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Study of the transfer of *Listeria monocytogenes* during the slaughter of cattle using molecular typing

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ABSTRACT

The introduction, transmission, and persistence of *Listeria monocytogenes* in Belgian beef slaughterhouses was investigated using genetic characterization. During slaughter, samples were taken of the hide, carcass, and environment to detect the pathogen. Remarkably, *L. monocytogenes* was massively present on the hide of incoming animals (93%; 112/120), regardless of their visual cleanliness, which implies high contamination pressure levels entering the slaughterhouses. Pathogen transfer via cross-contamination was conclusively confirmed in this study, with the same pulsotypes isolated from the hide, carcass, and environmental samples. Despite the important bacterial presence on the hide of incoming animals, most slaughterhouses succeeded in limiting the transfer as cause of carcass contamination. Persistence along the slaughter line seemed to be a more significant problem, as it was clearly linked to most of the *L. monocytogenes* positive carcasses. In one slaughterhouse, whole genome sequencing (WGS) revealed that the carcass splitter had been contaminating carcasses with the same strain belonging to CC9 for more than one year.

1. Introduction

Human listeriosis is one of the most severe foodborne illnesses in the EU, as it causes high rates of hospitalization and mortality, especially in high-risk populations. It currently occurs at a relatively low incidence rate (< 1 per 100,000 EU inhabitants) but, a significant upward trend has been observed since the start of EU monitoring in 2008. *Listeria monocytogenes* infections are most commonly reported in people over 64 years of age, and especially in the age group over 84 years old (EFSA and ECDC, 2019). More *Listeria* cases can be expected as the European population ages. Consumer habits, particularly increased consumption of ready-to-eat (RTE) food products, may also increase the risk.

The One Health 2018 Zoonoses Report (EFSA and ECDC, 2019) mentioned above reports an overall *L. monocytogenes* prevalence of 1.4% in RTE meat products examined between 2016 and 2018. The incidence of the pathogen was up to 3.1% in RTE meat products from bovine origin. Interestingly, the detection of *L. monocytogenes* was higher in samples collected in food processing plants than in retail. To prevent the introduction of *L. monocytogenes* in meat cutting plants and meat processing companies further along the agri-food chain, control of the pathogen at the slaughterhouse level is absolutely essential. *L. monocytogenes* is highly prevalent in cattle farm environments, ranging from 24% to almost 50% in previous studies, which implies a significant contamination risk in slaughterhouses via incoming animals (Esteban, Oporto, Aduriz, Juste, & Hurtado, 2009; Nightingale et al., 2004). In particular, bovine hides are proposed to contribute significantly to the *L. monocytogenes* contamination of carcasses by cross-contamination (Wieczorek, Dmowska, & Osek, 2012). Subsequently, contaminated carcasses were considered an important source for pathogen presence on the final meat product (Nastasijevic, Milanov, Velebit, Djordjevic, & Swift, 2017).

Also, the persistence of *L. monocytogenes* in the processing environment is considered the primary source of contamination (EFSA BIOHAZ Panel et al., 2018).

Despite the efforts made to control cross-contamination and avoid environmental persistence, we have recently confirmed that Belgian bovine slaughterhouses are still confronted with a significant presence of *L. monocytogenes* on carcasses at the end of the slaughter line

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(Demaître et al., 2020). Moreover, except for Wieczorek et al. (2012), few studies have been performed to determine contamination pressure via beef cattle entering slaughterhouses and the specific transfer to carcasses during slaughter. Contemporary molecular typing methods open interesting research pathways as they enable source attribution and persistence detection along the slaughter process and subsequent meat processing steps (EFSA BIOHAZ Panel et al., 2018). In addition to the conventional molecular subtyping techniques, including PFGE, used to characterize isolates, the discrimination power of WGS is now increasingly applied, also to investigate pathogen introduction and persistence in meat processing facilities (Fagerlund, Langsrud, & Møretrø, 2020; Hurley et al., 2019; Nastasijevic et al., 2017).

The present study aimed: to determine (1) the *L. monocytogenes* prevalence and genetic diversity along the beef slaughter process, to identify (2) contamination sources and pathogen transfer during the slaughter of cattle, and to assess (3) the correlation between visible hide cleanliness of incoming animals and the final pathogen's presence on carcasses before chilling.

2. Materials and methods

2.1. Sampling

Between November 2017 and May 2018, four Belgian cattle slaughterhouses (A-D) with a line speed of 20–35 animals h^{-1} were visited three times to collect samples from hides and carcasses. The time period between the first and the third sampling per slaughterhouse was max. 15 weeks. During each visit, ten randomly selected carcasses were followed throughout the slaughter process for sampling. The animals originated from over 35 different farms. Hide swabs were taken from both individual hind legs (left and right), the brisket, and the foreleg (approximately 400 cm^2 area per site) on the carcasses in a hanging position just before the manual dehiding of the corresponding sites started (Fig. 1A). Hide samples were taken along dehiding incisions. Brisket and foreleg hide swabs were taken from one half of the carcass, alternating left and right. At the end of the slaughter line but before chilling, the same carcass half was swabbed at the same three locations (approximately 400 cm^2 area per site, Fig. 1B). Swab samples were collected using sponge-sticks (3 M, St. Paul, MN, USA) pre-moistened with 20 ml of sterile Maximum Recovery Diluent (MRD; Oxoid, Basingstoke, UK). The time between taking the first hide sample and the last carcass swab of the concerned animal was approximately 45 min.

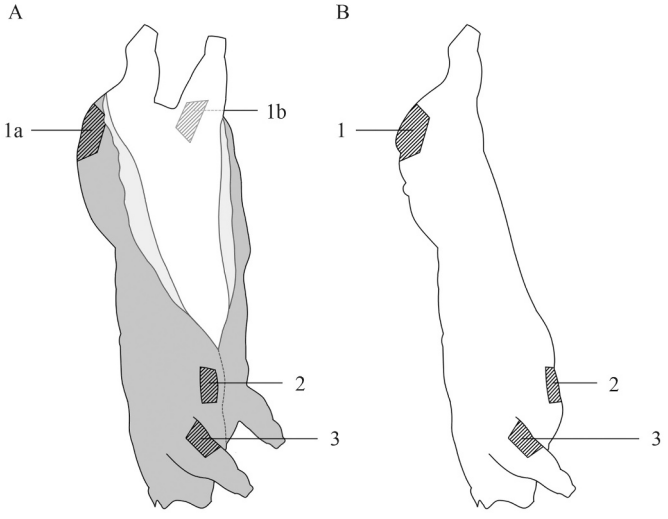

Fig. 1. A. Sampled sites on beef cattle hides: both individual left and right hind leg (1a, 1b); of one carcass half brisket (2) and inside foreleg (3). B. Sampled sites on corresponding carcasses: the same carcass half was swabbed at the same three locations (1, 2 and 3).

Additionally, 252 environmental samples were collected during these 12 sampling visits. The same number of samples was taken at three fixed time points per sampling day, i.e., just before sampling the first carcass, after sampling five carcasses, and after sampling the last carcass. Twenty-one samples were collected during each visit (3 × 4 knife blade samples, 3 × 1 evisceration platform sample, and 3 × 2 air samples). Knife blades and evisceration platforms were swabbed using sponge-sticks pre-moistened with 20 ml of sterile MRD. Sampled knives were all used at the manual predehiding steps and were swabbed on both sides (approx. 56 cm^2, $n = 144$). Good slaughterhouse practices require these knives to be placed in 82 °C water for disinfection between each use. Each knife was sampled twice: (1) immediately before use to check adequate disinfection ($n = 72$, 2) after the opening of the hide to determine whether pathogens were picked up (n = 72). Evisceration platforms (approx. 50 cm^2, $n = 36$) were swabbed at the contact surface where each carcass brisket collides with the device. Air samples (200 l/4 min.; n = 72) were taken within 2 m from the automatic hide puller with a Biotest Hycon RCS Air Sampler (BIOTEST AG, Frankfurt, Germany) containing a plate count agar media strip (PCA; Oxoid, Basingstoke, UK) upon which airborne microorganisms were collected for later analysis.

Based on the results of these samplings, in a follow-up study carcass splitters were intensively swabbed as part of additional research during one year (June 2018–June 2019). In slaughterhouses A, B, and D, the carcass splitter was initially sampled at 20 different locations after cleaning and disinfection immediately prior to production. Due to circumstances, it was not possible to sample the carcass splitter in slaughterhouse C. Per sampling day, 17 easily accessible sites, more specifically at the exterior of the carcass splitter, saw blade, and easy to reach visible interior surfaces, were sampled using sponge sticks pre-moistened with 20 ml MRD. Moreover, two hard-to-access sites on the inside of the carcass splitter (e.g., behind flywheel with saw blade) were sampled using cotton swab sticks (Cultiplast® swab LP Italiana, Milan, Italy) pre-moistened with 2 ml of sterile MRD. The last sample was taken from the outflowing (tap) water at the drain. After the initial screening, the carcass splitter was further monitored. Results in slaughterhouse A showed the need to remove the persistent presence of *L. monocytogenes* in the carcass splitter, resulting in two more samplings after cleaning and disinfection ($n = 20$) and once during production ($n = 17$) in the concerned slaughterhouse. The incoming clean tap cooling water of the carcass splitter at the device's water inlet was also sampled these times to determine whether the incoming water was *Listeria*-free. During this period, the device was subjected to a thorough cleaning and optimized disinfection as recommended by a specialized company.

All samples were transported under cooled conditions to the laboratory, where they were kept at 3 ± 2 °C and analyzed the same day.

2.2. Hide cleanliness

Information about the hide cleanliness of incoming animals was collected at the time of sampling. Cattle hides of selected animals were visually classified in terms of hide cleanliness at the level of the hind leg, brisket and foreleg just before the start of manual predehiding of the corresponding site. Hide cleanliness was evaluated according to 5 cleanliness categories previously described by the Irish Department of Agriculture and Livestock, using a scale of 1 (very clean) to 5 (very dirty) based on the amounts of adherent dirt/feces (McEvoy et al., 2000).

2.3. Microbiological analyses

Before analysis, swab samples were homogenized for 2 min using a Stomacher Lab Blender 400 (Seward Laboratory, London, United Kingdom). In addition to the analysis of the initial homogenate, further dilutions were analyzed to semi-quantify numbers in hide and quantify numbers in carcass samples.

For hide samples, the initial swab homogenate, as well as two

successive 10-fold dilutions, were analyzed for *L. monocytogenes* presence, namely 2 ml (10–99 CFU/400 cm^2) and 0.2 ml (>100 CFU/400 cm^2) from the homogenate. To these last two volumes, 18 ml half-Fraser broth was added and the detection of *L. monocytogenes* occurred as described below.

For *L. monocytogenes* enumeration in carcass swab samples, 1 ml of each homogenate was plated onto two Agar *Listeria* plates according to Ottaviani and Agosti (ALOA; Bio-Rad, Marnes-La-Coquette, France). For further dilution, 100 µl was plated using an Eddy Jet 2 spiral plater (IUL Neutec Group, Inc., Barcelona, Spain) onto ALOA agar plates, thus requiring subsampling of 2 ml of the homogenate. All plates were incubated at 37 °C for 48 h before enumeration. If present, suspect *L. monocytogenes* colonies were counted. The lower quantification limit was −1.30 log CFU/cm^2 (or 20 CFU/400 cm^2) for carcass swab samples.

To the remaining volumes of the initial homogenates (approx. 17 ml) from hide and carcass, the same volume of double concentrated half-Fraser broth was added. For samples taken from the environment, carcass splitters, and water samples, only detection of *L. monocytogenes* was performed. An additional 20 and 2 ml of half-Fraser broth was added to each sponge and cotton swab, respectively. Water samples were primarily enriched by adding the same volume of double concentrated half-Fraser broth.

All half-Fraser broths were incubated at 30 ± 1 °C for 24 ± 2 h. Then 0.1 ml from each incubated broth was transferred into 10 ml Fraser broth (Bio-Rad, Marnes-La-Coquette, France). After incubation at 37 ± 1 °C for 48 ± 2 h, a loopful (10 µl) was streaked onto an ALOA plate. Plates were incubated at 37 ± 1 °C for 24 ± 2 h. From each sample yielding suspect colonies on ALOA, one *L. monocytogenes* colony was picked and purified on ALOA plates. From ALOA plates used to quantify *L. monocytogenes* in carcass samples, also one suspect colony was picked for purification. Purified colonies were then streaked on Tryptone Soya Yeast Extract Agar (TSYEA) plates (TSA; Oxoid / YE [0.6%]; Oxoid) and incubated for 24 ± 2 h at 37 ± 1 °C. All isolates ($n = 330$) were stored in brain heart infusion broth (BHI; Oxoid) with glycerol (15%, vol/vol) at −80 °C for further testing.

2.4. Molecular analysis

From each positive sample, the isolate obtained after initial detection enrichment was genotyped by Pulsed-Field Gel Electrophoresis (PFGE) after being confirmed by multiplex PCR (Herman, De Ridder, & Vlaemynck, 1995) and serotyped (Borucki & Call, 2003). The same procedure was carried out for carcass samples from the direct platings. PFGE was performed according to the PulseNet standardized procedures (Graves & Swaminathan, 2001) with *Asc*I and *Apa*I enzymes (New England BioLabs, Ipswich, MA, USA). Similarities between *Asc*I and *Apa*I fingerprint patterns were calculated using BioNumerics version 7.6 software package (Applied Maths, Sint-Martens-Latem, Belgium) to assign pulsotypes from which multilocus sequence typing (MLST) information could subsequently be derived (Félix et al., 2018; Maury et al., 2016). Dendrograms were generated using a band-based Dice coefficient for similarity (optimization of 1%; position tolerance of 1%), and the Unweighted Pair Group Method with Average Linkages (UPGMA) for clustering of fingerprints. An 85% similarity was applied to differentiate pulsotypes (Demaître et al., 2020).

In order to confirm the persistence of the same strain, whole genome sequencing (WGS) for core genome MLST analysis (cgMLST) was performed on five carcass isolates, including one isolate from our previous study (Demaître et al., 2020). All samples that belonged to the same pulsotype and were repeatedly isolated from the slaughterhouse A over a period of 18 months. Genomic DNA of the selected isolates was extracted using the DNeasy Blood and Tissue Extraction Kit (Qiagen, Aarhus, Denmark). DNA extracts were further processed by Admera Health (NJ, USA) for fragmentation and library preparation (KAPA Hyper PCR-free library prep kit) and HiSeq sequencing (2 × 2.5 M paired-end reads, 2 × 150 bp). Read quality was evaluated using FastQC 0.11.5 (Andrews, 2010). Furthermore, the data was checked for contamination using Kraken2 version 2.0.8-beta against the standard database (Wood, Lu, & Langmead, 2019). Before continuing with the analysis, reads were randomly subsampled to not exceed a coverage of 100 times that of the median *L. monocytogenes* genome length (2.97 Mbp). Genome assembly was performed using SPAdes version 3.11.1 (Bankevich et al., 2012). Finally, the cgMLST profiles for the selected *L. monocytogenes* isolates were determined using chewBBACA 2.0.17.2 (Silva et al., 2018) with 1748 housekeeping genes defined in the Institut Pasteur *L. monocytogenes* cgMLST scheme (Moura et al., 2016). Allelic profiles were mutually compared between the strains to determine the number of allelic differences.

2.5. Statistical analysis

The relationship between *L. monocytogenes* prevalence on hide samples ($n = 330$) and hide cleanliness scoring data ($n = 292$) was assessed using linear regression models, including 'slaughterhouse' and 'carcass sites' as main effects and interaction terms. Confidence intervals for prevalence of *L. monocytogenes* were calculated using binomial distributions. All analyses were performed with RStudio version 1.2.5001 using R version 3.5.0 (R Core Team, R Foundation for Statistical Computing, Vienna, Austria); packages ggplot2, lme4 and effects were used to process the data.

3. Results

3.1. Hide samples

On average, 93% [(112/120); 95% CI: 89–98%] of the incoming animals were found to be externally contaminated by *L. monocytogenes* on at least one of the four sampled hide sites. In slaughterhouses A and B, all hides of incoming ruminants were contaminated with *L. monocytogenes* (100%; 30/30). For slaughterhouses C and D, the prevalence was 90% (27/30) and 83% (25/30), respectively. For the four slaughterhouses together, the prevalence found on hide sites were approximately the same: more specifically left hind leg 68% (81/120), right hind leg 65% (78/120), brisket 74% (89/120), and foreleg 68% (82/120).

Three predominant serogroups were found, namely IVb, IIb, and IIa, representing 36%, 32%, and 29% of the isolates, respectively. Other much less commonly found serogroups were IIc (2%) and IVa (1%).

Further molecular typing showed a large genetic heterogeneity among hide isolates on the slaughterhouse level (Fig. 2). The 330 *L. monocytogenes* isolates represented 35 pulsotypes; of these 26 were grouped into 24 CCs and 2 STs. In total, 7 CCs and one ST were more prevalent: CC1 (15%), CC77 (10%), CC224 (9%), CC6 (9%), CC5 (6%), CC37 (5%), CC59 (5%) and ST191 (4%). Together, these represented 62% (205/330) of all hide isolates and were also found in each slaughterhouse. In general, the diversity of CCs present on animals delivered across the different slaughterhouses was comparable (Fig. 2). Also, the vast majority of incoming animals (75%) carried at least two different clones at the sampled sites on their hides. In 13% of the animals, each of the four sampled sites carried a different CC. The percentage of *L. monocytogenes* positive samples per hide cleanliness category is shown in Table 1. The overall proportion of *L. monocytogenes* positive samples were similar across hide cleanliness categories and fluctuated between 65 and 73%. No significant correlation was found between the *L. monocytogenes* presence ($p = 0.109$) or semi-quantitative numbers of the pathogen ($p = 0.076$) and hide cleanliness scores. In most bovine hide samples (39%, 115/292), more than 100 CFU per 400 cm^2 could be found. In 31% (90/292) the numbers were between 10 and 99 CFU/400 cm^2 and in 30% (87/292) of the cases between 1 and 9 CFU/400 cm^2.

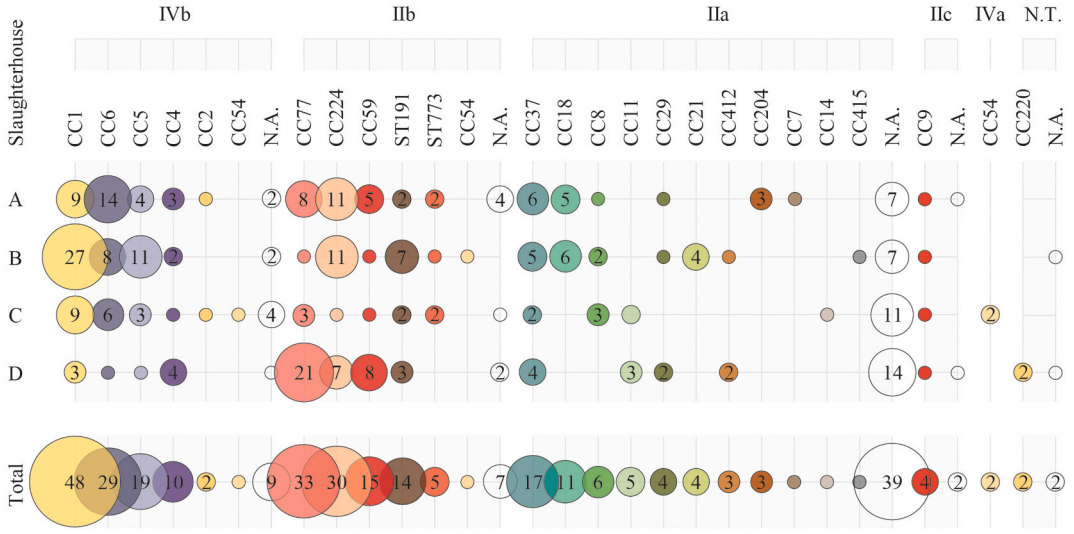

Fig. 2. Genetic diversity of *L. monocytogenes* from hide isolates (n = 330) according to serogroup and CCs. Dots are colored according to the CCs and dot sizes are proportional to the number of isolates within the cluster. N.A. = Not assigned. N.T. = Not typeable by PCR.

Table 1
Percentage of *L. monocytogenes* positive samples given per hide cleanliness category and per site. The scale for hide cleanliness ranged from 1 (very clean) to 5 (very dirty).

Carcass site	Cleanliness categories									
	1		2		3		4		4	
	N[a]	P (%)[b]	N[a]	P (%)[b]	N[a]	P (%)[b]	N[a]	P (%)[b]	N[a]	P (%)[b]
Hind leg (left)	17	10 (58,8)	31	22 (71,0)	34	26 (76,5)	25	15 (60,0)	11	7 (63,6)
Hind leg (right)	17	8 (47,1)	31	23 (74,2)	34	21 (61,8)	25	16 (64,0)	11	9 (81,8)
Brisket	23	20 (87,0)	23	16 (69,6)	18	14 (77,8)	28	22 (78,6)	27	17 (63,0)
Foreleg	38	31 (81,6)	32	20 (62,5)	20	13 (65,0)	21	14 (66,7)	8	4 (50,0)
All sites	95	69 (72,6)	117	81 (69,2)	106	74 (69,8)	99	68 (67,7)	57	37 (64,9)

[a] N=Number of samples.
[b] P=Proportion of *L. monocytogenes* positive samples.

3.2. Carcass samples

3.2.1. Overall results

L. monocytogenes could be isolated from 43% [(51/120); 95% CI: 34–51%] of the carcasses before chilling. The hind leg (29%; 35/120) was most contaminated, followed by the brisket (22%; 26/120) and the foreleg (13%; 15/120).

Seventy-six (76/360; 21%) *L. monocytogenes* isolates were collected from the carcasses, and four different serogroups were identified. Serogroup IIc was by far the most frequently encountered serogroup,

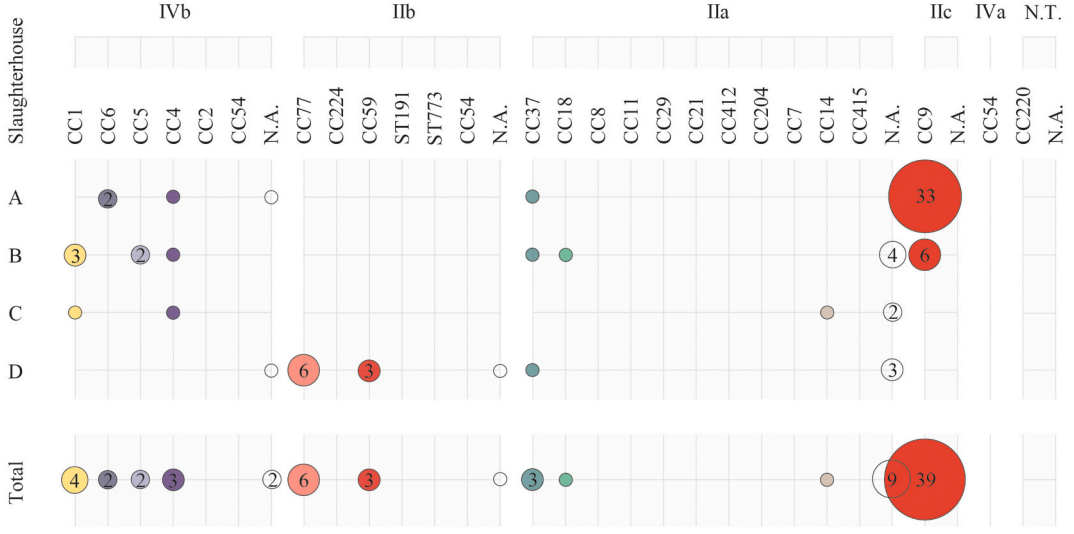

Fig. 3. Genetic diversity of *L. monocytogenes* from carcass isolates (*n* = 76) according to serogroup and CCs. Dots are colored according to the CCs and dot sizes are proportional to the number of isolates within the cluster. N.A. = Not assigned. N.T. = Not typeable by PCR.

accounting for 51% of the carcass isolates (39/76). The other serogroups were IIa (18%; 14/76), IVb (17%; 13/76), and IIb (13%; 10/76).

In total, 15 pulsotypes could be categorized within the isolates, 10 of which could be assigned to CCs (Fig. 3). The most prevalent pulsotype, corresponding to CC9 (serogroup IIc), accounted for 51% (39/76) of the strains. The remaining 9 (CC77, CC1, CC37, CC59, CC4, CC5, CC6, CC14, and CC18) together accounted for only 33%. In the specific cases CC1, CC6, CC37, CC59, and CC77, hide-meat transmission could be confirmed, ultimately resulting in 10% positive carcasses (Fig. 4). All isolates from samples with a *L. monocytogenes* contamination level of more than 20 CFU/400 cm^2 ($n = 9$) were identified as the same pulsotype and consequently belonged to the same CC of their corresponding isolates after enrichment.

3.2.2. Results at slaughterhouse level

Fig. 4 gives a detailed overview of *L. monocytogenes* isolates found per slaughterhouse. Noteworthy were the observed differences in the number of contaminated carcasses between the four slaughterhouses. In slaughterhouse A, 23 (77%) of the 30 sampled carcasses were contaminated with *L. monocytogenes*, in slaughterhouse B 15 carcasses (50%), in slaughterhouse C 5 carcasses (17%), and in slaughterhouse D 8 carcasses (27%).

Molecular characterization of carcass isolates showed striking differences between the slaughterhouses. In total, 38, 18, 5, and 15 isolates were recovered from the carcasses in slaughterhouses A, B, C, and D, respectively. Most notable was the dominant occurrence of CC9 (33 isolates) in slaughterhouse A, isolated from 87% (20/23) of the positive carcasses. This pulsotype is consistent with the mapped CC9. All sampled carcass locations represented this same CC over the three sampling days: the hind leg (16/38), the brisket (10/38), and the foreleg (7/38). Isolates belonging to other clones, particularly CC4, CC6, and CC37, were found only sporadically (11%; 4/38). Across the 38 carcass samples containing *L. monocytogenes* in this slaughterhouse, three samples had counts above 20 CFU/400 cm^2. In the range of 55–400 CFU/400 cm^2, all were CC9.

The same pulsotype was also found on 40% (6/15) of the *L. monocytogenes* positive carcasses in slaughterhouse B, representing one-third of the isolates (33%; 6/18). The CCs CC1, CC5, CC4, CC37, and CC18 together accounted for 44% of the strains isolated in this slaughterhouse. On carcasses from slaughterhouse B, three *L. monocytogenes* samples containing CC4, CC5, and CC9 barely exceeded the lower enumeration/quantification limit (20 CFU/400 cm^2).

CC9 was not identified from isolates in slaughterhouses C and D. In slaughterhouse C, only five isolates were recovered from five carcasses, and all belonged to different pulsotypes. Three could be assigned to CC1, CC4, and CC14, respectively. Genetic clones CC77 and CC59 were only found on carcasses in slaughterhouse D, during sampling day 3, and accounted for 60% (9/15) of the isolates collected in this slaughterhouse. One isolate corresponded to CC37. Three isolates of the above CCs had *L. monocytogenes* counts ranging from 20 to 60 CFU/400 cm^2.

3.3. Environmental samples

3.3.1. General results

In addition to hide and carcass samples, a total of 252 environmental samples were taken during sampling, 17 of which were positive for *L. monocytogenes* [7%; 95% CI: 4–10%] (Fig. 5). Knife blades sampled before making the first cut occasionally still contained *L. monocytogenes* (6%; 4/72). The pathogen was also recovered from seven samples taken from knives used after making the first cut during manual skinning (10%; 7/72). Twice a knife tested positive for *L. monocytogenes* both before and after cutting, but the genetic CCs were different before and after.

Four samples (11%; 4/36) taken from the contact point of the evisceration platform tested positive for *L. monocytogenes*. Another two *L. monocytogenes* positive samples (6%; 2/36) were detected in the air close to the automatic hide puller.

Serogroups IIa and IIb were most commonly identified in the environment with six isolates each (35%; 6/17) followed by serogroup IVb (18%; 3/17) and IIc (12%; 2/17). Further molecular typing revealed nine pulsotypes, seven of which were assigned to CC37, CC77, CC6, CC9, CC1, CC59, and CC224, which in total accounted for 89% of the environmental isolates analyzed.

3.3.2. Results at slaughterhouse level

The *L. monocytogenes* contamination in environmental samples was 16%, 5%, 0%, and 6%, respectively, for slaughterhouses A, B, C, and D.

In contrast to the predominant carcass contamination in slaughterhouse A with CC9, the results of the environmental samples did not indicate the presence of dominant isolate types (Fig. 5). Therefore, another source of carcass contamination was likely to be present. The presence of CC9 in all sampled sites, i.e., from hind to foreleg, together with the knowledge of carcass sampling in the same slaughterhouse in our previous study (Demaître et al., 2020) where eight different carcass sites were mostly contaminated with CC9, led to the hypothesis that the carcass splitter could be the source of this persistent contamination. Therefore, additional sampling was conducted to test for the presence of *L. monocytogenes* in the carcass splitter.

3.3.3. Carcass splitters

In slaughterhouses B and D, *L. monocytogenes* was not isolated from any swab samples ($n = 20$) taken from carcass splitters. However, other *Listeria* spp. were still present in 6 and 10 out of 20 samples, respectively, especially at hard-to-access sites.

L. monocytogenes was extensively isolated from the carcass splitter in slaughterhouse A (20/20). Samples of the cooling water taken directly from the device's water inlet were free of *L. monocytogenes* during the additional sampling days (0/3), while the outflowing water samples taken at the drain were almost always positive for the detection of the pathogen (2/3). After a thorough cleaning and adapted disinfection, the presence of *L. monocytogenes* in swab samples of the carcass splitter was reduced but still noticeably present: August 2018 47% (9/19) and March, 2019 32% (6/19). Samples taken from the splitter during production in September 2018 were all *L. monocytogenes* positive. All isolates belonged to serogroup IIc and the same pulsotype and could be attributed to CC9. The carcass splitter isolate was subjected to cgMLST and was subsequently confirmed to belong to ST9. It only differed up to seven allelic mismatches (Moura et al., 2016) from isolates found on carcasses in February 2017 and March 2018. In addition, the same strain was also isolated from a knife used in November 2017.

4. Discussion

The results show a remarkably high prevalence of *L. monocytogenes* on hides from cattle in Belgian slaughterhouses (93%), indicating a near-constant influx of *L. monocytogenes* into cattle slaughterhouses. To the best of our knowledge, this is the first time that a contamination pressure of this magnitude has been demonstrated, as nearly every incoming animal was externally contaminated with *L. monocytogenes*. Other studies reported a much lower prevalence of the pathogen on bovine hides in slaughterhouses, ranging from 10% to 27% (Guerini et al., 2007; Khen, Lynch, Carroll, Mcdowell, & Duffy, 2015; Rivera-betancourt et al., 2004; Wieczorek et al., 2012).

Multiple explanations for these contrasting findings are possible. First, in this study, multiple sites of the hides of the animals ($n = 4$) were sampled and individually analyzed. In contrast, the studies mentioned above focused on one specific site, usually the brisket, if mentioned. However, we still get a percentage of 74% positive animals if we only consider brisket results in our study. Second, sampling method and further enrichment may influence the results, as both abovementioned US studies, one significant area (1000–1700 cm^2) was sampled, but the initial sample volumes were aliquoted to include other parameters and

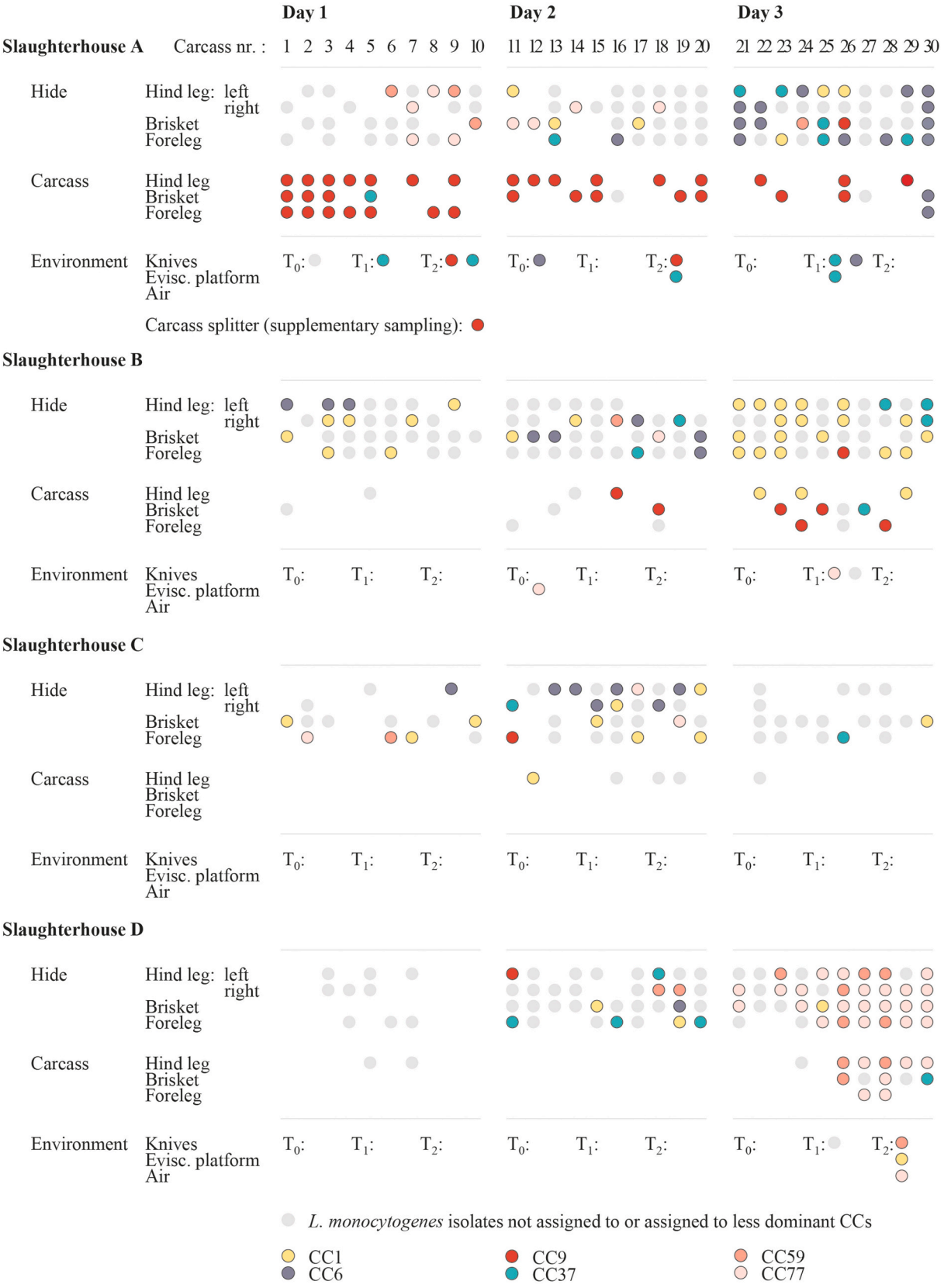

Fig. 4. Detailed overview of *L. monocytogenes* isolates found per slaughterhouse with attention for specific predominant genetic types confirming transfer and persistence. Results of each tracked animal are presented, per sampling day and per farm. Environmental *L. monocytogenes* samples are classified by time (T_0, T_1 and T_2).

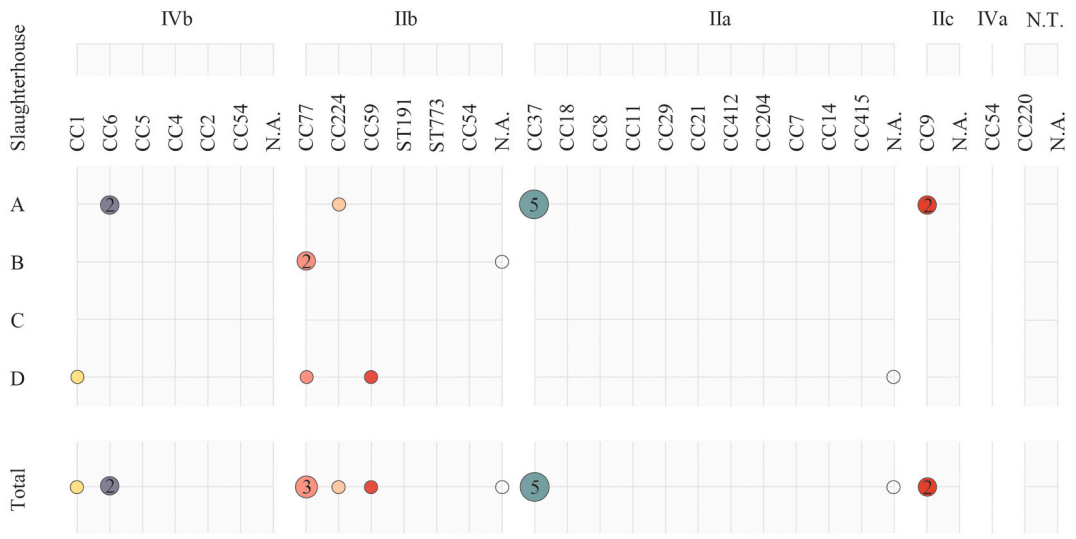

Fig. 5. Genetic diversity of *L. monocytogenes* from environmental isolates ($n = 17$) according to serogroup and CCs. Dots are colored according to the CCs and dot sizes are proportional to the number of isolates within the cluster. N.A. = Not assigned. N.T. = Not typeable by PCR.

were likely reduced. This could decrease the chance of *L. monocytogenes* detection when present in small numbers. Third, several factors may influence the prevalence of *L. monocytogenes* on the hides of the animals, such as differences in beef production systems, transport and lairage-related conditions, the origin of cattle presented for slaughter, and geographical variations in patterns of bovine carriage and shedding of *L. monocytogenes* (Guerini et al., 2007; Khen et al., 2015). Seasonality may also play a role. The study of Guerini et al. (2007) shows that *Listeria* is more prevalent on hides during the colder months. Also, fecal shedding of *L. monocytogenes* at cattle farms peaked in winter through silage-associated amplification in healthy ruminants, with fecal shedding rates exceeding 60% compared to less than 10% in summer (Nightingale et al., 2005). Although we cannot adjust for the seasonal effect within this study as the experiments ran in winter and spring, seasonality could partly explain the high prevalence found on bovine hides.The vast majority of *L. monocytogenes* isolates from bovine hides appear to belong to serogroups IVb, IIb, and IIa, which are commonly isolated from ruminants and seem to be widespread in farm environments (Esteban et al., 2009; Guerini et al., 2007; Khen et al., 2015; Orsi, Bakker, & Wiedmann, 2011; Wieczorek et al., 2012). The serotypes within these groups, specifically serotype 1/2a, 1/2b, and 4b, have been associated with 98% of all human listeriosis (EFSA BIOHAZ Panel et al., 2018). Further, PFGE analysis showed large genetic heterogeneity, confirming the environmental exposure of ruminants to a broad range of strains (Dreyer et al., 2016). Also, all assigned MLST clonal complexes (CCs) included one defined pulsotype. Within this broad variation, the results demonstrated the high prevalence of frequent pulsotypes corresponding with the following clonal complexes: CC1, CC77, CC224, CC6, CC5, CC37, and CC59. Most of these are human infection-associated clones belonging to lineage I, except for CC37 (Maury et al., 2016). CC1 is present in farm environments and is even strongly associated with listerial encephalitis in ruminants (Dreyer et al., 2016; Maury et al., 2019). CC37, CC59, and CC77 are associated and likely better adapted to the farm environment than to the meat production environment (Dreyer et al., 2016; Félix et al., 2018). Isolates of serogroups IIc, including CC9, and IVa were only rarely found on bovine hides. Likewise, CC9 was not, or only rarely, isolated in farm environments, confirming a lower adaptation to the primary sector (Dreyer et al., 2016; Félix et al., 2018).

Concerning visible dirtiness of bovine hides, visual cleanliness scores were not related to the occurrence of *L. monocytogenes* on hides. Excessively dirty animals do not, therefore, assure the presence of *L. monocytogenes* or vice versa. The few other studies on the relationship between visual hide cleanliness and microbial contamination of bovine hides report similar results for other microorganisms. Blagojevic, Antic, Ducic, and Buncic (2012) found no clear relationship between the visual hide cleanliness and the presence of *E. coli* O157 on hides. Even visually clean bovine hides, intuitively believed to have a lower microbial load, carry high numbers of bacteria, including pathogens (Antic et al., 2010). Several studies support the assumption that dirty animals yield an increased number of bacteria on the carcasses (Hauge, Nafstad, Rotterud, & Nesbakken, 2012; McEvoy et al., 2000). However, the study of Gill et al. (2004) reported inconsistent findings regarding visible and microbiological contamination of hides and carcasses and highlighted the importance of the skinning skills concerning the microbiological contamination of carcasses. With each incoming animal potentially externally contaminated with *L. monocytogenes*, regardless of the cleanliness of the animals, this study also emphasizes the importance of good hygienic practices during skinning.

Skinning practices have already been identified as a significant microbial contamination source, including *L. monocytogenes* (Arthur et al., 2010; Bell, 1997; Dickson & Anderson, 1992; Khen et al., 2015). Nevertheless, strain characterization in this study showed a less important direct transfer of *L. monocytogenes* from the heavily loaded hides from incoming animals to the carcasses, particularly in slaughterhouses B, C, and D (Fig. 4). According to Antic et al. (2010), carcass microflora is a relatively constant fraction of the hide microflora with low hide-to-meat transmission rates; this is consistent with our findings. The number of positive carcasses confirmed by hide-meat transmission cases by identical pulsotypes or even isolates found on the hide and carcasses that differed by up to five alleles in cgMLST was limited to 10% (Fig. 4). Also, environmental samples, others than carcass splitter, showed a low prevalence of *L. monocytogenes*, highlighting the application of proper skinning practices most of the time with even no transmission on some sampling days (Fig. 4).

On the other hand, at the end of the slaughtering process, a more critical source of carcass contamination was found. The same strain belonging to CC9 was often isolated from carcasses in slaughterhouse A. This strain was rarely present on animal hides. It has been reported before that CC9 seems to find favorable settlement conditions in the meat processing environment, persists in slaughterhouse environments, and is a hypovirulent clone associated with meat products (Fagerlund et al., 2020; Félix et al., 2018; Maury et al., 2019; Stoller, Stevens, Stephan, & Guldimann, 2019). It concerned a clone adaptation to this specific ecological niche, with a higher prevalence of stress resistance and benzalkonium chloride tolerance genes and a biofilm formation capacity, which may enhance its survival (Maury et al., 2019; Stoller

et al., 2019). Consequently, CC9 is a typical clone, particularly in Europe that is relatively more common in food, especially in meat products, than in human cases (Chenal-Francisque et al., 2011).

Further cgMLST analyses of selected CC9 isolates from slaughterhouse A confirmed the hypothesis of persistence of this strain in this slaughterhouse for more than one year and identified the carcass splitter as contamination source. These findings meet all the requirements described in the definition of persistent isolates by Hurley (2019). In that slaughterhouse CC9 contamination of every carcass from top to bottom was confirmed during splitting. Moreover, it proved challenging to eradicate its presence in the carcass splitter completely. The ability of CC9 to persist may indicate insufficient sanitation practices in this particular slaughterhouse and highlights the importance of thorough cleaning (including mechanical, thermal, and chemical aspects) before disinfection, which is the most effective way to detach adhered cells and biofilms (Gibson, Taylor, Hall, & Holah, 1999). However, carcass splitters have small spaces and narrow openings that are difficult to access during cleaning and disinfection, resulting in poor cleaning and inadequate disinfectant performance (Thévenot, Dernburg, & Vernozy-Rozand, 2006). The design of these devices is a compromise between functionality, efficiency, worker safety, and ease of cleaning and disinfection, where the latter may be more challenging to achieve for practical reasons. This may lead to persistence and biofilm formation problems, which was also the case in other slaughterhouses: Nastasijevic et al. (2017) isolated CC9 from the water drain at carcass splitting. The persistent presence of CC9 on the slaughter line for slaughterhouse A results in a higher number of positive carcasses at the end of the slaughter process compared to the other slaughterhouses. Also, the transmission of pathogens from contaminated knife blades, air, or evisceration platform may lead to carcasses to test positive, but to a much lesser extent than the persistence of CC9, at the carcass splitter. This demonstrates the importance of frequent environmental pathogen monitoring, removal, and avoidance in the future. Avoiding persistence will accordingly reduce contamination risks and persistence in successive processing units in the meat chain.

5. Conclusions

This study provides a comprehensive view of the introduction, transmission, and persistence of *L. monocytogenes* in some beef slaughterhouses in Belgium. Bovine hides appear to be a significant source of *L. monocytogenes* whose presence cannot be predicted from hide cleanliness. Sporadic transfer to carcasses during skinning practices therefore proved to be inevitable. Proper manual skinning practices are essential to minimize the transmission of pathogens to carcasses, as demonstrated by *L. monocytogenes*-free carcasses on some sampling days in certain slaughterhouses.

This study also shows that the persistence of *Listeria* contamination on the slaughter line was the main cause of the problem and accounted for the majority of the contaminated carcasses. This was due to the persistent presence of CC9, a hypovirulent clone associated with meat processing environments, in the carcass splitter in one of the slaughterhouses.

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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