be due to awareness and additional care being taken with the final RTE food products.

In parallel, a high diversity among strains isolated from FCS (six pulsotypes) and NFCS (nine pulsotypes) was observed, which may indicate introduction of new *L. monocytogenes* strains. Introduction of new strains could contribute to continuous contamination among processing areas, equipment, and raw meat despite the cleaning and disinfection procedures used at the time of the study. A high diversity of strains in a meat processing facility also was reported by Ortiz et al. *(36)* and Ferreira et al. *(17)*.

Multiplex PCR for serogroup determination of the 42 selected strains revealed that all isolates belonged to lineage II, comprising 35 strains of serogroup 1/2a or 3a and 7 strains of serogroup 1/2c or 3c. This finding is in agreement with those of other researchers, who have found that lineage II isolates are more prevalent than lineage I isolates in foods and food producing environments *(32, 35)*. Serogroup 1/2a-3a and 1/2c-3c isolates also have been found in a high percentage of samples from meat producing facilities and RTE meat products *(5)*.

For this study, persistence was defined as the occurrence of indistinguishable isolates (based on PFGE results) throughout the sampling period. Strains of two pulsotypes (T4 and T8) were isolated on all four sampling occasions and were considered persistent. Persistent isolates have also been isolated in previous studies in meat processing facilities *(18, 25, 34)*. Isolates of pulsotype T8 were obtained from chicken and pork raw meat, the processing environment, and final food products. Each of these raw materials could have contributed to contamination of the processing environment. However, the raw material also could have become contaminated from the processing environment. A more likely scenario is that this pulsotype is widely dispersed in different food types, as reported for other pulsotypes by Fox et al. *(19)*.

Various factors can contribute to the persistence of *L. monocytogenes* strains in food processing plants, including adhesion properties and resistance to disinfectants *(28)*. Adhesion is the first stage in biofilm formation. The differences in adhesion at the temperatures tested in this study could be directly correlated with the ability of *L. monocytogenes* isolates to grow at different temperatures. However, Djordjevic et al. *(10)* found no correlation between growth rate and the ability to form biofilms for various *L. monocytogenes* isolates. Without being able to see the cells, it is only possible to say that the cells are attached to the surface; a longer incubation time would be required for biofilm formation. Most isolates had stronger adhesion at 20°C than at 10 or 4°C. At 20°C, isolates of the persistent pulsotype T8 had relatively strong adhesion, but isolates of the persistent pulsotype T4 did not have stronger adhesion than did other strains that were not considered persistent. At 10°C, the most relevant temperature for the processing environment in which most isolates were obtained, isolates from pulsotypes T6 and T8 had the strongest adhesion, whereas at 4°C isolates of pulsotypes T6 and T1 had the strongest adhesion. Except for the pulsotype T6 isolate, which was not considered persistent, little difference was found in the adhesion ability of all isolates at 10 or 4°C. Therefore, adhesion ability does not seem to be a contributing factor in persistence. However, a more extensive sampling plan would be necessary to determine whether pulsotype T6 would be considered persistent in the food producing environment.

None of the isolates of the two persistent pulsotypes (7 T4 and 22 T8 strains) harbored the *bcrABC* or *qacH* genes but did harbor the SSI-1. Isolates of pulsotypes T1 and T2, which were not considered persistent, had *bcr*ABC but not *qacH*. Therefore, *bcrABC* and *qacH* do not appear to be contributing factors to persistence. As indicated by analysis of T4 and T8 isolates, SSI-1 may be involved in persistence. However, isolates of pulsotypes T1, T2, T3, T9, T10, and T11, which were not considered persistent, also had SSI-1. Ryan et al. *(39)* reported that SSI-1 was involved in acid and salt stress tolerance. In the present study, although SSI-1 may be involved in specific stress tolerance, it did not appear to be a contributory factor to the persistence of individual isolates.

Numerous studies have been conducted to investigate disinfectant tolerance of persistent and presumed nonpersistent *L. monocytogenes* strains, but the results have been contradictory *(1, 26)*. In the present study, no correlation was detected between persistence and increased disinfectant tolerance. The higher BC tolerance of two sporadic isolates could be explained by the presence of the *bcrABC* cassette, which is responsible for BC resistance *(10)*.

The work flow of the employees in a food producing plant may encourage spread of *L. monocytogenes (4, 38)*. In the processing facility studied, the daily duties of the employees included tasks at both the area producing raw meat and the area producing RTE foods, without a requirement for procedures that could reduce cross-contamination. From observations in the facility, four putative contamination routes were identified (Fig. 5) according to the work flow of the employees (routes A through C) or the design of the process (route D). The same employees handled both raw materials and final RTE meat products without any hygiene barriers between these two processes (route A). Such a procedure presents a high risk of cross-contamination from the raw materials to the RTE food via contamination of personnel, NFCS, or FCS. Another route (route B) was employees frequently crossing the raw meat production area to take vacuum-packaged RTE products to the storage area. Within the raw meat area, the same workers transported the raw meat from the cutting area through the production area to the brine injection and maturation area (route C). Route D was associated with the work flow for packaging of RTE meat products. After cooling, the RTE meat products were weighed in the delivery area and returned to the portioning-vacuuming area. The devacuumed RTE products from the delivery area were returned to the portioning–vacuum packaging area. Before portioning and vacuum packaging, some products were deposited in storage room 2, and after portioning and vacuum packaging, products were deposited in storage room 1.

Tracing *L. monocytogenes* T4 and T8 isolates in the processing facility revealed the cross-contamination between various areas of the production facility, as suggested by routes A, B, and C (Fig. 5). The primary source of pulsotype T4 could be raw materials, based on the fact that its presence was detected throughout the entire production line, starting with beef and pork meat (positive sample from the derinding machine), continuing with processing equipment (cutter), the floor in the poultry meat storage room, and a wall in the brine injection room, and ending in the vacuum packaging room, where this pulsotype was found on the slicing machine and the ceramic plaque of the vacuum machine. Damage to the conveyor belt material, which could provide a protected niche for *L. monocytogenes,* also was observed. The putative contamination routes A, B, and C also were confirmed by the spread of pulsotype T8 to almost all rooms of the processing facility (Fig. 5). Identification of pulsotype T8 on the smoked and boiled sausages and on the aluminum sticks used for hanging sausages could be the result of inefficient sanitizing of the rods.

Regarding infrequently isolated pulsotypes (e.g., T3 and T10), these strains may have entered the facility via raw

materials, but more extensive sampling may be required to confirm persistence of these strains. The presence of pulsotype T3 in the recirculated brine for injection of the ham and in the skinning machine on the last sampling occasion suggests a new contamination event, which could have originated from any of many sources. The contaminated brine is kept at 2 to 4°C and reused two or three times for ripening the meat used for ham preparation, with the possibility of multiple cross-contamination events.

To address the cross-contamination issues with staff work flows, a number of steps could be taken within this facility. The raw meat and RTE food processing areas should be completely separate. If staff must move from the raw meat area to the RTE area, they should pass through a hygiene barrier, including at least a hand washing facility, an area to change clothing, and a well-maintained foot bath. The sanitization procedure in place when this study was conducted was sanitization of the facility (excluding storage areas) with a 0.5% PA solution at room temperature after 16 h of operation. The PA solution was left on the surfaces for 10 min and then rinsed off with water. The storage areas were sanitized once per week. Because *L. monocytogenes* was isolated from the floors, walls, and storage areas, this procedure was inadequate for control of this pathogen. A more intensive sanitization procedure should be used, including attention to details of sanitization and rotation of disinfectants to avoid the establishment of tolerant strains in areas where the disinfectant concentration was lower than that recommended.

The number of *L. monocytogenes*–positive RTE food samples was high at sampling events I and II (20 and 60%, respectively), but at sampling events III and IV, no *L. monocytogenes* was detected in the RTE food samples. The results of the study were reported to the facility management team after each sampling event. The improvement in the results obtained for RTE food may be the result of corrective measures taken by the company to control *L. monocytogenes* in the processing environment. These measures included using different workers in the RTE food area and the production area (eliminating routes A and C), preventing worker access from the portioning–vacuum packaging area to the other areas of the factory (eliminating route B), and improving cleaning and washing procedures and installing a balance for weighing products in the vacuum packaging area (eliminating route D). Washing procedures were introduced after each working shift, especially in the areas were RTE meat products are manipulated, portioned, sliced, and/or vacuum packaged, and a UV lamp was installed in the portioning–vacuum packaging area. Training for the workers from all the departments also was implemented. Effective monitoring and sanitation programs in all food processing and especially meat processing facilities are strongly recommended *(3, 6, 13, 16, 19).*

One year after implementation of the corrective measures listed above, 13 samples were collected from the portioning–vacuuming packaging area on two occasions separated by 2 weeks. All 26 samples were negative for *L. monocytogenes* (data not shown), compared with 20 positive of 31 samples tested (64.5%) during the original study. Similar results were obtained by Dalmasso and Jordan *(9),* who found that sampling plans for processing environments can be effective for assessing the hygiene status of a processing facility and that implementation of corrective actions can improve hygiene with respect to *L. monocytogenes*.

The present study provides essential information about the need to understand putative cross-contamination routes of *L. monocytogenes* to improve hygiene measures in food production facilities and prevent contamination of RTE meat products. Contamination hotspots and factors promoting high levels of contamination were identified. Using this study as a template, the meat industry in Romania and many other countries should reassess the selection of raw materials, avoid incorrect handling of products, and personalize the sanitization plans for every facility according to processing needs. By identification of putative routes of cross-contamination in other food production facilities, a better understanding of contamination routes could be obtained, thereby contributing to development of strategies to prevent product recalls and listeriosis outbreaks.

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