Risk profiling *Listeria* in ready-to-eat foods

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ISBN: 978-1-905767-83-0

Publication date: July 2018

Acknowledgements

***safe*food** wishes to thank the project team of Dr R. H. Madden (the Agri-Food and Biosciences Institute), Dr M. Hutchison (Hutchison Scientific Limited), Dr K. Jordan (Teagasc) and Professor N. Corcionivoschi (the Agri-Food and Biosciences Institute).

We also acknowledge the contribution of the participating companies for the staff time and effort they made to support this project. Dr Roisin Lagan of the College of Agriculture, Food and Rural Enterprise assisted by using her industrial expertise to bring food companies into the programme. Ms Pam Scates (the Agri-Food and Biosciences Institute) played a significant role by coordinating the in-plant sampling programme, supporting the participating companies and managing the sample analysis at the Institute. Dr. Ozan Gundogdu of the London School of Hygiene and Tropical Medicine carried out the analysis by whole genome sequencing.

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Executive summary

Clinical invasive infection by ***Listeria monocytogenes*** bacteria is called **listeriosis**. Listeriosis causes flu-like symptoms and it is rare in healthy people. However, there are groups that are vulnerable to invasive infection. These include the immunocompromised (people who do not respond normally to infection because their immune system is weakened), elderly people and pregnant women. The mortality or death rate for *Listeria monocytogenes* infections was 12.7% across 26 European Union Member States and Norway for the period January 2010 to January 2012.

In terms of the number of microbiological incidents in food reported in Ireland each year, those involving *Listeria* are the second most frequent, Incidents involving *Salmonella* are the most frequently reported. (*Salmonella* infection causes diarrhoea, cramps, vomiting and fever). Approximately 30% of incidents involving *Listeria* reported between 2005 and 2011 involved ready-to-eat sliced meats. Whilst few of these incidents were associated with known cases of listeriosis, this food type was linked to 2 United Kingdom-wide listeriosis outbreaks in 2009 and 2010.

Listeria monocytogenes can be introduced into meat- and fish-processing environments by several routes, including the organism being present on contaminated ingredients such as raw meat, fish and packing materials. *Listeria monocytogenes* is widespread in the environment. Food-processing environments are at continuous risk of colonisation by this bacterium.

Legally, the levels of *Listeria monocytogenes* in a ready-to-eat food product must not exceed 100 bacterial colony-forming units per gram during its shelf-life (European Commission Regulation No. [EC] 2073/2005).

This research project involved

- Risk profiling *Listeria* in ready-to-eat foods
- Investigation of control strategies and practical interventions for food processors in Northern Ireland.

The study was undertaken to complement an ongoing study supported by the Republic of Ireland's Department of Agriculture, Food and the Marine under the Food Institutional Research Measure project number 11F008. Both studies had the aim of monitoring the occurrence and persistence of *Listeria monocytogenes* in foods and environments of food-processing facilities.

This study involved the active participation of 24 food business operators, all based in Northern Ireland. Most were classified as small to medium-sized enterprises.

The sampling part of the study took place over a period of 18 months, from July 2015 until November 2016. It involved approximately 2,250 analyses and examinations.

- Participants swabbed 6 specific sites in their premises.
- Participants sent these environmental samples, plus 2 samples of their final food products, to the Food Science Branch at the Agri-Food and Biosciences Institute, Belfast, for analysis to isolate (look for and identify) *Listeria monocytogenes* colonies of bacteria.
- *Listeria monocytogenes* "isolates" – the separated bacterial strains – obtained were subjected to pulsed-field gel electrophoresis (a highly accurate means of detecting the presence of organisms) at Teagasc, Moorepark.
- Isolates were also subjected to whole genome sequencing, which gives an extra level of discrimination (the ability to distinguish between organisms), at the London School of Hygiene and Tropical Medicine.
- Data obtained was analysed to gain information on the relatedness (the similarities) and potential pathogenicity (the ability to cause disease) of the isolated strains, which would indicate whether they are significant in terms of human health.
- In addition, the physical properties (the acidity and a_w) of 130 foodstuffs were determined.
- The ability of the foods to support the growth of *Listeria monocytogenes* at normal refrigeration temperature, and at "abuse" temperatures (conditions that are considered too hot or too cold for storing the foods safely), was estimated.
- The presence of genes enabling isolates to tolerate sanitiser used in the food industry was investigated.

Overall, 1,594 samples were submitted for analysis to detect *Listeria monocytogenes*.

- *Listeria monocytogenes* was found in 4.6% of food samples and 6.3% of environmental swab samples, with 96 isolates obtained.
- Half of the food business operators submitted samples in which no *Listeria monocytogenes* was detected.
- Two meat products were submitted that exceeded the legal limit for *Listeria monocytogenes* of 100 colony-forming units per gram.
- *Listeria monocytogenes* was mainly found in premises producing processed mushrooms, cooked meats or sandwiches. The latter 2 products were implicated previously in outbreaks of listeriosis.
- In food business facilities, floors, drains, trolley wheels, boots and chill surfaces yielded 81% of the *Listeria monocytogenes* isolates, confirming the findings of previous studies.
- Significantly more environmental samples were positive for *Listeria monocytogenes* in the warmer months (May, July and September) but no underlying cause was defined for this.
- Based on the physical properties measured for 130 food samples (their acidity, or "pH", and their a_w), 111 would support the growth of *Listeria monocytogenes*. At 4 degrees Celsius the initial

level of contamination would have to be more than 1 colony-forming unit per 10 grams for the legal limit to be exceeded within a week. However, at the abuse temperature of 8 degrees Celsius, 77.5% of foods could support growth from this low level to more than the legal limit, within a week. This shows that an effective "cold chain" (a temperature-controlled food supply chain), and appropriate shelf-life limits, are essential for the potential safety of some products.

- Analysis of the whole genome sequence data showed that all *Listeria monocytogenes* isolates carried the genes *inl*A and *act*A, which are involved in pathogenicity, or virulence (the ability of the organism to cause disease).
- In addition, 71% of *Listeria monocytogenes* isolates carried *qac*H. This gene gives the organism resistance to quaternary ammonium compounds, commonly used in sanitisers, which may help explain the persistence of some strains in food business premises.
- Multilocus sequence typing was undertaken using the whole genome sequence data. This molecular typing technique allows for detailed comparisons to be made. It showed that recurrence of specific sequence types or strains of bacteria occurred in some premises over periods of months, or even over a year. Further, the same sequence type was isolated from both environmental swabs and food products in some food businesses, indicating cross-contamination.
- Ninety-eight per cent of the *Listeria monocytogenes* isolates obtained in this study were of the same sequence types as have been isolated in clinical cases of listeriosis in the United Kingdom.
- Comparison of the pulsotypes – the strains of bacteria distinguished by the pulsed-field gel electrophoresis study – showed recurrence and cross-contamination within the food-processing facilities. When compared with a database of pulsotypes found in the Republic of Ireland, it was seen that some Northern Ireland isolates were identical to those found in the Republic of Ireland, including clinical isolates (pure microbial strains).
- Finally, a detailed questionnaire survey intended to assess control strategies and practical interventions in participating food businesses was returned by 54% of recipients. There is not enough data to make recommendations regarding control measures. On-site audits would be required to define any noticeable and relevant differences in control practices that give rise to the results observed in this study.

Key project recommendations

1. The study included a detailed questionnaire survey for assessing control strategies and practical interventions to control *Listeria monocytogenes* in food businesses. This yielded no differences between premises in which *Listeria monocytogenes* (*L. monocytogenes*) was present and those in which it was absent.
   It is therefore recommended that a follow-up study is undertaken to audit premises, based on the results of this study. This will define key differences between the facilities where no *L. monocytogenes* was found and those with significant prevalence, so that better guidance can be supplied to food business operators (FBOs).

2. The study found that most of the foods could potentially support growth of *L. monocytogenes* and that the *L. monocytogenes* obtained in this study were potential human pathogens.
   This finding should be communicated to all participating FBOs, and disseminated to relevant authorities.

1 Background

This project involved;

- Risk profiling of *Listeria* in ready-to-eat (RTE) foods
- Investigation of control strategies and practical interventions for food processors in Northern Ireland (NI).

The study was undertaken to complement a study supported by the Republic of Ireland's (ROI) Department of Agriculture, Food and the Marine under the Food Institutional Research Measure (FIRM) project number 11F008. Both studies had the aim of monitoring the occurrence and persistence of *L. monocytogenes* in foods and environments of food-processing facilities. A closely coupled research programme resulted, with this study directly benefitting from the experiences of the staff in the ROI, and adding to the data gathered, to enable information from across the island of Ireland to be compiled and considered.

This report deals with the study concerning *L. monocytogenes* in NI only.

2 Project aims and objectives and methodology

Project aims and objectives

The study was based on achieving a sequence of specific aims and objectives:

- Carry out a full and thorough review of literature (published material) around *Listeria monocytogenes* in the food-processing environment, and existing control strategies.
- Harness the resources of the College of Agriculture, Food and Rural Enterprise (CAFRE) and the ***safe***food *Listeria* Knowledge Network to contact food-processing facilities across NI and request their participation in the sampling part of the study.
- Recruit 24 food business operators (FBOs) producing RTE foods in NI to the programme.
- Train participants to take swab samples in their premises every 2 months at 6 defined locations and dispatch the swabs to the Agri-Food and Biosciences Institute (AFBI), in addition to 2 food samples.
- All samples to be analysed by AFBI for the presence of *Listeria monocytogenes*,
- All food samples to undergo enumeration of *Listeria monocytogenes* at AFBI, with identification using API® *Listeria*.
- Analyse selected foodstuffs to determine their acidity or "pH" and a_w, so that the potential for the foodstuffs sampled to support the growth of *L. monocytogenes* can be determined using the ComBase *L. monocytogenes* predicted growth model.
- Invite all participants to complete questionnaires to assess their premises and practices for factors relevant to the control of *L. monocytogenes*.
- Confirmed isolates of *L. monocytogenes* to be genotyped by being subjected to pulsed-field gel electrophoresis (PFGE), as the normal typing method for *Listeria*.
- Confirmed isolates of *L. monocytogenes* to be subjected to whole genome sequencing (WGS), to allow typing of the isolates and further comparison between strains.

Having met these aims and objectives, the project team analysed all available data to complete a final report to ***safe***food.

It is intended that this report will allow the participating FBOs, and others across the island of Ireland, to be informed of the risk presented by *L. monocytogenes* in food premises.

Project methodology

Literature review

A review of the literature relating to *L. monocytogenes* contamination of cooked sliced meat and cold smoked fish was undertaken from a variety of academic and other sources. The review literature was identified using a systematic and reproducible approach.

- The likelihood of cooked sliced meat contamination by *L. monocytogenes* is examined in the review. Key production and processing practices that could influence the prevalence of contamination by *L. monocytogenes* and numbers of incidents associated with ready-to-eat food through the processing and retailing chain are also investigated. This is a very comprehensive review and is published separately.

Recruitment and training of food business operators

Dr Roisin Lagan (CAFRE, Cookstown) led the recruitment of FBOs to the programme. In total, 24 companies participated in this study.

- Four half-day workshops were undertaken between 15 and 28 April, 2015, at the CAFRE. All participating FBOs provided sketch plans of their premises and marked the sites to be sampled during the programme.
- The supply of sampling kits to the FBOs and the subsequent analysis of the samples was managed by Ms Pam Scates (AFBI). A video of sampling procedures was prepared and the internet address of this was provided to participants.
- Sampling for the project commenced in July 2015. A mid-term feedback session for the FBOs was held at CAFRE in February 2016. Participating FBOs were informed of their sample results throughout the project.

Microbiological analyses

All microbiological media were supplied by Thermo Fisher Scientific Oxoid Ltd (Basingstoke RG24 8PW, England) unless stated otherwise. Sampling kits (see Appendix 1) were despatched to FBOs 2 weeks before the target sampling date.

- The samples were sent from the food businesses by courier on the day they were taken, to arrive at AFBI the next morning.
- Methods of analysis used, and the local methodology reference number (used on local ISO 17025 certification), were,
    - For detection of any *Listeria* species present, including *L. monocytogenes*: (based on BS EN ISO 11290-1:1997+A1:2004)

- For the enumeration of any *Listeria* species present, including *L. monocytogenes*: As described in BS EN ISO 11290-2:1998.

- Briefly, for the detection of *Listeria* species in food samples:
    1. Half Fraser Supplements (SR0166) were added to Fraser Broth Base. The nutrition supplements are intended to boost the growth of *L. monocytogenes*, to make it easier to detect the pathogen.
    2. The mixture was blended for 2 minutes (Colworth Stomacher® 400 circulator, Seward Limited, Worthing, West Sussex BN14 8HQ, England) then allowed to stand for 1 hour.
    3. Samples were then plated (0.1 ml) onto plates of a colour-producing growing medium to detect and count *L. moncytogenes*. The medium used was "agar *Listeria* according to Ottaviani and Agosti" (ALOA CM1084+SR0226). (Agar is a substance that comes from algae or seaweed, and is often used as a medium in which to grow, or "culture", microorganisms.)
    4. The plated samples were incubated at 30 °C for 24 hours.
    5. Next, 0.1 ml was added to 10.0 ml Fraser Broth, with full strength Fraser Supplements (SR0156), which was then incubated at 37 °C for 48 hours.
    6. Plates of ALOA growing medium were "streaked" from the broths after incubation. (Streaking is a method of diluting and separating bacterial cultures so that individual organisms can grow into identifiable colonies of distinct strains.)
    7. The streaked plated samples were incubated at 37 °C for 48 hours, with examination after 24 hours and 48 hours.
    8. Where plates yielded presumptive *Listeria,* 5 colonies were purified and confirmed using API® *Listeria*.

- Briefly, for the detection of *Listeria* species in the food-processing environmental swabs:
    - Fraser Broth Base (90 ml) with half-strength supplements was added to the swab, in the same bag that had been used to transport the swab to the laboratory.
    - The sample was then incubated and processed as described (steps 3-8) for the detection of *Listeria* species in foods, above.

- Briefly, for the enumeration of *Listeria* species in food samples:
    - Twenty-five grams (g) of sample was added to 225 millilitres (ml) Fraser Broth Base (CM0895, without selective supplements). (The broth is a nutrient-rich liquid in which pathogens are grown, or cultured. Nutrient supplements can be added to this to boost the growth of selected organisms.)
    - The mixture was blended for 2 minutes (Colworth Stomacher® 400 circulator, Seward Limited, Worthing, West Sussex BN14 8HQ, England) then allowed to stand for 1 hour.
    - Samples were then plated (0.1 ml) onto plates of ALOA CM1084+SR0226.

- The plated samples were incubated at 37 degrees Celsius (°C) for 48 hours, and examined after 24 hours and 48 hours.
- Plates with less than 150 typical colonies of bacteria were counted. Where plates yielded presumptive (likely but unconfirmed) *L. monocytogenes,* 5 colonies were purified and confirmed as *L. monocytogenes*, using API® *Listeria* (bioMérieux UK Limited, Basingstoke RG22 6HY, England).
- The final count was obtained by multiplying the presumptive count by the percentage of confirmed *L. monocytogenes* colonies.

- All *L. monocytogenes* isolates were stored at minus 80 °C prior to further study.

Questionnaire survey of food business operators' premises and practices

After 8 sets of samples had been received, a questionnaire (see Appendix 2) was sent to all FBOs, to obtain information about the premises and the practices relevant to the control of *L. monocytogenes*.

Follow-up phone calls to contact staff at all premises were made in an effort to maximise the number of forms returned.

Pulsed-field gel electrophoresis typing

Pulsed-field gel electrophoresis typing is a highly accurate means of detecting the presence of organisms by applying a changing electrical current to a gel "matrix". This matrix is the substance in which the organisms are held and subsequently separated for identification during the test.

1) Deoxyribonucleic acid ("DNA", which carries the genetic instructions for a cell's function, growth, development and reproduction) was prepared from bacterial cells "washed" in 0.9% phosphate-buffered saline, a salty solution that helps maintain a constant pH.
2) The cells were mixed 1:1 in a solution of 2% weight by volume (w/v) agarose (CleanCut®, Bio-Rad, Hemel Hempstead, England). (Agarose is a type of sugar. It is the main constituent of agar and is used to make the gel. A "2% w/v" solution contains 2% of a substance by weight as against the total volume of the solution.)
3) The mixture was "lysed", a process in which the cell walls break down in a purpose-made solution – the "buffer" – and so release the particles contained inside. This took place during 2 overnight incubations in 2 ml of exclusion-based lysis preparation (ESP) buffer (0.5 moles per litre [mol/l] ethylenediaminetetraacetic acid [EDTA] [E5134; Sigma-Aldrich/Merck], pH 9.0 plus 1% N-laurylsarcosine [L5777; Sigma-Aldrich/Merck]) containing 250 micrograms per millilitre (µg/ml) proteinase K, to aid digestion, (P2308; Sigma-Aldrich/Merck) at 56 °C.
4) Analysis was performed by digesting the whole genomic DNA of the bacteria, encased in 1.0% w/v agarose with the "restriction enzymes" *Asc*I and *Apa*I, in 2 separate treatments, following the International Standard PulseNet protocol (PulseNet USA, 2013). (Restriction enzymes break strands of DNA into pieces at specific points.)

5) The DNA was separated using a CHEF-DR III® (Bio-Rad, Hemel Hempstead, England) pulsed-field electrophoresis apparatus. An initial switch time – the length of time that the electrical field is pulsed in a single direction – of 4.0 seconds was ramped to a final switch time of 40.0 seconds over a period of 18 hours. The voltage was 6 volts per centimetre (V/cm) of gel using an electrical field application angle of 120 degrees. Molecular weight standards of concatemers of lambda phage were included to allow the normalisation of separation conditions between batches.
6) Bands were visualised under ultraviolet (UV) light at a wavelength of 305 nanometres (nm) after staining with ethidium bromide, a fluorescent marker.
7) Isolate similarity dendrograms ("tree" diagrams that illustrate genetic similarities between strains) were generated using BioNumerics version 5.10 software (Applied Maths NV, Kortrijk, Belgium), by the unweighted pair group method with arithmetic mean (UPGMA) with tolerance and optimisation settings of 1%, as previously described by Fox and colleagues (2012).

Whole genome sequencing

Whole genome sequencing is the process of determining the entire DNA sequence of an organism's genome at a single time. This allows a highly accurate way of identifying organisms and their level of similarity to each other.

- The DNA for analysis was extracted from all isolates using a PureLink® Genomic DNA Kit (Thermo Fisher Scientific, Paisley PA4 9RF, Scotland) following the manufacturer's instructions.
- The genome sequencing of all *L. monocytogenes* isolates was performed as described by Ugarte-Ruiz and colleagues (2015) using Illumina® MiSeq® 2 × 250 base pairs (bp) paired-end sequencing.
- To analyse the data quality FastQC software (Babraham Bioinformatics, Cambridge, England) was used (Andrews, 2016).
- To evaluate the sequencing reads, Trimmomatic software (v0.32, Usadellab.org) was used with the parameters
    - "leading" and "trailing" setting of 3
    - "slidingwindow" setting of 4:20
    - "minlength" of 36 nucleotides

  (Bolger et al., 2014).
- Burrows–Wheeler Aligner software (BWA-MEM v0.7.7-r441) was used to map the reads using the genome sequence of *L. monocytogenes* EGD (HG421741) as described by Li and Durbin (2009).
- VelvetOptimiser software (v2.2.5) using n50 optimisation was used to perform sequence assembly (Zerbino and Birney, 2008; Gladman and Seeman, 2012).
- The reference strain *L. monocytogenes* EGD (HG421741) was used to complete contigs (maps of overlapping DNA or fragments of DNA) using ABACAS software (v1.3.1) (Assefa et al., 2009).

- Genome annotation was provided by using Prokka software (Seemann 2014).
- To read the genomes, Artemis and Artemis Comparison Tool (ACT) software were used (Carver et al., 2012).

Statistical analyses

All statistical analyses were undertaken by Mr Alan Gordon, of the Biometrics and Information Systems Branch, AFBI, using Genstat Release 18.1 for Windows (VSN International Limited, Hemel Hempstead HP2 4TP, England).

3 Results

Survey for *Listeria monocytogenes*

Participating FBOs were provided with sampling kits by the AFBI, and access to a training video. The CAFRE provided support where required, including appropriate training to the food-processing companies. The samples were sent to the AFBI, who undertook the analyses required to determine the presence – and for foodstuffs also the numbers – of *L. monocytogenes*. Premises undertook sampling on alternate months for a period of 18 months, from July 2015 until November 2016. This involved sending 6 environmental swabs plus 2 food samples, as per the FIRM study, to the AFBI lab for the isolation and confirmation of *L. monocytogenes*.

Overall, 24 FBOs submitted 1,592 samples for analysis. These consisted of 1,197 swabs and 395 food samples.

- Seventy-six food environment swabs (6.3%) and 18 food samples (4.6%) yielded *L. monocytogenes*.
- Two samples of cooked meat had more than 100 colony-forming units per gram (cfu/g) of *L. monocytogenes*.
- The positive samples came from 12 premises – 50% of the participating FBOs.

Food products that yielded no *Listeria monocytogenes*

The products of the food businesses that yielded no *Listeria monocytogenes* are given in Table 1.

Table 1: Food products that yielded no *Listeria monocytogenes*

Food products that yield no *Listeria Monocytogenes*
Cheese snack foods
Chicken and egg products
Gluten-free salads, meat and vegetarian ready-meals, stuffing
Hot smoked organic salmon
Ice cream A
Ice cream B
Pasta sauces
Pates and ready meals
Savoury and sweet pastry products
Snacks: sprouted seeds, gluten-free and nut-free roasted seeds and snacks
Vegetable convenience ready-to-eat products
Yoghurt

Number of isolates obtained from environmental swabs and food products in premises that yielded *Listeria monocytogenes*, by product type manufactured

The product types manufactured in premises where *L. monocytogenes* was isolated from submitted samples are shown in Table 2. The source of each isolate is shown in terms of environmental swabs and food products.

- The 6 premises at the top of Table 2 yielded 80% of the isolates obtained.
- Positive food samples were received from 21% of all FBOs.

Table 2: Number of isolates obtained from environmental swabs and food products in premises that yielded *Listeria monocytogenes*, by product type manufactured from food business operators* premises

Food product type manufactured	Environmental swabs	Food products
Processed mushrooms	12	7
Cooked meats A: Pulled chicken, turkey and beef	17	2
Sandwiches A: Rolls and wraps	10	2
Baked goods: Cakes, pies, sausage rolls, traybakes	10	
Cooked meats B: Pork from fresh and cured meat	5	5[1]
Sandwiches B: Salads, wraps and snacks	7	
Salads: Green and pasta salads, chicken tuna and egg mixes	5	
Ready-to-eat raw fruit pieces in consumer packs	5	
Sandwiches C: Pasta and salad bowls, coleslaw, potato salad		4
Ready-to-eat processed fish or shellfish	2	
Ready-to-eat vegetable products	2	
Sandwiches D	1	

[1]Three food samples were positive. Two of these samples yielded *L. monocytogenes* isolates from both enrichment cultures and enumeration plates.

Occurrence of *Listeria monocytogenes* by category of food product manufactured

The categories of food processors from which *L. monocytogenes* was obtained are shown in Table 3.

Table 3: Occurrence of *Listeria monocytogenes* by category of food product manufactured

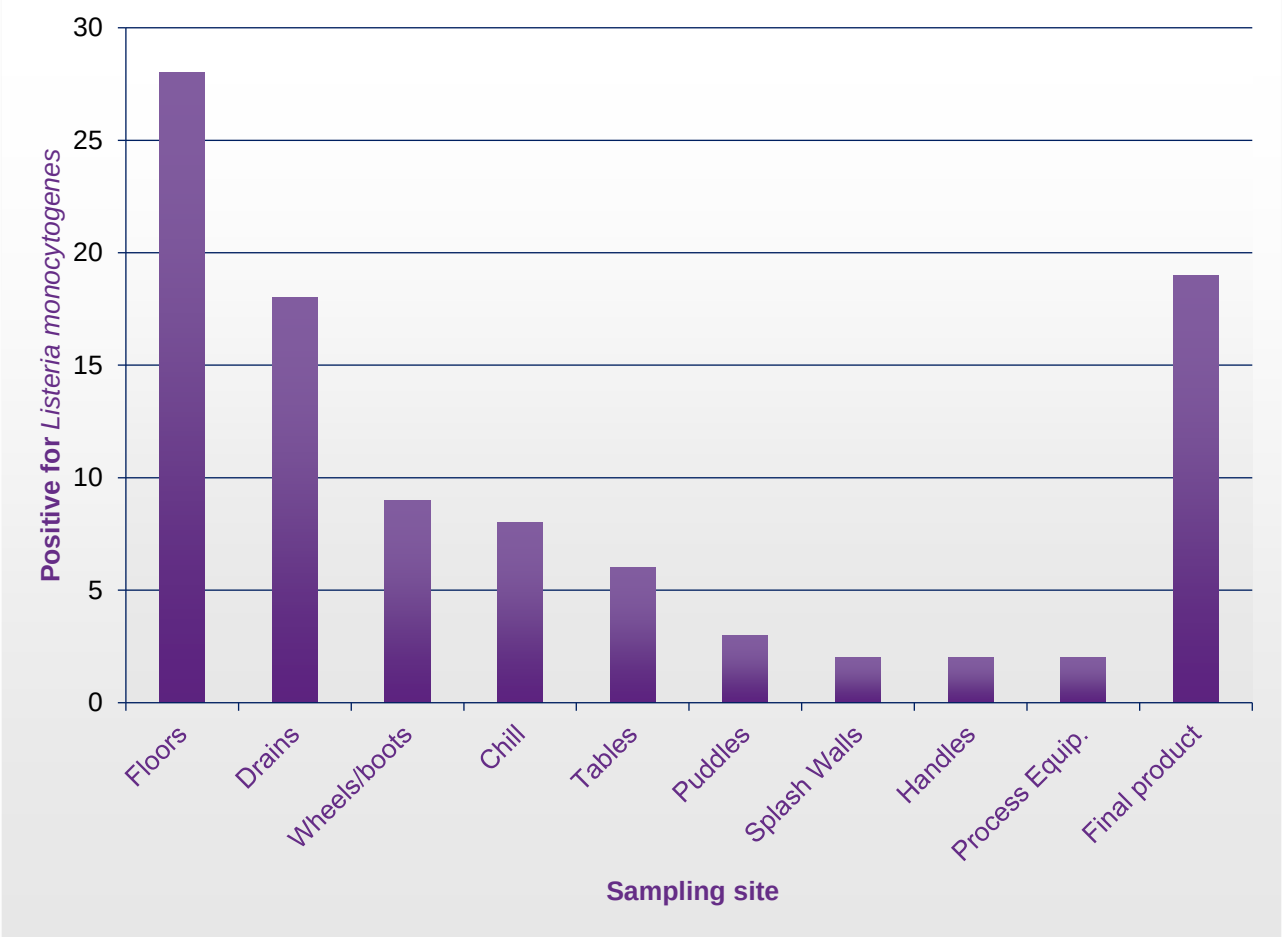
Food product category	Total number of samples	Number of samples positive for *Listeria monocytogenes*	Percentage of samples positive for *Listeria monocytogenes* (%)
Meat	225	27	12.0%
Sandwiches	286	24	8.4%
Baked goods	128	10	7.8%
Horticulture	398	26	6.5%
Salads	136	5	3.7%
Seafood	144	2	1.4%
Dairy	281	0	0.0%

Environmental swabs

The locations from which the *L. monocytogenes* isolates were obtained are shown as Figure 1.

- The 4 most frequently contaminated sites yielded 81% of isolates.

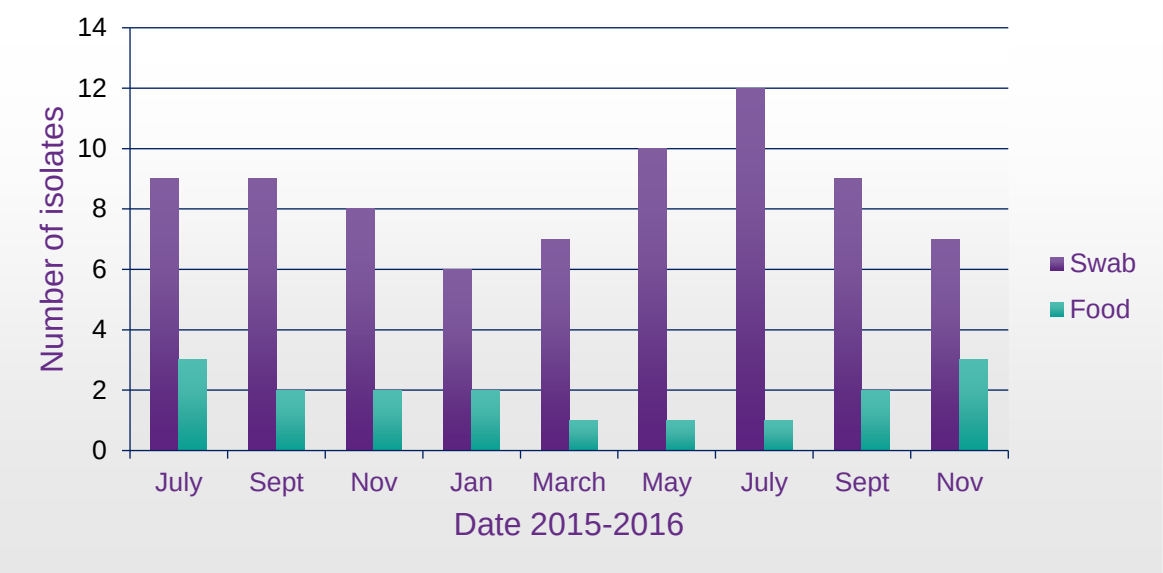
Figure 1: Locations from which *Listeria monocytogenes* was isolated



The isolates obtained during an 18-month sampling schedule, and the distribution of isolates with time, are shown as Figure 2.

- No statistically significant association was found between the numbers of *L. monocytogenes*-positive food samples and the seasons of summer (May, July, September) and winter (November, January, March).
- However, for environmental samples the difference was significant: $p = 0.007$, by one-way analysis of variance. (One-way analysis of variance, or "one-way ANOVA" is a technique used to assess differences between unrelated groups of data.)

Figure 2: Isolations of *Listeria monocytogenes* over the 9 isolation events of the survey



Food product samples

To determine the potential for the foodstuffs sampled (Table 1, Table 2) to support the growth of *L. monocytogenes* a total of 130 food samples were analysed to measure their pH and a_w.

- This data was fed into the ComBase *L. monocytogenes* computer growth prediction model (http://browser.combase.cc) to obtain an estimate of the mean or average generation time – the time it takes to complete one generation of an organism – at 3 storage temperatures: 4 °C, 6 °C and 8 °C.
- Nineteen samples were predicted not to support the growth of *L. monocytogenes*, 14 of these due to their low pH (below 4.60) and 5 due to their low a_w (below 0.974).

The pH and a_w values for the remaining 111 foods are shown in Figure 3.

For these, the doubling time at each of the 3 temperatures was calculated. To assess and compare the potential of each foodstuff to support the growth of *L. monocytogenes*, an arbitrary growth parameter was chosen. This was the ability of the food to support 10 generations of growth, equivalent to approximately 1,000-fold growth in numbers of bacteria, in 7 days or less. This corresponded to growth of bacteria from 1 cell per 10 grams to the legal limit of these foodstuffs: 100 cfu/g.

- Over 80% of the 111 samples in which *L. monocytogenes* was predicted to grow had a pH greater than 5.5, and over 86% had an a_w greater than 0.97.
- At 4 °C none of the 111 foodstuffs that were predicted to support growth of *L. monocytogenes* could achieve 10 generations in a week.
- However, at 6 °C 55.0% of foods could exceed 10 generations of growth in a week.
- At 8 °C 77.5% of foods could exceed 10 generations of growth in a week.

These results were reported to the relevant companies. Advice was provided where necessary to deal with the information supplied, such as discussion of the requirements of the European Community Regulation (EC) 2073/2005 (2005) on microbiological criteria for foodstuffs.

Food business operators

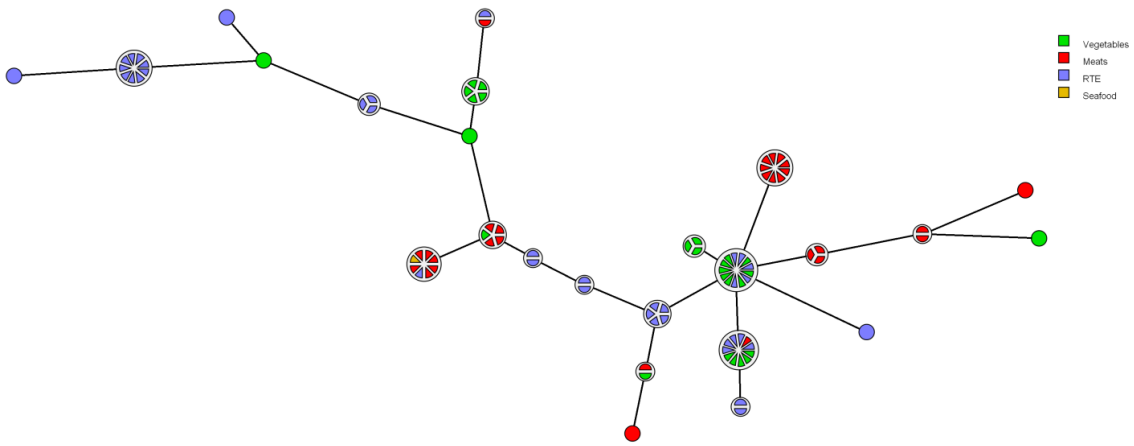
Listeria monocytogenes isolates from all positive samples were purified and genotyped using PFGE, to produce a DNA fingerprint. The resulting profiles were stored and analysed using BioNumerics software. Isolates exhibiting the same PFGE profile are grouped together in pulsotypes, and these similarities can be used to connect isolates from food, environmental and clinical samples (pure microbiological strains). The PFGE typing process was carried out to allow the *L. monocytogenes* obtained in this study to be directly compared with those obtained in studies undertaken in the ROI.

Figure 3 shows a minimum spanning tree graph, representing the PFGE profiles of all the isolates. The different colours represent different food categories. Within a circle, each segment represents an isolate, while the circle represents a pulsotype where the isolates show more than 90% similarity. The length of the line between the circles represents the distance of the relationship between the pulsotypes/isolates. Pulsotypes with only one isolate are represented by a circle with no segments.

- There were 25 pulsotypes identified from the 94 isolates examined. (Two of the isolates were not recoverable.)
- Of the 25 pulsotypes, there were 8 with a single isolate and 8 with more than 4 isolates.
- Six of the pulsotypes had isolates from more than one food sector, indicating possible cross-contamination between food sectors.

Table 4 gives more detail on pulsotypes shared across food sectors.

Figure 3: Minimum spanning tree graph summarising the data from the pulsed-field gel elctrophoresis profiles of the 94 *Listeria monocytogenes* isolates



For each of the food business operators' premises that tested positive for *L. monocytogenes*, Table 4 shows the number of pulsotypes, number of persistent pulsotypes and the number of pulsotypes shared between different food sectors. A "persistent" pulsotype was defined as repeated identification of an isolate of the same pulsotype over a period longer than 6 months.

- Of the 12 premises where *L. monocytogenes* were identified, more than 14 of the 25 pulsotypes were identified in 3 premises.
- These 3 premises also shared similar pulsotypes.

On one hand, this shows the diversity of the isolates obtained at these food manufacturing premises; however, it also shows a degree of similarity in the isolates from the different food sectors. This could indicate cross-contamination between the manufacturers, but could also indicate the isolation of related strains from unrelated sources. Leong and colleagues (2017) also found related strains from unrelated sources, including strains from different countries that had indistinguishable pulsotypes.

Further studies analysing the WGS data in more detail may help to resolve this issue of relatedness between strains and sources.

Table 4: Number of pulsotypes of *Listeria monocytogenes* obtained from different food sectors

Food sector	Number of pulsotypes	Number of persistent pulsotypes	Number of pulsotypes shared with other companies
Meats 1	8	3	4
Ready-to-eat 1	5	3	5
Ready-to-eat 2	4	1	4
Meats 2	2	1	0
Ready-to-eat 3	4	3	1
Ready-to-eat 4	3	1	1
Vegetables 1	2	1	1
Ready-to-eat 5	1	1	1
Vegetables 2	2	0	2
Seafood	1	0	1
Vegetables 3	6	1	3
Ready-to-eat 5	1	0	0

- Persistent pulsotypes were identified at 9 of the 12 premises that tested positive for *L. monocytogenes*.
- Of the 25 pulsotypes, 7 were persistent.

Persistence of *L. monocytogenes* in the food-processing environment presents a risk of cross-contamination to the food being produced. Indeed, cross-contamination was seen where 5 pulsotypes were found in the processing environment and on food, involving 3 food business operators. It is possible that the cross-contamination could have been from the food to the processing environment (rather than the other way round) but the PFGE method used cannot distinguish this. Either way, the principle of cross-contamination was shown.

The PFGE profiles obtained in this study were compared with a database of profiles obtained during the ROI FIRM study (Leong et al., 2017). This allowed a comparison of *L. monocytogenes* isolates from across the island of Ireland.

- There were no similarities found at the level of more than 90% similarity between the NI isolates and the isolates from the ROI FIRM project.

The pulsotypes obtained in this study were compared with about 2,500 PFGE profiles at Teagasc, Moorepark.

- Of the 25 pulsotypes obtained in this study, 10 were similar to profiles in the Moorepark collection. These included strains from Ireland, Austria, Romania, the Czech Republic, Turkey and Australia (data not shown).

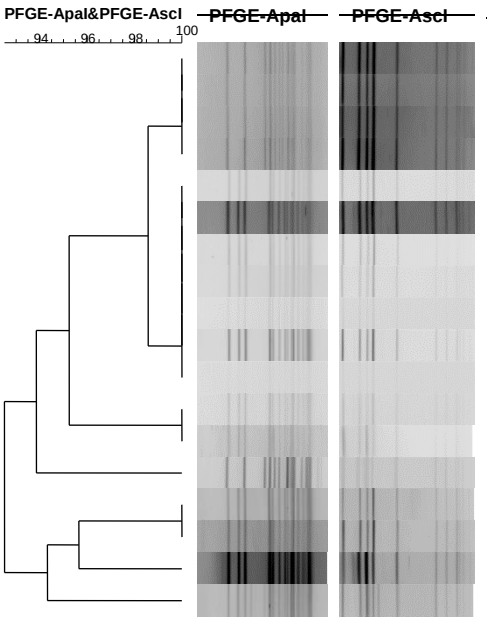
On the island of Ireland, the mushroom industry is a "cross-border" industry – some companies have premises in both NI and the ROI. For that reason, comparison between isolates from an ROI mushroom-based project and the current project were made.

- Seven of the pulsotypes from the current project were identified in the mushroom isolates from the ROI.

Figure 4 shows an example of some of these profiles, as well as comparisons with isolates from other food types.

- This could indicate that there are links between NI and ROI companies.

Figure 4: Pulsed-field gel electrophoresis profiles showing relatedness between Listeria monocytogenes from mushroom producers in the Republic of Ireland (A and B) compared with food producers in Northern Ireland: A mushroom producer (Northern Ireland), a sandwich maker (Sandwich A) and a salad producer (Salad A)



PFGE-ApaI&PFGE-AscI				
2716	.	148 drain water	RoI A	
2718	1/2a 3a	242 Floor H9	RoI B	
2714	1/2a 3a	145 bin R13	RoI A	
2713	1/2a 3a	144 drain R13	RoI A	
2947	.	173D	NI	16/09/2016
2700	.	110C	NI	23/03/2016
2950	.	173A	NI	16/09/2016
2946	.	173C	NI	16/09/2016
2549	.	87B	Sandwich A	03/02/2016
2948B	.	152D	NI	05/08/2016
2548	.	84G		03/02/2016
2547	.	84E	NI	03/02/2016
2539	.	55B	Sandwich A	13/11/2015
2205	1/2a 3a	1.2.1 Ladder	RoI A	
2931	.	173H	NI	16/09/2016
2512	.	8B	Sandwich A	10/07/2015
2522	.	22B	Sandwich A	02/09/2015
2922	.	157B	Salad A	05/08/2016

Note: The dates given are those on which the Northern Ireland isolates were stored. The scale shows the percentage (%) similarity between profiles.

Pathogenicity and persistence

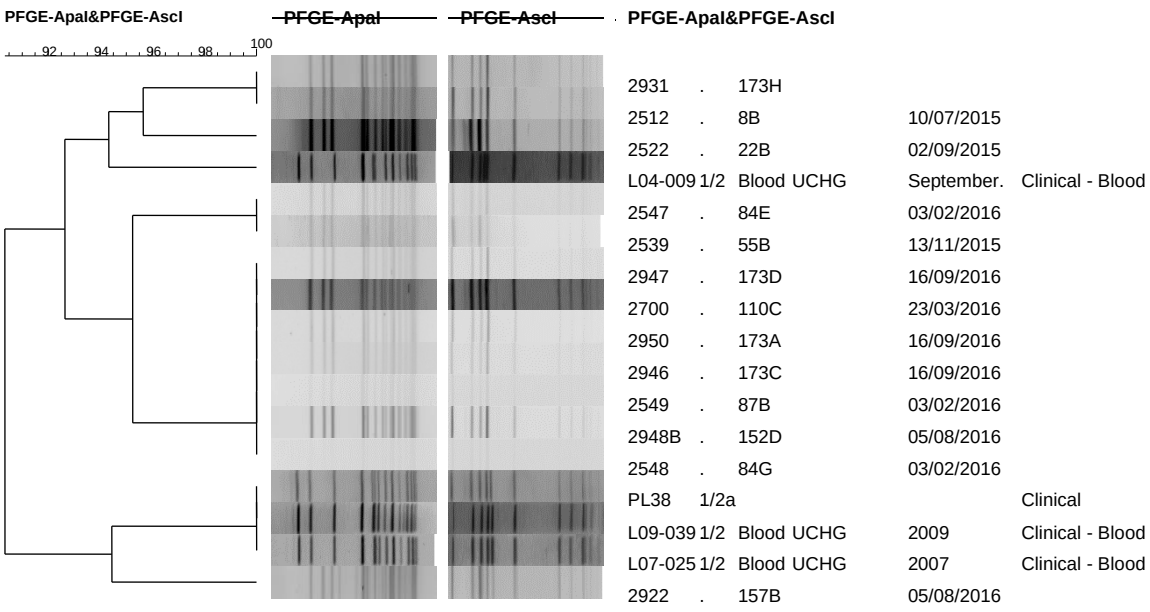
A comparison between the pulsotypes from this study and those of clinical isolates from the ROI was made.

Seven of the pulsotypes identified in this project were similar to pulsotypes from the ROI clinical isolates at a level of more than 90% similarity.

An example is shown in Figure 5.

This indicates that the strains could cause disease. However, in the absence of epidemiological data (which relates to the occurrence and distribution of disease) no link can be made between food and disease.

Figure 5: Pulsed-field gel electrophoresis profiles of *Listeria monocytogenes* from this study compared to 4 clinical isolates from the Republic of Ireland



Note: The scale shows the percentage (%) similarity between the profiles.

Subsequently, DNA was extracted and the isolates were subjected to WGS to allow multilocus sequence (MLS) types (Parisi et al., 2010) to be determined, as well as to determine the presence of genes indicative of pathogenic properties.

Multilocus sequence typing (MLST) is a molecular typing technique whereby a number of well-chosen housekeeping genes (loci) are sequenced, and allows detailed comparison between strains. Whole genome sequencing allows MLST to be carried out more easily than by the traditional MLST method.

- Ninety-one isolates yielded acceptable WGS. (Five sequences were not of acceptable quality.).
- Twelve sequence types (were found, which are shown in Figure 6.
- The 6 most common sequence types – that is, half of those found – comprised 87% of the typed isolates. At least 6 isolates were obtained for each of these 6 sequence types.

Figure 6: Prevalence of the 12 multilocus sequence types determined for 91 *Listeria monocytogenes* isolates

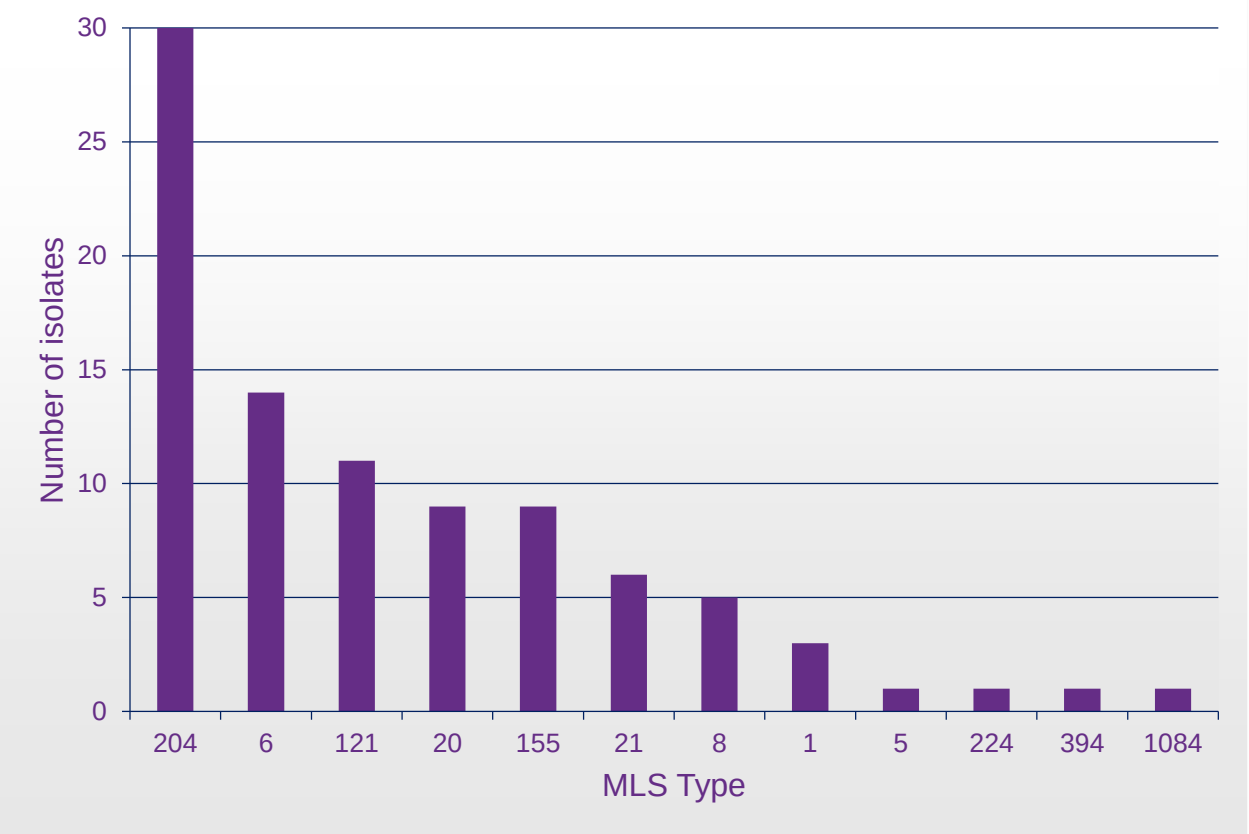


Figure 6 Prevalence of the 12 multilocus sequence types determined for 91 *Listeria monocytogenes* isolates

The genome sequences were then analysed for the presence of 4 genes that are "virulence markers". These are internalin A (*inl*A); actin assembly protein (*act*A); *Listeria* pathogenicity island 3 (LIPI-3); and *Listeria* pathogenicity island 4 (LIPI-4).

The sequences were also analysed for the presence of 3 genes that are markers for stress tolerance. These are stress survival islet 1 (SSI-1); a resistance cassette, which contributes to resistance to quaternary ammonium compounds such as those commonly used in sanitisers (*bcr*ABC); and quaternary ammonium compound-resistance protein (*qac*H) (Fox et al., 2016).

- All 91 isolates carried complete genes for *inl*A and *act*A.
- Sixty-five isolates (71.4%) carried genes for *qac*H.
- None of the isolates carried LIPI-3, LIPI-4 or *bcr*ABC (see Appendix 3).

The recurrence of *L. monocytogenes* sequence types in individual premises was then studied. Given the limited nature of this study, the term "recurrent" was applied to any sequence type isolated from a single FBO on more than one occasion.

- Five sequence types were obtained from individual FBO premises more than once.
- Sequence types ST8, ST20 and ST21 recurred in one food production plant each, ST 121 in 2 plants, ST 6 in 3 plants and ST204 in 4 plants, shown in Table 5.

Table 5: sequence types isolated from individual food business operators' premises on more than 1 occasion

Food product type manufactured	Total number of sequence types	Number of recurrent sequence types	Recurrent sequence types
Processed mushrooms	8	2	ST8, ST204
Cooked meats A: Pulled chicken, turkey and beef	8	4	ST6, ST20, ST121, ST204
Sandwiches A: Rolls and wraps	4	1	ST204
Cooked meats B: Pork from fresh and cured meat	7	1	ST204
Baked goods: Cakes, pies, sausage rolls and traybakes	6	2	ST21, ST121
Sandwiches B: Salads, wraps and snacks	4	1	ST6
Salads: Green and pasta salads, chicken tuna and egg mixes	4	0	
Sandwiches C: Pasta and salad bowls, coleslaw, potato salad	3	1	ST6
Ready-to-eat raw fruit pieces in consumer packs	2	1	ST155

The dates of the isolations of ST204 in the premises of a mushroom processor and of a sandwich producer (type A) are shown in Table 6 as examples to illustrate the persistence seen.

Table 6: Dates on which *Listeria monocytogenes* ST204 was isolated from samples taken in 2 food businesses, showing recurrence

Date	Number of *Listeria monocytogenes*-positive samples isolated in food business operators' premises	
	Mushroom processor	Sandwich A
July 2015		1
September 2015	1	
November 2015	1	1
January 2016	1	1
March 2016	1	1
May 2016		
July 2016	2	
September 2016	3	2
November 2016		2

Questionnaire survey of food business operators' premises and practices

All premises were sent a questionnaire about their premises and practices (Appendix 2). Follow-up contact was made, in some cases repeatedly, in an effort to maximise the number of returned completed questionnaires.

- Returned questionnaires were received from 13 food business operators – 54% of participants.
- Of the 13 food business operators, 5 had submitted no samples yielding *L. monocytogenes*.
- The remaining 8 participants submitted an average of 7.7 *L. monocytogenes*-positive samples during the study.
- The questionnaire yielded approximately 75 answers per respondent (a total of 975 records), which were statistically analysed.
- No significant differences between positive and negative premises were seen, but with a small sample size it is not possible to draw definitive conclusions.

Washing and sanitising products used in food business operators' premises

The questions generating the widest variety of responses from participants related to the cleaning and sanitising products used.

Table 7 shows the wide range of products used, illustrating the lack of consistency in approaches to cleaning.

Table 7: Washing and sanitising products as reported by 13 food business operators

Food business operator	Detergent for washing	Disinfectants for sanitising
A (Used cleaning in place [CIP] system)	Diversey® CIPTECH caustic detergent, then chlorinated foam detergent	Quaternary ammonium compound disinfectant
B	Incidin® Oxyfoam detergent	Diversey® Quatdet Clear
C	Topmaxx 123, Topmaxx 314,	Oxonia Activ®, MIP (in CIP)
D	CL1	Triquart AM
E	Fairy® Liquid, Diversey® Divosan® TC86	Bival International Sanitiser
F	Lazer FG, Quattro	Lazer FG, Quattro
G	F104 Amphoclen	F104 Amphoclen
H	Topmaxx	Topax 99
I	Holfoam Acid, chlorofoam	Tribac
J	K18 alkaline detergent	B2275 sanitiser
K	Chlorfoam Plus, Holfoam Acid, sodium hypochlorite, Nopac, V Clean	Perbac OPD, Tribac
L[1]	Ecolab Regain	Desguard 20
M[1]	Ecolab Regain	Desguard 20

[1] Two separate premises, producing different products, were managed by the same company and therefore used the same products in each. Samples from FBOs A to E were all *L. monocytogenes*-negative; the rest yielded positive test results.

Discussion

1. This study was designed and managed to be comparable with the ROI Food Institutional Research Measure (FIRM) project number 11F008, some of whose results have been published (Leong et al., 2014, 2017).
    - Swabs of the production environment submitted by the FBOs in NI in this study had a slightly higher prevalence of *L. monocytogenes* – 6.2% – than that seen in the ROI, which fell from 4.7% to 3.4% over a period of 3 years (Leong et al., 2017).
    - The prevalence of *L. monocytogenes* in foods in the ROI fell from 5.1% to 2.7% over the same period, whilst in NI in this study it was 4.6%.

2. Given the relatively small number of participating FBOs in NI, and the range of products produced (Table 1, Table 2), it is problematic to group the companies into the 4 categories used by Leong and colleagues; however, some differences were seen.
    - In NI none of the 4 companies producing dairy products (Table 1) submitted any positive samples (number [n] = 281), whilst in the ROI 3.8% of 1,920 samples were positive. Dairy products are amongst those most commonly implicated in outbreaks of listeriosis (Kiss et al., 2006; Almeida et al., 2013).
    - In NI 12% of the samples from 2 meat processors were positive (Table 3), in contrast to the ROI where the corresponding figure for meat processors was 4.3% (Leong et al., 2017). However, the difference here may be due to the difference between categorisation used in this study and that used in the ROI. Meat products have been responsible for major outbreaks, such as one in Canada in 2008 that caused 57 cases of listeriosis and 24 deaths (Thomas et al., 2015).

3. In this study, companies handling a wide range of product categories, such as sandwich makers and baked goods companies, have been identified as such, rather than attempting to categorise them more narrowly. In the ROI study all food processors were grouped into only 4 categories: dairy, meat, seafood or vegetables.
    - For seafood, similar results were obtained from both NI and the ROI: 1.4% of NI samples (n = 144) tested positive for *L. monocytogenes* (Table 3) whilst in the ROI the corresponding figure was 1.7% (n = 1,621).
    - Further meaningful comparisons are not possible due to the different approaches to categorising the FBOs involved.

4. The locations from which *L. monocytogenes* was obtained during this study (Figure 1) can be seen to be those commonly contaminated, such as floors and drains (Kells and Gilmour, 2004; Schoder et al., 2012; Leong et al., 2014; Ruckerl et al., 2014).
5.
    - The prevalence was not consistent over the 18-month period of the study (Figure 2); however, the environmental samples were more often positive during the warmer months of May to September and the difference was statistically significant ($p = 0.007$). This was in contrast to studies undertaken in the ROI (Leong et al., 2017), where no seasonality was found. No overt cause for this observation was determined.
    - In contrast, the statistical analyses showed no seasonal effects with regard to the food samples. Seasonality of contamination has been reported in dairy products (Meyer-Broseta et al., 2003) but no dairy products were positive in this study.

Raising the temperature slightly to 6 °C would cause 55% of samples to fail the test, and 77.5% would fail at 8 °C.

1. Having shown that *L. monocytogenes* was present in half of the FBOs participating in this study, the potential for the food products being prepared to support its growth was investigated using the ComBase online growth modelling tool.
    - Only 19 products of the 130 analysed could not support the growth of *L. monocytogenes*. This was mostly due to the low pH of products such as yoghurts, fruit products and coleslaw, which all had a pH below 4.4. The pH in cheeses has been seen to rise post–production due to the actions of the endogenous (naturally resident) microflora (Schoder et al., 2013). However, it would be unlikely to occur in the products studied, without obvious spoilage taking place, and so they would be safe for their normal shelf life.
    - The remaining 111 food samples – 85% – all had pH and a_w values that would support the growth of *L. monocytogenes* at 4 °C, 6 °C and 8 °C according to the ComBase model.

    Growth in the foodstuffs was categorised using the arbitrary criterion of calculating the time for 10 generations of microbial growth (approximately a 1,000-fold increase in numbers) using the generation time predicted by ComBase, and determining if it was less than 7 days.

    - At 4 °C none of the 111 foodstuffs would support such growth.

- However, raising the temperature slightly to 6 °C would cause 55% of samples to fail the test, and 77.5% would fail at 8 °C

> Only nineteen products of the 130 analysed could not support the growth of *L. monocytogenes*.

- This information was communicated to participants because they should be undertaking testing to meet Commission Regulation (EC) No 2073/2005, which states:

As necessary, the food business operators responsible for the manufacture of the product shall conduct studies in accordance with Annex II in order to investigate compliance with the criteria throughout the shelf-life. In particular, this applies to ready-to-eat foods that are able to support the growth of Listeria monocytogenes *and that may pose a* Listeria monocytogenes *risk for public health.*

Guidance for FBOs undertaking such studies has been published (Beaufort et al., 2014).

The temperature range studied was based on the observation that most of the FBOs were small to medium-sized enterprises (SMEs) and would therefore sell their product directly, or through SME vendors. A UK-wide microbiological study of RTE meats at SME premises (Madden et al., 2014) found that the mean temperature of the pre-packed meats on retail display was 6.80 °C, with 71.3% of samples above the industry guideline of 5 °C, and 32.7% being stored above 8 °C.

- As such, most of the food products analysed in this study could, should they become contaminated with *L. monocytogenes* and subject to abuse temperatures already seen in retail premises, exceed the safe level cited in Commission Regulation (EC) No 2073/2005 as 100 cfu/g, based on an initial contamination of one *L. monocytogenes* per 10 grams of product.
- This finding emphasises the importance of ensuring that SMEs producing RTE foods have appropriate systems in place to exclude *L. monocytogenes* from their premises and from products that are likely to support *L. monocytogenes* growth.

2. In this study the efficacy of contamination control systems was assessed by seeking to isolate *L. monocytogenes* from both the FBOs premises and the food products produced.

This finding emphasises the importance of ensuring that SMEs producing RTE foods have appropriate systems in place to exclude *L. monocytogenes* from their premises and from products that are likely to support *L. monocytogenes* growth.

- Two samples of meat products were found to exceed the limit of 100 cfu/g in July 2015, but all other food samples (n = 393) had less than the legal limit.

All strains were subjected to genotyping by WGS and PFGE to allow the recurrence of *L. monocytogenes* strains in FBO premises to be studied. Analysis of WGS sequence data allowed MLST to be undertaken and sequence types were determined for 91 isolates (Figure 3).

- Recurrence was seen with the 7 most common sequence types (Table 4), comprising 92% of WGS isolates.
- The intervals between isolation events (the date when the samples were collected) for ST6 in 2 sandwich producer FBOs were 2 and 4 months respectively, whilst in a cooked meats producer ST6 recurred after 14 months. Recurrence of *L. monocytogenes* in meat-processing plants in NI has been previously reported (Harvey and Gilmour, 1994) and also observed in other countries (Autio et al., 2003; Currie et al., 2015).

Table 5 shows the recurrence of the most common sequence type found in this study, ST204, in the 2 FBOs that yielded the most positive samples.

- It can be seen that ST204 recurred and possibly persisted over a period greater than one year in both premises.
- As a widespread environmental soil contaminant *L. monocytogenes* is known to be present in mushroom-processing environments, and can persist, although improved sanitation can be effective (Murugesan et al., 2015). The degree of contamination may also be dependent on the species of mushroom being cultivated (Venturini et al., 2011), with the main commercial types grown in Ireland (*Agaricus bisporus*) appearing not to support the growth of *L. monocytogenes* (Leong et al., 2015). However, in the processed products analysed in this study growth appeared possible.

- The relatively frequent isolation of ST204 from the mushroom-processing environment was a cause for concern.
- It should also be noted that on 2 occasion's samples of breadcrumbs used in the preparation of the final product were found to contain ST204. It was not possible to determine if this arose from cross-contamination within premises, or if the *L. monocytogenes* was already present when the ingredient was delivered. Cross-contamination from the processing environment has been previously reported (Ivanek et al., 2004; McCollum et al., 2013; Leong et al., 2017), and has been implicated in historical outbreaks of listeriosis (Currie et al., 2015).

> Certain locations within premises were frequently contaminated. Swabs labelled "floor in front of sink" from one premises tested positive on 4 occasions, and from another premises on 8 out of 9 occasions. In these cases, all staff using the sink would potentially contaminate their footwear and carry *Listeria* to other areas within the premises.

The sequence type results further supported the potential for cross-contamination in some premises as they showed that certain sites were frequently contaminated,

- Swabs labelled "floor in front of sink" from a single FBO premises tested positive on 4 occasions, and from another on 8 out of 9 occasions. Therefore all staff using the sink would potentially contaminate their footwear and carry *Listeria* to other areas within the premises.
- It would be expected that being provided with this information would assist the FBOs in the application of their cleaning regimes but the repeated contamination of this site suggests the problem was persistence. It should be noted that the latter FBO manufactured sandwiches and on 2 occasions the same sequence type, ST204, was isolated from both the floor and food product, which is strongly indicative that cross-contamination could be occurring.

Sandwiches can have significant *Listeria* contamination (Cossu et al., 2016), and are known to be implicated in cases of listeriosis (Silk et al., 2014). Therefore the frequency of isolations of

L. monocytogenes would, again, give cause for concern. However, based on a study of 15 ST204 isolates, this sequence type was reported as being mainly an environmental isolate (Fox et al., 2016).

Some information listing 16 sequence types of Irish isolates of *L. monocytogenes* (14 from humans and 4 from food-processing businesses, of which 2 were shared) was made available to this study and this also showed that ST204 was an isolate typically found in the food-processing environment, with no corresponding clinical isolations being made to date. However, clinical isolates of 5 sequence types identified in this study were reported: ST1, ST6, ST8, ST20 and ST121. These comprised 46% of the isolates sequence-typed in this study, showing that many of the isolates obtained in this study are potential human pathogens.

3. Information was also supplied by the Gastrointestinal Bacteria Reference Unit (GBRU), National Infection Service, Public Health England, and is presented as Figure 7 and Appendix 4. These are the results of WGS of *L. monocytogenes* isolates undertaken since April 2016.
   The GBRU is usually sent 5 picks of each food isolate and food isolates from routine testing, disease outbreak investigations and surveys. Since multiple isolates can be taken from a single sample the number of isolates is not directly proportional to the prevalence of a sequence type.
   - All of the sequence types reported to the GBRU as being isolated from people were also found in FBOs in this study, where they comprised 98% of the isolates.
   - ST204, the most common sequence type determined in this study, was found in human infections in the UK.
   - Determining the true pathogenic abilities of the isolates is beyond the scope of this study. However, further work on the WGS could be subsequently undertaken as this can be related to pathogenic properties (Chen et al., 2016).

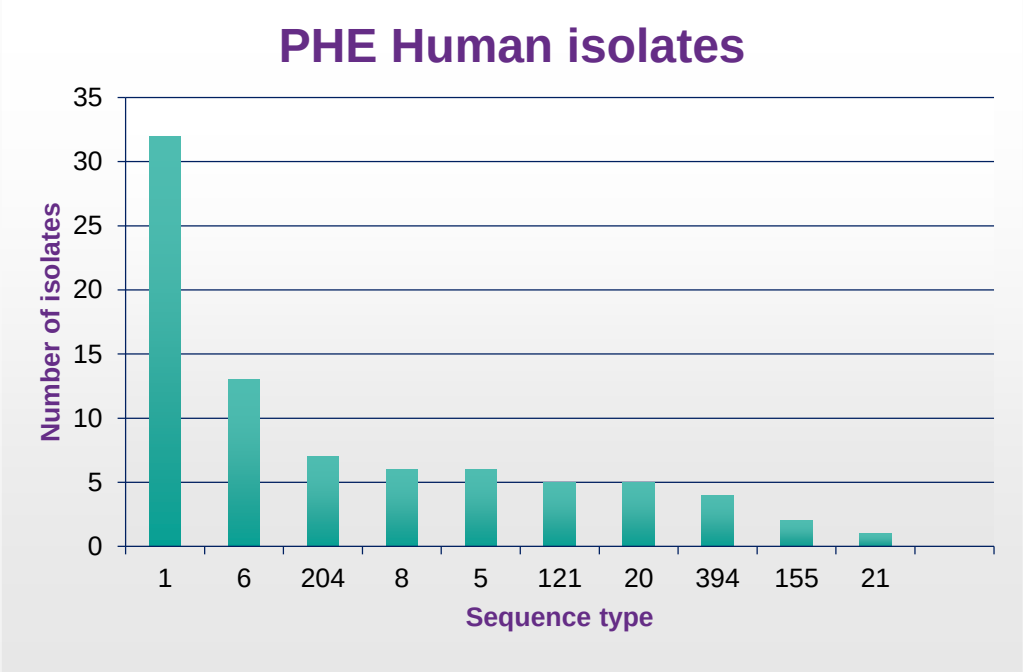


Figure 7 Prevalence of sequence types of *Listeria monocytogenes* isolated from humans in the United Kingdom since April 2016

- It should be noted that isolates with a given sequence type are not genetically identical. For example, 86% of the ST204 isolates carried the *qac*H gene, and were therefore different from the 14% which did not carry it. Therefore, despite only limited genetic studies being undertaken, differences were noted within sequence types.
- Overall, most isolates for which WGS data was obtained (71%) carried the *qac*H gene, which confers resistance to quaternary ammonium compounds. These are the basis of many sanitisers used in the food industry (Sidhu et al., 2002).
- The high rate of carriage of *qac*H by ST204 found in this study contrasts with its absence in the 15 isolates described in the report of Fox and colleagues (2016). This finding reflects genetic selection favouring the property of resistance to QACs in food-processing environments. It may also contribute to the high prevalence of the sequence type found in this study.

4. In addition to the screening of the WGS sequences, PFGE studies were undertaken to allow the *L. monocytogenes* obtained in this study to be directly compared with those obtained in studies undertaken in the ROI.
- High degrees of similarity were seen between some NI isolates and ROI clinical isolates (Figure 6). This is further support for the view, based on sequence types, that *L. monocytogenes* found in NI food-processing plants could be potentially pathogenic.
- The PFGE profiles also illustrated patterns of recurrence and cross-contamination as found with the sequence types.
- Similar PFGE types were observed in mushroom-processing facilities in both NI and the ROI (Figure 5), and these were also shared with other FBOs. Work with the mushroom industry is ongoing in the ROI and may later clarify reasons for the frequent isolation of specific types of *L. monocytogenes* from these type of premises. For example, adaption of the organism may allow the colonisation of the premises (Autio et al., 2003); or the introduction of specific types of *L. monocytogenes* with the raw materials may be the problem (Ruckerl et al., 2014).

5. To assess procedures for the control of *L. monocytogenes* a questionnaire was submitted to all participating FBOs (Appendix 2).
- Statistical analysis of the responses to the questionnaire found no significant differences between the 5 companies which yielded no *L. monocytogenes*, and the 8 in which it was found. This suggests that the FBOs all have appropriate procedures in place but that their implementation may differ.
- To discover significant differences in control practices it would be necessary to attend some of the FBOs. This would allow researchers to observe the practices being implemented, and discuss them with the relevant staff, rather than accepting responses from the staff member responsible for completing the questionnaire.

6. During this study staff from the AFBI and CAFRE actively collaborated with 24 FBOs in NI, and obtained a considerable number of *L. monocytogenes* isolates.

- The results obtained showed that most participating FBOs can produce RTE food free from *L. monocytogenes*, and many were able to keep their production facility free from this pathogen. Most FBOs appear to have determined effective control strategies and practical interventions.
- Where *L. monocytogenes* was found, FBOs were provided with relevant assistance.

To conclude, this study has contributed to the production of safer RTE foods produced by SMEs in NI.

4 Key findings

1. Half of the participating FBOs submitted samples yielding *L. monocytogenes*.

2. Of 1,197 environmental swabs 6.3% were positive, whilst of 397 food samples 4.6% were positive for *L. monocytogenes*.

3. The 3 product categories of FBOs yielding most *L. monocytogenes* were processed mushrooms, cooked meats and sandwiches.

4. Swabs of floors, drains, wheels and boots were most frequently positive, and environmental samples were more likely to be positive in warmer months.

5. Based on their pH and a_w most food samples submitted (85%) would support the growth of *L. monocytogenes*, as estimated using the ComBase mathematical model.

6. Using an arbitrary growth measure (the ability of a food to support 10 generations of growth in 7 days or less) most products would not support **significant** growth of *L. monocytogenes* at 4 °C. However, at 8 °C (frequently seen in an assessment of SME chilled meats in the UK) most would support growth of *L. monocytogenes* (77%).

7. Whole genome sequencing (WGS) analysis showed that 71.4% of isolates carried genes conferring resistance to quaternary ammonium compounds, which are used as the basis of many sanitisers.

8. Whole genome sequencing allowed MLST to be undertaken. This showed that recurrence of sequence types occurred in several premises. It also suggested cross-contamination of the food product and the processing environment.

9. Most isolates (98%) shared a sequence type with *L. monocytogenes* strains that have been isolated from clinical cases in the UK.

10. Pulsed-field gel electrophoresis profiling of isolates supported the observations on recurrence and cross-contamination in FBOs. The analysis showed that both food and clinical isolates obtained in the ROI were very similar to isolates obtained from the FBOs.

11. Analysis of the information supplied by 54% of FBOs in a detailed questionnaire failed to show any statistically significant differences between the 8 *Listeria*-positive and 5 *Listeria*-negative FBOs. This suggests visits to FBOs to audit strategies and interventions would be needed to determine best practices.

5 Conclusions

1. Half of the 24 participating SME FBOs submitted samples yielding *L. monocytogenes*. The FBOs who completed a questionnaire (54% of all participants) all had documented procedures in place in order to control *L. monocytogenes*. However, 6.3% of environmental swabs and 4.6% of food samples analysed carried this pathogen, and therefore the FBOs producing these foods were reliant on an effective cold chain to ensure their products were safe and met legal requirements. Previous studies have shown that SME vendors do not maintain an effective cold chain.

2. The environmental isolates were mainly obtained from floors and drains, which are known "hotspots" for the detection of *L. monocytogenes*. More such isolations were made in warmer months but the cause of this could not be determined. Most isolates of *L. monocytogenes* were obtained from suppliers of cooked meats and sandwiches, which are known vehicles in *L. monocytogenes* outbreaks.

3. Genetic characterisation of the isolates following WGS showed that 2 genes that indicate pathogenicity were present in all isolates. In addition, a gene conferring resistance to sanitising products was common, especially in the most common MLS type found: ST204. The MLS data also indicated that recurrence of specific sequence types occurred in several premises, and that cross-contamination between the processing environment and final product may have occurred. Genotyping by PFGE supported these observations.

4. Comparison of sequence types found in this study with those reported in people in the UK showed that 98% of local isolates were of the same sequence type as was found in UK clinical isolates. This indication of potential pathogenicity was supported by the PFGE data from this study, which showed that identical PFGE profiles were exhibited by clinical isolates in the ROI. As such, *L. monocytogenes* of a potentially pathogenic nature was found in food products from 21% of FBOs, but only 2 food samples exceeded the legal limit. The WGS and PFGE datasets will be utilised for further study, beyond the scope of this study.

5. Certain premises were seen to control *L. monocytogenes* contamination, whereas it recurred in others. No significant differences in control strategies or practical interventions, as assessed by a questionnaire, could be found to account for this observation. On-site visits to audit practices would be required to determine any noticeable and relevant differences in contamination control strategies.

6. Many of the *L. monocytogenes* found in FBOs in NI appeared to be potential pathogens, and 4.6% of food samples carried these organisms.

6 References

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7 Appendices

Appendix 1: Sampling kits provided to food business operators

Figure 3 Sampling kits provided to food business operators

The sampling kits consisted of

- Six sponge stick swabs (3M, Cain Road, Bracknell, Berkshire RG12 8HT, England)
- Ten ml pH-neutralising buffer
- Two ice packs (to be frozen on site)
- Two sterile food sample containers

- Instruction sheets

Appendix 2: Questionnaire survey of food business operators' premises and practices, showing examples of responses

SECTION 1: Business details & quality management	
Full name of the company	
Business address	
Number of employees	

Product range	
GENERAL	
Was the manufacturing premises purpose built for food handling?	Yes
Is there a Quality Team in place with defined responsibilities? Please, give brief details.	Technical manager Quality Group lead Quality admin Compliance officer Quality graduate Samples technician
Are workers trained in aspects of food safety and quality and are they skilled on the job? Please, give brief details.	All employees receive induction when first on site then within 3 months are given basic food hygiene training. There is also annual GMP refreshers.
HACCP, food safety and food quality	

Are you accredited to a recognised food safety/quality standard?	Yes
If not, do you have fully documented company policies and procedures to control food quality and safety?	N/A
Do you have a certified HACCP manual?	Yes
Has your HACCP system been independently audited?	Yes
At what frequency is the HACCP system audited to ensure its continued effectiveness?	As a minimum once a year or when a new piece of equipment or new product is introduced.
Are procedures in place to monitor critical limits in the HACCP plan?	Yes
Are specifications available for: Raw materials; Finished products; Packaging; Cleaning solutions? Please, give brief details.	Specifications for Packaging, Raw Material and finished products. MSDS in the COSHH folder for all cleaning solutions and SOPs on how to use cleaning solutions in the factory environment.
Independently of this project, is final product tested for presence of *L. monocytogenes*? How frequently? How many positives did you find in the last 12 months?	On a weekly basis one product from each production line is taken and sent for Listeria mono testing. Also on a 2 year schedule all products on site get tested via shelf life testing for Listeria Mono. No positives in the last 12 months.

Independently of this project, do you test environmental swabs for presence of *L. monocytogenes*? How frequently? How many positives did you find in the last 12 months?	All productions areas are swabbed on a monthly basis for TVC, coli and Listeria Mono. This will cover contact and non-contact equipment and floor/drain areas. 1 Fail in the last 12 months – Floor Brush – 5 days retests all back in spec.

SECTION 2: Manufacturing arrangements	
Are the premises located close to farming activities?	Yes
Has any building, renovation or modification work taken place in the last 12 months?	Yes
Does the design of the process flow from intake to dispatch prevent the contamination of raw material, packaging, intermediate and finished products?	Yes
Is there segregation between high and low risk operations to minimise the risk of product contamination?	Yes
Is there a preventative maintenance programme in place?	Yes
Are walls designed, finished & maintained to prevent the accumulation of dirt and mould growth?	Yes
Are the junctions between the walls and the floor sloped to facilitate cleaning?	Yes
Are there openings in the walls/ceilings through which animals, insects and birds could enter the production area?	No

Are floors made of alkali and acid resistant material?	Yes
Do the floors have sufficient slope to avoid water stagnation?	Yes
Are drains adequately designed to handle the volume of waste water?	Yes
Are the drains cleaned and maintained regularly? State frequency & methods in high risk and low risk areas.	Yes The drains are cleaned on a nightly basis.
Are changing rooms provided for workers, staff, visitors or contractors for changing into working clothes before entering the production area?	Yes
Are there visible "Wash Hands" instructions available before entering the production areas?	Yes
Are washbasins available and suitably located? Please, give brief details.	Yes Sinks are located in all changing rooms during the PPE changing process and also at the entry to all production areas.
Is Personal Protection Equipment (PPE) available?	Yes
Is protective clothing laundered effectively and on a regular basis? By whom and what method is used?	Yes Berensden Laundries
Is separate PPE worn in low and high risk areas? Are they colour coded/segregated?	Yes
Is suitable footwear worn within the factory environment?	Yes
Is hand wash and hand sanitiser provided at critical areas?	Yes

Please describe briefly policies & procedures which must be followed by employees in case of illness.	If anyone feels sick or has been sick they must notify their manager/TL immediately They will be sent home. They must notify the TL when returning back to work and they follow the below guidelines - If a person has had more than 1 bout of diarrhoea and/or vomiting which has lasted for longer than 24 hours they must be symptom free for 48 hours before they can be allowed return to work. If this person is Fit to return to work before they are 48 hours clear. Their Duties must be assessed as to where they will commence work. This person must be assessed as to what Area of Work they can return to, for example if they are a Food handler they can only be placed in an area where product is completed packaged, although they must be briefed that they MUST practice good personal hygiene, especially washing their hands thoroughly after using the toilet, so as not to spread the infection to other workers. A return to work questionnaire must be filled in and completed before the operator commences work. Procedures and policy are held by HR.
Are hand/glove swabs carried out on staff? Please describe briefly.	Yes All staff employees, maintenance, managers etc. are swabbed on a monthly basis.
Is there a pest control system implemented?	Yes

SECTION 3: Cleaning and disinfection	
Do you have a documented cleaning schedule for all areas of the factory?	Yes
Is the cleaning schedule being used in practice?	Yes
How often is cleaning carried out at the premises? Provide brief details on the frequency, time intervals, etc.	Cleaning will be taken on all production lines every night after production run. There is an external cleaning crew. SOPs dedicated for cleaning for each line and equipment.
Who undertakes cleaning at the premises?	External cleaning crew.
Who carries out training of cleaning staff?	They have their own training and also the site carries out chemical, pest awareness, GMP, glass and Perspex etc. training.
Is the cleaning process supervised & audited by Quality team?	Yes
Do you follow a six steps cleaning schedule (preclean – physical, wash – detergent, rinse, sanitise, final rinse, dry)?	Yes
What physical procedures do you use for the preclean step?	List the activities carried out.
SOP – Gross clean before	Break Lines Down Blow down all debris Brush up

SOP – Pre rinse	Cover all electrical equipment Using hot water hose Visual check to inspect your cleaning standard of equipment
What detergents do you use for the wash step?	List all products utilised.
Commercial name	Procedure followed (time of exposure, etc.)
OXYFOAM	*Water temperature to be 45 °C – 55 °C *Detergent OXYFOAM Concentration should be between 3.0% – 5.0% *Contact time minimum 12 minutes
What disinfectants do you use for the sanitise step?	List all products utilised.
Commercial name	Procedure followed (time of exposure, etc.)
*Quatdet Clear	*Quatdet Clear 1 % Concentration with cold water Left on equipment to air dry
How do you perform the rinsing steps?	List the activities carried out including times, temperatures, etc.
	Using water hose Final removal of Small particles & Foam Check equipment
Do you perform a dry step?	List the activities carried out.

Yes	Once the post rinse is carried out and before sanitising all the equipment and areas are blown down.
How do you evaluate the effectiveness of your cleaning and disinfection programme?	Morning and nightly audits on the cleaning crew ATP swabs after cleaning Listeria swabs Aerial plates
Do you test the concentration of cleaning solutions if they are diluted for use?	Yes
Do you take surface swabs to evaluate cleaning process? Please provide frequency and kind of microbiological analysis performed.	ATP swabs – Daily TVC and Coli swab – Monthly Listeria swabs – Monthly

Thank you for completing the survey. This will contribute to understanding management practices that contribute to controlling *L. monocytogenes* in the processing environment.

Appendix 3: Screening of whole genome sequences for genes related to virulence or stress tolerance

Overall, 71.4% of the sequenced isolates carried the gene *qac*H, which confers resistance to quaternary ammonium compounds.

Table 8: Results of screening of whole genome sequences for genes related to virulence or stress tolerance

Isolate code	Genes screened for in whole genome sequencing						
	Virulence marker genes				Stress tolerance marker genes		
	*inl*A	*act*A	LIPI-3	LIPI-4	SSI-1	*bcr*ABC	*qac*H
LR15/2C	+[1]	+	-[2]	-	-	-	+
LR15/2E	+	+	-	-	-	-	-
LR15/5A	+	+	-	-	-	-	+
LR15/6D	+	+	-	-	-	-	+
LR15/8B	+	+	-	-	-	-	+
LR15/13B	+	+	-	-	-	-	+
LR15/15E	+	+	-	-	-	-	-
LR15/15G	+	+	-	-	-	-	+
LR15/15G	+	+	-	-	-	-	-
LR15/15H	+	+	-	-	-	-	+
LR15/15H	+	+	-	-	-	-	+
LR15/16A	+	+	-	-	-	-	+
LR15/16B	+	+	-	-	-	-	+
LR15/16G	+	+	-	-	-	-	+
LR15/22B	+	+	-	-	-	-	-
LR15/30C	+	+	-	-	-	-	+
LR15/31A	+	+	-	-	-	-	-
LR15/35A	+	+	-	-	-	-	-
LR15/35D	+	+	-	-	-	-	-
LR15/35E	+	+	-	-	-	-	-
LR15/37H	+	+	-	-	-	-	+
LR15/39A	+	+	-	-	-	-	+
LR15/39B	+	+	-	-	-	-	+
LR15/39D	+	+	-	-	-	-	-

LR15/39H	+	+	-	-	-	-	+
LR15/45B	+	+	-	-	-	-	+
LR15/45D	+	+	-	-	-	-	+
LR15/45E	+	+	-	-	-	-	+
LR15/45F	+	+	-	-	-	-	+
LR15/45H	+	+	-	-	-	-	+
LR15/46E	+	+	-	-	-	-	+
LR15/55B	+	+	-	-	-	-	+
LR15/56F	+	+	-	-	-	-	+
LR15/56H	+	+	-	-	-	-	+
LR15/65B	+	+	-	-	-	-	+
LR15/68E	+	+	-	-	-	-	-
LR15/76H	+	+	-	-	-	-	+
LR15/82A	+	+	-	-	-	-	+
LR15/82H	+	+	-	-	-	-	+
LR15/84E	+	+	-	-	-	-	+
LR15/84G	+	+	-	-	-	-	+
LR15/87B	+	+	-	-	-	-	+
LR15/90B	+	+	-	-	-	-	+
LR15/91B	+	+	-	-	-	-	-
LR15/97A	+	+	-	-	-	-	-
LR15/97D	+	+	-	-	-	-	-
LR15/97E	+	+	-	-	-	-	-
LR15/100D	+	+	-	-	-	-	+
LR15/101G	+	+	-	-	-	-	+
LR15/101H	+	+	-	-	-	-	-
LR15/110C	+	+	-	-	-	-	-
LR15/113A	+	+	-	-	-	-	-
LR15/113B	+	+	-	-	-	-	-
LR15/117B	+	+	-	-	-	-	-
LR15/117C	+	+	-	-	-	-	-
LR15/119E	+	+	-	-	-	-	-
LR15/125A	+	+	-	-	-	-	+
LR15/125B	+	+	-	-	-	-	+
LR15/125D	+	+	-	-	-	-	-

LR15/126A	+	+	-	-	-	-	+
LR15/133H	+	+	-	-	-	-	-
LR15/135A	+	+	-	-	-	-	+
LR15/142A	+	+	-	-	-	-	+
LR15/142D	+	+	-	-	-	-	+
LR15/146C	+	+	-	-	-	-	-
LR15/146E	+	+	-	-	-	-	+
LR15/148D	+	+	-	-	-	-	+
LR15/150A	+	+	-	-	-	-	+
LR15/150B	+	+	-	-	-	-	+
LR15/150F	+	+	-	-	-	-	+
LR15/152D	+	+	-	-	-	-	+
LR15/152H	+	+	-	-	-	-	+
LR15/153E	+	+	-	-	-	-	+
LR15/156B	+	+	-	-	-	-	+
LR15/157B	+	+	-	-	-	-	+
LR15/163A	+	+	-	-	-	-	+
LR15/167H	+	+	-	-	-	-	+
LR15/168B	+	+	-	-	-	-	+
LR15/168G	+	+	-	-	-	-	+
LR15/171A	+	+	-	-	-	-	+
LR15/171D	+	+	-	-	-	-	+
LR15/173A	+	+	-	-	-	-	+
LR15/173C	+	+	-	-	-	-	+
LR15/173D	+	+	-	-	-	-	-
LR15/173H	+	+	-	-	-	-	+
LR15/175B	+	+	-	-	-	-	+
LR15/183H	+	+	-	-	-	-	-
LR15/196E	+	+	-	-	-	-	+
LR15/197C	+	+	-	-	-	-	+
LR15/197E	+	+	-	-	-	-	+
LR15/197F	+	+	-	-	-	-	+

[1] Plus sign (+) indicates the gene is present.

[2] Minus sign (-) indicates the gene is absent.

Appendix 4: Sequence types of *Listeria monocytogenes* found in the United Kingdom and identified from whole genome sequencing

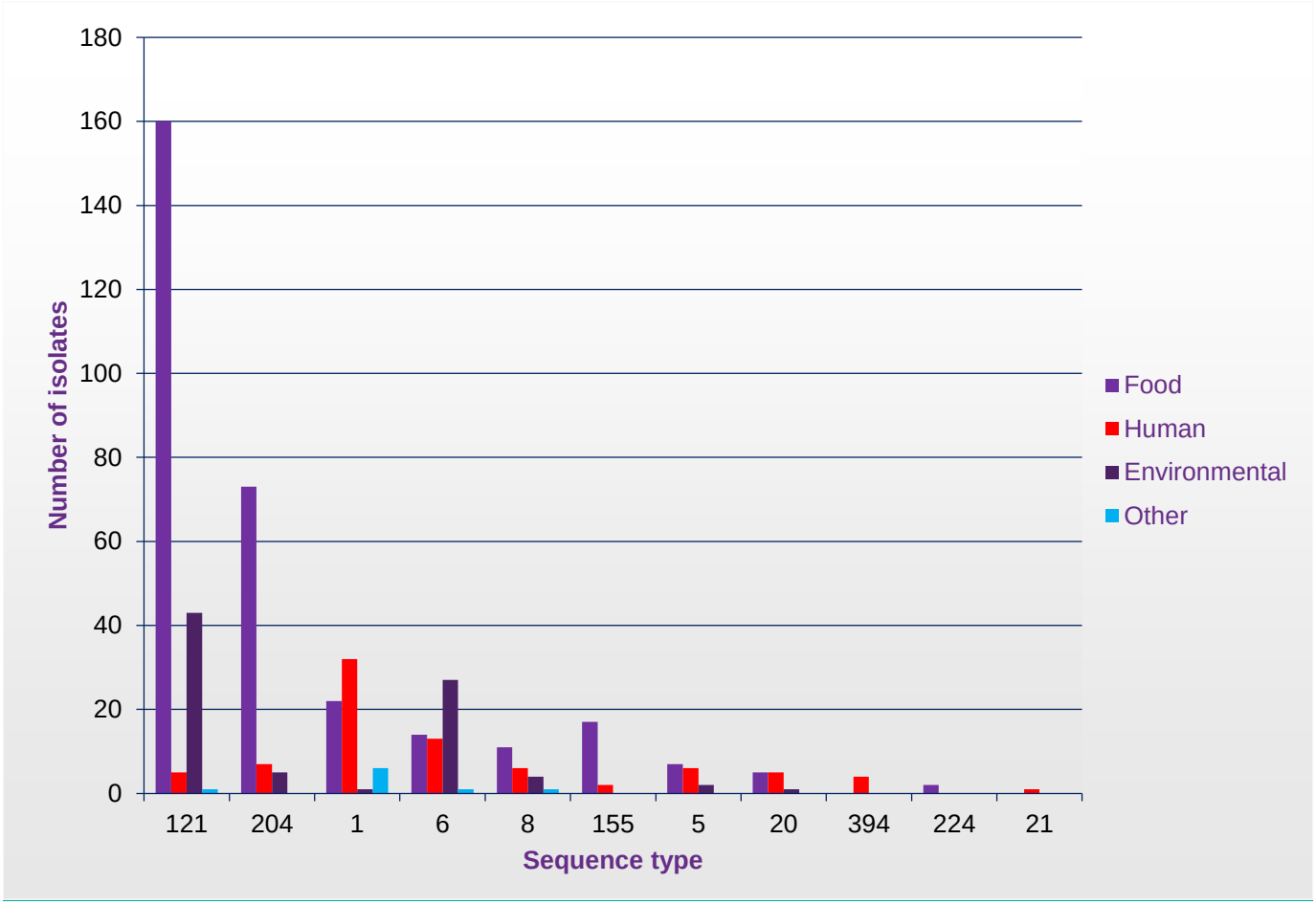

Figure 4 Sequence types of *Listeria monocytogenes* found in the United Kingdom and identified from whole genome sequencing

safefood

7 Eastgate Avenue, Eastgate, Little Island, Co.Cork, T45 RX01
7 Ascall an Gheata Thoir, An tOiléan Beag, Co. Chorcaí, TT45 RX01
7 Aistyett Avenue, Aistyett, Wee Isle, Co. Cork, T45 RX01

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