Genomic analysis of high copy-number sequences for the targeted detection of *Listeria* species using a flow-through surveillance system

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Abstract
The bacterial foodborne pathogen *Listeria monocytogenes* has been implicated in fresh produce outbreaks with a significant economic impact. Given that *L. monocytogenes* is widespread in the environment, food production facilities constantly monitor for the presence of *Listeria* species. To develop a surveillance platform for food processing facilities, this study conducted a comparative genomic analysis for the identification of conserved high copy sequences in the ribosomal RNA of *Listeria* species. Simulated folding was performed to assess RNA accessibility in the identified genomic regions targeted for detection, and the developed singleplex assay accurately detected cell amounts lower than 5 cells, while no signals were detected for non-targeted bacteria. The singleplex assay was subsequently tested with a flow-through system, consisting of a DNA aptamer-capture step, followed by sample concentration and mechanical lysis for the detection of *Listeria* species. Validation experiments indicated the continuous flow-through system accurately detected *Listeria* species at low cell concentrations.

Keywords  
*Listeria* · Food safety · Fresh produce · RNA · Foodborne pathogen · Genomes

Introduction
Foodborne pathogens are responsible for a consistent level of human illness that poses a substantial public health and economic burden, resulting in an economic burden of $15.5 billion annually (Hoffmann et al. 2015). Listeriosis, a serious infection caused by eating food contaminated with the bacterium *Listeria monocytogenes*, has been recognized as an important public health problem in the United States, and the annual economic impact of listeriosis in the United States alone is estimated at over US$2.8 billion. There has been an estimated 1500 listeriosis cases each year, and of those, approximately 300 cases have resulted in death (Hoffmann et al. 2012; Scallan et al. 2011). In particular, *L. monocytogenes* is responsible for systemic listeriosis with an approximate 30% mortality rate in susceptible populations of pregnant women, neonates, elderly, or immunocompromised patients (Buchanan et al. 2017; Hoffmann and Scallan Walter 2019; Scallan et al. 2011).

While *L. monocytogenes* is considered one of the major foodborne pathogens that cause listeriosis in humans and animals (Pesavento et al. 2010), other *Listeria* species, which include *Listeria grayi*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, and *Listeria welshimeri*, have been occasionally implicated in human clinical case reports, primarily in individuals with suppressed immune functions and/or underlying illnesses (Korsak and Szuplewska 2016). *Listeria* species are widely distributed in various environments, including soil, water, vegetation, animal feed, farm environments, food processing environments, urban and suburban environments (Korsak and Szuplewska 2016). *L. monocytogenes* and *L. innocua* have been the most prevalent species of *Listeria* found in urban environments, produce production, preharvest environments, retail environments, and processing environments (Estrada et al. 2020). Recently, high profile outbreaks of *L. monocytogenes* have been associated with deli meats, fresh produce, and ready-to-eat foods (Churchill et al. 2019). Other *Listeria* species have also been found in ready-to-eat, raw or unprocessed foods (Arslan and Özdemir 2020; Guerra et al. 2001; Pesavento et al. 2010; Soriano et al. 2001; Zeinali et al. 2017), as well as in food...
processors (Huang et al. 2007; Korsak and Szuplewska 2016) and other food products (Chen et al. 2009; Pesavento et al. 2010). Given that L. monocytogenes is widespread in the environment, food production facilities constantly monitor and control for the presence of Listeria species on surfaces. Listeria species are considered a broad indicator of the conditions potentially favorable for L. monocytogenes growth and survival in the environment (Brouillette et al. 2014; The United Fresh Food Safety & Technology Council 2018; Zoellner et al. 2018). Using a broad indicator group, such as screening for all Listeria species (genus level), increases the chances of finding L. monocytogenes niches and reacting in an effective manner to mitigate the prevalence of this pathogen in a food production facility (Brouillette et al. 2014; The United Fresh Food Safety & Technology Council 2018; Zoellner et al. 2018).

Given that conventional culture-based assays are labor intensive and time consuming, several methodologies have been developed for the identification of foodborne pathogens, which can be further classified into nucleic acid-based, biosensor-based and immunological-based methods (Chen et al. 2017; Law et al. 2015). In particular, nucleic acid-based methods, employing real-time quantitative PCR (qPCR), have become preferred methods for the detection and quantification of Listeria species due to their simplicity, high sensitivity and specificity, and low risk of contamination due to the lack of post-processing steps for obtaining the detectable signal when compared to conventional PCR (Chen et al. 2017; Gasanov et al. 2005). Recently, several multiplex qPCR assays have become available for the identification of multiple Listeria species by targeting genes required for virulence and regulatory functions including those coding for bacteriolytic properties (iap), phosphatidylinositol phospholipase C (plcB), sucrose-specific enzyme (scrA) as well as putative internalin and oxidoreductase, and N-acetyl muramidase proteins (Chen et al. 2017; Hage et al. 2014; Hein et al. 2001; Hitchins et al. 2017). Other qPCR assays have been developed by targeting the 23S ribosomal DNA or non-coding RNA specific to the Listeria genus; however, these qPCR assays require multiple sets of primers and probes to be multiplexed to enable the detection of various Listeria species (Chen et al. 2017; Petrauskene et al. 2017; Rodríguez-Lázaro et al. 2004).

In the past years, there has been a significant increase in the availability of bacterial genome sequences in public databases (Land et al. 2015; Sayers et al. 2020). These recent developments would consequently enable the improvement of the new design of singleplex qPCR assays for detecting multiple strains of Listeria species by allowing better identification of specific and accessible genomic regions to be inclusive in detecting the targeted species while still being exclusive in discriminating against non-Listeria strains. Given that ribosomal RNA (rRNA) is present in large quantities and multiple copies in bacteria, in particular in the Listeria genome (Glasner et al. 2001; Milner et al. 2001), new approaches on the development of molecular assays for detecting foodborne pathogens have chosen RNA as the targeted analyte (Lively et al. 2013), which offers a reliable and highly sensitive detection of Listeria species. In the present study, a comparative genomic analysis was conducted for evaluating high copy-number sequences to enable the targeted and specific detection of Listeria species. By conducting an in silico analysis with a dynamic programming algorithm, simulated folding was performed to assess the accessibility of multicopy targeted regions in the rRNA for optimal detection. This nucleic acid-based assay, a singleplex assay, was further validated by conducting inclusivity tests for Listeria species and exclusivity tests for non-targeted environmental bacterial strains, belonging to the Bacillus, Citrobacter, Enterobacter, Pseudomonas, Salmonella and Shigella genera. As a proof-of-concept for the development of an in-process detection system for foodborne pathogens in food processing facilities, this nucleic acid-based assay was tested in conjunction with a flow-through system, consisting of aptamer-capture step, followed by sample concentration and mechanical lysis for the detection of Listeria species at low cell concentrations.

Materials and methods

Bacterial strains and culture conditions

The Listeria strains used in the present study (Table 1) were streaked for isolation on BBL™ Trypticase™ Soy Agar (Becton, Dickinson and Co., Franklin Lakes, NJ) with 0.6% Bacto™ Yeast Extract (Becton, Dickinson and Co.) (Hitchins et al. 2017). Listeria species liquid cultures were grown in BD™ Tryptic Soy Broth (Becton, Dickinson and Co.), and liquid cultures were grown in LB broth overnight with constant shaking (225 rpm) at 37 °C. Non-targeted bacterial strains (Table 1) were streaked for isolation on Luria–Bertani agar (LB; Becton, Dickinson, and Co.), and liquid cultures were grown in LB broth overnight with constant shaking (225 rpm) incubated at 37 °C for strains belonging to the genera Citrobacter, Escherichia, Salmonella, and Shigella and incubated at 28 °C for strains belonging the genera Bacillus, Enterobacter, and Pseudomonas.

Comparative genomics and in silico analysis for oligonucleotide design

To design oligonucleotides targeting the rRNA operon (rrn operon), 59 ribosome sequences of 19 Listeria species including the common Listeria species: L. innocua, L. ivanovii, L. grayi, L. monocytogenes, and L. welshimeri.
Table 1  Bacterial reference strains used in this study

| Species                          | Strain   | Other designation\(^a\) | Source              | Location     | Reference or provider\(^b\) |
|----------------------------------|----------|--------------------------|---------------------|--------------|-----------------------------|
| *Bacillus cereus*                | RM3190   | N/A                      | Soil                | USA          | (Friedman et al. 2006)      |
|                                  | RM5141   | 6A1                      | Soil                | USA          | *Bacillus Genetic Stock Center* |
|                                  | RM5142   | 6A2                      | Soil                | USA          | *Bacillus Genetic Stock Center* |
|                                  | RM5143   | 6A3                      | Soil                | USA          | *Bacillus Genetic Stock Center* |
|                                  | RM20582  | ATCC 14,579              | Soil                | USA          | (Ivanova et al. 2003)       |
| *Bacillus maroccanus*            | RM3191   | N/A                      | Soil                | USA          | Laboratory Collection       |
| *Bacillus subtilis* subspecies *spizizenii* | RM19627  | ATCC 6633                | Soil                | USA          | (Waleh and Ingraham 1976)   |
| *Citrobacter braakii*            | RM7359   | ATCC 43,162              | Human               | USA          | (Shanks et al. 2012)        |
| *Citrobacter freundii*           | RM4680   | N/A                      | Lettuce             | USA          | Laboratory Collection       |
| *Citrobacter youngae*            | RM3642   | N/A                      | Moss                | USA          | (Bettelheim et al. 1993)    |
| *Escherichia coli*               | RM5034   | ATCC 29,425              | N/A                 | Basel        | (Quiñones et al. 2011)      |
| Enteropathogenic *Escherichia coli* O55:H7 | RM13616  | 99,107 (90)              | Human               | Brazil       | (Kyle et al. 2012)          |
| *Enterobacter asburiae*          | RM3638   | N/A                      | Soil                | USA          | (Cooley et al. 2003)        |
| *Enterobacter cloacae*           | RM9194   | P-1242                   | Spinach             | USA          | (Cooley et al. 2007)        |
|                                  | RM9195   | P-1248                   | Lettuce             | USA          | (Cooley et al. 2007)        |
| *Listeria grayi*                 | RM2207   | ATCC 19,120              | Rodent feces        | USA          | (Rocourt et al. 1992)       |
|                                  | RM2208   | ATCC 25,401              | Corn                | USA          | (Rocourt et al. 1992)       |
|                                  | RM3344   | BA54                     | Ice cream           | USA          | Laboratory Collection       |
| *Listeria innocua*               | RM2209   | ATCC 33,090              | Cattle              | USA          | (Rosimin et al. 2016)       |
|                                  | RM2438   | ATCC 51,742              | Cabbage             | USA          | (Niemira et al. 2003)       |
|                                  | RM2442   | CDHS# 90E01283-L-11      | Shellfish           | USA          | Laboratory Collection       |
|                                  | RM3320   | SEA 15b60                | Lettuce             | USA          | Laboratory Collection       |
| *Listeria ivanovii*              | RM2206   | ATCC 19,119              | Sheep               | Bulgaria     | (Longhi et al. 2014)        |
|                                  | RM3096   | ATCC 700,402             | N/A                 | USA          | (Charlemroj et al. 2019)    |
|                                  | RM3325   | SEA 15a96                | Cheese              | USA          | Laboratory Collection       |
| *Listeria monocytogenes*         | RM2199   | F2379                    | Human               | USA          | (Gorski et al. 2006)        |
|                                  | RM3022   | ATCC 19,116              | Chicken             | England      | (Wei et al. 2019)           |
|                                  | RM3106   | N/A                      | Processing plant    | USA          | (Gorski et al. 2006)        |
|                                  | RM3871   | Isolate# 33,123          | Floor drain         | USA          | (Liu et al. 2018)           |
|                                  | RM17178  | FN-290–1                 | Water               | USA          | (Cooley et al. 2014)        |
| *Listeria seeligeri*             | RM2211   | ATCC 35,967              | Soil                | Germany      | (Ryu et al. 2013)           |
|                                  | RM3321   | SEA 3126                 | Cheese              | USA          | Laboratory Collection       |
|                                  | RM3322   | SEA 2232                 | Fish                | USA          | Laboratory Collection       |
| *Listeria welshimeri*            | RM2210   | ATCC 35,897              | Decaying plant material | USA       | (Ryu et al. 2013)           |
|                                  | RM3323   | SEA 15b05                | Cheese              | USA          | Laboratory Collection       |
|                                  | RM3324   | SEA 3639                 | Fish roe            | USA          | Laboratory Collection       |
| *Pseudomonas chlororaphis*       | RM2107   | FC4                      | Cilantro            | USA          | (Brandl and Mandrell 2002)  |
| *Pseudomonas syringae*           | RM1952   | B728a                    | Bean                | USA          | (Feil et al. 2005)          |
|                                  | RM14153  | N/A                      | Lettuce             | USA          | Laboratory Collection       |
| *Salmonella enterica* serovar Kentucky | RM7890 | MH67589                 | Ground chicken      | USA          | (Fagerquist and Zaragoza 2018) |
| *Shiga toxin-producing* *Escherichia coli* O104:H4 | RM14735 | 11EN0819                | Human               | Germany      | (Cooley et al. 2013)        |
| *Shiga toxin-producing* *Escherichia coli* O157:H7 | RM2084 | EDL-933; DEC 4f          | Meat                | USA          | (Quiñones et al. 2012)       |
| *Shigella boydii*                | RM7079   | 6326/9                   | N/A                 | Germany      | Laboratory Collection       |
| *Shigella flexneri*              | RM7078   | SEA 13,618               | Human               | USA          | Laboratory Collection       |
| *Shigella sonnei*                | RM7077   | SEA 136,012              | Human               | USA          | Laboratory Collection       |

\(^{a}\) N/A, Not available

\(^{b}\) Laboratory Collection at U.S. Department of Agriculture, Agricultural Research Services, Western Regional Research Center, Produce Safety & Microbiology Unit, 800 Buchanan Street, Albany, CA 94,710 USA. The *Bacillus* Genetic Stock Center, Biological Sciences 556, 484 West 12th Avenue, Columbus, OH 43,210 USA
L. monocytogenes strains with all known serovars were included for the design (Doumith et al. 2004). The ribosome sequence of L. monocytogenes strain EGD-e (GenBank Accession No. CP023861) was chosen as a model ribosome sequence (Glaser et al. 2001). Non-targeted bacterial sequences were selected by searching the top non-Listeria matches with the model ribosome sequence. Additional bacterial strains belonging to a diverse genera such as Citrobacter, Enterobacter, and Pseudomonas, commonly found as resident bacteria in agricultural and food processing environments (Dees et al. 2015; Møretrø and Langsrud 2017; Orsi and Wiedmann 2016; Williams and Marco 2014; Williams et al. 2013), were also added to the non-targeted list of genomes examined. Simulated folding of the model sequence was performed to find regions of high RNA accessibility with unfolding energy < 20 kcal/mol using Visual-OMP™ software package (DNA Software, Inc., Ann Arbor, MI). The target sequences and non-target sequences were aligned using Geneious Software (Biomatters, Ltd., Auckland, New Zealand). Regions of high RNA accessibility in the model ribosome sequence with the most heterogeneity with an average of less than 80% match with the reference sequence were selected as target regions for the primer design using RealTimeDesign™ qPCR Assay Design Software (LGC, Biosearch Technologies, Petaluma, CA). The use of the RealTimeDesign™ software allowed the design with the proprietary BHQplus® probes incorporating a duplex-stabilizing technology (LGC, Biosearch Technologies) to elevate the melting temperature and enhance target specificity (Kutyavin 2008). The default of least restrictive parameters for RealTimeDesign™ software were used in the design (Sowers et al. 2005, 2006); however, for some regions, the default parameters were modified to design oligonucleotides with increased specificity to the model target. In particular, the minimum primer temperature was lowered from 65 °C to 60 °C, and the maximum probe temperature was increased from 72 °C to 77 °C. Additionally, the maximum internal stability was increased to 0.6 from 0.0. In some instances, runs of four guanidine bases were allowed to improve specificity. Three guanidine and/or cytosine bases were allowed at the 3’ end of the oligonucleotide, and the maximum percentage of the guanidine bases in the oligonucleotide probes was increased to 40%.

Nucleic acid amplifications

Using a unidirectional workflow previously employed for experimental procedures with foodborne enteric viruses (Quintones et al. 2017), the amplification by reverse transcription-quantitative PCR (RT-qPCR) was performed in a 20 µl reaction mixture containing 5 µl of 4 × TaqPath™ 1-Step Multiplex Master Mix (Applied Biosystems, Foster City, CA), 0.5 µM of each forward and reverse BH1 primer, 0.1 µM of BH1 probe (LGC, Biosearch Technologies) and 1 µl purified RNA or 5 µl crude lysate. The reaction mixtures were placed in a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems) with the following parameters: RT step at 54 °C for 15 min, hot start at 95 °C for 2 min followed by 40 cycles each of denaturation at 95 °C for 3 s and annealing and extension at 64 °C for 30 s. Cycle threshold (Ct) values were analyzed using the QuantStudio™ Design & Analysis software (Applied Biosystems). The performance of the BH1 oligonucleotide-based assay was compared with a DNA-based assay, MicroSEQ™ Listeria monocytogenes Detection kit (Applied Biosystems) with tenfold serial dilutions of L. monocytogenes cell lysate. The estimated cell concentrations in the suspension were determined by optical density and confirmed by viable bacterial colony count on solid media.

Design of a continuous flow-through detection system

For the design and validation of the continuous flow-through detection system, L. grayi strain RM2208 was used as the model organism (Table 1). In detail, samples were blended with capture buffer (0.2 × PBS, 2 mM magnesium chloride, and 0.2% Triton X-100) and filtered into a sample container using a Whirl–Pak® sterile sampling bag with 330 µm filter membrane (B01547; Nasco, Fort Atkinson, WI) to remove...
particulates. A biocompatible Masterflex™ peristaltic pump (Masterflex™ Model 7550–60; Cole-Parmer Instrument Co., Vernon Hills, IL) introduced the liquid sample into a Tenn Jr. temperature-controlled chamber (Model TLJR; Tenney Environmental, Parsippany, NJ) (Medin et al. 2020). Fluideic valves (Model SMC LVM205R-6B1; SMC Pneumatics, Yorba Linda, CA) moved homogenized sample through a depth filter, consisting of glass beads (100 µm diameter; BioSpec Products, Inc., Bartlesville, OK) having non-fouling surface properties (Medin et al. 2020). The Listeria cells were then captured in the column using aptamer-functionalyzed soda-lime glass beads (100 µm diameter; BioSpec Products, Inc.) with silane surface modification for attachment of the oligonucleotide sequences, serving as a spacer sequence (5′-GGTTTTTGTGGAAAGTTGTTTTTTTTTTTTTT-3′) for aptamer extension and a tether sequence (5′-CAA CTTTCAAAACAAAACCTTTTTTTTTT-Amino C6-3′) for aptamer surface attachment (Medin et al. 2020; Quiñones et al. 2020). The release of the sequestered Listeria cells from the column using release buffer (1 M NaCl, 100 mM EDTA, 0.1% Tween 20, 0.05% SDS, 30 mM NaHCO3, pH 10.3–10.4) (Medin et al. 2020). After potential inhibitors were removed as waste, the released Listeria cells were mechanically lysed using an ultrasonic transducer (Black & Decker, Baltimore, MD) and yttrium-stabilized zirconia beads (100 µm diameter; Pingxiang Chemshun Ceramics Co. Ltd., Pingxiang City, Jiangxi Province, China). An aliquot of the lysate was subjected to further amplification by RT-qPCR by using the BH1 oligonucleotide assay. To determine the capture efficiency of the aptamer-functionalyzed column, the capture column was conditioned by adding approximately 30 ml of capture buffer through the column at 15 ml/min using a Masterflex™ peristaltic pump (Cole-Parmer Instrument Co.), and the conditioning flow-through was discarded. A 10 ml Listeria cell suspension, diluted to a final concentration of approximately 1.6 × 10⁵ CFU/ml, was added to the capture column. The entire flow-through was collected, and the L. greyi cell concentration was enumerated. The capture efficiency to the aptamer column was determined as the fraction of Listeria cells from the starting cell suspension added to the capture column that were not detected in the flow-through cell count, as previously described (Medin et al. 2020).

**Statistical analysis**

Statistical significance in the detected Ct values was determined by performing two-tailed Fisher’s exact test using R statistical software (version 3.0.1; R Foundation for Statistical Computing, Vienna, Austria) (Fisher 1935; Mehta and Patel 1986). Descriptive statistical analyses and one-way analysis of variance (ANOVA) were performed using the Analysis ToolPak in Microsoft® Excel® version 2002 for Office 365 ProPlus (Microsoft Corporation, Redmond, WA). P-values of ≤ 0.005 were considered statistically significant.

**Results and discussion**

**Design strategy of the oligonucleotide-based assay for detecting Listeria species**

As a suitable targeted region, rRNA has been considered for the development of detection assays as it is found to be represented in the bacterial genome in multiple copies (Livezey et al. 2013; Milner et al. 2001). In particular, whole-genome analysis revealed that a single L. monocytogenes cell contains 6 copies of the rRNA (rrn) operon (Glaser et al. 2001), and expression studies estimated that approximately 600–25,000 copies of the ribosomes were detected per cell (Milner et al. 2001). These findings have indicated that targeting rRNA is more than enough for a reliable amplification of nucleic acids to enable detection of the targeted pathogen at low cell concentrations. To initiate the design of oligonucleotides, approximately 200 genomic entries of distinct species of Listeria and non-targeted bacterial species were aligned and compared, and regions in the ribosome sequences with the most heterogeneity between the Listeria sequences and non-target sequences were selected for the probe design (Fig. 1). The distinct species of Listeria for the comparative genomic analysis included Listeria aquatica, Listeria booriniae, Listeria cornellensis, Listeria costaricensis, Listeria goaensis, Listeria fleischmannii, Listeria floridensis, Listeria grandensis, L. grayi, L. innocua, L. ivanovii, Listeria marthii, L. monocytogenes, Listeria newyorkensis, Listeria riparia, Listeria rocourtiae, L. seeligeri, Listeria thailandensis, Listeria welinstephanensis, and L. welshimeri.

The in silico analyses were performed with algorithms using the nearest-neighbor model coupled with multi-state equilibrium model for conducting simulated folding to assess RNA accessibility in the identified genomic regions targeted for detection (Fig. 1a). The results revealed multiple locations in the genome with an optimal target complexity as well as target unfolding. As shown in Fig. 1a, analysis of the 16S rRNA in L. monocytogenes revealed that the targeted region for the oligonucleotide design had an unfolding delta G calculation to be approximately 14 K to 16 K cal/mol when compared to most sites in the examined region with much higher values of 26–28 K cal/mol. To reduce any false positives due to low levels of specificity in the oligonucleotide design, the complexity of the targeted regions was also further examined (Fig. 1a). The analysis of optimal genome locations was further assessed to select the best sites for designing the targeted oligonucleotides (Fig. 1a, circles), and these selected sites for the design of the oligonucleotides were found to have optimal complexity scores.
between 0.8 and 1, indicating relative accessibility and complexity of regions within the target sequences (Nielsen et al. 2003). Additionally, the in silico mismatch was examined by comparing a collection of various *Listeria* species genome sequences with other bacterial non-targets, commonly present from soil, water, and plant surfaces (Fig. 1b). The sequence alignment to non-targeted bacterial species, included *Bacillus* species, *Escherichia* species, *Pseudomonas* species, and *Citrobacter* species, representing non-targeted bacterial species from agricultural environments (Dees et al. 2015; Orsi and Wiedmann 2016; Williams and Marco 2014; Williams et al. 2013).

To increase the level of desired target specificity at the genus level, the oligonucleotide design targeted the *rrn* operon comprising of the 16S and 23S rRNA gene clusters in the *Listeria* genome, a region commonly used as a microbial molecular marker for phylogenetic classification of bacterial species (Woese 1987). The design employed base analogs to promote nucleic acid duplex stabilizing, enabling an elevated melting temperature of the oligonucleotides (Kutyavin 2008). Additional standard criteria were also employed for developing the nucleic acid amplification assays, which consisted of examining for the G–C content, annealing temperatures, and self-hybridization (Sowers et al. 2005, 2006). The in silico analysis resulted in a total of six oligonucleotide sets with a G–C content ranging from 36.8% to 57.9% and annealing temperatures ranging from 55.4 °C to 76.6 °C (Table 2).

**Assay specificity for target RNA detection in *Listeria* species**

As a preliminary screening test, the various candidates of designed oligonucleotides sets were subjected to an initial inclusivity test using *L. monocytogenes* as a representative targeted species, and an exclusivity test was conducted using *B. cereus* as a representative non-targeted species, which was based on previous whole-genome comparisons suggesting that high conservation in the genome organization is specific for *Bacillus* and *Listeria* (Buchrieser et al. 2003). Amplifications for detecting *L. monocytogenes* with 10 fg of RNA as template, corresponding to 10 cell equivalents (Milner et al. 2001), resulted in Ct values, ranging from 28.5 to 33.7 for all of the oligonucleotide sets (Table 2). However, only the BH1 oligonucleotide set was found to show no cross reactivity for detecting *B. cereus* in the exclusivity test even at template amounts that were 100-fold when compared to the targeted *Listeria* species. The other candidate oligonucleotide sets showed a positive reaction resulting in Ct values ranging from 16.4 to 32.5 (Table 2). Based on these initial
observations determining the specificity of the amplification reaction, the subsequent validation tests employed the BH1 oligonucleotide set.

The performance of the BH1 oligonucleotide-based assay, targeting multicopy sequences in the rRNA, was further compared to a DNA-based assay, which targets a single copy of an essential gene (rnpB) in *Listeria* species (Mandin et al. 2007; Petrauskene et al. 2017; Yusuf et al. 2010) (Fig. 2). In particular, cell lysates of *L. monocytogenes* outbreak strain RM2199 were prepared at various tenfold dilutions, ranging from 5000 to 5 cell equivalents (Fig. 2). Aliquots of the cell lysate were subjected to amplification by performing RT-qPCR using the BH1 oligonucleotide-based assay or just PCR using the DNA-based assay targeting rnpB. As shown in Fig. 2, the RNA-based assay with the BH1 probe set resulted in Ct values of 22.7 and 26.9 at the 5000 and 500 cell equivalents, respectively. By contrast, the DNA-based assay resulted in Ct values of 29.1 and 32.1 at the 5000 and 500 cell equivalents, respectively. Interestingly, the BH1 oligonucleotide-based assay still resulted in Ct values of 29.1 and 31.6 at the 50 and 5 cell equivalents, respectively; however, at these lower amounts of *Listeria* cell equivalents, the DNA-based assay yielded no detectable or Ct values > 35, considered to be a threshold detection limit for gene amplification (McCall et al. 2014). When compared to the DNA-based assay tested, the RNA-based assay, using the BH1 oligonucleotide set, reliably detected cell amounts lower than 5 cells, and these detection sensitivities were statistically

| Oligonucleotide name | Sequence (5' → 3') | rRNA target | Ampli- | Annealing temperature (C°) | GC% | Ct values\(^c\) |
|---------------------|--------------------|-------------|--------|------------------------|-----|----------------|
|                     |                    |             | con size (bp) |                        |     | 10 fg *Listeria* | 1 ng *Bacillus* |
| L1R1-Fwd            | GTTACGATTGTTGCAAGGTTAAG CGGAAA | 23S       | 126      | 65.2                   | 40.0% | 40.0%          |
| L1R1-Rev            | ACCCTGTACTCGACATTTG | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| L1R1-Probe          | FAM-GCGGAGCGTGAGGAAGC-G-Q1 | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| BH1-Fwd             | CCGGATACCTGTACTCGACATTTG | 23S       | 126      | 65.2                   | 40.0% | 40.0%          |
| BH1-Rev             | GGGGGATACCTGTACTCGACATTTG | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| BH1-Probe           | FAM-CACACTCGTCAGAGATT | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| BH2-Fwd             | CTATACGATACCTGTACTCGACATTTG | 23S       | 126      | 65.2                   | 40.0% | 40.0%          |
| BH2-Rev             | GGGGGATACCTGTACTCGACATTTG | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| BH2-Probe           | FAM-CACACTCGTCAGAGATT | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| BH3-Fwd             | TGGGATACCTGTACTCGACATTTG | 23S       | 126      | 65.2                   | 40.0% | 40.0%          |
| BH3-Rev             | GGGGGATACCTGTACTCGACATTTG | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| BH3-Probe           | FAM-CACACTCGTCAGAGATT | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| BH4-Fwd             | AGGTGATACCTGTACTCGACATTTG | 23S       | 126      | 65.2                   | 40.0% | 40.0%          |
| BH4-Rev             | GGGGGATACCTGTACTCGACATTTG | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| BH4-Probe           | FAM-CACACTCGTCAGAGATT | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| BH5-Fwd             | GATAAGGATACCTGTACTCGACATTTG | 23S       | 126      | 65.2                   | 40.0% | 40.0%          |
| BH5-Rev             | GGGGGATACCTGTACTCGACATTTG | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
| BH5-Probe           | FAM-CACACTCGTCAGAGATT | 16S       | 93       | 65.3                   | 57.9% | 30.9           |
significant (Fisher’s Exact Test, \( P \)-value = 0.002). These findings revealed that targeting multicopy RNA sequences significantly improved the detection capabilities of the assay for \( L. \) monocytogenes, and the results showed that the BH1 oligonucleotide-based assay was found to be at least 100 times more sensitive than the DNA-based assay targeting a single copy gene.

To further validate the detection capabilities of the assay, the specificity of the BH1 oligonucleotide set was further expanded by testing for \( Listeria \) species other than \( L. \) monocytogenes. Other additional tested species were \( L. \) innocua, \( L. \) ivanovii, \( L. \) welshimeri, and \( L. \) seeligeri, which have been potentially implicated in causing illness to immunocompromised individuals (Korsak and Szuplewska 2016) and have been recovered from ready-to-eat foods or raw/unprocessed foods (Arslan and Özdemir 2020; Guerra et al. 2001; Pesavento et al. 2010; Soriano et al. 2001; Zeinali et al. 2017) as well as in food processing facilities (Huang et al. 2007; Korsak and Szuplewska 2016), and food products (Chen et al. 2009; Pesavento et al. 2010). The inclusivity test was conducted using RNA (100 pg) from \( Listeria \) species, corresponding to an estimated 100–150 cell equivalents of RNA per amplification reaction (Glaser et al. 2001; Guerra et al. 2001; Milner et al. 2001) (Fig. 3). These experiments also examined for cross hybridization of the BH1 oligonucleotide set with excess of non-target RNA (20 ng), which is equivalent to approximately 2 million non-target cells and 4 billion copies of non-target RNA sequences, using an estimated million cell equivalent of RNA per test (Milner et al. 2001) from environmental bacterial species from soil, water, and plant surfaces (Dees et al. 2015; Williams and Marco 2014; Williams et al. 2013) or other foodborne pathogens (Fig. 3). The results indicated a high level of specificity for detecting various \( Listeria \) species with Ct values ranging from 19.9 to 25.0, showing a statistically significant difference (Fisher’s Exact Test, \( P \)-value = 0.0006) when compared to the lack of detected signals for non-targeted environmental and pathogenic bacterial strains.

Based on recent evidence indicating that the residential bacterial communities are able to persist over time on food production surfaces (Møretø and Langsrud 2017), additional experiments examined the effect of various amounts of non-target RNA on the efficiency of the amplification of the \( Listeria \) sequences. Purified target RNA from \( L. \) monocytogenes was co-incubated in the presence of various amounts of excess non-target RNA from \( B. \) cereus, a persistent species in food industry environments (Møretø and Langsrud 2017) and displaying a sequence similarity greater than 90% in the 16S ribosome region (Sallen et al. 1996). As shown in Fig. 4, the results indicated that no significant differences in the Ct values were observed with values ranging from 27.8 to 28.3, corresponding to the signal detected for \( L. \) monocytogenes strain RM2199 in the presence of excess non-target RNA from \( B. \) cereus strain ATCC 14,579. To determine the effect of the various tested conditions on the amplification efficiency of the target, the slope of the curve at the pre-inflection point was examined (Guescini et al. 2013). Analysis of the amplification curve resulted in no significant change in the slope of the curve under the various conditions tested, and a one-way ANOVA.
test indicated that the differences between the tested conditions, varying the amounts of non-target template, were not statistically significant ($df = 4$, $F = 3.266$, $F$ critical = 5.192). These observations demonstrated that the efficiency of the amplification specific for *Listeria* was not adversely affected by addition of the non-target template.

**Design and validation of a continuous flow-through system for *Listeria* species detection**

To incorporate the use of the BH1 oligonucleotide-based assay with an improved detection platform, a flow-through system was designed for the real-time surveillance of *Listeria* species in food processing facilities (Medin et al. 2020). By avoiding the need for sample preparation processes utilizing multistage centrifugation to concentrate the targeted pathogen, the present study replaced the input centrifugation with a flow-through system that included a depth filter to remove large particulate matter while permitting target pathogen to pass through a DNA aptamer-functionalized capture column that specifically bound *Listeria* cells (Fig. 5). In detail, the filtered sample was introduced into a temperature-controlled chamber (10 °C–12 °C), and fluidic valves moved homogenized sample to a depth filter, which removed large particulate matter present in the collected sample, while still permitting target pathogen to pass through to a microbead capture column that specifically bound *Listeria* cells (Fig. 5). The specific binding in the capture column was
achieved by the use of DNA aptamers (Suh et al. 2014; Suh and Jaykus 2013), which are oligonucleotide molecules that bind to specific epitopes that are presented on the bacterial cell surface and have been proposed for pathogen capture as a low-cost alternative to antibodies (Teng et al. 2016). The use of DNA aptamers enabled Listeria cell capturing without the need for small particle filtering, which can be subject to membrane fouling when isolating bacterial pathogens from food and environmental samples (Ferrari et al. 2019; Kearns et al. 2019; Li et al. 2013; Zhang et al. 2018). To improve the capturing of the Listeria cells with the DNA aptamer (Suh et al. 2014; Suh and Jaykus 2013), additional sequences, a spacer sequence for aptamer extension and a tether sequence for aptamer surface attachment (see Material and Methods)(Quiñones et al. 2020), were designed and added to the aptamer to enable binding to the capture column and prevent the aggregation of the aptamer for efficient release of the target bacterial cell from the aptamer (Medin et al. 2020; Quiñones et al. 2020). A fluidic valve was used to bypass the depth filter, and potential inhibitors were removed as waste. The captured Listeria cells were released from the aptamer-functionalized column, and the liquid sample volume with the Listeria cells was reduced and further subjected to mechanical lysis and amplification with the BH1 oligonucleotide-based assay (Fig. 5). In summary, the designed flow-through system included a sample preparation process with DNA aptamers to specifically capture the targeted Listeria cells away from the contaminating matrix and other non-targeted organisms and inhibitors prior to nucleic acid amplification and analysis.

As a proof-of-concept, the ability of the flow-through system for efficiently capturing and detecting Listeria cells was further examined. To improve the capture binding efficiency of the Listeria cells, the capture time in the flow-through system was optimized by considering the function of the cross-sectional area of the flow channel and the surface area of the capture surface (Medin et al. 2020). In addition, the aptamer-capture column employed a functionalized surface chemistry based on soda-lime glass surfaces (Medin et al. 2020), one of the least expensive and most-commonly used glass types for many applications. By flowing cell suspensions of L. grayi, a non-pathogenic Listeria species found in raw and ready-to-eat foods (Orsi and Wiedmann 2016; Soriano et al. 2001), through the capture column, the results indicated a 78% capture efficiency of the targeted L. grayi strain RM2208. Given that the tested DNA aptamer may display some affinity for closely related species (Suh et al. 2014; Suh and Jaykus 2013; Teng et al. 2016), Listeria cells captured via aptamer were differentiated using the BH1 oligonucleotide set. To further validate the detection sensitivities of the

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**Fig. 5** Schematic diagram of the sample processing steps in the continuous flow-through system. Sample was blended with capture buffer, filtered to remove large particulates, and introduced into a temperature-controlled chamber using a peristaltic pump. Fluidic valves moved homogenized sample to a depth filter, and Listeria cells in the sample were captured with an aptamer-functionalized column. Potential inhibitors were removed as waste, and the cells were subjected to mechanical lysis. The lysed cells were collected and subjected to further amplification by RT-qPCR.
flow-through system, *L. grayi* strain RM2208 cell suspensions, consisting of various amounts at or below the infectious dose, based on an estimated infectious dose of *Listeria* at about 10,000 cells (Pouillot et al. 2016), were determined by traditional plate enumeration on selective solid media. After elution from the capture and ultrasonic mechanical lysis, the results revealed Ct values of 12.3, 16.3, 20.9, and 24.9 for *L. grayi* cell amounts that were 100 times above the infectious dose, 10 times above the infectious dose, at the infectious dose, and 15 times below the infectious dose, respectively (Fig. 6). Further analysis of the Ct values using the tested serial dilutions of the *Listeria* cell suspensions were used to calculate the efficiency of the amplification (Kubista et al. 2006), and the analysis revealed that the efficiency of the amplification for detecting *Listeria* with the flow-through system in conjunction with the BH1 oligonucleotide set had an estimated amplification efficiency of 0.9235 (R = 0.99955; slope -3.52). These findings indicated that this integrated flow-through system has the capabilities to accurately and sensitively detect *Listeria* species.

Conclusions

In conclusion, the present study developed a singleplex RT-qPCR assay using the BH1 oligonucleotide set for the accurate detection of multiple *Listeria* species. Previously published assays for *Listeria* species detection employed qPCR and were developed by targeting *iap, plcB,* and *scrA* genes, required for virulence and regulatory functions, as well as putative internalin and oxidoreductase, and N-acetylmuramidase proteins (Chen et al. 2017; Hage et al. 2014; Hein et al. 2001; Hitchins et al. 2017). Other qPCR assays have been documented, which target the 23S ribosomal DNA or non-coding RNA specific to the *Listeria* genus (Chen et al. 2017; Petruuskene et al. 2017; Rodríguez-Lázaro et al. 2004). The disadvantage of these published assays is the requirement of multiple set of primers and probes to be multiplexed per amplification reaction to enable the detection of various *Listeria* species.

As an alternative to DNA-based methods by employing qPCR, the present study developed a RT-qPCR assay that detected rRNA in *Listeria* species. The advantage of using rRNA as the target in *Listeria* species identification is that the rRNA genes are present in this pathogen at all stages of growth, accumulate mutations at a slow constant rate, and contain sequences that enable discrimination of this targeted pathogen at the genus level (Espejo and Plaza 2018; Milner et al. 2001). Additionally, analysis of the *Listeria* genome revealed large amounts of rRNA (600–25,000 copies per cell) (Milner et al. 2001), which makes rRNA a better target for a sensitive detection at low cell concentrations when compared with one copy of genomic DNA for a targeted gene. The explosive use of genome sequencing has resulted in an increase in the growth of data (Land et al. 2015; Sayers et al. 2020), which has consequently improved the in silico sequence analyses for the identification of rRNA sequences that are highly specific for the targeted pathogen. In the

![Fig. 6](image-url) Detection sensitivity of the oligonucleotide-based assay with the continuous flow-through system. The Y-axis shows the relative fluorescence (ΔRn), the X-axis shows the number of amplification cycles. Representative data are shown for samples containing *L. grayi* strain RM2208 at equivalent amounts of 100 times above the *Listeria* infectious dose (circles), at 10 times above the *Listeria* infectious dose (diamonds), at the *Listeria* infectious dose (triangles), at 15 times below the *Listeria* infectious dose (squares). The negative control is indicated by the gray line. The detection threshold is indicated by the dashed line.
present study, the designed singleplex RT-qPCR assay using the BH1 oligonucleotide assay, targeting 23S rRNA, resulted in the reliable detection of less than 5 *Listeria* cells, and this detection was at least 100 times more sensitive than the DNA-based assay targeting a single copy gene. Furthermore, the BH1 oligonucleotide assay results showed no cross-reactivity for detecting non-*Listeria* strains in the exclusivity test even at template amounts that were 100-fold when compared to the targeted *Listeria* species.

The use of enrichment in routine surveillance testing is traditionally performed to amplify the target organism exponentially by as much as a millionfold so that detection is possible (Law et al. 2015). To improve the detection of pathogens from samples, various reports have documented on using microfiltration to reduce large samples to a small volume (Li et al. 2013). However, small particle filtering can be subject to membrane fouling when isolating bacterial pathogens from food and environmental samples, resulting in reduced levels of detection sensitivities (Kearns et al. 2019; Li et al. 2013; Zhang et al. 2018). In the present study, the flow-through system did not use membrane filtration but instead used an adaptation of depth filtering, a processing stage previously used for preventing filter clogging (Murakami 2012), followed by the use of an aptamer-functionalized column for successfully capturing and concentrating the targeted *Listeria* cells from samples. The findings from the present study indicated the flow-through system accurately detected *Listeria* cells at cell concentrations that were 15 times below the infectious dose. Due to the non-uniform distribution of microorganisms, current methods employing small sample sizes can result in undetected pathogens, and the lack of accurate detection of the tested foodborne pathogen can lead to subsequent outbreaks (Capobianco et al. 2021; Kearns et al. 2019). To continue to meet the needs of the food safety industry and regulators (Kaplan et al. 2014; Kuiper and Paoletti 2015), further research is aimed at optimizing the high binding efficiency of the flow-through system, documented in the present study, to optimize the rapid, low-cost capture of *Listeria* species using increased sample size over a range of environmental and food samples. Moreover, recent preliminary evidence demonstrated that the flow-through system accurately detected *Listeria* at low cell concentrations, ranging from 3.5 CFU/ml to 1000 CFU/ml, in samples collected at a food processing facility (Quiñones et al. 2019, 2020) and these challenging samples were characterized by containing contaminants commonly found in soils and agricultural products, including debris and humic acids, which are known inhibitors of nucleic acid amplification (Law et al. 2015; Opel et al. 2010; Schrader et al. 2012). These observations indicate that the high capture efficiency and strong binding affinity of aptamers towards the targeted *Listeria* cells enable aggressive washes to remove inhibitors and substances that negatively impact nucleic acid amplification and would thus enable the detection of *Listeria* cells in other types of samples such as complex food matrices, known to contain PCR inhibitory substances (Schrader et al. 2012). Future studies will also explore the increased sampling size of the method, described in the present study, to improve the statistical significance of the testing procedure and to enable a sampling process that is more accurate and representative of the entire agricultural field, providing an added value to the food industry.

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**Author contributions** BQ, VSDG and DLM conceived and designed the experiments. BQ, VSDG and DLM provided important advice, reagents, and analysis tools to the design of the test. BGL and DLM conducted bioinformatics analyses. JCY, VSDG and BGL performed the experiments. BQ, JCY, VSDG, BGL and DLM analyzed the data. BQ, JCY and BGL wrote the manuscript. All authors read, reviewed and approved the final version of the manuscript.

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**Availability of data and material** The material analyzed during the current study is available from the corresponding author on reasonable request.

**Declarations**

**Conflicts of interest** Authors BQ, JCY and BGL have no relevant financial or non-financial interests to declare that are relevant to the content of this article. Authors VSDG and DLM are employed by SnapDNA-BioNEMS, Inc. (Mountain View, CA, USA).

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