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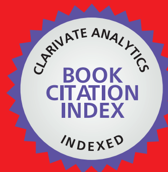

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Chapter

Real-Time Quantitative PCR as a Tool for Monitoring Microbiological Quality of Food

Amanda Teixeira Sampaio Lopes and Bianca Mendes Maciel

Abstract

Microbiological parameters of food provide quality information regarding the processing, storage, and distribution conditions, shelf life, as well as whether the food poses a health risk to the population. In this context, the concern with food safety is a competitive advantage, as the pressure of consumers, who are increasingly interested and concerned about what they are consuming, directs the trade to reach the quality of products and services offered. With regard to microbiological analyses, researchers have been developing sensitive techniques to produce rapid results, since traditional methods of microbiological culture are time-consuming and very laborious. Thus, the real-time quantitative PCR technique (qPCR) offers the possibility of quantifying the total bacterial DNA in a food sample without the need of the microbial growth step. That is, the result can be expressed on the same day, and it is possible to perform a simultaneous quantification of more than one pathogen in a single assay. Therefore, it can be a useful tool for monitoring microbiological quality in food industries. In this chapter, we will present the advantages and disadvantages of this methodology for food microbiology emphasizing the challenge of differentiating viable cells from nonviable cells.

Keywords: pathogen quantification, food microbiology, food safety, quantitative microbial risk assessment, propidium monoazide qPCR, ethidium monoazide qPCR

1. Introduction

The quality term has undergone variations over time. In the last century, the food quality was expressed only by the intrinsic and extrinsic characteristics of different individual units of a product which would determine its acceptability [1]. Currently, the term quality has already a broader aspect: it is related to the ability to plan and develop continuous actions during all stages of processing, aiming at maintaining the product characteristics and performance to reach the requirements that satisfy the needs and expectations of the consumer [2]. Thus, food companies seek to achieve more and more the quality standard of their products that will be delivered to the final consumer. In turn, the perception of quality by consumers is closely linked to the attributes they most value: nutrition and food safety.

Food safety practices aim to ensure the appropriate physical, chemical, and microbiological conditions for product quality. For food industries, the safety aspect is always a determining factor about quality, since any problem can compromise the consumer health, culminating in serious financial losses and diminishing the reliability of their products. Thus, the industry advocates the application of food safety management system in the entire food production chain, as a preventative approach toward identifying, preventing, and reducing foodborne hazards, to ensure the food is safe for consumption and with nutritional value. Only then, the total quality of food can be reached [3–5].

One of the main parameters that determine the food quality is its microbiological characteristic, since microbial contamination is responsible for most foodborne disease (FBD) outbreaks in worldwide, affecting individuals of all ages, particularly children under 5 years of age and persons living in low-income regions of the world [6]. The microbiological evaluation is performed with the objective of establishing the absence of pathogens or their toxins and to enumerate total or indicator microorganisms that provide information about the conditions of processing, storage, distribution, shelf life, and the health risk of the population [7]. As such examples, we can cite the increased numbers of *Staphylococcus aureus* when detected in a food processing step might imply in contamination by handling, the increased numbers of *Escherichia coli* might suggest fecal contamination, and the detection of *Salmonella* spp. might indicate that the processing has not been able to eliminate pathogenic microorganisms [8].

The microbiological quality should be effectively focused on traceability, with emphasis on the “farm-to-fork” approach, reaching the entire food chain. For this, rapid diagnostic methods are highly recommended so that early interventions of control strategies can be applied, ensuring the consumer’s health and reducing the financial losses of the industry, as well as the costs with public health in cases of FBD outbreaks. In addition, these methods are essential for assessment of food safety objectives (maximum levels of hazards at the point of consumption) in food safety management, which require results in a shorter time than those obtained by culture cultivation [9]. Among rapid methods, real-time quantitative polymerase chain reaction (qPCR) has been shown to be a good tool for monitoring microbiological quality of food, since this technique is evolving to improve the sensitivity and specificity in detection and quantification of pathogens. According to “MIQE guidelines” [10], we chose to use the abbreviation qPCR for real-time quantitative PCR in entire chapter, avoiding confusion with other abbreviations that designate reverse transcription-qPCR (RT-qPCR).

2. Advantages and disadvantages of real-time quantitative PCR for microbiological assessment of food quality

For more than a century, the identification and isolation of pathogens in food and clinical samples were performed exclusively by microbiological culture techniques. The analyses use a wide variety of selective, nonselective, and differential media. The suspect microbial colonies in these media are selected and isolated and need to go through yet another confirmation step, the biochemical tests. If a pathogen is detected, serological typing and more detailed biochemical tests are performed, and the data from these tests facilitate epidemiological analyses. However, even though these conventional methods are valuable, there is a great need of time (around 1 week) and material, making this technique inadequate in the event of a food outbreak. One of the main criticisms of conventional methods is that the results are available relatively late in clinical disease, limiting the overall value of

the test. Treatment decisions are usually based on the clinical severity of the disease prior to receipt of confirmation of isolation of the microbial culture. This long period to diagnose foodborne diseases by traditional microbiological methods may have an impact on the clinical pathway for each patient. However, for isolation and identification of bacterial pathogens transmitted by food, these classical methods are still considered as the "gold standard," especially by regulatory agencies, since they are harmonized methods in worldwide [7, 11, 12].

In the last decades, several alternative methods have been developed with the purpose of producing fast microbiological results to ensure food safety and allowing manipulation of multiple samples in the same analysis [12]. These methods are based on chromogenic culture media, immunoassays for antigen detection, bacteriophage analysis, biosensors, or molecular methods that detect nucleic acids [11–15]. Among the molecular methods, polymerase chain reaction (PCR) is the most versatile and widely used amplification technique [12].

2.1 Principle of real-time quantitative PCR techniques

The real-time quantitative PCR technique (qPCR) is a variant of conventional PCR and offers the possibility of quantifying the pathogen DNA in a sample in real time, without the need of microbial growth steps. That is, the result can be expressed on the same day. In addition, it is possible to perform multiplex analyses, allowing simultaneous quantification of more than one pathogen in the same assay [16]. For absolute quantification of pathogens, it is necessary to design a standard curve through serial dilutions of a known amount of target DNA [17]. In this curve, the lowest DNA amount detectable by the technique may be included (< 10 copies of a target gene) to attest its sensitivity. The high sensitivity, specificity, and speed of results have allowed qPCR to be widely used for specific pathogen quantification in which microbial amount is low.

The use of qPCR, by reducing the time associated with generating quantitative data, offers the potential to increase the robustness of the quantitative microbial risk assessment, thus allowing a subsequent early intervention of control strategies. The quantification of a pathogen in a food product and the prevalence of contamination are important parts of the quantitative microbial risk assessment modeling process, because it needs to determine the probability of exposure as well as the amount of exposure to a pathogen [18].

The quantification through qPCR is based on the exponential increase of the initial DNA amount during PCR amplification cycles. After amplifying a specific sequence, the amplification progress is monitored in real time using fluorescence technology. As soon as the fluorescent signal reaches a threshold level, correlation with the amount of original target sequence occurs, thus allowing DNA quantification in a sample. In addition, the final product may further be characterized by gradual raising temperature during a melt curve to determine the "melt temperature." This point is reached when half of the DNA strand is on single strand and the other half on double strands. It depends on the length and composition of nucleotide sequence of the target gene, which increases the specificity of technique [19].

Among fluorescent reagents, the DNA intercalating agents (such as SYBR Green) and hydrolysis probes (also known as TaqMan™ probes) are the most popularly used. SYBR Green dye is a nonspecific detection system that promotes intercalation, followed by surface binding to double strands of newly amplified DNA [20]. As the DNA is amplified, the fluorescent signal is emitted by the reagent and detected by the equipment. As any DNA amplification can be detected and quantified, to help ensure the reaction specificity, the melt curve of the amplified product can be analyzed to determine the melt temperature (Tm). If there are two

or Tm peaks, it is suggested that more than one sequence was amplified, and one may not be the specific DNA target [19] or even primer dimer.

The principle of detection system using the hydrolysis probes is based on Förster resonance energy transfer, when a non-radiative energy is transferred from a fluorescent donor (the fluorophore) to a lower energy acceptor (the quencher) via long-range dipole-dipole interactions [21, 22]. It occurs because the hydrolysis probes are small dual-labeled oligonucleotide sequences: in one side, it is labeled by a specific fluorophore, and the other side by the quencher. As the fluorophore and quencher are in close proximity, the quencher adsorbs the reporter fluorophore signal. When the DNA amplification occurs during qPCR reaction, the probe is hydrolyzed by the Taq DNA polymerase, due to its 5′-nuclease activity, and the fluorophore and quencher are separated, emitting fluorescence that corresponds to specific amplification of the target DNA [19]. The great advantage of qPCR using hydrolysis probes is that when the probes are labeled with fluorophores that emit fluorescence at different wavelengths, there is the possibility of performing a multiplex qPCR reaction in which more than two targets are detected and quantified simultaneously in a specific way [23]; therefore, it is a good alternative for use as a rapid test in large number of samples, providing real-time results, and to diminish the cost of analyses.

2.2 qPCR *versus* traditional culture method in food microbiology

Some studies comparing qPCR and microbial culture observed that qPCR for the detection of a single pathogen (singleplex assay) demonstrated to be statistically more sensitive than the conventional technique. Real-time PCR assay specific for detection of *Salmonella enterica* serotype enteritidis analyzed 422 naturally contaminated environmental samples from integrated poultry houses, being the same samples also evaluated by traditional microbiology. The diagnostic sensitivity of the qPCR assay for these samples was significantly higher than those using the culture method. In addition, the result of real-time PCR was obtained in 2 days, while the traditional method took 4–8 days [24]. Another study comparing standard culture methods, conventional PCR, and real-time PCR for the detection of *Listeria monocytogenes* in milk, cheese, fresh vegetables, and raw meat showed that the real-time PCR assay was statistically more sensitive, reducing the time of analysis and laborious work [25]. The targeted gene coding for a protein of the ribosome large subunit was used in qPCR for quantifying Enterobacteriaceae in 51 food products naturally contaminated. The results showed high specificity to differentiate Enterobacteriaceae of non-Enterobacteriaceae based on the cycle threshold (Ct) values; by comparing qPCR and culture methods, only a < 1log difference between methods was obtained in 81.8% of these samples [26]. In seafood products and sediments, conventional PCR, real-time PCR, and culture methods were used to detect pathogenic *Vibrio* spp. (*V. parahaemolyticus*, *V. cholerae*, and *V. alginolyticus*) in 113 fish, 83 clams, 30 seawater samples, and 21 sediment samples. Of the 247 samples analyzed, 41.3% were positive for traditional microbiological method, while 51% were positive for the molecular methods, without prior isolation of pathogens [27].

However, by using multiplex qPCR assay for detection/quantification of more than one pathogen, the sensitivity of the technique may decrease compared to the traditional culture technique (or even compared with singleplex assay), probably due to the competitive nature of the process [8]. In our lab, we compared multiplex qPCR assay for quantification of *Escherichia coli, Staphylococcus aureus,* and *Salmonella* spp. with singleplex assays (by hydrolysis probes and by SYBR Green) in 28 naturally contaminated oyster samples containing pools of 40 oysters collected from natural estuarine environment (1120 in total). The multiplex assay presented lower sensitivity and higher specificity than both singleplex assays

(data not published). This can be caused by the competition of the primers by the reagents available in the reaction mix or by the non-varying concentrations of the reaction components (which are used in the same way in the singleplex and multiplex reactions). In addition, the amplification of one target DNA present in the reaction can be overcome by more efficient amplification of other targets (including nonspecific products), thereby reducing the efficiency of the multiplex reaction and consequently decreasing its sensitivity [28]. The same methodology was applied in different food matrices (ground beef, milk, and oyster samples) artificially contaminated by *E coli, S. aureus*, and *Salmonella enteritidis*. Differences <1log in *E. coli* and *S. aureus* quantities were observed comparing multiplex qPCR and traditional culture method in milk and ground beef, with no statistic difference. However, in oyster samples, the multiplex qPCR demonstrated to be more sensitive than culture methods for *E. coli* quantification [8]. Thus, we can affirm that the food matrix can interfere in the sensitivity of the results due to the intrinsic nature of PCR inhibitors present in such food.

	Singleplex qPCR	**Multiplex qPCR**	**Traditional culture**
Advantages			
Shorter analysis time	++	+++	0
Specificity	+++	++	+
Sensitivity	+++	+	++
Reproducibility	+++	+++	++
Monitoring the results in real time	+++	+++	0
Simultaneous quantification of different pathogens	0	+++	0
Distinguishing of living cells from dead cells	0	0	+++
Detection of “viable but non-culturable” (VBNC) microorganisms	+++	++	0
Colony isolation for further genotyping/phenotyping analysis	0	0	+++
Potential of automation	+++	+++	0
Standardized method in worldwide	+	0	+++
“Gold standard” for regulatory agencies	0	0	+++
Fast screening of large number of samples	+++	++	0
Useful for microbiological quality control	+++	+++	+
Useful for the quantitative microbial risk assessment	+++	++	+
Disadvantages			
Cost of material, equipment, and infrastructure	−−−	−−−	−−−
Competitive amplification (decrease of the efficiency)	0	−−−	0
Interference of food sample	−−−	−−−	0
Labor-intensive analysis	−−−	−−−	−−−
Need for qualified personnel	−−−	−−−	−−−

**Based on Refs. [8, 12].*
(+) advantage score; (−) disadvantage score; (0) no score for such characteristic.

Table 1.
Advantage and disadvantage scores of real-time quantitative PCR (singleplex/multiplex qPCR) and traditional culture methods for microbiological analysis of food.*

To increase sensitivity, a pre-enrichment step may be applied prior to qPCR reaction. However, this stage favors microbial growth making it impossible to quantify the pathogens in the original sample; only their detection is possible [29]. Therefore, for simultaneous quantification of pathogens in food, multiplex qPCR can be a potential tool for rapid screening of large number of samples in food industries, leading to faster product release for sale [8].

The high cost of equipment investment and its maintenance can be an obstacle to qPCR implementation in routine food analysis laboratories. We must not forget the training of skilled labor. This is because, despite the potential of automation of the technique, the interpretation of the results must be done in a thorough way, so that the "noises" produced by the technique are not interpreted as real signals. However, what really limits the use of this technique in microbiological analysis of foods is the impossibility of distinguishing living cells from dead cells [30]. That is, this technique is able of amplifying any target DNA present in the sample, even being from nonviable cells, which can generate false-positive results by overestimating the number of pathogens present in the food. The **Table 1** summarizes some advantages and disadvantages of qPCR (singleplex and multiplex) and traditional culture methods for microbiological analysis of food.

3. Potential of qPCR for the monitoring microbiological quality of foods: the challenge of differentiating viable cells from nonviable cells

The changes in consumption, diversity, and food mobility, due to globalization, world population growth, and increasing purchasing power, have increased the need of analyzing food qualitatively and quantitatively, especially from the perspective of standardization, authentication, and certification. In this sense, real-time PCR is undergoing continuous improvement and becoming a method present in food analysis both to detect and quantify pathogens, allergens, and plant species or animals that are present in food, with high sensitivity and specificity. Many fluorescent probes are available, and nowadays, nanoparticles are opening up new diagnostic opportunities using this methodology due to it high sensitivity and providing results in a short time [31].

As already mentioned, the inability of qPCR to differentiate viable cells from nonviable (dead) cells is one of its main limitations in microbiological food analysis [30]. As DNA persists in samples even after the cell have lost its viability, the DNA-based detection methods cannot differentiate whether positive signals originate from living or dead bacterial targets. Thus, in order to detect only viable microorganisms in foods, DNA intercalating dyes, such as propidium monoazide (PMA) or ethidium monoazide (EMA), have been used in a step prior to PCR methods (**Table 2**). These agents selectively penetrate in damaged cell membranes and cross-link to DNA, thereby reducing the amplification capacity of the DNA template [32]. Both EMA and PMA are being used for detection of viable cells from different human pathogens, including those that assume the physiological status of "viable but non-culturable" (VBNC), such as *Campylobacter jejuni, Escherichia coli, Helicobacter pylori, Klebsiella pneumoniae, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella typhimurium*, *Shigella dysenteriae*, and *Vibrio cholerae*, which may be viable, but cannot grow outside their natural habitat [33].

PMA has been reported to be more effective than EMA in eliminating qPCR signals from dead cells [32]. Studies comparing EMA and PMA have shown that EMA can also penetrate in living cells of some bacterial species, such as *Anoxybacillus flavithermus* [34], *Staphylococcus aureus, Listeria monocytogenes, Micrococcus luteus, Mycobacterium avium, Streptococcus sobrinus*, and *Escherichia coli* O157: H7 [32],

Food matrix	Microorganisms	Cell viability dye-PCR method	References
Meat			
Chicken breasts and legs	*Campylobacter jejuni*	EMA-qPCR	[37]
Chicken rinses and egg broth	*Salmonella* spp.	EMA-qPCR	[38]
Poultry	*Campylobacter jejuni*; *Campylobacter coli*	EMA/PMA-qPCR	[39]
Chicken carcasses	*Campylobacter* spp.	PMA-qPCR	[40]
Ground beef	*E. coli* O157:H7	EMA-qPCR	[41]
		PMA-qPCR	[42]
	Salmonella spp.	PMA-qPCR	[43]
Broiler carcass rinses	*Campylobacter jejuni; Campylobacter coli*	PMA-qPCR	[44]
Meat products	*Staphylococcus aureus*	PMA-qPCR	[45]
Meat exudates	*Listeria monocytogenes*	PMA-qPCR	[46]
Frozen and chilled broiler carcasses	*Campylobacter* spp.	PMA-qPCR	[47]
Ground beef meatballs	*E. coli* O157:H7*	PMA-qPCR	[48]
Dairy products			
Gouda cheese	*Listeria monocytogenes*	EMA-qPCR	[49]
Infant formula	*Cronobacter sakazakii*	EMA-qPCR	[50]
Pasteurized milk	Coliform bacteria; Enterobacteriaceae	EMA/PMA-qPCR	[51]
UHT milk	*Bacillus sporothermodurans*	PMA-semi-nested PCR	[52]
	Bacillus cereus group	PMA-qPCR	[53]
Milk powder	*Staphylococcus aureus*	PMA-qPCR	[45]
Ice cream	*Salmonella typhimurium*	PMA-qPCR	[54]
Milk and milk products	*Cronobacter sakazakii; Bacillus cereus*; *Salmonella* spp.	PMA-multiplex qPCR	[55]
Milk	*E. coli* O157: H7; *Salmonella* spp.	PMA-multiplex qPCR	[56]
Probiotic yogurt	*Bifidobacterium*	EMA-qPCR	[57]
	Lactobacillus paracasei	PMA-qPCR	[58]
Seafood			
Fish fillets	16S rDNA	EMA-qPCR	[59, 60]
		PMA-qPCR	[61]
Raw seafood (oyster, scallop, shrimp, and crab)	*Vibrio parahaemolyticus*	PMA-qPCR	[62]
Raw shrimp	*Vibrio parahaemolyticus; Listeria monocytogenes*	PMA-multiplex qPCR	[63]
Shrimp, pomfret fish, and scallop	*Vibrio parahaemolyticus**	PMA-qPCR	[64]
Smoked salmon juice	*Listeria monocytogenes*	PMA-qPCR	[46]

Food matrix	Microorganisms	Cell viability dye-PCR method	References
Water and vegetables			
Water	*Campylobacter jejuni; Campylobacter coli*	EMA-qPCR	[65]
Lettuce	*Salmonella typhimurium*	PMA-qPCR	[66]
Lettuce and soya sprouts	*E. coli* O157:H7	PMA-qPCR	[67]
Fresh spinach	*Salmonella* spp.	PMA-qPCR	[43]

**Bacterial culture in physiological status of "viable but nonculturable" (VBNC).*

Table 2.
Summary of the studies using PMA or EMA prior to PCR methods for microbiological analysis applied in different food matrices.

causing loss of genomic DNA during extraction [35] and reducing the efficiency of PCR. However, PMA has been shown to be highly selective in penetrating only bacterial cells with compromised membrane integrity, but not in cells with intact cell membranes. After the DNA intercalation of nonviable cells, the azide group, present in the dye molecule, forms a covalent grid and when exposed to halogen light makes the DNA insoluble, which results in its loss during the extraction process of the genomic DNA. Thus, exposing a bacterial population composed of living and dead cells to PMA treatment results in the selective removal of DNA from dead cells [32]. Nevertheless, the dose of PMA must be carefully adjusted because this reagent becomes increasingly toxic to cells at higher concentrations. It is important to note that the cost of method may become prohibitive in the case of increasing concentration of PMA for its use in different food matrix, or its use in large scale [36].

4. Conclusion

The qPCR came with the intention of reducing the time of analysis and laborious work of the microbiological culture method. The analysis of a food sample performed by qPCR allows the monitoring of amplification while it runs; therefore, it does not need to perform any postreaction processing, such as the electrophoresis gel, allowing results available in around 2 h. Nevertheless, the difficulty of distinguishing living cells from dead cells is the great obstacle when using this methodology as routine food analysis laboratories. In this way, the pretreatment of food samples using PMA (or EMA) aims at eliminating false-positive results, as it only allows the quantification of viable cells. Thus, the PMA/EMA-qPCR promises to be a valuable tool in food safety management and microbiological quality control, especially as a method for quantitative microbial risk assessment. It is critical, therefore, that assays are comparatively evaluated in different food matrices for the detection and quantification of different pathogens and their reproducibility must be validated with intralaboratory experiments to ensure their effectiveness in the intended testing situation prior to implementation.

Acknowledgements

The authors thank Universidade Estadual de Santa Cruz for the financial support. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES), Finance Code 001, for the scholarship granted to Amanda Teixeira Sampaio Lopes.

Conflict of interest

The authors declare no conflict of interest.

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