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# Detection of foodborne pathogens by qPCR: A practical approach for food industry applications

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**Abstract:** Microbiological analysis of food is an integrated part of microbial safety management in the food chain. Monitoring and controlling foodborne pathogens are traditionally carried out by conventional microbiological methods based on culture-dependent approaches in control laboratories and private companies. However, polymerase chain reaction (PCR) has revolutionized microbiological analysis allowing detection of pathogenic microorganisms in food, without the necessity of classical isolation and identification. However, at present, PCR and quantitative polymerase chain reaction (qPCR) are essential analytical tools for researchers working in the field of foodborne pathogens. This manuscript reviews recently described qPCR methods applied for foodborne bacteria detection, serving as economical, safe, and reliable alternatives for application in the food industry and control laboratories. Multiplex qPCR, which allows the simultaneous detection of more than one pathogen in one single reaction, saving considerable effort, time, and money, is emphasized in the article.

**Subjects: Science; Microbiology; Food Science & Technology; Food Analysis; Food Microbiology**

**Keywords: PCR; qPCR; pathogen; detection; industry**

## 1. Introduction

In order to ensure food safety, it is necessary to consider all aspects of the food production chain. The free movement of safe and wholesome food is an essential aspect of the internal market and contributes significantly to the health and well-being of citizens, and to their social and economic interests. A high level of protection of human life and health should be assured in the pursuit of community policies (EC, 2002).

## ABOUT THE AUTHORS

The authors perform their research activity in the Food Safety Division and Microbiology and Bioassays Laboratory of ANFACO-CECOPESCA. Here, they have developed an extensive work on development of new methods and implementations, in order to provide fast and reliable tools which are intended for food industry, with the final goal of enhancing food safety, by determining the cause specific problems such as marine biotoxins and foodborne pathogens detection, control, reduction, and/or elimination from their food products.

## PUBLIC INTEREST STATEMENT

This manuscript is intended to provide readers, especially for those who develop their professional activity related in the food industry, a general idea about how molecular methods, particularly qPCR, may be applied for controlling foodborne pathogens. Two main fields are covered, on the one hand, a more scientifically oriented overview of PCR/qPCR is included, summarizing from the technique fundamentals to primer/probe design and advanced multiplex applications. On the other hand, a detailed description of available methodologies is provided for the different bacterial pathogens covered, intended to be helpful for people looking for direct industry applications.

During the year 2012, the number of food alerts and notifications received through the European alert information system or Rapid Alert system for Food and Feed (http://www.eftasurv.int) has risen to a total of 2,801 for food products. This number includes different contaminants, of which 462 (16%) have been alerts, 869 (31.5%) have been information notifications, and 1476 (52.5%) were rejections in border. Microbiological contamination corresponds to 21% of notifications, that *Salmonella* spp. has the highest risk and causes 49% of the microbiological contamination (Agroalimentaria, 2012). All these notifications and risks have led to extensive analyses of foodstuffs before their release and distribution to the market.

Networking of laboratories of excellence, at regional and/or interregional level, with the aim of ensuring continuous monitoring of food safety, could play an important role in the prevention of potential health risks for consumers as well as of unnecessary economic costs. In this sense, the availability of quick and quality assays allows giving precise results in a few hours and results as a great economic and safe advantage. Among microbiological risks that can lead to foodborne diseases, *Salmonella*, *Listeria monocytogenes,* and *Escherichia coli* are pathogenic micro-organisms that keep rising in numbers through recent years (Commission, 2011). According to several ISOs: ISO 6579, ISO 11290-1 and -2, and ISO 16654 for *Salmonella*, *L. monocytogenes,* and *E. coli,* respectively (ISO, 1996, 1998, 2001, 2003) complete analyses for these pathogens can take from two to seven days since the sample arrives to the laboratory. This period can be longer if the pathogenic serotype have to be determined, what means that foodstuffs have to be immobilized and waiting for the release until obtaining the final results. It is worth to mention that if the food is fresh, it can be completely spoiled and even if is a ready-to-eat product, it can loss the quality and not be allowed for consumption. In the case of products with a short shelf life, if the immobilization is very long, the foodstuffs can reach the expiry date or be close to that with all the consequent commercial problems that can prevent to place these products in the market and all derived economic losses included those that have to be paid due to the destruction of food.

All these facts highlight the necessity of the development of fast, feasible, and accurate analytical methods in order to allow a product to be released immediately or, at least, in a very short period of time, to prevent or decrease no necessary costs and food spoilage. Figure 1 shows a schematic comparison of traditional culture methods and quantitative polymerase chain reaction (qPCR) detection methods.

## 2. Impact of multiplex real-time polymerase chain reaction in routine food control

The food microbiological testing is experiencing an exciting momentum due to the development in the analytical field of new and accurate approaches. Polymerase chain reaction (PCR) has revolutionized traditional microbiological analysis by allowing detection of pathogenic micro-organisms directly in the food, without the necessity of classical isolation and identification. Since its discovery it has became an essential analytical tool for researchers working in the foodborne pathogens field (Wernars, Delfgou, Soentoro, & Notermans, 1991; Wernars, Heuvelman, Chakraborty, & Notermans, 1991). This technique was used in more than 300,000 scientific publications, and was applied in different areas of research, including food safety, due to its versatility, specificity, and sensitivity. In recent years, PCR has been successfully applied in food science for microorganisms identification (Rodriguez-Lazaro et al., 2007), for detection of ingredients in food products (Lopez-Calleja et al., 2013) and different meats and fish species identification (Chapela et al., 2002; Mackie et al., 1999; Quinteiro et al., 1998; Rodríguez-Lázaro, Cook, & Hernández, 2013; Soares, Amaral, Oliveira, & Mafra, 2013; Sotelo et al., 2001; Zhang, 2013).

### *2.1. PCR and multiplex PCR*

Conventional PCR can be described as an exponential amplification of a DNA fragment. Its principle is based on the mechanism of DNA replication *in vivo*, i.e. double-stranded DNA (dsDNA) is denatured to single-stranded DNA (ssDNA) and duplicated by a DNA polymerase (Higuchi, Fockler, Dollinger, & Watson, 1993). Usually, PCR is performed using one pair of primers to amplify a specific sequence; however, multiplex PCR allows the simultaneous amplification of more than one target sequence in

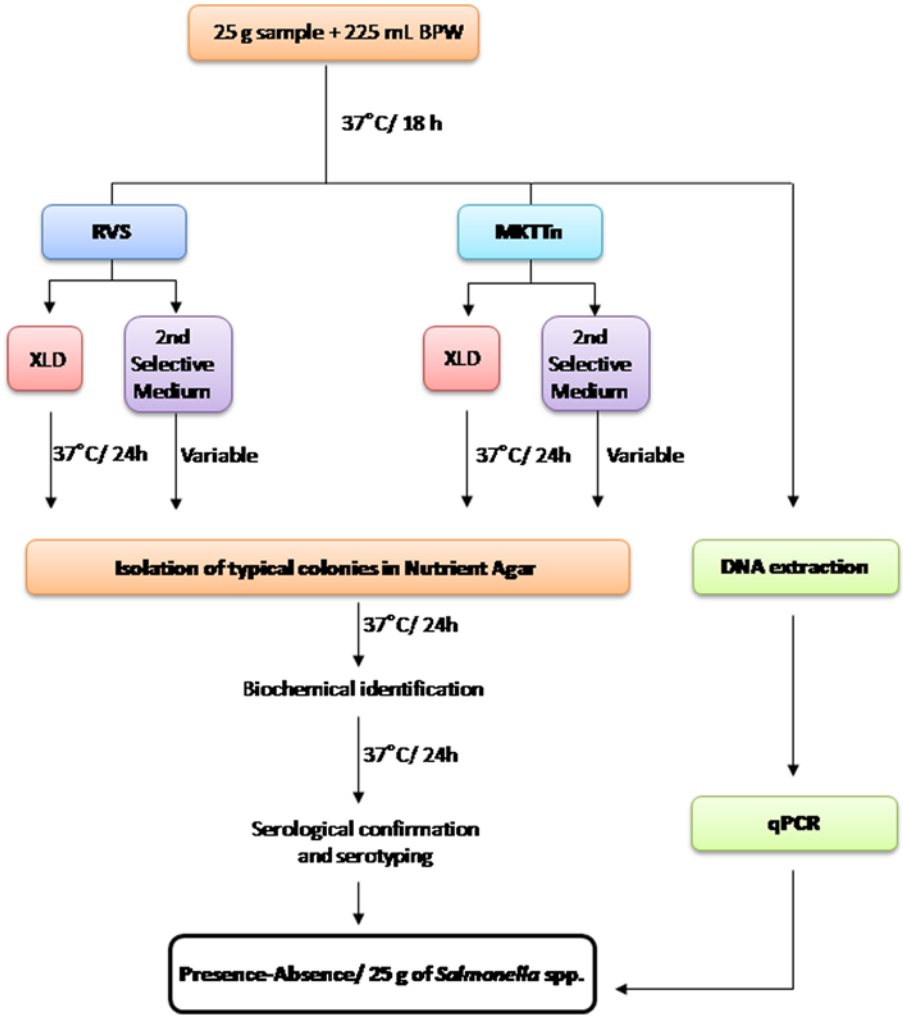


**Figure 1. Schematic comparison of traditional culture methods and qPCR detection methods.**

Notes: BPW: Buffered Peptone Water; RVS: Rappaport-Vassiliadis Soya; XLD: Xylose Lysine Deoxycholate agar; MKTTn: Muller–Kauffmann Tetrathionate-Novobiocin Broth.

a single reaction by using different sets of primers (Elnifro, Ashshi, Cooper, & Klapper, 2000). This option saves considerable time and effort, and decreases the number of reactions that need to be performed to detect the desired targets in the sample. However, the presence of many PCR primers in a single tube can cause some problems, such as the increased formation of misprimed PCR products, known as "primer dimers," and the amplification of unspecific DNA fragments. Primers should be chosen with similar annealing temperatures for multiplex PCR assays. Also, the lengths of amplified products should be similar, since large differences in the lengths of the target DNAs may favor the amplification of shorter targets over the longer ones, which results in different yields of amplified products. In standard PCR protocols, amplification products are detected by gel electrophoresis using UV luminescence and the ethidium bromide double-stranded DNA intercalating dye or similar non-toxic compounds. These procedures have the big disadvantage of relying on end-point analysis; therefore, amplification products cannot give quantitative information regarding the initial amount of target molecules (Rodríguez-Lázaro & Hernández, 2013).

### *2.2. qPCR and multiplex qPCR*

Higuchi and collaborators (Higuchi, Dollinger, Walsh, & Griffith, 1992; Higuchi et al., 1993) constructed a system that detects PCR products as soon as they accumulate. This was the beginning of the real time or qPCR, also known as the second generation of PCR. qPCR allows the monitoring of the amplification process in real time by using fluorescence, and not only at the end of the reaction as occurs in conventional PCR. Its major advantages are that it is a fast and easy-to-perform analysis,

the extremely wide dynamic range of quantification (more than eight orders of magnitude), and the significantly higher reliability and sensitivity of the results compared to conventional PCR. Since the first scientific work published in 1996 (Heid, Stevens, Livak, & Williams, 1996), the number of publications where qPCR is used has increased nearly exponentially. Specific detection and quantification of pathogens in food by qPCR has been evaluated for a wide variety of micro-organisms, with emphasis on the main foodborne pathogens responsible for important medical and economic outbreaks: *Salmonella* spp., *L. monocytogenes*, *E. coli* O157:H7, and *Staphylococcus aureus*, among others (Rodríguez-Lázaro et al., 2013).

The major advantage of this molecular method with respect to standard methods is the shorter time required to obtain reliable results. Typical culture methods as ISO or FDA Bacteriological Analytical Manual—for *Shigella* spp., *E. coli* O157, and different human pathogenic *Vibrio* spp. (*V. cholerae*, *V. parahemolyticus,* and *V. vulnificus*) detection—need two days to guarantee a negative result. Regarding *Salmonella* spp. and *L. monocytogenes,* three and five days are needed for a definitive negative result. In contrast, when a positive result is found, the duration of the analyses is greatly extended as additional isolation and identification steps must be performed. However, recent studies have demonstrated that times can be significantly shortened to only 24 h by optimizing qPCR methods for most of these bacteria (Chapela et al., 2010; Garrido, Chapela, Ferreira, et al., 2012; Garrido, Chapela, Román, et al., 2012; Garrido, Chapela, Román, Fajardo, Lago, et al., 2013).

### *2.3. Digital PCR*

Digital PCR (dPCR) allows the precise quantification of nucleic acids, facilitating the measurement of small percentage differences and quantification of rare variants. It was reported to be more reproducible and less susceptible to inhibition than qPCR. Its main applications are: rare variant measurement, molecular counting, and applications that need higher precision. Due to its growing interest, a "Minimum Information for Publication of Quantitative dPCR Experiments" guide has been recently published (Huggett et al., 2013).

### *2.4. Limit of detection and limit of quantification of qPCR*

Concerning the limit of detection (LOD) of qPCR, it was evaluated in several multiplex studies targeting different types of bacteria (including Gram positive and Gram negative) from different origins. Obtained results were similar to traditional culture methods or even lower, with values in general below 10 CFU/25 g, with the added advantage that, with multiplex qPCR, several targets were detected simultaneously (Garrido, Chapela, Ferreira, et al., 2012; Garrido, Chapela, Román, et al., 2012; Garrido, Chapela, Román, Fajardo, Lago, et al., 2013). Low levels of target pathogens can also be detected in food matrices contaminated by other dominant micro-organisms. Garrido and collaborators demonstrated in two independent studies that *L. monocytogenes* could be successfully enriched and detected in the presence of high initial numbers of other interfering bacteria, generally above $10^6$ CFU/25 g (Garrido, Chapela, Román, Fajardo, Lago, et al., 2013).

However, in a great number of cases, the detection and quantification limits obtained without enrichment of the food samples prior to performing qPCR were in the range of 102–103 CFU/g (or ml) (Hierro, Esteve-Zarzoso, Gonzalez, Mas, & Guillamon, 2006; Takahashi et al., 2009). Therefore, in order to meet the microbiological criteria required by international legislations for foodstuffs, it is sometimes necessary to associate qPCR with an enrichment step of a few hours.

When qPCR was used, not only for detection, but also as a quantitative tool, linear quantifications were reported over a large range of at least five logs and very good correlations with plate counts were obtained. In some cases, however, discrepancies between microbiological counts and qPCR have been reported with higher bacterial counts with the molecular method (Martínez-Blanch, Sánchez, Garay, & Aznar, 2009). Several reasons can explain the differences: (1) the presence of intact DNA from dead cells, (2) the presence of viable but non-culturable forms, which can be quantified by qPCR but not by plate counts, (3) the fact that one CFU on plate might be generated from more than one cell, and (4) the use of PCR primers targeting varying numbers of multicopy genes

(e.g. 16S rRNA). An important distinction should be made between LOD and quantification limits (LOQ). This distinction may lead to confusing comparisons between sensitivities of different methods. The LOD is the lowest population of micro-organisms that can be detected by the method. The LOQ is the minimal population that can be accurately quantified. Usually, LOD is lower than LOQ, meaning that when qPCR gives a positive signal, the amount of template is too low and provides a quantification cycle (Cq) that falls out of the linear range of quantification curves. When quantifying pathogens in food, the lower LOQ in the food matrix should be considered and not the lower LOQ obtained from pure cultures because it takes into account the efficiency of nucleic acid extraction and possible interactions of food components with PCR amplification. In agreement with EN ISO 22174:2005, a standard for PCR application in order to detect foodborne pathogens, and with experts in PCR/qPCR (Hoorfar et al., 2003; Hoorfar, Cook, et al., 2004; Hoorfar, Malorny, et al., 2004; ISO, 2005), an increasing number of studies have included an internal amplification control to qPCR protocols, to avoid negative deviations (Calvó, Martínez-Planells, Pardos-Bosch, & Garcia-Gil, 2008; Garrido, Chapela, Ferreira, et al., 2012; Garrido, Chapela, Román, et al., 2012; Garrido, Chapela, Román, Fajardo, Lago, et al., 2013; Jofré et al., 2005; Nordstrom, Vickery, Blackstone, Murray, & DePaola, 2007).

Nowadays, the current trend is moving towards identification of several pathogens in the same reaction tube, by applying multiplex amplification. Some qPCR technologies are particularly adapted to multiplexing. For instance, using TaqMan chemistry, several sequence-specific probes can be labeled with different fluorophores and many targets can be co-amplified and quantified within a single reaction. By contrast, in theory, the widely used SYBR Green chemistry does not allow multiplexing reactions because binding takes place non-specifically in the presence of DNA. However, some authors have circumvented the problem and successfully proposed multiplex qPCR protocols with SYBR Green by performing subsequent melting curve analysis. Distinct melt curves were obtained for each target micro-organism (Wehrle, Didier, Moravek, Dietrich, & Märtlbauer, 2010). Therefore, multiplex qPCR is an interesting tool to quickly detect different genera or species which are potentially present in the same food matrices.

qPCR has revolutionized the molecular approaches in food microbiology; specific micro-organisms can be quantified in food, and there is no need for post-amplification treatment of the samples, such as gel electrophoresis, saving time and efforts. Currently, a high number of qPCR chemistries are commercially available. These can be divided into those that are not sequence specific, such as DNA minor groove-binding dyes, and those that are sequence specific and that allow simultaneous detection and confirmation of the target amplicon during the PCR reaction (Rodríguez-Lázaro et al., 2013).

### *2.5. qPCR primers and probes design*

When a qPCR assay is designed, the most important parameters are the amplicon length and the Tm of the primers and probe. The optimal amplicon length should be less than 150 bp; however, amplicons up to 300 bp amplify efficiently. Shorter amplicons amplify more efficiently than longer ones and are more tolerant to suboptimal reaction conditions. See Table 1 for details regarding TaqMan® probes assays that may be extensive to other type of qPCR methods.

**Table 1. General guide for qPCR hydrolysis probes design**

| | |
|---|---|
| Primers length | 20–24 bp |
| Primers Tm | 60 ± 2°C |
| Amplicon size | 140–300 bp |
| Probe length | 20–24 bp |
| Probe Tm | Primer Tm + 10°C |
| Additional considerations | Avoid "G" in 5′ end, secondary and repetitive structures |
| | Probe placed no more than 10 bp from the forward primer and not less than 2 bp |

Notes: Tm is the melting temperature; "G" is the guanine; and "bp" is the base pairs.

## 3. Importance of sample preparation methods

### *3.1. Enrichment broths*

The combination of microbiological enrichment and molecular pathogen detection is a useful link between traditional and molecular microbiology. The advantages of both methods add up, and the disadvantages are partly eliminated. Then, the LOD of qPCR is far exceeded after a successful enrichment. Short enrichments, if performed in an appropriate way, are mostly sufficient to provide enough DNA for detection (Rossmanith & Wagner, 2010). In general, pathogenic bacteria are present in foods in very low numbers; thus, an enrichment step to increase their number is crucial in order to guarantee the achievement of legal limits (absence in 25 g for most bacterial pathogens). Extensive evaluation of optimal enrichment conditions, as well as incubation times and DNA extraction protocols has been approached for different pathogens (Gattuso et al., 2014; Rodríguez-Lázaro, Gonzalez-García, Gattuso, Gianfranceschi, & Hernandez, 2014).

When facing multipathogen detection, a popular approach is to select different broths for the enrichment of every pathogen; then DNA is extracted and multiplex qPCR is performed (Elizaquível, Gabaldón, & Aznar, 2011; Jofré et al., 2005; Ruiz-Rueda, Soler, & Calvo, 2010). An alternative is the use of a common broth for all the target pathogens, but the broth design and its performance must be carefully evaluated. In this sense, one of the first broths designed for several bacteria was the Universal Pre-enrichment Broth (UPB) for the simultaneous recovery of *Salmonella* spp. and *L. monocytogenes* (Bailey & Cox, 1992). This broth, which is still in use (Bhagwat, 2004; Hammack, Amaguana, Johnson, & Andrews, 2003), does not contain any inhibitory substance, allowing a better recovery of the bacteria due to its high buffer concentration. Previous studies have demonstrated that methods which incorporate a non-selective enrichment step gave better results than completely selective media, specially for the recovery of slow growing bacteria such as *L. monocytyogenes* (Rijpens & Herman, 2004). Following this concept, Kawasaki et al. developed a similar broth named N17 (Kawasaki et al., 2005) which has been applied in different studies for recovery of *Salmonella* spp., *L. monocytogenes,* and *E. coli* O157 (Kamisaki-Horikoshi et al., 2011; Kawasaki et al., 2009, 2010, 2011; Mahmuda & Kawamoto, 2007). The N17 broth was modified in later studies by independent laboratories for an optimal performance by eliminating dextrose (Omiccioli, Amagliani, Brandi, & Magnani, 2009), and later on, the final pH of the broth was also modified by adjusting the buffering salts concentrations. This optimized broth was successfully applied for the simultaneous enrichment of *Salmonella* spp., *L. monocytogenes*, *Shigella* spp., and *E. coli* O157 (Garrido, Chapela, Román, et al., 2012; Garrido, Chapela, Román, Fajardo, Lago, et al., 2013). Excellent results were obtained with the modified version of N17 broth with all the bacteria tested, obtaining colony counts higher than $10^6$ CFU/ml for all microorganisms tested. Another non-selective enrichment broth recently developed is the simultaneous enrichment broth (SEB) developed by Kobayashi et al. SEB was applied for the simultaneous recovery of *Salmonella* spp., *L. monocytogenes*, *S. aureus*, *Bacillus cereus*, *Vibrio parahaemolyticus,* and *E. coli* O157:H7; and its performance was compared to tryptic soy broth (TSB) (Kobayashi et al., 2009). Even though satisfactory results were obtained when compared against TSB, further studies need to be performed with natural and spiked food and environmental samples.

A different approach was done by other researchers who developed selective enrichment broths for the simultaneous recovery of different bacteria, like the SEB SEL that included four antimicrobial agents—acriflavine, cycloheximide, fosfomycin, and nalidixic acid. SEL showed comparable results to UPB (Kim & Bhunia, 2008) and it was successfully applied for the recovery of *Salmonella*, *L. monocytogenes,* and *E. coli* O157 in meat products (Suo, He, Tu, & Shi, 2010).

### *3.2. DNA extraction protocols*

Inhibition of the PCR amplification reaction is one of the most limiting factors of the PCR-based methods and it can cause complete reaction failure, leading to false negative results or reducing sensitivity of detection. Inhibition might be driven by interference with the cell lysis necessary for DNA extraction, by nucleic acid degradation or capture, and by inhibition of the polymerase activity

necessary for amplification of the target DNA. Organic and phenolic compounds and polysaccharides are also possible inhibitors (Elizaquivel & Aznar, 2008). In this sense, a critical factor when facing multipathogen detection is the selection of an appropriate DNA extraction protocol. In addition, the type of bacteria of interest must be kept in mind as it is more difficult to extract DNA from Gram positive bacteria, than from Gram negative. For Gram negative bacteria, a thermal cell lysis in the presence of a chelating resin—e.g. 6–10% (w/v) Chelex-100 suspension—is usually enough to obtain good-quality DNA (Garrido, Chapela, Román, et al., 2012; Kim & Cho, 2010; Malorny, Huehn, Dieckmann, Kramer, & Helmuth, 2009; Rodriguez-Lazaro, Jofre, Aymerich, Hugas, & Pla, 2004). Reyes-Escogido et al. developed a chelex-based method that replaced the boiling water bath by a microwave heating procedure, as the thermal lysis source, and by adding proteinase K and RNase A obtained large amounts of highly pure DNA from Gram positive and negative bacteria (Reyes-Escogido et al., 2010); but this method has been scarcely used.

In-house developed protocols have shown appropriate results regarding the quantity and purity of DNA extracted from pure and mixed cultures, as well as from natural food and environmental samples. Kawasaki et al. developed and compared a DNA extraction protocol based on the use of a strong chaotropic agent (guanidine isothiocyanate) combined with a surfactant agent (Tween 20) and an enzymatic lysis step (combination of lysozyme and achromopeptidase enhanced the enzymatic extraction) (Kawasaki et al., 2005). This protocol was successfully applied in different studies and compared against other in-house protocols, showing similar or better results, specially focused on Gram positive bacteria (Garrido, Chapela, Román, Fajardo, Lago, et al., 2013; Garrido, Chapela, Román, Fajardo, Vieites, et al., 2013; Germini, Masola, Carnevali, & Marchelli, 2009; Mahmuda & Kawamoto, 2007). Later studies reported that DNA extraction may be enhanced by the addition of a filtration step through a 11–5 µm sterile micropore filter (Kawasaki et al., 2009; Rodriguez-Lazaro, Jofre, et al., 2004), being this in agreement with other studies reporting that the presence of food particles should be avoided (Malorny et al., 2009) as it may have different effects in the qPCR reaction, like the Cq of the final fluorescence obtained (Wright et al., 2007).

On the opposite side of in-house protocols, are commercial DNA extraction kits. Different studies have been applied and several of these kits were compared, like PrepMan Ultra® (Applied Biosystems), InstaGene® (BioRad), DNeasy Tissue Kit® (Quiagen), UltraClean Microbial DNA isolation kit® (MoBio), NucleoSpin Tissue Kit® (Machery-Nagel), Nexttec kit for genomic DNA from Bacteria® (Nexttec), QIAmp DNA stool mini kit® (Quiagen), and High Pure PCR template® (Roche), among others (Elizaquivel & Aznar, 2008; Flekna, Schneeweiss, Smulders, Wagner, & Hein, 2007). Out of these, PrepMan Ultra® and InstaGene® have been extensively applied, especially with Gram negative bacteria. The DNeasy Tissue Kit is one of the most frequently selected kit as it has proven suitable for different types of samples and with different types of micro-organisms (Elizaquivel & Aznar, 2008; Elizaquível et al., 2011; Jofré et al., 2005; Kobayashi et al., 2009; McGuinness et al., 2009).

## 4. Target genes and micro-organisms

The bacterial ribosomal operon (16S rRNA, 23S rRNA, and intergenic spacer (IGS) region) has been frequently used as target for PCR amplification. It is ubiquitous, bears both variable and highly conserved sequences, is easily available from public databases for many species, and often results in sensitive detection due to its multicopy nature. Detailed sequence analysis has demonstrated that the 16S rRNA gene is suitable for accurate PCR identification of many pathogens (Chakravorty, Helb, Burday, Connell, & Alland, 2007). While its discriminating power might be sufficient for some genera and species, it is not always enough to distinguish closely related species (e.g. for enterococci or *B. cereus* group members). Therefore, other housekeeping genes have been studied, as well as functional genes involved in virulence or metabolism. Greater availability of genome sequences, generalization of high-throughput molecular tools and progress in computational genomics are facilitating the set up of new qPCR protocols based on a large variety of genes, thus increasing test specificity.

Nowadays, bacterial foodborne pathogen detection by PCR and qPCR is based on the detection of one or multiple specific genes; thus, correct selection of the target along with the design/selection

of the primers and probes is a key factor for the correct identification of bacterial hazards. For every single pathogenic bacterium, there is a plethora of possible gene targets; thus lack of consensus is a general situation, even though a higher degree of agreement may be observed for the detection of certain micro-organisms.

According to the FoodNet, in 2012, 19,531 cases of infection were laboratory confirmed in the US, being the main bacterial pathogens identified *Salmonella* spp. (16.42%), *Campylobacter* spp. (14.30%), *Shigella* spp. (4.50%), Shiga-toxin-producing serotypes (STEC) non-O157 (1.16%), STEC O157 (1.12%), *Vibrio* spp. (0.41%), and *Listeria* (0.25%) (CDC, 2013). These data highlight the importance that these pathogens still represent for human health, even some have been studied for long time.

### *4.1. Legislated pathogens according to Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs*

#### *4.1.1. Salmonella spp.*

Nowadays, several alternatives exist for the specific detection of *Salmonella* spp. These include the *bipA* which encodes a protein of the guanosine -5′-triphosphate (GTP)-elongation binding family that displays global modulating properties by regulating a wide variety of downstream processes (Calvó et al., 2008), the *fimC*, which is involved in the synthesis of type 1 fimbria, and has been successfully applied for the detection of the species *S. enterica* (Krascsenicsová, Piknová, Kaclíková, & Kuchta, 2008; Piknová, Kaclíková, Pangallo, Polek, & Kuchta, 2005), the *ttrRSBCA* locus, which is located near the *Salmonella* pathogenicity island 2 at centisome 30.5. It is required for tetrathionate respiration in *Salmonella* (Hyeon et al., 2010; Malorny et al., 2004). Delibato et al., reported a European interlaboratory validation based on the method described by Josefsen et al., (Delibato et al., 2014; Josefsen, Krause, Hansen, & Hoorfar, 2007). Another gene used for *Salmonella* detection is the *iagA*, the invasion associated gene (Chua & Bhagwat, 2009).

One of the most frequently used genes to perform the detection of *Salmonella* spp. is the *invA*. This gene allows the entrance of the bacterium in the intestinal epithelial cells. Even though previous authors have described the existence of strains lacking this gene, like *S*. Saintpaul, while others stated, in the case of this strain, that they could successfully detect it. It was hypothesized that strains lacking this gene could not penetrate the intestinal cells, thus not being able to cause disease. A similar situation is observed with serovars Litchfield and Senftenberg, although, in general, absence of *invA* seems to be rare (Anderson et al., 2011; Calvó et al., 2008; Cheng et al., 2008; D'Urso et al., 2009; González-Escalona, Brown, & Zhang, 2012; Jofré et al., 2005; Kimura et al., 1999; Malorny, Hoorfar, Bunge, & Helmuth, 2003; Malorny et al., 2004; Rahn et al., 1992). Even though these slight discrepancies were observed by different authors, independent laboratories have selected this target for interlaboratory validation of different PCR and qPCR methods like the study of Malorny et al. (2003, 2004) in Europe, or the one carried out by Cheng et al. in the USA (Cheng et al., 2008; Cheng, Van, Lin, & Ruby, 2009).

Recently, Malorny et al. (2007) developed a qPCR method for the specific detection of serovar Enteritidis. This is of special relevance, since in the last amendment of a European Regulation 2073/2005 ((EC), 2005; (EU), 2011), it is specifically pointed out that the investigation of *Salmonella* spp. in chicken meat should be restricted to serovars Enteritidis and Typhimurium. The method targeted the Prot6e gene located on the *S*. Enteritidis specific 60-kb virulence plasmid. Even though the gene is promising, further studies should be performed, due to the fact that it fails to detect 4 out of the 79 isolates tested as they lacked the plasmid. Previous studies targeted the *sefA* gene encoding a fimbrial antigen termed SEF14. This gene is exclusively unique to *Salmonella* serogroup D serovars such as Gallinarum, Pullorum, Dublin, Rostock, Semban, and Typhi, and consequently not specific enough to identify only *S*. Enteritidis. Another conventional PCR assay used a chromosomal located fragment, called SdfI which has a high exclusivity but did not detect phage types 6A, 9A, 11, 16, 20, and 27 and was only tested on pure cultures (Malorny et al., 2007).

*4.1.2. Listeria monocytogenes*

Concerning *L. monocytogenes,* the most widely used targets for its specific detection are the genes *hlyA* which encodes for the listeriolisin O (Elizaquível et al., 2011; Kobayashi et al., 2009; Navas et al., 2006; Omiccioli et al., 2009; Rodriguez-Lazaro, Hernández, et al., 2004; Ruiz-Rueda et al., 2010; Suo et al., 2010), and the *prfA* which is a central virulence regulator of this bacterium (D'Urso et al., 2009; Jofré et al., 2005; Rossmanith, Krassnig, Wagner, & Hein, 2006). Additionally, the *iap* gene, coding the protein p60 essential for cell viability, has also been applied (Flekna, Stefanic, et al., 2007; Mukhopadhyay & Mukhopadhyay, 2007; Wuenscher, Köhler, Bubert, Gerike, & Goebel, 1993).

The most popular targets are *hlyA* and *prfA*. Regarding the former, the qPCR method described by Rodriguez-Lazaro, Jofre, et al. (2004) was included in a European interlaboratory assay to evaluate different methodologies' performance (Dalmasso et al., 2014). Later on, the same group of laboratories performed its validation (Gianfranceschi et al., 2014). Concerning *prfA*, it was selected for an interlaboratory validation study involving several laboratories in Europe (D'Agostino et al., 2004). In a later different report, a specific fluorescent probe was designed in order to adapt the method to qPCR (Rossmanith et al., 2006) and extensively evaluated with natural samples (Rossmanith, Mester, Wagner, & Schoder, 2010). Primers and probe targeting *prfA* were recently adopted in an in-house multiplex qPCR method for the simultaneous detection of *Salmonella* spp., *E. coli* O157, and *L. monocytogenes* returning comparable results to those of the original studies where *L. monocytogenes* was the only target (Garrido, Chapela, Román, Fajardo, Vieites, et al., 2013).

### *4.2. Emerging pathogens*

*4.2.1. Campylobacter spp.*

*Campylobacter* spp. Detection has been performed in two different ways, either the main pathogenic species (*C. jejuni*, *C. coli,* and *C. lari*) or the complete genus. Different genes have been described for each species like *hipO* (gene encoding the hippuricase), *mapA* (membrane-associated protein), *rpoB* (gene encoding the β-subunit of the RNA polymerase), and the VS1 sequence of *C. jejuni*'s genome (Debretsion, Habtemariam, Wilson, Nganwa, & Yehualaeshet, 2007; He et al., 2010; Mayr et al., 2010; Rantsiou, Lamberti, & Cocolin, 2010; Yang, Jiang, Huang, Zhu, & Yin, 2003).

Regarding *C. lari,* the *gyrA* (a fragment of the gyrase A subunit gene) and *pepT* (gene encoding a peptidase) have been used for the specific detection of this species.

Finally, the *ceuE* (periplasmic substrate binding protein) and the *cdtA* (gene encoding a subunit of cytolethal toxin, an important virulence factor) have been applied (He et al., 2010; Mayr et al., 2010). As it can be observed, there is not much agreement on the PCR targets for the detection of these species. Typical differentiation of *C. jejuni*, from *C. coli*, is based on hippurate hydrolysis, but atypical strains exist, thus Toplak et al. combined a *hipO* and *ccoN* (encoding the cytochromo c oxidase of *C. jejuni*), from *C. coli cadF* (fibronectin-binding protein) for the simultaneous detection of both species (Toplak, Kovac, Piskernik, Mozina, & Jersek, 2011).

The second alternative, detection of genus *Campylobacter* spp. without differentiating the species, may be an ideal approach from a risk assessment point of view, specially focused on thermotolerant campylobacters (*C. jejuni*, *C. coli,* and *C. lari*). Many studies have successfully applied different primers targeting 16S rRNA, specific for genus *Campylobacter* and/or thermotolerant campylobacters (Botteldoorn et al., 2008; Josefsen, Cook, et al., 2004; Josefsen, Jacobsen, & Hoorfar, 2004; Leblanc-Maridor et al., 2011; Lubeck, Cook, Wagner, Fach, & Hoorfar, 2003; Lubeck, Wolffs, et al., 2003; Wolffs, Norling, Hoorfar, Griffiths, & Radstrom, 2005).

Due to the fact that the group of thermotolerant campylobacters are the main human hazard, 16S rRNA was selected as the optimal target in an interlaboratory study performed in Europe (Josefsen, Cook, et al., 2004; Josefsen, Jacobsen, et al., 2004; Lubeck, Cook, et al., 2003; Lubeck, Wolffs, et al., 2003). A later study developed an appropriate fluorescent probe suitable for its application along with the European-validated primers (Josefsen, Jacobsen, et al., 2004).

*4.2.2. Escherichia coli O157 and other STEC*

In 2004, Perelle et al. designed a method for the identification of the most frequent clinical serotypes of Shiga-toxin producing *E. coli*, which included O26, O55, O91, O103, O111, O113, O145, and O157:H7. For this purpose, genes *stx1*, *stx2*, *rfbE* (lipopolysaccharide O-antigen synthesis gene for O157), *wbdI* (O111), *wzx* (flippase gene for O26), *wzy* (O-antigen polymerase specific for O113), *wzy* (O91), *wbgN* (glycosyltransferase gene specific for O55), *ihpI* (based on the polymorphism between the genes encoding the putative adhesin from "O-island 29" of STEC O157 and STEC O145 O145), *eae* (adherence intimin protein for O103), and *fliC* H7 (flagellar antigen H7) were successfully applied (DebRoy et al., 2004; Gonzales et al., 2011; Kagkli et al., 2011; Perelle, Dilasser, Grout, & Fach, 2003, 2004; Samuel, Hogbin, Wang, & Reeves, 2004). Out of these, genes *stx1* and *stx2* (Shiga-toxin genes 1 and 2) are considered two major virulence factors for *E. coli* O157 and other STEC (Bonetta et al., 2011; Prendergast et al., 2011) and the *eae* gene has also been applied for serotype O157 detection by other authors (Adiguzel et al., 2012; Franz, Klerks, De Vos, Termorshuizen, & van Bruggen, 2007; Fratamico & DebRoy, 2010; Kawasaki et al., 2010; Zhang et al., 2009).

Different combinations of several genes have been published and alternative targets have been selected, like *hlyA* (hemolysin) in combination with *fliC*, *stx1*, *stx2*, *eae,* and *rfbE*(Bai, Shi, & Nagaraja, 2010). Other options included the *uidA* (β-glucuronidase gene) which was only found in O157:H7 and H- but not other O157 (Al-Ajmi et al., 2006; Elizaquível et al., 2011).

A large outbreak caused by Shiga-toxin 2 (*stx2*)-producing enterohemorrhagic *E. coli* (EHEC) O104:H4 that involved 4,000 cases, including 855 patients with hemolytic-uremic syndrome (HUS) and 53 fatalities, occurred in Germany from May to July 2011. A combination of the previously mentioned genes was specifically optimized and combined for the detection of this specific serotype of STEC including $wzy_{O104}$, *stx2,* and $fliC_{H4}$ (Zhang et al., 2012).

The wide variety of possible targets is mainly associated with high number of possible serotypes included in the group STEC. Due to the fact that the most prevalent serotype associated with human infection was O157, a European validation study, gene *rfbE* was selected for the specific detection of this serotype independently of the "H" antigen. In the validation study, special attention was focused on the specificity of the primers due to the fact that this gene is also present in the genus *Vibrio* spp. As expected, after careful design and optimization of the method, optimal results were obtained (Abdulmawjood et al., 2003; Abdulmawjood et al., 2004).

*4.2.3. Yersinia enterocolitica*

Regarding *Yersinia enterocolitica*, it was observed that few studies performed the detection at the species level 16S rRNA (Lantz et al., 1998; Wolffs, Knutsson, Norling, & Radstrom, 2004). Most studies are focused on the detection of pathogenic strains of this species. There is a high degree of consensus on the application of genes *ail* (attachment invasion locus, which mediates cell invasion) as well as plasmid genes such as *yadA* (whose product is involved in autoagglutination, serum resistance, and adhesion) (Cheyne, Van Dyke, Anderson, & Huck, 2010; Jourdan, Johnson, & Wesley, 2000; Lambertz, Nilsson, Hallanvuo, & Lindblad, 2008; Lantz et al., 1998; Najdenski, Heyndrickx, Herman, Werbrouck, & Van Coillie, 2012; Wiemer et al., 2011).

Additionally, other genes like *inv* (invasive gene, which mediates cell invasion), *ystA* (which is responsible for the production of a heat-stable enterotoxin in virulent *Y. enterocolitica*), *ystB* and *ystC* (which have been observed to encode an enterotoxin present mainly in avirulent *Y. enterocolitica*), *rfbC* (which can be used to identify pathogenic *Y. enterocolitica* O:3 strains), and *virF* or *lcrF* (for *Y. enterocolitica* and *Y. pseudotuberculosis*, respectively, which encodes transcriptional activators of the *yop* regulon) have also been used (Zheng, Sun, Mao, & Jiang, 2008).

*4.2.4. Shigella spp.*

A high degree of homogeneity was observed for the detection of *Shigella* spp., both by PCR and by qPCR. The most commonly used target is the *ipaH* gene, the invasion plasmid antigen H. This gene

was also observed in some strains of entero invasive *E. coli* (EIEC) (Gómez-Duarte, Bai, & Newell, 2009; Hsu et al., 2010; Li, Zhuang, & Mustapha, 2005; Thiem et al., 2004; Wang, Li, & Mustapha, 2007). It was included in an in-house multiplex qPCR validation method which simultaneously detected *Salmonella* spp., *Shigella* spp., and *L. monocytogenes*. Excellent results were obtained with natural and spiked samples regarding relative sensitivity, relative specificity, and relative accuracy for the detection of *Shigella* spp. as well as for the other two bacteria (Garrido, Chapela, Román, et al., 2012).

#### *4.2.5. Vibrio cholerae*

The most widely used genes for the detection of this species are *toxR* (the transcriptional regulator for the genes encoding the major outer membrane porins OmpU and OmpT) and *ompW* (outermembrane protein) (Baron, Chevalier, & Lesne, 2007; Goel, Tamrakar, Nema, Kamboj, & Singh, 2005; Gubala & Proll, 2006; Nandi et al., 2000; Neogi et al., 2010; Sheikh, Goodarzi, & Aslani, 2012; Shuan Ju Teh et al., 2009), even though recently the *lolB* (outermembrane lipoprotein) gene has also been described as an optimal target for *V. cholerae* detection (Chua et al., 2011; Lalitha et al., 2008). Out of these, the most extensively applied and evaluated gene was the *ompW*. Further studies need to be performed with the other targets.

The most important pathogenic gene associated with *V. cholerae* is *ctxA* (gene coding for the A subunit of the cholera toxin) (Blackstone et al., 2007; Chapela et al., 2010; Fedio et al., 2007), and also *hly* (coding for a hemolysin) (Huang et al., 2009; Lyon, 2001; Saravanan, Sanath Kumar, Karunasagar, & Karunasagar, 2007; Shuan Ju Teh et al., 2009), *tcpA* (toxin-coregulated pilus A) (Sharma & Chaturvedi, 2006; Sheikh et al., 2012), and the *zot* (zonula ocludens toxin) (Ang et al., 2010).

#### *4.2.6. Vibrio parahaemolyticus*

Detection of *V. parahaemolyticus* has been accomplished by the detection of the *toxR* gene and the *tlh* (thermolabile hemolysin) (Croci et al., 2007; Neogi et al., 2010; Nordstrom et al., 2007; Parveen et al., 2008; Rizvi & Bej, 2010; Rizvi, Panicker, Myers, & Bej, 2006). Virulence of *V. parahaemoyliticus* has historically been associated with the presence of two hemolysins encoded in the *tdh* and *trh* genes (thermostable direct hemolysin and hemolysin related with tdh) (Blackstone et al., 2003; Garrido, Chapela, Ferreira, et al., 2012; Nordstrom et al., 2007). Recently, the type three secretion system has been observed to be related with the pathogenic potential of the species (Hiyoshi, Kodama, Iida, & Honda, 2010; Pineyro et al., 2010).

Multiplex qPCR methods have been developed for the simultaneous detection of *tlh*, *tdh,* and *trh* (Nordstrom et al., 2007; Ward & Bej, 2006). These methods successfully detected *V. parahemolyticus* in food and environmental samples.

#### *4.2.7. Vibrio vulnificus*

Uniformity has been observed for the detection of *V. vulnificus* at the species level, where *vvhA* (exotoxin hemolysin/cytolysin) (Campbell & Wright, 2003; Neogi et al., 2010; Panicker & Bej, 2005; Wright et al., 2007) is one of the most frequently applied targets. This homogeneity is not observed when trying to assess the pathogenic potential of the bacterium, where several targets have been proposed, such as the *rtxA*, *vcgC*/E (virulence-correlated gene clinical/environmental), and 16S rRNA type A (environmental) and type B (clinical) (Drake, Whitney, Levine, DePaola, & Jaykus, 2010; Rosche, Binder, & Oliver, 2010; Rosche, Yano, & Oliver, 2005). Recently, a polimorfism in the *pilF* gene has been described as a good marker of the pathogenic potential of *V. vulnificus* (Baker-Austin et al., 2012; Fajardo et al., 2014; Garrido-Maestu, Chapela, Román, Vieites, & Cabado, 2014; Roig, Sanjuan, Llorens, & Amaro, 2010).

Multiplex qPCR methods for the simultaneous detection of total and pathogenic *V. vulnificus* are scarce (Garrido-Maestu et al., 2014). Most methods combined specific gene targets for the detection at the species level *V. vulnificus*, *V. cholerae,* and *V. parahemolyticus* (Bauer & Rørvik, 2007; Garrido-Maestu, Chapela, Peñaranda, Vieites, & Cabado, 2014; Izumiya et al., 2011; Neogi et al., 2010; Panicker, Call, Krug, & Bej, 2004).

## 5. Commercial kits available for multiplex qPCR

At present, a wide variety of commercial qPCR kits are available for detection of single micro-organisms from different companies as iQ-Check from Bio-Rad, DuPont Qualicon™ BAX®, foodproof® Salmonella from Merck-Millipore, AnDiaTec® from Roche or MicroSEQ® from Applied Biosystems. All these brands have validated solutions for *Salmonella* spp., *L. monocytogenes*, *Campylobacter* spp., and *Legionella*, among others. The problem arises when a multiplex qPCR approach is desired, as the commercial kits are scarce. A possible solution may be the selection of open-formula studies that have gone through an in-house validation where parameters like specificity of the primers and probes, LOD, diagnostic specificity, diagnostic sensitivity, and diagnostic accuracy are evaluated against a reference method. In this context, a very common combination of bacterial pathogens found in the literature include simultaneous detection of *Salmonella* spp. and *L. monocytogenes* (Badosa, Chico, Pla, Pares, & Montesinos, 2009; Chua & Bhagwat, 2009; Garrido, Chapela, Román, Fajardo, Lago, et al., 2013; Jofré et al., 2005; Singh, Batish, & Grover, 2011). This combination should be more useful when the method also includes of *E. coli* O157 (Elizaquível et al., 2011; Garrido, Chapela, Román, Fajardo, Vieites, et al., 2013; Kawasaki et al., 2010; Omiccioli et al., 2009) or others food-borne pathogens like *Shigella* spp. (Garrido, Chapela, Román, et al., 2012; Wang, Li, & Mustapha, 2007).

## 6. Future challenges

An international standard guideline (ISO 16140:2003) was prepared by CEN/TC275 in collaboration with the Technical Committee ISO/TC34 to establish the general principle and technical procedure for the validation of alternative methods in the field of microbiological analysis of food, animal feeding stuffs, and environmental and veterinary samples. Many of the performance criteria are well recognized and accepted by numerous internationally recognized organizations for standardization (ISO, EMEA, Codex Alimentarius, etc.). There is a series of concepts that define an analytical method and that are applied to diagnostic qPCR. The main concepts are: linearity, accuracy, precision, sensitivity, and specificity. Progressive introduction of automated equipment for qPCR has enabled it to become a high-throughput method. The numerous examples cited in this review and their recentness illustrate the current interest for qPCR methods in food microbiology. However, not all fields of application are equally developed yet, in particular, the multiplex strategies as described above. There is an obvious lack of consensus considering the appropriate approach to perform the experiments and interpret the data (Bustin et al., 2009, 2010; Huggett et al., 2013).

## 7. Conclusions

Molecular techniques applied in food microbiology are very promising alternatives to classical cultures, biochemical and serological identification and typification methods. Among them, PCR and real-time PCR have been successfully used, not only in control laboratories, but also in research to gain deeper knowledge in the biology and pathogenesis of bacterial species.

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**References**

Abdulmawjood, A., Bülte, M., Cook, N., Roth, S., Schönenbrücher, H., & Hoorfar, J. (2003). Toward an international standard for PCR-based detection of *Escherichia coli* O157. *Journal of Microbiological Methods, 55*, 775–786. http://dx.doi.org/10.1016/j.mimet.2003.08.012
Abdulmawjood, A., Bulte, M., Roth, S., Schonenbrucher, H., Cook, N., Heuvelink, A. E., & Hoorfar, J. (2004). Development, validation, and standardization of

polymerase chain reaction-based detection of *E. coli* O157. *Journal of Aoac International, 87*, 596–603.
Adiguzel, G., Gulluce, M., Bozoglu, C., Yanmis, D., Gormez, A., Atasever, M., & Adiguzel, A. (2012). Molecular characterization of *Escherichia coli* O157:H7 from retail beef in Erzurum, Turkey. *Journal of Pure and Applied Microbiology, 6*, 1033–1041.
Agroalimentaria, E. F. V. p. l. S. (2012). *Informe RASFF*.
Al-Ajmi, D., Padmanabha, J., Denman, S. E., Gilbert, R. A., Al Jassim, R. A. M., & McSweeney, C. S. (2006). Evaluation of a PCR detection method for *Escherichia coli* O157:H7/H-bovine faecal samples. *Letters in Applied Microbiology, 42*, 386–391. http://dx.doi.org/10.1111/lam.2006.42.issue-4
Anderson, A., Pietsch, K., Zucker, R., Mayr, A., Müller-Hohe, E., Messelhäusser, U., … Huber, I. (2011). Validation of a duplex real-time PCR for the detection of *Salmonella* spp. in different food products. *Food Analytical Methods, 4*, 259–267. http://dx.doi.org/10.1007/s12161-010-9142-8
Ang, G. Y., Yu, C. Y., Balqis, K., Elina, H. T., Azura, H., Hani, M. H., & Yean, C. Y. (2010). Molecular evidence of cholera outbreak caused by a toxigenic *Vibrio cholerae* O1 El Tor variant strain in Kelantan, Malaysia. *Journal of Clinical Microbiology, 48*, 3963–3969. http://dx.doi.org/10.1128/JCM.01086-10
Badosa, E., Chico, N., Pla, M., Parés, D., & Montesinos, E. (2009). Evaluation of ISO enrichment real-time PCR methods with internal amplification control for detection of *Listeria monocytogenes* and *Salmonella enterica* in fresh fruit and vegetables. *Letters in Applied Microbiology, 49*, 105–111. http://dx.doi.org/10.1111/lam.2009.49.issue-1
Bai, J., Shi, X., & Nagaraja, T. G. (2010). A multiplex PCR procedure for the detection of six major virulence genes in *Escherichia coli* O157:H7. *Journal of Microbiological Methods, 82*, 85–89. http://dx.doi.org/10.1016/j.mimet.2010.05.003
Bailey, J. S., & Cox, N. A. (1992). Universal preenrichment broth for the simultaneous detection of *Salmonella* and *Listeria* in foods. *Journal of Food Protection, 55*, 256–259.
Baker-Austin, C., Lemm, E., Hartnell, R., Lowther, J., Onley, R., Amaro, C., … Lees, D. (2012). *pilF* polymorphism-based real-time PCR to distinguish *Vibrio vulnificus* strains of human health relevance. *Food Microbiology, 30*, 17–23.
Baron, S., Chevalier, S., & Lesne, J. (2007). *Vibrio cholerae* in the environment: A simple method for reliable identification of the species. *Journal of Health Population and Nutrition, 25*, 312–318.
Bauer, A., & Rørvik, L. M. (2007). A novel multiplex PCR for the identification of *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*. *Letters in Applied Microbiology, 45*, 371–375. http://dx.doi.org/10.1111/lam.2007.45.issue-4
Bhagwat, A. A. (2004). Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. *Food Microbiology, 21*, 73–78. http://dx.doi.org/10.1016/S0740-0020(03)00020-0
Blackstone, G. M., Nordstrom, J. L., Bowen, M. D., Meyer, R. F., Imbro, P., & DePaola, A. (2007). Use of a real time PCR assay for detection of the *ctxA* gene of *Vibrio cholerae* in an environmental survey of Mobile Bay. *Journal of Microbiological Methods, 68*, 254–259. http://dx.doi.org/10.1016/j.mimet.2006.08.006
Blackstone, G. M., Nordstrom, J. L., Vickery, M. C. L., Bowen, M. D., Meyer, R. F., & DePaola, A. (2003). Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. *Journal of Microbiological Methods, 53*, 149–155. http://dx.doi.org/10.1016/S0167-7012(03)00020-4
Bonetta, S., Borelli, E., Bonetta, S., Conio, O., Palumbo, F., & Carraro, E. (2011). Development of a PCR protocol for the detection of *Escherichia coli* O157:H7 and *Salmonella* spp. in surface water. *Environmental Monitoring and Assessment, 177*, 493–503. http://dx.doi.org/10.1007/s10661-010-1650-x
Botteldoorn, N., Van Coillie, E., Piessens, V., Rasschaert, G., Debruyne, L., Heyndrickx, M., … Messens, W. (2008). Quantification of *Campylobacter* spp. in chicken carcass rinse by real-time PCR. *Journal of Applied Microbiology, 105*, 1909–1918. http://dx.doi.org/10.1111/jam.2008.105.issue-6
Bustin, S. A., Beaulieu, J.-F., Huggett, J., Jaggi, R., Kibenge, F. S. B., Olsvik, P. A., & Toegel, S. (2010). MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology, 11*, 74.
Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., … Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry, 55*, 611–622. http://dx.doi.org/10.1373/clinchem.2008.112797
Calvó, L., Martínez-Planells, A., Pardos-Bosch, J., & Garcia-Gil, L. J. (2008). A new real-time PCR assay for the specific detection of *Salmonella* spp. targeting the *bipA* gene. *Food Analytical Methods, 1*, 236–242. http://dx.doi.org/10.1007/s12161-007-9008-x
Campbell, M. S., & Wright, A. C. (2003). Real-time PCR analysis of *Vibrio vulnificus* from oysters. *Applied and Environmental Microbiology, 69*, 7137–7144. http://dx.doi.org/10.1128/AEM.69.12.7137-7144.2003
CDC. (2013). Incidence and trends of infection with pathogens transmitted commonly through food-foodborne diseases active surveillance network, 10 US sites, 1996–2012. *MMWR. Morbidity and mortality weekly report, 62*, 283–287.
Chakravorty, S., Helb, D., Burday, M., Connell, N., & Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Journal of Microbiological Methods, 69*, 330–339. http://dx.doi.org/10.1016/j.mimet.2007.02.005
Chapela, M. J., Fajardo, P., Garrido, A., Cabado, A. G., Ferreira, M., Lago, J., & Vieites, J. M. (2010). Comparison between a TaqMan polymerase chain reaction assay and a culture method for ctx-positive *Vibrio cholerae* detection. *Journal of Agricultural and Food Chemistry, 58*, 4051–4055. http://dx.doi.org/10.1021/jf903658k
Chapela, M. J., Sotelo, C. G., Calo-Mata, P., Perez-Martin, R. I., Rehbein, H., Hold, G. L., … Santos, A. T. (2002). Identification of cephalopod species (Ommastrephidae and Loliginidae) in seafood products by forensically informative nucleotide sequencing (FINS). *Journal of Food Science, 67*, 1672–1676. http://dx.doi.org/10.1111/jfds.2002.67.issue-5
Cheng, C. M., Lin, W., Van, K. T., Phan, L., Tran, N. N., & Farmer, D. (2008). Rapid detection of *Salmonella* in foods using real-time PCR. *Journal of Food Protection, 71*, 2436–2441.
Cheng, C. M., Van, K. T., Lin, W., & Ruby, R. M. (2009). Interlaboratory validation of a real-time PCR 24-hour rapid method for detection of *Salmonella* in foods. *Journal of Food Protection, 72*, 945–951.
Cheyne, B., Van Dyke, M., Anderson, W., & Huck, P. (2010). The detection of *Yersinia enterocolitica* in surface water by quantitative PCR amplification of the *ail* and *yadA* genes. *Journal of Water and Health, 8*, 487–499. http://dx.doi.org/10.2166/wh.2009.215
Chua, A. L., Elina, H. T., Lim, B. H., Yean, C. Y., Ravichandran, M., & Lalitha, P. (2011). Development of a dry reagent-based triplex PCR for the detection of toxigenic and non-toxigenic *Vibrio cholerae*. *Journal of Medical Microbiology, 60*, 481–485. http://dx.doi.org/10.1099/jmm.0.027433-0
Chua, T., & Bhagwat, A. A. (2009). A rapid and simple DNA extraction procedure to detect *Salmonella* spp. and *Listeria monocytogenes* from fresh produce using real-time PCR. *Food Analytical Methods, 2*, 96–101. http://dx.doi.org/10.1007/s12161-008-9032-5
Commission, E. (2011). *RASFF-Annual Report*.
Croci, L., Suffredini, E., Cozzi, L., Toti, L., Ottaviani, D., Pruzzo, C., … Mioni, R. (2007). Comparison of different biochemical

and molecular methods for the identification of *Vibrio parahaemolyticus*. *Journal of Applied Microbiology, 102*, 229–237. http://dx.doi.org/10.1111/jam.2007.102.issue-1

D'Agostino, M., Wagner, M., Vazquez-Boland, J. A., Kuchta, T., Karpiskova, R., Hoorfar, J., … Cook, N. (2004). A validated PCR-based method to detect *Listeria monocytogenes* using raw milk as a food model—Towards an international standard. *Journal of Food Protection, 67*, 1646–1655.

D'Urso, O. F., Poltronieri, P., Marsigliante, S., Storelli, C., Hernández, M., & Rodríguez-Lázaro, D. (2009). A filtration-based real-time PCR method for the quantitative detection of viable *Salmonella enterica* and *Listeria monocytogenes* in food samples. *Food Microbiology, 26*, 311–316. http://dx.doi.org/10.1016/j.fm.2008.12.006

Dalmasso, M., Bolocan, A. S., Hernandez, M., Kapetanakou, A. E., Kuchta, T., Manios, S. G., … Jordan, K. (2014). Comparison of polymerase chain reaction methods and plating for analysis of enriched cultures of *Listeria monocytogenes* when using the ISO11290-1 method. *Journal of Microbiological Methods, 98*, 8–14. http://dx.doi.org/10.1016/j.mimet.2013.12.018

Debretsion, A., Habtemariam, T., Wilson, S., Nganwa, D., & Yehualaeshet, T. (2007). Real-time PCR assay for rapid detection and quantification of *Campylobacter jejuni* on chicken rinses from poultry processing plant. *Molecular and Cellular Probes, 21*, 177–181. http://dx.doi.org/10.1016/j.mcp.2006.10.006

DebRoy, C., Roberts, E., Kundrat, J., Davis, M. A., Briggs, C. E., & Fratamico, P. M. (2004). Detection of *Escherichia coli* serogroups O26 and O113 by PCR amplification of the wzx and wzy genes. *Applied and Environmental Microbiology, 70*, 1830–1832. http://dx.doi.org/10.1128/AEM.70.3.1830-1832.2004

Delibato, E., Rodriguez-Lázaro, D., Gianfranceschi, M., De Cesare, A., Comin, D., Gattuso, A., … De Medici, D. (2014). European validation of real-time PCR method for detection of *Salmonella* spp. in pork meat. *International Journal of Food Microbiology, 184*, 134–138.

Drake, S. L., Whitney, B., Levine, J. F., DePaola, A., & Jaykus, L.-A. (2010). Correlation of mannitol fermentation with virulence-associated genotypic characteristics in *Vibrio vulnificus* isolates from oysters and water samples in the Gulf of Mexico. *Foodborne Pathogens and Disease, 7*, 97–101. http://dx.doi.org/10.1089/fpd.2009.0362

(EC), C. R. (2002). Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January2002. Laying down the general principles and requirements of food law, establishing the European food safety authorityand laying down procedures in matters of food safety. *Official Journal of the European Communities, Official Journal of the European Union, 178/2002*.

(EC), C. R. (2005). Microbiological criteria for foodstuffs. *Official Journal of the European Union, 2073/2005*.

Elizaquivel, P., & Aznar, R. (2008). Comparison of four commercial DNA extraction kits for PCR detection of *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7, and *Staphylococcus aureus* in fresh, minimally processed vegetables. *Journal of Food Protection, 71*, 2110–2114.

Elizaquível, P., Gabaldón, J. A., & Aznar, R. (2011). 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, 22*, 158–164. http://dx.doi.org/10.1016/j.foodcont.2010.05.018

Elnifro, E. M., Ashshi, A. M., Cooper, R. J., & Klapper, P. E. (2000). Multiplex PCR: Optimization and application in diagnostic virology. *Clinical Microbiology Reviews*, 13, 559–570. http://dx.doi.org/10.1128/CMR.13.4.559-570.2000

(EU), C. R. (2011). Amending Annex II to Regulation (EC) No. 2160/2003 of the European parliament and of the council and annex I to Commission Regulation (EC) No. 2073/2005 as regards *Salmonella* in fresh poultry meat. *Official Journal of the European Union, 1086/2011*.

Fajardo, P., Atanassova, M., Garrido-Maestu, A., Wortner-Smith, T., Cotterill, J., & Cabado, A. G. (2014). Bacteria isolated from shellfish digestive gland with antipathogenic activity as candidates to increase the efficiency of shellfish depuration process. *Food Control, 46*, 272–281. http://dx.doi.org/10.1016/j.foodcont.2014.05.038

Fedio, W., Blackstone, G. M., Kikuta-Oshima, L., Wendakoon, C., McGrath, T. H., & DePaola, A. (2007). Rapid detection of the *Vibrio cholerae ctx* gene in food enrichments using real-time polymerase chain reaction. *Journal of Aoac International, 90*, 1278–1283.

Flekna, G., Schneeweiss, W., Smulders, F. J. M., Wagner, M., & Hein, I. (2007). Real-time PCR method with statistical analysis to compare the potential of DNA isolation methods to remove PCR inhibitors from samples for diagnostic PCR. *Molecular and Cellular Probes, 21*, 282–287. http://dx.doi.org/10.1016/j.mcp.2007.02.001

Flekna, G., Štefanič, P., Wagner, M., Smulders, F. J. M., Možina, S. S., & Hein, I. (2007). Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide (EMA) compromises EMA/real-time PCR. *Research in Microbiology, 158*, 405–412. http://dx.doi.org/10.1016/j.resmic.2007.02.008

Franz, E., Klerks, M. A., De Vos, O. J., Termorshuizen, A. J., & van Bruggen, A. H. C. (2007). Prevalence of Shiga toxin-producing *Escherichia coli stx1*, *stx2*, *eaeA*, and *rfbE* genes and survival of *E. coli* O157:H7 in manure from organic and low-input conventional dairy farms. *Applied and Environmental Microbiology, 73*, 2180–2190. http://dx.doi.org/10.1128/AEM.01950-06

Fratamico, P. M., & DebRoy, C. (2010). Detection of *Escherichia coli* O157:H7 in food using real-time multiplex PCR assays targeting the *stx 1*, *stx 2*, *wzy O157*, and the *fliC h7* or *eae* genes. *Food Analytical Methods, 3*, 330–337. http://dx.doi.org/10.1007/s12161-010-9140-x

Garrido, A., Chapela, M. J., Ferreira, M., Atanassova, M., Fajardo, P., Lago, J., … Cabado, A. G. (2012). Development of a multiplex real-time PCR method for pathogenic *Vibrio parahaemolyticus* detection (*tdh*+ and *trh*+). *Food Control, 24*, 128–135. http://dx.doi.org/10.1016/j.foodcont.2011.09.015

Garrido, A., Chapela, M.-J., Román, B., Fajardo, P., Lago, J., Vieites, J. M., & Cabado, A. G. (2013). A new multiplex real-time PCR developed method for *Salmonella* spp. and *Listeria monocytogenes* detection in food and environmental samples. *Food Control, 30*, 76–85. http://dx.doi.org/10.1016/j.foodcont.2012.06.029

Garrido, A., Chapela, M.-J., Román, B., Fajardo, P., Vieites, J. M., & Cabado, A. G. (2013). 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, 164*, 92–98. http://dx.doi.org/10.1016/j.ijfoodmicro.2013.03.024

Garrido, A., Chapela, M. J., Román, B., Ferreira, M., Lago, J., Vieites, J. M., & Cabado, A. G. (2012). Development of a multiplex real-time PCR method for simultaneous detection of *Salmonella enterica*, *Shigella flexneri* and *Listeria monocytogenes* in processed food samples. *European Food Research and Technology, 234*, 571–580. http://dx.doi.org/10.1007/s00217-012-1665-3

Garrido-Maestu, A., Chapela, M.-J., Peñaranda, E., Vieites, J. M., & Cabado, A. G. (2014). In-house validation of novel multiplex real-time PCR gene combination for the simultaneous detection of the main human pathogenic vibrios (*Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*). *Food Control, 37*, 371–379. http://dx.doi.org/10.1016/j.foodcont.2013.09.026

Garrido-Maestu, A., Chapela, M. J., Román, B., Vieites, J. M., & Cabado, A. G. (2014). Application of a novel pathogenicity marker in a multiplex real-time PCR method to assess total and pathogenic *Vibrio vulnificus* in food and environmental samples. *Food Control, 35*, 274–283. http://dx.doi.org/10.1016/j.foodcont.2013.07.007

Gattuso, A., Gianfranceschi, M. V., Sonnessa, M., Delibato, E., Marchesan, M., Hernandez, M., … Rodriguez-Lazaro, D. (2014). Optimization of a real time PCR based method for the detection of *Listeria monocytogenes* in pork meat. *International Journal of Food Microbiology,184*, 106–108.

Germini, A., Masola, A., Carnevali, P., & Marchelli, R. (2009). Simultaneous detection of *Escherichia coli* O175:H7, *Salmonella* spp., and *Listeria monocytogenes* by multiplex PCR. *Food Control, 20*, 733–738. http://dx.doi.org/10.1016/j.foodcont.2008.09.010

Gianfranceschi, M. V., Rodriguez-Lazaro, D., Hernandez, M., Gonzalez-Garcia, P., Comin, D., Gattuso, A., … De Medici, D. (2014). European validation of a real-time PCR-based method for detection of *Listeria monocytogenes* in soft cheese. *International Journal of Food Microbiology, 184*, 128–133.

Goel, A. K., Tamrakar, A. K., Nema, V., Kamboj, D. V., & Singh, L. (2005). Detection of viable toxigenic *Vibrio cholerae* from environmental water sources by direct cell duplex PCR assay. *World Journal of Microbiology & Biotechnology, 21*, 973–976.

Gómez-Duarte, O. G., Bai, J., & Newell, E. (2009). Detection of *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Yersinia enterocolitica*, *Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by 3-reaction multiplex polymerase chain reaction. *Diagnostic Microbiology and Infectious Disease, 63*, 1–9. http://dx.doi.org/10.1016/j.diagmicrobio.2008.09.006

Gonzales, T. K., Kulow, M., Park, D. J., Kaspar, C. W., Anklam, K. S., Pertzborn, K. M., et al. (2011). A high-throughput open-array qPCR gene panel to identify, virulotype, and subtype O157 and non-O157 enterohemorrhagic *Escherichia coli*. *Molecular and Cellular Probes, 25*, 222–230. http://dx.doi.org/10.1016/j.mcp.2011.08.004

González-Escalona, N., Brown, E. W., & Zhang, G. (2012). Development and evaluation of a multiplex real-time PCR (qPCR) assay targeting *ttrRSBCA* locus and *invA* gene for accurate detection of *Salmonella* spp. in fresh produce and eggs. *Food Research International, 48*, 202–208.

Gubala, A. J., & Proll, D. F. (2006). Molecular-beacon multiplex real-time PCR assay for detection of *Vibrio cholerae*. *Applied and Environmental Microbiology, 72*, 6424–6428. http://dx.doi.org/10.1128/AEM.02597-05

Hammack, T. S., Amaguana, R. M., Johnson, M. L., & Andrews, W. H. (2003). Effectiveness of universal pre-enrichment broth for recovery of *Salmonella* from selected dairy foods. *Journal of Aoac International, 86*, 714–718.

He, Y., Yao, X., Gunther, N. W., Xie, Y., Tu, S.-I., & Shi, X. (2010). Simultaneous detection and differentiation of *Campylobacter jejuni*, *C. coli*, and *C. lari* in chickens using a multiplex real-time PCR assay. *Food Analytical Methods, 3*, 321–329. http://dx.doi.org/10.1007/s12161-010-9136-6

Heid, C. A., Stevens, J., Livak, K. J., & Williams, P. M. (1996). Real time quantitative PCR. *Genome Research, 6*, 986–994. http://dx.doi.org/10.1101/gr.6.10.986

Hierro, N., Esteve-Zarzoso, B., Gonzalez, A., Mas, A., & Guillamon, J. M. (2006). Real-time quantitative PCR (QPCR) and reverse transcription-QPCR for detection and enumeration of total yeasts in wine. *Applied and Environmental Microbiology, 72*, 7148–7155. http://dx.doi.org/10.1128/AEM.00388-06

Higuchi, R., Dollinger, G., Walsh, P. S., & Griffith, R. (1992). Simultaneous amplification and detection of specific DNA-sequences. *Bio/Technology, 10*, 413–417. http://dx.doi.org/10.1038/nbt0492-413

Higuchi, R., Fockler, C., Dollinger, G., & Watson, R. (1993). Kinetic PCR analysis: Real-time monitoring of DNA amplification reactions. *Bio/Technology, 11*, 1026–1030. http://dx.doi.org/10.1038/nbt0993-1026

Hiyoshi, H., Kodama, T., Iida, T., & Honda, T. (2010). Contribution of *Vibrio parahaemolyticus* virulence factors to cytotoxicity, enterotoxicity, and lethality in mice. *Infection and Immunity, 78*, 1772–1780. http://dx.doi.org/10.1128/IAI.01051-09

Hoorfar, J., Cook, N., Malorny, B., Wagner, M., De Medici, D., Abdulmawjood, A., & Fach, P. (2003). Making internal amplification control mandatory for diagnostic PCR. *Journal of Clinical Microbiology, 41*, 5835–5835. http://dx.doi.org/10.1128/JCM.41.12.5835.2003

Hoorfar, J., Cook, N., Malorny, B., Wagner, M., De Medici, D., Abdulmawjood, A., & Fach, P. (2004). Letter to the editor. *Journal of Applied Microbiology, 96*, 221–222. http://dx.doi.org/10.1046/j.1365-2672.2003.02188.x

Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., & Fach, P. (2004). Practical considerations in design of internal amplification controls for diagnostic PCR assays. *Journal of Clinical Microbiology, 42*, 1863–1868. http://dx.doi.org/10.1128/JCM.42.5.1863-1868.2004

Hsu, B. M., Wu, S. F., Huang, S. W., Tseng, Y. J., Ji, D. D., Chen, J. S., & Shih, F. C. (2010). Differentiation and identification of *Shigella* spp. and enteroinvasive *Escherichia coli* in environmental waters by a molecular method and biochemical test. *Water Research, 44*, 949–955. http://dx.doi.org/10.1016/j.watres.2009.10.004

Huang, J., Zhu, Y., Wen, H., Zhang, J., Huang, S., Niu, J., & Li, Q. (2009). Quadruplex real-time PCR assay for detection and identification of *Vibrio cholerae* O1 and O139 strains and determination of their toxigenic potential. *Applied and Environmental Microbiology, 75*, 6981–6985. http://dx.doi.org/10.1128/AEM.00517-09

Huggett, J. F., Foy, C. A., Benes, V., Emslie, K., Garson, J. A., Haynes, R., … Bustin, S. A. (2013). The digital MIQE guidelines: Minimum information for publication of quantitative digital PCR experiments. *Clinical Chemistry, 59*, 892–902. http://dx.doi.org/10.1373/clinchem.2013.206375

Hyeon, J. Y., Hwang, I. G., Kwak, H. S., Park, C., Choi, I. S., & Seo, K. H. (2010). Evaluation of PCR inhibitory effect of enrichment broths and comparison of DNA extraction methods for detection of *Salmonella* Enteritidis using real-time PCR assay. *Journal of Veterinary Science, 11*, 143–149. http://dx.doi.org/10.4142/jvs.2010.11.2.143

ISO. (1996). *Microbiology of food and animal feeding stuffs—Horizontal method for the detection and enumeration of Listeria monocytogenes-Part 1: Detection method* (Vol. 11290-1:1996).

ISO. (1998). *Microbiology of food and animal feeding stuffs—Horizontal method for the detection and enumeration of Listeria monocytogenes-Part 2: Enumeration method* (Vol. ISO 11290-2:1998).

ISO. (2001). *Microbiology of food and animal feeding stuffs—Horizontal method for the detection of Escherichia coli O157* (Vol. 16654:2001).

ISO. (2003). *Microbiology of food and animal feeding stuffs—Horizontal method for the detection of Salmonella spp.* (Vol. 6579:2002).

ISO. (2005). *Microbiology of food and animal feeding stuffs—Polymerase chain reaction (PCR) for the detection of food-borne pathogens* (General requirements and definitions). *22174*, 19.

Izumiya, H., Matsumoto, K., Yahiro, S., Lee, J., Morita, M., Yamamoto, S., … Ohnishi, M. (2011). Multiplex PCR assay for identification of three major pathogenic *Vibrio* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio*

*vulnificus*. *Molecular and Cellular Probes, 25*, 174–176. http://dx.doi.org/10.1016/j.mcp.2011.04.004

Jofré, A., Martin, B., Garriga, M., Hugas, M., Pla, M., Rodríguez-Lázaro, D., & Aymerich, T. (2005). Simultaneous detection of *Listeria monocytogenes* and *Salmonella* by multiplex PCR in cooked ham. *Food Microbiology, 22*, 109–115. http://dx.doi.org/10.1016/j.fm.2004.04.009

Josefsen, M. H., Cook, N., D'Agostino, M., Hansen, F., Wagner, M., Demnerova, K., ... Hoorfar, J. (2004). Validation of a PCR-based method for detection of food-borne thermotolerant campylobacters in a multicenter collaborative trial. *Applied and Environmental Microbiology, 70*, 4379–4383. http://dx.doi.org/10.1128/AEM.70.7.4379-4383.2004

Josefsen, M. H., Jacobsen, N. R., & Hoorfar, J. (2004). Enrichment followed by quantitative PCR both for rapid detection and as a tool for quantitative risk assessment of food-borne thermotolerant campylobacters. *Applied and Environmental Microbiology, 70*, 3588–3592. http://dx.doi.org/10.1128/AEM.70.6.3588-3592.2004

Josefsen, M. H., Krause, M., Hansen, F., & Hoorfar, J. (2007). Optimization of a 12-h TaqMan PCR-based method for detection of *Salmonella* bacteria in meat. *Applied and Environmental Microbiology, 73*, 3040–3048. http://dx.doi.org/10.1128/AEM.02823-06

Jourdan, A. D., Johnson, S. C., & Wesley, I. V. (2000). Development of a fluorogenic 5' nuclease PCR assay for detection of the ail gene of pathogenic *Yersinia enterocolitica*. *Applied and Environmental Microbiology, 66*, 3750–3755. http://dx.doi.org/10.1128/AEM.66.9.3750-3755.2000

Kagkli, D. M., Weber, T. P., Van den Bulcke, M., Folloni, S., Tozzoli, R., Morabito, S., ... Van den Eede, G. (2011). Application of the modular approach to an in-house validation study of real-time PCR methods for the detection and serogroup determination of verocytotoxigenic *Escherichia coli*. *Applied and Environmental Microbiology, 77*, 6954–6963. http://dx.doi.org/10.1128/AEM.05357-11

Kamisaki-Horikoshi, N., Okada, Y., Takeshita, K., Sameshima, T., Kawasaki, S., Kawamoto, S., & Fratamico, P. M. (2011). Evaluation of TA10 broth for recovery of heat- and freeze-injured *Salmonella* from beef. *Journal of Aoac International, 94*, 857–862.

Kawasaki, S., Fratamico, P. M., Horikoshi, N., Okada, Y., Takeshita, K., Sameshima, T., & Kawamoto, S. (2009). Evaluation of a multiplex PCR system for simultaneous detection of *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in foods and in food subjected to freezing. *Foodborne Pathogens and Disease, 6*, 81–89. http://dx.doi.org/10.1089/fpd.2008.0153

Kawasaki, S., Fratamico, P. M., Horikoshi, N., Okada, Y., Takeshita, K., Sameshima, T., & Kawamoto, S. (2010). Multiplex real-time polymerase chain reaction assay for simultaneous detection and quantification of *Salmonella* species, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in ground pork samples. *Foodborne Pathogens and Disease, 7*, 549–554. http://dx.doi.org/10.1089/fpd.2009.0465

Kawasaki, S., Fratamico, P. M., Kamisaki-Horikoshi, N., Okada, Y., Takeshita, K., Sameshima, T., & Kawamoto, S. (2011). Development of the multiplex PCR detection kit for *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7. *Jarq-Japan Agricultural Research Quarterly, 45*, 77–81.

Kawasaki, S., Horikoshi, N., Okada, Y., Takeshita, K., Sameshima, T., & Kawamoto, S. (2005). Multiplex PCR for simultaneous detection of *Salmonella spp.*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in meat samples. *Journal of Food Protection, 68*, 551–556.

Kim, H. C., & Bhunia, A. K. (2008). SEL, a selective enrichment broth for simultaneous growth of *Salmonella* enterica, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. *Applied and Environmental Microbiology, 74*, 4853–4866. http://dx.doi.org/10.1128/AEM.02756-07

Kim, H.-J., & Cho, J.-C. (2010). Simple and rapid detection of *Listeria monocytogenes* in fruit juice by real-time PCR without enrichment culture. *Food Control, 21*, 1419–1423. http://dx.doi.org/10.1016/j.foodcont.2010.04.006

Kimura, B., Kawasaki, S., Fujii, T., Kusunoki, J., Itoh, T., & Flood, S. J. A. (1999). Evaluation of TaqMan PCR assay for detecting *Salmonella* in raw meat and shrimp. *Journal of Food Protection, 62*, 329–335.

Kobayashi, H., Kubota, J., Fujihara, K., Honjoh, K., Iio, M., Fujiki, N., ... Miyamoto, T. (2009). Simultaneous enrichment of *Salmonella* spp, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes* by single broth and screening of the pathogens by multiplex real-time PCR. *Food Science and Technology Research, 15*, 427–438. http://dx.doi.org/10.3136/fstr.15.427

Krascsenicsová, K., Piknová, L., Kaclíková, E., & Kuchta, T. (2008). Detection of *Salmonella enterica* in food using two-step enrichment and real-time polymerase chain reaction. *Letters in Applied Microbiology, 46*, 483–487. http://dx.doi.org/10.1111/j.1472-765X.2008.02342.x

Lalitha, P., Sid Suraiya, M. N., Lim, K. L., Lee, S. Y., Nur Haslindawaty, A. R., Chan, Y. Y., ... Ravichandran, M. (2008). Analysis of *lolB* gene sequence and its use in the development of a PCR assay for the detection of *Vibrio cholerae*. *Journal of Microbiological Methods, 75*, 142–144. http://dx.doi.org/10.1016/j.mimet.2008.05.001

Lambertz, S. T., Nilsson, C., Hallanvuo, S., & Lindblad, M. (2008). Real-time PCR method for detection of pathogenic *Yersinia enterocolitica* in food. *Applied and Environmental Microbiology, 74*, 6060–6067. http://dx.doi.org/10.1128/AEM.00405-08

Lantz, P. G., Knutsson, R., Blixt, Y., Al-Soud, W., Borch, E., & Rådström, P. (1998). Detection of pathogenic *Yersinia enterocolitica* in enrichment media and pork by a multiplex PCR: A study of sample preparation and PCR-inhibitory components. *International Journal of Food Microbiology, 45*, 93–105. http://dx.doi.org/10.1016/S0168-1605(98)00152-4

Leblanc-Maridor, M., Garénaux, A., Beaudeau, F., Chidaine, B., Seegers, H., Denis, M., & Belloc, C. (2011). Quantification of *Campylobacter* spp. in pig feces by direct real-time PCR with an internal control of extraction and amplification. *Journal of Microbiological Methods, 85*, 53–61. http://dx.doi.org/10.1016/j.mimet.2011.01.013

Li, Y., Zhuang, S., & Mustapha, A. (2005). Application of a multiplex PCR for the simultaneous detection of *Escherichia coli* O157:H7, *Salmonella* and *Shigella* in raw and ready-to-eat meat products. *Meat Science, 71*, 402–406. http://dx.doi.org/10.1016/j.meatsci.2005.04.013

López-Calleja, I., de la Cruz, S., Pegels, N., González, I., García, T., & Martín, R. (2013). Development of a real time PCR assay for detection of allergenic trace amounts of peanut (*Arachis hypogaea*) in processed foods. *Food Control, 30*, 480–490. http://dx.doi.org/10.1016/j.foodcont.2012.09.017

Lubeck, P. S., Cook, N., Wagner, M., Fach, P., & Hoorfar, J. (2003). Toward an international standard for PCR-based detection of food-borne thermotolerant campylobacters: Validation in a multicenter collaborative trial. *Applied and Environmental Microbiology, 69*, 5670–5672. http://dx.doi.org/10.1128/AEM.69.9.5670-5672.2003

Lubeck, P. S., Wolffs, P., On, S. L. W., Ahrens, P., Radstrom, P., & Hoorfar, J. (2003). Toward an international standard for PCR-based detection of food-borne thermotolerant campylobacters: Assay development and analytical validation. *Applied and Environmental Microbiology, 69*, 5664–5669. http://dx.doi.org/10.1128/AEM.69.9.5664-5669.2003

Lyon, W. J. (2001). TaqMan PCR for detection of *Vibrio cholerae* O1, O139, non-O1, and non-O139 in pure cultures, raw oysters, and synthetic seawater. *Applied and Environmental Microbiology, 67*, 4685–4693. http://dx.doi.org/10.1128/AEM.67.10.4685-4693.2001

Mackie, I. M., Pryde, S. E., Gonzales-Sotelo, C., Medina, I., Perez-Martin, R., Quinteiro, J., … Rehbein, R. (1999). Challenges in the identification of species of canned fish. *Trends in Food Science & Technology, 10*, 9–14.

Mahmuda, Y., & Kawamoto, K. S. (2007). Evaluation of multiplex PCR system for simultaneous detection of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella enteritidis* in shrimp samples. *Bangladesh Journal of Microbiology, 24*, 42–46.

Malorny, B., Bunge, C., & Helmuth, R. (2007). A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. *Journal of Microbiological Methods, 70*, 245–251. http://dx.doi.org/10.1016/j.mimet.2007.04.013

Malorny, B., Hoorfar, J., Bunge, C., & Helmuth, R. (2003). Multicenter validation of the analytical accuracy of *Salmonella* PCR: Towards an international standard. *Applied and Environmental Microbiology, 69*, 290–296. http://dx.doi.org/10.1128/AEM.69.1.290-296.2003

Malorny, B., Huehn, S., Dieckmann, R., Krämer, N., & Helmuth, R. (2009). Polymerase chain reaction for the rapid detection and serovar identification of *Salmonella* in food and feeding stuff. *Food Analytical Methods, 2*, 81–95. http://dx.doi.org/10.1007/s12161-008-9057-9

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., & Helmuth, R. (2004). Diagnostic real-time PCR for detection of *Salmonella* in food. *Applied and Environmental Microbiology, 70*, 7046–7052. http://dx.doi.org/10.1128/AEM.70.12.7046-7052.2004

Martínez-Blanch, J. F., Sánchez, G., Garay, E., & Aznar, R. (2009). Development of a real-time PCR assay for detection and quantification of enterotoxigenic members of *Bacillus cereus* group in food samples. *International Journal of Food Microbiology, 135*, 15–21. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.07.013

Mayr, A. M., Lick, S., Bauer, J., Thaerigen, D., Busch, U., & Huber, I. (2010). Rapid detection and differentiation of *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* in food, using multiplex real-time PCR. *Journal of Food Protection, 73*, 241–250.

McGuinness, S., McCabe, E., O'Regan, E., Dolan, A., Duffy, G., Burgess, C., … O'Grady, J. (2009). Development and validation of a rapid real-time PCR based method for the specific detection of *Salmonella* on fresh meat. *Meat Science, 83*, 555–562. http://dx.doi.org/10.1016/j.meatsci.2009.07.004

Mukhopadhyay, A., Mukhopadhyay, K., & U. (2007). Novel multiplex PCR approaches for the simultaneous detection of human pathogens: *Escherichia coli* 0157:H7 and *Listeria monocytogenes*. *Journal of Microbiological Methods, 68*, 193–200. http://dx.doi.org/10.1016/j.mimet.2006.07.009

Najdenski, H., Heyndrickx, M., Herman, L., Werbrouck, H., & Van Coillie, E. (2012). Quantification of *Yersinia enterocolitica* in raw milk using qPCR. *Veterinary Microbiology, 160*, 428–434. http://dx.doi.org/10.1016/j.vetmic.2012.06.013

Nandi, B., Nandy, R. K., Mukhopadhyay, S., Nair, G. B., Shimada, T., & Ghose, A. C. (2000). Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW. *Journal of Clinical Microbiology, 38*, 4145–4151.

Navas, J., Ortiz, S., Lopez, P., Jantzen, M. M., Lopez, V., & Martinez-Suarez, J. V. (2006). Evaluation of effects of primary and secondary enrichment for the detection of listeria monocytogenes by real-time PCR in retail ground chicken meat. *Foodborne Pathogens and Disease, 3*, 347–354. http://dx.doi.org/10.1089/fpd.2006.3.347

Neogi, S. B., Chowdhury, N., Asakura, M., Hinenoya, A., Haldar, S., Saidi, S. M., … Yamasaki, S. (2010). A highly sensitive and specific multiplex PCR assay for simultaneous detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*. *Letters in Applied Microbiology, 51*, 293–300. http://dx.doi.org/10.1111/lam.2010.51.issue-3

Nordstrom, J. L., Vickery, M. C. L., Blackstone, G. M., Murray, S. L., & DePaola, A. (2007). Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Applied and Environmental Microbiology, 73*, 5840–5847. http://dx.doi.org/10.1128/AEM.00460-07

Omiccioli, E., Amagliani, G., Brandi, G., & Magnani, M. (2009). A new platform for real-time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. *Food Microbiology, 26*, 615–622. http://dx.doi.org/10.1016/j.fm.2009.04.008

Panicker, G., & Bej, A. K. (2005). Real-time PCR detection of *Vibrio vulnificus* in oysters: Comparison of oligonucleotide primers and probes targeting *vvhA*. *Applied and Environmental Microbiology, 71*, 5702–5709. http://dx.doi.org/10.1128/AEM.71.10.5702-5709.2005

Panicker, G., Call, D. R., Krug, M. J., & Bej, A. K. (2004). Detection of pathogenic *Vibrio* spp. in shellfish by using multiplex PCR and DNA microarrays. *Applied and Environmental Microbiology, 70*, 7436–7444. http://dx.doi.org/10.1128/AEM.70.12.7436-7444.2004

Parveen, S., Hettiarachchi, K. A., Bowers, J. C., Jones, J. L., Tamplin, M. L., McKay, R., … DePaola, A. (2008). Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *International Journal of Food Microbiology, 128*, 354–361. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.09.019

Perelle, S., Dilasser, F., Grout, J., & Fach, P. (2003). Development of a 5′ nuclease PCR assay for detecting Shiga toxin-producing *Escherichia coli* O145 based on the identification of an "O-island 29" homologue. *Journal of Applied Microbiology, 94*, 587–594. http://dx.doi.org/10.1046/j.1365-2672.2003.01872.x

Perelle, S., Dilasser, F., Grout, J. L., & Fach, P. (2004). 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, 18*, 185–192. http://dx.doi.org/10.1016/j.mcp.2003.12.004

Piknová, L., Kaclíková, E., Pangallo, D., Polek, B., & Kuchta, T. (2005). Quantification of *Salmonella* by 5′-nuclease real-time polymerase chain reaction targeted to fimC gene. *Current Microbiology, 50*, 38–42. http://dx.doi.org/10.1007/s00284-004-4374-8

Pineyro, P., Zhou, X. H., Orfe, L. H., Friel, P. J., Lahmers, K., & Call, D. R. (2010). Development of two animal models to study the function of *Vibrio parahaemolyticus* type III secretion systems. *Infection and Immunity, 78*, 4551–4559. http://dx.doi.org/10.1128/IAI.00461-10

Prendergast, D. M., Lendrum, L., Pearce, R., Ball, C., McLernon, J., O'Grady, D., … Gutierrez, M. (2011). Verocytotoxigenic *Escherichia coli* O157 in beef and sheep abattoirs in Ireland and characterisation of isolates by pulsed-field gel electrophoresis and multi-locus variable number of tandem repeat analysis. *International Journal of Food Microbiology, 144*, 519–527. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.11.012

Quinteiro, J., Sotelo, C. G., Rehbein, H., Pryde, S. E., Medina, I., Pérez-Martín, R. I., … Mackie, I. M. (1998). Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR–restriction fragment length polymorphism

methodologies in species identification of canned tuna. *Journal of Agricultural and Food Chemistry, 46*, 1662–1669. http://dx.doi.org/10.1021/jf970552+

Rahn, K., De Grandis, S. A., Clarke, R. C., McEwen, S. A., Galán, J. E., Ginocchio, C., … Gyles, C. (1992). Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Molecular and Cellular Probes, 6*, 271–279. http://dx.doi.org/10.1016/0890-8508(92)90002-F

Rantsiou, K., Lamberti, C., & Cocolin, L. (2010). Survey of *Campylobacter jejuni* in retail chicken meat products by application of a quantitative PCR protocol. *International Journal of Food Microbiology, 141*, S75–S79. http://dx.doi.org/10.1016/j.ijfoodmicro.2010.02.002

Reyes-Escogido, L., Balam-Chi, M., Rodríguez-Buenfil, I., Valdés, J., Kameyama, L., & Martínez-Pérez, F. (2010). Purification of bacterial genomic DNA in less than 20 min using chelex-100 microwave: Examples from strains of lactic acid bacteria isolated from soil samples. *Antonie van Leeuwenhoek, 98*, 465–474. http://dx.doi.org/10.1007/s10482-010-9462-0

Rijpens, N., & Herman, L. (2004). Comparison of selective and nonselective primary enrichments for the detection of *Listeria monocytogenes* in cheese. *International Journal of Food Microbiology, 94*, 15–22. http://dx.doi.org/10.1016/j.ijfoodmicro.2003.10.016

Rizvi, A. V., & Bej, A. K. (2010). Multiplexed real-time PCR amplification of *tlh*, *tdh* and *trh* genes in *Vibrio parahaemolyticus* and its rapid detection in shellfish and Gulf of Mexico water. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 98*, 279–290. http://dx.doi.org/10.1007/s10482-010-9436-2

Rizvi, A. V., Panicker, G., Myers, M. L., & Bej, A. K. (2006). Detection of pandemic *Vibrio parahaemolyticus* O3:K6 serovar in Gulf of Mexico water and shellfish using real-time PCR with Taqman (R) fluorescent probes. *FEMS Microbiology Letters, 262*, 185–192. http://dx.doi.org/10.1111/fml.2006.262.issue-2

Rodríguez-Lázaro, D., Cook, N., & Hernández, M. (2013). Real-time PCR in food science: PCR diagnostics. *Current Issues in Molecular Biology, 15*, 39–44.

Rodríguez-Lázaro, D., Gonzalez-García, P., Gattuso, A., Gianfranceschi, M. V., & Hernandez, M. (2014). Reducing time in the analysis of *Listeria monocytogenes* in meat, dairy and vegetable products. *International Journal of Food Microbiology, 184*, 98–105.

Rodríguez-Lázaro, D., & Hernández, M. (2013). Real-time PCR in food science: Introduction. *Current Issues in Molecular Biolog, 15*, 25–38.

Rodriguez-Lazaro, D., Hernandez, M., Scortti, M., Esteve, T., Vazquez-Boland, J. A., & Pla, M. (2004). 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, 70*, 1366–1377. http://dx.doi.org/10.1128/AEM.70.3.1366-1377.2004

Rodriguez-Lazaro, D., Jofre, A., Aymerich, T., Hugas, M., & Pla, M. (2004). Rapid quantitative detection of *Listeria monocytogenes* in meat products by real-time PCR. *Applied and Environmental Microbiology, 70*, 6299–6301. http://dx.doi.org/10.1128/AEM.70.10.6299-6301.2004

Rodriguez-Lazaro, D., Lombard, B., Smith, H., Rzezutka, A., D'Agostino, M., Helmuth, R., … Cook, N. (2007). Trends in analytical methodology in food safety and quality: Monitoring microorganisms and genetically modified organisms. *Trends in Food Science & Technology, 18*, 306–319.

Roig, F. J., Sanjuan, E., Llorens, A., & Amaro, C. (2010). *pilF* polymorphism-based PCR to distinguish *Vibrio vulnificus* strains potentially dangerous to public health. *Applied and Environmental Microbiology, 76*, 1328–1333. http://dx.doi.org/10.1128/AEM.01042-09

Rosche, T. M., Binder, E. A., & Oliver, J. D. (2010). *Vibrio vulnificus* genome suggests two distinct ecotypes. *Environmental Microbiology Reports, 2*, 128–132. http://dx.doi.org/10.1111/j.1758-2229.2009.00119.x

Rosche, T. M., Yano, Y., & Oliver, J. D. (2005). A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. *Microbiology and Immunology, 49*, 381–389. http://dx.doi.org/10.1111/mim.2005.49.issue-4

Rossmanith, P., Krassnig, M., Wagner, M., & Hein, I. (2006). Detection of *Listeria monocytogenes* in food using a combined enrichment/real-time PCR method targeting the *prfA* gene. *Research in Microbiology, 157*, 763–771. http://dx.doi.org/10.1016/j.resmic.2006.03.003

Rossmanith, P., & Wagner, M. (2010). The challenge to quantify *Listeria monocytogenes*-a model leading to new aspects in molecular biological food pathogen detection. *Journal of Applied Microbiology, 110*, 605–617.

Rossmanith, P., Mester, P., Wagner, M., & Schoder, D. (2010). Demonstration of the effective performance of a combined enrichment/real-time PCR method targeting the *prfA* gene of *Listeria monocytogenes* by testing fresh naturally contaminated acid curd cheese. *Letters in Applied Microbiology, 51*, 480–484. http://dx.doi.org/10.1111/j.1472-765X.2010.02925.x

Ruiz-Rueda, O., Soler, M., & Calvo, L. (2010). Multiplex real-time PCR for the simultaneous detection of *Salmonella* spp. and *Listeria monocytogenes* in food samples. *Food Analytical Methods, 4*, 131–138.

Samuel, G., Hogbin, J.-P., Wang, L., & Reeves, P. R. (2004). Relationships of the *Escherichia coli* O157, O111, and O55 O-antigen gene clusters with those of *Salmonella* enterica and *Citrobacter freundii*, which express identical O antigens. *Journal of Bacteriology, 186*, 6536–6543. http://dx.doi.org/10.1128/JB.186.19.6536-6543.2004

Saravanan, V., Sanath Kumar, H., Karunasagar, I., & Karunasagar, I. (2007). Putative virulence genes of *Vibrio cholerae* from seafoods and the coastal environment of Southwest India. *International Journal of Food Microbiology, 119*, 329–333. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.08.023

Sharma, A., & Chaturvedi, A. N. (2006). Prevalence of virulence genes (*ctxA*, *stn*, *OmpW* and *tcpA*) among non-O1 *Vibrio cholerae* isolated from fresh water environment. *International Journal of Hygiene and Environmental Health, 209*, 521–526. http://dx.doi.org/10.1016/j.ijheh.2006.06.005

Sheikh, A. F., Goodarzi, H., & Aslani, S. (2012). Identification of *Vibrio cholerae* pathogenicity island (*ctxA*, *OmpW* and *tcpA*) in non-O139 and non-O1 *V. cholerae* strains isolated from Karun River in Ahvaz, Iran. *African Journal of Microbiology Research, 6*, 1185–1189.

Shuan Ju Teh, C., Lin Thong, K., Tein Ngoi, S., Ahmad, N., Balakrish Nair, G., & Ramamurthy, T. (2009). Molecular characterization of serogrouping and virulence genes of Malaysian *Vibrio cholerae* isolated from different sources. *The Journal of General and Applied Microbiology, 55*, 419–425. http://dx.doi.org/10.2323/jgam.55.419

Singh, J., Batish, V. K., Grover, S. (2011). Simultaneous detection of *Listeria monocytogenes* and *Salmonella* spp. in dairy products using real time PCR-melt curve analysis. *Journal of Food Science and Technology, 49*, 234–239.

Soares, S., Amaral, J. S., Oliveira, M. B. P. P., & Mafra, I. (2013). A SYBR green real-time PCR assay to detect and quantify pork meat in processed poultry meat products. *Meat Science, 94*, 115–120. http://dx.doi.org/10.1016/j.meatsci.2012.12.012

Sotelo, C. G., Calo-Mata, P., Chapela, M. J., Pérez-Martín, R. I., Rehbein, H., Hold, G. L., … Santos, A. T. (2001). Identification

of flatfish (*Pleuronectiforme*) species using DNA-based techniques. *Journal of Agricultural and Food Chemistry, 49*, 4562–4569. http://dx.doi.org/10.1021/jf010452a

Suo, B., He, Y. P., Tu, S. I., & Shi, X. M. (2010). A multiplex real-time polymerase chain reaction for simultaneous detection of *Salmonella spp.*, *Escherichia coli* O157, and *Listeria monocytogenes* in meat products. *Foodborne Pathogens and Disease, 7*, 619–628. http://dx.doi.org/10.1089/fpd.2009.0430

Takahashi, H., Kimura, B., Tanaka, Y., Shinozaki, J., Suda, T., & Fujii, T. (2009). Real-time PCR and enrichment culture for sensitive detection and enumeration of *Escherichia coli*. *Journal of Microbiological Methods, 79*, 124–127. http://dx.doi.org/10.1016/j.mimet.2009.08.002

Thiem, V. D., Sethabutr, O., von Seidlein, L., Van Tung, T., Canh, D. G., Chien, B. T., ... Trach, D. D. (2004). Detection of *Shigella* by a PCR assay targeting the ipaH gene suggests increased prevalence of shigellosis in Nha Trang, Vietnam. *Journal of Clinical Microbiology, 42*, 2031–2035. http://dx.doi.org/10.1128/JCM.42.5.2031-2035.2004

Toplak, N., Kovac, M., Piskernik, S., Mozina, S. S., & Jersek, B. (2011). Detection and quantification of *Campylobacter jejuni* and *Campylobacter coli* using real-time multiplex PCR. *Journal of Applied Microbiology, 112*, 752–764.

Wang, L. X., Li, Y., & Mustapha, A. (2007). Rapid and simultaneous quantitation of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* in ground beef by multiplex real-time PCR and immunomagnetic separation. *Journal of Food Protection, 70*(6), 1366–1372.

Ward, L. N., & Bej, A. K. (2006). Detection of *Vibrio parahaemolyticus* in shellfish by Use of multiplexed real-time PCR with TaqMan fluorescent probes. *Applied and Environmental Microbiology, 72*, 2031–2042. http://dx.doi.org/10.1128/AEM.72.3.2031-2042.2006

Wehrle, E., Didier, A., Moravek, M., Dietrich, R., & Märtlbauer, E. (2010). Detection of *Bacillus cereus* with enteropathogenic potential by multiplex real-time PCR based on SYBR green I. *Molecular and Cellular Probes, 24*, 124–130. http://dx.doi.org/10.1016/j.mcp.2009.11.004

Wernars, K., Delfgou, E., Soentoro, P. S., & Notermans, S. (1991). Successful approach for detection of low numbers of enterotoxigenic *Escherichia coli* in minced meat by using the polymerase chain-reaction. *Applied and Environmental Microbiology, 57*, 1914–1919.

Wernars, K., Heuvelman, C. J., Chakraborty, T., & Notermans, S. H. W. (1991). Use of the polymerase chain reaction for direct detection of *Listeria monocytogenes* in soft cheese. *Journal of Applied Bacteriology, 70*, 121–126. http://dx.doi.org/10.1111/jam.1991.70.issue-2

Wiemer, D., Loderstaedt, U., von Wulffen, H., Priesnitz, S., Fischer, M., Tannich, E., & Hagen, R. M. (2011). Real-time multiplex PCR for simultaneous detection of *Campylobacter jejuni*, *Salmonella*, *Shigella* and *Yersinia* species in fecal samples. *International Journal of Medical Microbiology, 301*, 577–584. http://dx.doi.org/10.1016/j.ijmm.2011.06.001

Wolffs, P., Knutsson, R., Norling, B., & Radstrom, P. (2004). Rapid quantification of *Yersinia enterocolitica* in pork samples by a novel sample preparation method, flotation, prior to real-time PCR. *Journal of Clinical Microbiology, 42*, 1042–1047. http://dx.doi.org/10.1128/JCM.42.3.1042-1047.2004

Wolffs, P., Norling, B., Hoorfar, J., Griffiths, M., & Radstrom, P. (2005). Quantification of *Campylobacter* spp. in chicken rinse samples by using flotation prior to real-time PCR. *Applied and Environmental Microbiology, 71*, 5759–5764. http://dx.doi.org/10.1128/AEM.71.10.5759-5764.2005

Wright, A. C., Garrido, V., Debuex, G., Farrell-Evans, M., Mudbidri, A. A., & Otwell, W. S. (2007). Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of *Vibrio vulnificus* bacteria. *Applied and Environmental Microbiology, 73*, 7477–7481. http://dx.doi.org/10.1128/AEM.01118-07

Wuenscher, M. D., Köhler, S., Bubert, A., Gerike, U., & Goebel, W. (1993). The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. *Journal of bacteriology, 175*, 3491–3501.

Yang, C. B., Jiang, Y., Huang, K. H., Zhu, C. Q., & Yin, Y. L. (2003). Application of real-time PCR for quantitative detection of *Campylobacter jejuni* in poultry, milk and environmental water. *FEMS Immunology & Medical Microbiology, 38*, 265–271. http://dx.doi.org/10.1016/S0928-8244(03)00168-8

Zhang, C. (2013). Semi-nested multiplex PCR enhanced method sensitivity of species detection in further-processed meats. *Food Control, 31*, 326–330. http://dx.doi.org/10.1016/j.foodcont.2012.11.002

Zhang, D., Zhang, H., Yang, L., Guo, J., Li, X., & Feng, Y. (2009). Simultaneous detection of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica* and *Escherichia coli* O157:H7 in food samples using multiplex PCR method. *Journal of Food Safety, 29*, 348–363. http://dx.doi.org/10.1111/jfs.2009.29.issue-3

Zhang, W., Bielaszewska, M., Bauwens, A., Fruth, A., Mellmann, A., & Karch, H. (2012). Real-time multiplex PCR for detecting Shiga toxin 2-producing *Escherichia coli* O104:H4 in human stools. *Journal of Clinical Microbiology, 50*, 1752–1754. http://dx.doi.org/10.1128/JCM.06817-11

Zheng, H., Sun, Y., Mao, Z., & Jiang, B. (2008). Investigation of virulence genes in clinical isolates of *Yersinia enterocolitica*. *FEMS Immunology & Medical Microbiology, 53*, 368–374. http://dx.doi.org/10.1111/fim.2008.53.issue-3