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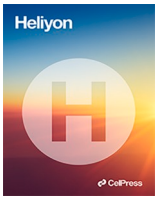

Genetic population structure of *Listeria monocytogenes* strains isolated from salmon and trout sectors in France

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ABSTRACT

Smoked salmon and smoked trout are ready-to-eat and potentially contaminated with the pathogenic bacterium *Listeria monocytogenes* making them high risk for the consumer. This raises questions about the presence of hypervirulent or persistent strains in the salmon and trout industries. Knowledge of the genetic diversity of circulating strains in these sectors is essential to evaluate the risk associated with this pathogen and improve food safety. We analyzed the genetic structure of 698 strains of *L. monocytogenes* isolated from 2006 to 2017 in France, based on their serogroup, lineage and clonal complexes (CCs) determined by Multilocus sequence typing (MLST). Most of the CCs were identified by mapping the strains PFGE profiles and a novel high-throughput real-time PCR method for CC identification. We identified thirteen CCs and one sequence type (ST) with variable distribution in salmon and trout samples (food, environment). The three most prevalent CCs were CC121, CC26 and CC204. Strains from ST191 and CC54 were detected for the first time in these sectors, while less than 0.6% of the isolates belonged to the hyper-virulent CC1, CC6 and CC20. No CC was exclusively associated with the salmon sector. This project allowed us to assess the population diversity of CCs of *L. monocytogenes* in the salmon and trout industries.

1. Introduction

Listeria monocytogenes is a Gram-positive bacterium that is small and ubiquitous, and causes a rare but serious foodborne disease called listeriosis. The disease has a lethality rate of 20–30% and mainly affects at-risk populations such as the elderly, pregnant women and their newborns, and the immunocompromised [1]. It is important to note that 99% of cases of listeriosis are due to the ingestion of contaminated food (foodborne transmission) which causes a major threat to consumer health and results in significant economic losses [2].

Since the 1980's, *L. monocytogenes* has been incriminated in several epidemics at an international level, particularly related to seafood products. From 2010 to 2017, four epidemics of *L. monocytogenes* associated with the consumption of fish or fishery products were reported to the EFSA by Germany (one epidemic in 2010) and Denmark (three epidemics in 2010, 2014 and 2017) resulting in a total of 44 cases [3].

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Recently, Food Standards Scotland (FSS) and the Food Standards Agency (FSA) have reported a listeriosis outbreak that has affected 15 people since October 2020 in England, Scotland, and Wales. Nine patients have become ill since January 2022 following the consumption of ready-to-eat smoked fish [4]. *L. monocytogenes* has been reported to be frequently present in raw materials and processing environments for smoked salmon and trout industries around the world [5]. The bacterium is particularly resistant to many extreme conditions, since it can grow in a wide range of pH values, at low water activity and at refrigerator temperature [6]. The transformation processes encountered in the salmon and trout industries do not represent an efficient decontamination strategy to definitively eliminate *L. monocytogenes*. For example, cold smoking (generally at a temperature below 30 °C) does not represent an effective destruction step for *L. monocytogenes* [7]. Regarding the process environment, several authors reported that inadequate cleaning and disinfection operations did not kill *L. monocytogenes* cells adhered on surfaces but induced a viable but non-culturable state [8,9].

Both within and outside an epidemic context, the study of the genetic diversity of *L. monocytogenes* holds paramount importance. It enables the tracing of food contamination sources and the identification of critical points in the production chain where the bacterium can propagate. This facilitates the implementation of specific measures to reduce contamination risks and target control interventions more precisely. Furthermore, it allows for the assessment of the risk to human health by identifying the predominant CCs within the fishery product industry. This evaluation is essential to determine if these strains are associated with more severe infections or greater resistance to treatments, thus guiding prevention and control strategies accordingly. The study of the genetic diversity of *L. monocytogenes* in the fishery product industry also plays a pivotal role in monitoring epidemiological trends of this bacterium. By tracking the evolution of CCs over time, the emergence of new strain types or specific CCs associated with contamination or infection episodes can be detected. This surveillance enables proactive measures to prevent the spread of virulent strains and adapt control measures based on observed trends.

To this end, various typing methods are used to trace the sources of food contamination and to characterize the isolates of *L. monocytogenes*. Agglutination serotyping is considered as the first level of discrimination between isolates and can differentiate 13 serotypes. For many years, pulsed field electrophoresis (PFGE) has been the gold standard for subtyping *L. monocytogenes* [10]. Another key typing approach is multi-locus sequence typing (MLST) based on standardized nomenclature derived from the sequences of seven household genes [11]. The unique combinations of alleles from MLST analysis determine strain sequence types (ST) which are then grouped into CCs. This nomenclature is now officially used to describe the genetic profile of *L. monocytogenes* populations [11–14]. Several authors have highlighted phenotypic specificities according to the CC of *L. monocytogenes.* For instance, certain CCs (such as CC1, CC2, CC4 and CC6) were preferentially associated with clinical cases of listeriosis in humans [12]. Fritsch et al. (2018) provided also global overview on strain virulence based on their distribution in food product and human clinical cases. Likewise, Stoller et al. [15] and Maury et al. [12] showed that CC9 and CC121 were more specific to the food sector and had a predisposition to form biofilms, although according to the biofilm trial performed the results can vary (Lee et al., 2019). More recently, whole genome sequencing (WGS) based typing of *L. monocytogenes* has become a very powerful tool and more and more public agencies are employing this method for national surveillance, outbreak detection or source tracking of listeriosis [16–18]. However, this technique is costly and time-consuming, leading to the development of alternative approaches. Recently, the MLST/PFGE cluster mapping technique has been developed. This technique involves mapping PFGE profiles to databases of PFGE profiles assigned to known CCs [13]. This technique has the advantage of bridging the gap between the old PFGE profiles and the new nomenclature of *L. monocytogenes* CC, thereby improving data comparability and facilitating result interpretation. This technique has proven effective, for instance, in assessing the genetic diversity within the pig and pork production chain in France [13]. More recently, Felix et al. [19] have developed a novel high-throughput real-time PCR methods, enabling the detection of the 30 most commonly found CCs of *L. monocytogenes* in European food samples. This advancement has significantly reduced the cost and genetic analysis expenses associated with *L. monocytogenes.*

Few studies have been conducted on the genetic diversity of *L. monocytogenes* in France, specifically on seafood products. In this sector, salmon and trout are of particular importance as they represent the most consumed seafood products in France in terms of both volume and value [20]. Previous studies on the French salmon and trout sectors have primarily focused on a limited number of strains, exclusively of food origin, during the period from 2001 to 2016 [13,21]. To date, there is a lack of extensive data regarding the genetic structure of *L. monocytogenes* strains populations isolated from a diverse range of samples originating from the French salmon and trout sectors, including both food and environmental samples from industrial facilities. Therefore, the main objective of this study was to identify the genetic structure of a large number of *L. monocytogenes* isolates obtained from food and environmental samples collected in the French salmon and trout sectors between 2006 and 2017. To achieve this goal, two innovative tools were employed: the MLST/PFGE cluster mapping technique and the high-throughput real-time PCR technique. The underlying objective of this study was to determine the predominant CCs within the French seafood industry and evaluate associated characteristics such as a propensity for causing more severe infections, specific resistance profiles, or persistence. Through this approach, we aim to fill the knowledge gap in this field and contribute to a better understanding of the genetic dynamics of *L. monocytogenes* in the French salmon and trout sectors.

2. Materials and methods

2.1. Strains panel

A panel of 698 specific strains of *L. monocytogenes*, isolated from the trout and salmon sector, was utilized in this study (Supplementary Table 1). These isolates were collected over a period from 2006 to 2017, as part of various research projects conducted by the Anses-B3PA unit (Boulogne-sur-Mer, France). The entire panel was employed without any prior selection, thus encompassing the diversity of PFGE profiles, isolation frequency, and sample types (processed foods, raw materials, environment, before and after

cleaning and disinfection operations, etc.). The isolates originated from four different samples types: salmon food (n = 646), trout food (n = 29) and salmon processing environment (n = 23).

2.2. Molecular serotyping PCR

All strains were subjected to molecular serotyping using multiplex PCR, as described by Doumith et al. [22] and adapted by Tresse et al. [23]. The targeted DNA fragments, including the five genes (*lmo0737*, *lmo1118*, ORF2819, ORF2110, *prfA* and *prs*), were amplified from a single colony of each strain grown on Trypticase soy agar supplemented with 6 g/L yeast extract (TSAYe, Oxoid, Dardilly, France) for 24 h at 37 °C, and resuspended in 50 μL of sterile water. DNA extraction was performed using In-stagene matrix (Biorad, Marnes la Coquette, France), followed by PCR amplification, which included an initial denaturation step at 94 °C for 3 min; 35 cycles at 94 °C for 40 s, 53 °C for 45 s, and 72 °C for 1 min 15 s; and a final cycle at 72 °C for 7 min, using a mastercycler personal thermocycler (Eppendorf, Sartrouville, France). Finally, the PCR products were separated as previously described by Doumith et al. [22].

2.3. Mapping of MLST/PFGE Clusters

To perform Pulsed-field gel electrophoresis (PFGE) analyses, chromosomal DNA was extracted using the standardized protocol of the CDC [24] and digested by *Apa*I (Ozyme, Saint-Quentin en Yvelines, France) and *Asc*I (Ozyme) restriction enzymes. The restriction fragments were separated in a 1% SeaKem Gold agarose in a CHEF-DRIII (Bio-Rad SA) apparatus. The banding patterns were visualized under UV light And the PFGE-types were analyzed using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). The generated bands were analyzed according to the standard operation procedure for PFGE results interpretation described by Roussel et al. [25]. The pattern comparison was performed using Dice coefficient with a position tolerance of 1% and the unweighted pair group method with arithmetic average (UPGMA). The CCs were deduced from the PFGE profiles, using the mapping protocol detailed by Felix et al. [13].

2.4. High-throughput real-time PCR assay for the clonal complex determination

The CC identification were performed according to Felix et al. [19] by high-throughput real-time PCR. Briefly, thirty-four sets of primers and probes were utilized. One primer and probe set was selected for each of the thirty targeted CCs, except for four (CC14, CC1, CC37, and CC121) for which two sets were designed to discriminate mutational subdivisions within each CC. The analyses were performed using the BioMark HD system, a high-throughput real-time microfluidic PCR system developed by Fluidigm (San Francisco, CA, USA). For each primer and probe set, a 6 μL PCR mixture was prepared by combining 4.5 μM of primers, 2 μM of probes, and Fluidigm's 2 × loading reagent. Additionally, a 6 μL sample mixture was prepared for each sample by mixing 3 μL of PerfeCTa qPCR ToughMix Low Rox (Quantabio, Beverly, MA, USA), 0.3 μL of sample loading reagent (Fluidigm), and 2.7 μL of diluted DNA (0.1–1 ng/μL) obtained from the *L. monocytogenes* strains under analysis. Subsequently, 5 μL of the PCR mixture and 5 μL of the sample mixture were transferred to the chip inlets and loaded using the Integrated Fluidic Circuits (IFC) controller. Once loaded, the chip was placed in the BioMark instrument. The PCR was carried out with an initial step of 10 min at 95 °C, followed by 40 cycles consisting of 95 °C for 15 s and 60 °C for 1 min. Each PCR included positive controls comprising reference strains of *L. monocytogenes*, as well as negative controls without DNA. Internal controls were also included, utilizing a pBluescript II SK plasmid mixture carrying PCR amplicons flanked by 50 bp segments on each side.

3. Results and discussion

3.1. Serogroup distribution

The majority of the strains (96.8%) belonged to lineage II, with 95.6% of strains belonging serogroup IIa (including 663 strains from serotype 1/2a), and 1.3% belonging serogroup IIc (Table 1). Large part of these strains were isolated from the salmon sector. The predominance of the serogroup IIa in the *L. monocytogenes* panel studied was in agreement with the observations of Wieczorek and Osek [26] and Skowron et al. [27] in Poland, where 75.9%–83.3% of isolates belonged to serogroup IIa, 2.7%–20.7% to serogroupe IIb and 3.4%–16% of the samples contained strains from serogroup IVb. Similar results were reported in Italy [28,29], in Ireland [30] and

Table 1
Distribution of the 698 strains isolated from salmon and trout sectors according to their lineage and serogroup.

Lineage	Serogroup	Sample nature
I (22 strains)	IIb (12 strains)	Salmon food (6 strains)
		Trout food (6 strains)
	IVb (10 strains)	Trout food (10 strains)
II (676 strains)	IIa (667 strains)	salmon food (640 strains) and salmon industrial environment (23 strains)
		Trout food (4 strains)
	IIc (9 strains)	Trout food (9 strains)

in Iceland [31]. To our knowledge, serogroup IIc has not been previously detected in the trout sector. We also characterized the lineage I for 3.2% of *L. monocytogenes* strains with 1.7% and 1.4% of strains belonging serogroup IIb and IVb respectively. Serogroups IIb and IVb have been previously identified in salmon sector [27,29]. Serogroup IIb has already been detected in rainbow trout from fish farms in lakes and sea areas around Finland [32], and several authors have reported a prevalent presence of serogroup IVb in the trout industry [33–35]. Serogroup IVb, particularlyserotype 4b, is known to be frequently associated with clinical cases of listeriosis. Indeed, Miettinen et al. [36] characterized isolates of *L. monocytogenes* involved in an outbreak linked to the consumption of vacuum-packed cold-smoked rainbow trout resulting in febrile gastroenteritis in five healthy individuals.

3.2. Clonal complexes distribution

A total of 698 *L. monocytogenes* isolates were characterized using PFGE in order to elucidate the genetic relationship between them (Fig. 1). The PFGE analysis combining the two restriction enzymes *Apa*I and *Asc*I resulted in 27 different restriction PFGE profiles that showed a high diversity of pulsotypes within the strains panel. In total, the mapping MLST/PFGE Clusters determined the CCs of 541 out of the 698 *L. monocytogenes* strains (CC204, CC121, CC37, CC9, CC7, CC6, CC5, CC3, ST191, CC54, CC20 and CC1). One hundred and fifty seven strains were not assigned to a CC by the mapping MLST/PFGE, and were characterized by high-throughput real-time PCR which allowed the identification of 2 additional CCs (CC26 (n = 155) and CC31 (n = 2)) and confirmed the CC121 for one strain.

Globally, 13 CCs and one ST were identified (Table 2 and Fig. 2). The majority of lineage II strains were isolated from the salmon industry either from food samples or from industrial environments (95%), with 8 CCs being defined. The three most prevalent CCs were CC121 (62%), CC26 (22%) and CC204 (9%). Lee et al. [37] and Stoller et al. [15] described the CC26 that was shown to have a higher capacity to form biofilms and CC204 was described as preponderant in food industry and as a carrier of biocide resistance genes. CC121 was determined for 9 different PFGE profiles and has been described as highly dominant in the fishery product in France [13,38], presumed persistent, biofilm producing and hypovirulent [5,12,39,40]. Our founding demonstrated that these CCs were primarily associated with the salmon sector. These results are in agreement with the observations of Wagner et al. [41] who characterized 307

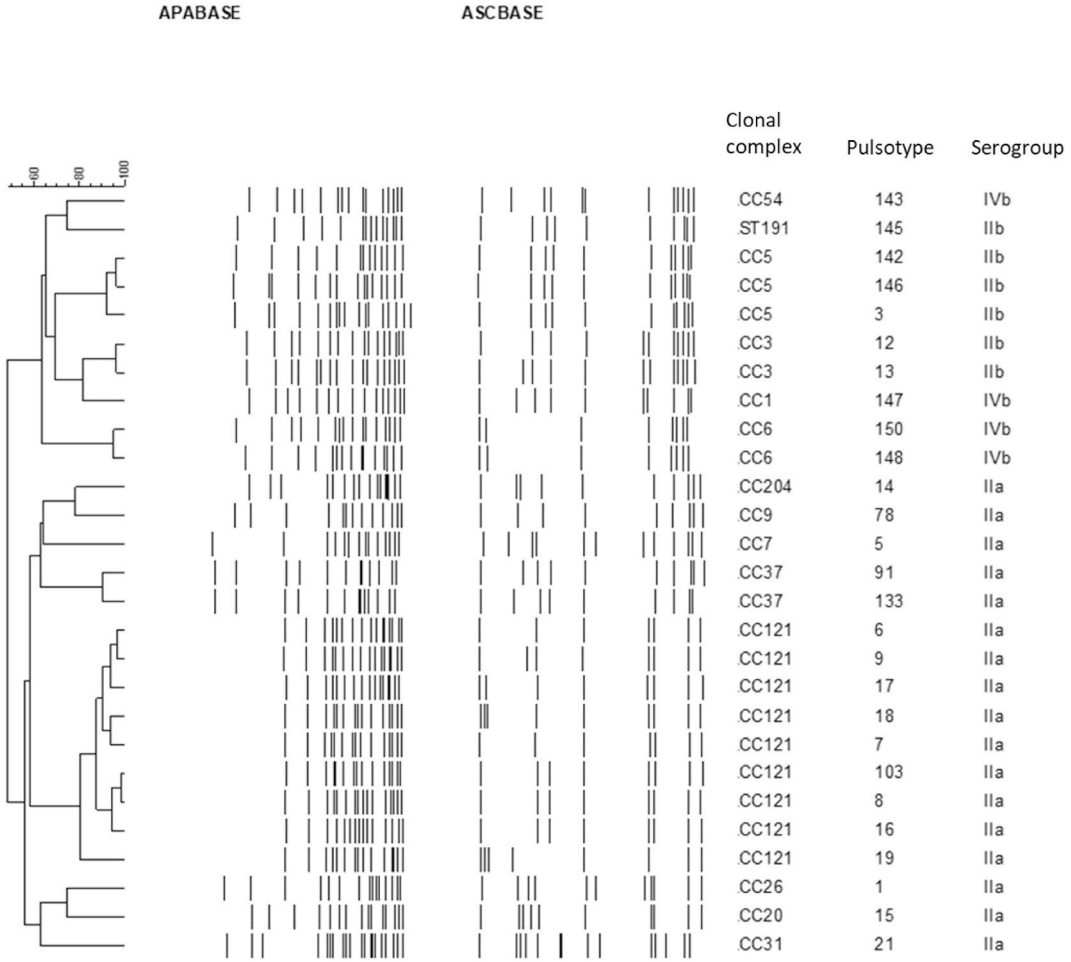

Fig. 1. Dendrogram showing similarities among the representative *L. monocytogenes* PFGE profiles obtained with *Apa*I and *Asc*I.

Table 2
Distribution of the 698 strains isolated from salmon, trout production sectors and environment according to their clonal complex.

Serogroup	Clonal complex (CC) or Sequence type (ST)	Salmon food	Trout food	Salmon industrial environment	Total
IIa	CC121	420	0	15	435
	CC26	155	0	0	155
	CC204	58	0	7	65
	CC7	6	0	0	6
	CC37	0	4	0	4
	CC20	1	0	0	1
	CC31	0	0	1	1
IIb	CC3	5	0	0	5
	CC5	1	4	0	5
	ST191	0	2	0	2
IIc	CC9	0	9	0	9
IVb	CC54	0	2	0	2
	CC1	0	4	0	4
	CC6	0	4	0	4
	Total	646	29	23	698

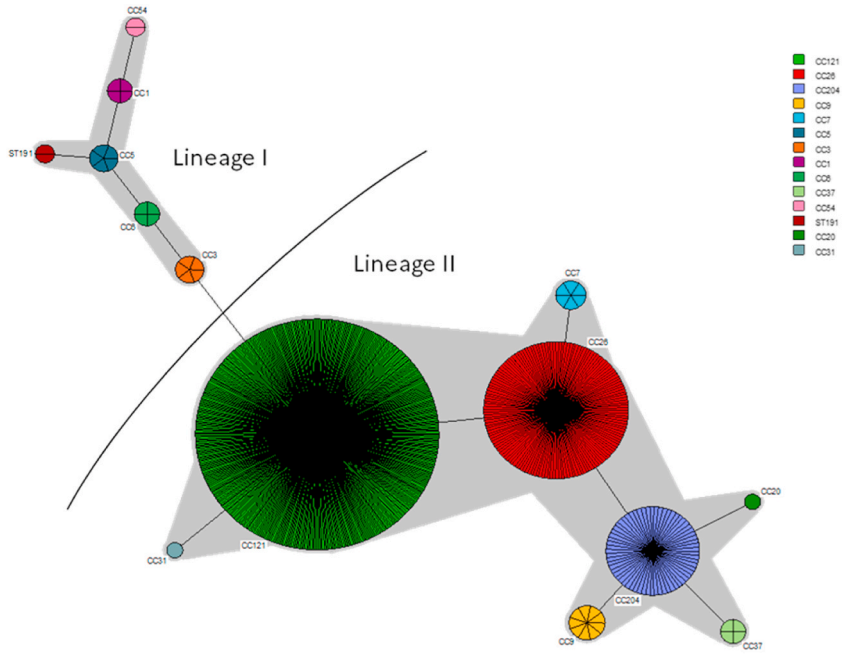

Fig. 2. Minimum spanning tree of MLST data for 698 *L. monocytogenes* isolates. Each circle represents one ST and each fragment of pie chart corresponds to single isolates. The size of the circle is proportional to the number of isolates with that Sequence type (ST). The grey zones gather isolates of the same lineage. Connecting lines infer phylogenetic relatedness in terms of number of allelic differences.

L. monocytogenes strains from the Norwegian meat and salmon processing industry. The authors identified 64 CC121 strains isolated from salmon sectors. Palma et al. [21] also identified CC121 and CC204 of *L. monocytogenes* strains isolated from salmon and shrimp samples. The CC121 was also identified in salmon products by Wieczorek et al. [42] in addition to CC6. Among the lineage II strains isolated from trout, CC9 (1.2%) belonging to serogroup IIc was identified in our study. Previously, CC9 had only been described in the meat sector and is known for its high capacity to form biofilms [37,43]. The majority of lineage I strains was isolated in trout industry (2.3%), and 5 CCs and one ST being detected. CC54 and ST191 have not been previously reported in the seafood sector, and limited information is available in the scientific literature regarding these ST and CC. We also identified *L. monocytogenes* strains from lineage I including CC1 (0.6% of strains) and CC6 (0.6% of strains), exclusively in the trout sector. Fagerlund et al. [43] identified *L. monocytogenes* CC1 in salmon processing plant environments in Norway. Both CC1 and CC6 have previously been classified as hypervirulent by several authors [12,40]. However, assigning the "hypervirulence" trait to *L. monocytogenes* strains should be done with caution. The concept of "hypervirulence" is used for CCs that are more frequently associated with human clinical listeriosis compared to their prevalence in food, while the term "hypovirulence" is used for CCs associated with food, food processing environments, persistence, and low frequencies in human clinical listeriosis [40]. Despite the evidence of a heterogeneous pathogenicity

among *L. monocytogenes*, all isolates are considered to be of equal risk and relevance as a foodborne pathogen by food safety and regulatory authorities [41]. CC8 and CC89 are associated with listeriosis outbreaks incriminating the consumption of fishery products but were not detected in our study [44–47]. A minority of isolates belonging to CC3, CC5, CC7, CC31, CC37, CC54 and ST191 were identified in our study. A few information is available on these CCs. CC3, CC7, CC31, and CC37 have already been described in the Norwegian salmon industry [41,43].

Globally, this study provides genetic structure data of *L. monocytogenes* strains in the salmon and trout sector in France and can provide valuable information to strengthen the control of listeria-related risks and guide control measures. The widespread use of these methods is expected to contribute to: (i) Identification of predominant strains: By knowing the most frequently isolated CCs and STs in the salmon and trout sectors, control efforts can be targeted towards these specific strains. For instance, if a particular CC or ST is associated with a higher prevalence of *Listeria*, more specific control measures can be implemented to reduce contamination. (ii) Understanding virulence and persistence characteristics: Certain CCs or STs of *L. monocytogenes* may exhibit specific virulence or persistence characteristics. By identifying the strains present in the salmon and trout sector, the associated risks linked to these characteristics can be assessed, and appropriate control strategies can be developed. For example, if a CC is known to form biofilms or to be hypovirulent, specific cleaning and disinfection measures can be implemented to reduce the persistence of that particular strain. (iii) Surveillance and early detection: By monitoring the prevalence of CCs and STs in the salmon and trout sector over time, potential changes or emergence of *L. monocytogenes* strains can be detected. This enables swift actions to control these strains before they become a major problem. (iv) Adaptation of control strategies: The genetic structure data of *L. monocytogenes* strains can also help in adjusting existing control strategies. If certain CCs or STs exhibit resistance to specific biocides or antiseptics, disinfection protocols can be reviewed, and effective alternatives can be adopted.

It is important to acknowledge the limitations of this study. Firstly, with regard to the techniques employed, the MLST/PFGE cluster mapping and the high-throughput real-time PCR from Felix et al. [19] do not encompass the entire MLST diversity described within the *L. monocytogenes* species. These techniques only allow for typing of the most prevalent CCs found worldwide in food products. However, this limitation did not hinder our study since the combined use of these two techniques facilitated the systematic assignment of a CC to the 698 strains examined. Furthermore, it is noteworthy that the study focused exclusively on isolates obtained from the French salmon and trout industry. Consequently, caution should be exercised when extrapolating the results to other food sectors or geographical regions. The findings may not provide a comprehensive representation of the diversity and distribution of *L. monocytogenes* strains in various other food industries. Our study primarily relied on information pertaining to CCs and sequence types (STs) for strain identification. Although CCs and STs offer valuable insights into strain relatedness, they do not capture the entirety of the genetic diversity present within *L. monocytogenes*. Whole genome sequencing (WGS) analysis could furnish more detailed information regarding the genetic characteristics of the strains. Certain CCs, such as CC54 and ST191, have limited information available in the scientific literature, rendering the evaluation of their potential risks or characteristics in the seafood sector challenging. Further research is required to comprehend the epidemiological significance of these less-studied CCs. Additionally, we noted the classification of CC1 and CC6 as “hypervirulent” based on existing literature. However, caution must be exercised when attributing hypervirulence characteristics to *L. monocytogenes* strains. The concept of hypervirulence is founded on a higher prevalence of certain CCs in human clinical listeriosis cases compared to their occurrence in food sources. Further studies are necessary to gain a better understanding of the pathogenicity and virulence potential exhibited by different CCs. Lastly, it is important to highlight that CCs associated with listeriosis outbreaks (such as CC8 and CC89) were not detected in our study. The absence of these CCs in the study results could be attributed to specific sampling methods or regional factors and does not necessarily indicate their absence in the seafood sector.

4. Conclusions

In conclusion, we identified 13 CCs and one ST with the first description in the salmon and trout sector of ST191 and CC54. One to four isolates belonged to CCs considered as “hypervirulent” and frequently isolated from clinical infection cases (CC1, CC6), and especially the CC1 which was reported in an outbreak involving fishery products. Sixty percent of the isolates belonged to CC121 and 1.2% to CC9. Theses CCs are considered as hypovirulent and more prevalent in food industries. In perspective, it is important to continue this study on the strains isolated more recently in the seafood sector to continue to manage the risk *L. monocytogenes* in this sector.

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Author contribution statement

Thomas Brauge, Graziella Midelet: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Guylaine Leleu, Karine Capitaine: Performed the experiments.

Aurélie Hanin: Analyzed and interpreted the data; Wrote the paper.

Benjamin Felix: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

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.2023.e18154.

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