International Journal of Food Microbiology 347 (2021) 109166

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Genomic diversity of *Listeria monocytogenes* isolates from seafood, horticulture and factory environments in New Zealand

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ARTICLE INFO

Keywords:
Genomic diversity
Listeria monocytogenes
Seafood
Horticulture and factory environments
PFGE
MLST
WGS
New Zealand

ABSTRACT

Listeria monocytogenes is a foodborne human pathogen that causes systemic infection, fetal-placental infection in pregnant women causing abortion and stillbirth and meningoencephalitis in elderly and immunocompromised individuals. This study aimed to analyse *L. monocytogenes* from different sources from New Zealand (NZ) and to compare them with international strains. We used pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and whole-genome single nucleotide polymorphisms (SNP) to study the population structure of the NZ *L. monocytogenes* isolates and their relationship with the international strains. The NZ isolates formed unique clusters in PFGE, MLST and whole-genome SNP comparisons compared to the international isolates for which data were available. PFGE identified 31 *Asc*I and 29 *Apa*I PFGE patterns with indistinguishable pulsotypes being present in seafood, horticultural products and environmental samples. Apart from the Asc0002:Apa0002 pulsotype which was distributed across different sources, other pulsotypes were site or factory associated. Whole-genome analysis of 200 randomly selected *L. monocytogenes* isolates revealed that lineage II dominated the NZ *L. monocytogenes* populations. MLST comparison of international and NZ isolates with lineage II accounted for 89% (177 of 200) of the total *L. monocytogenes* population, while the international representation was 45.3% (1674 of 3473). Rarefaction analysis showed that sequence type richness was greater in NZ isolates compared to international trend, however, it should be noted that NZ isolates predominantly came from seafood, horticulture and their respective processing environments or factories, unlike international isolates where there was a good mixture of clinical, food and environmental isolates.

1. Introduction

Listeria monocytogenes is an important facultative intracellular pathogen that has been implicated as an etiological agent for severe foodborne disease outbreaks in the past decades (Farber and Peterkin, 1991; FDA, 2003). *L. monocytogenes* are non-sporulating, rod-shaped facultative anaerobic bacteria that are ubiquitous in nature. It has been isolated from a range of ecological niches and sources including soil, water, fresh produce, ready-to-eat food products, fecal matter and decaying material (Allerberger, 2003; FDA, 2003; Freitag et al., 2009). *L. monocytogenes* is robust in nature and capable of surviving in a range of harsh environmental conditions such as salt (up to 10%), acid (pH 4.1–9.0) and temperature (0 °C–45 °C) (Thevenot et al., 2006). *L. monocytogenes* can form biofilms on food-processing surfaces. This allows it to persist over time, posing major challenges to particularly food industries (Gandhi and Chikindas, 2007). Human listeriosis is caused by *L. monocytogenes* as a result of consuming contaminated raw or processed foods including milk and dairy-based products, meat, vegetables, seafood and ready-to-eat food products (Farber and Peterkin, 1991; Thevenot et al., 2006). The mortality rates of listeriosis have been recorded as up to 20–30% but

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https://doi.org/10.1016/j.ijfoodmicro.2021.109166
Received 19 August 2020; Received in revised form 28 February 2021; Accepted 6 March 2021
Available online 17 March 2021

its incidence is low (EFSA, 2014). High-risk populations include pregnant women, the elderly (>65 years old), immunocompromised individuals (Cossart and Toledo-Arana, 2008). The clinical symptoms include gastroenteritis, septicaemia, meningitis, meningoencephalitis, stillbirths and abortion (Cossart and Toledo-Arana, 2008; Swaminathan and Gerner-Smidt, 2007). There was a significant increase in the incidence of listeriosis (8.6%) in Europe between 2008 and 2013 and, in 2015 over 2200 cases were reported in Europe, which has renamed listeriosis as a re-emerging public health concern in developed countries; furthermore the number of disability-adjusted life years (DALYs) for listeriosis case numbers were 208 (95% UI: 192–226) in 2012 and 252 (95% UI: 200–307) in 2014 which indicated an increasing trend (reviewed by de Noordhout et al., 2014a; EFSA, 2014; Maertens de Noordhout et al., 2017). In New Zealand (NZ), there were 30 mandatory notifications (0.6 cases per 100,000 population) in 2018, a higher population-adjusted rate than other regions of the world (de Noordhout et al., 2014b). Of the 30 cases, just five were reported to be perinatal and there were two non-perinatal deaths (Pattis et al., 2019).

L. monocytogenes isolates can be subtyped into four genetic lineages, such as I, II, III and IV that are often isolated from overlapping ecological niches (Nightingale, 2010; Orsi et al., 2011; Wang et al., 2019). These lineages have been aligned with serovar clusters as lineage I, comprising serotypes 1/2b, 3b, 3c, and 4b; lineage II, comprising serotypes 1/2a, 1/2c, and 3a; and lineage III, comprising serotypes 4a and 4c (Nadon et al., 2001). Lineage I is predominantly associated with human infections and has been isolated from clinical samples (Gray et al., 2004; Kathariou, 2002; Maury et al., 2016; McLauchlin, 1990). Members of lineage II have also been implicated in human listeriosis outbreaks (Gaul et al., 2013; Knabel et al., 2012; Lopez-Valladares et al., 2017), isolated predominantly from food sources and food-processing plants and/or environments or factories (Cruz et al., 2014; Nadon et al., 2001; Nowak et al., 2017). Lineages III and IV have been mostly isolated from animal sources (Nadon et al., 2001) and recently lineage IV has been identified to harbour pathogenic island LIPI-4 (Wang et al., 2019).

Different strategies have been employed for subtyping *L. monocytogenes* strains and Pulsed Field Gel Electrophoresis (PFGE) was used as the gold standard method for epidemiological investigations within PulseNet, USA (Brosch et al., 1996; Brosch et al., 1991; Jamali and Thong, 2014) with the European and the US (CDC) surveillance systems using it widely for *L. monocytogenes* outbreak investigations (CDC, 2016; EFSA, 2014). However, the discriminatory power of PFGE was accepted to be suboptimal for accurate epidemiological investigations, even when using multiple enzymes (Gerner-Smidt et al., 2006; Liebana et al., 2002; Schjørring et al., 2016). Furthermore, whole-genome sequencing (WGS) superseded PFGE for outbreak investigations (CDC, 2016). MultiLocus Sequence Typing (MLST) characterisation tool utilises the internal fragments of housekeeping genes (*abc*Z, *bgl*A, *cat*, *dap*E, *dat*, *ldh*, *lhk*A for *L. monocytogenes*) and this has been widely used to investigate population structure in many bacterial species including *L. monocytogenes* (Chenal-Francisque et al., 2011b; Haase et al., 2011; Henri et al., 2016a, b; Maury et al., 2016; Salcedo et al., 2003). WGS-Single Nucleotide Polymorphism (SNP), whole-genome (wg) MLST), core genome (cg) SNP and cgMLST have recently been applied for surveillance and source tracking of *L. monocytogenes* (Chen et al., 2017; Jackson et al., 2016; Moura et al., 2017). Furthermore, the SNP approach that involves mapping against a reference genome (Pightling et al., 2014) has been used for regulatory and outbreak investigation purposes (inclusive of listeriosis) by the US Food and Drug Administration (FDA) (Davis et al., 2015), Denmark (Anonymous, 2014; Kvistholm Jensen et al., 2016) and the UK (Awofisayo-Okuyelu et al., 2016).

Although *L. monocytogenes* is ubiquitous, little is known about the sources attributable to listeriosis illnesses in NZ as many cases are sporadic (Cruz et al., 2014), accounting for an estimated 87.8% (95% CI: 57.9–98.5%) of foodborne listeriosis in 2018 (Pattis et al., 2019). Foodborne listeriosis outbreaks have been attributed to seafood and horticultural products (Brett et al., 1998; Callejon et al., 2015; Schjorring et al., 2017). These are significant industries in NZ with growing export targets (Aitken and Warrington, 2018; Seafood New Zealand, 2019). Previous studies have identified persistent *L. monocytogenes* strains in NZ food-processing environments and factories (Cruz et al., 2014; Nowak et al., 2017), however, the strains were rarely isolated from the final product (Cruz and Fletcher, 2011). The presence of these strains at the factories but rarely on the product raises questions around the impact of these strains on food safety and public health. Furthermore, given that NZ suffers from a considerable number of foodborne illnesses and foodborne listeriosis has been notified as the most severe (cost per case of perinatal listeriosis is $NZ380,000) (Lake et al., 2010; Lim et al., 2012), it is prudent to investigate the genetic characteristics of *L. monocytogenes* from food and the respective food-processing environments. Comparing these to similar strains outside NZ could provide insights into their distribution around the globe and their potential impact on food safety and on public health. Therefore, this present study aimed to compare the genetic characteristics of *L. monocytogenes* isolates from NZ seafood and horticultural sources and their respective processing or factory environments with those from publicly available international isolates to better understand their distribution, genetic diversity and population structure in comparison with the international isolates.

2. Material and methods

2.1. *L. monocytogenes* isolates

A total of 306 *L. monocytogenes* isolates from seafood and seafood processing environments (for convenience, hereafter the processing environments are referred to as factories) and 53 horticultural isolates were revived from the Food Safety Culture Collection, at The New Zealand Institute for Plant & Food Research Limited, Auckland. This study included isolates collected between 1997 and 2016 from different food matrices of seafood (salmon, mussels, mackerel, oysters), seafood factories, fruit, vegetables and horticulture produce packing factories (the details are presented in the Data in Brief article elsewhere in Table D3 (Mohan et al., 2021)).

2.2. Pulsed-field gel electrophoresis

All *L. monocytogenes* isolates were revived in Brain Heart Infusion (BHI) (Difco, Becton, Dickinson and Co., Sparks, MD, USA) agar plates for 24 h at 37 °C. The purity was checked and a colony was inoculated into 10 mL of BHI broth. The optical density of the overnight cultures was checked at 610 nm wavelength using a MicroScan Turbidity Meter (Siemens, West Sacramento, CA, USA). PFGE of *L. monocytogenes* isolates was performed using the US Center for Disease Control and Prevention (CDC) Pulsenet protocol (Graves and Swaminathan, 2001).

The run conditions consisted of a linear ramping factor with pulse times from 4.0 to 40.0 s at 14 °C and 120 °C, applied for 18 h. For reference or molecular ladder, *Xba*I (Roche, Sigma-Aldrich, USA)-digested plugs of *Salmonella enterica* serovar Braenderup (CDCH9812) were used. After the electrophoresis run, the gels were stained with ethidium bromide (Bio-Rad, NZ) and the images were captured using the GelDoc-documentation system (Bio-Rad, Hercules, CA, USA). The TIFF formatted gel images were analyzed using Infoquest™FP fingerprint analysis software (Bio-Rad, Hercules, CA, USA). The images were checked and processed before assigning a pulsotype. The fingerprints were analyzed for identical fingerprints (pulsotypes that have matching fingerprints) and dissimilar fingerprints which were determined by the Dice coefficient and a band position tolerance of 1%. Dendrograms were generated using an unweighted pair group method with arithmetic mean (UPGMA) by combining the *Apa*I and *Asc*I restriction patterns, and the pulsotypes were assigned with a unique number by comparing the patterns with the NZ *L. monocytogenes* database: the ESR (Aotearoa PulseNet, New Zealand) PFGE database (Gilpin, 2006). The fingerprints

were submitted to the ESR PFGE database (Gilpin, 2006). When an *Asc*I fingerprint from an isolate matched an *Asc*I fingerprint in the ESR database, the corresponding matching *Asc*I number was allocated to that fingerprint, however, when the *Apa*I fingerprint from the same isolate did not match any of the *Apa*I fingerprints, *Apa*I fingerprint was named "New". The same principle was applied to the non-matching *Asc*I fingerprints from the isolates, for example, the pulsotype of an isolate with matching *Asc*I pattern 02 and a non-matching *Apa*I pattern was named as AscI: ApaI = 02: New and vice-versa. If both fingerprints were not matching the isolate's pulsotype was named "New: New". The NZ pulsotypes were compared with 96 international isolates obtained from the PFGE database available with the ESR PFGE database, NZ (Gilpin, 2006). The international isolates consisted of a mixture of strains from food sources (70%) and human clinical cases (30%).

2.3. DNA extraction for whole-genome sequencing

The *L. monocytogenes* isolates were randomly selected from the culture collection, representing 152 PFGE typed isolates and another 48 isolates without PFGE typing (total of 200 isolates). These isolates were revived in BHI agar plates for 24 h at 37 °C and the purity confirmed using Chromogenic agar plates (ChromAgar™ *Listeria*, CHROMagar, 75006, Paris) for halo formation. Single colonies from ChromAgar™ *Listeria* plates were selected and cultured in tryptic soy broth plus 0.6% yeast extract (TSBYE, Bacto™, BD, Spark, USA). Whole-genome sequencing of the confirmed *L. monocytogenes* isolates was performed using DNA extracted from each isolate which was prepared using the DNeasy Blood and tissue kit (Qiagen, Hilden, Germany). DNA concentrations were determined using Nanodrop (Thermo Fisher Scientific, Waltham, MA. USA) and PicoGreen® (Quant-iT; Thermo Fisher Scientific). Sequencing libraries containing 1 ng of DNA were prepared using Nextera XT chemistry (Illumina, San Diego, CA, USA) for 250 bp paired-end sequencing run on a MiSeq (Illumina, San Diego, CA, USA) sequencer, according to the manufacturer's recommendations. Sequence quality was evaluated on a per genome basis using the Nullarbor pipeline (Seemann et al., 2018) and BioNumerics Version 7.6.3 (Applied Maths, Belgium). The lineage, sub-lineage, seven-loci multilocus sequence type (ST) were inferred from WGS data and defined by using international nomenclature using the Bigsdb. Pasteur.fr database (Moura et al., 2016).

2.4. Multilocus sequence typing

The MLST alleles used to characterize all the compared *L. monocytogenes* isolates were retrieved from WGS data and were based on the seven housekeeping genes: *abc*Z (ABC transporter), *bgl*A (beta-glucosidase), *cat* (catalase), *dap*E (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (Lactate dehydrogenase), and *lhk*A (histidine kinase) (Salcedo et al., 2003). The allele numbers for the corresponding alleles were assigned using BioNumerics, v 7.1 that has the inbuilt *L. monocytogenes* Pasteur MLST scheme. The Pasteur international database of *L. monocytogenes* MLST (PubMLST, 1998) was used to investigate the representation of NZ MLST types internationally. The Pasteur international MLST and whole genome database was accessed to compare NZ isolates where the database possessed 3473 isolates at the time of access. These isolates were used to conduct a comparative analysis for sequence type (ST) richness and diversity indices in comparison with NZ isolates (Chenal-Francisque et al., 2011a; Moura et al., 2016; Ragon et al., 2008).

2.5. Sequence Type richness and diversity based on ST

Sequence Type richness in the NZ *L. monocytogenes* isolates based on the STs were analyzed using rarefaction analysis. Rarefaction analysis was carried out using R version 5.1, package Vegan (R, 2018) that has the inbuilt rarefaction function by using the frequency of STs (Gormley et al., 2008) encountered in NZ seafood and horticultural sources. The frequency distribution of each *L. monocytogenes* ST and their respective lineages were summarized to describe the populations of *L. monocytogenes*. The STs were grouped based on the lineages they belonged to and the curves were classified to represent the lineages and the number of STs present in each lineage. Diversity indices (Simpson index 1-D and Shannon index) were measured using PAleontological STatistics (PAST) software program (Hammer et al., 2001) to analyse the ST richness in *L. monocytogenes* populations in NZ food sources. Simpson index 1-D provides the measure of evenness of the community and this scales from 0 to 1. A measure of 0 indicates that all taxa (in this study, it is the STs) are equally present and 1 indicates that one ST dominates the given population. Shannon index accounts for the number of individuals as well as number of STs and a 0 denotes a single taxon accounting for high values or frequency and values above 0 represents that the population comprises of many taxa, each with few individuals ST.

2.6. Whole-genome phylogenetic analyses using core-genome SNP

The NZ isolates (n = 200) were compared with publicly available international *L. monocytogenes* whole genomes. A total of 1256 from lineage I, 1133 from lineage II and 50 from lineage III from fully sequenced international isolates from GenBank were used to compare with NZ isolates for phylogenomic diversity. The genomes were clustered using core genome single nucleotide polymorphisms (cgSNPs) using parSNP (Treangen et al., 2014). The genomes were categorised into different lineages and their phylogenetic relationships were analyzed. The trees produced by parSNP were visualized using Treegraph v2 (Stover and Muller, 2010) and Figtree (Rambaut, 2018). MEGA 7 was used to construct maximum likelihood trees of parSNP data from the genomes. A minimum spanning tree (MST) was created using the conventional MLST allelic profiles from NZ isolates and these were in turn compared with the international MLST profiles in the Pasteur PubMLST database using the Bionumerics software package Version 7.6.3 (Applied Maths, Belgium). An advanced cluster analysis was carried out using the MST for categorical data option from the predefined templates and sequence type was used as the data type. The whole genomes sequences were deposited in the European Nucleotide Archive (Tabe D5 (Mohan et al., 2021; Vliet, 2018)).

2.7. Data in Brief article

Detailed sample data description and additional results associated with the data analysis from this research work are provided in the Data in Brief article (Mohan et al., 2021). The respective tables are referenced appropriately in the manuscript.

3. Results

3.1. Genetic diversity based on Pulse Field Gel Electrophoresis comparison

PFGE discriminated isolates into 31 previously recognized *Asc*I and 29 previously recognized *Apa*I PFGE patterns, which when combined generated 55 different AscI: *Apa*I patterns (pulsotypes, Table 1). Individual restriction patterns and their frequency are detailed in Table D2 in the Data in Brief article elsewhere (Mohan et al., 2021). Fig. 1 shows the dendrogram generated using UPGMA by combining the *Apa*I and *Asc*I restriction patterns into pulsotypes that were assigned by comparing the patterns with the NZ *L. monocytogenes* database that is based on internationally recognized pulsotypes. Examining the dendrogram makes it clear that the NZ isolates from both horticultural and seafood operators clustered quite separately from the international isolates. Although a few international isolates were found in the clusters of NZ isolates, the NZ isolates mostly stood out as unique clusters from the main international clusters. There was also unique clustering among the NZ isolates where the isolates from the seafood operator clustered

Table 1
The *Asc*I and *Apa*I patterns found in different seafood and horticultural operator and crops. A, B, C, D, E are five different seafood operator, H and O represent multiple operators of two different horticultural crops while U is a single vegetable operator. If any one of the fingerprints, either *Asc*I or *Apa*I from an isolate did not match any of the *Asc*I and *Apa*I patterns in the ESR PFGE database, they were named "New" and, if both *Asc*I and *Apa*I fingerprints from an isolate did not match with any of the corresponding fingerprints in the database the pulsotype of the isolate was named "New: New".

Pulsotypes	Factories								
*Asc*I:*Apa*I	A	B	C	D	E	H	O	U	Grand total
01:01	1					1			2
02:02	4	3				3			1
02:New	1	1							2
03:12	1								1
04:06	1	1							2
08:72	1		1			3	1	2	8
14:04					1				1
23:12		1							1
23:31		1							1
23:43		1							1
23:61						1			1
23:New								1	1
35:27		3			1				4
38:14	11	26	3			1		1	42
38:02		1							1
38:32	1	1							2
38:60		4			2				6
38:80			2		1				3
40:48		1	2		1				4
40:New		1							1
43:26		2						1	3
46:58		1							1
48:14	1								1
48:41	1	6							7
51:32	27	7		1			1	2	38
51:73			1						1
51:76	1								1
51:82	2								2
55:57		1							1
59:42							1		1
59:46	1				1				2
63:31			1					1	2
64:37						1			1
65:14		1							1
65:02	11	98	3			1	1		114
65:32	1								1
65:New		1	1						2
69:02	1				1		2		4
69:New							1		1
70:02	1								1
74:59		1							1
77:32		1							1
78:06	1								1
83:14	1								1
83:42	2	1	2		1				15
83:72							1		1
84:32		1							1
85:31		1							1
86:75			1						1
87:79	1								1
88:41		1							1
89:81	1								1
90:06	1								1
New:02		1				2			12
New:New	2	21	1			17			41
Grand total	77	208	18	1	9	30	8	8	359

together and, those from the horticultural operator clustered separately from the seafood origin isolates.

The distribution of the pulsotypes is tabulated in Table 1 with details of operator or crops. (The distribution of PFGE pulsotypes in the food sources from NZ is shown in the Data in Brief article elsewhere in Table D2 (Mohan et al., 2021)). Fifty-five pulsotypes were identified in 359 *L. monocytogenes* isolates from food sources and factories with 8 isolates common to both seafood and horticultural operator or factories. Thirty-one of the pulsotypes were only found in a single isolate while pulsotype 65:02 was found in 114 isolates including 98 from seafood operator B. Such occurrences of multiple isolates with the same pulsotypes being found in particular operator show that these pulsotypes were residents of those particular sites and/or factories and in the case of 65:02, 38:14 and 51:32, these had previously been shown to persist in the particular mussel factories for at least 6 months. In general, the most common pulsotypes overall were most widely distributed across the different operator/crops with pulsotypes 65:02, 38:14, and 51:32 (represented by 114, 42 and 38 isolates respectively) all being found in five of the eight operators/crops and pulsotypes New: New and 83:42 (represented by 41 and 15 isolates respectively) being found in four operators/crops. This suggests that where these particular pulsotypes were found was not especially related to the type of operator/crop but more to their overall high prevalence in NZ. The exception was pulsotype 08:72 which, although only represented by eight isolates, was found in five different operators/crops.

3.2. MLST analysis and interrogation of MLST database

The random selection from the PFGE typed and un-typed collection provided a total number of 200 *L. monocytogenes* isolates. Table D1 from the Data in Brief article presented elsewhere provides details on the sample unique IDs, the sources from where they were collected, MLST sequence types (ST), clonal complexes (CC), the lineages and the pulsotypes (Mohan et al., 2021). Lineage II was the predominant lineage that dominated the NZ *L. monocytogenes* population accounting for 89% (177 of 200). The isolates formed 23 clonal complexes and 25 sequence types out of 200 isolates characterised. In contrast, the international isolates showed an overall proportion of lineage II of only 45.3% (1674 of 3473). The distribution of lineage II internationally and in NZ was further compared based on the source of isolation, where all environments were taken into consideration inclusive of seafood and/or horticultural processing factories, seafood and/or horticulture processing equipment (inside the factories), natural environments (soil, fruit picking bags, water and wastewater) and production environments. Table 2 shows the distribution of lineage II in different environments internationally and in NZ. Furthermore, the rest of the other NZ sources including food sources (n = 36), one clinical isolate (due to contaminated mussel (Brett et al., 1998)) and one of an unknown source, the majority (25/36) also belonged to lineage II (Table 2).

The phylogenetic diversity index, Simpson 1-D measures for NZ isolates were 0.80 for lineage I, 0.75 for lineage II and 0.00 for lineage III, respectively. There were only six isolates in lineage III. The Shannon-H index was 1.76 for lineage I, 1.91 for lineage II and 0.00 for lineage III, respectively (0 denotes a single taxon being at high frequency and values above 0 represent that the population comprises many STs each ST being represented at different frequencies). The Simpson and Shannon indices for the international isolates are presented in the Data in Brief article elsewhere in Table D4 (Mohan et al., 2021). The 95% confidence intervals of the NZ lineage I indices overlapped with those of the international isolates for environmental and food isolates but there was less diversity in the NZ lineage II and III isolates than for the international isolates. The *L. monocytogenes* international population from human clinical cases showed a Shannon_H index of 4.1 for lineage II, 3.6 for environmental isolates and 3.8 for food isolates.

The MLST database was interrogated for the prevalence of all the STs that were isolated from this study. Table 3 details the prevalence of the NZ isolated STs and compares these with where they have been found internationally. ST1262 (CC1) was not found in the database, which implies that it is a new ST isolated from NZ. ST120 and ST204 were predominantly found in Oceania and most of the STs were prevalent in Europe in general (PubMLST, 1998).

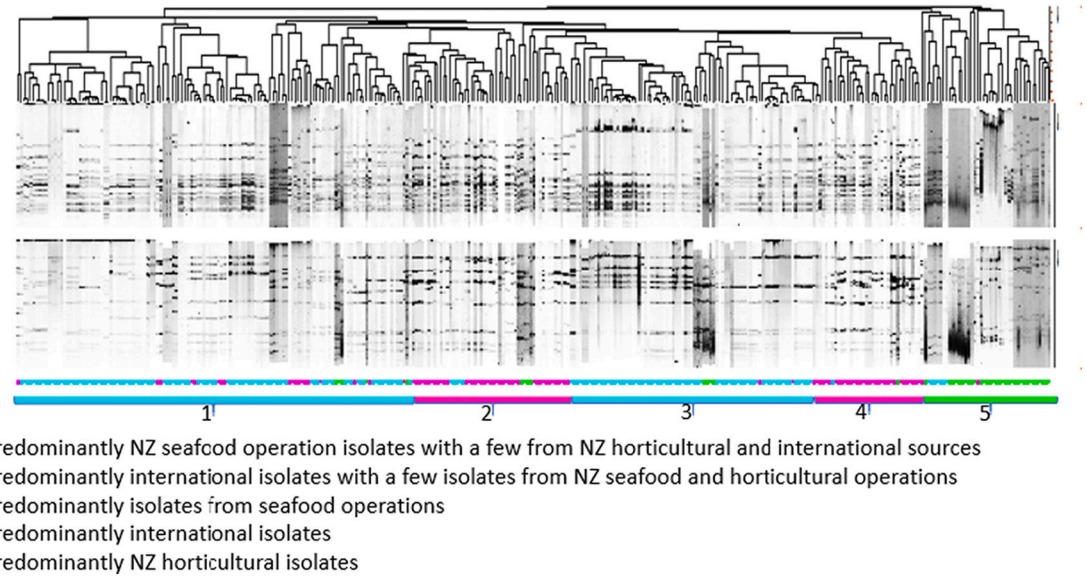

Fig. 1. Dendrogram of the pulsotypes from 359 New Zealand isolates and 96 international isolates constructed using Infoquests™ FP Molecular genetic fingerprint analysis software (Bio-Rad). The fingerprints created by *Apa*I and *Asc*I restriction enzymes and the dendrogram was determined by the Dice coefficient with a band position tolerance of 1% using an unweighted pair group method with arithmetic mean (UPGMA). The international isolates comprised food and human clinical cases pulsotypes. Turquoise spots represent isolates from seafood operations; green represents isolates from horticultural facilities and pink color represents the international isolates from different sources. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Frequency distribution of *Listeria monocytogenes* lineages I, II and III from different sources internationally and in NZ. The sources include natural and production environment, feed and food for international and New Zealand isolates.

Source	Country	Lineage I	Lineage II	Lineage III	Total
Natural and production environment	Africa	3	–	–	4
	Asia	10	1	–	10
	Central America and the Caribbean		1	–	1
	Europe	88	79	–	167
	No info	38	13	–	52
	North America	89	113	3	195
	NZ	8	152	4	164
	Oceania	13	4	–	17
	South America	75	40	–	115
Feed	Asia	1	–	–	1
	Europe	29	44	–	73
	No info	11	26	–	37
	Oceania		1	–	1
Food	Africa	9	4	–	13
	Asia	80	105	2	187
	Europe	80	153	–	233
	Middle East	4	2	–	6
	No info	2	9	–	11
	North America	28	34	4	66
	NZ[1]	9	25	2	36
	Oceania	99	113	1	213
	South America	48	30	–	78
	Unknown	2	10	2	14

[1] The 36 NZ "Food" isolates include one clinical strain from a smoked mussel outbreak (Lineage II) and one from an unknown source (Lineage I).

3.3. Rarefaction analysis of NZ and international lineages

The ST richness of the NZ *L. monocytogenes* populations was analyzed and the frequency distribution of each *L. monocytogenes* ST and their respective lineages were plotted in Fig. 2a. The x-axis in the rarefaction plot represents the number of samples used in the analysis and the y axis represents the different number of genotypes identified in the given number of samples. NZ isolates were compared with the international environmental isolates as the majority of the NZ isolates were retrieved from food-processing factory environments. These isolates could be transferred onto products during processing and packaging. The rarefaction analysis demonstrated the over-representation of lineage II and the ST richness in lineage II in NZ food and their environmental sources as opposed to other lineages (Fig. 2a). In contrast, equal representation of the *L. monocytogenes* lineages I and II was observed in the *L. monocytogenes* MLST Pasteur database (Fig. 2b). Furthermore, in the other international sources, the trend was slightly different: the food sources possessed greater numbers of lineage II STs (Fig. 2c) while human clinical cases were dominated by lineage I STs (Fig. 2d). In combination with the diversity indices, the rarefaction analysis indicated the taxonomic richness in the NZ lineage II cluster.

3.4. Minimum spanning tree of L. monocytogenes MLST types

Fig. 3 shows the minimum spanning tree (MST) of the NZ and the international isolates publicly available in the MLST database. The majority of the isolates from NZ belonged to lineage II. It is also interesting to consider the ramification of having the lineage III isolates among the lineage I and II isolates. The MST diagram shows clear demarcation of two different evolutionary directions for lineage I and lineage II, where a few isolates that belonged to lineage III emerge from lineage II and a few isolates emerge from lineage I. Fig. 4 shows the frequency distribution of different ST in NZ factories and crops. ST120 was predominantly found in factory B and, ST321 and ST399 dominated factory A. In contrast, factory C harbored a variety of genotypes including ST399, ST9, ST31, ST120 and ST321 and similarly factory G harbored a mixture of different genotypes. This might imply that either these factories handle samples from different sources or simply that no particular resident *L. monocytogenes* population had been established in these factories.

3.5. Phylogenetic comparison of whole genomes

On comparing the whole genome sequences, the NZ isolates formed unique clusters in SNP analysis (Fig. 5) as had been observed in the PFGE

Table 3
The prevalence of New Zealand isolated *Listeria monocytogenes* STs globally and the information on the prevalence as retrieved from the *L. monocytogenes* MLST Pasteur database. STs that are bold and underlined represent the STs that are predominant in Oceania, which includes New Zealand.

New Zealand MLST			Number of NZ isolates	NZ source	Presence and dominance in other regions	International sources
Lineage	ST	CC				
I	1	CC1	5	Factory	Europe, North America, Oceania, Asia	Human, food, production environment, animal
I	2	CC2	3	Vegetable	Europe	Human
I	3	CC3	4	Smoked Salmon	Europe, France	Human
I	4	CC4	2	Fruit	Europe	Human, animal
II	7	CC7	4	Factory	Europe	Human, animal
II	9	CC9	2	Factory	Europe	Human
II	12	CC7	1	Factory	North America	Unknown
II	26	CC26	1	Factory	Europe	Natural environment, animal, human
II	31	CC31	1	Factory	Europe	Food
II	101	CC101	5	Factory	Oceania, North America, Europe, Africa	Animal, human, food
II	**120**	**CC8**	78	Vegetable	North America, **Oceania-predominant**	Human-predominant, food
II	155	CC155	15	Factory	Europe, Oceania, North America, Asia-predominant	Food-predominant, animal, human
II	193	CC193	1	Factory	Europe, Asia	Food, food production environment-predominant
II	**204**	**CC204**	8	Factory	**Oceania-predominant**, North America, Europe	Food environment-predominant, food, natural environment
I	224	CC224	1	Factory	Europe, North America	Human, natural environment, food, production environment
III	299	CC131	6	Factory	Asia, Oceania	Human, food
II	307	CC307	1	Factory	Asia, Oceania	Human, food
II	321	CC321	24	Factory	North America-predominant, Oceania, Asia	Production environment, natural environment, food, human
I	324	CC288	1	Factory	Oceania	Human, production environment, food
II	394	CC415	1	Factory	Europe	Natural environment
II	399	CC14	19	Factory	Oceania	No source
II	424	CC20	1	Factory	Oceania	Human
II	451	CC451	2	Factory	Europe	Human
II	706	CC229	13	Factory	Oceania	Food
I	1262	CC1	1	Factory	No records	No records

and MLST data. Although the overall topology was similar in these two methods, the clustering patterns of NZ isolates with the international isolates differed slightly. A total of 177 isolates from 200 NZ isolates belonged to lineage II and 17 belonged to lineage I and six belonged to lineage III, as predicted by MLST. For comparison, the international and NZ isolates were analyzed based on different lineages and the lineages were compared independently in Fig. 5A, B and C.

It should be noted that most NZ isolates formed unique clusters borne on independent branches in the SNP trees, although a few isolates were found to cluster amidst the international isolates. There is no dominant MLST-type or genetic cluster of NZ isolates. One relatively large set (n = 86) of NZ isolates grouped on a branch with international isolates that were from multiple sources in lineage II, with this branch not representative of any predominant source. NZ lineage III isolates formed an independent cluster standing out from the rest of international lineage III isolates except for one isolate that shared a common neighbor from the food sourced international isolates.

4. Discussion

Molecular subtyping of bacterial pathogens has improved our understanding of bacteria in a variety of contexts including surveillance of pathogens and their dynamics, their sources, reservoirs, movement, population structure and establishment in certain niches. The current study reports the use of different molecular characterisation methods (PFGE, MLST and WGS (cgSNP)) to understand the population structure and the diversity of *L. monocytogenes* seafood and horticultural strains in NZ compared to international strains.

PFGE, MLST and WGS SNP methods showed that NZ *L. monocytogenes* populations are unique for the international set of strains tested compared to the rest of the world. The PFGE pulsotypes showed that NZ *L. monocytogenes* isolates demonstrated clear clustering between isolates from horticulture produce and their processing factory environments and the seafood and seafood processing factory environments. However, there were indistinguishable pulsotypes not adhering to any existing pattern present among the isolates from both sources that could not be defined clearly to a pulsotype.

Some of the populations showed high factory association by being persistently present over time in the factories from where they were collected. This trend was previously reported in NZ by Nowak et al. (2017) who investigated the association of *L. monocytogenes* with seafood processing premises and identified genetic traits associated with persistence and other characteristics including biofilm formation, heat resistance and motility. This indicated that these populations may be residents of that geographical location and/or well adapted in that niche. Furthermore, studies have shown that the presence of *L. monocytogenes* in products is often due to the presence of the pathogen in the processing environment (Autio et al., 1999; Fletcher et al., 1994). In this study, although the *L. monocytogenes* populations comprised predominantly food-processing environmental (factory) sources, a significant number of samples clustered with food sources (Fig. 1).

A total of 359 isolates were typed by PFGE and while most of the 55 PFGE pulsotypes were only represented by one or two isolates, four pulsotypes dominated (Ascl:Apal types 38:14, 51:32, 65:02 and New:New). There were more than 30 isolates of each of these and together they accounted for 65% of the isolates (Table 1). These were also most widely distributed, each being isolated from four (New:New) or five of the eight factories or crops. This suggests that these isolates are ubiquitous in the NZ environment and must be robust enough to survive in different environmental conditions (Table 1). In a previous study (24), all tested isolates (n = 12) of pulsotype 51:32 were shown to have a truncated *InlA* and reduced ability to invade human epithelial cells. In contrast to the widely distributed pulsotypes, others appeared to be more confined to particular sources. For example, 38:60 (n = 6) was only found in two seafood factories and New:02 (N = 12) was only found in one factory and one horticultural crop (Table 2). Some sources had very diverse pulsotypes, for example, the nine isolates from factory E were of eight different pulsotypes. The entry of trucks, entry of raw

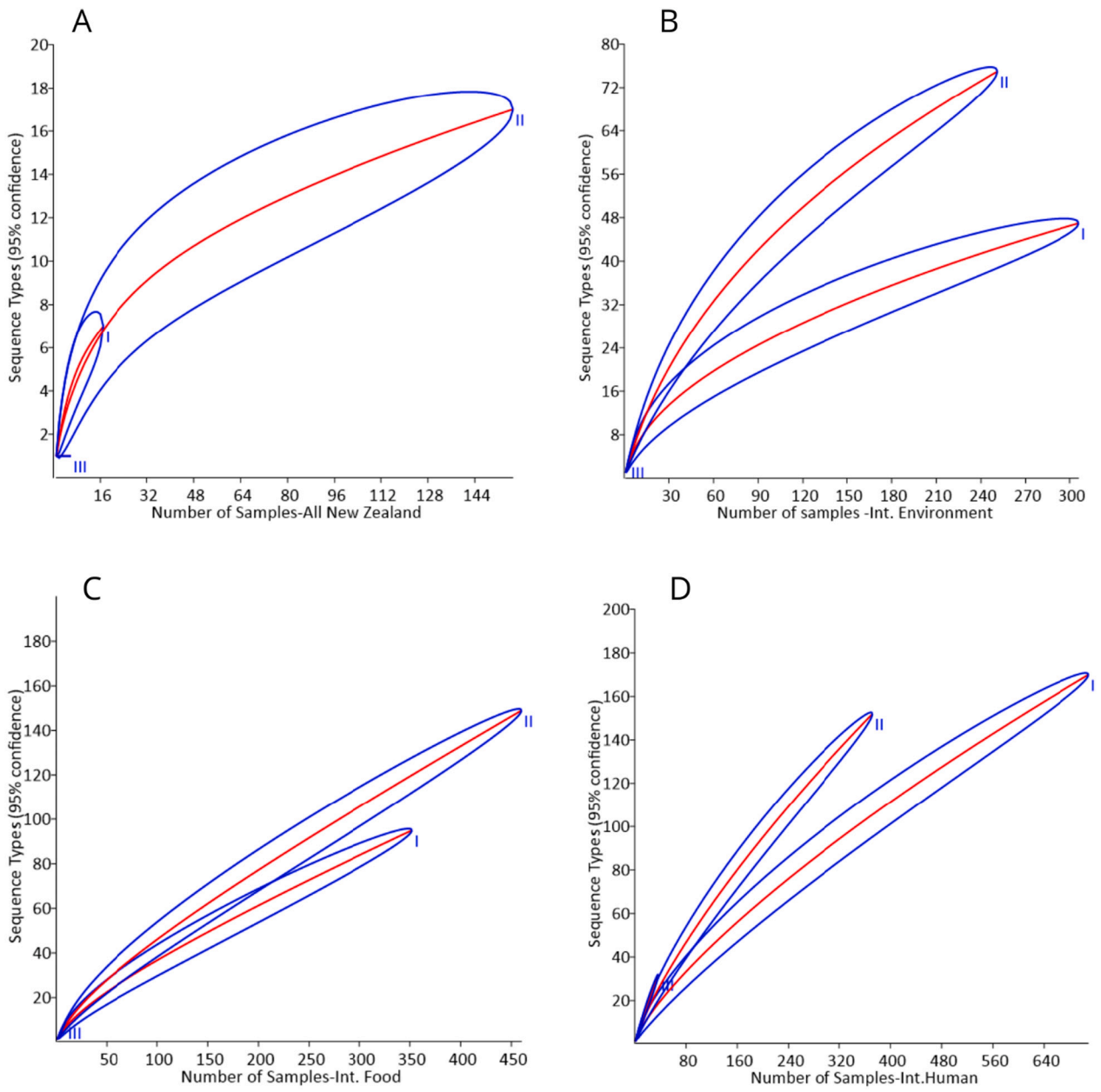

Fig. 2. Rarefaction analysis of the NZ *L. monocytogenes* populations by using the frequency of MLST STs encountered in NZ seafood and horticultural sources. The x-axis in the rarefaction plot represents the number of samples used in the analysis and the y axis represents the different number of genotypes (STs) identified in the given number of samples. The red lines in the charts represent the mean and the blue lines around it represent the upper and the lower limits at 95% confidence interval. The roman letters I, II and III in each chart denote the lineage. In A: the chart includes all New Zealand isolates; B includes all international environmental isolates excluding NZ isolates (Int. Environment); C includes international food isolates (Int. Food) and 2D includes international human listeriosis isolates (Int. Human). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

products from different places, utensils and personnel from the different environment may facilitate the entry of different strains into a factory environment. Some sources were dominated by particular pulsotypes, for example, 47% of the isolates from factory B (n = 208) were 65:02 and 57% of crop H (n = 30) were New:New. Such pulsotypes likely found a particular niche in the environment from which they typically spread to colonize other sites in the factory or supply chain. Although PFGE used to be the gold standard for typing *L. monocytogenes* (Graves and Swaminathan, 2001), due to its inherent limitations, recent studies suggest employing sequence-based typing or characterising tools is a better approach (Moura et al., 2017).

The underlying mechanisms for *L. monocytogenes* distribution and niche adaption have not been fully understood, however, Maury et al. (2019) suggested virulence to be the main player for niche adaptation. They stated that hypervirulent strains to be strongly associated with dairy products and gut lumen while the hypovirulent strains to be associated with food-processing environments (Maury et al., 2019). Based on MLST, Clonal complex 1 (CC1) has been reported as a hypervirulent gut lumen associated strain. Besides, other studies have identified and defined seven clonal complexes (CCs) as epidemic clones (EC) that cause disease outbreaks these being CC1:ECI; CC2:ECIV; CC5:ECVI; CC6:ECII; CC7:ECVII; CC8:ECV; CC11:ECIII) (Cantinelli et al., 2013; Chenal-Francisque et al., 2011b; Knabel et al., 2012; Ragon et al., 2008). In the current study, epidemic clones (EC in bold fonts were involved in outbreaks) CC1 (EC), CC101, CC14, CC14, CC155, CC193, CC2 (EC), CC204, CC224, CC307, CC31, CC321, CC7(EC), CC8 (EC) and CC9 were

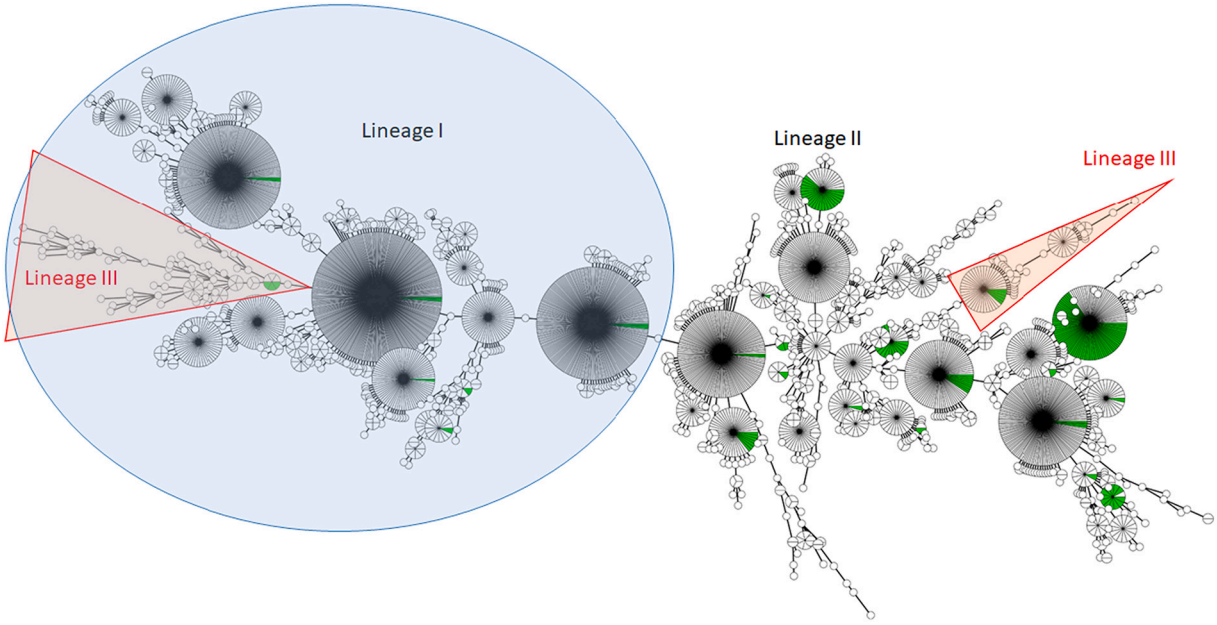

Fig. 3. Minimum spanning tree analysis of multilocus sequence types (ST) of *Listeria monocytogenes.* The green color in the pie charts represents New Zealand isolates. The blue big circle represents Lineage I and the colorless clusters without boundary lines represent Lineage II and the red triangles represent Lineage III. There are two clusters of lineage three isolates observed, one emerging from lineage I and another cluster emerging from lineage II marked in red font. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

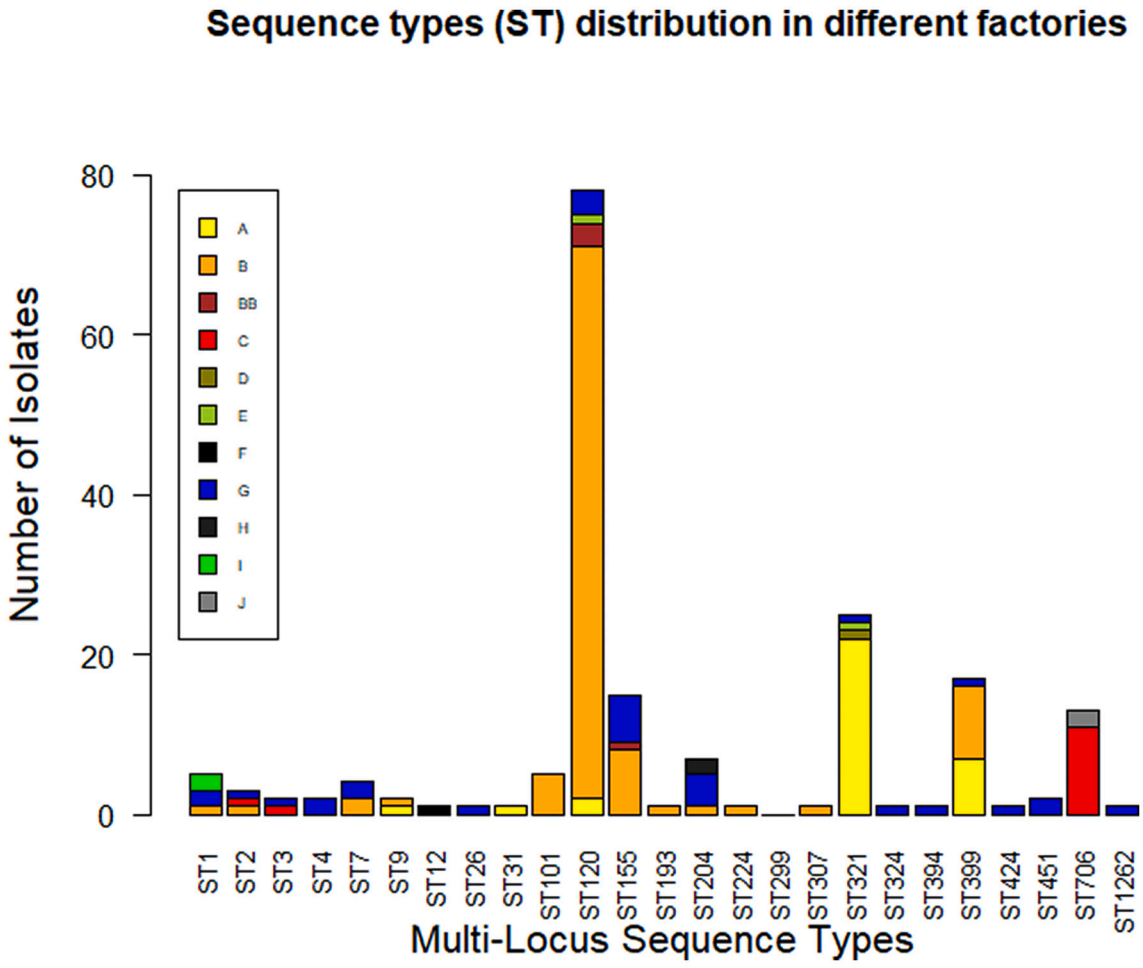

Fig. 4. The frequency distribution of *L. monocytogenes* STs in different factories. The factories are coded as A, B, BB, C, D, E, F, G, H, I and J. A, B, BB, C, D, E, F and G are different seafood processing operations while H, I and J are different horticultural crops and operations.

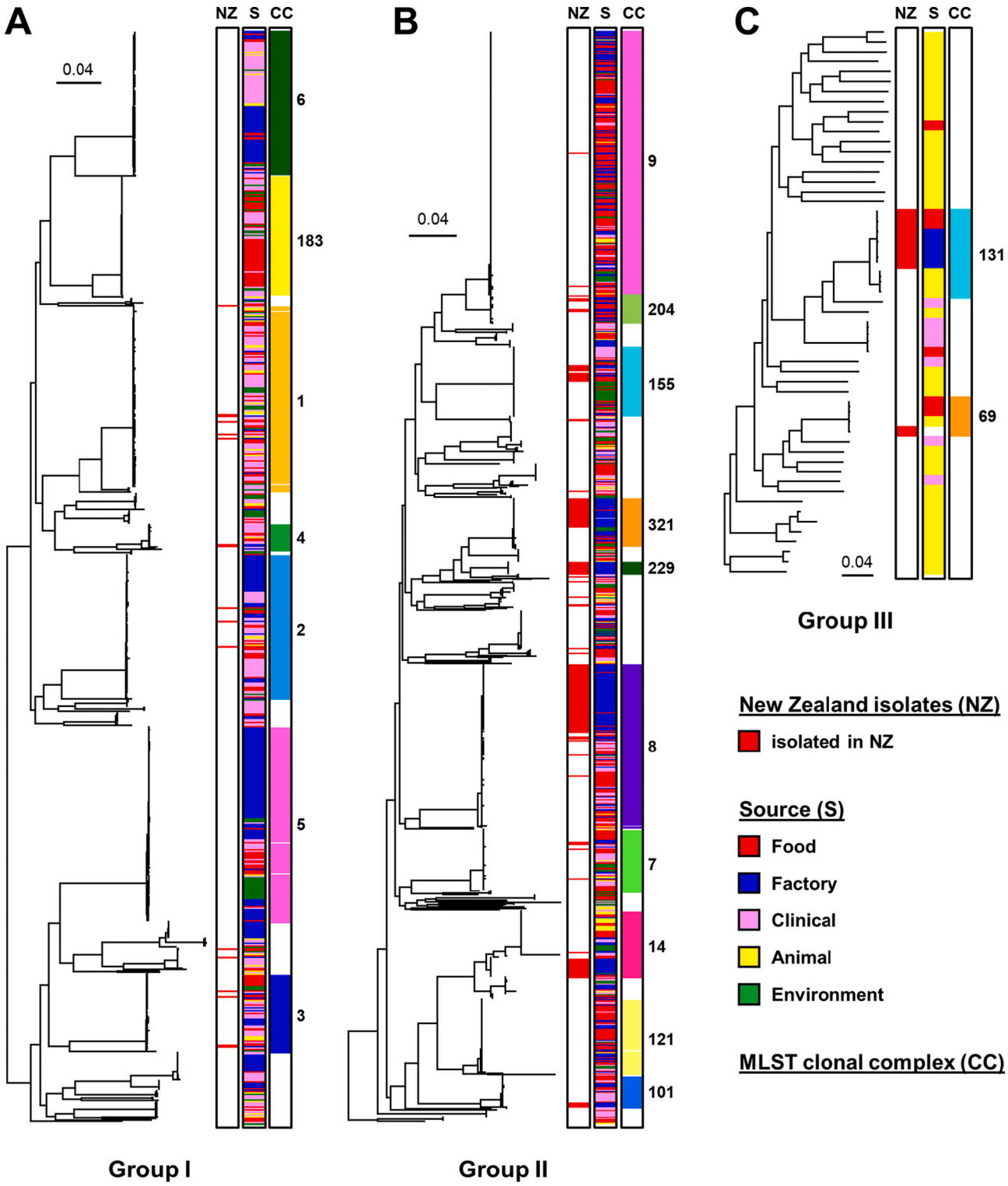

Fig. 5. Comparison of core genome single nucleotide polymorphisms (SNPs) of *L. monocytogenes* isolates from New Zealand and international sources. Trees were generated using ParSNP, and isolates highlighted for NZ isolation (NZ, the first bar, red), isolation source category (S, second bar, legend provided in the figure) and multilocus sequence type clonal cluster (MLST-CC, third bar). The specific clonal complexes are indicated by color and labeled. Panel A shows *L. monocytogenes* Lineage I, Panel B shows *L. monocytogenes* Lineage II, and Panel C shows *L. monocytogenes* Lineage III. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

isolated. The presence of CC1, CC2, CC7 and CC8 that caused outbreaks elsewhere among NZ isolates brings potential public health concerns. It is also very intriguing to observe the presence of another ancient EC, the CC101. Although common in the mid-1950s, CC101 became very rare until recently when it may have "begun to re-emerge" and ST38 of CC101 was implicated in 31/132 cases of listeriosis between 2006 and 2010 in Lombardy, Italy (Haase et al., 2014; Mammina et al., 2013). There was no ST38 in this study, however, all five CC101 isolates were characterised as ST101 (The details are presented in the Data in Brief article elsewhere in Table D1 (Mohan et al., 2021)). Haase et al., (Haase

et al., 2014) concluded that from a historical perspective, CCs present in the 1950s are not ancestral founders but they persisted and increased in frequency over decades and are now frequently being isolated from all source categories (Haase et al., 2014).

The ST distribution patterns varied at different factories. CC8 (ST120) was found to dominate in factory B (Fig. 4). ST120 (CC8) (and single-locus variants of it) is one of the ECs (ECVII) (Knabel et al., 2012) and was shown to be very successful in persisting among human patients and environmental niches in Canada (Knabel et al., 2012). CC8 has also been found in a variety of products such as meat, fruits and vegetables, mixed products and from unknown sources (Maury et al., 2019). ST120 has also been found to be resistant to benzalkonium chloride (Meier et al., 2017). The over-representation of ST120 in factory B suggests that ST120 may have persisted in that factory environment longer than other strains and may have been well adapted to that factory environment which may be correlated to the development of resistance towards sanitizers and sanitising protcols used in that factory.

ST9 has been isolated predominantly from food environments (Henri et al., 2016a, b; Martin et al., 2014; Maury et al., 2019) and has been attributed to causing human listeriosis in France (Henri, Felix, et al., 2016). The present study isolated ST9 from food-processing environments, indicating the likelihood of it being a public health risk if transferred onto products. Previous NZ studies on the association of *L. monocytogenes* isolates with processing premises have shown the environmental isolates often being found on the products (Nowak et al., 2017). Furthermore, in this study ST3 (CC3) and ST1 (CC1) were isolated from both food sources and their environments. ST3 has previously been encountered in ready-to-eat food (RTE) in China (Wu et al., 2016), from human clinical cases in Northern Spain (Pérez-Trallero et al., 2014) and in Australia (Jennison et al., 2017). ST1 (CC1) has previously been identified in Poland among human clinical cases (Kuch et al., 2018) and it has been reported to be spread globally (Chenal-Francisque et al., 2011asuggesting that these STs may be highly virulent. ST1 has also been isolated from RTE in China (Wu et al., 2016), dairy and ruminant lumen (Maury et al., 2019) and it is interesting to see ST1 being isolated from food processing factories in this study.

The majority of the STs (15/25) identified in the current study were also reported in Europe (Table 3). ST120 and ST204 dominated in Oceania regions and ST12 was only found in horticultural crop, while ST204 (n = 8) was most commonly found in Factory E. Factory G harbored the most diverse genotypes found in the study which implied that either the factories A and G handle samples from different sources or there is no particular persistent *L. monocytogenes* population in these factories.

MST of NZ isolates and the international isolates combined, showed lineage II dominating over all the other three lineages (Fig. 3) with the majority of NZ isolates falling into lineage II. Lineage II strains accounted for 89% of NZ strains (177 of 200) while lineage II represented only 45.3% (1674 of 3473) of the international *L. monocytogenes* population (PubMLST, 1998). Although the sample size of the NZ *L. monocytogenes* population is small compared to the international population, it is striking that 24/36 food isolates belonged to lineage II (Table 3). The diversity indices indicated the lineage II in the NZ population was quite diverse compared to the international lineage II populations while the isolates from human clinical cases had greater diversity compared to food and environmental sources in the international population. Moreover, the rarefaction analysis showed that the distribution of strains in lineage I and II in the international population was similar compared to the NZ population (Fig. 2a, b, c & d).

Considering the advantages of WGS through numerous proof-of-concept studies (Halbedel et al., 2018; Hilliard et al., 2018; Kwong et al., 2016; Lüth et al., 2018; Moura et al., 2017; Stasiewicz et al., 2015), we employed WGS SNP for understanding the *L. monocytogenes* diversity and population structure, their association with different sources in NZ and their international prevalence. When we compared the whole genomes with international genomes, NZ isolates closely clustered among themselves into distinct branches confirming the observations from the PFGE and MLST (Figs. 1 and 3). Given that the majority of the NZ isolates are from food-processing factory environments, it was expected that their genomes would cluster with international environmental sources, but quite a number grouped with isolates from food sources as well as human clinical cases. Nonetheless, NZ isolates stood out as distinct clusters and this perhaps indicate that their genomic characteristics need further investigation in terms of virulence and other genomic islands including phages and prophages.

CgSNP analysis of NZ lineage I isolates (Fig. 5a) showed good dispersion of grouping with international food, clinical and factory isolates. Given that lineage I is considered to be virulent and strongly associated with human infections (Gray et al., 2004; Kathariou, 2002; Maury et al., 2016; McLauchlin, 1990), their presence in processing environments represents a particular public health risk. The current study shows that lineage I *L. monocytogenes* strains are capable of colonizing factory and/or environmental niches, which is concerning as lineage I strains have been found to possess more invasive phenotypes compared to other lineages and their human gut colonizing genetic traits are robust (Pirone-Davies et al., 2018). This warrants further exploration of these NZ lineage I strains as well as targeted control measures to prevent them from being transferred on to products. Although lineage II has been reported to be an environmentally associated lineage (Cruz et al., 2014; Nadon et al., 2001; Nowak et al., 2017), its risk to human health cannot be neglected as lineage II isolates are capable of causing human infections and have been implicated in human listeriosis outbreaks (Gaul et al., 2013; Knabel et al., 2012; Lopez-Valladares et al., 2017). The current study showed that NZ lineage II isolates clustered with all different sources of international lineage II isolates (Fig. 5B). There were large clusters associated with international lineage II isolate from food, factory and multiple sources. However, there were also several smaller clusters found grouping with human clinical cases. *L. monocytogenes* within the lineage II group have been previously reported to be weakly clonal, undergoing frequent recombination and their genomes have increased genomic plasticity compared to Lineage I (den Bakker et al., 2008; Pirone-Davies et al., 2018). Their plasticity enabled them to adapt to different environments (den Bakker et al., 2008) and as expected, in the current study, NZ lineage II isolates were genetically diverse, grouping with isolates from different sources from the international database. This trait of lineage II is important to be understood better as their adaptation mechanisms play a crucial part in designing control measures that will mitigate the food safety risks due to *L. monocytogenes* lineage II strains.

Furthermore, a study identified eight genes that are significantly associated with food isolates in lineage II (Pirone-Davies et al., 2018) and one other previous study identified lineage II isolates being resistant to benzalkonium chloride (Moura et al., 2016). Although these eight genes are also present in lineage I they occur at a lower frequency (Pirone-Davies et al., 2018). Of these eight genes, two genes have been identified to cause resistance to cadmium (*cad*A and *cad*C), one gene for multi-drug resistance (*ebrB*) and one gene for quaternary ammonium compounds (*qac*). All of these resistant genes are plasmid-borne and these plasmids are associated with lineage II food isolates and these genes have been identified to be scattered in lineage I outbreak strains' genomes. This genetic characteristic of lineage II is concerning and *L. monocytogenes* genomes have to be studied in greater detail to better understand the movement and/or transmission of these genes to other lineages which is very important for food safety risk mitigation. Lineage II food isolates may be the sources of these plasmids transmitting them to other lineages.

Lineages III and IV have been isolated mostly from animal sources (Nadon et al., 2001) in previous studies and in the current study, the majority of *L. monocytogenes* were isolated from horticultural sources. However, using SNP analysis showed that the NZ isolates clustered with international human clinical isolates apart from one that clustered with food isolates (Fig. 5C). Although SNP has provided a significant level of

discrimination, gene-based comparison of NZ isolates will shed more lights into the genetic basis of persistence, resistance and niche adaptation.

Overall, to conclude, the current study has evaluated different molecular characterisation techniques for characterising *L. monocytogenes* isolated from different food processing factories from New Zealand. The characterised isolates, in turn, were compared with international *L. monocytogenes* isolates to better understand the population structure and the global distribution of *L. monocytogenes* strains. Although PFGE can be used as an initial screening tool that provided relative discrimination between strains, MLST provided greater phylogenetic inferences and less ambiguous clustering and WGS-based typing (cgSNP) allowed improved discriminatory power over PFGE. NZ isolates appear to be unique when compared to international populations, although some clustered among global isolates, the majority of the isolates formed distinct clusters on the phylogenomic trees. The current study also discovered the presence of epidemic clones and historic CCs in New Zealand that were isolated back in the 1950s which brings major public health concerns. The genomic comparisons also showed that although most of the NZ isolates came from food-processing and packing environments (factories), some isolates genetically clustered alongside international human outbreak and clinical isolates suggesting that human clinical isolates and food processing factory *L. monocytogenes* isolates have to be investigated simultaneously to establish the link between factory isolates and human clinical cases. One of the limitations of this study is that, due to confidentiality protocols, this study could not access clinical isolates which would have provided a better understanding of the population structure and distribution of *L. monocytogenes* in food, factories, and clinical cases.

Declaration of competing interest

The authors declare that there are no competing interests or conflict of interest in this research work and the manuscript publication.

Acknowledgements

The authors are grateful to Mark Bradbury, Falk Kalamorz. Susan Marshall and Monica Holland for reviewing and editing the manuscript. This work was funded by The New Zealand Ministry of Business, Innovation, and Employment (CAWX0301) and Plant & Food Research's Discovery Science and Strategic Science Investment Funds, Future Consumer Foods Programme (1921).

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