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Luca Cocolin[1]*, Andreja Rajkovic[2,3], Kalliopi Rantisou[1], Mieke Uyttendaele[2]

[1]DIVAPRA, Faculty of Agriculture, University of Turin, Italy

[2]Laboratory of Food Microbiology and Food Preservation, Faculty of Bio-Science Engineering, Ghent University, Belgium

[3]Department of Food Safety and Food Quality Management, Faculty of Agriculture, Belgrade University, Serbia

*Address for correspondence: Via Leonardo da Vinci 44, 10095 Grugliasco, Torino, Italy. Tel. 0039/011/670-8553, Fax 0039/011/670-8549, email: lucasimone.cocolin@unito.it

Abstract

The field of food microbiology and safety has experienced an exciting time in the last 10 years. New methods have been introduced to assess safety of foods by targeting pathogenic microorganisms without the need for cultivation. The most prominent innovation in this field is represented by the Polymerase Chain Reaction. From the 90s, PCR has seen a tremendous increase in its applications, and from the conventional, qualitative format, nowadays it became quantitative. In this paper we describe its principles and applications in food safety and we critically evaluate its benefits as well as aspects requiring further improvement.

Introduction

Microbiological safety of food is ensured both by the implementation of hygiene practices and Hazard Analysis Critical Control Points (HACCP) systems as preventive strategy, and by testing the compliance of foods against set microbiological criteria as a control measure. Individual Food Business Operators (FBO) use microbiological analysis for assessment of the performance of their food safety management strategies (Jacxsens, Kussaga, Luning, Van der Spiegel, Devlieghere & Uyttendaele, 2009) and competent authorities use microbiological testing for the purpose of monitoring of the actual situation and trend analysis in order to detect emerging risks. Food-borne pathogens represent one of the key safety concerns for all stakeholders in the food chain. While in the past, safety was considered a quality aspect of the food, nowadays it is a pre-requisite for commercialization of foods. Moreover, at European level in January 1, 2006, a food legislation entered into force (EU Regulation 2073/2005 last amended by 1441/2007 [Anonymous, 2005 and 2006]), imposing obligatory, scientifically determined and risk-assessment-based microbiological criteria, for foods intended for human consumption. Although highly important, both practically and as a statement of the Community determination to guard safety of the consumer, the EC 2073/2005 comprises microbiological limits for only few pathogens-food product combinations.

Following the technological developments in the field of biotechnology and instrumentation, new methods for detection and quantification of food-borne pathogens became available, thereby offering new

possibilities for the safety assessment of foodstuffs. In this paper we will focus on the description of the principles of the quantitative PCR and a critical evaluation of its application in the field of food microbiology for the detection and quantification of food-borne pathogens will be presented.

PCR and its increasingly important role in food diagnostics in the last decennia

Microbiological analysis in foods is an integrated part of management of microbial safety in the food chain. The monitoring and control of food-borne pathogens is traditionally carried out by conventional microbiological methods based on culture-dependent approaches. In other words the presence of food-borne pathogens is revealed by their capability to grow in enrichment and selective media, and form colonies on selective/differential agar plates. These methods include identification of pure culture isolates using series of morphological, biochemical, immunological and other tests. From the '80s, significant efforts have been dedicated by researchers in order to develop improved culture media for the detection and isolation of food-borne pathogens and, especially in the '90s, a number of chromogenic media have been developed, able to presumptively identify the pathogenic microorganisms directly on the plate, based on the specific aspect and color of the colony (Manafi, 2000). These evolutions are still continued. The accuracy of such culture-dependent methods, however, has been criticized in the last years, particularly due to the fact that injured and stressed cells are not always easily recovered, thereby are not able to grow in the selective media and, as a consequence, false negative results can be obtained. Moreover, low numbers of pathogenic microorganisms are not always detected, especially in the case of fastidious food-borne pathogens, i.e. *Campylobacter jejuni* (Habib, Sampers, Uyttendaele, Berkvens & De Zutter, 2008; Rantsiou, Lamberti & Cocolin, 2010). Lastly, another important drawback of the traditional methods is the long time required to obtain the results.

The food microbiological testing is experiencing an exciting *momentum* due to the introduction in the analytical field of new approaches that do not necessarily require growth of the microorganism for its detection. Particularly relevant in the analysis of food-borne pathogens are the methods based on the molecular biology. More specifically, at the end of the '90s, Kary Mullis invented the polymerase chain

reaction (PCR) (Mullis, Faloona, Scharf, Saiki, Horn & Erlich, 1986) a method that revolutionized traditional microbiological analysis by allowing a culture-independent detection directly in the food, without the necessity for classical isolation and identification of the pathogenic microorganism (yet, this may still be advisable for particular purposes, such as characterization of the antibiotic resistance, virulence, growth and survival properties of isolates, as well as their typing for epidemiological studies [Shen et al., 2006; Felicio, Hogg, Gibbs, Teixeira & Wiedmann, 2007]). From its first introduction in the field of food safety (Wernars, Delfogou, Soentoro & Notermans, 1991; Widjojoatmodjo, Fluit, Torensma, Keller & Verhoef, 1991), PCR became an essential analytical tool for researchers working with food-borne pathogens and an impressive number of papers are nowadays still being published.

Second generation PCR: the quantitative PCR principle

Conventional PCR protocols, where the amplification products are detected by gel electrophoresis using UV luminescence and the ethidium bromide double strand DNA intercalating dye, have the big disadvantage of being restricted to research labs. This is mainly due to the fact that there is a need for skills and experience for the execution and the interpretation of PCR results. Furthermore standardization of the whole procedure (from sampling to result interpretation) is challenging. In addition, conventional PCR relies on end-point analysis, therefore amplification products cannot give quantitative information regarding the initial amount of target molecules.

Higuchi, Dollinger, Walsh and Griffith (1992) and Higuchi, Fockler, Dollinger and Watson (1993) pioneered the analysis of PCR kinetics by constructing a system that detects PCR products as they accumulate. The PCR became a method that allows quantification, following technological advancements. In the late ‘90s, instruments able to detect the PCR product while it is synthesized and amplified, became available, making possible quantitative analysis by PCR protocols. This technique, called quantitative PCR (qPCR), or Real time PCR (Rt PCR), revolutionized the molecular approaches in food microbiology. With this method, not only one specific microorganism can be quantified in food, but it is also possible to study its behavior as a consequence of the influence of the environment (i.e food composition, temperature, pH, oxygen, etc.) by

studying expression of suitable target genes. Moreover, since the process is monitored in real time, there is no need for post-amplification treatment of the samples, such as gel electrophoresis, saving time needed to obtain the results of the analysis. A graphical overview of the application of qPCR in food microbiology is presented in Figure 1.

Currently, a 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 they allow simultaneous detection and confirmation of the target amplicon during the PCR reaction (McKillip & Drake, 2004).

Double-Stranded DNA Binding Dyes

Hoeschst 33258 is an example of a minor groove binding dye whose fluorescence increases when bound to double-stranded DNA (Searle & Embrey, 1990). Perkin Elmer Biosystems has developed conditions that permit the use of the SYBR® Green I dye in PCR without inhibition and with increased sensitivity compared to ethidium bromide. Both the advantage and disadvantage of using a DNA binding dye for real-time detection of PCR are that the dye allows detection of any double-stranded DNA generated during PCR. On the positive side, this means versatility because the same dye can be used to detect any amplified product. Thus, any PCR amplification can be monitored simply by including the generic DNA binding dye with the other PCR reagents. On the negative side, both specific and non-specific products generate signal. Therefore, any mis-priming events that lead to spurious bands observed on electrophoresis gels will generate false positive signal when a generic DNA binding dye is used for real-time detection.

Today, research and development have resulted in a number of new intercalating and saturated dyes (SYBR® GreenER, SYTO® 9, EvaGreen®, LCGreen®, CHROMOFY) that give higher fluorescence readouts and reduce the risk of primer–dimer formation. In theory, the sensitivity of the assay should be increased, because threshold cycle value acquisition can take place at earlier cycles.

Another aspect of using DNA binding dyes is that multiple dyes bind to a single amplified molecule. This increases the sensitivity for detecting amplification products. A consequence of multiple dye binding is that

the amount of signal is dependent on the mass of double-stranded DNA produced in the reaction. Thus, if the amplification efficiencies are the same, amplification of a longer product will generate more signal than a shorter one.

Fluorogenic Probes

Real-time systems for PCR were improved by probe-based PCR product detection. In contrast to the non-specific double-strand binding dyes, an alternative method, the 5' nuclease assay, provides a real-time method for detecting only specific amplification products. Holland, Abramson, Watson and Gelfand (1991) were the first to demonstrate that cleavage of a target probe during PCR by the 5' nuclease activity of *Taq* DNA polymerase could be used to detect amplification of the target-specific product. With fluorophores attached to either end of an oligonucleotide, sophisticated probes are engineered to fluoresce only upon disrupting a molecule's geometry (Lee, Connell & Bloch, 1993). The signal emitted by such probes is carefully regulated by a molecular interaction called Förster Resonance Energy Transfer (FRET), a quenching mechanism phenomenon first described in the 1940s (Broll, 2010), in which quenching can occur over a fairly long distance (100Å or even more), depending on the fluorescence dye and quencher used, causing the signal from one modification to be immediately captured by the other, rather than be released as fluorescence. Alternatively, the dyes may be also physically binding one another to extinguish the signal through static quenching. Either way, the signal from the first cannot escape the grasp of the second until they become separated (Sowers, 2009). Probe design and synthesis has been simplified by the finding that adequate quenching is observed for probes with the reporter at the 5' end and the quencher at the 3' end (Livak, Flood, Marmaro, Giusti & Deetz, 1995). Examples of probes with two dyes are the hydrolysis probes, popularly called TaqMan® probes (Holland et al., 1991), molecular beacons (Tyagi & Kramer, 1996), hybridization probes (Caplin, Rasmussen, Bernard & Wittwer, 1999) and the scorpion probes (Kubista et al., 2006). In the case of the hydrolysis probes, if the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of *Taq* DNA polymerase as this primer is extended. This cleavage of the probe separates the reporter dye from the quencher dye,

increasing the reporter dye signal. Cleavage removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process. Additional reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon produced. Recently, in order to increase the specificity of these probes the TaqMan®MGB probes have been proposed. DNA probes with conjugated minor groove binder (MGB) groups form extremely stable duplexes with single-stranded DNA targets, allowing shorter probes to be used for hybridization based assays. In comparison with unmodified DNA, MGB probes have higher melting temperature (T_m) and increased specificity (Kutyavin et al., 2000). For the molecular beacons and the scorpion probes, their hybridization on the target sequence during the annealing step provokes a geometrical rearrangement of the structure of the molecule pulling apart the reported from the quencher, voiding the extinguishing of the signal thereby allowing fluorescence.

The advantage of fluorogenic probes over DNA binding dyes is that specific hybridization between probe and target is required to generate fluorescent signal. Thus, with fluorogenic probes, non-specific amplification due to mis-priming or primer-dimer artifact does not generate signal. Another benefit of fluorogenic probes is that they can be labeled with different, and from each other distinguishable, reporter dyes. By using probes differentially labeled, amplification of two distinct sequences can be detected in a single PCR reaction. Multiplex real-time PCR approaches clearly have several advantages, but are limited by the availability of fluorescent dye combinations, the spectral overlap between dyes, and the need to assure specific amplification and the same PCR efficiency for all targets.

The fidelity of a qPCR assay is measured by its specificity, low background fluorescence, steep fluorescence increase, high amplification efficiency, and high level plateau. The absolute dynamic range of the detectable fluorescence (maximal plateau minus background fluorescence) should be maximally extended in a qPCR assay. Introduction of new chemistries and also enzyme, hardware, plastic ware, and cycling procedures improvements have resulted in qPCR finding its way for widespread application in the detection of food-borne pathogens (Pfaffl, 2009).

Opening opportunities to bring the qPCR technique to routine labs for pathogen testing

By a PCR-based method, the target pathogen is detected independently of its physiological state, thereby stressed or injured cells are picked up by the method, avoiding the false negative results described above for the traditional microbiological methods and as such PCR screening may better reflect the true prevalence of pathogens in surveys. The time required for the qPCR detection is overall much shorter if compared to the cultural methods. The PCR step alone can take as short time as 40 minutes with specific fast chemistries but most commonly a run takes ca. 90 minutes and thus time to result, including DNA extraction and interpretation of qPCR outcome, is usually obtained in ca. 3-4 hours. If an enrichment is carried out, a step that is deemed necessary for the detection of low numbers of pathogenic microorganisms (1-100 colony forming units [cfu]/10g) and that usually takes up minimum 6h up to 24h, overall responses on the presence of food-borne pathogens by PCR are referred to as next day results which is still considered rapid in food diagnostics.

The PCR method, on the condition of the right primer set chosen and PCR reaction well optimized, is extremely specific for the target pathogen and in this way false positive results can be avoided. As such it is advocated that upon a positive detection signal further isolation and confirmation of the pathogen is not needed. However, European Validation bodies still recommend the confirmation of a positive signal by isolation of the pathogen, also considering the fact that qPCR positive results with Ct values > 40 (overall indication of very low levels of DNA near the theoretical detection limit) should be interpreted with care (Stals et al., 2009).

There are some inherent characteristics of the underlying principles of PCR that need to be taken into consideration for full acceptance and functionality of qPCR in food diagnostics. Since the target for the amplification is mainly represented by DNA, a molecule characterized by high stability also after cell death, a positive result by PCR does not necessarily indicate the presence of alive pathogenic microorganisms in foods and thus a potential public health risk. This pitfall of the PCR has been addressed in different ways. It is generally accepted that if positive results are obtained after an enrichment step, this implies the

presence of alive populations that were able to multiply, and their DNA was subsequently amplified by PCR (Rossen, Holstrom, Olsen & Rasmussen, 1991). A prior (short) enrichment step in non-selective or selective media is part of most of the commercial PCR kits' usual protocol for food pathogens' presence/absence testing. Nonetheless, even the mere presence of dead cells by PCR indicate that somewhere along the food production line there has been a breach of food safety measures e.g. initial high contamination of raw materials. A number of approaches have being designed in PCR itself to overcome detection of dead cells, such as detection of mRNA in reverse-transcriptase PCR format and exclusion of dead cells on the basis of their permeable membrane to intercalating dyes, such as ethidium bromide monoazide, in e.g. EMA-PCR format. None of the approaches was completely and always successful (Nogva, Dromtorp, Nissen & Rudi, 2003).

A drawback to the uptake of PCR by analytical labs in the food industry is the significant investment required (certainly an issue at internal FBO laboratories of SMEs) in terms of equipment, infrastructure as well as lab flow adjustments and trained personnel, although in the last years, the market has seen a significant decrease in the prices of the thermal cyclers, dedicated to the amplification of the DNA, and associated PCR reagents.

Sample preparation, a prerequisite for reliable qPCR application in the food labs for food-borne pathogen testing

The terms "rapid" and "sensitive" in the description of the PCR technique as a detection method for food-borne pathogens are relative. PCR, as such, is taking only approx. 90-120 minutes. However, PCR detects genomic DNA copies in a PCR tube, rather than bacterial cells in foods as requested by legal criteria. For detection of low numbers i.e. 1 to 10 cells per 25 g, a prior cultural enrichment procedure is normally applied. The minimum time needed for the prior enrichment to enable a positive PCR detection might be calculated from the mean generation time at optimum temperature in the enrichment medium, ca. 20-30 minutes, and shows that ca. 4-5 hours of prior enrichment is needed to obtain a positive test result, if starting from low numbers. However, it should also be mentioned that pathogens in foods are often

stressed and/or sub-lethally injured, due to processing and preservation techniques (salting, acidification, freezing, packaging, etc.), requiring an increased lag phase (2-3h) prior to the exponential growth phase, prolonging the minimum time to detection (Jasson, Uyttendaele, Rajkovic & Debevere, 2007). Therefore, a 6-8 h enrichment is considered as a prerequisite for reliable detection of low numbers of pathogens. The enrichment time may be further prolonged (commonly used overnight enrichment) for slower growing organisms such as *L. monocytogenes* or if selective (less growth supporting) broths are used for enrichment (Jasson, Rajkovic, Debevere & Uyttendaele, 2009). Shortened enrichment procedures, overlooking the need to repair sub-lethally injured cells may result in a true risk of not reaching the detection limit of the subsequent PCR technique and can lead to false negative results (Jasson, Rajkovic, Baert, Debevere & Uyttendaele, 2009).

On the other hand, PCR methods have another hurdle that should be tackled and that is still playing against the fully blown adoption for pathogen detection in the food chain. That has mostly to do with the relatively low levels of pathogens present in difficult-to-analyze foods. A crucial issue in the application of PCR for pathogen screening is the worry that negative qPCR results are observed due to inhibition by food constituents, that in some instances are difficult to eliminate when performing the nucleic acids extraction protocols prior to amplification. Methods applied to separate and concentrate microorganisms from foods (and their inhibitory components) are reviewed in a great detail elsewhere (Brehm-Stecher, Young, Jaykus & Tortorello, 2009). In latest years, sample preparation rather than the actual PCR technique have been shown to be the bottleneck of the molecular detection approach. It has been indicated that complex and laborious methods do not always have a better recovery (Baert, Uyttendaele & Debevere, 2007, Baert, Uyttendaele & Debevere, 2008). The goal of improving sample preparation, as part of a more rapid detection technique, is the improvement of the speed, the cost, automation and user friendliness while maintaining a high recovery of cells. Focus is actually put on concentration protocols of bacteria or DNA from larger volumes e.g the use of bacteriophage-based capture (Bennett, Davids, Vlahodimou, Banks & Betts, 1997), the use of nanoparticles (Yang, Mi, Cao, Zhang, Fan & Hu, 2008), devices enabling upscaling of present available capture methods (Warren, Yuk & Schneider, 2007). Fukushima, Katsube, Hata, Kishi and

Fujiwara (2007) reported a sample preparation method combining filtration, low- and high-speed centrifugation and buoyant density centrifugation to concentrate food pathogens up to 250-fold without enrichment and detected loads as low as 10 to 10^3 cfu/g. Alternatives are sought to direct detection and quantification of microbial loads to avoid destructive and cumbersome sample preparation in food analysis. Although this makes a compromise to accurate quantification it offers good screening and semi-quantitative information. Gomez, Pagnon, Egea-Cortines, Artes, & Weiss (2010) reported use of qPCR to quantify total aerobic count on fresh produce by using centrifugation water (CW), obtained during processing, instead of fresh produce matrix itself. On an average, 35% of the natural bacterial population and 64% of inoculated bacteria were recovered in the CW. Taking into account log CFU/g values it becomes clear that bacterial number in CW was proportional to initial lettuce contamination suggesting that application of qPCR on CW allows a narrow estimation of lettuce contamination.

Often, the sample preparation is based on commercially available kits that are specifically designed for a specific target. However, sample preparation methods should be also selected depending upon the food matrices under analysis and the sensitivity of the test required. Protocols selected are always a compromise between cost, rapidity and universal application with regard to the food matrix. Nevertheless, in the concentration of the cells (or DNA), care has to be taken not to concentrate potential interfering factors from the food matrix. This would jeopardize the efficient functioning of the qPCR reaction, which is prone to inhibition, and in turn might lead to false negative results. Different inhibitory components can result from the food matrix, growth media (Rossen, Norskov, Holmstrom & Rasmussen, 1992), and nucleic acids extraction reagents (Rodriguez-Lazaro et al., 2007). It has been suggested to add PCR facilitators to overcome PCR inhibition such as BSA, Triton X-100, Tween 20 (Malorny, Huehn, Dieckmann, Kramer & Helmuth, 2009). The inclusion of an internal amplification control in every PCR tube is also strongly recommended to check on inhibitory compounds present in the food sample's DNA extract (Anonymous, 2006).

Application of qPCR for enumeration of pathogens in foods

A prerequisite for the application of qPCR to quantify food-borne pathogens in foods can be obtained through the generation of calibration curves. Calibration curves associate the parameter of cfu/g or ml, commonly used in traditional microbiological analysis, with the Ct value, the threshold cycle in qPCR. However, in order to maintain the correlation between the numbers of bacteria in the food and the enumeration by PCR, a prior enrichment step should be avoided. Although, a short pre-enrichment step, followed by qPCR, has been suggested to obtain quantitative data of *Salmonella* in meat (Malorny et al., 2009).

For application of qPCR in food diagnostics it should be stressed out that calibration curves must be created in food matrices, because only in this way all steps involved in the sample preparation and amplification are taken into account. It would be experimentally not correct to create standard curves for the quantification of a microorganism in a specific food, by diluting a pure culture, extracting the DNA from each dilution and then performing the qPCR. Moreover, in order to have a direct correlation of the quantification with an internationally accepted way to report microbial counts, standard curves cannot be constructed from DNA dilutions, as is the usual approach in molecular biology applications, and the results should not be reported as genome equivalents. Making calibration curves with pure cultures or genomic DNA will neglect the inference that the food matrices have when extracting the DNA by co-extracting inhibitory compounds and thus affecting the efficiency of the PCR reaction. Such types of calibration curves would result in a wrong estimation of the microbial load. It should be pointed out that calibration curves should be constructed for various types of foods as usually there is variation in extraction efficiency from different sources. DNA extraction that is functional for one food type may not necessarily be applicable to others as its performance may depend upon the food composition. Therefore, tailored, case-by-case protocols for extraction of DNA are needed, and the development of qPCR approaches for enumeration of pathogens in food can be a real challenge. It is recommended to include, with each series of targeted food samples subjected to qPCR, a process control (cells inoculated in the food item/suspension taken up in parallel during the extraction of the food item under investigation) in the extraction step. Using process controls will provide information on the efficiency of the extraction and the PCR procedure. When designing

calibration curves one needs to keep in mind PCRs insensitivity to physiological status of cells, while the destiny of cells (dying, surviving and growing) in food samples will depend on their physiological status. Therefore, cultures used for determination of calibration curves still need to be as realistically stressed as possible.

Quantification of genomic copies by qPCR and counting cfu by classical culture based methods result in data expressed in different units, the latter being a key feature defined by legislation. The correlation between these two targets could potentially be established to allow appropriate data interpretation. It has to be noted that the correlation between genomic copies by PCR and cfu is not straightforward and not permanent (e.g. species and strain based variations, growth phase based variations etc.). The detection of genomic copies can result in overestimation of present bacterial cells due to multiple copies of targeted genetic fragments (Seo, Valentin-Bon & Brackett, 2006). In addition, the challenge of the multiple chromosomes in some bacterial species can lead to several-fold overestimation of present bacterial cells (Akerlund, Nordstrom & Bernander, 1995; Saier, 2008; Sherratt, 2003).

In spite of the potentials offered by qPCR, often its limit of quantification does not comply with the maximum tolerable level imposed by law. The explanation for this relatively low performance of the qPCR is not related with the PCR technique itself (which is intrinsically sensitive) but rather with the problems of sample preparation and DNA extraction from food samples. As mentioned above, this relates with PCR inhibitors, present in food but also to the use of small input volumes to PCR versus large test portions to be analyzed. The quantification limit of qPCR as reported above are still too high for practical application of pathogen enumeration, since for most samples taken along the food chain, the contamination is low (usually less than 100 cells/g). From this point of view one could question the use of qPCR for quantitative purposes. For this reason, the major application of qPCR is for qualitative, presence/absence testing, performed after an enrichment step. In this context, qPCR gives the advantage to avoid post-processing handling. Since gel electrophoresis is not necessary to analyze the results of the amplification process, the time requested to obtain the qualitative result is shorter when compared to conventional PCR. Only in few cases (if compared with the total number of papers published in the last 5 years) authors put efforts in

developing qPCR protocols that could be used for quantification purposes without enrichment. Some examples of these studies are reported in Table 1, where the target organism, the food matrices in which the microorganism was quantified, the chemistry used for quantification and the detection limit are reported.

Final remarks and future perspectives

The use of molecular biology in food microbiology is probably one of the best examples of how applied science, such as food diagnostics, gets revolutionized and driven by advances in basic sciences. Although many rapid and sensitive methods have been developed and their applications described (Jasson, Jacxsens, Luning, Rajkovic & Uyttendaele, 2010), PCR in its different forms, remains currently the most important one. As reported in this paper, the field of food safety has seen the introduction of PCR methods for detection and quantification of pathogenic microorganisms. These approaches allow for a better recognition of the contaminated foods, thereby better protection of the consumer can be achieved. It is important to highlight that methods, such as qPCR, can be applied throughout the food chain, in order to monitor and quantify food-borne pathogens in any step of food production. If in the last 5 years an increasing number of papers have been published reporting quantification of pathogenic microorganisms in final food products, it is only very recently that the exploitation of qPCR to control their presence in food chains and production plants has been described. Alessandria, Rantsiou, Dolci and Cocolin (2010) and Osés, Rantsiou, Cocolin, Jaime and Rovira (2010) used qPCR to follow the routes of contamination of *L. monocytogenes* and STEC *E. coli* in a dairy plant and in the lamb food chain, respectively. These applications showed how qPCR can be used as an important diagnostic tool to identify weak points during production, which can lead to a contaminated food product. Even more importantly, the evidence of viable but not culturable cells on the surfaces of the equipment and in some production steps (for example the salting in brine for the cheese production) was reported. This aspect should be stressed out particularly because intervention strategies can be implemented only if FBOs realize that there are weak points that should be corrected.

Food microbiologists have lost their initial hesitation to this “high tech” technique and accepted that PCR is a complementary tool to set rapid diagnostic test methods for microbiological analysis of foods. Progressive introduction of automated equipment for qPCR has enabled it to become a high through-put method. Further evolutions comprise the miniaturization of the PCR method e.g. by Lab on a Chip (LOC) technology that integrates sample concentration, PCR amplification and detection and might bring the PCR technique closer to on-field diagnostics.

The shortcomings of the qPCR methodology in food diagnostics are still intriguing scientific attention and wide-scale efforts are made around the globe to bring PCR based methods another step closer to overall applicability in diagnostics of food-borne pathogens. However, the experience so far indicates that also these methods, just like currently employed golden standard classical cultural methods, suffer from imperfections that will be driving force for further improvements and permanent look out for a better assay.

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Table 1: Examples of studies in which a quantification of the target microorganism was done by the mean of qPCR without enrichment

Microorganism	Food matrix	qPCR chemistry	Limit of quantification	Reference
Bacillus cereus	Liquid egg Infant formula	MGB probe	60 cfu/ml	Martinez-Blach, Sanchez, Garay & Aznar, 2009
Campylobacter jejuni	Chicken meat	SYBR Green	10 cfu/g	Rantsiou et al., 2010
Escherichia coli O157:H7	Milk Dairy foods	Scorpion probe	10^3 cfu/g	Singh, Batish & Grover, 2009
Escherichia coli O157:H7	Vegetables leaf	Taqman probe	2.3 x 10^3 cfu/g	Ibekwe, Shouse & Grieve, 2006
Klebsiella pneumoniae	Infant formula	SYBR Green	2x10^3 cfu/25 g	Sun, Wu, Qiu, Jin, Wang & Li, 2010
Listeria monocytogenes	Meat and meat products Milk and milk products Ready to eat salads	Taqman probe	10^3 to 10^4 cfu/ml or g	Rantsiou, Alessandria, Urso, Dolci & Cocolin, 2008
Listeria monocytogenes	Salmon	Taqman probe	10^3 cfu/g	Rodriguez-Lazaro, Jofré, Aymerich, Garriga & Pla, 2005
Listeria monocytogenes	Meat	Taqman probe	10^3 cfu/g	Rodriguez-Lazaro Jofré, Aymerich, Hugas & Pla, 2004
Listeria monocytogenes	Salads	Hybridization probes	10 cfu/g	Berrada, Soriano, Pico & Manes, 2006
Mycobacterium avium subsp. *paratuberculosis*	Milk	Taqman probe	5 to 6 cells/ml	Slana, Kralik, Kralova & Pavlik, 2008
Salmonella enterica *Listeria monocytogenes*	Yogurt	SYBR Green	10 cells/10 g	D'Urso, Poltronieri, Marsigliante, Storelli, Hernandez & Rodriguez-Lazaro, 2009
Salmonella enterica	Pork meat (pig carcass swab)	Taqman probe	4.4 x 10^2 cfu/ml	Lofstrom, Schelin, Norling, Vigre, Hoorfar & Radstrom, 2010
Shigella spp.	Buffer rinse of 15 different fresh produce*	Taqman probe	0.4 to 16 cfu/100 ml	Lin, Cheng, & Van, 2010
Vibrio vulnificus	Shellfish Drinkwater	SYBR Green	10^2 to 10^3 cells/g or ml	Gordon, Vickery, DePaola, Staley & Harwood, 2008

*Although the inoculation was done in rinsing buffer and on produce surface, it still covers most of the sample preparation steps except the efficacy
of the rinse step.

Figure 1. Schematic and simplified presentation of quantitative PCR application work-flow in microbial food analysis (modified from Kretzer, Biebl, & Miller, 2008).