



## *In vitro* and *in vivo* characterisation of *Listeria monocytogenes* outbreak isolates

Alexandros Ch Stratakos<sup>a,c,1</sup>, Umer Zeeshan Ijaz<sup>b</sup>, Patrick Ward<sup>c</sup>, Mark Linton<sup>a</sup>, Carmel Kelly<sup>a</sup>, Laurette Pinkerton<sup>a</sup>, Pam Scates<sup>a</sup>, Jane McBride<sup>a</sup>, Ioan Pet<sup>d</sup>, Adriana Criste<sup>e</sup>, Ducu Stef<sup>f</sup>, Jillian M. Couto<sup>b</sup>, William T. Sloan<sup>b</sup>, Nick Dorrell<sup>g</sup>, Brendan W. Wren<sup>g</sup>, Lavinia Stef<sup>d,\*\*\*</sup>, Ozan Gundogdu<sup>g,\*\*</sup>, Nicolae Corcionivoschi<sup>a,d,e,\*</sup>

<sup>a</sup> Bacteriology Branch, Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, UK

<sup>b</sup> School of Engineering, University of Glasgow, Glasgow, G12 8LT, UK

<sup>c</sup> Aurlant, NovaUCD, Dublin, Ireland

<sup>d</sup> School of Biengineering of Animal Science Resources, Banat University of Animal Sciences and Veterinary Medicine – King Michael I of Romania, Timisoara, Romania

<sup>e</sup> School of Animal Science and Biotechnology, University of Animal Sciences and Veterinary Medicine, Cluj Napoca, Romania

<sup>f</sup> Faculty of Food Engineering, Banat University of Animal Sciences and Veterinary Medicine – King Michael I of Romania, Timisoara, Romania

<sup>g</sup> Faculty of Infectious & Tropical Diseases, London School of Hygiene and Tropical Medicine, 11 Keppel Street, London, WC1E 7HT, UK

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### ABSTRACT

Listeriosis is an important food-borne disease responsible for high rates of morbidity and mortality. *L. monocytogenes* has been the cause of several foodborne outbreaks and its ability to adapt and survive in a wide range of environmental conditions makes eradication difficult. Many *L. monocytogenes* strains are avirulent but have the ability to increase their virulence if exposed to environmental stresses. The aim of this study was to explain the observed increase in virulence of outbreak *L. monocytogenes* isolates by using phenotypic assays and whole genome sequencing. Four *L. monocytogenes* isolates from sweetcorn and one isolate from a raw milk (control) were sequenced and characterised using a range of phenotypic assays. The four *L. monocytogenes* sweetcorn isolates displayed a significant increase for *in vitro* adhesion and invasion of epithelial cells compared to the control isolate. They also showed a higher level of colonisation of the liver and spleen *in vivo*. In addition, the four *L. monocytogenes* isolates displayed an increased ability to form biofilms, resist heat stress and resist a combination of antimicrobials. Investigation of the genomes of the four *L. monocytogenes* sweetcorn isolates identified Single Nucleotide Polymorphisms (SNPs) in genes, which may have a role in the observed phenotypes characteristic of these strains, particularly in response to survival properties within the environment or in terms of virulence. We highlight the importance of combining whole genomic sequencing with phenotypic characterisation as a key element in the investigation of outbreaks of foodborne pathogens.

### 1. Introduction

In 2018 the European Food Safety Authority (EFSA) released a report on an invasive *Listeria monocytogenes* outbreak which had caused lethal human infections, with the source initially attributed to Hungarian frozen sweetcorn and other frozen vegetables (European Food Safety A and E, 2015). Whole-genome sequencing (WGS) confirmed the link between the human isolates and frozen sweetcorn isolates and so in 2018 the Hungarian authorities banned the marketing of all frozen vegetables (European Food

Safety A and E, 2015). The authorities in the United Kingdom and other countries in the European Union took a similar action. In order to understand their virulence, we have isolated *L. monocytogenes* from commercially available frozen processed sweetcorn from Hungary and performed genotypic and phenotypic characterisation. Currently WGS represents a powerful tool for molecular epidemiological studies; however, associating genotype with phenotype, is often lacking. Many foodborne pathogenic bacteria will behave differently upon colonisation of the human host and by coupling genome sequencing with phenotypic experimental data, our understanding

\* Corresponding author. Bacteriology Branch, Veterinary Sciences Division, Agri-Food and Biosciences Institute, Belfast, UK.

\*\* Corresponding author.

\*\*\* Corresponding author.

E-mail addresses: [Alexandros.stratakos@uwe.ac.uk](mailto:Alexandros.stratakos@uwe.ac.uk) (A.C. Stratakos), [lavi\\_stef@animalsci-tm.ro](mailto:lavi_stef@animalsci-tm.ro) (L. Stef), [ozan.gundogdu@lshtm.ac.uk](mailto:ozan.gundogdu@lshtm.ac.uk) (O. Gundogdu), [Nicolae.corcionivoschi@afbini.gov.uk](mailto:Nicolae.corcionivoschi@afbini.gov.uk) (N. Corcionivoschi).

<sup>1</sup> University of the West of England (UWE Bristol), Bristol, BS16 1QY, UK.

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of bacterial pathogenesis and survival can vastly improve. A variety of genes involved in pathogenicity and survival in the host are likely to be activated only *in vivo*, which strengthens the importance of scrutinizing outbreak isolates in experimental infections (Chiang, Mekalanos, & Holden, 1999).

*L. monocytogenes* is an intracellular foodborne pathogen that can cause listeriosis, which is linked to high morbidity and mortality in infants, pregnant women, elderly and immunocompromised individuals. Listeriosis is considered a public health concern worldwide, with an increasing number of incidents in Europe (Denny & McLauchlin, 2008; European Food Safety Authority, 2015). Although gastroenteritis is the most common manifestation of listeriosis, it can also cause septicaemia, meningitis and death (Vazquez-Boland et al., 2001). The most severe forms of the disease are a result of the ability of the pathogen to circumvent important physiological barriers. Once inside a host cell, several specialised bacterial factors enable the pathogen to multiply and spread to different host tissues (Camejo et al., 2011). Antibiotics can be used to successfully treat *L. monocytogenes* infection, however, the mortality rate can reach 20–30%, which is the highest fatality rate of any foodborne pathogen (Lieberman & Higgins, 2010; Swaminathan & Gerner-Smidt, 2007). *L. monocytogenes* is considered an ubiquitous environmental bacterium (Hellberg & Chu, 2016) able to withstand different physicochemical stresses and can persist in a variety of niches. The high stress tolerance characteristic of *L. monocytogenes* enables it to successfully persist in food processing environments and also to effectively pass and survive from food into the gastrointestinal tract of mammalian hosts and subsequently cause disease. Phenotypic data in conjunction with gene expression data have shown that the sigma B factor ( $\sigma^B$ ) of *L. monocytogenes* regulates many stress related responses in non-host associated environmental stress conditions (e.g. acid, osmotic, oxidative and cold stresses) (Chaturongakul, Raengpradub, Wiedmann, & Boor, 2008; Raengpradub, Wiedmann, & Boor, 2008). Upon invasion into the potential host a set of virulence genes that permit invasion and spread of the pathogen, which are regulated by PrfA, a master transcriptional regulator, are expressed (NicAogain & O'Byrne, 2016). In the EU, if a ready-to-eat food product allows the growth of this pathogen then the producer must show that *L. monocytogenes* will not reach counts above 100 CFU/g during its shelf life (Commission Regulation (EC) No 2073/2005). However, the U.S.A. has adopted a zero tolerance approach for all ready-to-eat food products (Hingston et al., 2017). Recent listeriosis outbreaks have been associated with various foodstuffs such as meat, cheese and fresh produce (Bolocan et al., 2016; Zhu, Gooneratne, & Hussain, 2017). An outbreak of invasive *L. monocytogenes* infection was confirmed by WGS and was linked to frozen sweetcorn, environmentally contaminated in a food-processing factory in Hungary. This outbreak has been ongoing in Austria, Denmark, Finland, Sweden and the United Kingdom since 2015, and has resulted in 47 confirmed cases and 9 deaths (European Food Safety Authority, 2018).

In order to understand the pathogenic potential of these *L. monocytogenes* isolates we have performed comparative genomic analysis in addition to phenotypic assays to correlate genotypic and phenotypic markers for a virulence profile of these *L. monocytogenes* outbreak isolates. Our study explores the resistance of these *L. monocytogenes* outbreak isolates to the major stresses encountered during host invasion and the establishment of a disease phenotype.

## 2. Material and methods

### 2.1. Isolation procedure

The *L. monocytogenes* isolates (OT171, OT172, OT173 and OT174) were obtained from commercial sweetcorn produced and packed in Hungary at the time of the outbreak. Each positive sample had > 100 CFU/g. The detection of *L. monocytogenes* was performed by following BS EN ISO 11290e1:1996/Amd 1:2004 specifications. Briefly, for the sweetcorn samples (of Hungarian origin), 25 g of sample were added to 225 ml of Fraser broth base (CM0895 without selective supplements), blended for 2 min (Colworth 400, Seward Limited, Worthing, U.K), then allowed to stand for 1 h. The samples were then plated (0.1 ml) onto plates of ALOA agar and incubated (37 °C for 48 h), with examination after 24 h and 48 h. Plates with less than 150 typical colonies were counted. Where plates yielded presumptive *L. monocytogenes*, five colonies were purified and confirmed using

API Listeria (bio-Merieux Ltd, Basingstoke, U.K), and the final count obtained by multiplying the presumptive count by the percentage of confirmed *L. monocytogenes* colonies. Four sweetcorn isolates were obtained from four separate sweetcorn samples; OT171, OT172, OT173 and OT174. For appropriate comparison purposes we have used the environmental isolate, FMT 1750 as a reference strain, which was initially isolated from a dairy processing factory (Harvey & Gilmour, 1992). All OT171-4 isolates and the FMT 1750 reference strain were sequenced and uploaded to the EBI ENA database (Accession number PRJEB31285).

### 2.2. Infection of mice by natural feeding

The infection model used was previously described (D'Orazio, 2014). Briefly, prior to infection, mice (C57BL/6J) were fasted for one day and the bedding was replaced with wire bedding to avoid coprophagy, but with water *ad libitum*. Contaminated bread pieces ( $10^6$ – $10^7$  CFU/piece of OT171, OT172, OT173, OT174 and FMT 1750) were introduced into the cage of each mouse. All mice consumed the bread within 3 min. For some experiments, a sub-lethal concentration of 0.5% Auranta 3009 (Sima et al., 2018) was included in the drinking water. After 3 days, infected mice were sacrificed by cervical dislocation and their livers and spleens harvested. Prior to microbiological analysis, all the collected organs were weighed, necropsies performed on all dead mice. Tests were performed to confirm infection with *L. monocytogenes*. Organs were manually homogenized in PBS, serially diluted and plated onto BHI agar plates containing *Listeria* selective supplement in order to investigate bacterial translocation. All experiments were approved by the Animal Research Committee according to the legislation in place (Law 471/2002 and government ordinance 437/2002) and under the supervision of National Sanitary Veterinary Agency. The ethics committee of Banat University of Agricultural Sciences and Veterinary Medicine – King Michael I of Romania, approved this work.

### 2.3. Cytokine production

The IL-6, INF- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-10 concentrations from blood and organs was determined at 18 h using a commercially available enzyme-linked immunosorbent assay systems from Quantikine (R&D Systems, U.K). Results of cytokine concentrations were expressed as pg/ml or ng/ml. Blood was collected by cardiac puncture and serum was obtained by centrifugation at 2800 rcf for 5 min. Each study run included wells with no bacteria in the HCT-8 cell.

### 2.4. Plaque-forming assay

Plaque-forming assay was performed as previously described (Roche et al., 2001). Briefly, HCT-8 cells were trypsinized and deposited per well in a 96-well tissue culture plate (Roche Diagnostics Ltd., Burgess Hill, UK). Plates were then incubated for 3 days with antibiotics followed by incubation for 24 h without antibiotics. *L. monocytogenes* isolates were grown overnight on Trypticase Soy Agar with 0.6% Yeast Extract (TSA-YE) and then suspended in complete medium to a concentration of  $10^5$  CFU/ml. HCT-8 monolayers were infected with a dilution series of  $10^2$ – $10^7$  *L. monocytogenes*/well and incubated for 2 h at 37 °C. The number of viable bacteria was assessed by duplicate plating of the appropriate dilutions onto TSA-YE and incubated at 37 °C for 24 h. Following the removal of the infected supernatants, cell monolayers were incubated for 1.5 h with media containing 100 mg gentamicin/ml (Sigma-Aldrich, Gillingham, U.K). Each well was then covered with media containing 10 mg gentamicin/ml and 2.5% (m/v) agarose (Invitrogen, Paisley, U.K). In order to prevent starvation, liquid media was added on top of the agar media followed by incubation for 24–48 h at 37 °C under 5% (v/v) CO $_2$ . After 48 h of incubation, enumeration of the plaques was confirmed.

### 2.5. In vitro adhesion and invasion assays

The ability of *L. monocytogenes* isolates to adhere to and invade HCT-8 cells was determined by previously described procedures (Corcionivoschi et al., 2012), with minor modifications. Briefly, HCT-8 cell monolayers were

grown overnight in a 5% CO<sub>2</sub> atmosphere at 37 °C in RPMI1640 supplemented with 10% fetal calf serum (Lonza, Analab Ltd., U.K) in 12-well tissue culture plates seeded with approximately  $4.3 \times 10^5$  cells per well. Prior to adhesion and invasion assays, the monolayers were washed three times in RPMI1640 followed by the addition of 1 ml of RPMI1640 containing 10% FCS to each well. For some experiments 0.5% Auranta 3001 was included in the tissue culture media. Bacterial cultures were re-suspended in 1 ml of RPMI1640 to an OD<sub>580</sub> of 2.0 for all isolates. For the adherence assays the cells were infected with 0.1 ml of bacterial culture, followed by incubation at 37 °C for 2 h in 5% CO<sub>2</sub>. After incubation, non-adherent bacteria were removed by three washes with 3 ml of PBS. The cells were lysed with 1 ml of 1% (v/v) Triton X-100 for 10 min at 37 °C followed by serial dilutions and plating on BHI agar. The invasion assays started with the addition of 1 ml of fresh RPMI1640 medium containing 10% FCS and 100 mg of gentamicin/ml to each well. After 2 h incubation at 37 °C the tissue culture cells were washed three times in 3 ml of RPMI1640 and lysed with 1 ml of 1% (v/v) Triton X-100 for 10 min at 37 °C.

## 2.6. Microtiter plate biofilm assay

The biofilm assay was performed as previously described (Djordjevic, Wiedmann, & McLandsborough, 2002). Briefly, 100 µl of culture containing each of the five *L. monocytogenes* isolates (approximately 10<sup>6</sup> CFU) were transferred into a 96 well plate. After a 36 h incubation, the growth media was removed from the wells and the wells were washed five times with sterile distilled water to remove loosely attached bacteria. The plates were dried at room temperature for 45 min and each well was stained with 150 µl of 1% crystal violet solution in water for another 45 min at room temperature. Subsequently, the washing step was repeated five times. In order to quantify the biofilm formation, 150 µl of 95% ethanol were added to de-stain the wells. 100 µl from each well was then transferred to a new plate and the absorbance of the solution was determined at OD<sub>595</sub> nm with a microplate reader (FLUOstar Omega, BMG Labtech, U.K).

## 2.7. Resistance to heat

*L. monocytogenes* isolates were grown on BHI broth at 37 °C for 48 h. Cells were harvested from 10 ml of broth by centrifugation and the pellet washed twice in PBS. The washed pellet was re-suspended in the same volume of BHI broth. A 10<sup>-2</sup> dilution of this suspension was prepared in BHI broth. A glass 5 ml test-tube containing 1350 µl of sterile BHI broth was pre-warmed to 60 °C (temperature was monitored using digital thermometer fitted with a K-type thermocouple placed in a blank containing the same volume of BHI broth). A 150 µl aliquot of each *L. monocytogenes* suspension was added to the pre-warmed BHI broth to give approximately 10<sup>6</sup> CFU/ml. The exact counts were determined after appropriate dilutions in maximum recovery diluent (MRD) (Oxoid, Basingstoke, U.K). When the temperature had reached 60 ± 0.5 °C (mean come-up time 10.5 s) the inoculated broths were held at this temperature for 5 min, then cooled immediately in iced water. *L. monocytogenes* were enumerated in heat-treated samples by preparing a suitable dilution series in MRD and spread-plating 100 µl of the appropriate dilutions in duplicate onto TSAYE. Plates were incubated for 48 h at 37 °C

## 2.8. Resistance to hydrogen peroxide

Hydrogen peroxide resistance was determined as previously described (Gundogdu et al., 2011). A stationary phase culture was prepared for each strain in brain-heart infusion (BHI) broth (Oxoid, Basingstoke, U.K). Cells were harvested from 10 ml of broth by centrifugation and the pellet was washed twice in PBS. The washed pellet was resuspended in the same volume of BHI broth. A 10<sup>-2</sup> dilution of this suspension was prepared in BHI broth. An aliquot of each *L. monocytogenes* suspension was inoculated to new BHI broths to give approximately 10<sup>6</sup> CFU/ml. The exact counts were determined after appropriate dilutions in MRD. Hydrogen peroxide was added at a final concentration of 420 mM and incubated for 15 min at 37 °C. Untreated controls were also prepared. Subsequently, appropriate 10-fold

dilutions were prepared in MRD. An aliquot of 100 µl of each of the 10-fold dilutions was spread plated on TSAYE. The dilutions and plating out, in duplicate, were performed immediately to avoid any residual hydrogen peroxide inactivation (Metselaar et al., 2015). Plates were incubated for 48 h at 37 °C.

## 2.9. Resistance to pH

*L. monocytogenes* isolates were grown in BHI broth to stationary phase. Cells were harvested from 10 ml of broth by centrifugation and the pellet was washed twice in PBS. The washed pellet was resuspended in the same volume of BHI broth. A 10<sup>-2</sup> dilution of this suspension was prepared in BHI broth. An aliquot of each *L. monocytogenes* suspension was inoculated to new BHI broths to give approximately 10<sup>6</sup> CFU/ml. The exact counts were determined after appropriate dilutions in MRD. The pH of BHI broths had been previously adjusted to 3.5 using hydrochloric acid. Untreated controls were also prepared. The cultures were incubated for 15 min at 37 °C. Subsequently, appropriate 10-fold dilutions were prepared in MRD. An aliquot of 100 µl of each of the 10-fold dilutions was spread plated, in duplicate, on TSAYE. Plates were incubated for 48 h at 37 °C

## 2.10. Resistance to commercial antimicrobial mixtures

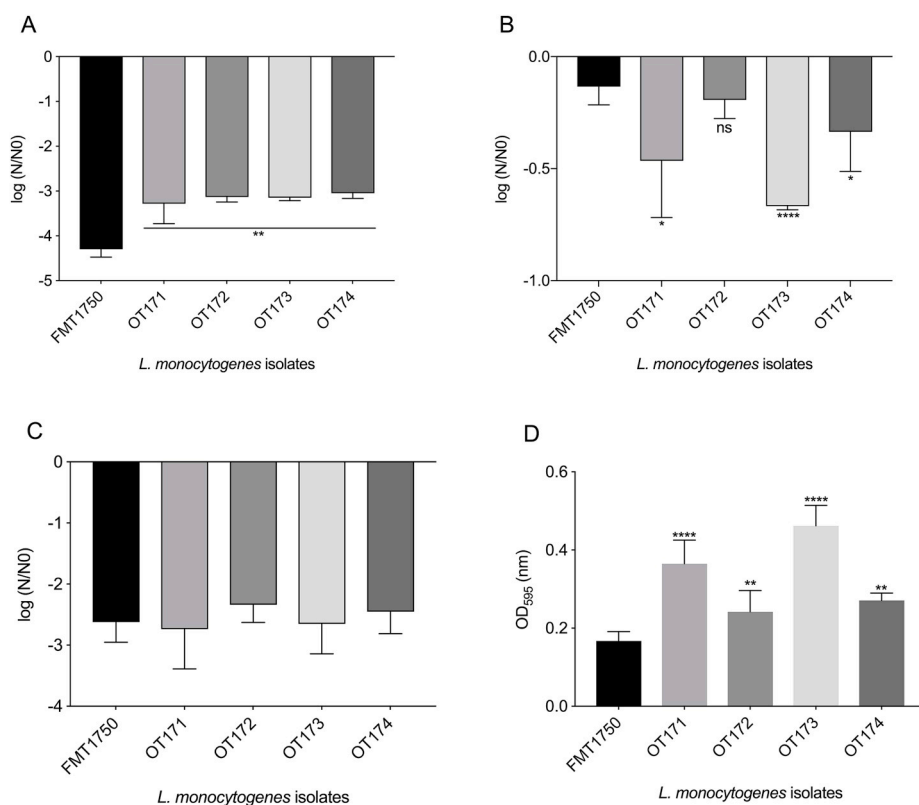
Resistance to the commercial antimicrobial, Auranta 3009 (supplied by Auranta-Envirotech Innovative Products Ltd and contains lactic and citric acid, glycerine-based emulsifying agent, sodium hydroxide, sodium chloride, citrus extract (6%), grape seed extract (2%), oregano extract (1%).) was determined as for hydrogen peroxide. The antimicrobial was added at a final concentration of 2% (v/v) and incubated for 90 min at 37 °C. Subsequently, appropriate 10-fold dilutions were prepared in MRD. An aliquot of 100 µl of each of the 10-fold dilutions was spread plated, in duplicate, on TSAYE. Untreated controls were also prepared. Plates were incubated for 48 h at 37 °C.

## 2.11. Listeriolysin detection by western blotting

Overnight cultures of all *L. monocytogenes* isolates were set up in Mueller-Hinton Broth and incubated aerobically at 37 °C overnight. For each isolate, the OD<sub>600</sub> was adjusted to 0.4. 1 ml of each culture was centrifuged, the supernatant was removed, and the resulting pellet used for Western Blotting. 200 µl of 25 mM Tris Buffer, containing 0.02 g/ml lysozyme (Invitrogen, Paisley, U.K), were added to each pellet and re-suspended. All samples were placed in a heating block at 37 °C for 90 min and subsequently sonicated for 80 min. The proteins were separated by electrophoresis in 12% Bis-Tris gels (Invitrogen, Paisley, U.K). Subsequently proteins were transferred on PVDF membranes and detected after overnight incubation with specific listeriolysin (LLO) antibody (1:20000). Anti-rabbit HRP-conjugated antibody was used as a secondary antibody (1:10000) (Santa Cruz Biotechnologies, Germany).

## 2.12. Whole genome sequencing

DNA was extracted from all isolates using a PureLink Genomic DNA Kit (ThermoFisher Scientific, Paisley, U.K) as per the manufacturer's instructions. The whole genome sequencing of all *L. monocytogenes* isolates was performed as previously described (Ugarte-Ruiz et al., 2014) using Illumina MiSeq 2 × 250 bp paired-end sequencing. To analyse the data quality, FastQC was used (Andrews, 2010). To trim and crop the sequencing reads, Trimmomatic was used with the following parameters: (v0.32) 'leading' and 'trailing' setting of 3, a 'slidingwindow' setting of 4:20 and a 'minlength' of 36 nucleotides (Bolger, Lohse, & Usadel, 2014). 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 & Durbin (2010). VelvetOptimiser (v2.2.5) using n50 optimization was used to perform assembly (Zerbino & Birney, 2008). The reference strain *L. monocytogenes* EGD (HG421741) was used to complete contigs using ABACAS (v1.3.1) (Assefa, Keane, Otto, Newbold, & Berriman, 2009). Multi locus sequence type (MLST) was determined using PubMLST (<https://github.com/tseemann/mlst>). Genome



**Fig. 1. *L. monocytogenes* resistance to stressors and biofilm formation.** (A) Heat resistance of *L. monocytogenes* strains in BHI broth, (B) pH resistance, (C) H<sub>2</sub>O<sub>2</sub> resistance, (D) biofilm formation. Results are expressed as log reduction (N0: control counts; N: counts after stress exposure). Error bars represent the standard deviation. Asterisks indicate significant differences (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001; \*\*\*\*p < 0.0001).

annotation was provided by using Prokka (Seemann, 2014). To read the genomes, and assess them for presence of specific genes, Artemis and ACT software were used (Carver, Harris, Berriman, Parkhill, & McQuillan, 2012). Pan genome analysis was performed using Roary (Page et al., 2015) and visualised using Phandango (Hadfield et al., 2017). Phylogenetic analysis was performed using the alignments generated from Roary following by ClustalX (Larkin et al., 2007) and visualised using FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

### 2.13. SNP calling

We downloaded fully annotated *L. monocytogenes* (HG421741) from NCBI (FASTA sequence and GENBANK file). We next mapped the five samples (FMT 1750, OT171-4) using BWA (Li & Durbin, 2010) and samtools (Li et al., 2009) against the reference genome to obtain the mapped BAM files. LoFreq (Wilm et al., 2012) was then utilised to generate SNPs in VCF format. Additionally, we used popoolation2 (Kofler, Pandey, & Schlötterer, 2011) to perform a Cochran-Mantel-Haenszel test (cmh-test.pl) for repeated tests of independence for every SNP. To do so, we made the VCF files produced by LoFreq compatible with popoolation2 (that accepts a proprietary nsync format) and for this purpose we wrote a custom script (lofreq2sync.py). We then used filt-Zerosync.py (from popoolation2) to pre-process/filter out any SNPs that were not expressed in all the samples. The cmh-test.pl was then used by comparing the outbreak samples (OT171, OT172, OT173, OT174) against FMT 1750, by using -min-count 10 -min-coverage 10 -max-coverage 5000 and -min-logpvalue 3 to identify SNPs that were significantly different (p-value ≤ 0.001 in CMH test) between FMT 1750 and the test strains (OT171-174). Following this, we extracted the genes sequences by writing a custom script (gbExtractFeatures.py) to extract the genes (from GENBANK file) hitting the SNPs. Having extracted the FASTA sequences for the genes hitting the SNPs, we used BLAST2GO (Conesa et al., 2005) to obtain GO Ontologies associated with the genes affected by the SNPs. These are given in Supplementary Table 2. The custom scripts utilised in this study are available at <http://userweb.eng.gla.ac.uk/umer.ijaz/#bioinformatics>.

### 2.14. Statistical analysis

Data are presented as mean ± standard deviation (SD). All experiments represent at least three biological replicates performed in triplicate in each experiment. Statistical analyses were performed using Prism software (GraphPad Software). Variables were compared using Student's *t*-test.

## 3. Results

### 3.1. Stress tolerance and phenotypic analysis of *L. monocytogenes* isolates

All OT171-4 isolates were evaluated for their capacity to survive under high heat, low pH and hydrogen peroxide resistance (Fig. 1). The four *L. monocytogenes* sweetcorn isolates displayed similar resistance to heat (3–3.2 log CFU/ml reduction after 5 min at 60 °C) (Fig. 1a) however, the FMT 1750 presents significantly lower heat resistance. For acid tolerance (pH), there were significant differences in resistance between some isolates except for the OT172 isolate (Fig. 1b). The resistance of all five isolates to hydrogen peroxide (420 mM for 15 min) was also determined (Fig. 1c) and showed very similar resistance levels between all isolates with the reductions ranging from 2.3 to 2.7 log CFU/ml (Fig. 1c). Significant differences were observed in the case of biofilm formation for OT171-4 isolates when compared to the FMT 1750 reference strain (Fig. 1d).

### 3.2. In vitro invasion assay and plaque formation

The ability of OT171-4 isolates to invade HCT-8 epithelial cells was investigated using FMT 1750 as a reference strain (Fig. 2). The ability of the OT171-4 isolates to adhere to HCT-8 cells is significantly enhanced compared to the FMT 1750 reference strain as shown in Fig. 2a (p < 0.001). *L. monocytogenes* OT171-4 isolates were also significantly more invasive than FMT 1750 (Fig. 2b). The listeriolysin levels, as detected by western blotting, displayed an increased production of LLO in the OT171-4 isolates, which may explain the increased ability to attach and invade epithelial cells (Fig. 2f). In addition to these adhesion and invasion properties, we also performed similar experiments in the presence of an antimicrobial mixture

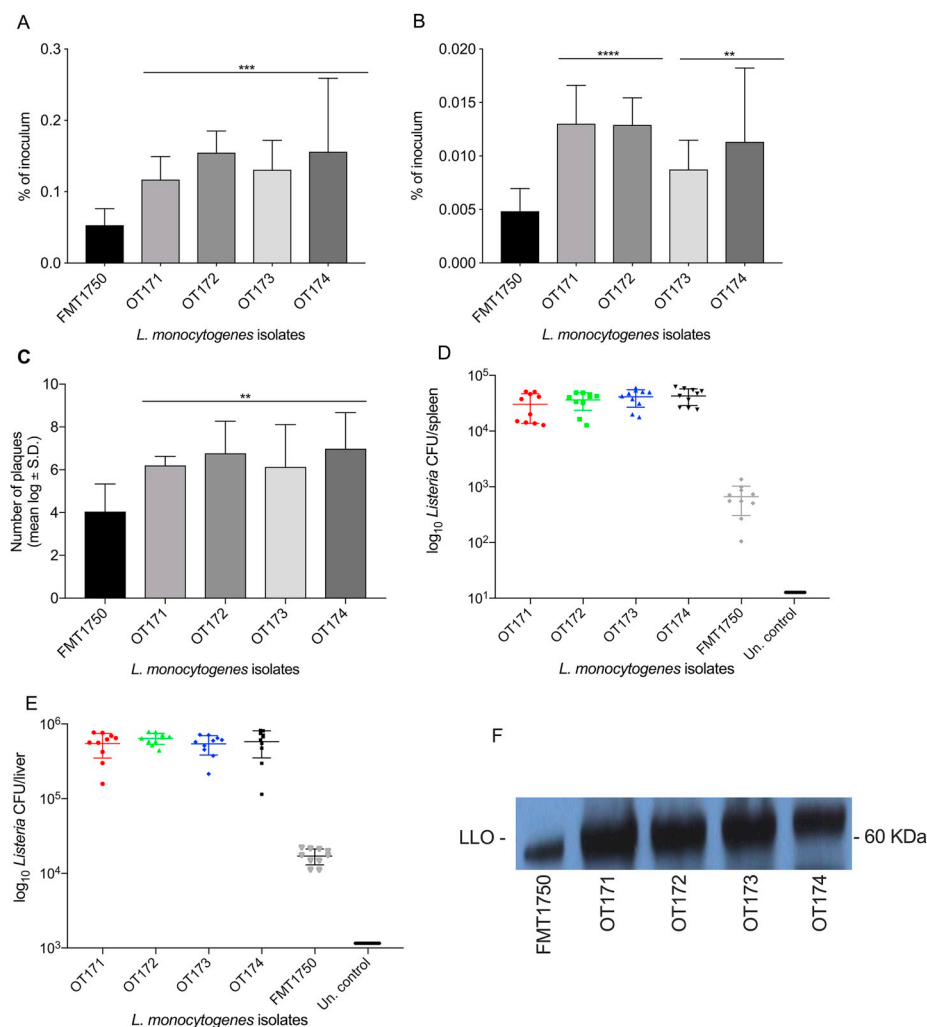
(Auranta 3009). Here, the OT171-4 isolates are less resistant to a mixture of antimicrobials (Fig. 3d) and at sub-lethal concentrations the antimicrobial mixture was able to reduce the total adherence of the OT171-4 isolates to the HCT-8 cells compared to the FMT 1750 reference strain (Fig. 3c). We have also investigated the resistance of OT171-4 isolates to a number of antibiotics and the results indicate similar susceptibilities for all OT171-4 isolates and the FMT 1750 reference strain (Supplementary Table 3).

In order to confirm the results of the cell-based assays we have performed the plaque-forming assay (PFA) using HCT-8 cells. The PFA results allowed us to test the ability of the *L. monocytogenes* isolates to form plaques 24 h after cell infection in 96-well tissue culture plates (Fig. 2c). These results correlate with the adhesion and invasion assays described in Fig. 2a and b where the OT171-4 isolates displayed significant increase in plaque formation when compared to the FMT 1750 reference strain.

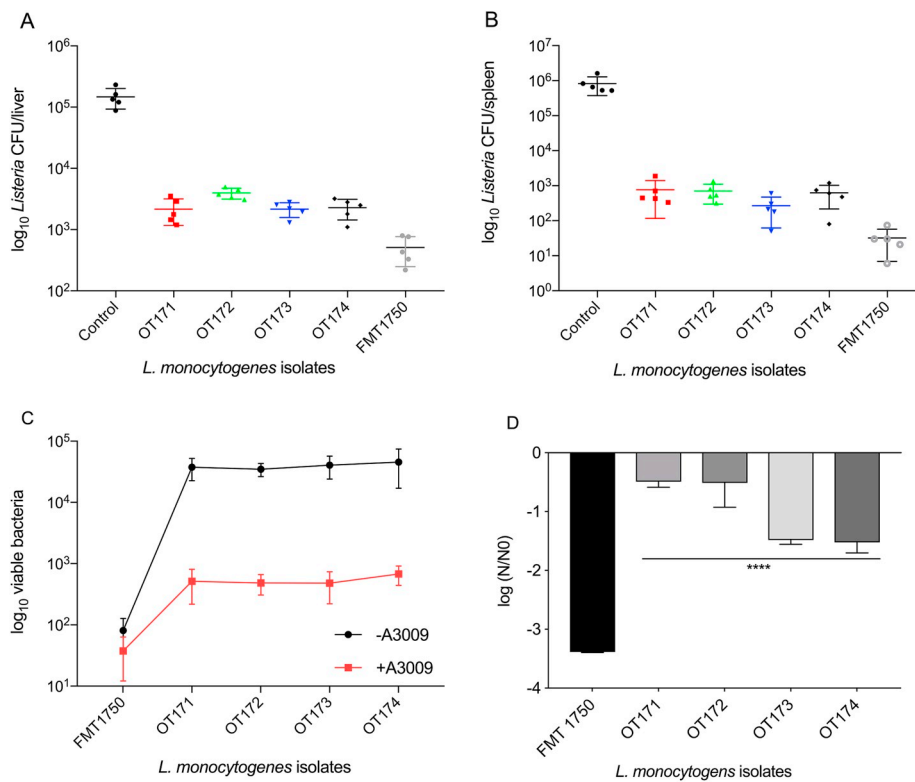
### 3.3. In vivo infection assay and immune response

In order to further confirm the increased *in vitro* virulence, we performed *in vivo* infection experiments using a mouse infection model as describes in the material and methods. At 3 h post infection, OT171-4 isolates were detected at approximately 5 log<sub>10</sub> CFU/spleen (Fig. 2d) and at 6 log CFU/liver (Fig. 2e). The infection levels for the FMT 1750 reference strain were detectable at approximately 3 log CFU/spleen (Fig. 2d) and 4 log CFU/liver (Fig. 2e). In all cases, the mice survived a maximum of four days. The natural antimicrobial mixture (Auranta 3009) has also shown a significant effect reducing the infection levels *in vivo* (Fig. 3a and b) on liver and spleen in a mouse infection model. The difference in virulence was also reflected at host defence level by investigating the detrimental and defencing cytokines

produced during infection. Investigation of TNFα production (Fig. 4a) in the blood of the infected mice revealed significantly higher levels for the OT171-4 isolates, including FMT 1750 (p < 0.05), when compared to the un-infected control. However, the levels of significance are higher for OT171 (p < 0.01), OT172 (p < 0.001) and OT174 (p < 0.01) when compared to the un-infected control. In the spleen, the TNFα levels ranged between 1000 and 2000 pg/ml for the FMT 1750 reference strain and the un-infected control, whereas for OT171-4 isolates (p < 0.001) were detected over 2000 pg/ml (Fig. 4a). In addition, OT171-4 isolates and the FMT 1750 reference strain had significantly higher levels of TNFα detected in the liver (Fig. 4a). In the liver, the detected levels of IFNγ were higher for the OT171-4 isolates compared to the FMT1750 reference strain whose levels did not significantly differ with respect to the uninfected control (Fig. 4b). The levels of IFNγ (Fig. 4b) were significantly higher in the blood of the infected mice for OT171-4 isolates when compared to both the un-infected controls and FMT 1750 reference strain (p < 0.001). In the spleen, the levels were also significantly higher for OT171-4 isolates, however infection with FMT 1750 resulted in a smaller increase (p < 0.05) (Fig. 4b). In the liver, the detected levels were higher for the OT171-4 isolates, however were not significant for the FMT 1750 reference strain (Fig. 4b). For IL6, when compared to the un-infected control, there was a significant increase in detection within the blood for all OT171-4 isolates and the FMT 1750 reference strain (Fig. 4c). Significance was also recorded for the spleen infection (p < 0.001) and the liver (p < 0.05) when comparing all OT171-4 isolates to the un-infected control (Fig. 3c). We next investigated the expression levels of IL4 (Fig. 4d) and IL10 (Fig. 4e) in the blood and as expected, because all OT171-4 isolates caused an infection, the concentrations were significantly lower when compared to the un-infected control (p < 0.001).



**Fig. 2. In vitro and in vivo infection abilities, LLO secretion and plaque formation of *L. monocytogenes* isolates.** *In vitro* adherence (A), invasion (B) of HCT-8 cells of *L. monocytogenes* sweetcorn isolates compared to the FMT1750 reference strain. Panel C, represents the number of plaques with the results corresponding to the mean standard deviation (S.D). Panels D (spleen) and E (liver) show the levels of *in vivo* infection expressed as log<sub>10</sub> CFU/ml. The levels of LLO production between strains is presented in panel F. Error bars represent the standard deviation. Asterisks indicate significant differences (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001; \*\*\*\*p < 0.0001).



**Fig. 3. Virulence abilities in the presence of antimicrobial mixtures.** *Listeria* isolates load in liver (A) and spleen (B) of infected mice at 3 days post infection in presence/absence of Auranta 3009. The control in panels A and B represents infected mice in the absence of Auranta 3009. Panel C describes the ability of the OT171-4 *L. monocytogenes* isolates to attach to HCT-8 cells *in vitro* in the presence of 0.5% Auranta 3009. Panel D shows the resistance of *L. monocytogenes* isolates to Auranta 3009 in BHI broth. Results are expressed as log reduction (N0: control counts; N: counts after stress exposure). Error bars represent the standard deviation. Asterisks indicate significant differences (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

### 3.4. Comparative genomics of the OT171-4 *L. monocytogenes* isolates

Following WGS, phylogenetic analysis of the OT171-4 isolates, the FMT 1750 reference strain and 45 additional publicly available *L. monocytogenes* genome sequences were investigated bioinformatically (Fig. 5, Supplementary Table 1). Phylogenetic analysis identified the four *L. monocytogenes* isolates (OT171-4) had diverged from the FMT 1750 reference strain, in agreement with these being isolated from a different source (sweetcorn versus dairy processing facility). Of the four OT strains, OT172-4 formed a distinct cluster that appeared as separate in comparison to the OT171 isolate. This grouping coincides with their serotype classification (OT172-4 Serotype 8; OT171 Serotype 398). Analysis of the four OT171-4 isolates and the FMT 1750 reference strain was performed using Roary (Page et al., 2015) (Supplementary Fig. 1). To investigate this further, we analysed SNP locations and identified those statistically significant based on CMH tests and further functional resolution using GO ontology (Supplementary Table 2).

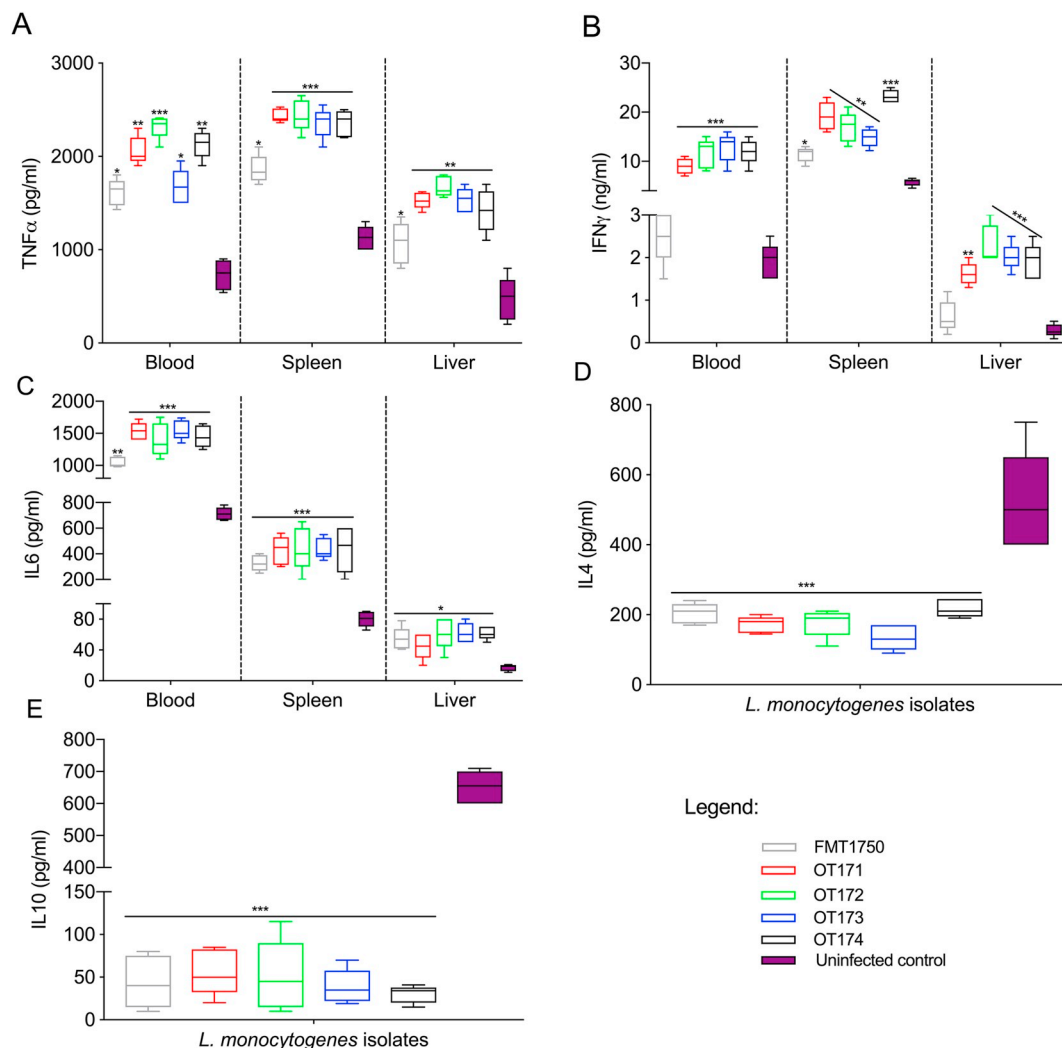
## 4. Discussion

*L. monocytogenes* can be found in diverse ecological niches: natural and farm environments, animals, food and humans (Vivant et al., 2013a, 2013b) and is able to survive for long periods of time in unfavourable environments conditions that do not allow the microorganism to grow (Carpentier & Cerf, 2011). This constant exposure to environmental factors make bacterial circulation difficult to trace (Felix et al., 2018). Many *L. monocytogenes* are avirulent but have the ability to increase their virulence if exposed to environmental stresses (Lammerding, Glass, Gendron-Fitzpatrick, & Doyle, 1992; McLauchlin, 1990). *L. monocytogenes* has caused episodes of human listeriosis throughout the world, with isolates reported in North and South America, Europe, Africa, Asia, and Oceania (Orsi & Wiedmann, 2016). In recent years, bacterial whole genome sequencing and analysis has become more accessible with the development of next-generation sequencing methods (Deurenberg et al., 2017). The use of this technology, in conjunction with phenotypic molecular experimental assays can provide us with the necessary data to better understand what makes such strains survive, thrive and become outbreak strains. This work investigated the phenotypic virulence characteristics and the genetic basis for increased stress

resistance of *L. monocytogenes* outbreak isolates.

Our initial phenotypic assays allowed us to test a range of environmental associated stress conditions, with OT171-4 isolates displaying a higher level of adhesion and invasion, *in vitro*, compared to the reference isolate. Also, the *in vivo* experimental data showed that the four new isolates displayed significantly higher virulence properties when compared to the reference strain as the colonisation levels for the sweetcorn isolates were higher for both the liver and the spleen. It is important to note that the OT171-4 sweetcorn isolates all showed similar levels of colonisation in both organs. The *in vivo* findings reveal that the sweetcorn isolates have increased capacity at translocating from the gastrointestinal tract to other organs of mice or simply are more capable of surviving in the gastrointestinal tract. The *in vitro* resistance results showed that all isolates used in this study were resistant to acidic conditions (Fig. 1b). In addition, the OT171-4 sweetcorn isolates also had the same susceptibility to oxidative stress, which could suggest that increased survivability in the gastrointestinal tract is not the determining factor that influences colonisation.

Successful infection by *Listeria* spp. is facilitated by internalins, especially internalin A (InIA) known for its role in internalisation in epithelial cells (Lecuit, Ohayon, Braun, Mengaud, & Cossart, 1997). Structural changes could increase/decrease the ability of InIA to promote internalisation and our study indicates that the InIA of the OT171-4 isolates show potential SNP modifications (Lecuit et al., 1997). This is also mirrored for the biofilm and heat phenotypic data where the OT171-4 sweetcorn isolates were all significantly different to the FMT 1750 reference strain. One of the most common virulence bacterial traits, affected by pH and temperature, is the ability to produce a biofilm (Hostacka, Ciznar, & Stefkovicova, 2010) which in *L. monocytogenes* can be affected by many other environmental parameters as well (Moretro & Langsrud, 2004). In *L. monocytogenes*, several genes have been identified which play a role in heat resistance including *dnaK*, *dnaJ*, *groES*, *groEL*, ATP-dependent *Clp* proteases, and ABC transporters (Kazmierczak, Mithoe, Boor, & Wiedmann, 2003). SNP analysis of the four sweetcorn isolates compared to the reference strain revealed significant changes in the ATP binding ABC transporters (Supplementary Table 2) suggesting the possible involvement in the accumulation of osmolytes as a consequence of exposure to salt, acid and cold stress (Sleator, Wouters, Gahan, Abee, & Hill, 2001; Wemkamp-Kamphuis et al., 2004). The



**Fig. 4.** Kinetics of cytokine production in infected mice. Endogenous TNF- $\alpha$  (A), IFN- $\gamma$  (B), IL-6 (C), IL4 (D) and IL10 (E) in the bloodstream, spleen and liver of infected mice at 3 h post infection. Error bars represent the standard deviation. Asterisks indicate significant differences (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

observed effect of temperature could be explained through the changes induced by the DNA gyrase subunit A (Supplementary Table 2). It has been shown that changes in the level of DNA supercoiling overlap with an exposure to temperature stress, peroxide stress, and osmotic shock (Cheung, Badarinarayana, Selinger, Janse, & Church, 2003). Under certain stresses including pH and oxidative stress ( $H_2O_2$ ) the OT171-4 sweetcorn isolates display similar phenotypes to the FMT 1750 reference strain.

The commercial antimicrobial mixture (Auranta 3009), decreased the virulence of the OT171-4 sweetcorn isolates and also of the reference strain (Fig. 3A and B). In contrast, *in vitro* susceptibility to the antimicrobial mixture was significantly different between the sweetcorn and reference strain isolate whereby the OT171-4 sweetcorn isolates were more resistant to the antimicrobial. (Fig. 3D). Natural antimicrobials or mixtures of antimicrobials have been shown effective in reducing the virulence of bacterial pathogens *in vitro* and *in vivo* (Sima et al., 2018; Stratakos et al., 2018). In *L. monocytogenes*, essential oils and citric acid are known to downregulate the expression of genes involved in glycolysis, stress response and protein synthesis after exposure to environmental stresses (Braschi et al., 2018). Our study shows a similar pattern as Auranta 3009 was able to reduce the *in vitro* virulence of the OT171-4 sweetcorn isolates. Auranta 3009 was also able to reduce the *in vivo* virulence, since the liver and spleen colonisation observed was at very similar levels compared to the control isolate. However, the presence of the antimicrobial liver and spleen colonisation was significantly higher for the sweetcorn isolates. At genome level, the SNP analysis revealed changes in genes involved in oxidative stress defence (e.g. catalase)

and virulence factors (e.g. polysaccharide biosynthesis proteins) suggesting that these compounds could be effective, though not lethal.

Host resistance against *L. monocytogenes*, both in humans and experimental animals, is dependent on both early non-specific defence as well as late T lymphocyte mediated defence regulated by endogenous cytokines (Queiroz, Quadros, & Santos, 2000).  $\gamma$ -interferon (IFN- $\gamma$ ) tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6) play important roles in host resistance against *L. monocytogenes* infection. In contrast, IL-4 and IL-10 have shown a detrimental role in resistance (Hoge et al., 2013; Nakane et al., 1999; Pamer, 2004). The data here show a significant increase in the expression of host detrimental (IFN- $\gamma$ , TNF- $\alpha$  and IL-6) cytokines in the case of all four OT171-4 sweetcorn isolates, suggesting the involvement of bacterial proteins with a role in triggering an immunological response. More specifically, *L. monocytogenes* caused pronounced systemic IFN- $\gamma$ , TNF- $\alpha$  and IL-6 production during infection with all the isolates used in this study, however, production was significantly higher for OT171-4 isolates compared to the reference isolate. IL-4 and IL-10 were found in significantly lower levels in infected mice, which was observed for all the isolates.

SNP analysis identified flagellar hook protein FlgE as a potential SNP modified gene between OT171-4 sweetcorn isolates and the reference strain. Mutations occurring in the flagellar hook could affect their ability to act as proinflammatory mediator (Shen et al., 2017). An additional gene picked up by the SNP analysis in the OT171-4 sweetcorn isolates is ethanolamine utilization (*eutL*), a gene that provides bacterial pathogens with

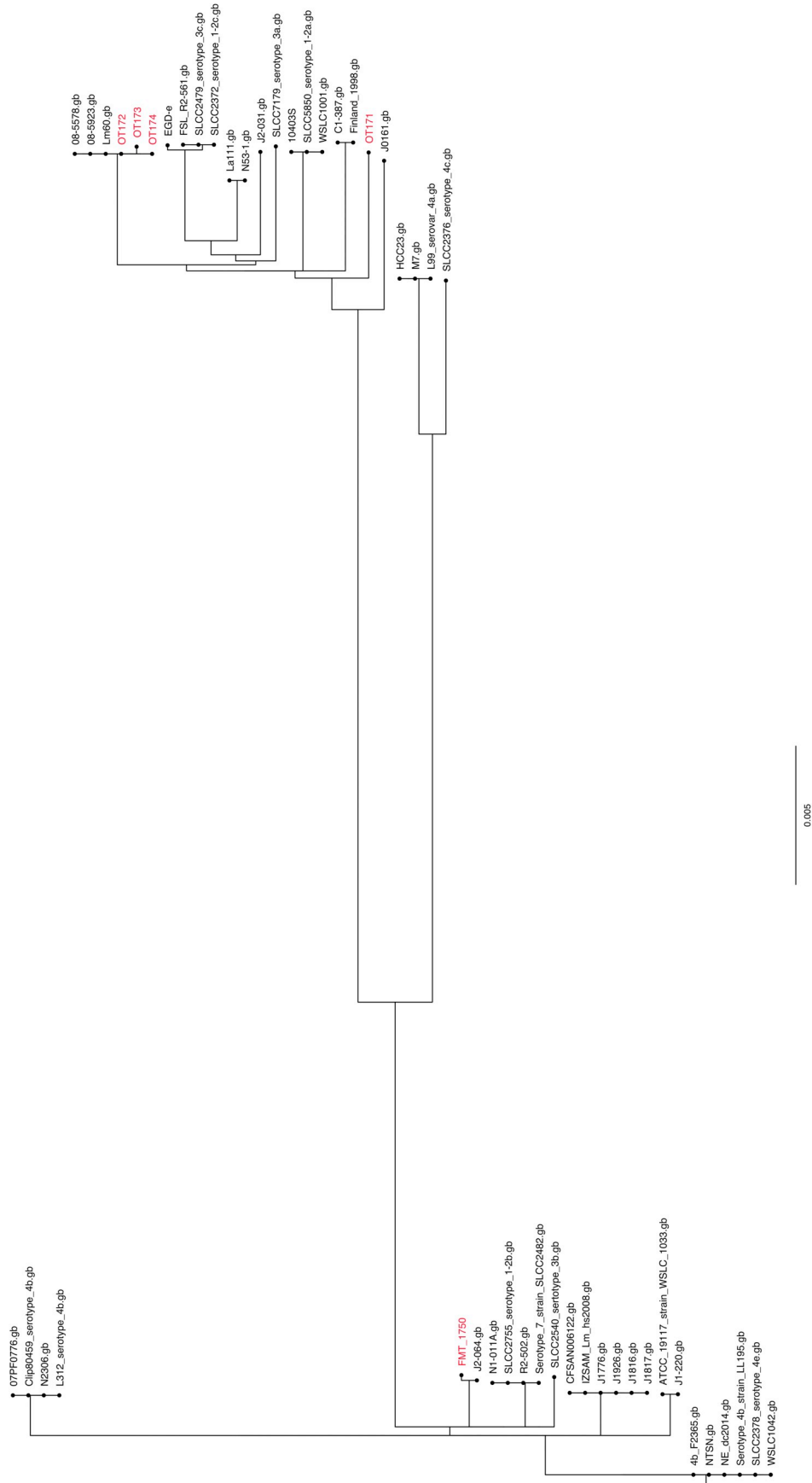


Fig. 5. Phylogenetic tree generated from binary presence and absence of accessory genes. Phylogenetic analysis of all four OT1171-4 isolates, the FMT 1750 reference strain and 45 additional publicly available *L. monocytogenes* genomes (Supplementary Table 1). Phylogenetic tree was created using Roary and ClustalX. Visualisation was performed using FigTree software.



the ability to utilize host or diet originated ethanolamine as carbon source contributing in this way to pathogenesis (Kaval & Garsin, 2018; Roof & Roth, 1988). These genes are upregulated in *L. monocytogenes* when used to infect the gut of a mouse infection model suggesting their involvement in pathogenesis (Toledo-Arana et al., 2009). Interestingly this pathway also links to our results on the immune response during *in vivo* infection, which has resulted in the upregulation of detrimental cytokines. This immunogenic role has been shown in *Pseudomonas aeruginosa*, where acetate can be formed because of ethanolamine breakdown and can interact with a G-protein coupled receptor to modulate innate immunity and inflammation at gut level (Maslowski et al., 2009; Starai, Garrity, & Escalante-Semerena, 2005). Another set of genes also involved in nutrient uptake and predicted as mutated by the SNP analysis in the present study is precorrin-3B C(17)-methyltransferase and sirohydrochlorin cobaltochelataase. These genes are involved in cobalt metabolism providing *Listeria* spp., *in vivo*, with the ability to access those ions and support their nutrient requirement and survival needs in anaerobic conditions (Camejo et al., 2009; Moore et al., 2013).

SNP analysis revealed transketolase (TKT) as affected significantly between the OT171-4 sweetcorn isolates and the reference strain. *Salmonella* spp., strains lacking transketolase have been described as avirulent in mice suggesting their possible polar effect in bacterial pathogenesis (Shaw et al., 2018). Increased resistance to induced stresses in the OT171-4 isolates may also be related to these significant changes in the TKT enzymes, since it has been shown that they could be beneficial during stressful conditions encountered during infection (Varghese, Tang, & Imlay, 2003). In *L. monocytogenes*, the metabolism of the intracellularly growing bacteria is significantly different from that of extracellular bacteria and the TKT enzymes have been shown to be involved in the pentose phosphate pathway intracellularly providing access to a carbon source by degrading glucose during infection of macrophages (Eylert et al., 2008). The SNP analysis also revealed significant differences between the OT171-4 sweetcorn isolates and the reference strain, this time referring to the cell wall metabolism sensor histidine kinase. We have also linked these changes to the observed increased resistance to the environmental stimuli since these sensors are a part of two-component signal transduction systems involved in adaptive responses in bacteria (Beier & Gross, 2006; West & Stock, 2001). In *B. pertussis* this histidine kinase receptor is known to stimulate virulence gene expression at normal body temperature, but being switched off at low temperatures (Cotter & Jones, 2003). Highly involved in bacterial nutrient metabolism and virulence are also the 6-phospho-beta-glucosidase enzymes required for energy generation. In pathogenic bacteria such as *Listeria*, the bacterium will rely on the host to produce the required energy for growth and virulence using this type of enzymes (Terra, Zhi, Kahya, Andrew, & Yesilkaya, 2016). Our SNP analysis indicates that in the OT171-4 sweetcorn isolates this gene is significantly mutated compared to the control with a possible impact on its metabolic activity.

## 5. Conclusion

In summary, survival during stress including food-related stresses suggests that *L. monocytogenes* is well equipped to endure the associated stresses in the gastrointestinal tract (Archer, 1996; Lee, Lin, Hall, Bearson, & Foster, 1995) and we have recently shown that motility, an important virulent factor in bacteria, can be influenced by the environment of origin (Stratakos et al., 2019). Here we aimed to characterise and help understand why certain outbreak strains can persist within the environment and which properties enable them to demonstrate enhanced survival and virulence. Using a combination of genotypic and phenotypic methods, we have identified similar phenotypic properties such as *in vitro* adhesion and invasion, *in vivo* liver and spleen colonisation, biofilm formation and resistance to heat for all four *L. monocytogenes* outbreak strains (OT171-4) which have differed significantly from a reference strain (FMT 1750). Using SNP analysis we have investigated what genetic changes may be causing these phenotypic observations. We have highlighted the importance of combining whole genomic sequencing strategies in conjunction with phenotypic methods as a key approach in the investigation of listeriosis and these principles could be applied to other foodborne outbreak pathogens.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2019.106784>.

## Author contributions

ACS, UZI, CK, ML, LP, AC, IP, JM, PS, DS and LS conceived the design and performed the experiments. ACS, IP, NC, LS, UZI, JMC and OG analysed the data. NC, PW, OG and LS contributed reagents, materials, and analysis tools. NC, UZI, ACS, JMC, WTS, ND, BWW and OG wrote the paper. All authors read and approved the final manuscript.

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