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(Article begins on next page)

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Detection, Quantification and Vitality of *Listeria monocytogenes* in Food as determined by Quantitative PCR

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Running title: Real-Time PCR for *Listeria monocytogenes* detection in food

ABSTRACT

In this paper we describe the development of a quantitative PCR (qPCR) technique to detect, quantify and define the vitality of *Listeria monocytogenes* in foods. The method was based on the amplification of the intergenic region spacer (IGS) between the 16S and 23S rRNA genes. A panel of more that 100 strains of *Listeria* spp. and non-*Listeria* was used in order to verify the specificity of the primers and Taqman probe and amplification signals were obtained only when *L. monocytogenes* DNA and RNA were loaded in the qPCR mix. Standard curves were constructed in several food matrices (milk, meat, soft cheese, fermented sausage, cured ham and ready-to-eat salad). The quantification limit was of 10^3-10^4 cfu/g or ml, while for the determination of vitality it was 10^4-10^5 cfu/g or ml. After an overnight enrichment in BHI at 37°C also 10 cfu/g or ml could be detected in all the matrices used in this study. When we applied the protocol to food samples collected from the market or from small food processing plants, on a total number of 66 samples, 4 fresh cheeses from raw milk gave positive results prior to the overnight incubation, while 9 samples, of which only one represented by fresh meat and the others by cheeses from raw milk, were positive after the enrichment. Out of the 4 positive samples, only one could be quantified and it was determined to contain $4x10^3$ cfu/g.

Key-words: *Listeria monocytogenes*, Real-Time PCR, detection, quantification, vitality

1. INTRODUCTION

Listeria monocytogenes is recognized worldwide as one of the most important food-borne pathogens of concern for the food industries. It is a ubiquitous microorganism and it is commonly isolated from foods of animal origin, mainly meat and milk products (Schuchat et al., 1991), but it can be also found in fresh produce, such as salads (Berrada et al., 2006). However, human listeriosis outbreaks are most often associated with ready-to-eat food products that are consumed without prior cooking (Ryser, 1999). Ingestion of foods contaminated with *L. monocytogenes* can result in listerioris, a severe infectious disease characterized by meningoencephalitis, abortion, septicemia, and a high fatality rate (30%). Listeriosis predominantly affects certain risk groups, including pregnant women, newborns, elderly people and immunocompromised patients (Kathariou, 2002; McLauchlin et al., 2004). However, recent reports of a noninvasive form of listeriosis that causes febrile gastroenteritis clearly indicate that persons with no predisposing conditions may be affected (Franciosa et al., 2001). The food safety regulations of most of the countries tolerate no *L. monocytogenes* in ready-to-eat foods (Gallagher et al., 2003), although the minimal infection dose is generally higher than 100 viable cells (Roberts et al., 1996). From January 1st 2006, the new Commission Regulation (EC) No. 2073/2005 entered into force in the European Union , that set the limit of 100 colony forming units (cfu) per g or ml for ready-to-eat products. For foods produced for specific subgroups of the population that are at risk, the absence in 25 g or ml is required.

The use of molecular methods for detection and identification of *L. monocytogenes* dates back to 1990s (Niederhauser et al., 1992; Wang et al., 1992). In the last 10 years a considerable number of studies, exploiting molecular methods, have been produced and in 2005 an extensive review by Gasanov et al., (2005) was published collecting the available information. Nowadays, due to technological advancements, we are experiencing a new era in

which the polymerase chain reaction (PCR), from qualitative assay is becoming quantitative. Lately several papers have been published on the use of quantitative PCR (qPCR) for the enumeration of *L. monocytogenes* in food samples (Berrada et al., 2006; Rodriguez-Lazaro et al., 2005; Rodriguez-Lazaro et al., 2004; Rossmanith et al., 2006; Rudi et al., 2005).

The goal of our study was to develop a qPCR protocol for the detection, quantification and definition of the vitality of *L. monocytogenes* in food samples. A couple of primers and a Taqman probe were designed on the 16S-23S intergenic region and after optimization of the amplification conditions we obtained high specificity towards *L. monocytogenes*. DNA and RNA were extracted from several food matrices artificially inoculated with serial dilutions of *L. monocytogenes* and standard curves were created. These were used in the following step of quantification of *L. monocytogenes* in food samples obtained from the market or from small producers in the Piedmont region, in the Northwest part of Italy.

2. MATERIALS AND METHODS

2.1 Bacterial strains

Listeria monocytogenes NCTC 7979 (serotype 1/2a), NCTC 10887 (serotype 1/2b), NCTC 9862 (serotype 1/2c) and NCTC 10527 (serotype 4b) were used in order to optimize the amplification conditions. Moreover, *Listeria innocua* DSMZ 20649, *Listeria ivanovii* DSMZ 20750, *Listeria seeligeri* DSMZ 20751, *Listeria welshimeri* DSMZ 20650 and other bacterial species commonly isolated from foods were selected and used in the qPCR to assess the specificity of the protocol. In particular, *Lactobacillus sakei, Staphylococcus xylosus, Lactococcus lactis, Streptococcus thermophylus, Enterococcus faecium, Pseudomonas aeruginosa, Serratia marcescens, Bacillus cereus, Escherichia coli* and *Campylobacter jejuni* were chosen. At least 2 strains for each species were tested. The non-*Listeria* strains were all coming from the collection of the Department of Food Science, University of Udine, Italy, and they were identified by sequencing the V1-V3 region of the 16S rRNA gene as previously described (Cocolin et al., 2004). Final evaluation of the protocol developed was performed by testing the *Listeria* strains described by Cocolin et al., (2002) and Cocolin et al., (2005). MRS, M17 and BHI broths (Oxoid, Milan, Italy) were used to culture the strains prior to DNA extraction.

2.2 DNA extraction from cultures

One ml of an overnight culture was centrifuged at 13,400 rpm for 5 min at 4°C and resuspended in 200 µl of sterile water. Ten µl of proteinase K (25 mg/ml, Sigma, Milan, Italy) were added and the tubes were incubated at 65°C for 1 h and 100°C for 10 min. After a centrifugation at 13,400 rpm for 10 min at 4°C, the supernatant was transferred to a new tube and the DNA was quantified by using the NanoDrop Instrument (NanoDrop Technologies, Wilmington, DE, USA) and diluted at 100 ng/µl.

2.3 Extraction of nucleic acids from food samples

For solid foods, 10 grams were diluted in 40 ml of BHI broth (Oxoid) in a stomacher bag and homogenized in a stomacher machine (PBI International, Milan, Italy) for 1 min. About 25 ml of the homogenate were transferred to a 50 ml sterile tube and the debris was let to deposit for about 5 min. For liquid foods, 10 ml were mixed with the BHI broth directly in the 50 ml tube. For the extraction of the DNA, a further 1 to 10 dilution in water was carried out and 1 ml was used, while for the RNA extraction 1 ml of the homogenate was used without further dilution. After centrifugation at 13,400 rpm for 10 min at 4°C, the pellets were subjected to the nucleic acids extraction by using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions. The nucleic acids were resuspended in 50 μl of sterile water and the solution was split in two aliquots, one for the DNA and the second for the RNA analysis. For the digestion of the RNA, the DNAse free-RNAse from Roche Diagnostics (Milan, Italy) was used, while to eliminate the DNA, the Turbo-DNAse from Ambion (Foster City, CA, USA) was employed. The presence of residual DNA in the RNA preparation was evaluated by qPCR. In case of positive signals, a second treatment was performed.

2.4 Oligonucleotides

The PCR primers and the Taqman probe, shown in Table 1, were designed after alignment of the 16S-23S intergenic spacers (IGS) of the *Listeria* members. Sequences with accession numbers U57912, U57913, U57915, U57916, U57917 and U57918 for *L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimerii* and *Listeria grayi*, respectively, were aligned by using the ClustalW program, available at http://www.ebi.ac.uk/clustalw/. Primers and probe were synthesized by Sigma. The Taqman probe was 5' labeled with FAM and 3'

quenched with TAMRA.

2.5 Amplification conditions

Amplifications were performed in a final volume of 25 µl in the Chromo4 Real-Time PCR Detection System (Biorad, Milan, Italy). One µl of DNA, extracted as described above, was amplified with the specific primers and TaqMan probe. The Fluomix for probe kit of Euroclone (Celbio, Milan, Italy) was used with a $MgCl_2$ concentration of 8 mM and the primers were added at a final concentration of 400 nM while the probe at 250 nM. The amplification cycle was as follows: initial denaturation at 95°C for 10 min, 95°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec. The cycle was repeated 50 times. For quantitative reverse transcription PCR (qRT-PCR), 9 µl of RNA were reverse transcribed using the M-MLV enzyme (Promega, Milan, Italy) as suggested by the manufacturer and 1 µl of cDNA was loaded in the qPCR reaction.

2.6 Construction of standard curves

Standard curves were constructed from serially diluted cells of *L. monocytogenes* in water and in several food matrices. Soft cheese, fermented sausage, cured ham and ready-to-eat salad, as well as minced meat and milk, were considered. Serial dilutions of an overnight culture in BHI broth of *L. monocytogenes*, containing approximately 10^9 cells/ml, were prepared and inoculated in 10 g or ml of food sample. The final concentration of the cells in the food samples were from 10^8 to 10 cells/ml. BHI broth (40 ml) was added to the artificially contaminated samples and homogenized in a stomacher machine. The overnight culture of *L. monocytogenes* used to contaminate the food samples was enumerated on BHI agar to determine the exact colony forming unit (cfu) spiked in the samples. DNA and RNA were extracted and amplified as described above. The signals produced (threshold cycle, C_t) by the

serial dilutions of *L. monocytogenes* in water and in the various food matrices were plotted against the Log_{10} cfu and the standard curves were constructed. Correlation coefficients (R^2) and efficiency of amplification were calculated as previously described (Higuchi et al., 1993). The food samples were also incubated at 37°C overnight and the next day, 1 ml of the enriched broth, diluted ten times, was collected and the DNA was extracted and amplified. The absence of *L. monocytogenes* in the samples used to construct the standard curve was assessed by ISO/DIS method (1990).

2.7 Food samples

The protocols developed were used to determine the presence of *L. monocytogenes* in food samples collected from local producers in the Piedmont region (Northwest of Italy) and from the market. The samples were represented by fresh meat (20 samples), fresh sausages (2 samples), fermented sausages (2 samples), fresh cheeses (31 samples) and ripened cheeses (11 samples). The cheese samples collected in the study were all produced in small plants and made from raw milk. All the samples (10 g) were homogenized with 40 ml of BHI broth and 1 ml was mixed with 9 ml water before an aliquot (1 ml) was subjected to DNA extraction and quantitative amplification as described above. A pellet originating from one ml of the undiluted sample was saved at -20°C covered with RNA later (Ambion) and it was used for the quantification of the vitality if needed. In addition, after an overnight enrichment at 37°C, 1 ml was collected and after a 1 to 10 dilution, the pellet, obtained by centrifugation, was saved at -20°C for DNA extraction. Both at time zero (T_0) and after enrichment (T_{24}), aliquots were plated or streaked, respectively, onto Palcam agar (Oxoid) for detection of *L. monocytogenes* by classical microbiological techniques.

3. RESULTS

3.1 Optimization of the qPCR protocol

Initial optimization of the qPCR cycle and of the qPCR mix was done using as control strains the *Listeria* spp. coming from the international collections and described in the materials and methods. In particular, each single test was run considering the 4 serotypes of *L. monocytogenes* used in this study (1/2a, 1/2b, 1/2c and 4b) and 4 other members of the *Listeria* genus, namely *L. ivanovii, L. innocua, L. seeligeri* and *L. welshimeri*. Several commercial available kits for qPCR amplification were evaluated. For the purposes of this study we concluded that the kit described in the materials and methods gave the best results in terms of specificity and sensitivity (data not shown). After several attempts adjusting mainly the temperature of annealing, the concentrations of the primers and probe and the concentration of $MgCl_2$, a qPCR protocol that could only amplify *L. monocytogenes* was developed. The conditions that allowed reaching this result were as follows: temperature of annealing of 56°C, concentration of the primers 400 nM, concentration of the probe 250 nM and concentration of the $MgCl_2$ of 8 mM. In the qPCR we obtained signals for *L. monocytogenes*, but not for the other species of listeriae. Moreover, all the serotypes tested were characterized by similar C_t, not differing more than 2 cycles. The parameters described here were first used in order to verify the specificity of the assay, testing several bacteria that are commonly found in foods of different origins. None of the strains used produced an amplification signal, thereby confirming the specificity of the method developed. In order to validate the qPCR we lastly checked its inclusively and exclusivity by amplification of a large panel of *Listeria* spp. isolated, identified and characterized in previous studies (Cocolin et al., 2002; Cocolin et al., 2005). About 100 strains, mainly represented by *L. monocytogenes*, but including also non-*L. monocytogenes* listeriae, were subjected to DNA extraction and qPCR. The results obtained confirmed the robustness of the method. All the *L. monocytogenes* tested

were correctly amplified, while the other strains of *Listeria* spp. did not give any amplification signal (data not shown).

3.2 Standard curves

In order to quantify *L. monocytogenes* cells in food samples, several standard curves were created considering the food matrices where there is a high incidence of *L. monocytogenes*. In particular fresh meat and milk, meat and milk products (fermented sausage, cured ham and soft cheese) and ready-to-eat salad were considered in this study. One standard curve was also created starting from serially diluted cells in water. Both DNA and RNA were extracted and subjected to qPCR and qRT-PCR, respectively, in order to construct the standard curves. When cells were diluted in water (Fig. 1), the linearity range was from 10^8 to 10^2 cfu/ml, covering 6 orders of magnitude. The efficiency was of 95% and the correlation coefficient (R^2) was of 0.970 (Tab. 2). In Figures 2, 3 and 4 the standard curves for meat and meat products, milk and soft cheese and ready-to-eat salad are reported, respectively. When the food matrices were artificially contaminated with serially diluted cells of *L. monocytogenes* and the DNA was extracted and amplified, the linearity range decreased with respect to the cells in water. For almost all of the foods considered in this study we could not quantify less than 10^3 cfu/g or ml, while for fresh meat this limit was increased to 10^4 cfu/g. Also the efficiency changed significantly. Only for milk and cured ham, they were close to 100%, while for all the other matrices they were far from this values (Tab. 2). At RNA level, amplification signals were obtained only when a number of cfu higher than 10^5 was inoculated in the food matrices (data not shown). Only in meat it was possible to detect 10^4 cfu/g. As a result, this method allows the definition of the vitality of *L. monocytogenes* in food samples only when the contamination level is higher than 10^4-10^5 cfu/g or ml. As reported for the DNA standard curves, also for the RNA curves the efficiencies were different

based on the matrix used, however the R^2 value was always acceptable (>0.930) (data not shown). The results obtained in terms of quantification limit, efficiency of amplification and coefficient of correlation were confirmed by at least three independent experiments.

After the enrichment in BHI broth at 37°C overnight, positive amplification signal were recorded for all the matrices tested also when only 10 cfu/g or ml were present in the samples prior to enrichment.

3.3 Application of the qPCR protocol for the detection and quantification of L. monocytogenes in food samples

The results obtained analyzing 66 food samples collected from the market or from small producers in the Piedmont region are shown in Table 3. Only 4 samples were positive without the enrichment, while after the overnight period at 37°C, the number increased to 9. Fresh cheeses represented the 4 positive samples and when subjected to quantification only one gave signals in the linear range of the standard curve. The *L. monocytogenes* load was determined to be $4x10^3$ cfu/g. Of the 9 positive samples after enrichment, only one was fresh meat and all the others were again cheeses. Surprisingly none of the Palcam plates streaked or spread presented suspected colonies of *L. monocytogenes*.

4. DISCUSSION

The aim of this study was the development of qPCR protocols in order to detect, quantify and define the vitality of *L. monocytogenes* in foodstuffs. In the last couple of years several papers have been published proposing different protocols for the detection and quantification, but only few examples are available to quantify live *L. monocytogenes* cells without enrichment steps. Here we attempted to work at DNA and RNA level in order to be able to quantify both the total cell number or only the viable cells, respectively. We could not rely on the amplification of specific virulence genes from *L. monocytogenes*, such as *hly* (Guilbaud et al., 2005; Rodriguez-Lazaro and Hernandez, 2006), *inlA* (Navas et al., 2006) or *prfA* (Rossmanith et al., 2006) to define the number of alive cells because, as previously reported (Bohne et al., 1996; Leimeister-Wächter et al., 1992), these genes are under the regulation of environmental factors such as the growth medium composition or the temperature. For this reason we decided to target the amplification on the ribosomal RNA genes, in a way that we could have a direct indication of the vitality based on the specific rRNA presence. After alignment of the rRNA operon of the *Listeria* species, a high level of homology was observed. Enough divergence was found in the IGS region between the 16S and 23S rRNA genes that allowed us to design two primers and a Taqman probe, specific for *L. monocytogenes* (Tab. 1). The protocol, after optimization, resulted to be highly specific for *L. monocytogenes* only, since no amplification signal was obtained when DNA or RNA extracted from other *Listeria* spp. or non-*Listeria* spp. was used in the qPCR protocol.

The following experimental step carried out was the construction of standard curves inoculating serial dilutions of *L. monocytogenes* in several food matrices. Moreover, cells diluted in water were also processed in order to calculate the efficiency of amplification and the coefficient of correlation in a system that does not contain any inhibitor. At DNA level, as

shown in Figure 1 and Table 2, the values obtained for cells in water resulted to be satisfactory, underlining the suitability of the method developed for the quantitative amplification of *L. monocytogenes*. When the standard curves were prepared from DNA of *L. monocytogenes* dilutions inoculated in food matrices, a food-dependent result was observed. While the quantification limit was calculated to be 10^3 cfu/g or ml in almost all of the foods considered in this study (only for fresh meat it was 10^4 cfu/g), the efficiencies of amplification were different, being the worst for the soft cheese, fresh meat and fermented sausages. However, the R^2 values for all the matrices were acceptable. The differential efficiency found in this study should be explained by considering a different capability of the kit used to purify the nucleic acids from the food matrices, thereby eliminating qPCR inhibitors. In this study we decided to process food samples with a kit produced by Epicenter, after several trials in which either mechanic/enzymatic treatment as described by Rantsiou et al., (2004), chelex-100 based DNA purification (Rodriguez-Lazaro et al., 2004) or other commercial kits (DNeasy Blood & Tissue Kit, Qiagen) were tested. The best results in terms of efficiency, linearity range and quantification limit, were obtained by using the kit from Epicenter (data not shown). An increase in the sensitivity of the protocol may be obtained by pre-processing the food sample before nucleic acid extraction. Indeed, recently Fukushima et al., (2007) proposed a buoyant density gradient centrifugation as concentration method for 12 food-borne pathogens. The detection limit of the protocol varied from 10 to 10^3 cfu/g, showing good applicability for *Salmonella* and *C. jejuni* for which the detection of 10 to 10^2 cfu/g in naturally contaminated chicken was obtained in 3 h. However, the results in terms of quantification limit obtained here are in agreement with reports of other authors who developed qPCR protocols to quantify *L. monocytogenes* in meat (Rodriguez-Lazaro et al., 2004) and in salmon products (Rodriguez-Lazaro et al., 2005).

At RNA level, the results obtained from the construction of standard curves in different food matrices, allowed the quantification of viable cells of *L. monocytogenes* only if loads were higher than 10^4-10^5 cfu/g or ml. This result may be due to the expression pattern of the IGS region, used for the specific amplification, during growth of *L. monocytogenes*. In order to verify this hypothesis, the expression of the IGS region was monitored during growth and it was determined that IGS expression is decreasing during the stationary phase (data not shown). Since all the standard curves were constructed inoculating cell dilutions prepared from overnight cultures, we assume that the mRNA related to the IGS was already in low quantity and this is explaining the poor sensitivity of the method. We looked into other genes in order to increase the sensitivity of the protocol, but considering normally used housekeeping genes, such as 16S rRNA gene or *rpoB* gene, *L. monocytogenes* could not be differentiated from other *Listeria* spp. An alternative qPCR method for the detection of viable and dead *L. monocytogenes* has been proposed by Rudi et al., (2005), who used an ethidium monoazide bromide (EMA)-PCR. In this study the combined growth and qPCR complemented by the EMA-PCR enabled semi quantitative detection of low levels of culturable cells. Here, the problem related to the low sensitivity of the method at RNA level was surpassed considering the results obtained after the enrichment step.

If we take into consideration the results of the sensitivity at DNA level obtained after an overnight enrichment, we can assess that the qPCR protocol is able to determine the presence of at least 10 cfu/ml or g of alive *L. monocytogenes* in foods. In this context, Navas et al., (2006) reported an increase of the sensitivity of detection of *L. monocytogenes* by qPCR when a secondary enrichment is performed. From a value of 37% positive samples after the primary enrichment, the percentage increased to 70% after the second enrichment, suggesting the possibility to use a second enrichment step when very low numbers of *L. monocytogenes* are expected. However, in the last case the time needed for the analysis is increasing and partially

eliminates the benefits related with the use of qPCR for detection and quantification of *L. monocytogenes* in food.

Lastly, we tested the developed qPCR protocol in food samples collected from the market or from small processing plants. As shown in Table 3, the highest incidence of *L. monocytogenes* was found in cheeses produced from raw milk, where 8 samples on a total number of 41 resulted positive. Of these, 4 gave amplification signals also without enrichment, but only for one we could quantify the load. This sample was a fresh cheese that is commercialized 8 hours after production. In our opinion, the cheese samples for which quantification was not possible, contained a number of *L. monocytogenes* very close to the quantification limit defined as 10^3 cfu/g or ml. Only 1 meat sample was found positive after the enrichment step. The results obtained here are highlighting once more the risk associated with the consumption of raw milk and raw milk cheeses as recently reaffirmed (US FDA/CFSAN, 2007). The results obtained by qPCR did not agree with the cultural method. As a matter of fact, the plates of Palcam agar that were spread or streaked before and after the enrichment in BHI broth, respectively, did not show any *L. monocytogenes* suspected colony. However, it is important to note that we did not use the official method for the isolation of *L. monocytogenes* from food. The main reason why this was not performed was the known and described inhibitory effect of the Fraser broth, normally used in the pre-enrichment steps, on the PCR method (Rossen et al., 1992). Since the protocol developed showed high specificity towards *L. monocytogenes* we interpret the results as false negative of the cultural method. This is possibly due to strong competition of other microorganisms present in the food samples and able to proliferate in the BHI used for the enrichment, that did not allow the detection on the plates of *L. monocytogenes*.

The approach described in this paper represents a contribution to the state of the art of the quantification of *L. monocytogenes* in food samples by qPCR. It can be used in order to monitor the presence and persistence of this pathogen in different food matrices. Moreover it gives the possibility to quantify as low as 10^3-10^4 cfu/g or ml and determine viable populations of at least 10 cfu/g or ml after an enrichment step.

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Table 1. Primers and probes specific for *L. monocytogenes*

Primer name	Sequence (5'-3')	Concentration in the PCR mix
IGS 1	GGCCTATAGCTCAGCTGGTTA	400 nM
IGS 2	GCTGAGCTAAGGCCCCGTAAA	400 nM
Probe IGS	FAM-ATAAGAAATACAAATAATCAT-TAMRA	250 nM

Table 2. Efficiencies of amplification (%) and R^2 of the standard curves in different matrices

Matrix	**Efficiency (%)**	**R^2**
Water	95	0.970
Milk	101	0.961
Soft cheese	77	0.977
Fresh meat	68	0.984
Cured ham	107	0.993
Fermented sausages	119	0.987
Ready-to-eat salad	88	0.993

Table 3. Results obtained for the detection and quantification of *L. monocytogenes* in food samples collected from the market and from small food producers

Food samples	N° of samples	qPCR signals[a]			
		T_0		T_{24}	
		+	-	+	-
Fresh meat	20	0	20	1	19
Fresh sausages	2	0	2	0	2
Fermented sausages	2	0	2	0	2
Fresh cheeses	31	4[b]	27	8	23
Ripened cheeses	11	0	11	0	11
Total	66	4	62	9	57

[a]T_0, without enrichment; T_{24}, after enrichment at 37°C in BHI broth overnight

[b]Only for one sample the quantification was possible and it resulted to be $4x10^3$ cfu/g

FIGURE LEGENDS

Figure 1. DNA standard curve of *L. monocytogenes* cell dilutions in water.

Figure 2. DNA standard curve of *L. monocytogenes* cell dilutions in meat (A), cured ham (B) and fermented sausages (C).

Figure 3. DNA standard curve of *L. monocytogenes* cell dilutions in milk (A) and soft cheese (B).

Figure 4. DNA standard curve of *L. monocytogenes* cell dilutions in ready-to-eat salad.

Figure 1

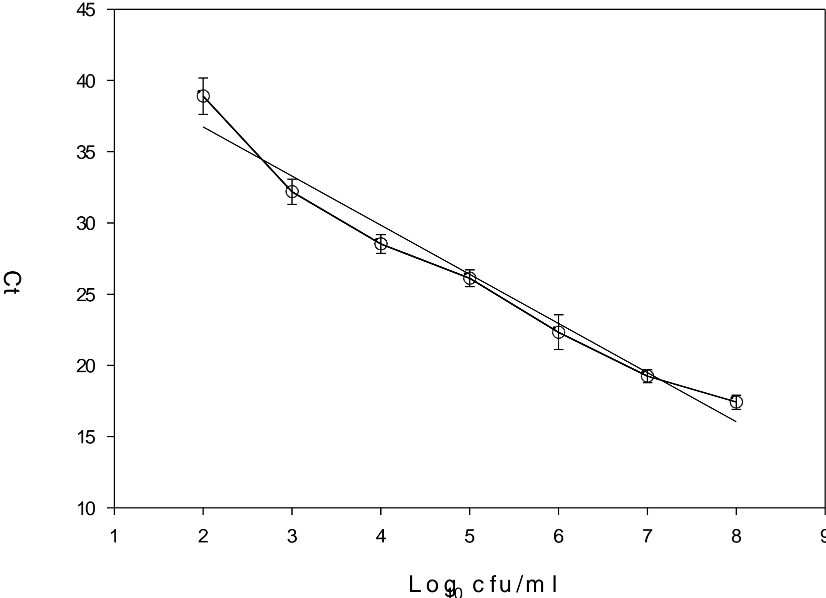

Figure 2

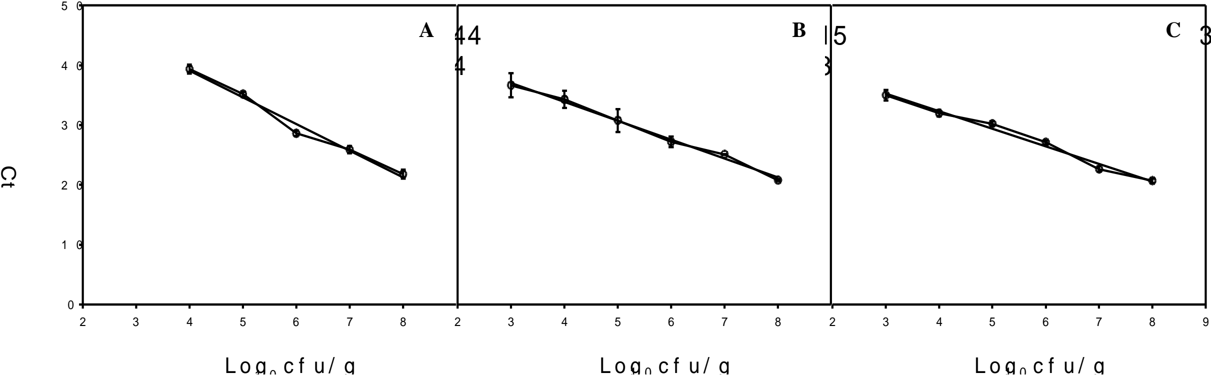

Figure 3

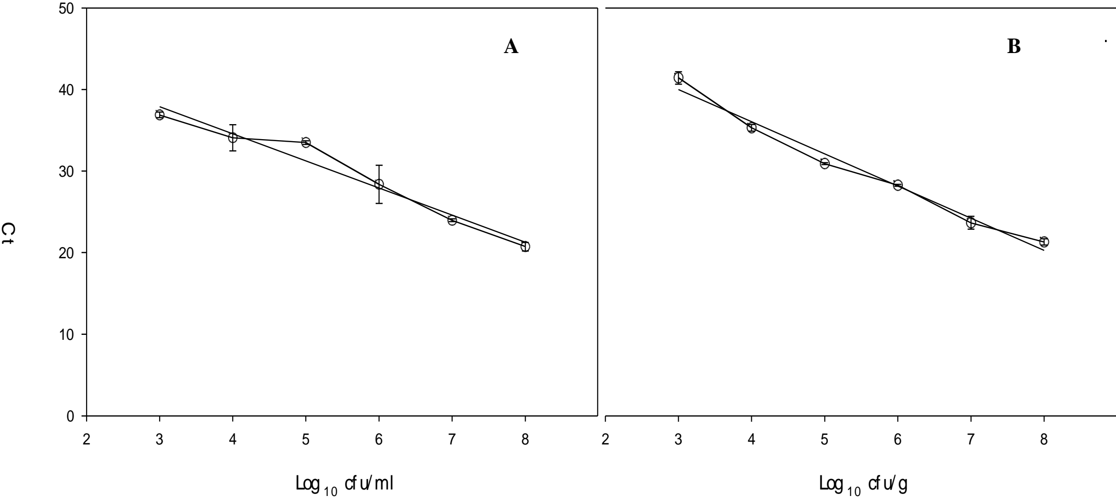

Figure 4