Evaluation of two loop-mediated isothermal amplification methods for the detection of *Salmonella* Enteritidis and *Listeria monocytogenes* in artificially contaminated ready-to-eat fresh produce

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Abstract

In the present study, the effectiveness of two loop-mediated isothermal amplification (LAMP) assays was evaluated. Samples of romaine lettuce, strawberries, cherry tomatoes, green onions and sour berries were inoculated with known dilutions (10^0-10^8 CFU/g of produce) of *S.* Enteritidis and *L. monocytogenes.* With LAMP, assay pathogens can be detected in less than 60 min. The limits of detection of *S.* Enteritidis and *L. monocytogenes* depended on the food sample tested and on the presence of enrichment step. After enrichment steps, all food samples were found positive even at low initial pathogen levels. The developed LAMP, assays, are expected to become a valuable, robust, innovative, powerful, cheap and fast monitoring tool, which can be extensively used for routine analysis, and screening of contaminated foods by the food industry and the Public Food Health Authorities.

Introduction

The consumption of ready-to-eat (RTE) produce, such as salads, fresh fruits and raw vegetables has been increased significantly. RTE fresh produce can become a vehicle for the transmission of pathogens capable of causing human illness (Abadias *et al*., 2008). Numerous outbreaks in RTE fresh produce have been reported due to extensive human handling during their preparation or by cross-contamination (Escalona *et al*., 2010). *L. monocytogenes* and *Salmonella* spp. are important pathogens detected in vegetables and fruits (Beuchat, 1999; Bracket, 1999). *Salmonella* has been shown to survive or grow in a wide variety of products (Hanning *et al*., 2009). An increasing number of product-associated foodborne outbreaks in the United States associated with bacterial contamination are primarily from *Salmonella* (Tauxe *et al*., 1997; Harris *et al*., 2003). *L. monocytogenes* has been isolated from 15% of environmental samples in New York, emphasizing the prevalence of this pathogen on product fields (Strawn *et al*., 2013). Prevalence estimates reported in the literature for products grown in the United States range from 0% to more than 35% (Hoelzer *et al*., 2014). *Salmonella* remains the most frequently detected causative agent in the food-borne outbreaks reported (22.5 % of total outbreaks). For *Listeria* foodborne outbreaks a total of 13 outbreaks have been reported in 2013, which was slightly higher than in the previous years (EFSA and ECDC, 2015).

Their control and prevention is essential throughout the food chain, as they can be related with many foodborne outbreaks registered in the European Union (European Food Safety Authority, 2012). Moreover, in case of foodborne outbreaks they cause high mortality rates within high-risk population groups (Kotzekidou, 2013). The fatality rate of *L. monocytogenes* is relatively rare but can reach 30% (Ponniah *et al*., 2010).

Salmonellosis constitutes a major foodborne infectious disease worldwide and is caused by 2500 serovars. It is most often attributed to the consumption of contaminated foods such as poultry, beef, pork, eggs, milk, seafood, nut products, and fresh products (Techathuvanan *et al*., 2010; Wang *et al*., 2008). *S. enterica* serovar Enteritidis is often associated with salmonellosis outbreaks (Techathuvanan and D'Souza, 2012). Traditionally, *Salmonella* detection includes pre-enrichment, then selective enrichment, culture plating, biochemical and serological tests, which in total require approximately 5 days for completion (Kokkinos *et al*., 2014). Therefore, it is time consuming, cost and labor intensive to meet the criteria for food safety control in routine analysis in food companies (Techathuvanan *et al*., 2010).

Listeriosis is another major foodborne infectious disease that has the potential to cause serious life-threatening diseases, like septicemia, meningitis, meningoencephalitis and abortion (Dogany, 2003). Listeriosis can lead to a severe illness for individuals with compromised immune systems as the elderly, pregnant women, and young children (Ahmed *et al*., 2015). In USA approximately 1600 illnesses and 260 deaths have been caused each year due to listeriosis (Scallan *et al*., 2011). It is well known that a *zero tolerance* policy for *L. monocytogenes* in RTE fresh product exists (Gombas *et al*., 2003). However, for RTE foods which are able to support the growth of *L. monocytogenes*, EU Regulation 2073 specifies that the 100 CFU/g criterion *applies if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 CFU/g throughout the shelf-life* and the *absence in 25 g* criterion applies only when the manufacturer is *not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 CFU/mL throughout the shelf-life* (Koutsoumanis and Angelidis, 2007; EFSA, 2007). Traditionally, food companies test the existence of *L. monocytogenes* with routine culture methods, which demand at least 5 days for completion. The lack of any heating step prior to consumption of the RTE produce emphasizes the need for taking appropriate sanitary measures for the prevention and reduction of the microbial load, but simultaneously without altering the *fresh* status and the organoleptic and quality properties of the products, intended to be consumed raw (Baert *et al*., 2009). Increased public awareness related to health and economic impacts of foodborne diseases has resulted in greater efforts in order to develop sensitive, rapid and inexpensive screening methods of pathogens detection (Wang *et al*., 2008). To satisfy the expected rapidity, molecular methods have been developed. However, they still require time and expensive, sophisticated equipment, as well as expertise (Techathuvanan *et al*., 2011). Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification assay that is rapid, specific, and simple, and can be selected as a new alternative for molecular diagnosis (Notomi *et al*., 2000). The advantages

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of LAMP are high specificity and sensitivity, simple operation, and low cost, which constitutes a potentially valuable tool for rapid diagnosis of food-borne pathogens (Shao *et al*., 2011). LAMP works under isothermal conditions between 60 and 65°C resulting in 10^9 copies of target DNA within an hour (Parida *et al*., 2008). The aim of the present study was the evaluation of two specific LAMP assays which are simple, cost-effective, one-step, single-tube, for the detection of *S.* Enteritidis and *L. monocytogenes* seperately, on a series of artificially inoculated fresh ready-to-eat (RTE) produce samples. The main objective of our study was the application and further evaluation of two diagnostic LAMP-based assays, and the simultaneous-complementary testing with classic ISO methods for the detection of different concentrations of *S.* Enteritidis and *L. monocytogenes* in RTE produce, and their potential use in routine food analysis by the food industry and the Public Food Authorities.

Materials and Methods

Bacterial strains

Bacterial strains used were *S.* Enteritidis NCTC 6676 and *L. monocytogenes* NCTC 11994 [Health Protection Agency (HPA), Colingdale, UK]. Lenticules with the microorganisms were rehydrated in 9 mL of peptone saline (0.1%) (Oxoid, Basingstoke, UK), and after 20 min, working cultures were streaked onto Tryptic Soy Agar (TSA; Oxoid), incubated at 37°C for 24 h, and stored at 4°C.

Culture preparation

Each bacterial type was cultured in 20 mL Tryptone Soya Broth (TSB; Merck KGaA, Darmstadt, Germany) at 37°C for 17 h, harvested by centrifugation at 4000 x g for 20 min at 4°C and washed three times with buffered peptone water (BPW; Oxoid). The final pellets were resuspended in BPW, corresponding to approximately 10^8-10^9 CFU/mL.

Food samples collection and processing

Five different RTE fresh produce, were purchased from a local supermarket (Patras, Greece) the day of the experiment, and stored under refrigerated conditions (4°C) until the time of the experiment. Fresh RTE produce, such as romaine lettuce (*Lactuca sativa L. var. longifolia*), strawberries (*Fragaria x ananassa*), cherry tomatoes (*Solanum lycopersicum var. cerasiforme*), green onions (*Allium spp*), and sour berries (*Prunus cerasus*), were used for the specificity and evaluation tests of the developed LAMP assay.

Sample inoculation

All RTE fresh produce samples were rinsed with sterile water to remove some of the natural microbiota or any other matter (*e.g.* soil) before treatment (Bermúdez-Aguirre and Barbosa-Cánovas, 2013). For the inoculation of the samples, a spot-inoculation method was applied to inoculate the bacteria on their surface. Briefly, 100 μL of *S.* Enteritidis and *L. monocytogenes* corresponding to concentrations of 10^8 CFU/g to 10^0 CFU/g (which were determined by culture method) were spotted separately with a micropipette on 10 different areas of the surface of each RTE fresh produce sample weighing 10g, in order to simulate real conditions. After spiking, the samples were dried, for 1 hour at 22±2°C, to allow bacterial attachment. All processes were performed in a class II biosafety cabinet (Cytair 155; FluFrance, Wissous, France).

Culture-based confirmation of *Salmonella* spp. detection

The method used was based on protocol ISO 6579:2002 (ISO, 2002), with slight modifications. Artificially inoculated RTE fresh produce (10 g) were added to 90 mL Buffered Peptone Water (BPW; Oxoid). Secondary enrichment was performed using only Rappaport-Vassiliadis medium (RVS broth; Oxoid). Finally, plating out was performed using xylose lysine desoxycholate agar (XLD; Oxoid). At the same time a LAMP-friendly secondary enrichment with YPCE broth (Knuttson *et al*., 2002) was assessed, with incubation for 24h at 37°C (Figure 1).

Culture-based confirmation of *Listeria* spp. detection

The method used was based on protocol ISO 11290-1:1996 (ISO, 1996). Artificially inoculated RTE fresh produce (10 g) were added to 90 mL Half Fraser Broth (HFB) (Oxoid) and enrichment for 22-24h at 30°C was followed. Secondary enrichment was performed with Fraser Broth (FB) (Oxoid) for 24h at 37°C. Finally, plating out was performed using Oxford Listeria selective agar (Oxford Listeria; Oxoid). Samples from different stages (samples without enrichment, samples from first enrichment, and samples from secondary enrichment) were taken for culture and LAMP assays (Figure 2). *L. monocytogenes* nucleic acids were extracted using the Genomic DNA from tissue (Nucleospin tissue; Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.

Sample preparation for loop-mediated isothermal amplification methods

Buffered Peptone Water (90 mL) and Half Fraser Broth (90 mL) were added to 10 g of each RTE fresh produce sample prior inoculation with *S.* Enteritidis and *L. monocytogenes*, respectively. The samples were homogenized for *Salmonella* and *Listeria* testing, respectively. Sample was taken at day 0 (fresh produce sample with PBW or HFB). Then, 100μL were added to 100ml of selective enrichment broth YPCE and enrichment at 37°C for 24-48h (day 1 and day 2 respectively) followed for Salmonella detection. The LAMP friendly enrichment broth was prepared according to D'Agostino *et al*. (2015). For *L. monocytogenes* detection, enrichment of samples with HFB at 37°C for 24h (day 1) followed and finally 0.1 μL of HFB was added to 10ml of FB and the samples were enriched at 37°C for 24h (day 2).

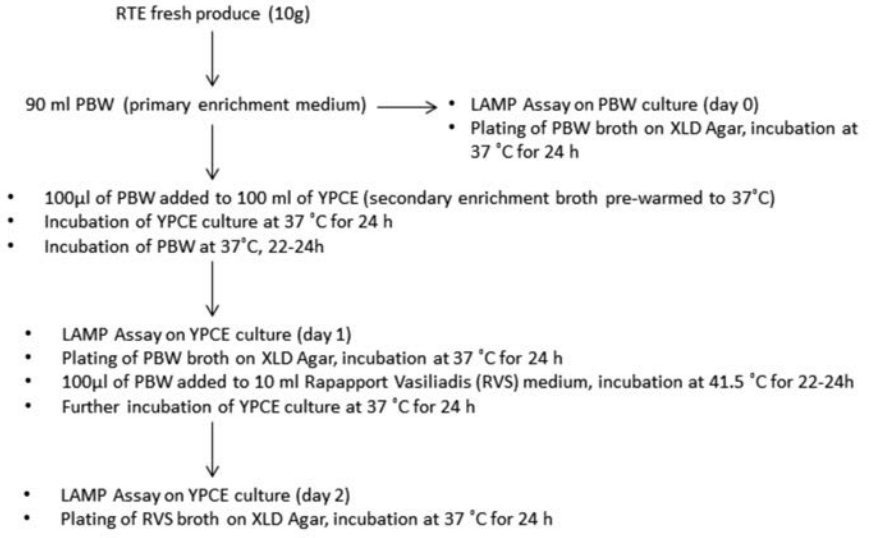

Figure 1. Flowchart of loop-mediated isothermal amplification/International Organization for Standardization methods for *Salmonella* Enteritidis detection.

Loop-mediated isothermal amplification assay for *Salmonella* Enteritidis detection

In this study, six primers (two inner primers, two outer primers and two loop primers), targeting *Salmonella* enterica invasion protein (invA) gene were used for the LAMP reactions (Hara-Kudo *et al*., 2005; Ziros *et al*., 2012). The reaction was carried out in a total of 25μL and contained 16μL of Tin Isothermal Mastermix, 25 μM invASalm FIP, 25 μM invASalm BIP, 5 μM invASalmF3, 5 μM invASalmB3, 12,5 μM invASalmF-Loop, 12,5 μM invASalmB-Loop and 3μL of template DNA (D'Agostino *et al*., 2015). The primers were high-performance liquid chromatography-purified. The thermal profile of the reaction was the following: 95°C for 2 min (cell lysis), followed by 65°C for 50 min (nucleic acid amplification). Amplicon annealing profiling was performed by heating to 98°C then cooling to 80°C at a rate of 0.05°C sec-1. The samples were analyzed using a LightCycler Nano Instrument (Roche, Basel, Switzerland). Positive and negative controls were included in each run. Aliquots of 10 μL of LAMP products were electrophoresed on 2% agarose gels and were visualized by ethidium bromide (Sigma, St. Louis, MO, USA) staining. The amplified products were also detected by adding 1 μL of 1000 X SYBR green dye to each reaction tube. After incubation for 15 min in the dark at room temperature, a yellowish green colour indicated a positive reaction, while a reddish orange (the colour of the unbound dye) indicated a negative reaction.

Loop-mediated isothermal amplification assay for *Listeria monocytogenes* detection

This study used six primers (two inner primers, two outer primers and two loop primers) targeting *hly*A gene of *L. monocytogenes*. The primers were high-performance liquid chromatography-purified. Positive and negative controls were included in each run. The LAMP reaction was carried out in a total volume of 25 μL. The optimal conditions as well as the thermal profile were based on the assay of Wang *et al*. (2012). The samples were analyzed using a LightCycler Nano Instrument (Roche). Aliquots of 10 μL of LAMP products were electrophoresed on 2% agarose gels and were visualized by ethidium bromide (Sigma) staining with UV light transillumination. The amplified products were also detected by adding 1 μL of 1000 X SYBR green dye to each reaction tube. After incubation for 15 min in the dark at room temperature, a yellowish green colour indicated a positive reaction, while a reddish orange (the colour of the unbound dye) indicated a negative reaction.

Prevention of polymerase chain reaction carryover contamination

To avoid any LAMP carryover contamination strict laboratory practices were followed throughout the experimental procedure. The manipulations before the LAMP assay (DNA isolation and LAMP set-up) were performed in a clean UV room that was separated from the LAMP PCR machine and the post-LAMP processing area. Negative controls were run with all assays, and no indications of contamination were detected. Attention was paid when the caps of the used reaction tubes were opened for the addition of SYBR green dye or subsequent electrophoresis.

Specificity and sensitivity of the loop-mediated isothermal amplification assays

The specificity of the developed LAMP assays in real-world situations were evaluated in the context of the present study by analyzing different fresh produce matrices, after inoculation of non-*Salmonella* and non-*Listeria* DNA. Strains other than *Salmonella* and *Listeria* (human adenovirus-35, hAdV35; human adenovirus 40/41, hAdV40/41; *E. coli*, NCTC 9001) were selected in order to check if cross-reaction was observed in LAMP assays. The negative controls which have been used in the study were the following: i) for every batch of analyzed food samples, a control sample (non-inoculated with the target microorganism) was tested to investigate any potential initial contamination or contamination during the enrichment procedures; ii) for every LAMP assay, two negative controls with the aforementioned non-target microorganisms, and one additional negative control consisting of water (ddH2O) (instead of the target microorganism), were used. In addition, ten-fold dilutions of *Salmonella* and *Listeria*, which were previously quantified by culture methods, were inoculated in RTE fresh produce samples to determine the sensitivity of the assay for food analysis. Finally, for measuring the limit of detection (LOD) of the LAMP assay, RTE fresh produce samples were tested in triplicate and the lowest concentration of colony forming units (CFUs) was taken as the limit when all of the triplicate samples (for each dilution) were positive.

Statistical analysis

All experiments were carried out in triplicates. During each experiment two samples were run in agarose gels (2%) or tested after the addition of SYBR Green. The data for the detection of two pathogens with LAMP assays were analyzed for statistical significance using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Results were compared by an analysis of variance (ANOVA) followed by Tukey's method with a significance level of $P<0.05$.

Results and Discussion

In the present study, different RTE fresh produce (romaine lettuce, strawberries, cherry tomatoes, green onions, sour berries) were tested for detection of 2 pathogens (*S.* Enteritidis and *L. monocytogenes*) that were prior inoculated on the food samples.

The specific isothermal amplification of the DNA of *Salmonella* and *Listeria* strains on food samples generated ladder-like pattern bands on agarose gel. No amplification was observed in LAMP reactions without template DNA (negative control) and in the control reactions with non-*Salmonella/Listeria* DNA. LAMP assay successfully detected the aforementioned bacteria in food samples within 60 min, when no enrichment step was followed. However, when enrichment steps were used, the detection limit was higher for both methods (Tables 1

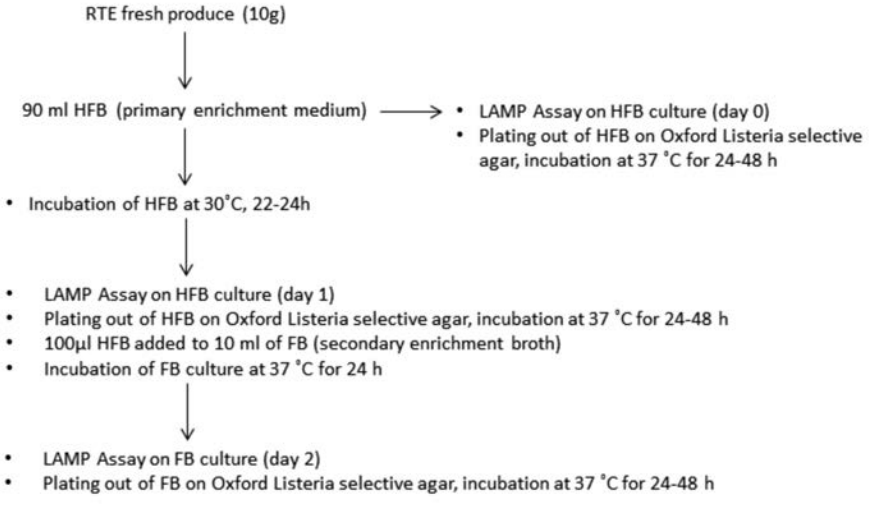

Figure 2. Flowchart of loop-mediated isothermal amplification/International Organization for Standardization methods for *Listeria monocytogenes* detection.

and 2) and the total assay time was 25 h and 49 h for primary and secondary enrichment steps, respectively. Moreover, there was no visual detection of LAMP products after SYBR Green addition and observation under UV light (Figure 3) or difference between the LAMP results detected by agarose gel electrophoresis of LAMP products (Figure 4).

The LAMP-based methods are important for analysis of fresh RTE produce, through screening of uncontaminated or contaminated samples in approximately 24-48 hours less than the standard culture-based method alone (data not shown). It is known that it is necessary for the food companies to certify the fresh RTE products as *Salmonella*-free or *Listeria*-free before the products are released to the market. Until now, the time till the final confirmation of the result using the standard cultivation methods, is not in favor of the companies as it is linked with high costs and time consumption.

DNA amplification and reading of results require minimum equipment, thus the technique has great potential for use in diagnosis of foodborne diseases. Simple heating methods, such as chemical heaters or thermal bottles, have been recently designed for removing the dependence upon stable electricity and allowing for LAMP to be conducted at any time

Table 1. Loop-mediated isothermal amplification results of different ready to eat fresh produce samples prior inoculated with different dilutions of *S. Enteritidis* during a 3-day period.

RTE fresh produce	Corresponding initial inoculum (CFU/g)	LAMP-SALM (day 0)	LAMP-SALM (day 1)	LAMP-SALM (day 2)
Romaine lettuce	3*10^0	-	+	+
	3*10^1	-	+	+
	3*10^2	-	+	+
	3*10^3	-	+	+
	3*10^4	+	+	+
	3*10^5	+	+	+
	3*10^6	+	+	+
	3*10^7	+	+	+
	3*10^8	+	+	+
Strawberries				
	1*10^0	-	+	+
	1*10^1	-	+	+
	1*10^2	-	+	+
	1*10^3	-	+	+
	1*10^4	-	+	+
	1*10^5	-	+	+
	1*10^6	-	+	+
	1*10^7	+	+	+
	1*10^8	+	+	+
Cherry tomatoes				
	2*10^0	-	+	+
	2*10^1	-	+	+
	2*10^2	-	+	+
	2*10^3	-	+	+
	2*10^4	-	+	+
	2*10^5	+	+	+
	2*10^6	+	+	+
	2*10^7	+	+	+
	2*10^8	+	+	+
Green onions				
	2*10^0	-	+	+
	2*10^1	-	+	+
	2*10^2	-	+	+
	2 *10^3	-	+	+
	2*10^4	+	+	+
	2*10^5	+	+	+
	2*10^6	+	+	+
	2*10^7	+	+	+
	2*10^8	+	+	+
Sour berries	3*10^0	-	+	+
	3*10^1	-	+	+
	3*10^2	-	+	+
	3*10^3	-	+	+
	3*10^4	-	+	+
	3*10^5	+	+	+
	3*10^6	+	+	+
	3*10^7	+	+	+
	3*10^8	+	+	+

RTE, ready to eat; CFU, colony-forming units; LAMP, loop-mediated isothermal amplifications; SALM, *Salmonella*. Day 0, fresh produce sample with PBW without enrichment; day 1, fresh produce sample in YPCE enriched at 37°C for 24h; day 2, fresh produce sample in YPCE enriched at 37°C for 48h.

in any setting (Curtis *et al*., 2012; Hatano *et al*., 2010). As a consequence, LAMP assay has all the characteristics required of real-time assays along with simple operation for easy adaptability on-site.

LAMP has the advantages of simplicity, rapidity, specificity, cost-effectiveness and higher amplification efficiency. Furthermore, since DNA amplification and reading of results require minimum equipment, the technique has great potential for use in low-income countries (Njiru, 2012; Mori *et al*., 2013).

The specificity and sensitivity of the LAMP assays for *Salmonella* and *L. monocytogenes* detection have been previously evaluated (Hara-Kudo *et al*., 2005; Ziros *et al*., 2012; Wang *et al*., 2012). The analytical sensitivity of the LAMP assays for both bacteria depended on the enrichment steps that were followed. Two-step enrichment procedures were used for each pathogen as this can indicate the viability of a presumptive positive (D' Agostino *et al*., 2015). After an enrichment step is performed, only a detection result is possible. Any reference to concentrations of target microorganisms throughout this study refers to initial microbial load.

Table 2. Loop-mediated isothermal amplification results of different ready to eat fresh produce samples prior inoculated with different dilutions of *L. monocytogenes* during a 3-day period.

RTE fresh produce	Corresponding initial inoculum (CFU/g)	LAMP-LIS (day 0)	LAMP-LIS (day 1)	LAMP-LIS (day 2)
Romaine lettuce	1*10^0	-	-	+
	1*10^1	-	+	+
	1*10^2	-	+	+
	1*10^3	-	+	+
	1*10^4	-	+	+
	1*10^5	-	+	+
	1*10^6	-	+	+
	1*10^7	+	+	+
	1*10^8	+	+	+
Strawberries				
	2*10^0	-	-	+
	2*10^1	-	-	+
	2*10^2	-	-	+
	2*10^3	-	-	+
	2*10^4	-	+	+
	2*10^5	-	+	+
	2*10^6	-	+	+
	2*10^7	+	+	+
	2*10^8	+	+	+
Cherry tomatoes				
	1*10^0	-	-	+
	1*10^1	-	-	+
	1*10^2	-	+	+
	1*10^3	-	+	+
	1*10^4	-	+	+
	1*10^5	-	+	+
	1*10^6	-	+	+
	1*10^7	-	+	+
	1*10^8	+	+	+
Green onions				
	2*10^0	-	-	+
	2*10^1	-	-	+
	2*10^2	-	-	+
	2*10^3	-	+	+
	2*10^4	-	+	+
	2*10^5	-	+	+
	2*10^6	-	+	+
	2*10^7	+	+	+
	2*10^8	+	+	+
Sour berries	1*10^0	-	-	+
	1*10^1	-	-	+
	1*10^2	-	-	+
	1*10^3	-	-	+
	1*10^4	-	+	+
	1*10^5	-	+	+
	1*10^6	+	+	+
	1*10^7	+	+	+
	1*10^8	+	+	+

RTE, ready to eat; CFU, colony-forming units; LAMP, loop-mediated isothermal amplifications; LIS, *Listeria*. Day 0, fresh produce sample with PBW without enrichment; day 1, fresh produce sample in YPCE enriched at 37°C for 24h; day 2, fresh produce sample in YPCE enriched at 37°C for 48h.

The specific isothermal amplification of the DNA of *Salmonella* and *Listeria* on food samples generated ladder-like pattern bands on agarose gel. No amplification was observed in LAMP reactions without template DNA (negative control) and in the control reactions with non-*Salmonella* and non-*Listeria* DNA. There was no difference between the LAMP results detected by agarose gel electrophoresis of LAMP products or visual detection of LAMP products after SYBR Green addition and observation under UV light (Figures 3 and 4).

Ten-fold dilutions of 10^0 to 10^8 CFU/g of *S.* Enteritidis and *L. monocytogenes* in the selected food matrices were used for the sensitivity assays. The analytical sensitivity of the LAMP assay for each food pathogen and each food sample, with and without enrichment was estimated and is illustrated in Tables 1 and 2.

The two enrichment steps that were used for each pathogen increase the possibility of detecting viable cells originally existing in the sample to detectable amounts and increased the sensitivity of the assay (D'Agostino *et al*., 2015). The likelihood that a LAMP signal will be derived from viable *Salmonella* can be demonstrated arithmetically (D'Agostino *et al*., 2015). The LAMP assay for *Salmonella* can detect ~400 cells per reaction (D' Agostino *et al*., 2015), which is in accordance with the results of the present study for romaine lettuce, and onions. Whereas a higher detection limit (approximately 1 log higher) was found for cherry tomatoes, sour berries and strawberries (Table 1). The nature of different food types (smoother or porous surfaces, pH, *etc*.) could be the reason for the recorded differences in the detection limit. Further studies are needed in order to reveal the mechanisms under these observations. All the enrichment steps exhibited statistically significant results when tested with Paired Samples T-test ($P<0.05$).

The LAMP assay for *Listeria* exhibited a higher detection limit (3-4 log higher) compared to *Salmonella* in all RTE fresh produce, which imposed the necessity of the two enrichment steps. The higher detection level for *L. monocytogenes* when a longer period of enrichment was used can be explained by the longer generation time of this organism and indicates that selective enrichment with FB is necessary for detection of low initial numbers of cells of this species (Burtscher *et al*., 1999). Moreover, since *L. monocytogenes* is a Gram positive bacterium, *L. monocytogenes* cells are more difficult to extract, which makes DNA recovery less efficient (Burtscher *et al*., 1999). Obviously, the detection limit of low numbers of cells was increased when the second enrichment broth (FB) was used and incubation for 2 days was followed (Table 2). However, considerably less time than the classic procedures is required, which usually takes at least 4 days (data not shown). In *Salmonella* LAMP assays, the use of the thermostable heat stable enzyme Thermodesulfotator indicus (Tin) DNA polymerase enzyme allowed the direct addition of the whole *Salmonella* cells to the LAMP reactions. This is a very important aspect for decreasing time in food companies (D' Agostino *et al*., 2015). However, in *Listeria* LAMP assays the nucleic acid extraction was necessary as no amplification was observed in any sample without prior nucleic acid extraction, compared to *Salmonella*. Moreover, the boiling extraction method (Wu *et al*., 2014) exhibited faint signals in gel electrophoresis, which is in accordance with Amagliani *et al*. (2007), thus a Nucleospin tissue KIT was selected for *L. monocytogenes* detection, inoculated on RTE fresh produce (data not shown).

In the present study, the robustness of the LAMP assays for the analysis of food samples was shown by the detection of the target Gram negative and Gram positive bacteria in RTE food samples with smoother surface, like cherry tomatoes, green onions, and lettuce, without enrichment step. The detection of *Salmonella* and *Listeria* was found to be higher (approximately 2-4 log), compared to that of the samples tested after first and second enrichment step. Moreover, the results for both pathogens were found to be significant ($P=0.046$), when tested with one-way Anova test, for all the RTE fresh produce tested.

For instance, the detection without enrichment procedure was proved to be slightly lower (approximately 4-7 log) compared to that of the samples tested after an enrichment of 1st and 2nd day. Especially, in more complex food samples (strawberries, sour cherries), which can be categorized as soft fruits with porous surfaces and with rich sugar content, the target DNA was detected only when high levels of initial contamination existed for both pathogens. On the other hand, the detection was achieved in lower levels for vegetables with more firm surfaces, such as lettuce, cherry tomatoes (Dubois *et al*., 2007; do Nascimento, 2015). However, the results for RTE fresh produce were not significant ($P>0.05$) for both pathogens tested and for all enrichment steps. This imposes the implementation of enrichment steps, which for *Salmonella,* one day enrichment was found to be sufficient, whereas for *Listeria*, two enrichment days were important for the detection of lower levels of pathogen (Tables 1 and 2).

Different researchers have studied LAMP assays for *Salmonella* detection in RTE fresh produce like lettuce, tomatoes and milk (Zhang *et al*., 2011; Yang *et al*., 2015; Wu *et al*., 2015). More precisely Zhang *et al.* (2011) have found the detection limit of *L. monocytogenes* in lettuce (2 CFU/25g) and tomato (19 CFU/25g). Whereas, the detection limit of *L. monocytogenes* using the ISO 11290-1:1996 method (Amd. 2004) was found to be 5-100 CFU/25g (Zunabovic *et al*., 2011). Other researchers have studied the detection limit of *L. monocytogenes* in food samples like chicken

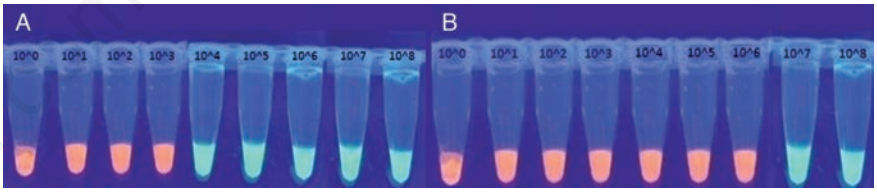

Figure 3. Loop-mediated isothermal amplification products after SYBR green addition and observation under ultra-violet light: ready to eat romaine lettuce at day 0 for *Salmonella* Enteritidis A) and *Listeria monocytogenes* (B).

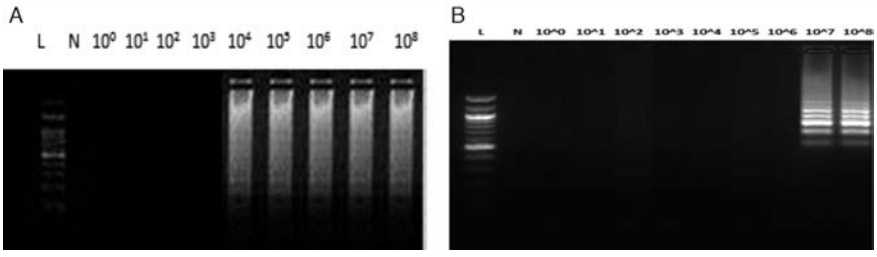

Figure 4. Loop-mediated isothermal amplification results detected by agarose gel electrophoresis: ready to eat romaine lettuce at day 0 for *Salmonella* Enteritidis A) and *Listeria monocytogenes* (B). L, 100bp Ladder; N, negative control; 10^0-10^8, decimal dilutions of Salmonella Enteritidis loop-mediated isothermal amplification amplicons.

and milk (Wu *et al*., 2014; Wang *et al*., 2011; Tang *et al*., 2011).

The present study did not aim at proposing LAMP as an alternative to ISO methods, but aimed to highlight the potential of LAMP assays to complement ISO methods in order to enhance their potential use in routine food analysis by the food industry and the Public Food Authorities. LAMP-based assays may be used as sensitive, specific and cheap tools for a fast initial screening of food samples for the target microorganisms of interest. Negative samples may be rapidly released to the market, while positive samples may be further analyzed and confirmed by ISO methods.

This is to the best of our knowledge the first report on the evaluation of -one Gram negative (*Salmonella*) and one Gram positive (*Listeria*) bacterium-specific LAMP assays on different food samples. This is the first time that LAMP assays are used for pathogen detection in five RTE fresh produce.

Conclusions

The results showed that *Salmonella* spp. and *L. monocytogenes* were detected successfully on the five fresh RTE fresh produce samples tested (romaine lettuce, strawberries, cherry tomatoes, green onions, sour berries) by both LAMP assays and conventional culture assays. The greatest advantage of the proposed LAMP assays is the reduction of the required analysis time depending on the initial contamination of the products, compared to the classical culture methods. The developed LAMP assays are expected to become a valuable tool and be used extensively for routine analysis and screening of contaminated foods by the food industry and the Public Health Authorities.

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