Heliyon 10 (2024) e28662

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Distribution of *Listeria* spp., and *Listeria monocytogenes* in micro- and small-scale meat product processing plants

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ARTICLE INFO

Keywords:
Environmental pathogen monitoring
Listeria monocytogenes
Meat products
Ready-to-eat food
Listeria spp

ABSTRACT

Listeriosis is a disease caused by *L. monocytogenes*, a relevant microorganism as a causative agent of foodborne diseases - FBD. This study aimed to evaluate the distribution of *Listeria* spp., and *L. monocytogenes* in different production areas in two small plants (A and B) and two micro-food processing plants (C and D) producing meat derivatives, located in different cities of Colombia. The methodology implemented was *i.* The analysis of sampling points is based on a harmonised tool. *ii.* Four samplings in each production plant between 2019 and 2020. *iii.* Isolation and identification of microorganisms through conventional microbiology, a semi-automated system, molecular serotyping and clonal characterisation by ERIC-PCR. *L. monocytogenes* frequency in the production plants belonging to the study ranged between 5.9 and 28.6 %; for *Listeria* spp., plants A and D had isolated, plant A had the highest proportion, while for *L. monocytogenes* geno-serotypes found were: 1/2a, 1/2c, 4a-4c, 4b, 4d - 4e, with geno-serotype 4b as the most frequent. Furthermore, possible persistent isolates were detected in plant C as the feasible sources of contamination, based on failures in flow management, raw material contaminated with *L. monocytogenes,* lack of standardised cooking processes and transfer of the microorganism through equipment and surfaces. Finally, in three of the four production plants assayed, *L. monocytogenes* or *Listeria* spp. were present in the packaging area in some of the samples taken during the study, which calls for increased and frequent monitoring, as well as constant technical support for the control of *L. monocytogenes* in micro and small-scale production plants.

1. Introduction

L. monocytogenes became more relevant as a causative agent of foodborne illness (FBD) compared between listeriosis, salmonellosis and campylobacteriosis, observing that cases of listeriosis were not decreasing. In addition, the organism is more prevalent in ready-to-eat foods [1], demonstrating that *L. monocytogenes* is an increasing problem. Moreover, the globalisation of food supply chains has contributed to the increased occurrence of outbreaks [2], allowing the spread of the organism and thus increasing the need for

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https://doi.org/10.1016/j.heliyon.2024.e28662
Received 1 August 2023; Received in revised form 19 March 2024; Accepted 21 March 2024
Available online 22 March 2024

monitoring during production.

Products contamination with *Listeria* spp. can occur during food processing due to cross-contamination during the use of previously contaminated utensils, such as trays and cutters, by operators [3] and also due to design flaws in equipment such as tanks, racks, conveyor belts and ventilation systems, as well as inefficient implementation of cleaning and disinfection programmes [4,5]. Furthermore, the ubiquitous and psychrotrophic nature of *L. monocytogenes* allows it to survive and grow in refrigeration, difficulting the control and management of the microorganism. Therefore, *L. monocytogenes* can be introduced repeatedly into the production process; this scenario could be worse by persistent isolates (in the production plant environment) can reside in niches where eradication is inefficient, becoming a constant source of contamination. Furthermore, cleaning and disinfection programmes are not always effective in controlling *L. monocytogenes*, which creates a risk for food producers (ready-to-eat), because they include heat treatments before consumption [4,6,7].

In addition to the above, small food companies have more pronounced periods of contamination, compared to large ones, due in part to limited economic resources, which prevents or delays the implementation of corrective measures to combat contamination, favouring the persistence of the microorganism [4]. However, large food production plants (ready-to-eat) have the economic muscle to develop an effective sampling plan designed by trained personnel [8], allowing better monitoring and control of risks.

As a strategy for the detection of *Listeria* spp., as part of the sampling plan, it is necessary to make hygienic zoning to identify i) non-food processing areas (where must be met the basic sanitary requirements, but not Good Manufacturing Practices (GMP) such as offices, employee areas and maintenance areas), ii) transition areas (areas that allow passage to GMP areas, may include an entrance or door, iii) GMP compliance areas (must meet basic GMP requirements, with special care in handling product flow, linear traffic, and attention to preventive controls) and iv) primary pathogen control areas (areas where the finished product is exposed to the environment, thus requiring a strict control) [9] (Food Safety Preventive Control Alliance; https://www.ifsh.iit.edu/fspca/fspca-preventive-controls-human-food; access 08- 08-2022).

The identification, characterisation of the zones and the design of a sampling plan allow the dynamics of *Listeria* spp. to be monitored, especially *L. monocytogenes*. However, identification of the genus and species is not sufficient, considering that Whole Genome Sequencing-WGS studies have shown that some subtypes of *L. monocytogenes* may be associated with a specific supply chain, products or locations, but other subtypes may be geographically distributed and related to different sources [10], which makes it necessary to implement molecular tools to differentiate between isolates.

Microbiological identification of *L. monocytogenes* isolated from surface sampling is the focus of hygiene monitoring, environmental sampling and biohazard analysis programmes [11]. Hence, the stages of sample collection, sample processing and analysis of results are under constant scrutiny. Two sampling techniques are available: (i) the friction technique using a swab, gauze pad and sponge, and (ii) the contact technique using a contact plate, contact blade and petrifilms^{TM}. In the detection of *L. monocytogenes* in the study by Brauge et al. (2020), no significant differences were observed in the recovery of the microorganism by the contact methods; however, it is necessary to take into account the characteristics of the area to study when selecting the sampling method. Also, the most appropriate neutraliser selection (depending on the biocides used in the company) is recommended for sample collection if disinfectant residues are expected to be present to avoid their inhibitory effect on the growth of microorganisms [12,13].

In Colombia, meat production occurs in micro-, small-, medium- and large-size food processing plants, with the majority being small and medium-sized food processing plants [14]. The objective of this research was to evaluate the distribution of *Listeria* spp. and *L. monocytogenes* in different production areas in two small plants (A and B) and two micro-size plants (C and D) of meat derivatives production; located in Colombian cities between 2500 and 2800 m above sea level (m.a.s.l.), to elucidate the possible routes of contamination in each of them, in the hope that this approach, could be reproduced in similar plants in other regions and countries of the continent.

2. Materials and methods

2.1. Company descriptions

The study was carried out in four production plants, considered micro and small food processing plants according to Colombian regulations [14]; these are hereinafter referred to as meat products processing plants A, B, C and D. The production lines of each are in Table 1 described.

Table 1
Meat product processing plants, size and quantity of processing lines.

Meat product processing plants	Processing plants scale	Total number of meat products (Number of types of meat products ready-to-eat and the other meat products)	Number of processing lines on the same day
A	Small	7 (6 ready-to-eat y 1 precooked)	3
B		6 (4 ready-to-eat y 2 precooked)	3
C	Micro	1 (1 ready-to-eat)	1
D		6 (6 ready-to-eat)	2

Table 2
Sampling points, classified as surfaces, equipment and personnel deployed in each hygiene zoning area.

Equipment and utensils			
Sampling point	Transition area	GMP Area	Pathogen control area
Fans		X	X
Drip pans		X	X
Hoist chain bags			X
Racks that are not used for exposed finished product		X	
Racks that are used for exposed finished product			X
Trolleys, lifters, forklifts and pallet jacks		X	
Carts		X	X
Trash cans		X	X
Bowl cutters		X	X
Knife		X	X
Soap dispensers		X	X
In-floor weighing equipment and floor scales		X	X
Cleaning equipment			X
Exterior of ice makers			X
Platform		X	
Maintenance tools		X	X
Hoses (and hose holders)		X	X
Ice maker used for ice that comes in contact with exposed finished product		X	X
Packaging machines			X
Skinning machines		X	
Mixers		X	
Meat grinders		X	
Other Equipment (e.g., vats, tanks, tables) that comes in contact with product after CCP			X
Smoke sticks			X
Peelers		X	
Choppers		X	
Vacuum sealers			X
Cutting boards used for finished product			X
Sorting tables that contact finished product			X
Meat slicer			X
Thermometers, Thermocouples, etc. That contact finished products		X	X
Surfaces			
Sampling point	**Transition area**	**GMP Area**	**Pathogen control area**
Floor mats	X	X	X
Band Saws		X	
Railings in finished producto areas			X
Brine chiller chamber/tunnel		X	
Conduits, electrical boxes, conduit-in-casing			X
Air return cover			X
Electrical outlet covers			X
Drains			X
Scales			X
Flaps or strip curtains used in areas where exposed finished product is present		X	X
Handwash sinks		X	X
Framework and non-food contact areas of food contact equipment			X
Sorting tables (surfaces that contact finished product)			X
Loading Dock			X
Walls		X	X
Portable steps/stools/ladders		X	X
Foot pedals			X
Footbaths		X	X
Floor		X	X
Cooler doors and other doors not used for transport of open finished product		X	X
Ramp		X	X
Fillers			X
Break room and locker room area floors	X		X
Conveyor systems, belt and other food contact sites			X
Air blower			X
Hopper Surface		X	
Control switch/HMI screens on equipment close to FCS		X	X
Ceiling in production area		X	X
Conveyor systems			X
Pipelines and exposed overhead piping in área with exposed product			X
Floor/wall junctions		X	X
Windows			X
Dock bumpers			X

(*continued on next page*)

Table 2 (*continued*)

Equipment and utensils			
Sampling point	Transition area	GMP Area	Pathogen control area
Visible areas possible contamination in the break room		X	
Staff			
Sampling point	**Transition area**	**GMP Area**	**Pathogen control area**
Boots/footwear worn by personnel who handle exposed product	X		X
Aprons that contact finished product			X
Boots/footwear worn by personnel who do not handle exposed product			X
Equipment and utensils			
Sampling point	Transition area	GMP Area	Pathogen control area
Fans		X	X
Drip pans		X	X
Hoist chain bags			X
Racks that are not used for exposed finished product		X	
Racks that are used for exposed finished product			X
Trolleys, lifters, forklifts and pallet jacks		X	
Carts		X	X
Trash cans		X	X
Bowl cutters		X	X
Knife		X	X
Soap dispensers		X	X
In-floor weighing equipment and floor scales		X	X
Cleaning equipment			X
Exterior of ice makers			X
Platform		X	
Maintenance tools		X	X
Hoses (and hose holders)		X	X
Ice maker used for ice that comes in contact with exposed finished product		X	X
Packaging machines			X
Skinning machines		X	
Mixers		X	
Meat grinders		X	
Other Equipment (e.g., vats, tanks, tables) that comes in contact with product after CCP			X
Smoke sticks			X
Peelers		X	
Choppers		X	
Vacuum sealers			X
Cutting boards used for finished product			X
Sorting tables that contact finished product			X
Meat slicer			X
Thermometers, Thermocouples, etc. That contact finished products		X	X
Surfaces			
Sampling point	**Transition area**	**GMP Area**	**Pathogen control area**
Floor mats	X	X	X
Band Saws		X	
Railings in finished producto areas			X
Brine chiller chamber/tunnel		X	
Conduits, electrical boxes, conduit-in-casing			X
Air return cover			X
Electrical outlet covers			X
Drains			X
Scales			X
Flaps or strip curtains used in areas where exposed finished product is present		X	X
Handwash sinks		X	X
Framework and non-food contact areas of food contact equipment			X
Sorting tables (surfaces that contact finished product)			X
Loading Dock			X
Walls		X	X
Portable steps/stools/ladders		X	X
Foot pedals			X
Footbaths		X	X
Floor		X	X
Cooler doors and other doors not used for transport of open finished product		X	X
Ramp		X	X
Fillers			X
Break room and locker room area floors	X		X
Conveyor systems, belt and other food contact sites			X

(*continued on next page*)

Table 2 (*continued*)

Equipment and utensils			
Sampling point	Transition area	GMP Area	Pathogen control area
Air blower			X
Hopper Surface		X	
Control switch/HMI screens on equipment close to FCS		X	X
Ceiling in production area		X	X
Conveyor systems			X
Pipelines and exposed overhead piping in área with exposed product			X
Floor/wall junctions		X	X
Windows			X
Dock bumpers			X
Visible areas possible contamination in the break room		X	
Staff			
Sampling point	**Transition area**	**GMP Area**	**Pathogen control area**
Boots/footwear worn by personnel who handle exposed product	X		X
Aprons that contact finished product			X
Boots/footwear worn by personnel who do not handle exposed product			X

2.2. Recognition of the production plants

Four visits were made to the production plants at different times of the production days, on various days, to get to know the areas, equipment and personnel in each of them. The first sampling was before the cleaning and disinfection at the beginning of the production day. The hygienic zoning of the plant and the possible sampling points detection in each area were done by following the work by Simmons and Wiedmann (2018). The zoning and sampling points (according to EPM) allow classifications as verification site (VS), indicator site (IS), niche (Ni) or transfer point (TP) (Table 2) [15]. Finally, the raw materials handling-the personnel- and the finished product -flows were known.

2.3. Sampling description

2.3.1. Samplings number and samples taken for each plant

Four samples were taken at each plant between February 2019 and December 2020 at "clean" (finished product handling) and "dirty" (raw material handling) locations to detect cross-contamination.

A standard methodology allows for defining the sampling points to assess the detection of microorganisms at the sites described in Table 2 [15], a tool that also favoured the analysis of each positive sample; sampling occurred at different times (Table 3): the first sampling was before the start of the working day to assess the effectiveness of the cleaning and disinfection process in the production plant [15]; subsequently, samples 2-4 were taken during the processing day, 4 h after the sanitation activities (on different days) [16,17] (Table 3); this allowed a constant evaluation of the dynamics of the isolates found in each of the plants, at the different sampling times (The time between samplings was longer than 30 days).

The number of samples was not based on statistical methods because the implementation of the formulas requires prior information on the prevalence and or a minimum level of contamination [17,18] and in all the plants belonging to the project, it is the first time that investigations have been carried out in search of *Listeria* spp., furthermore, in the studies carried out as a requirement of the entity in charge of inspection, surveillance and control, there are no records of the microorganism, possibly due to the low number of samples processed or the lack of increased monitoring as indicated in some studies [19].

Plant A: environmental samples taken were 119, of which 36 (30.25 %) were CS and 83 (69.75 %) of NFCS (Supplementary Material 1.1).

Plant B: environmental samples taken were 102, of which 41 (40.20 %) were CS and 61 (59.80 %) of NFCS (Supplementary Material 1.2).

Table 3
Number of samples per plant.

Meat products processing plants	Environmental Sampling						Raw material and Finished Product (samples number)	Total, samples
	Samples number					Total, samples		
A	0	57	21	21	20	119	69	188
B	0	44	20	15	23	102	53	155
C	12	37	16	15	15	83	38	133
D	0	33	16	15	15	79	39	118
Total	12	171	73	66	73	383	199	594
Sampling moment	**Pilot study**	**M1**	**M2**	**M3**	**M4**			

Plant C: environmental samples taken were 92 (83 taken during the four samplings and 9 in the pilot study), of which 22 (23.91 %) were CS and 70 (76.09 %) of NFCS (Supplementary Material 1.3).

Plant D: environmental samples taken were 79, of which 36 (45.57 %) were CS and 43 (54.43 %) of NFCS (Supplementary Material 1.4).

2.3.2. Sampling description

2.3.2.1. Surface samples. Samples collection involved sterile sponge bags (3 M Sponge stick), which were hydrated with 25 mL of a neutralising buffer solution (Trypticase Azolectin Tween Broth Base - TAT Broth, BD Difco (20 gL^{-1} Pancreatic Digest of Casein, 5 gL^{-1} Soy Lecithin, 40 mL Polysorbate 20)).

The sampling areas were 1000 cm^2 (for large surfaces), e.g. flats samples; sampling from discontinuous surfaces (e.g. washbasin) or on all working surfaces of a short design (e.g. flat dryer), the sampling area was 645 cm^2.

2.3.2.2. Equipment sampling. Depending on the type of equipment, samples involved sponges or swabs; in the case of swabs, were moistened with 5 mL of neutralising buffer solution (Trypticase Azolectin Tween Broth - TAT Broth, BD Difco (20 gL^{-1} Pancreatic Digest of Casein, 5 gL^{-1} soy lecithin, 40 mL polysorbate 20)).

In the case of samples taken with sponges 25 mL of Buffered Peptonated Water (3 M) was added for a final volume of 50 mL, and in the case of swabs, 5 mL of Peptonated Water (3 M) allows to reach a final volume of 10 mL; subsequently, homogenised and stored, to be transported in less than 24 h to the Food Microbiology Laboratory of the Pontificia Universidad Javeriana, Bogotá D.C., Colombia.

Sampling is usually done from the "cleanest" to the "dirtiest" sectors to avoid cross-contamination [16]; however, due to the structural layout of some plants, this rule could not be followed, as can be seen in the analysis of individual plants below.

2.3.2.3. Finished product samples. Samples of the finished product (at least 50 g) were from two times: on the same working day as the environmental samples and at an additional sampling carried out for finished product collection (during the COVID-19 pandemic declaration).

2.4. Sample processing for the detection of Listeria spp.

For *Listeria* spp. and *L. monocytogenes*, ISO 11290-1 allows presumptive isolates detection. ISO 11290-1 indicates a primary enrichment phase in Half-Fraser broth at 30 °C for 24–26 h, followed by a secondary enrichment in Fraser broth at 37 °C for 24 h, Followed by seeding on Ottaviani and Agosti agar (37 °C, 24 h) and a second selective medium (incubation conditions according to the manufacturer's specifications). Finally, presumptive colonies of *Listeria* spp. were on a non-selective Tryptone Soya Yeast Extract Agar (TSYEA), isolated after incubation for 24 h at 37 °C [20].

Characteristic colonies of *Listeria* spp. on TSYEA were by microscopy, catalase, Voges-Proskauer and motility at 25 °C assayed. For L. *monocytogenes*, haemolysis on blood agar, carbohydrate usage by L-Rhamnose and D-Xylose and the CAMP test were also necessary.

The rapid detection method "3 M Listeria Molecular Detection Assay 2″ were used, following the instructions of the company; subsequently, all suspect isolates were on Trypticase Soy Agar with Yeast Extract - TSAYE (15.0 gL^{-1} Pancreatic Digest of Casein, 5.0 gL^{-1} Enzymatic digest of Soya Bean, 5.0 gL^{-1} NaCl, 15.0 gL^{-1} Agar-agar) and Ottaviani agar cultured, for following confirmation with molecular tests.

2.5. Molecular characterisation

2.5.1. Molecular identification of L. monocytogenes and Listeria spp.

In a Multiplex-PCR to identify the isolates of *L. monocytogenes and Listeria* spp., two sets of primers L1-Forward (CTC CAT AAA GGT GAC CCT) and U1-Reverse (CAG CMG CCG CGG TAA TWC) allow yielding a product of 938 bp, identifying the Listeria genus. LF-Forward (CAA ACG TTA ACA ACG CAG TA) and LR-Reverse (TCC AGA GTG ATC GAT GTT AA) yield a product of 750 bp, amplifying the *hly*A gene, typical for monocytogenes specie, [21]. The final reaction volume was 35 μl, composed of 1X PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM dNTPs, 20 pmol of primers and 2U of TaqDNA polymerase (recombinant Taq DNA Polymerase (5 U/μL), Thermo Scientific). Five μl of DNA (~100 ng) were for amplification. Temperatures setting were: 95 °C x 1′; (94 °C × 30s, 51 °C × 20s, 72 °C × 30s) 40 Cycles; 72 °C x 8′ [21].

2.5.2. Grouping by divisions the L. monocytogenes isolates

The isolates of *L. monocytogenes* were sorted by divisions by using a Multiplex-PCR, by using two pairs of primers: D1-Forward (CGA TAT TTT ATC TAC TTT GTC A), D1-Reverse (TTG CTC CAA AGC AGG GCA T). These primers allow to yield a product of 214 bp; classifying isolates into division I (geno-serotypes 1/2b, 3b, 4b, 4d and 4e) or division III (geno-serotypes 4a and 4c). Primers D2 Forward (GCG GAG AAA GCT ATC GCA), and Reverse (TTG TTC AAA CAT AGG GCT A), yield a product of 140 bp and classify the isolates into the division II (geno-serotypes 1/2a, 1/2c, 3a and 3c). PCR temperatures setting were: 95 °C x 3′; (95 °C × 30s; 59 °C × 30s; 72 °C x 1′) 25 Cycles; 72 °C x 10′ [21].

2.5.3. PCR Serotyping

Once classified by divisions, the isolates belonging to division II were subtyped using the FlaA primer set: Forward (TTA CTA GAT CAA ACT GCT CC) and Reverse (AAG AAA AGC CCC TCG TCC), to generate a product of 538 bp; characteristic of geno-serotypes 1/2a and 3a; the absence of amplification indicated the presence of geno-serotypes 1/2c or 3c. PCR temperatures setting were: 95 °C x 3′; (95 °C × 30s, 54 °C × 30s; 72 °C x 1′)25 Cycles; 72 °C x 10′ [21].

Isolates grouped into divisions I and III, were subtyped with the GLT primer set: Forward (AAA GTG AGT TCT TAC GAG ATT T) and Reverse (AAT TAG GAA ATC GAC CTT CT) to obtain a product of 483 bp that identifies geno-serotypes 1/2b and 3b. PCR temperatures setting were 95 °C x 3′; (95 °C × 30s, 45 °C × 30s; 72 °C x 1′) 25 Cycles; 72 °C x 10′ [21].

The reaction mixture for D1/D2, FlaA and GLT consisted of: 25 μl reaction volume, 50 pmol/μl of each primer, 1U of Taq DNA polymerase (recombinant Taq DNA Polymerase (5 U/μL), Thermo Scientific), 1X of PCR Buffer, 0.2 mm of each dNTP, 2.5 mM $MgCl_2$ and 5 μl (~100 ng) of sample DNA [21].

Isolates that did not yield the 483 bp band with GLT primer set, were considered as geno-serotype 4 and thus further subtyped with primers MAMA-C (LM4/LMB): Forward (CAG TTG CAA GCG CTT GGA GT) and Reverse (GTA AGT CTC CGA GGT TGC AA) yielding an amplification product of 268 bp, that identifies geno-serotypes 4a and 4c [21]. The strains lacking the amplification were geno-serotype 4 (b, d or e). The reaction volume was 50 μl containing 0.5 μmol of each primer, 2U Taq DNA polymerase (recombinant Taq DNA Polymerase (5 U/μL), Thermo Scientific), 1X PCR buffer, 200 μM of each dNTP, 2.0 mM $MgCl_2$ and 2 μl (~50 ng) of sample DNA [21]. MAMA-C PCR temperatures setting were: 95 °C x 10′; (95 °C × 30s, 55 °C x 1′, 72 °C x 1′)40 cycles; 72 °C x 10′.

2.5.3.1. PCR for iap gen amplification. *L. monocytogenes* isolates classified as geno-serotype 1/2a and 3a due to positive amplification with FlaA (538 bp) and those that do not amplify with FlaA (1/2c or 3c), and the isolates that did not amplify with MAMA-C were considered as geno-serotype 4b, 4d or 4e. Them were subtype with a single PCR, using a primer set CLM1-Forward (ACA GCT GGG ATT GCG GT) and CLM2-Reverse (CCC AGC CAG AGC CGT GGA) which were located within *iap* gen of *L. monocytogenes* to amplify a 1395 bp fragment of this gen. The reaction volume was 50 μl containing 0.1 pmol of each primer, 1.25U Taq DNA polymerase (recombinant Taq DNA Polymerase (5 U/μL), Thermo Scientific), 1X PCR buffer, 0.125 mM of each dNTP, 1.5 mM $MgCl_2$ and 5 μl (~100 ng) of sample DNA. PCR temperatures setting were 95 °C x 5′; (95 °C × 90s, 54 °C × 60s; 72 °C x 3′)35 Cycles; 72 °C x 7′ [22,23].

2.5.3.2. Restriction enzyme analysis of the amplification product (PCR-REA) for confirmation of geno-serotypes 1/2a, 1/2c and 4b. Two μl of each CLM1/CLM2 PCR-products were digested with *Hind*III restriction enzyme (New England BioLabs), following the instructions of the manufacturer [22,23]. Restriction fragments of 693, 425 and 277 bp will be typical of geno-serotypes 1/2a, 1/2c whereas restriction fragments of 1118 and 277 bp will be characteristic of geno-serotype 4b.

2.6. Clonal characterization

ERIC1R (ATGTAAGCTCCTGGGGATTCAC and ERIC2 (AAGTAAGTGACTGGGGTGAGCG primers) [24] were used for the ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus) of all the isolates. For the amplification mixture we used 200 ng of template DNA in 30 μl of a solution containing 25 pmol of each primer, 0.25 mM of each dNTP, 2.5 mM $MgCl_2$ and 1U (recombinant Taq DNA Polymerase (5 U/μL), Thermo Scientific). PCR temperatures setting were 95 °C x 5′; (90 °C × 30s, 46 °C × 30s, 49 °C × 30s, 72 °C x1′)30 Cycles; 72 °C x 8′ [24–26].

2.6.1. Analysis of ERIC-PCR products

The gel resulting from electrophoresis was photographed under UV light and image analysis was performed with GelAnalyzer 19.1 and NTSYS-pc V 2.20 software. DNA fingerprint positions were the presence of a DNA band (score "1″) and the absence of a DNA band (score "0″). Fingerprint similarity was calculated by Dice's similarity coefficient with a 1% band position tolerance, and dendrograms were generated by the unweighted pair group method using the arithmetic mean (UPGMA) [27–32]; the cut-off points in dendrograms were established by the NTSYS-pc V 2.20 software, according to the profile of the trees generated and analysed to detect the number of clusters and sub-clusters; the value of the cut-off point in each dendrogram varies.

3. Results

3.1. Frequency of Listeria monocytogenes and Listeria spp. in production sites

594 samples from the four production plants were processed during the study. After evaluation of the sampling points in plant A of

Table 4
Frequency of L. *monocytogenes* and *Listeria* spp., at each of the production sites.

Meat Products processing plants	Positive samples for *L. monocytogenes*	Frequence (%)	Positive samples for *Listeria* spp.	Frequence (%)
A (n = 188)	12	6.4	22	11.7
B (n = 155)	11	7.1	9	5.8
C (n = 133)	38	28.6	9	6.8
D (n = 118)	7	5.9	0	0

the 188 samples taken, 119 (63.3 %) were environmental and 69 (36.7 %) food; raw material and finished product. In plant B of the 155 samples taken, 102 (65.8 %) were environmental and 53 (34.2 %) food. In plant C of the 133 samples taken, 95 (71.4 %) were environmental and 38 (28.6 %) food. Finally, in plant D of the 118 samples taken, 79 (66.9 %) were environmental and 39 (33.1 %) were food (Table 3).

Table 4 shows the frequency of *L. monocytogenes* and *Listeria* spp., in each of the production plants. The highest frequency of *L. monocytogenes* was detected in plant C (micro-enterprise) while for *Listeria* spp., it was in plant A (small enterprise). On the other hand, the lowest frequencies for *L. monocytogenes* and *Listeria* spp., were found in plant D (micro-enterprise).

3.2. Analysis of the distribution of L. monocytogenes and Listeria spp., in small-scale production plants (plants A and B)

3.2.1. Plant A

Plant A has three areas where Good Manufacturing Practices (GMP) must be complied with and two areas for pathogen control. Furthermore, a point of possible cross-contamination was found at the exit of one of the GMP areas in the cooking zone, due to the entry of raw material and the exit of post-cooking product. The highest detection of *L. monocytogenes* isolates was found in the first sampling,

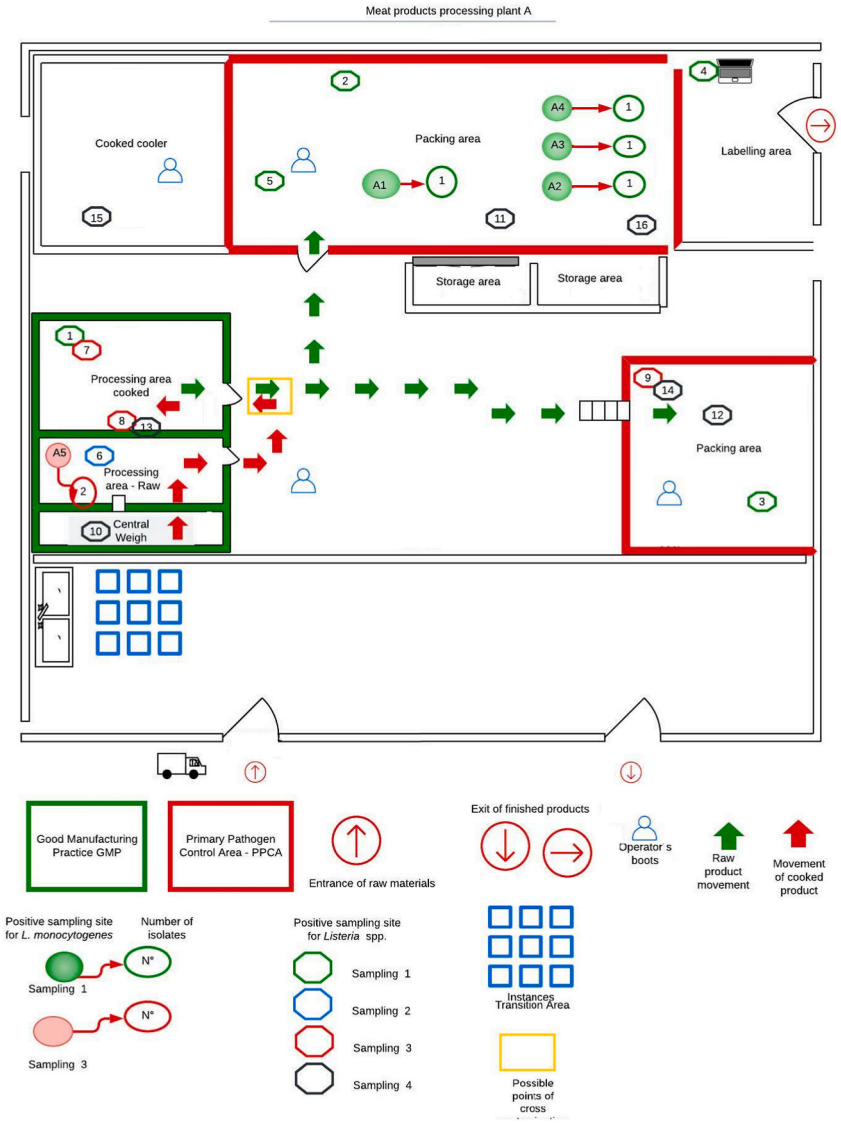


Fig. 1. Plant A, positive sampling sites for *L. monocytogenes*, circled, A1: table used for the finished product, A2: flaps labelling area, A3: flats, A4: windows, A5: saw. On the other hand, for Listeria spp., which are marked in hexagons were: 1: pressure washer, 2: air conditioning door, 3: knives, 4: printer, 5: boots, 6: production area saw, 7: pressure washer, 8: production area drains, 9: cooling area drain, 10: weighing area scale, 11: chopping machine, 12: trolleys, 13: production area drains, 14: cooling area drain, 15: cold room flaps - packaging area, 16: windows - packaging area.

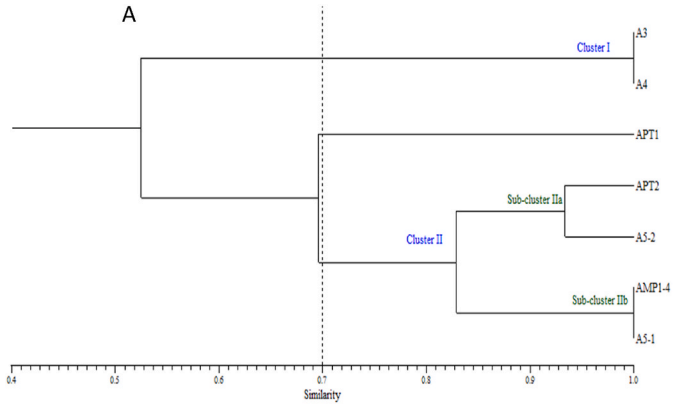
on the other hand, the frequency of *Listeria* spp. was observed in all the samplings carried out in the production plant in the raw and pathogen control areas (Fig. 1). Likewise, *Listeria* spp., was also found in the packaging area, in the labelling area and on the boots of an operator.

Of the 12 *L. monocytogenes* positive samples, 22 isolates were obtained, distributed as follows: six from environmental samples A1 (1), A2 (1), A3 (1), A4 (1) and A5 (2) and sixteen isolates from food, classified as: raw material 5 isolates (AMP1 to AMP5) and from finished product 11: APT 1 (1), APT 2 (1), APT 3 (1), APT 4 (1), APT 5 (1), APT 6 (6).

3.2.2. Plant B

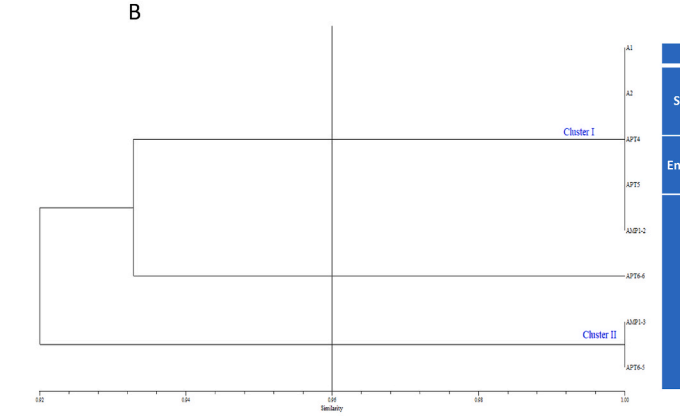
Plant B has three GMP areas and two finished product areas; one of which handles pre-cooked products and the other is used for finished product packaging. The flow management of this production plant occurs in an "L" shape with large areas without physical

A



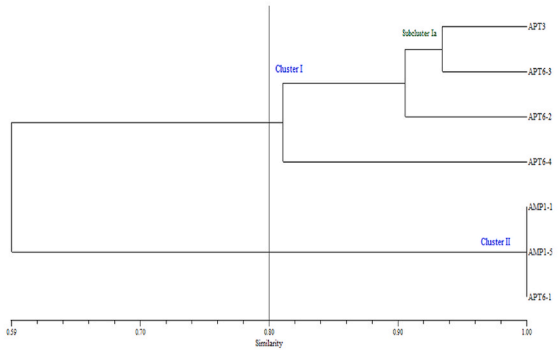
No typeable					
Sample type	Isolate	Sampling site for *L. monocytogenes*	Sampling	Zone	Classification
Food	APT3	APT3	1	-	Finished product
	APT6-3	APT6	2	-	Finished product
	APT6-2	APT6	2	-	Finished product
	APT6-4	APT6	2	-	Finished product
	AMP1-1	AMP1	2	-	Raw material
	AMP1-5	AMP1	2	-	Raw material
	APT6-1	APT6	2	-	Finished product

B



Serotype 4b					
Sample type	Isolate	Sampling site for *L. monocytogenes*	Sampling	Zone	Classification
Environmental	A1	A1	1	1	IS/TP
	A2	A2	1	3	
Food	APT4	APT4	1	-	Finished product
	APT5	APT5	1	-	
	AMP1-2	AMP1	2	-	Raw material
	APT6-6	APT6	2	-	Finished product
	AMP1-3	AMP1	2	-	Raw material
	APT6-5	APT6	2	-	Finished product

C



No typeable					
Sample type	Isolate	Sampling site for L. monocytogenes	Sampling	Zone	Classification
Food	APT3	APT3	1	-	Finished product
	APT6-3	APT6	2	-	Finished product
	APT6-2	APT6	2	-	Finished product
	APT6-4	APT6	2	-	Finished product
	AMP1-1	AMP1	2	-	Raw material
	AMP1-5	AMP1	2	-	Raw material
	APT6-1	APT6	2	-	Finished product

Fig. 2. Plant A, dendrograms of similarity coefficients and information of *L. monocytogenes* isolates, A. **Serotype 4ac**; B. serotype 4b; C. No typeable. A1: table used for finished product, A2: flaps labelling area, A3: flats, A4: windows, A5: saw.

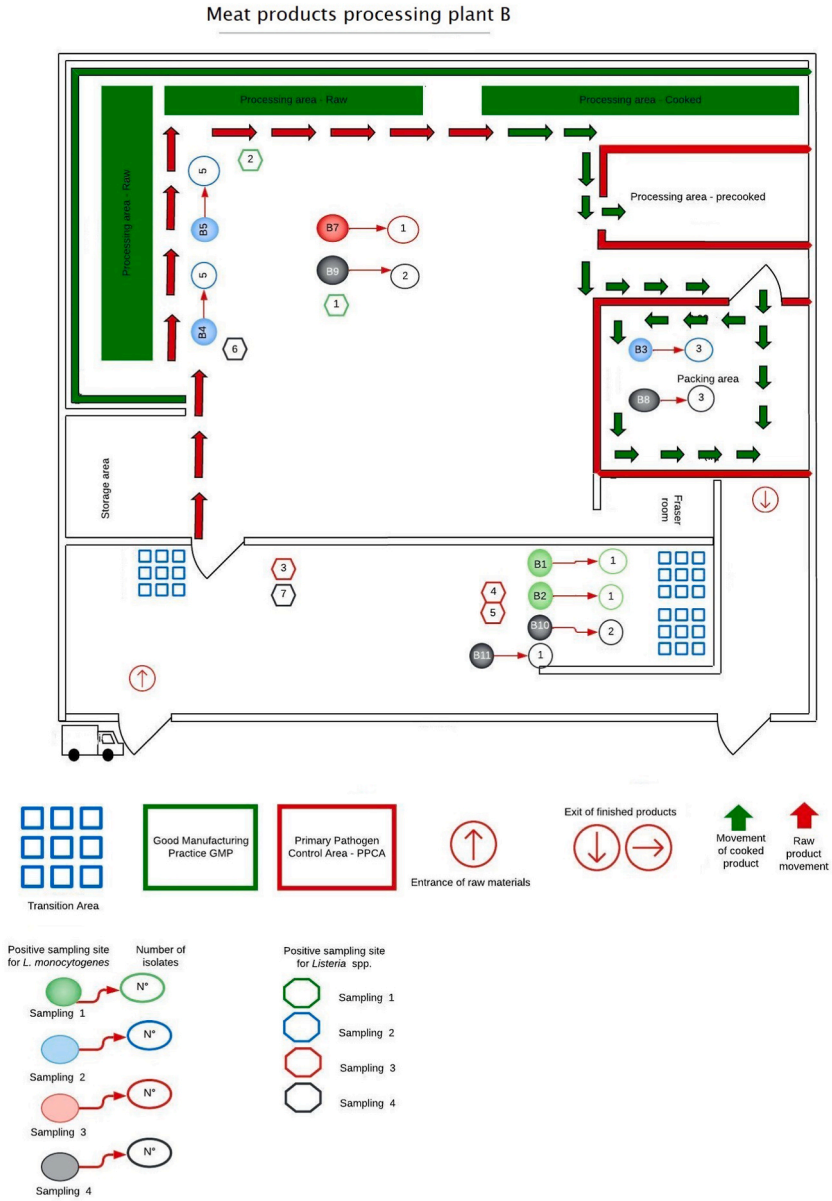
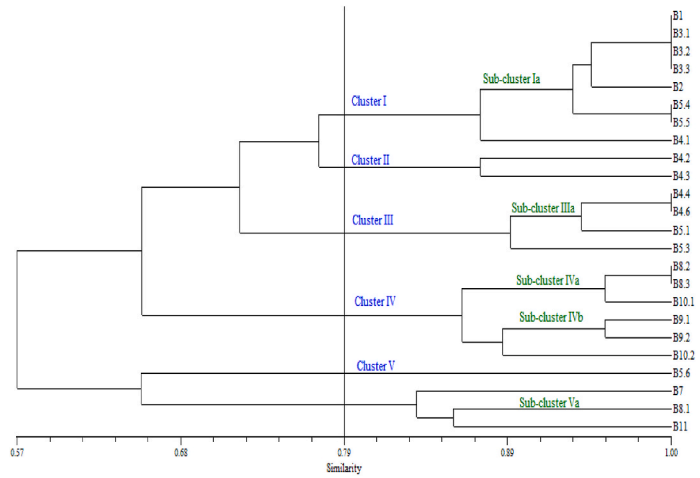


Fig. 3. Plant B, positive sampling sites for *L. monocytogenes*, circled. B1: footbaths, B2: cleaning equipment, B3: cutting machine in the packaging area, B4: cutter in the production area, B5: mixer in the production area, B6: saw, B7: drains, B8: tables for cutting the finished product, B9: drains, B10: footbaths, B11: cleaning equipment. On the other hand, for *Listeria* spp., which are marked in hexagons were: 1: drains, 2: hose, 3: drainage, reception of raw material, 4: footbaths, 5: cleaning equipment, 6: mill, 7: drainage, reception of raw material.



No typeable					
Sample type	Isolate	Sampling site for *L. monocytogenes*	Sampling	Zone	Classification
Environmental	B1	B1	1	3	IS/TP
	B3.1	B3	2	1	VS/TP
	B3.2	B3	2	1	VS/TP
	B3.3	B3	2	1	VS/TP
	B2	B2	1	3	IS/Ni
	B5.4	B5	2	1	VS/TP
	B5.5	B5	2	1	VS/TP
	B4.1	B4	2	1	VS/TP
	B4.2	B4	2	1	VS/TP
	B4.3	B4	2	1	VS/TP
	B4.4	B4	2	1	VS/TP
	B4.6	B4	2	1	VS/TP
	B5.1	B5	2	1	VS/TP
	B5.3	B5	2	1	VS/TP
	B8.2	B8	4	1	VS/TP
	B8.3	B8	4	1	VS/TP
	B10.1	B10	4	3	IS/Ni
	B9.1	B9	4	3	IS/Ni
	B9.2	B9	4	3	IS/Ni
	B10.2	B10	4	3	IS/Ni
	B5.6	B5	2	1	VS/TP
	B7	B7	3	3	IS/Ni
	B8.1	B8	4	1	VS/TP
	B11	B11	4	3	IS/Ni

Fig. 4. Plant B, a dendrogram of similarity coefficients and information of *L. monocytogenes* isolates, *A*: Not typeable. B1: footbaths, B2: cleaning equipment, B3: cutter in packaging area, B4: cutter in production area, B5: mixer in production area, B6: saw, B7: drains, B8: tables for cutting finished product, B9: drains, B10: footbaths, B11: cleaning equipment. Moreover, the letters in colours indicate green for the first sampling, blue second sampling, red third sampling and black fourth sampling. The acronym: VS refers to the verification site, IS: is the indicator site, Ni is the niche and TP is the transfer point.

separation. The presence of *L. monocytogenes* was detected in all four samplings, with the transition zone having the highest number of isolates (Fig. 3).

In plant B, 11 samples were positive and 27 isolates were obtained from them, all of them of environmental origin, 1 isolate was identified from samples B1, B2, B6, B7 and B11, 2 isolates in each of the samples B9 and B10, 3 isolates in B3 and B8; finally, 6 isolates from samples B4 and B5. Furthermore, from samples B4 (production area cutter) and B5 (production area mixer), 6 isolates were obtained in each sample; 1 of these was classified as geno-serotype 4b and 5 as non-typable. Finally, from sample B6 (sample taken from the saw) geno-serotype 1/2 c was identified, the remaining isolates were not typeable (Fig. 4). Fig. 3 shows the layout of plant B, which has an L-shaped workflow distribution, where the input of raw materials and the output of the finished product is carried out in different areas, avoiding cross-contamination. Additionally, in the external part of the production area, there are two transition areas with handwashing and foot foaming stations, frequently described in the literature for these areas [9]. However, in one of them, cleaning and disinfection materials such as brooms and cleaning rags are stored, favouring, as it is a humid area, conditions conducive to the growth of microorganisms.

3.3. Analysis of the distribution of L. monocytogenes and Listeria spp. in micro-scale production plants (plants C and D)

3.3.1. Plant C

The production plant C has a GMP area and a pathogen control area, furthermore, three cross-contamination points were identified and *L. monocytogenes* was isolated in all samples, with the labelling area being a repeat offender. When comparing the four production plants, the highest frequency of *L. monocytogenes* isolates was found in plant C.

The total number of isolates was 65, of which 1 isolate was obtained from samples (C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C16, C17, C18, C20, C24, C25, C26, C27 and C28), 4 isolates from C19, 5 isolates from samples C14, C21 and C22, 6 from C12, C15 and C23 and 8 from C13. The predominant geno-serotype was 4b with 27 isolates, followed by 1/2 a, 1/2 c with 21 isolates (Fig. 6). At this plant, 22/92 (23.9 %) belonged to CS and 70 to NFCS (76.1 %) (Supplementary Material 1.3). This plant had the highest *L. monocytogenes* and *Listeria* spp., positive samples in all environmental sampling. The sampling points for *L. monocytogenes* or *Listeria* spp., frequently isolated, were transition area, specifically the footbath (C8, C16 and C20), drip trays (C4 and C14), cleaning equipment (C10, C17 and C21), drying rails in the finished product area (C3, C9, C26 and 6), on the flat; the lower area of the drying rails (C7, C15, and C27), in the drain near the drying rails (C18 and C22) and the pressure washer (C23 and 1).

3.3.2. Plant D

Plant D has two pathogen control areas and two GMP-compliant areas, all are physically separated and a potential cross-contamination point was defined, the areas were large and had a smoking area. The frequency of *L. monocytogenes* was the lowest

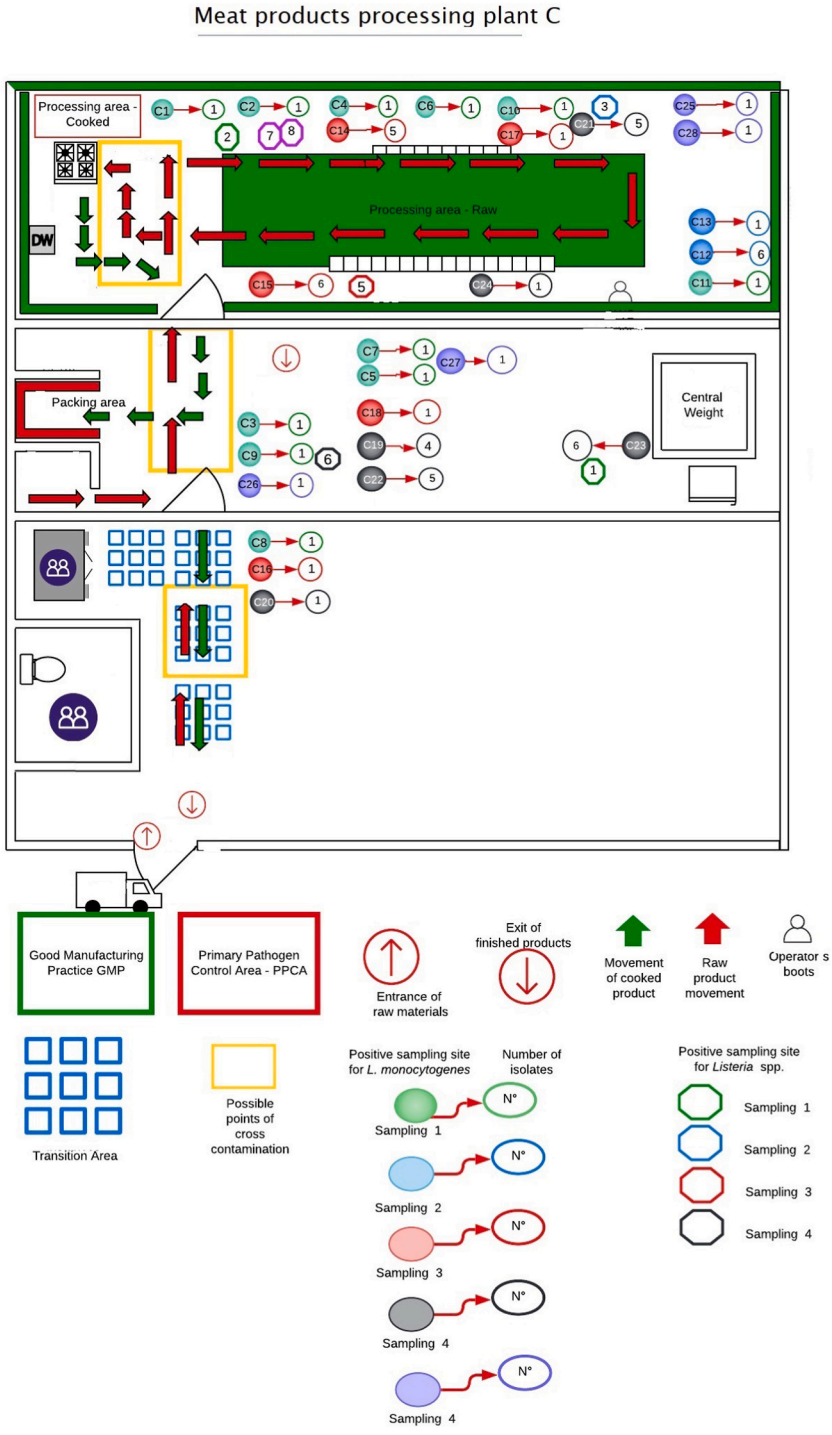
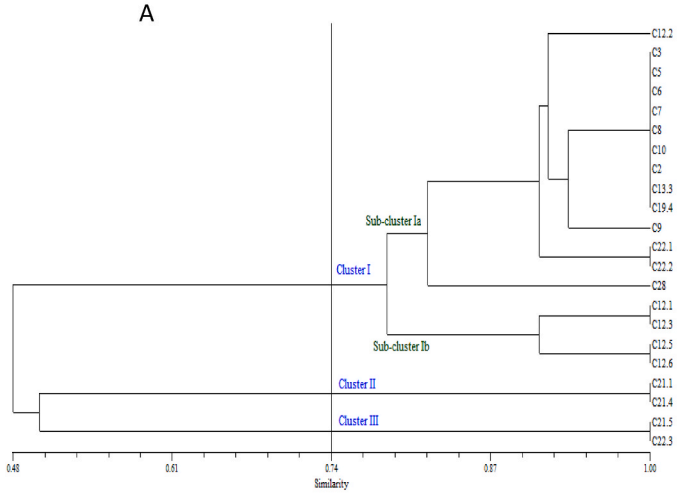


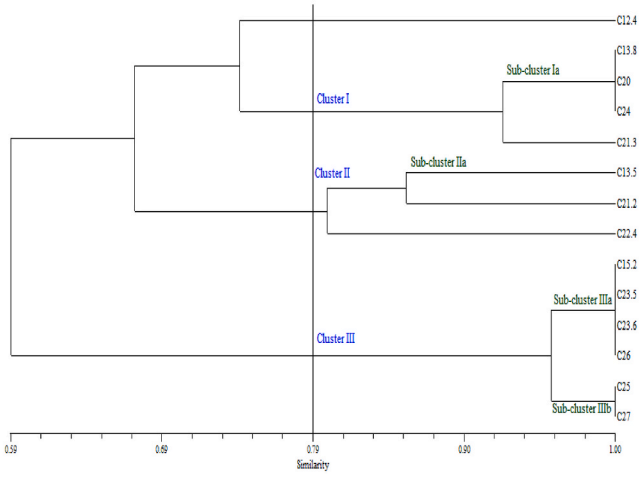
Fig. 5. Plant C, sampling sites positive for *L. monocytogenes*, circled. C1: mill, C2: knives, C3: railings in finished product areas, C4: drip pans, C5: frames and non-food contact areas, C6: drainage, C7: flats, C8: footbaths, C9: railings in finished product areas, C10: cleaning equipment, C11: bibs worn by staff, C12: finished product cutting boards, C13: drain 2, C14: drip pans, C15: flats, C16: footbaths, C17: cleaning equipment, C18: drain 3, C19: flats, C20: footbaths, C21:cleaning equipment, C22: drain 3, C23: pressure washer, C24: post-molding tub, C25: tie-down machine, C26: railings in finished product areas, C27: flat, C28: dishwasher. On the other hand, for Listeria spp., which are marked in hexagons were: 1: pressure washer, 2: hopper surface, 3: cleaning equipment, 4: drainage, 5: drainage, 6: railings in finished product areas, 7: mill, 8: packer.

A



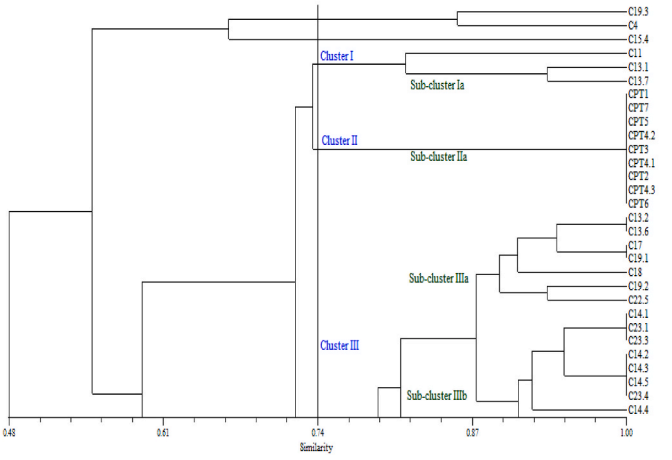
Serotype 1/2a, 1/2c					
Sample type	Isolate	Sampling site for *L. monocytogenes*	Sampling	Zone	Classification
Environmental	C12.2	C12	2	1	VS/TP
	C3	C3	1	2	IS/TP
	C5	C5	1	3	IS/Ni
	C6	C6	1	3	IS/Ni
	C7	C7	1	3	IS/TP
	C8	C8	1	4	IS/Ni
	C10	C10	1	3	IS/TP
	C2	C2	1	1	VS/TP
	C13.3	C13	2	3	IS/Ni
	C19.4	C19	4	3	IS/TP
	C9	C9	1	2	IS/TP
	C22.1	C22	4	3	IS/Ni
	C22.2	C22	4	3	IS/Ni
	C28	C28	Pilot study	3	IS/TP
	C12.1	C12	2	1	VS/TP
	C12.3	C12	2	1	VS/TP
	C12.5	C12	2	1	VS/TP
	C12.6	C12	2	1	VS/TP
	C21.1	C21	4	3	IS/Ni
	C21.4	C21	4	3	IS/Ni
	C21.5	C21	4	3	IS/Ni
	C22.3	C22	4	3	IS/Ni

B



No typeable					
Sample type	Isolate	Sampling site for *L. monocytogenes*	Sampling	Zone	Classification
Environmental	C12.4	C12	2	1	VS/TP
	C13.8	C13	2	3	IS/Ni
	C20	C20	4	4	VS/Ni
	C24	C24	Pilot study	3	IS/TP
	C21.3	C21	4	3	IS/Ni
	C13.5	C13	2	3	IS/Ni
	C21.2	C21	4	3	IS/Ni
	C22.4	C22	4	3	IS/Ni
	C15.2	C15	3	3	IS/TP
	C23.5	C23	4	3	IS/Ni
	C23.6	C23	4	3	IS/Ni
	C26	C26	Pilot study	2	IS/TP
	C25	C25	Pilot study	1	VS/Ni
	C27	C27	Pilot study	1	VS/TP

C



No typeable					
Sample type	Isolate	Sampling site for *L. monocytogenes*	Sampling	Zone	Classification
Environmental	C12.4	C12	2	1	VS/TP
	C13.8	C13	2	3	IS/Ni
	C20	C20	4	4	VS/Ni
	C24	C24	Pilot study	3	IS/TP
	C21.3	C21	4	3	IS/Ni
	C13.5	C13	2	3	IS/Ni
	C21.2	C21	4	3	IS/Ni
	C22.4	C22	4	3	IS/Ni
	C15.2	C15	3	3	IS/TP
	C23.5	C23	4	3	IS/Ni
	C23.6	C23	4	3	IS/Ni
	C26	C26	Pilot study	2	IS/TP
	C25	C25	Pilot study	1	VS/Ni
	C27	C27	Pilot study	1	VS/TP

(caption on next page)

Fig. 6. Plant C, dendrograms of similarity coefficients and information of *L. monocytogenes* isolates, A: Serotype 1/2a, 1/2c; B: Not typeable; C: serotype 4b. C1: mill, C2: knives, C3: railings in finished product areas, C4: drip trays, C5: frames and non-food contact areas, C6: drainage, C7: flats, C8: footbaths, C9: railings in finished product areas, C10: cleaning equipment, C11: overalls used by personnel, C12: cutting boards for finished product, C13: drain 2, C14: drip pans, C15: flats, C16: footbaths, C17: cleaning equipment, C18: drain 3, C19: flats, C20: footbaths, C21:cleaning equipment, C22: drain 3, C23: pressure washer, C24: post-molding tub, C25: tie-down machine, C26: railings in finished product areas, C27: flat, C28: dishwasher. Coloured letters indicate, green first sampling, blue second sampling, red third sampling and black fourth sampling. The abbreviations: VS stands for verification site, IS: indicator site, Ni: Niche, TP: transfer point.

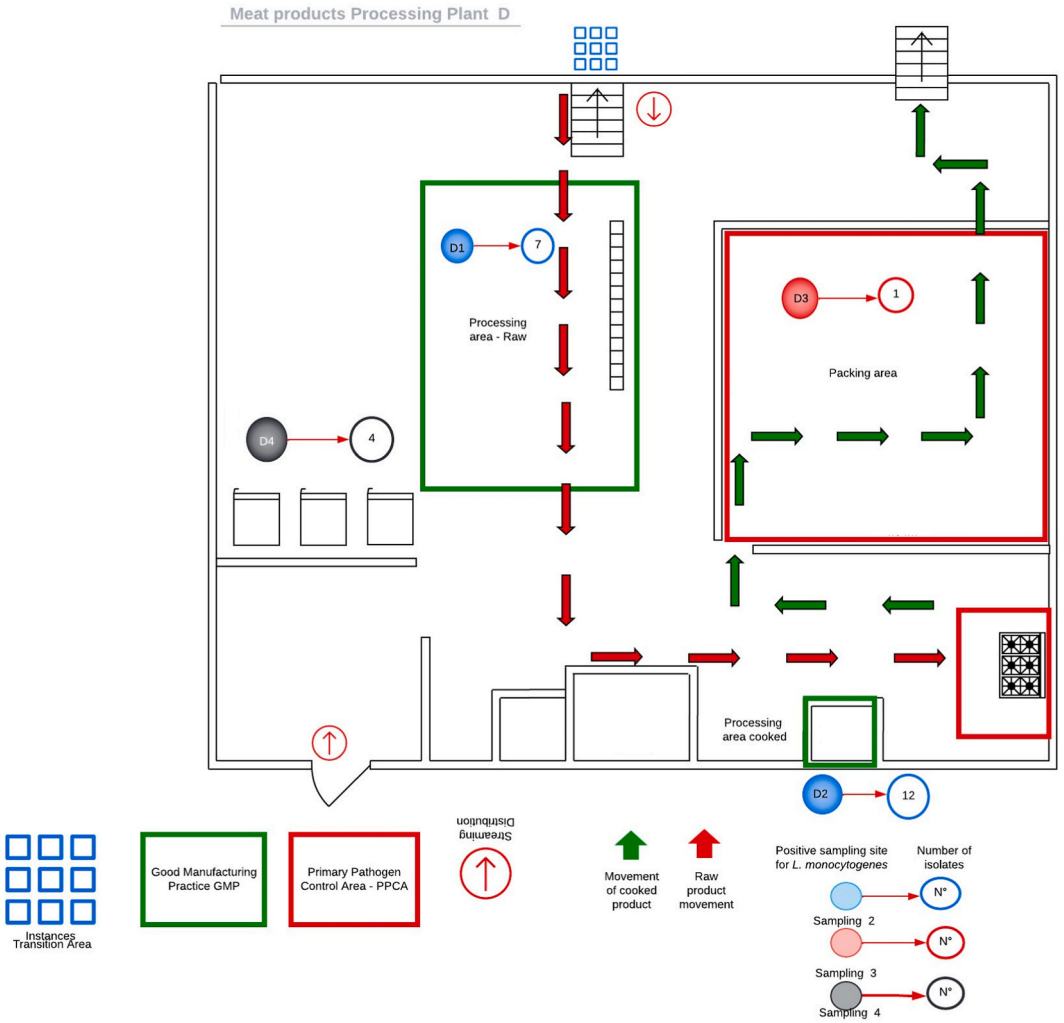
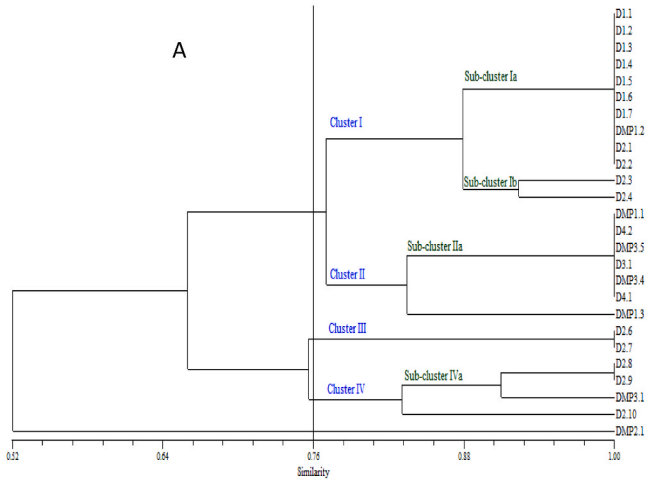


Fig. 7. Plant D, positive sampling sites for *L. monocytogenes*, circled. D1: spatula, D2: production well, D3: flat - packaging area, D4: raw material freezer.

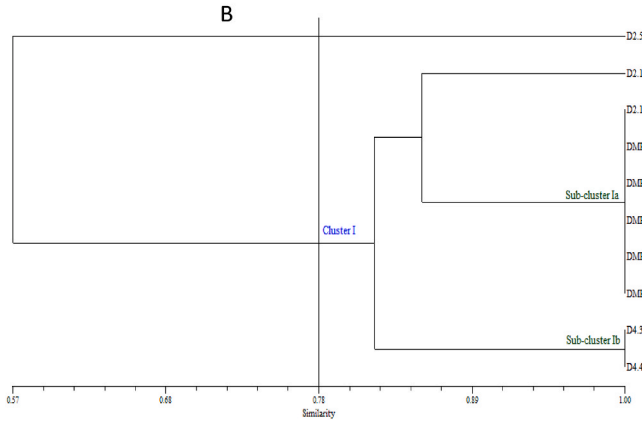
and no *Listeria* spp., isolates were obtained, (Fig. 7).

In the seven positive samples, 36 isolates were obtained, distributed as follows: 4 environmental: D1 (7), D2 (12), D3 (1) and D4 (4) and 3 from raw material DMP1 (6), DMP2 (1) and DMP3 (5). Finally, 26 isolates were typed as geno-serotype 4b and 10 were non-typable. *L. monocytogenes* positive samples were isolated from the production environment (D1 - spatula, D2 - pot in the processing area, D3 - cooking area floor and D4 - raw material freezer) and the raw material (DMP1-DMP3); samples from one sampling, (Fig. 8).



Serotype 4b

Sample type	Isolate	Sampling site for *L. monocytogenes*	Sampling	Zone	Classification
Environmental	D1.1	D1	2	1	IS/Ni
	D1.2	D1	2	1	IS/Ni
	D1.3	D1	2	1	IS/Ni
	D1.4	D1	2	1	IS/Ni
	D1.5	D1	2	1	IS/Ni
	D1.6	D1	2	1	IS/Ni
	D1.7	D1	2	1	IS/Ni
Food	DMP1.2	DMP1	2	-	Raw material
Environmental	D2.1	D2	2	3	VS/Ni
	D2.2	D2	2	3	VS/Ni
	D2.3	D2	2	3	VS/Ni
	D2.4	D2	2	3	VS/Ni
Food	DMP1.1	DMP1	2	-	Raw material
Environmental	D4.2	D4	4	3	IS/Ni
Food	DMP3.5	DMP3	2	-	Raw material
Environmental	D3.1	D3	3	3	VS/TP
Food	DMP3.4	DMP3	2	-	Raw material
Environmental	D4.1	D4	4	3	IS/Ni
Food	DMP1.3	DMP1	2	-	Raw material
Environmental	D2.6	D2	2	3	VS/Ni
Environmental	D2.7	D2	2	3	VS/Ni
Environmental	D2.8	D2	2	3	VS/Ni
Environmental	D2.9	D2	2	3	VS/Ni
Food	DMP3.1	DMP3	2	-	Raw material
Environmental	D2.10	D2	2	3	VS/Ni
Environmental	DMP2.1	DMP2	2	-	Raw material



No typeable

Sample type	Isolate	Sampling site for *L. monocytogenes*	Sampling	Zone	Classification
Environmental	D2.5	D2	2	3	VS/Ni
	D2.11	D2	2	3	VS/Ni
	D2.12	D2	2	3	VS/Ni
Food	DMP1.4	DMP1	2	-	Raw material
	DMP1.5	DMP1	2	-	
	DMP1.6	DMP1	2	-	
	DMP3.3	DMP3	2	-	
	DMP3.2	DMP3	2	-	
Environmental	D4.3	D4	4	3	IS/Ni
	D4.4	D4	4	3	IS/Ni

Fig. 8. Plant D, dendrograms of similarity coefficients and information of *L. monocytogenes* isolates, A: Serotype 4b; B: Not typeable. D1: spatula, D2: production well, D3: flat - packaging area, D4: raw material freezer. Coloured letters indicate, green first sampling, blue second sampling, red third sampling and black fourth sampling. The abbreviations: VS stands for verification site, IS: indicator site, Ni: Niche, TP: transfer point.

Table 5
Frequency of L. *monocytogenes* and *Listeria* spp. at each production site.

Meat Products processing plants	Serotype frequence (%)					
	1/2a	1/2c	4a-4c	4b	4d-4e	No typeable
A (n = 22)			7 (31.8)	8 (36.4)		7 (31.8)
B (n = 27)		1 (3.7)		2 (7.4)		24 (88.9)
C (n = 65)	21 (32.3)	1 (1.5)	1 (1.5)	27 (41.5)	1 (1.5)	14 (21.5)
D (n = 36)				26 (72.2)		10 (27.8)
Total (n = 150)	21 (14)	2 (1.3)	8 (5.3)	63 (42)	1 (0.7)	55 (36.7)

3.4. Frequency of geno-serotypes in production plants

The study yielded 150 isolates, with the highest frequency of geno-serotypeable isolates found for geno-serotype 4b, followed by 1/2a (Table 5).

4. Discussion

L. monocytogenes is present in various environments, both agricultural, urban and food processing plants, being a contaminating microorganism of finished food products in production plants, which often leads to the foodstuff withdrawal from the market. For example, in Spain, there was a registered outbreak and the only one associated with widely distributed commercial products, such as industrial-larded meat, where it was possible to genetically associate isolates from the processing environment with those detected in clinical samples ("*Alerta epidemiológica España*" 2015–2018; https://www.sanidad.gob.es/profesionales/saludPublica/ccayes/alertasActual/listeriosis/home.htm; accessed el 01-17-2023). On the other hand, in the United States, a recent outbreak was caused by deli meat and cheese (https://www.cdc.gov/listeria/outbreaks/deli-11-22/index.html; accessed 22-09-2022) and in South Africa, between June 2017 and April 2018, an outbreak (initially of unknown origin) subsequently linked to the consumption of polony (ready-to-eat processed meat); the pathogen was also present in the production environment [33]. Hence the importance of detection and monitoring of *L. monocytogenes* in production plants (the reason for our research).

On the other hand, laboratory tools for food diagnostics must be rapid, accurate and sensitive, allowing for the detection of the presence or absence of pathogens as soon as possible and to release or not the batch of food produced. However, microbial culture (inexpensive and easy to use) allows pathogens detection and identification in a sample and follow-up [34].

In the present study, two methodologies were used, a rapid one, due to the agility in obtaining the results [35] and the one established in ISO 11290-1, which allowed the identification of *L. monocytogenes* and other Listeria species, the latter serving as an indicator of the existence of an environment conducive to the growth of *L. monocytogenes* or to the masking of the pathogen [18].

4.1. Distribution analysis of Listeria spp., and Listeria monocytogenes geno-serotypes

4.1.1. Plants environmental (surfaces and equipment) and food isolates (raw material or finished product) distribution

4.1.1.1. Plant A. The GMP areas in Plant A are: i) raw material reception (where raw material reception and ingredients weighed occurred), ii) raw processing (where the production of sausages occurs) and iii) cooking (where the last pathogen control process occurs). This area is one of the most susceptible to Listeria contamination due to the entry of raw products and the exit of cooked products, which favours cross-contamination (Fig. 1). This potential point of cross-contamination is the last point of pathogen control. Failure to separate (non-contact) raw and cooked food could result in RTE (ready-to-eat) foods contaminated with pathogenic microorganisms from the environment, including *Listeria monocytogenes* and or *Listeria* spp; a situation that has been previously reported in several meat processing plants, especially for pigs [36,37]. In this sense, the infrastructure of the plant (old or modern) also has an influence, as modern plants have advantages over old ones, as they have a different spatial distribution, which prevents cross-contamination between areas, as occurs in plant A of this study (Fig. 1) [38].

The positive samples for *Listeria* spp. were distributed in all areas of the production plant, demonstrating that suitable environmental conditions existed for the growth of *L. monocytogenes*. The finding of the microorganism in the pathogen control area, detected in the first sampling before the start of the working day, demonstrated possible failures in the cleaning and disinfection process [16]. Additionally, one of the areas considered as "dirty", where GMP must be complied with, is the band saw, which is for cutting raw material during processing; a similar splitting saw equipment has been a point of transmission of Listeria in meat processing plants and slaughterhouse of raw beef, poultry, pig [39–42]. However, according to Simmons and Wiedmann (2018), this sampling point can be classified as a niche [15]. In the case of this production plant, the detection of *Listeria* spp., was positive in two different samplings; *L. monocytogenes* detection occurred in one sample and *Listeria* spp. in the other. The equipment contamination probably occurred due to the entry of contaminated raw material (Fig. 1), which is due to a sanitary design flaw that can be common in this type of production plant.

Other places where Listeria was detected (in more than one sampling) were the pressure washer, the drain of the production and the cooling areas. Aerosols are frequently formed at these sites, favouring microorganisms' dissemination in the production plant [43–45]. However, there is a discussion on the definition of drains as sampling points, both in favour, because they are an indicator of the environment in the production plant, and against, because being recognised as a niche does not allow precise detection for monitoring the pathogen in the plant [19] (Fig. 1).

In the CS, the positive sampling points for *L. monocytogenes* were the band saw and the finished product table, designated as an immediate risk for contamination of meat and meat by-products [38]. In the case of NFCS, the positive points were flats, windows and flaps or strip curtains, all of which are part of the pathogen control zone; NFCS are typical harborage sites, hence their importance in the food industry. Flats are a point of dissemination of Listeria, a worsened condition when are damp, as was the case in the production plant under study [44]. Similarly, windows have also been reported as a positive sampling point for the isolation of the microorganism, according to Fate et al. (2021); these authors describe in their study that very-small food processing plants may have a shortage of equipment and personnel, a crucial factor affecting cleaning and disinfection processes [46].

4.1.1.2. Plant B. At this plant, *L. monocytogenes* isolates were more frequent than *Listeria* spp. (Table 4). The areas where the microorganism was recurrent were the footbath, the drains (located in the centre of the production plant) and the cleaning equipment (Fig. 4). The finding of recurrent microorganisms (in some cases persistent) shows their survival ability, even in adverse environmental conditions, such as during food processing, temperature, and a_w, and insufficient cleaning and disinfection schedules. Some authors have related persistent isolates to the ability to adapt to refrigeration temperatures and highly acidic and highly salty environments, as well as to the ability to form biofilms on a variety of abiotic surfaces, enhancing the microorganisms' resistance to dehydration and to cleaning and disinfection processes [47,48].

4.1.1.3. Plant C. Plant C has a large GMP area for product processing; however, outside, there is the packaging area, which is small and uncontrolled temperature, for drying the finished product. Consequently, due to this distribution, the transfer of raw material and finished product share the same areas, which allowed the detection of three possible cross-contamination areas, the transition area, the production area, and the packaging area (Fig. 5). Among the recognised routes of dissemination of Listeria are: entry into the processing environment through contaminated raw material, poor practices of food handlers, and failures in the management of flows within the plant or through contaminated vehicles [49,50]. Production plant C has flaws in the design of raw material, personnel and finished product flows, which could lead to the dissemination of the microorganism in the environment, considered the most frequent form of contamination of the finished product [51].

4.1.1.4. Plant D. Plant D was the microenterprise with ample area, in which the production and processing of meat products is not constant, only twice a month, a frequency that increases during the Christmas season. The plant layout has a large and separate GMP and pathogen control area for each process, as the cooking area, in which we found a cross-contamination zone due to the entry of raw material and the exit of the cooked product (Fig. 7). *L. monocytogenes* has an ample spectrum of natural reservoirs, which implies a competitive advantage over other microorganisms and facilitates the possibility of persisting in food processing environments [52]. One way for transferring *L. monocytogenes* is through wet surfaces, hence the need to monitor water storage sites or material washing areas, such as the processing area. Another form of cross-contamination is through raw material or utensils in contact, causing the microorganism to be disseminated [53]. However, despite the *L. monocytogenes* on the spatula used during processing and on the raw material, the pathogen was not from the finished product detected.

4.1.2. Molecular serotyping characterisation of plants isolates

4.1.2.1. Plant A. In production Plant A, both in the environment and in the finished product, the most frequent geno-serotype of *L. monocytogenes* was 4b (42 %); this geno-serotype has been frequently detected in cases of food outbreaks [54]. The finding of the geno-serotype is of concern, even more so in RTE, being a risk for consumers (Fig. 2B). The raw material sample (AMP1) had five characteristic Listeria isolates, of which 1 (20 %) was geno-serotype *L. monocytogenes* 4ac, 2 (40 %) were *L. monocytogenes* 4b and 2 (40 %) were non-typeable *L. monocytogenes*; demonstrating the geno-serotype variability that can be found in a single sample and supporting the need to isolate as many suspected colonies of the genus as possible.

4.1.2.2. Plant B. In the production Plant B, the 27 isolates obtained belonged to environmental samples, of which 24 (89 %) could not be geno-serotyped by the methodology used, 2 (7.4 %) belonged to geno-serotype 4b and 1 (3.7 %) to geno-serotype 1/2c.

4.1.2.3. Plant C. Plant C has a large GMP area for product processing; however, outside, there is the packaging area, which is small and uncontrolled temperature, for drying the finished product. Consequently, due to this distribution, the transfer of raw material and finished product share the same areas, which allowed the detection of three possible cross-contamination areas, the transition area, the production area, and the packaging area (Fig. 5).

This plant has the highest *L. monocytogenes* and *Listeria* spp., positive samples in all environmental sampling. Among the recognised routes of dissemination of Listeria are: entry into the processing environment through contaminated raw material, poor practices of food handlers, and failures in the management of flows within the plant or through contaminated vehicles [49,50]. Production plant C has flaws in the design of raw material, personnel and finished product flows, which could lead to the dissemination of the microorganism in the environment, considered the most frequent form of contamination of the finished product [51].

4.1.2.4. Plant D. Plant D was the microenterprise with ample area, in which the production and processing of meat products is not constant, only twice a month, a frequency that increases during the Christmas season. The plant layout has a large and separate GMP and pathogen control area for each process, as the cooking area, in which we found a cross-contamination zone due to the entry of raw material and the exit of the cooked product (Fig. 7).

L. monocytogenes has an ample spectrum of natural reservoirs, which implies a competitive advantage over other microorganisms and facilitates the possibility of persisting in food processing environments [52]. One way for transferring *L. monocytogenes* is through wet surfaces, hence the need to monitor water storage sites or material washing areas, such as the processing area. Another form of cross-contamination is through raw material or utensils in contact, causing the microorganism to be disseminated [53]. However, despite the *L. monocytogenes* on the spatula used during processing and on the raw material, the pathogen was not from the finished product detected.

4.1.3. ERIC-PCR of plants isolates

4.1.3.1. Plant A, isolate's ERIC-PCR. ERIC PCR analysis allowed the grouping of the isolates by genetic similarity within each geno-serotype. Fig. 2A (geno-serotype 4ac), Fig. 2B (geno-serotype 4b) and Fig. 2C (non-typeable isolates) showed 2 clusters also.

Of the geno-serotype 4ac isolates (Fig. 2A), hose from flats (A3) and windows (A4) were genetically similar; sampling points were in the same area (cluster I) and classified as niches. A similar situation occurred with geno-serotype 4b isolates from the finished product table (A1) and flaps (A2) (Fig. 2B), both sampling points defined as TP/IS according to Simmons and Wiedmann (2018); it is noteworthy that although flaps or strip curtains used in areas where finished product are exposed are sampling point recommended by EFSA, it is not frequent to identify them as a sampling point in literature [15]. The scientific literature on this point as a sampling site is scarce, and there are no publications about *L. monocytogenes* in meat derivative production plants. All sampling points: A1, A2, A3, and A4 in the packaging presented air flows to maintain the temperature (Fig. 1); a report by Berrang et al. (2013) describes this area as " … *that a hose spray or flooding of a flat drain can result in the creation of aerosolized bacteria, specifically Listeria* … ", it is necessary to study if the dissemination of the microorganism is occurring by this route [44].

4.1.3.2. Plant B, isolate's ERIC-PCR. The analysis by ERIC-PCR with the non-typeable isolates (Fig. 4), grouped in 5 clusters with a similarity higher than 0.79; of these, subcluster IA grouped seven isolates, of which B1 - footbath; from sample 1, B3.1, B3.2 and B3.3 - cutter; sample 2, cluster III, subcluster IIIA, with isolates B4. 4 and B4.6- cutter; from sample 2, cluster IV, subcluster IVA isolates B8.2, B8.3 - Tables for cutting finished product and from subcluster, IVB isolates B9.1, B9.2 from drains, the groupings presented a similarity index of 1.00, suggesting that the isolates could be of the same type.

In other studies, using techniques such as wgSNP and MLST, a high degree of genetic diversity of *L. monocytogenes* has been demonstrated independently of sampling sites [55]; genetic variability could demonstrate why some isolates were not typeable.

Some authors [56] demonstrated the ability of pathogenic and contaminating bacteria to survive in disinfecting footbaths with chlorine and suggest that these should be administered carefully and alternated with other disinfectants at adequate concentrations and have an established hygienic monitoring frequency. It is noted that this area is generally not assumed to be safe in production plants and is rarely sampled, them this study points out the importance of including it as a sampling point. On the other hand, *L. monocytogenes* can adhere to various materials (characteristic of persistent isolates) [57] that compromise processing equipment such as the slicer [51] and promote cross-contamination.

Several factors can affect the cross-transfer of pathogens during slicing, including the concentration of the pathogen on the product and the surface of the slicer, feed composition, cutting force, blade speed and other factors such as temperature, surface topography and contact time [58], favouring possible contamination scenarios such as i) contamination of the slicer blade [51], ii) contamination of the product to the slicer blade and iii) the formation of micro-colonies on the surface of products for slice. On the other side, products with high-fat content facilitate contamination of the equipment by leaving excessive fat smearing. Also, it is necessary to clarify that the transfer is a discrete phenomenon, even though the slicing process is carried out sequentially [51,57,58].

4.1.3.3. Plant C, isolate's ERIC-PCR. ERIC PCR analysis grouped each geno-serotype: Fig. 6A for geno-serotypes 1/2a, 1/2c, formed by 3 clusters. Fig. 6B shows the grouping of non-typeable isolates in 3 clusters. In Fig. 6C, geno-serotype 4b isolates generated 3 clusters. As in other production plants, we found different geno-serotypes in the same sample: C12 (tables for cutting finished product), C13 (drainage in the processing area), C19 (flats in the packaging area), C22 (drainage in the packaging area), C21 (cleaning equipment), C15 (flats) and C23 (pressure washer).

In Fig. 6A for geno-serotypes 1/2a and 1/2c, isolates grouped in subcluster IB, cluster II and cluster III have a similarity index of 1.0 but were from the same sampling process, for which were considered as a "transient" or "transient persistent", isolate considered as a microorganism that enters the production environment but eliminated through cleaning and disinfection efforts [50]. On the other hand, a division of subcluster IA into two branches occurred, where isolates distributed in the processing area (C2 - knives, C6 - drain 1, C10-cleaning equipment and C13 - drain 2), packaging area (C3 - metal rails used for drying of the finished product, C5 - frames and non-food contact areas, C7 and C19 - flats in the packing area in two different samplings) and the transition zone (C8 - footbaths) come from several sampling times and have a similarity index of 1.0, considering possible similar isolation.

Fig. s 5 and 6A demonstrate that a similar isolate would be rotating through the mentioned areas and being at three different sampling times; it is an isolate with persistent characteristics, defined by some authors as a micro-organism that survives for an extended period [50]. However, calling it "persistent" would be hasty, as the concept of persistence is still widely discussed, and no consensus has been reached on its definition [59]. Additionally, it is crucial to monitor geno-serotypes 1/2a, 1/2b and 1/2c, as they have been recognised in cases of human listeriosis and may have competitive advantages over other geno-serotypes such as 4b in meat and meat products kept under refrigeration [60]. Therefore, positive sampling points in the packaging area are the most worrying, as this is where the ready-to-sell products are.

In Fig. 6C (geno-serotype 4b), subcluster IIA grouped nine isolates from the finished product. They all came from the same sample, and the similarity index was 1.00, showing that all isolates could have a common ancestor. Isolates with high similarity came from drainage samples (highest index 0.87) in sample 2 and from the bib of one of the food handlers in sample 1 (highest index 0.74). Food employees or food handlers can be vectors for transferring microorganisms, so boots, gloves and dungarees constant monitoring is necessary [36,50].

4.1.3.4. Plant D, isolate's ERIC-PCR. Fig. 8A shows the isolates grouped by ERIC-PCR belonging to geno-serotype 4b, cluster I,

subcluster IA grouped a higher number of isolates and a similarity index of 1.00 gathered: 7 isolates from sample D1 - spatula (possibly the same colony), one from raw material and two from the piggery located in the production area, all isolates were recovered during sampling 2. On the other hand, cluster II in the same geno-serotype, with a similarity index of 1.00, grouped isolates from raw material (sample 2), packaging area floor (sample 3) and raw material storage freezer (sample 4). A similar occurred in the non-typeable isolates (Fig. 8B); in Cluster I, Subcluster IA, isolates from raw material and the pot were grouped in a single cluster with a similarity index of 1.00, demonstrating the mobility of a similar strain (probably the same isolate), through the plant in samples 2, 3 and 4 and the genetic homogeneity of the recovered isolates.

The control of suppliers is one of the crucial aspects of pathogen control [61], as they can become the gateway to the plant due to the handling of the operators, who could be the vehicle for transferring microorganisms through the processing lines; in this respect, knowing that raw materials may contain listeria, the transport of raw materials within the plants should be limited to avoid cross-contamination [62].

4.2. Results comparison between production plants

L. monocytogenes and *Listeria* spp. were in all three studied production plants (A, B and C); only in micro-plant D, *Listeria* spp. was undetected. Considering the ubiquity of Listeria, the detection of positive samples during routine monitoring or outbreak investigation allows processors or investigators to perform a more extensive study to accurately identify potential routes of contamination to analyse the cause and perform corrective actions (to control the main contamination route(s)); crucial in small production plants, it also allows the description of the epidemiological distribution of *Listeria* spp., to be performed [63,64].

On the other hand, some *Listeria* spp., such as *L. innocua*, share physiological characteristics with *L. monocytogenes*, which is conducive to frequent coexistence in food and competition between strains. In this sense, in the initial phase of identification, during enrichment, different species can grow; in this way, the presence of *L. innocua* and other *Listeria* spp. can interfere with the detection and isolation of *L. monocytogenes* [65].

The sampling points with Listeria most frequently detected in all production plants were drains, cleaning equipment, footbaths, floors, finished product drying rails and mills (Supplementary Material 2). Drains were the points where Listeria was isolated most frequently, a sampling point also detected by Simmons and Wiedmann (2018), which was classified as a niche and transfer point, hence its importance.

4.2.1. Small production plants A and B

Small production plants have been responsible for some Listeriosis outbreaks in the United States and some European countries. However, studies in small and very small RTE meat plants have been few, perhaps because the importance of production and distribution of products at local and regional levels is sometimes not recognised [66], hence the relevance of having studies in this type of company.

The distribution of the infrastructure of production plants A and B is different. Plant A have an old architectural structure, which prevents continuous processes from occurring, which could lead to cross-contamination. In plant B, despite the proper product handling (L-shaped production flow), the problems were outside the GMP area, which became a source of dissemination of the microorganism. On the other hand, in studies comparing meat processing environments of large and small plants, no difference in community composition was observed; however, the authors point out that contamination of meat often depends on the environment in which it is handled and processed [67]; namely, in the case of meat product production plants, there are no similar studies.

Small plants A and B have 6–7 production lines, of which the majority are ready-to-eat products, and the others are pre-cooked. However, contamination of RTE products is almost always associated with the packaging process, subsequent handling or home use [68]; hence the concern that in plant B, the slicer and the tables (used for the finished product) were *L. monocytogenes* positive for, and at the plant A, the highest frequency of pathogens initially was detected in the packaging area.

The frequency ratio between *L. monocytogenes* and *Listeria* spp. was irregular in each plant. Plant A resulted, with the highest number of *Listeria* spp., while plant B had the highest frequency of *L. monocytogenes*. However, testing for *Listeria* spp. as an indicator of *L. monocytogenes* is a routinary practice; the relationship between *L. monocytogenes* and other *Listeria* spp. is still unknown because it is unknown whether there is an affinity between the pathogenic species and other Listeria species [66,69].

4.2.2. Microfood processing plants C and D

Microfood processing plants or very-small RTE meat plants have a different number of employees in each country; in the case of Colombia, until a few years ago, it involved a maximum of 10 employees; the resources in these production plants limit labour, space and financing for the purchase, operation and maintenance of decontamination methods; a similar situation has occurred in other studies [70]. Hence the need for continuous monitoring of production environments.

Plants C and D showed different scenarios for *L. monocytogenes* and *Listeria* spp. In the case of plant C, the frequency of *L. monocytogenes* was the highest in the whole study, while plant D had the lowest. On the other hand, the production frequency in plant C is five days a week, while in plant D, it is only twice a month, although the production levels of both plants increase in December. The variability in the forms of production and the presence of micro-organisms in these make it necessary to monitor the causative agents of TSE in small plants, as studies based on the farm-to-table concept have shown that contamination of end products usually occurs in processing plants or at distribution points and less frequently from the animal source [71].

On the other hand, production plant C was unique in which possibly persistent isolation occurred; a situation previously studied [59], in which no single genetic persistence mechanism has been detected for Listeria, while *L. monocytogenes* strains presented more

mutations related to hypovirulence and less invasive disease.

Finally, *L. monocytogenes* detection occurred in all production plants, highlighting problems in flow production management, cleaning and disinfection programmes [7,50,71]. In methodological terms, the contamination points searching favoured the strengthening sampling plans and application of appropriate intervention strategies for controlling *L. monocytogenes*, as demonstrated by the "*seek and destroy*" policy [36].

5. Conclusions

The methodology implemented in the study allowed continuous tracking of the microorganism throughout the production plants on contact and non-contact surfaces. The frequency of *L. monocytogenes* in the plants studied ranged from 5.9 to 28.6 %, with genoserotype 4b being the most frequent. On the other hand, the study detected possible transient isolates on plants A, B and D and persistent isolates on plant C. Among the possible causes of dissemination were: failures in flow management, raw material contaminated with *Listeria* spp. and *L. monocytogenes* and transfer of the microorganism from contaminated surfaces, which cleaning and disinfection programmes failures. The sampling point with the highest frequency of Listeria in the production plants was drainage, which becomes relevant when located in the packaging area. This area of pathogen control had isolates of *L. monocytogenes* and or *Listeria* spp., in all the production plants in the sampling carried out during the study; for this reason, we recommend strengthening the pathogen control programmes in micro or small production plants with due advice and based on the traceability of the microorganism using constant sampling methodologies, as proposed in the present study.

Funding

This research was funded by the "*Departamento Administrativo de Ciencia, Tecnología e Innovación*" (COLCIENCIAS, actually *Ministerio de Ciencia, Tecnología e Innovación de Colombia* MINCIENCIAS), call for proposals 733, contract No. FP 44842-186-2017 with "*Pontificia Universidad Javeriana*", (PUJ), Bogotá, D.C. Colombia. The APC was funded by "*Pontificia Universidad Javeriana, Bogotá D.C., Colombia*".

Data availability statement

Further details and information related to this research can be found at Figshare https://figshare.com/account/items/25435960/edit, (https://doi.org/10.6084/m9.figshare.25435960).

CRediT authorship contribution statement

Sandra M. Rincón-Gamboa: Writing – original draft, Investigation, Funding acquisition. **Raúl A. Poutou-Piñales:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Ana K. Carrascal-Camacho:** Writing – review & editing, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28662.

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