qPCR as a powerful tool for microbial food spoilage quantification: significance for food quality

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Abstract

The use of real time quantitative PCR (qPCR) has recently been extended to food science. The literature has mainly focused on its use in ensuring food safety. However, it offers a number of advantages with respect to the quantification of non-pathogenic food spoilage microorganisms. Indeed, qPCR may have a promising future in improving the quality of food products. The present review examines the use of qPCR in this area, the basis of the technique, the requirements that must be met for optimal qPCR assays to be performed, and the advantages it offers over other techniques.

Keywords: qPCR, food, quality, microorganisms, spoilage, microbial quantification.

Introduction

Real time quantitative PCR (qPCR) has recently entered service in the field of food science and technology. The rapid development of this technique is reflected in the increasing number of research articles and patents to be found in databases when 'qPCR' and 'food' are used as search keywords. Indeed, qPCR is now employed in the management of nearly all food safety and quality problems. One of the best developed and successful applications of qPCR is the detection and quantification of pathogens, including viruses, bacteria and eukaryotic microorganisms, as well as a number of parasites (Levin, 2004). It has also been used to monitor the presence of antibiotic resistance genes that might be transferred to pathogenic or commensal bacteria in cheese and other food-related scenarios (Manuzon et al., 2007). New applications include the detection of food ingredients (Tanabe et al., 2007) and ingredient fraud (Mafra et al., 2008), and the monitoring of unintended contamination of special foods (e.g., gluten-free foods) (Sandberg et al., 2003). It may also be used to study toxin-encoding genes (Fischer et al., 2007), to detect genetically modified organisms (Rodríguez-Lázaro et al., 2007), allergens (Koppel et al., 2009), and certain non-pathogenic spoilage microorganisms (NPSMs). Although the literature currently contains little on qPCR for the detection of NPSMs, research in this area is progressing and the use of the technique is likely to gain importance in the future.

NPSMs have different origins, but in general they are present at low concentration in the raw materials used or contaminate the food during its processing. Their numbers may grow during both processing and storage (Huis in´t Veld, 1996); it is generally recognized that the total absence of NPSMs is an unreachable goal. NPSMs have an important negative impact on food quality and therefore on their economic value. In some cases they can even have an impact on food safety. Their rapid identification is therefore of great importance to the food industry; early detection and quantification might allow appropriate actions be taken to avoid their negative impacts.

This review describes the basis of qPCR assays, the different markers that can be used in them, and the advantages of this technique over other microbiological and molecular methods. The requirements and challenges of qPCR quantitative detection of NPSMs are also analysed. Finally, a description of the qPCR assays currently available is provided, with special attention paid to those already on the market.

The basis and chemistry of qPCR

Culture-independent molecular methods have become recognised as powerful and reliable tools for use in ensuring food quality and safety. From PCR to DNA-arrays, a wide range of methods has been developed for use in the detection of foodborne microorganisms (for a review see Lauri and Mariani, 2009). However, the *quantification* of microbial populations in food matrices by molecular methods is becoming ever more necessary, especially with respect to certain spoilage microorganisms. In this respect, qPCR represents a powerful tool that could greatly help guarantee the safety and quality of foodstuffs. qPCR allows the progress of the PCR reaction to be monitored as it occurs in real time. Data are collected throughout the reaction, not just at the end point, and reactions are characterized by the cycle in which the amplification of a target DNA is detected rather than the amount of DNA product accumulated at the end. Such monitoring of the reaction has been made possible by the development of methods to fluorescently label the DNA synthesized in each cycle, and the measurement of this DNA by fluorescence detectors incorporated into thermocyclers. The cycle in which the fluorescence reaches the detection level of the instrument is known as the threshold cycle (Ct) and it is directly proportional to the initial copies of target DNA over a wide dynamic range (Logan et al., 2009).

The detection methods used in qPCR can be classified into two main groups: (i) non-specific methods that detect all double stranded DNA (dsDNA) produced in the reaction, and (ii) amplicon sequence-specific methods that distinguish target sequence amplifications from primer-dimers or non-specific amplifications.

The simplest and most used type of qPCR is based on non-specific quantification methods that involve DNA-binding fluorophores such as ethidium bromide, YO-PRO-I, SYBR green I, SYBR Gold, BEBO, BOXTO, LCGreen and SYTO9. These molecules are DNA minor-groove binders that emit a strong fluorescent signal only when associated with dsDNA and exposed to the appropriate wavelength of light. The use of these compounds requires no additional oligonucleotide design nor chemical conjugation, and they are minimally affected by small changes in the template sequence (Logan et al., 2009). However, the formation of primer-dimers is common and strongly

associated with the entry of the reaction into its plateau phase. The formation of these primer-dimers and other non-specific amplification products can hinder the interpretation of the results. These problems can be partially solved using software able to analyse the melting curve of the amplified DNA. If the qPCR reaction is fully optimised, a melting peak profile that represents a specific product can be produced. Based on the results of such analysis, non-specific fluorophores can also be used for the identification of microorganisms.

Fluorescent probes are used in methods to detect specific sequences. Their use adds an additional level of specificity to the amplification reaction. Different types of probe have been developed. Most of them are based on double-dye oligonucleotides that emit a signal only after hybridisation to the target DNA has occurred (molecular beacons, MGB Eclipse, Scorpions), or after their degradation by the 5'-3' exonuclease activity of DNA polymerase during the amplification process (TaqMan oligoprobe, TaqMan-MGB). A number of less commonly used probes are also available, e.g., universal template primer [UT], the Padlock probe, Qzyme, Resonsense light-up probes and Hy-Beacon probes (Logan et al., 2009).

Most qPCR applications are designed to detect DNA targets, although the detection of RNA molecules is also possible. Since the turnover of RNA is rapid, its detection means the producing microorganisms are viable. Two techniques are mainly used in the quantification of RNA: reverse transcription qPCR (RT-qPCR) and real time nucleic acid sequence-based amplification (RT-NASBA). In RT-qPCR a first step of retrotranscription is performed to synthesize cDNA, which is then used as a template in a standard qPCR amplification. RT-NASBA is an isothermal nucleic acid amplification method usually performed at 41ºC with steps involving the use of reverse transcriptase, RNA polymerase and RNAse H, followed by RNA quantification (Logan et al., 2009).

Requirements for accurate qPCR assays

Accuracy is of great importance in microbiological analyses of NPSMs. Reliable quantification depends on optimised and carefully performed qPCR reactions. The accuracy of qPCR is influenced by primer design, the quality of the template DNA, the presence of inhibitors (Edwards and Logan, 2009), and the handling and storage of

samples, primers, probes and enzymes (Dionisi et al. 2003). With food samples, special attention must be paid to the possible presence of inhibitors, and to the efficiency of DNA extraction. A thorough microbiological knowledge of the food in question, including its usual microbiota and potential contaminants, is also a prerequisite.

Once the microorganisms to be detected have been decided upon, a target gene must be selected and specific primers to recognise it must be designed. The selection of the target gene is of great importance. Targeting a gene highly conserved among different species can be used in broad-based detection strategies, while targeting a DNA sequence unique to a particular species or even strain can provide a highly specific test (Hanna et al., 2005). The best option when searching for spoilage organisms is to select a functional gene related to the spoilage effect. However, this is not always possible due to a lack of detailed genetic information. In such cases, the 16S rRNA sequence can be used to design specific primers and probes. However, it should be remembered that quantification is affected by the copy number of the 16S rRNA genes present. Their design should take into account that closely related organisms often share DNA sequences in the most conserved region, whereas species of the same genus may be distinguished by different DNA sequences in the variable regions (Woese et al., 1990).

Probes, primers, and PCR conditions should be optimised not just for a low detection limit (sensitivity) but also for a broad dynamic range (efficiency). The optimal concentration of each of the oligonucleotides used in the assay should also be optimised. The amount of DNA polymerase added is important as well: too little could lead to inefficient amplification and a loss of sensitivity (Edwards and Logan, 2009).

The results of qPCR analysis may be affected by PCR inhibitors present in food samples. Ideally, each sample should be serially diluted and tested in duplicate PCR runs to determine whether any inhibitors are present. However, the best alternative to this is the incorporation of an internal control (Levine, 2004). This should allow the presence of amplification inhibitors to be detected and is very useful in the identification of false negative results. In addition, internal controls can be used to detect the effect of the food matrix on the efficiency of qPCR assays. This is done by spiking the food to be analysed and performing the assay with serial dilutions of this food (Schneider et al., 2009).

qPCR compared with other detection methods

The introduction of strict food safety regulations made the availability of methods capable of reliably quantifying food contaminants essential – not only to detect contaminating microorganisms but to prevent economic losses due to false positives.

qPCR is fast, allows for quantitative analysis and requires no post-processing. In addition, it is economically viable, results are obtained quickly, and the number of microorganisms and the level to which they can be identified (genus, species or serotype) is expanding. The more traditional methods have many disadvantages compared to qPCR. For example, those based on the isolation and phenotypic characterization can be expensive and take days to complete (Fig. 1). Culture-based methods are laborious and a number of incubation conditions, such as an adequate temperature and an aerobic or anaerobic atmosphere, must normally be provided. Further, tests based on selective culture media often fail to detect certain strains within the target population, resulting in the underestimation of numbers. Moreover, since conventional methods are not able to detect non-cultivatable cells, stressed or weakened cells may need specific culture conditions to first recover before any quantification is possible. Even in food matrices, where cultivatable microorganisms are predominant, some 25-50% of the active microbial community may not be cultivatable (Justé et al., 2008). An alternative is the use of immunoassays to detect molecules such as sugar moieties or proteins, but these require the raising of specific antibodies and are not well suited to the detection of unwanted food ingredients in highly processed food, notably because proteins are less thermostable than DNA (Gachon, et al., 2004).

Despite the high sensitivity of qPCR methods, the concentration of spoilage microorganisms in food samples is sometimes below the detection limit. Previous enrichment culture is therefore commonly necessary, especially if one has to be absolutely sure that a microorganism is absent (Hanna et al., 2005). Nonetheless, enrichment times for qPCR are shorter than with other methods due to the technique's higher sensitivity (Martin et al., 2010) Traditional culture methods need between 2 or 3 days to detect microorganism that require a previous enrichment step, while qPCR may need less than 12 hours (Martin et al., 2010).

Another advantage of qPCR is that it can be used to indirectly measure the concentration of undesirable or even toxic compounds produced by spoilage microorganisms, such as biogenic amines (BAs). In some foods the presence of BAs indicates that hygiene during manufacture has been poor. BAs accumulate in food by the microbial decarboxylation of certain amino acids, and they can reach concentrations hazardous to health (Taylor, 1985). The quantification of BAs has traditionally been performed in final products, and using analytical methods that are often long and tedious. The capacity to detect BA-producing bacteria early, and to be able to check large numbers of samples in a short time, would, therefore, be of great advantage to the food industry. In addition, it would allowing appropriate interventions be undertaken before the BA concentration reaches unhealthy levels. Although microbiological screening methods based on media containing a pH indicator and conventional PCR methods have been proposed (Marcobal et al., 2006), none of them relate the presence of BA-producing microorganisms to the final BA content. However, a direct relationship has been established between qPCR results for BA-producers and the BA concentration of food samples (Ladero et al., 2008, 2010a). Indeed, a number of qPCR tests are available for use with different types of food matrix (Landete et al., 2011). Moreover, a threshold Ct has been established that allows the classification of samples as potentially dangerous (Ladero et al., 2010a).

qPCR for the quantitative detection of food spoilage microorganisms

Many studies have confirmed the value of qPCR as a rapid and reliable routine method that could be used in food manufacturing plants for detecting fastidious organisms (Table 1).

Spoilage microorganisms have been divided into broad categories based on certain phenotypic characteristics, but only in few cases are these related to the spoilage problems they cause. These groups include yeasts and moulds, Gram-negative rod shaped bacteria (e.g., *Pseudomonas, Aeromonas, Vibrio*), Gram-positive spore forming bacteria (e.g., *Bacillus* and *Clostridium* spp.), lactic acid bacteria (e.g., *Lactobacillus, Streptococcus, Leuconostoc*, and *Pediococcus* spp.), other Gram-positive bacteria (e.g.,

Brochotris thermospizucta, Micrococcus spp.), and bacteriophages. qPCR has been proposed as a means of detecting some of these groups (see Table 1), such as yeasts and moulds (Casey & Dobson, 2004). It can also be used to detect total viable bacteria (Lee and Levi, 2007), members of the phylum *Firmicutes* (Haakensen et al., 2008), thermophilic bacilli (Ruecker et al., 2006), strictly anaerobic bacteria of the class *Clostridia* (Juvonen et al., 2008), lactic acid bacteria (Haakensen et al., 2007), *Pseudomonas* (Reynisson et al., 2008), Gram negative histamine producers in fish products (Bjornsdottir-Butler et al., 2011), tyramine-producing strains (Ladero et al., 2010b) and bacteriophages of LAB involved in the spoilage of fermented dairy products (del Rio et al., 2006, 2008; Martín et al., 2008).

Several methods based on qPCR are also available for quantifying a number of bioindicators used to assess hygiene levels during food manufacture. Bioindicators are microorganisms, or groups of microorganisms, whose presence in given numbers indicates inadequate hygiene and the possible presence of pathogens (Mossel *et al*. 1995). In general, they are mainly used to assess food and drink quality (Jay 2001). Among these indicators are faecal bacteria such as coliforms, Bifidobacteria, enterococci, coliphages/enteroviruses. These are easily detected and can be used as markers of pathogenic, enteric, zoonotic agents (Jay, 2001). *Pseudomonas* is a psychotropic bacterium particularly involved in the spoilage of food stored at low temperatures, and is frequently used as an indicator (Jay et al., 2003).

The ability to test for specific spoilage microorganisms (SSOs) (Huis in´t Veld, 1996) is becoming increasing possible. Several qPCR assays (Table 1) have been developed for the identification of SSOs in different food matrices, such as *Clostridium tyrobutyricum* (López-Enríquez et al., 2007), *Pediococcus damnosus* ropy strains (Delaherche et al., 2004), and *Sacharamyces cerevisiae* (Martorell et al., 2005).

Other qPCR assays have been developed to monitor the growth of microorganisms with a major role in the production of fermented foods and beverages, such as LAB in dairy or meat fermentation (Martín et al, 2006), and yeast in the manufacture of fermented alcoholic beverages (Martorell et al., 2005). Nevertheless, these assays can also be used in situations in which these microorganisms are undesirable, for example the presence of yeast in many foods or elevated concentrations of LAB in certain drinks (Jespersen

and Jakobsen, 1996). In some cases, a fine line separates spoilage from beneficial activity. The decarboxylation of di- and tricarboxylic acids by LAB is a desirable step resulting in the production of compounds that enhance the organoleptic properties and/or the stability of finished fermented products (van Kranenburg et al., 2002). However, the decarboxylation of amino acids (e.g., histidine, tyrosine) leads to the production of BAs. There are now several qPCR assays that can detect and quantify LAB strains that produce BAs in different food matrices (Fernández et al., 2006, Nannelli et al., 2008; Torriani et al., 2008, Ladero et al., 2010a, b), as well as for the detection of histamine-producing gram-negative bacteria belonging to different genera (Bjornsdottir-Butler et al., 2011) (Table 1).

The detection of foodborne bacteria carrying antibiotic resistance genes is of increasing interest. There is growing evidence that the use of antibiotics in stock raising is leading to human pathogens developing resistance to them (Wang *et al*., 2006). Commensal bacteria, and the antibiotic resistance (AR) genes they harbour, can enter the human food chain through meat or milk products, or via foods grown in fields fertilized with animal manure or wastewater. Due to their enormous abundance, commensal bacteria can serve as a reservoir of AR genes and probably contribute to AR gene transfer among bacteria, including pathogenic bacteria (Johnston and Jaykus, 2004). qPCR has been used to monitor antimicrobial resistance in food and environmental scenarios (Manuzon et al., 2007).

qPCR has also been proposed for the detection of certain plant pathogens affecting crop marketability, e.g., *Fusarium graminearum* in cereal grains (Dyer et al., 2006), *Candidatus liberibacter solanacearum* in potato and tomato (Li et al., 2009), or Cucumber vein yellowing virus in plants (Picó et al., 2005).

Commercial qPCR kits for the detection of food spoilage microorganisms

Despite the power of qPCR to detect pathogens (Levin, 2004), and the advantages it offers in the detection of microorganisms of interest to the food industry - particularly in the monitoring of starter cultures for fermented foods (of great importance if high quality and safe final products are to be obtained) and in the quantification of probiotics in functional foods and beverages (Pennachia et al., 2009; Collado et al., 2009) - there

are still reservations about its routine use in food analysis. Certainly, its full introduction into the food industry will require the availability of adequately trained staff, the adaptation of the protocols described in the scientific literature to the everyday practice of analytical laboratories, and the availability of reagents in the form of kits at a reasonable price (particularly if qPCR is to be used by laboratories processing small numbers of samples).

To date, several commercial kits have been developed for the detection of the main foodborne pathogens. However, only a few have been produced for the determination of spoilage food microorganisms (Table 2) - although they are available for use in research laboratories (Fernández et al., 2006; Martínez-Blanch *et al*, 2009). Many oligonucleotides have also been designed for the identification of these kinds of microorganism, but few have been marketed (Table 1). Certainly, only a few commercial kits for the detection of NPSMs are available. Some of these allow the general detection of non-specific microorganisms in all kind of foods, e.g., the System BAX® (DuPont Qualicon, Wilmington, Delaware) kit for the detection of yeasts and moulds. The further development of kits for the routine detection of NPSMs could improve food production via the implementation of hazard analysis and critical control point (HACCP) systems.

The Foodproof® Detection System for *Enterobacteriaceae* (Merck, Darmstadt, Germany) is another important kit; the presence of *Enterobacteria* indicates poor hygiene practices in food manufacture. Some kits allow the detection of SSOs in certain foods. For example, the Primermix P1 Screening (Gen-ial, Troisdorf, Germany) and Foodproof® Beer Screening kit (Merck, Darmstadt, Germany) detects more than 25 spoilage organisms identified as contaminants of beer. The latter Foodproof® kit, developed and validated in cooperation with a number of large German breweries, has a sample processing time of just 30 min to 1 h, and an enrichment time of up to 18 h. The qPCR procedure itself takes about 2 h. Thus, the time to obtain results is shorter by several days compared to standard microbiological and biochemical methods.

Our group has developed and implemented two qPCR assays to detect *Streptococcus thermophilus* and *Lactobacillus bulgaricus* phages (del Río et al., 2008 and 2006 respectively), in a dairy company although they have not been marketed. Bacteriophage

infection of dairy starters is a major cause of milk fermentation failure (Neve and Teuber, 1991). A reliable system for their early detection in milk is vital given the magnitude of the problems they cause and their associated economic impact. A phage population of over 10^5 pfu ml^{-1} poses a serious risk of fermentation failure (Neve and Teuber, 1991); such numbers are, however, within the detection limits of qPCR. Multiplex qPCR is performed with an internal amplification control that uses specific primers and TaqMan-MGB probes for two different genes. In addition to the quantitative detection of phages, this allows the identification of *cos* and *pac* type *S. thermophilus* phages (del Rio., et al 2008). This method has been optimised for FAST technology (Applied Biosystems, California, USA), which reduces the assay time without loss of sensitivity. Thus, bacteriophages in milk samples can be detected, quantified and typified in about 30 min. The rapid, accurate identification of bacteriophages potentially able to attack starter cultures allows decisions concerning the final use of milk thus contaminated to be quickly taken. Such milk might be earmarked for use in processes in which phages are deactivated, processes that do not require starters, or processes that employ starter bacteria insensitive to the detected phage.

Challenges and future of qPCR in the quantitative detection of food spoilage microorganisms

Most of the technical challenges encountered in using qPCR with food matrices have been overcome (Levin et al., 2004; Hanna et al 2005). However, compared to its use in clinical testing, qPCR for the detection of spoilage microorganisms is still in early days. The main challenge is the many types of food that need to be tested, and the fact that many contain PCR inhibitors. The presence of inhibitors should be carefully checked for to ensure the accurate quantification of the target microorganisms (see *Requirements for accurate qPCR assays***).**

Ideally, food samples should be used directly as template providers. However, nucleic acids are sometimes better extracted from the food matrix. It should always be borne in mind, however, the quantification of a pathogen can vary by up to two log units depending of the lysis method used (Cheng and Griffiths, 2003). Extraction methods face two main challenges. The first is posed by heterogeneity of the matrix in terms of

its physical state (liquid or solid), texture, and composition (concentrations of proteins, sugars or fat), etc (a single extraction method valid for all foods and beverages is hard to come by). Independent of the matrix to be analysed, the nucleic acid extraction method must be efficient and result in preparations of repeatable quantity and quality (Demeke and Jenkins, 2010). Second, the initial concentration of spoilage microorganisms is generally low, and in fermented foods target organism need to be sought against a dense bacterial background (Jaykus, 2003).

Methods adapted for use with different foodstuffs must also be validated. In addition, validation may be required for legal reasons. One of the main inconveniences that the food industry encounters when trying to use qPCR technologies is that most of the available methods have not been adapted to ISO or APHA norms. It is of vital importance for the expansion of the use of qPCR in the food industry that both nucleic acid extraction methods and qPCR quantification methods be validated and standardized.

The qPCR quantification process could serve to help construct microbial growth prediction models. These could be very useful in the design of quantitative detection protocols, HACCP systems, and help in the making of accurate predictions of shelf life (Gram and Dalgaard, 2002).

The potential of qPCR should expand as the technology continues to develop. For instance, the capacity to combine several probes labelled in such a way that they can be differentiated and individually quantified within the same reaction opens up the horizon for new applications. Current commercial technologies can discriminate up to five different fluorescent dyes, potentially allowing for the simultaneous detection or four different organisms plus an internal control. Automation could also maximize efficiency by reducing assay times and the number of errors. The drawback of such systems is their initial price. Nevertheless, if qPCR becomes a routine technique in the food industry the cost of automation and per-sample testing should fall.

Conclusions

qPCR has proven its usefulness in basic microbiological research. Its capacity to amplify nucleic acids from a wide range of sample types makes it an ideal system for use in different microbiological disciplines (Mackay, 2004). In food microbiology and food safety it has aroused great interest (Hanna et al., 2005) and several commercial kits have been developed and validated as methods to detect food-borne pathogens.

qPCR for the quantification of food spoilage microorganism offers many advantages over other molecular techniques. Its versatility, speed, and sensitivity, together with its capacity to quantify the target organism within complex matrices, make this technique a promising tool that could be used to improve the safety and quality of food products.

The identification and quantification of spoilage microorganisms by conventional microbiological methods takes days, but with qPCR this can be done in a matter of hours. The ability to test up to 384 samples (in some systems) at a time, each of which can be multiplexed in order to detect various targets simultaneously, and with no need for post-amplification processing, reduces the workload and the time required to obtain results. The speed and efficiency of analysis may also improve as qPCR technology evolves. For instance, FAST technology can reduce assay times greatly, and automation could reduce errors and the number of personnel required to perform analyses.

An increasing number of applications are ready to be transferred from the laboratory to the food industry. However, the fact that most of them are not yet validated or written into norms will probably delay their introduction. Nevertheless, their routine use for screening and quantifying food spoilage microorganisms would help the food industry improve the safety and quality of its products.

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Figure captions

Figure 1: Comparison of conventional microbiological and qPCR methods for the quantification of eight spoilage microorganism in 10 food samples. The approximate time (six days *vs.* two days) and materials required (240 plates *vs.* 1 qPCR plate) for each type of analysis are indicated.

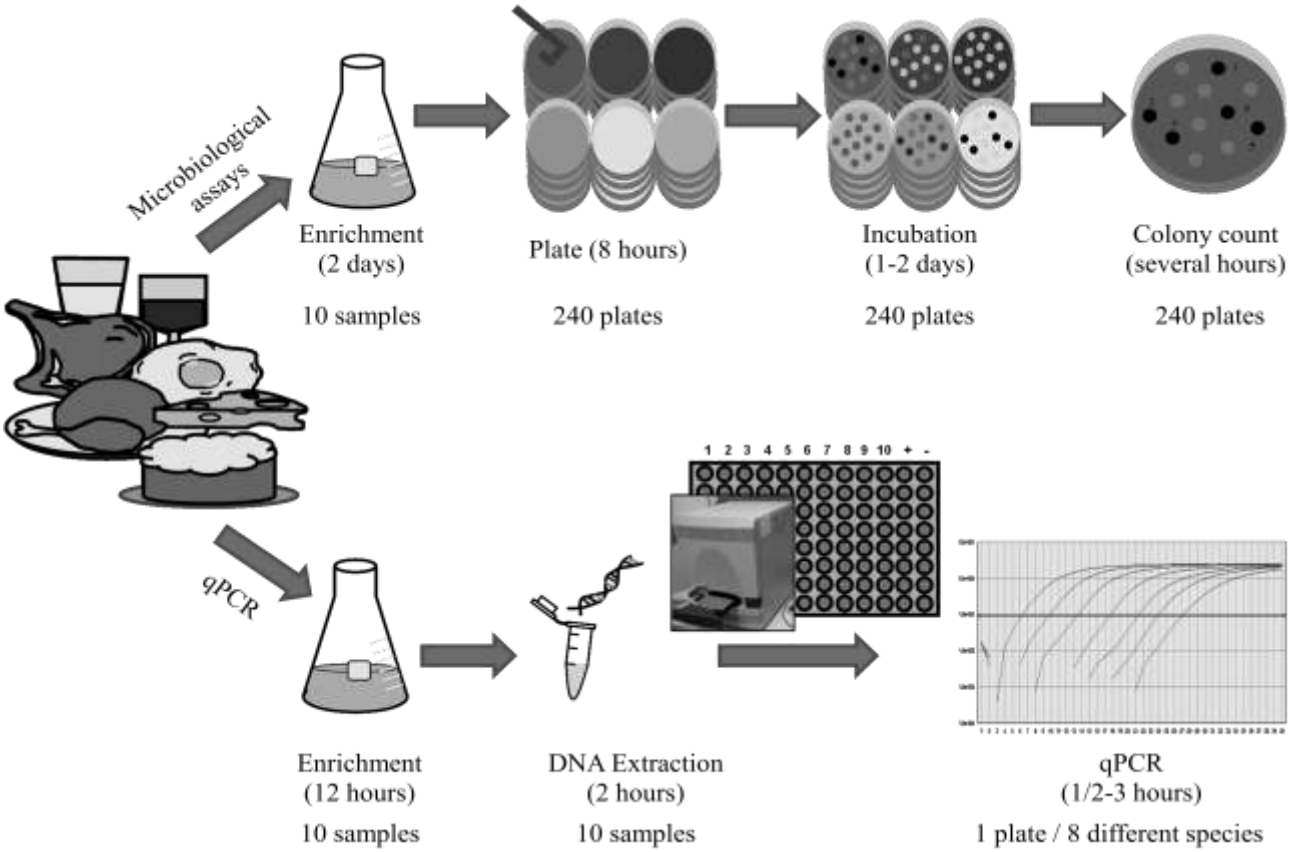

Table 1: Non-exhaustive list of qPCR assays described in the literature for the detection and quantification of NPSMs in food matrices. The gene target and fluorescent markers employed are indicated.

Microorganism	**gene target**	**qPCR fluorescent markers**	**Food**	**Reference**
Yeasts and Moulds				
General				
Yeasts and Moulds	*CS1*	SYBR Green	Fruit juice	Casey & Dobson, 2004
Yeasts	5.8S-ITS	SYBR Green	Orange juice	Renard et al., 2008
Moulds	18S *rRNA*	TaqMan probe	Orange juice	Wan et al., 2006
SSO				
Saccharomyces cerevisiae	RAPD-fragment	SYBR Green	Wine	Martorell et al., 2005
Brettanomyces	18S *rRNA*	TaqMan probe	Orange juice	Wan et al., 2006
Hanseniaspora sp.	D1/D2 26S *rRNA*	SYBR Green	Wine/Juice	Phister et al., 2007
Zygosaccharomyces bailli	26S *rRNA*	SYBR Green	Wine and Fruit juices	Rawsthorne & Phister, 2006
Dekkera bruxellensis	26S *rRNA*	SYBR Green	Wine	Phister & Mills, 2003
Bacteria				
General				
Total viable bacteria	16S *rRNA*	SYBR Green	Refrigerated fish	Lee & Levi, 2007
Firmicutes Phylum	16S *rRNA*	TaqMan probe	Beer	Haakensen et al., 2008
Strictly anaerobic bacteria (class *Clostridia*)	16S *rRNA*	SYBR Green	Beer	Juvonen et al., 2008

Acetic acid bacteria	16S *rRNA*	SYBR Green	Wine	González et al., 2006
Lactic acid bacteria	*horA*	TaqMan probe	Beer	Haakensen et al., 2007
Thermophilic bacilli	*spo0A*	SYBR Green	Milk powder	Ruecker et al., 2006
Enterobacteriaceae	*LacZ*	SYBR Green	Dairy products	Martín et al., 2010
SSO				
Brochothrix thermosphacta	16S *rRNA*	SYBR Green	Raw meat	Pennachia et al., 2009
Lactobacillus sakei	16-23 ITS	TaqMan probe	Meat/Fermented sausages	Martín et al., 2006
Pseudomonas	*carA*	SYBR Green	Fish	Reynisson et al., 2008
Brettanomyces bruxellensis	*Rad4*	SYBR Green	Wine	Delaherche et al., 2004
Pediococcus damnosus ropy strains	*dps*	SYBR Green	Wine	Delaherche et al., 2004
Xylella fastidiosa	16-23S ITS	TaqMan probe	Wine	Shaad et al., 2002
Obesumbacterium proteus	16S *rRNA*	SYBR Green	Beer	Koivula et al., 2006
Alicyclobacillus spp.	16S *rRNA*	TaqMan probe	Juice products	Connor et al., 2005
Gluconobacter *Gluconacetobacter*	16S *rRNA*	TaqMan probe	Electrolyte replacement drink	Gammont et al., 2007
Escherichia coli	*clpB*	Molecular Beacon NASBA	water	Heijnen & Medema, 2009
Bacillus spp.	*hblC*	Molecular Beacon NASBA	Milk	Gore et al., 2003
Clostridium tyrobutyricum	*fla*	TaqMan probe	Milk	López-Enríquez et al., 2007
Lactococcus lactis subsp cremoris	16S *rRNA*	SYBR Green	Milk fermented	Grattepanche et al., 2005
Enterococcus gilvus	*pheS*	TaqMan probe	Cheese	Zago et al., 2009
Viruses				
Enteric viruses	*NV*	TaqMan probe	Berries and vegetables	Butot et al., 2007
FRNA bacteriophages	NoV	TaqMan probe	Oysters	Flannery et al., 2009

Lactobacillus delbrueckii	*mur/lysA*	TaqMan probe	Milk	Martín et al., 2008
Streptococcus thermophilus	*orf1510/orf18*	TaqMan probe	Milk	del Rio et al., 2008
Gram + biogenic amine producers				
Histamine	*hdcA*	SYBR Green	Dairy products	Fernández et al., 2006
Putrescine	*odc/agdi*	SYBR Green	Wine	Nannelli et al., 2008
Tyramine	*tdc*	SYBR Green	Meat	Torriani et al., 2008
Gram - biogenic amine producers				
Histamine	*hdc*	TaqMan probe	Fish	Bjornsdottir-Butler et al., 2011

ITS: Internal transcribed spacer region including the 5.8 rRNA gene. RAPD: Random amplification polymorphic DNA

Table 2: qPCR commercial kits available for the detection and quantification of NPSMs in foods.

Kit name	**Target species**	**Food matrix**	**Sensitivity (cfu $ml^{-1)}$**	**Manufacturer**
System Bax associated	Yeasts and moulds	Prepared foods	*c* 10^4	DuPont Qualicon
Foodproof Detection System	*Enterobacteriaceae*	Prepared foods	*c* 10^1-10^3	Merck
Foodproof® Beer Screening kit	*Lactobacillus* *Megasphaera* *Pectinatus* *Pediococcus*	Beer	*c* 10^1-10^3	Merck
Primermix P1 Screening	*Acetobacter* *Lactobacillus* *Megasphaera* *Pectinatus* *Pediococcus* *Selenomonas*	Beer	$2x10^1$-$1x10^2$	Gen-ial

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