Research Article

A Fast, Reliable, and Sensitive Method for Detection and Quantification of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in Ready-to-Eat Fresh-Cut Products by MPN-qPCR

Pasquale Russo, Giuseppe Botticella, Vittorio Capozzi, Salvatore Massa, Giuseppe Spano, and Luciano Beneduce

*Department of Agriculture, Food and Environmental Sciences, University of Foggia, Via Napoli 25, 71122 Foggia, Italy*

Correspondence should be addressed to Luciano Beneduce; luciano.beneduce@unifg.it

Received 25 February 2014; Revised 29 April 2014; Accepted 30 April 2014; Published 15 May 2014

Academic Editor: Ola Olapade

Copyright © 2014 Pasquale Russo et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In the present work we developed a MPN quantitative real-time PCR (MPN-qPCR) method for a fast and reliable detection and quantification of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in minimally processed vegetables. In order to validate the proposed technique, the results were compared with conventional MPN followed by phenotypic and biochemical assays methods. When *L. monocytogenes* and *E. coli* O157:H7 were artificially inoculated in fresh-cut vegetables, a concentration as low as 1 CFU g\(^{-1}\) could be detected in 48 hours for both pathogens. qPCR alone allowed a limit of detection of 10\(^{-1}\) CFU g\(^{-1}\) after 2 hours of enrichment for *L. monocytogenes* and *E. coli* O157:H7. Since minimally processed ready-to-eat vegetables are characterized by very short shelf life, our method can potentially address the consistent reduction of time for microbial analysis, allowing a better management of quality control. Moreover, the occurrences of both pathogenic bacteria in mixed salad samples and fresh-cut melons were monitored in two production plants from the receipt of the raw materials to the early stages of shelf life. No sample was found to be contaminated by *L. monocytogenes*. One sample of raw mixed salad was found positive to an H7 enterohemorrhagic serotype.

1. Introduction

Ready-to-eat (RTE) fresh vegetables and fruits have become an established product in worldwide markets whose acceptance considerably increased in the last years. However, the growing popularity of these high quality fresh food products poses many safety concerns. Indeed, fresh-cut vegetables are a potential vehicle of transmission of foodborne pathogens, *Listeria monocytogenes* and *Escherichia coli* O157:H7 being among the most hazardous. Generally, infection with *L. monocytogenes* may cause mild febrile gastroenteritis, but in susceptible individuals, such as young, old, pregnant, and immune-compromised, invasive listeriosis can result in more serious diseases including meningitis, septicaemia, preterm birth, miscarriage, and rather high mortality rate [1]. *E. coli* O157:H7 is an enterohemorrhagic pathogen causing bloody diarrhoea and, in the worst cases, haemolytic uremic syndrome that can result in severe illness or even death. In the last years, outbreaks caused by the consumption of fresh vegetables contaminated with *E. coli* O157:H7 [2–6] or *L. monocytogenes* [7–10] have become increasingly recognized in developed countries. From a technological point of view, fresh-cut vegetables are considered “minimally processed food” and are hence characterized by the absence of treatment to break down the microbial load, with the exception of washing with chlorinated water and the compliance of the cold chain [11]. Fresh-cut contamination can occur at pre- and postharvest levels, with environment, irrigation water, handling of the product, and food plant uncleanness among the main critical points [12]. In particular, the ability of both foodborne pathogens to adhere to food and food-contact surfaces, thus forming or colonizing preexistent biofilm, represents a threat for the public health due to the risk of cross-contamination during food processing [13, 14]. Most recently, several food surveys throughout the world reported the detection of *L. monocytogenes* and *E. coli* O157:H7 in raw RTE vegetable salads sold at market retails [15–19]. From an industrial viewpoint, the prevalence of these events is a
serious hazard for fresh-cut producers because it involves economic losses due to the recall of the food from the market and damage to the company image, impacting its intangible capital. The current European regulation for fresh-cut vegetables stipulates the absence of *E. coli* O157:H7 in 25 g of food, while for *Listeria monocytogenes* a concentration of 100 CFU g⁻¹ is tolerated at the end of the shelf life [20]. Since minimally processed vegetables are generally characterized by short shelf life (7–15 days), rapid detection and quantification of human pathogens become a major challenge for producers as well as retail traders. Enumeration of *L. monocytogenes* and *E. coli* O157:H7 in food is generally done by the most probable number (MPN) method, which requires replicated dilution series of food in selective enrichment broth followed by plating on selective agar plates and subsequent biochemical assays for species identification [21]. Although MPN method has the advantage of enabling detection of the target pathogen even when it is present in low numbers, it is laborious and requires several days for confirmation of results. Therefore, in the last years, a considerable number of detection methods using faster molecular tools, mainly based on PCR techniques, have been proposed [16, 17, 21–23]. Currently, several genes have been suggested as targets for the molecular detection of *L. monocytogenes* [16, 24, 25] and *E. coli* O157:H7 [26]. The most recent advances in PCR methods are focused on the reduction of the limits of detection and quantification [27, 28], on the discrimination between dead and live cells [22] or the simultaneous detection of different foodborne pathogens [22, 27–29]. The performances of these methods were, in some cases, successfully compared with the official protocols or with diagnostic commercial kits [25, 28].

In the present work we integrated conventional MPN technique with qPCR, in order to preserve or explore the advantages of both methods, sensitivity of MPN, and reliability and quickness of qPCR. Moreover, we developed a qPCR enrichment-based method for a fast and reliable detection of *L. monocytogenes* and *E. coli* O157:H7 in minimally processed vegetables. In order to validate the proposed technique, the results were compared with conventional culture-dependent methods.

### 2. Materials and Methods

#### 2.1. Bacterial Strains and Growth Conditions. *Listeria monocytogenes* CECT 4031 and *Escherichia coli* O157:H7 CECT 4267 were purchased from the Spanish Type Culture Collection (CECT, Valencia, Spain). The non-*monocytogenes Listeria* spp. strains, *Listeria ivanovii* IZSP B45, and *Listeria innocua* IZSP B48 were kindly provided by Istituto Zooprofilattico Sperimentale di Puglia e Basilicata (IZPS, Foggia, Italy). Non-STEC *Escherichia coli* DSM 3423 was purchased from the German Collection of Microorganisms (DSMZ, Braunschweig, Germany). All strains were routinely cultured at 37°C in TSB broth (Oxoid, Hampshire, UK) until reaching mid exponential phase. *Lactobacillus plantarum* WCFS1 was used as additional negative control and grown at 30°C on MRS broth (Oxoid).

#### 2.2. Food Samples and Artificial Contamination. Minimally processed fresh-cut mix salads (lettuce, radicchio, and endive) were randomly purchased at local markets in Foggia (Italy) and stored at 4°C for a maximum of 24 h prior to analyses. All samples were investigated for the presence of *Listeria* spp. and *E. coli* O157:H7 as recommended by the ISO protocol 11290-1 from International Organization for Standardization [30]. All samples were negative for the presence of both pathogens and were used for subsequent artificial contamination experiments.

For artificial inoculation, *L. monocytogenes* CECT 4031 or *E. coli* O157:H7 CECT 4267 grown at middle exponential phase were added to the corresponding enrichment selective media used for the MPN assays (see below) in order to obtain a contamination level ranging from 0 to 3 Log CFU g⁻¹ of sample. For the qPCR detection without selective enrichment, salads samples were also inoculated at a level of 4 and 5 Log CFU g⁻¹.

#### 2.3. MPN Enumeration of *Listeria monocytogenes*. Salad samples (25 g) were added to 225 mL of Fraser Broth (Oxoid) supplemented with Fraser Selective Supplement SRO156E (Oxoid) and homogenized in a stomacher (BagFilter, Interscience, FR) for 2 min. Triplicate series of tubes containing decimal serial dilution from 10 to 10⁻⁵ grams of homogenate were incubated for 48 hours at 37°C in the same media. After incubation, aliquots of enrichment broth were taken from dark tubes (containing presumptive *Listeria* spp.) and streaked onto Oxford agar plates (Oxoid) and PALCAM agar (Oxoid). Plates were incubated at 37°C for 48 h and five typical colonies were picked for purification on TSA + 0.6% yeast extract plates (Oxoid). Then, plates were incubated for 24 h at 37°C and Gram-positive, catalase positive colonies were streaked on blood agar (37°C, 24 h). Hemolytic colonies were identified as *L. monocytogenes* using API Listeria strips (Biomerieux, Marcy l’Etoile, FR). The MPN value and 95% confidence intervals were determined by the number of positive tubes obtained in serial dilutions as reported by the USDA guidelines [31].

#### 2.4. MPN Enumeration of *Escherichia coli* O157:H7. Salad samples (25 g) were added to 225 mL TSB (Oxoid) supplemented with 20 mg L⁻¹ of novobiocin (Sigma, MO, US) and 1.12 g L⁻¹ of bile salts (Oxoid) as reported by Fusco et al. [32] and homogenized in a stomacher (Bag Mixer, Interscience) for 2 min. Triplicate series of tubes containing decimal serial dilution (from 10 to 10⁻⁵ grams of homogenate) were incubated for 48 h at 37°C. Turbid cultures were considered to be presumptive positive. Confirmation of presumptive *E. coli* O157:H7 was carried out by spread-plating 100 μL samples from each turbid tube onto tellurite-cefixime Sorbitol MacConkey agar plates (Becton Dickinson, Sparks, MD) supplemented with 200 μg mL⁻¹ ampicillin. Plates were incubated overnight at 37°C and colonies showing the typical morphology (colorless or neutral/gray with a smoky center and 1-2 mm in diameter) were considered positive. For further confirmation, a portion of each typical colony was picked...
2.5. DNA Extraction. Genomic DNA was extracted from dilution tubes considered for MPN enumeration by comparing two methods: the DNeasy Blood and Tissue kit (Qiagen, Milan, IT) according to the manufacturer’s instructions and the boiling method reported by de Oliveira et al. [16].

DNA was also extracted from artificially inoculated salads samples, after homogenization in the enrichment broth, after 0, 2, 4, 6, and 24 hours, respectively. Briefly, 10 mL of the homogenate were centrifuged for 10 min at 5,000 g. Supernatant was then discarded and DNA extracted from cell pellet using the DNeasy Blood and Tissue kit (Qiagen) following manufacturer’s instructions for Gram-positive or Gram-negative bacteria depending on the assayed microorganism.

DNA concentration was measured using a BioTek Eon spectrophotometer (BioTek, VT, USA) and its integrity checked by visualization on 1.2% agarose gels. Then, samples were stored at −20°C before analyses.

2.6. Real-Time PCR Conditions. L. monocytogenes specific primers and hybridization probe tagged with FAM fluorescent dye as designed by Rodriguez-Lázaro et al. [24] were used to amplify a 64-base pair fragment of the listeriolysin O gene (hlyA) (Table 1). E. coli O157:H7 was detected by using primers and probes targeting fliC H7 (encoding the flagellar antigen H7) and rfbE (coding for the antigen O157) genes [26], tugged with FAM and VIC fluorescent dye, respectively (Table 1). Primers and probes were synthesized by PRIMM Biotech (Milano, IT). qPCR assays were performed on an AB 7300 Real-Time PCR System (Life Technologies, Monza, IT). Amplification of the uidA gene was carried out by qPCR following the methods reported by Ram et al. [33]. Amplification of the stx2 gene was carried out following the method of Jinneman et al. [34]. Reaction total volumes were 20 μL; 3 μL of template DNA were added, to a real-time PCR mix containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 100 nM of each primer according to the manufacturer’s instructions. Cycling program was as follows: for uidA, initial denaturation at 95°C for 3 min followed by 45 cycles of 20 s at 95°C, 30 s at 54.5°C, and 30 s at 72°C. Cycling program for stx2 detection was as follows: 95°C for 10 min followed by 45 cycles of 10 s at 95°C and 30 s at 60°C.

2.7. Application of the qPCR Method to RTE Food Production Chain. Minimally processed fresh-cut samples representative of 2 production batches of rocket, mixed salad, and piel de sapo melons, respectively, were provided by two different production companies involved in fresh-cut vegetables and fruit companies. For each food sample, DNA was extracted by MPN-qPCR and culture-dependent methods at three different stages: raw, after processing, and at three days of shelf life at 4-5°C. All samples were used for demonstration activity only and not purchased by the companies involved.

3. Results

3.1. Optimizing Listeria monocytogenes qPCR Assay Conditions. In the present study, the qPCR method developed by Rodriguez-Lázaro et al. [24] was adapted to our purposes and employed in order to detect and quantify L. monocytogenes in artificially contaminated fresh-cut vegetables. Specificity of the assay was confirmed in all preliminary assays (data not shown), performed using 1 ng of genomic DNA from Listeria monocytogenes and non-monocytogenes strains as template.

Detection and quantification limits of the qPCR assay were investigated by using DNA extracted from overnight

| Target gene | Sequence (5'-3') | Fragment size | Reference |
|-------------|-----------------|---------------|-----------|
| hlyA        | CATGCCACCCACCGATCT | 64 bp | [24]       |
|             | ATCCGGTGTTCTTTTTCGA | |           |
| rfbE        | TTTCACTATTTTGGATGCTC | 88 bp | [26]       |
|             | CGATGAAGTCTCTGCAAGGGAT | |           |
| fliC H7     | CCACGACAGGTCTTTATGATCTGA | 96 bp | [26]       |
|             | CAACCTGATCCTTTATCGCCATTCC | |           |

Table 1: Primers and probes used in qPCR assays.
cultures of *Listeria monocytogenes* CECT 4031 strain. Serial dilutions of DNA were subjected to qPCR and a standard curve was constructed by using hlyA as target gene. Amplification profile of serial dilutions and standard curve are shown in Figure 1.

Amplification reactions were carried out with a range of DNA concentrations approximately corresponding to $1 \times 10^7$–$1 \times 10^9$ target molecules. With the target sequence being part of a single copy gene, number of target molecules was estimated as total DNA/mass of a single copy gene, number of target molecules was genome mass $\approx 2.94 \times 10^{-15}$ g. The standard curve showed a linear relationship, spanning 7 logs, between log input DNA and threshold cycle. The slope of the curve was $-3.27$, close to the theoretical optimum ($-3.32$), and corresponding to 102% amplification efficiency, calculated by the formula \( \text{Efficiency} = 10^{-\frac{1}{\text{slope}} - 1} \). Square regression coefficient was $R^2 = 0.999$ indicating that the qPCR assay is highly linear in the considered range. There was no overlapping of confidence intervals based on standard deviation of $C_T$ values down to $1 \times 10^5$ target molecules indicating that reliable quantification is possible to this limit. The LOD was found to be lower, corresponding to 5 target molecules (mean $C_T$ value: 37.8 and st. dev.: 3.7). This data led us to consider this qPCR assay suitable for development of MPN-qPCR and enrichment free detection protocols.

### 3.2. *Escherichia coli* O157:H7 qPCR Assay

We adapted the original protocol by Perelle et al. [26] for use in a duplex reaction but eventually found that, despite the duplex assay based on these primers and probe guaranteed high specificity, single reactions were more sensitive than duplex. Sensitivity was determined as limit of detection, namely, the lowest concentration at which 95% of the positive samples are detected. Duplex reaction showed a LOD that was about one order of magnitude higher than the LOD of single reactions (data not shown).

Therefore, we detected and quantified *E. coli* O157:H7 by adopting single reactions, performed at the same time and in the same experiment plate. Amplification reactions were carried out with a range of DNA concentrations approximately corresponding to $1.0 \times 10^{-15}$–$1.0 \times 10^9$ target molecules (single *E. coli* genome mass $\approx 6.13 \times 10^{-15}$ g), and two standard curves, one for each primer/probe system, were constructed. From now on in our exposition we will make reference to the less performing primer-probe system, targeting gene *fliC* H7, as it determines the detection limit of the assay. Standard curve and amplification plot for *fliC* H7 assay are shown in Figure 2.

The standard curve showed a linear relationship (spanning 6 logs) between log input DNA and threshold cycle (Figure 2). The slope of the curve was $-3.40$ and the square regression coefficient was $R^2 = 0.999$. Based on these data, efficiency of the assay is 96.84%. The estimated limit of quantitation (LOQ) is $1 \times 10^2$ target molecules. The estimated detection limit (LOD) was one order of magnitude lower, corresponding to 10 target molecules (mean $C_T$ value was 37.16 and st. dev. was 0.78).

### 3.3. *Listeria monocytogenes* and *E. coli* O157:H7 Enumeration by MPN-qPCR

In our experiments we used a MPN protocol integrated with qPCR in which presumptive positive MPN tubes are directly checked by qPCR for confirmation. In order to evaluate the reliability of our method on fresh-cut vegetables we applied our protocols on artificially inoculated salads (0 to $3 \log \text{CFU g}^{-1}$) with both pathogens. Salad samples not inoculated and previously assayed for both the target pathogens were used as negative control.

Enumeration of *Listeria monocytogenes* by the MPN method from artificially contaminated foods was performed after 48 h of incubation of triplicate serial dilutions in selective Fraser Broth. Aesculin hydrolysis in the positive tubes resulting in turning of culture medium to black allowed the definition of the three consecutive dilutions to determine the initial contamination by referring to the corresponding MPN tables [31]. Presumptive *L. monocytogenes* positive tubes were phenotypically and biochemically confirmed in all cases. Moreover, no typical *L. monocytogenes* colonies were observed on selective media when negative tubes from

---

**Figure 1:** Standard curve and amplification plot of the 64 bp hlyA gene fragment generated by qPCR amplification of serially diluted purified DNA of *Listeria monocytogenes* represented as log of genome equivalents/reaction. Trend line equation and the corresponding square regression coefficient ($R^2$) are shown.

**Figure 2:** Standard curve and amplification plot of the 96 bp fliC gene fragment by qPCR amplification of serially diluted purified DNA of *Escherichia coli* O157:H7 represented as log of genome equivalents/reaction. Trend line equation and the corresponding square regression coefficient ($R^2$) are shown.
the same dilutions were assayed. Black tubes from negative controls were never observed by MPN. This result was also confirmed by conventional techniques (Table 2).

*E. coli* O157:H7 MPN enumeration was determined considering positive the tubes showing turbidity after 48 h incubation in TSB supplemented with novobiocin and bile salts and further confirmed for the ability to grow on CT-SMAC. Differently from what is observed for *L. monocytogenes*, MPN enumeration of *E. coli* O157:H7 gave some presumptive positive results in control and in artificially contaminated samples (Table 2) that were not confirmed by conventional methods. Interestingly, although an incubation time of 48 h was used, bacterial growth was observed in all the positive tubes used for the MPN enumeration already after 24 h of enrichment. Although the DNA yield was higher when the commercial kit was used (data not shown), no discrepant results were observed using as template the DNA obtained from both the kit and the boiling method. Indeed, the amplification of *hlyA*, *fliC* H7, and *rfbE* genes by qPCR always confirmed the presence of pathogens only in the cultures positive to the conventional approaches while no signal was detected submitting DNA from negative tubes to the molecular analysis (Table 2).

| Theoretical inoculum (CFU g⁻¹) | 3-tube dilution¹ (mL homogenized) | Positive tubes (MPN) | True positive² | MPN (g⁻¹)³ (95% c.i.) | Positive (qPCR) |
|---------------------------------|----------------------------------|----------------------|----------------|------------------------|-----------------|
| **L. monocytogenes**             |                                  |                      |                |                        |                 |
| Control                         | 10/1/0.1                         | 0/0/0                | n.d.           | n.d.                   | n.d.            |
| 1                               | 10/1/0.1                         | 3/2/0                | 3/2/0          | 0.93 (0.23–3.80)       | 3/2/0           |
| 10                              | 1/0.1/0.01                       | 3/1/0                | 3/1/0          | 4.3 (0.90–18)          | 3/1/0           |
| 100                             | 0.1/0.01/0.001                   | 3/0/2                | 3/0/2          | 64 (17–180)           | 3/0/2           |
| 1000                            | 0.01/0.001/0.001                 | 3/1/1                | 3/1/1          | 750 (170–2000)        | 3/1/1           |
| **E. coli O157:H7**             |                                  |                      |                |                        |                 |
| Control                         | 10/1/0.1                         | 3/3/3                | n.d.           | n.d.                   | n.d.            |
| 1                               | 10/1/0.1                         | 3/3/3                | 3/1/2          | >11.0                  | 3/1/2           |
| 10                              | 1/0.1/0.01                       | 3/3/1                | 3/2/0          | 46 (9–200)            | 3/2/0           |
| 100                             | 0.1/0.01/0.001                   | 3/2/1                | 3/1/1          | 150 (37–420)          | 3/1/1           |
| 1000                            | 0.01/0.001/0.001                 | 3/2/0                | 3/2/0          | 930 (180–4200)        | 3/2/0           |

¹The reported dilution is referred to as the set of tubes considered for MPN enumeration.
²Confirmed by biochemical and immunological methods.
³Calculated on the basis of the true positive samples.

3.4. Detection of *L. monocytogenes* and *E. coli* O157:H7 by qPCR. With the aim to further reduce the time for the detection of both pathogens we assayed a qPCR approach not associated with MPN enumeration. For the evaluation of LOQ and LOD of the qPCR methods without MPN step, artificially contaminated fresh-cut vegetables used for MPN enumeration were incubated at 37°C and samples analyzed by qPCR after 0, 2, 4, 6, and 24 h of enrichment, respectively. As previously reported for vegetables matrices [35], DNA was extracted from 10 mL of homogenate by using the DNeasy Blood and Tissue kit (Qiagen). *L. monocytogenes* was detectable at contamination levels as low as 10 CFU g⁻¹ with two hours of enrichment (mean *C_T* value was 36.73 and st. dev. was 1.12). Limit of detection for *E. coli* O157:H7 was 10 CFU g⁻¹, after two hours of enrichment (mean *C_T* value was 37.11 and st. dev. was 2.48). Limit of quantitation (LOQ) was 10⁴ CFU g⁻¹ without selective enrichment for both pathogens (data not shown).

3.5. Screening of RTE Samples. Samples of ready-to-eat vegetables and fruits provided by two different companies were investigated for the presence of *Listeria monocytogenes* and *E. coli* O157:H7 by using both MPN-qPCR and conventional culture-dependent methods. Black tubes were not observed by MPN enumeration of all the samples, suggesting the absence of presumptive *Listeria* spp. Indeed, *L. monocytogenes* was never detected by qPCR nor conventional methods (data not shown). Compared to *L. monocytogenes* assays, *E. coli* O157:H7 gave some presumptive positive results after the enrichment step, but in no case they were confirmed by culture methods (Table 3). The amplification of *rfbE* gene by qPCR did not give positive signals in any sample. Interestingly, we found that one sample of mixed raw salad (prior to processing) was positive by using *flic* H7 as target gene (Table 3). For further investigation the sample was processed by qPCR targeting *uidA* (beta-glucuronidase gene) and *stx2* (shiga toxin 2 gene, shared by all enterohemorrhagic serotypes) and found to be positive for both (data not shown). Basing on the *uidA* and *stx2* assays, the sample was found to be contaminated by a strain of potentially pathogenic H7 serotype, other than O157, even though it was not possible to isolate the H7 serotype on culture media.

4. Discussion

Fresh-cut packaged fruits and vegetables sold in the market are generally considered a product of high quality and freshness. However, they may represent an underestimated public health risk due to the potential presence of pathogenic bacteria, like *Listeria monocytogenes* and *Escherichia coli*.
The existing regulations in Europe stipulate that the identification of these foodborne pathogens is carried out by culture-dependent methods. Nonetheless, an inexpensive analysis and a fast preliminary result based on a molecular approach could be remarkable for fresh-cut producers to integrate the conventional methods and thus allowing a promptly intervention on presumptive contaminated products and a more drastic sanitization of the plant. Therefore, a rapid detection of the pathogens should improve the internal quality control assessment ensuring a greater safety to the consumer.

In the last years, PCR-based techniques have been the subject of considerable focus and ISO guidelines have been established for the detection of foodborne pathogens (ISO 22174:2005, ISO/TS 20836:2005, ISO 20837:2006, and ISO 20838:2006). Particularly, real-time quantitative PCR is considered a method of choice for the detection and quantification of enteric bacteria, thus resulting in the growth of bacteria and/or E. coli strains different from O157:H7.

Therefore, in this study we aimed at the foodborne pathogens quantification by integrating MPN with qPCR, in order to considerably reduce the time required for confirmation by conventional phenotypic and biochemical assays. The first goal was the comparison of the results obtained by MPN-qPCR with those ones from culture-dependent methods. Our method enabled the detection and indirectly—quantification of L. monocytogenes and E. coli O157:H7 when inoculated a concentration of as low as 1 CFU g\(^{-1}\), after 24 hours of incubation in the corresponding selective media, with a gain of about 4 days compared to the standard culture method. It has to be remarked that in real conditions the pathogen cells on vegetable surfaces are submitted to several stresses such as washing with chlorinated water, low temperatures, and microbial competition, thus resulting in a slower recovery and growth rate. For this reason, an increase of the incubation time to 48 h is anyway recommended for MPN enumeration when real samples are analyzed. Only for E. coli O157:H7 false positive MPN results after the selective enrichment were recorded. The aspecific turbidity of MPN tubes from control and artificially contaminated samples was frequent above the theoretical inoculum of 10\(^2\) CFU g\(^{-1}\). We argue that Fraser is a selective medium with higher inhibition of non-Listeria spp., while resistance to selective agents such as bile salts and novobiocin is a more widely distributed feature between enteric bacteria, thus resulting in the growth of bacteria and/or E. coli strains different from O157:H7.

With the aim to further reduce the time for the detection of both pathogens we assayed a qPCR approach not associated with MPN enumeration by using as target DNA extracted after 0, 2, 4, and 6 h of enrichment in the corresponding selective media. We observed that after 2 h it was possible to detect L. monocytogenes and E. coli O157:H7 with a limit of 10 CFU g\(^{-1}\). Although quantitative PCR methods are increasingly being used to detect bacterial pathogens in food, detection limits rarely exceed 10\(^2\) - 10\(^3\) CFU g\(^{-1}\) [35]. More

---

**Table 3: Enumeration of E. coli O157:H7 in samples of rocket, mix salad and piel de sapo melons raw, processed, and at 3 days of shelf life by the MPN-qPCR method and using fliC H7 and rfbE as target genes.**

| Sample        | 3-tube dilution (mL homogenized) | Positive tubes (MPN) | Positive (conventional method) | Positive fliC H7 (qPCR) | Positive rfbE (qPCR) | MPN-qPCR |
|---------------|----------------------------------|----------------------|--------------------------------|------------------------|---------------------|----------|
| Rocket        |                                  |                      | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |
| Raw           | 0.1/0.01/0.001                   | 1/1/0                | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |
| Processed     | 1/0.1/0.01                      | 2/2/0                | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |
| 3-day shelf life | 1/0.1/0.01                  | 1/3/0                | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |
| Mix salad     |                                  |                      | n.d.                           | 0/1/0                  | 0/0/0               | +/-1     |
| Raw           | 0.1/0.01/0.001                   | 0/3/0                | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |
| Processed     | 1/0.1/0.01                      | 2/3/0                | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |
| 3-day shelf life | 1/0.1/0.01                  | 3/2/0                | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |
| Piel de sapo melons |                          |                      | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |
| Raw           | 1/0.1/0.01                      | 1/0/0                | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |
| Processed     | 10/1/0.1                       | 1/1/0                | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |
| 3-day shelf life | 10/1/0.1                   | 1/2/0                | n.d.                           | 0/0/0                  | 0/0/0               | n.d.     |

1 + for fliC (H7) gene and negative for rfb (O157) gene.
recently, a method based on the recovery and concentration allowed the simultaneous detection and quantification of 10^5 CFU g^-1 for \textit{L. monocytogenes} and \textit{E. coli} O157:H7 in parsley and salad [28]. However, detection values below 10 CFU mL^-1 were only achieved after selective enrichment step which required approximately 30 hours [16, 25, 29, 39]. In contrast, conventional methods require five days for determination of a negative result for \textit{L. monocytogenes} contamination while, if a positive test result occurs, additional days are required for biochemical tests to identify the species [40].

The robustness of the results obtained by qPCR approach is closely related to the efficient recovery of bacterial DNA. Furthermore, DNA quality is critical because the efficiency of PCR amplification can be reduced by inhibitors from the matrix. In the last years, several works compared different microbial DNA extraction techniques in order to optimize yield, time, and cost of the sample preparation process depending on the food [35, 41–43]. The commercial DNeasy Blood and Tissue kit has been reported as an efficient DNA purification method from vegetable matrices [27, 35]. Thus, in this study we used the same protocol to extract the DNA during the enrichment qPCR assays.

When we used the previously described boiling method for DNA extraction [16, 17], we found that, for mixed salad samples, the results of qPCR analyses were the same as that for DNA extracted with commercial kit. This result was possible since our method is based on DNA isolation from positive MPN tubes, in which vegetable debris is codiluted with bacterial cells, thus reducing the possible PCR inhibitors present in leafy vegetables. Therefore, with the aim to propose a method for routine analysis, we suggest the last as a suitable protocol in terms of cost, times for analysis, and handling.

Several authors reported the detection of \textit{L. monocytogenes} and \textit{E. coli} O157:H7 in fresh-cut vegetables and fruits sold at retail markets [18, 44, 45], but the occurrence of these foodborne pathogens during food processing has not been sufficiently investigated. Fresh-cut contaminations can occur at pre- and postharvest levels [12], and often an increase of \textit{L. monocytogenes} concentrations in fresh-cut vegetables stored under different conditions has been observed [46–48]. Therefore, our method was applied for the detection of \textit{L. monocytogenes} and O157:H7 to RTE food production chain, by analyzing 3 different products at 3 stages of processing (see Section 2). Rocket and mix salads were selected due to their worldwide spread on the market and fresh-cut melons, since contaminated cantaloupe were involved in the most important multistate listeriosis outbreak of the last years [7].

Our results showed that MPN-qPCR always matched the outcomes of the conventional methods supporting that it is a reliable approach to discriminate both positive and negative presumptive results from MPN enumeration.

This approach could be interesting for industrial purposes since enumeration of the pathogenic microorganisms can provide an estimation of the efficacy of sanitizers treatment and represent an alarm bell to reduce the risk of cross-contaminations in the plant.

Therefore, we believe that this work will contribute to confirming the effectiveness of molecular methods as a powerful tool to complement conventional methods for a rapid detection of relevant foodborne pathogens in the fresh-cut products.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Acknowledgments**

The research leading to these results has received funding from the European Union's Seventh Framework Programme for research, technological development, and demonstration under Grant agreement no. 289719 (Project "QUAFETY") and PONREC2007–2013, "Prodotti ortofruttioci ad alto contenuti in servizio: tecnologie per la qualità e nuovi prodotti" (OFR.AL.SER.). The authors are grateful to Dr. Giovanna Lasalandra, Istituto Zooprofilattico della Puglia e Basilicata, for providing them with \textit{Listeria ivanovii} and \textit{Listeria innocua} strains.

**References**

[1] J. Painter and L. Slutsker, “Listeriosis in humans,” in \textit{Listeria, Listeriosis and Food Safety}, E. T. Ryser and E. H. Marth, Eds., pp. 85–110, CRC Press-Taylor & Francis Group, Boca Raton, Fla, USA, 3rd edition, 2007.

[2] M. Cooley, D. Carycho, L. Crawford-Miksza et al., “Incidence and tracking of \textit{Escherichia coli} O157:H7 in a major produce production region in California,” \textit{PLoS ONE}, vol. 2, no. 11, Article ID e1159, 2007.

[3] I. Friesema, B. Schimmer, O. Steners et al., “STEC O157 outbreak in the Netherlands, September–October 2007,” \textit{Euro Surveillance}, vol. 12, no. 11, Article ID E071101.1, 2007.

[4] J. Grant, A. M. Wendelboe, A. Wendel et al., “Spinach-associated \textit{Escherichia coli} O157:H7 Outbreak, Utah and New Mexico, 2006,” \textit{Emerging Infectious Diseases}, vol. 14, no. 10, pp. 1633–1636, 2008.

[5] A. Süderström, P. Österberg, A. Lindqvist et al., “A large \textit{Escherichia coli} O157 outbreak in Sweden associated with locally produced lettuce,” \textit{Foodborne Pathogens and Disease}, vol. 5, no. 3, pp. 339–349, 2008.

[6] A. M. Wendel, D. H. Johnson, U. Sharapov et al., “Multistate outbreak of \textit{Escherichia coli} O157:H7 infection associated with consumption of packaged spinach, august-september 2006: the Wisconsin investigation,” \textit{Clinical Infectious Diseases}, vol. 48, no. 8, pp. 1079–1086, 2009.

[7] Centers for Disease Control and Prevention, “Multistate outbreak of listeriosis linked to whole cantaloupes from Jensen Farms, Colorado,” 2011, http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/.

[8] I. A. Gillespie, P. Mook, C. L. Little, K. Grant, and G. K. Adak, “\textit{Listeria monocytogenes} infection in the over-60s in England between 2005 and 2008: a retrospective case-control study utilizing market research panel data,” \textit{Foodborne Pathogens and Disease}, vol. 7, no. 11, pp. 1373–1379, 2010.

[9] C. L. Little, S. M. Pires, I. A. Gillespie, K. Grant, and G. L. Nichols, “Attribution of human \textit{Listeria monocytogenes} infections in England and wales to ready-to-eat food sources placed on the market: adaptation of the hald salmonella source.
European Commission, 2005.

European Commission Regulation 2073/2005 on Microbiological Criteria in Foodstuffs, European Commission, 2005.

E. C. P. De Martinis, R. E. Duvall, and A. D. Hitchins, “Real-time PCR detection of 16S rRNA genes speeds most-probable-number enumeration of foodborne Listeria monocytogenes,” Journal of Food Protection, vol. 70, no. 7, pp. 1650–1655, 2007.

P. Elizaziquel, G. Sanchez, and R. Aznar, “Quantitative detection of viable foodborne E. coli O157:H7, Listeria monocytogenes and Salmonella in fresh-cut vegetables combining propidium monoazide and real-time PCR,” Food Control, vol. 25, no. 2, pp. 704–708, 2012.

P. Kotzekidou, “Survey of Listeria monocytogenes, Salmonella spp. and Escherichia coli O157:H7 in raw ingredients and ready-to-eat products by commercial real-time PCR kits,” Food Microbiology, vol. 35, no. 2, pp. 86–91, 2013.

D. Rodriguez-Lazoaro, M. Hernandez, M. Scortti, T. Esteve, J. A. Vazquez-Boland, and M. Pla, “Quantitative detection of Listeria monocytogenes and Listeria innocua by real-time PCR: assessment of hly, iap, and lin02483 targets and AmpliFluor technology,” Applied and Environmental Microbiology, vol. 70, no. 3, pp. 1366–1377, 2004.

J. O’Grady, S. Sedano-Balbas, M. Maher, T. Smith, and T. Barry, “Rapid real-time PCR detection of Listeria monocytogenes in enriched food samples based on the ssaR gene, a novel diagnostic target,” Food Microbiology, vol. 25, no. 1, pp. 75–84, 2008.

S. Perelle, F. Dilasser, J. Grout, and P. Fach, “Detection by 5'-nuclease PCR of Shiga-toxin producing Escherichia coli O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world’s most frequent clinical cases,” Molecular and Cellular Probes, vol. 18, no. 3, pp. 185–192, 2004.

P. Elizaziquel, J. A. Gabaldon, and R. Aznar, “Quantification of Salmonella spp., Listeria monocytogenes and Escherichia coli O157:H7 in non-spiked food products and evaluation of real-time PCR as a diagnostic tool in routine food analysis,” Food Control, vol. 22, no. 2, pp. 158–164, 2011.

G. Sanchez, P. Elizaziquel, and R. Aznar, “A single method for recovery and concentration of enteric viruses and bacteria from fresh-cut vegetables,” International Journal of Food Microbiology, vol. 152, no. 1-2, pp. 9–13, 2012.

A. Garrido, M. J. Chapela, B. Roman, P. Fajardo, J. M. Vieites, and A. G. Cabado, “In-house validation of a multiplex real-time PCR method for simultaneous detection of Salmonella spp., Escherichia coli O157 and Listeria monocytogenes,” International Journal of Food Microbiology, vol. 164, no. 1, pp. 92–98, 2013.

S. Ram, P. Vajpayee, and R. Shanker, “Prevalence of antimicrobial-agent resistant shiga toxin and enterotoxin producing Escherichia coli in surface waters of river Ganga,” Environmental Science and Technology, vol. 41, no. 21, pp. 7383–7388, 2007.

K. C. Jinneman, K. J. Yoshitomi, and S. D. Weagant, “Multiplex real-time PCR method to identify shiga toxin genes stxl and stx2 and Escherichia coli O157:H7/H-serotype,” Applied and Environmental Microbiology, vol. 69, no. 10, pp. 6327–6333, 2003.

P. Elizaziquel and R. Aznar, “A multiplex RT-PCR reaction for simultaneous detection of Escherichia coli O157:H7, Salmonella spp. and Staphylococcus aureus on fresh, minimally processed vegetables,” Food Microbiology, vol. 25, no. 5, pp. 705–713, 2008.

G. A. Francis, A. Gallone, G. J. Nychas et al., “Factors affecting quality and safety of fresh-cut produce,” Critical Reviews in Food Science and Nutrition, vol. 52, no. 7, pp. 595–610, 2012.

F. Postollec, H. Falentin, S. Pavan, J. Combrisson, and D. Sohier, “Recent advances in quantitative PCR (qPCR) applications in food microbiology,” Food Microbiology, vol. 28, no. 5, pp. 848–861, 2011.

A. Jamshidi, S. Mohammadi, and A. Mohammadi, “Quantification of Escherichia coli o157:h7 in milk by most probable number -polymerase chain reaction (MPN-PCR) method,” African Journal of Microbiology Research, vol. 5, no. 26, pp. 4588–4591, 2011.
K. Oravcová, T. Kuchta, and E. Kacírková, “A novel real-time PCR-based method for the detection of *Listeria monocytogenes* in food,” *Letters in Applied Microbiology*, vol. 45, no. 5, pp. 568–573, 2007.

R. L. T. Churchill, H. Lee, and J. C. Hall, “Detection of *Listeria monocytogenes* and the toxin listeriolysin O in food,” *Journal of Microbiological Methods*, vol. 64, no. 2, pp. 141–170, 2006.

G. Amagliani, C. Giammarini, E. Omiccioli, G. Brandi, and M. Magnani, “Detection of *Listeria monocytogenes* using a commercial PCR kit and different DNA extraction methods,” *Food Control*, vol. 18, no. 9, pp. 1137–1142, 2007.

A. D. Pinto, V. Forte, M. C. Guastadisegni, C. Martino, F. P. Schena, and G. Tantillo, “A comparison of DNA extraction methods for food analysis,” *Food Control*, vol. 18, no. 1, pp. 76–80, 2007.

C. Jara, E. Mateo, J. M. Guillamón, M. J. Torija, and A. Mas, “Analysis of several methods for the extraction of high quality DNA from acetic acid bacteria in wine and vinegar for characterization by PCR-based methods,” *International Journal of Food Microbiology*, vol. 128, no. 2, pp. 336–341, 2008.

D. Althaus, E. Hofer, S. Corti, A. Julmi, and R. Stephan, “Bacteriological survey of ready-to-eat lettuce, fresh-cut fruit, and sprouts collected from the Swiss market,” *Journal of Food Protection*, vol. 75, no. 7, pp. 1338–1341, 2012.

A. S. Sant’Ana, M. C. Igarashi, M. Landgraf, M. T. Destro, and B. D. G. M. Franco, “Prevalence, populations and phenotypic and genotypic characteristics of *Listeria monocytogenes* isolated from ready-to-eat vegetables marketed in São Paulo, Brazil,” *International Journal of Food Microbiology*, vol. 155, no. 1-2, pp. 1–9, 2012.

A. S. Sant’Ana, M. S. Barbosa, M. T. Destro, M. Landgraf, and B. D. Franco, “Growth potential of *Salmonella* spp. and *Listeria monocytogenes* in nine types of ready-to-eat vegetables stored at variable temperature conditions during shelf-life,” *International Journal of Food Microbiology*, vol. 157, no. 1, pp. 52–58, 2012.

E. Likotrafiti, P. Smirniotis, A. Nastou, and J. Rhoades, “Effect of relative humidity and storage temperature on the behavior of *Listeria monocytogenes* on fresh vegetables,” *Journal of Food Safety*, vol. 33, no. 4, pp. 545–551, 2013.

J. P. Vandamm, D. Li, L. J. Harris, D. W. Schaffner, and M. D. Danyluk, “Fate of *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* on fresh-cut celery,” *Food Microbiology*, vol. 34, no. 1, pp. 151–157, 2013.