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Food Microbiology xxx (2011) 1–14

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Review

Recent advances in quantitative PCR (qPCR) applications in food microbiology

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A R T I C L E I N F O

Article history:
Received 17 November 2010
Received in revised form
14 February 2011
Accepted 21 February 2011
Available online xxx

Keywords:
Quantitative PCR
Reverse-transcription real-time PCR (RT-qPCR)
Food microbiology
Population dynamics

A B S T R A C T

Molecular methods are being increasingly applied to detect, quantify and study microbial populations in food or during food processes. Among these methods, PCR-based techniques have been the subject of considerable focus and ISO guidelines have been established for the detection of food-borne pathogens. More particularly, real-time quantitative PCR (qPCR) is considered as a method of choice for the detection and quantification of microorganisms. One of its major advantages is to be faster than conventional culture-based methods. It is also highly sensitive, specific and enables simultaneous detection of different microorganisms. Application of reverse-transcription-qPCR (RT-qPCR) to study population dynamics and activities through quantification of gene expression in food, by contrast with the use of qPCR, is just beginning. Provided that appropriate controls are included in the analyses, qPCR and RT-qPCR appear to be highly accurate and reliable for quantification of genes and gene expression. This review addresses some important technical aspects to be considered when using these techniques. Recent applications of qPCR and RT-qPCR in food microbiology are given. Some interesting applications such as risk analysis or studying the influence of industrial processes on gene expression and microbial activity are reported.

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1. Introduction

In the last two decades, culture-independent molecular approaches have undergone considerable development in microbial ecology. Techniques enabling analyses of total microbial communities have greatly improved our understanding of their composition, dynamics and activity (e.g. Wilmes and Bond, 2009; Zoetendal et al., 2008). A few years ago, a system based on quantitative PCR amplification of specific sequences was developed to rapidly quantify human intestinal bacteria (Yif-Scan proprietary system, Yakult Honsha Co, Ltd). In food microbiology, the first culture-independent application of molecular methods to a fermented food matrix was described in 1999 (Ampe et al., 1999). Nowadays, PCR-based methods, in particular quantitative PCR, are used predominantly to detect, identify and quantify either pathogens or beneficial populations such as fermenting microbes or probiotics (Le Dréan et al., 2010; Malorny et al., 2008; Masco et al., 2007). ISO standards have also been established and provide guidelines to qualitatively detect food-borne pathogens by PCR (ISO 22174:2005, ISO/TS 20836:2005, ISO 20837:2006, ISO 20838:2006). However, in comparison with environmental microbiology, the use of molecular tools applied to the study of population dynamics and gene expression in food is only starting (Falentin et al., 2010; Juste et al., 2008; Smith and Osborn, 2009). Recent publications have shown the possibility to follow the growth and activity of microbial populations in complex environments and highlight the potential of molecular approaches in assisting to control industrial processes (Hagi et al., 2010; Nakayama et al., 2007).

Compared with culture-based methods, PCR is faster, more sensitive and more specific and enables detection of sub-dominant populations, even in the absence of a selective enrichment medium and in the presence of other (dominant) populations. Moreover, it allows detection of dead cells or viable but non-cultivable cells. Real-time PCR (thereafter named qPCR for quantitative PCR) offers the possibility to quantify microbial populations through measurement of gene numbers. Combined with reverse transcription (RT), qPCR can also estimate transcript amounts, therefore providing data on microbial activity. Currently, qPCR and RT-qPCR have become the methods of choice to quantify genes and gene expression, respectively (Nolan et al., 2006). Nucleic acid isolation

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0740-0020/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2011.02.008

Please cite this article in press as: Postollec, F., et al., Recent advances in quantitative PCR (qPCR) applications in food microbiology, Food Microbiology (2011), doi:10.1016/j.fm.2011.02.008

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and qPCR preparation can be automated and, depending on the detection system, the molecular method can be relatively inexpensive and suitable for routine analysis. Compared with end-point PCR, qPCR and RT-qPCR (these two techniques will be hereafter defined with the single abbreviation (RT-)qPCR) do not require post-amplification manipulations, hence limiting the risk of contamination. In addition, they are more sensitive, and accurate template quantification is allowed over a wide dynamic range (7–8 log) (Bustin et al., 2005). However, due to very high sensitivity, (RT-)qPCR experiments should be carefully designed. Provided that proper controls are carried out, this technique appears to be the most accurate and reliable for genes or transcripts quantification (Bustin, 2009).

The qPCR and RT-qPCR technologies have been extensively described in other reviews (e.g. Heid et al., 1996; Kubista et al., 2006; VanGuilder et al., 2008; Wong and Medrano, 2005). In brief, similarly to end-point PCR, qPCR consists in a succession of amplification cycles in which the template nucleic acid is denatured, annealed with specific oligonucleotide primers, and extended to generate a complementary strand using a thermostable DNA polymerase. This results in exponential increase of amplicons (amplification products) that, in contrast with end-point PCR, can be monitored at every cycle (in real time) using a fluorescent reporter. The increase in fluorescence is plotted against the cycle number to generate the amplification curve, from which a quantification cycle Cq (often described as Ct for cycle threshold) value can be determined. Cq corresponds to the number of cycles for which the amount of fluorescence (hence, of template) is significantly higher than the background fluorescence. Therefore, the Cq value can be linked to the initial concentration of target nucleic acid and serves as a basis for absolute or relative template quantification (see below). Several detection chemistries are now available with well-described protocols (Wong and Medrano, 2005). As each of them is displaying specific characteristics, their choice will depend on the application. Currently in food microbiology, the two most popular detection systems are the DNA binding dye technology and the 5′ nuclease assay. While the first one is very well adapted to low-cost routine analyses (among other characteristics), the second technology enables the screening of multiple target genes within a single reaction (multiplex PCR).

First, this review highlights some important technical aspects to consider in food microbiology when designing or using (RT-)qPCR or when analyzing the results, with respect to the current scientific knowledge and also to our own field experience. In a second part, recent applications of (RT-)qPCR to quantify genes or transcripts in food samples are presented, with the aim to provide an overview about the possible range of applications of these methods.

2. Technical considerations for (RT-)qPCR implementation in food microbiology

In this section, we would like to point out some aspects of (RT-)qPCR protocols that are not often raised in technical papers and that are essential in food microbiology. Other "basic" aspects such as primer design, choice of reagents, etc. are not discussed here.

2.1. Quality of nucleic acid extracts

Nucleic acid extraction is the first step in the analysis process and sample quality is probably the most important component to ensure reproducibility of the analysis and to preserve the biological meaning (Bomjen et al., 1996; Bustin and Nolan, 2004). Nowadays, it is easy to isolate DNA with very high qualitative and quantitative yields. Most procedures employ commercial extraction kits, used as such or with some adaptations depending on the food matrix, with satisfactory results. By contrast with DNA, intact RNA extraction is more laborious, especially from complex or fatty food matrices. Some extraction methods compatible with subsequent RT-qPCR have been developed for various foods (de Wet et al., 2008; Hierro et al., 2006; Rantsiou et al., 2008; Ulve et al., 2008). Due to fast degradation, RNA should be quickly analyzed. Currently automated capillary-electrophoresis equipment (e.g. Bioanalyzer 2100, Agilent) is the most appropriate to determine sample quality. A RNA integrity number (RIN) can be calculated (Schroeder et al., 2006) to determine suitability of samples for RT-qPCR analysis (Fleige and Pfaffl, 2006). In spite of these technical breakthroughs, upstream steps of the detection procedure, i.e. sampling and sample preparation, often remain overlooked in comparison with the analytical part (Brehm-Stecher et al., 2009).

2.2. Detection chemistries

Several reporter systems are available. A description of their mode of action, advantages and limitations can be found elsewhere (e.g. Wong and Medrano, 2005). In food microbiology, essentially two detection chemistries are commonly used: the DNA binding dye assay using SYBR® Green as a fluorophore (Wittwer et al., 1997), and the hydrolysis probe method (or 5′ nuclease assay) (Gibson et al., 1996) mostly employing the TaqMan® probe (Applied Biosystems) assay. As SYBR® Green binding is not specific for a target sequence this system can be readily used for different gene assays, is flexible, inexpensive, and accurate results can be obtained provided validation of the specificity by melt curve (or dissociation curve) analysis. The TaqMan® chemistry is more expensive than DNA binding dye assays, but presence of the hydrolysis probe ensures that only specific amplicons is measured. In addition, multiplexing reactions are possible, although their set up requires an important optimization phase.

2.3. Quantification methods

Accurate quantification is of prime importance for most food microbiology applications. Absolute quantification is based on comparison of Cq values with a standard curve generated from amplification of known amounts of the target gene. This method requires similar amplification efficiencies (see below) for all samples and standards. Therefore, the standard curve template must be carefully chosen (Dhanasekaran et al., 2010; Leong et al., 2007; Malorny et al., 2008; Whelan et al., 2003). Relative quantification is used to estimate changes in gene expression. It is based on the use of an external standard or a reference sample. The quantification results are expressed as a target/reference ratio. Several mathematical models have been set up (see for review (Wong and Medrano, 2005)). Depending on the quantification method chosen different results can be observed (Cikos et al., 2007). Compared to absolute quantification, relative quantification is simpler as it does not necessitate setting up a reliable standard to be included in every PCR. However, it can be applied only to the samples run within the same PCR. To compare different PCRs, a reference control must be included in every run (Wong and Medrano, 2005).

Amplification efficiency is important to consider when relative quantification is performed, as many PCR do not display ideal efficiency (presence of inhibitors, nucleotide variability). It is recommended to calculate and report amplification efficiency values for each amplicon (Smith and Osborn, 2009; Tuomi et al., 2010), especially when Cq values are to be compared between different samples originating from different food matrices, or when different strains are quantified.

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Table 1
Some applications of (RT)-qPCR in food microbiology.

Microorganism	Target gene	Application	Test characteristics	Food matrix	Reference
qPCR studies					
Salmonella spp.	*invA*	Detection	Enrichment + qPCR – TaqMan®, IAC[a] DL[b]: ≤2.5 CFU/25 g salmon and minced meat, 5 CFU/25 g chicken meat, 5 CFU/25 ml milk	Artificially contaminated chicken meat, minced meat, salmon, raw milk	(Hein et al., 2006)
Salmonella spp.	*invA*	Detection	Enrichment + qPCR – LightCycler® hybridization probes, IAC DL: <5 cells/25 g	Artificially contaminated fish, minced beef, raw milk Naturally contaminated raw milk and meat	(Perelle et al., 2004)
Salmonella spp.	*invA*	Detection	Enrichment + qPCR – TaqMan® DL: 0.08 or 0.2 CFU/g (24 h-enrichment or 48 h-enrichment)	Artificially contaminated mashed potatoes, soft cheese, chilli powder, chocolate, eggs, sprouts, apple juice, fish, shrimp, ground beef, ground chicken	(Cheng et al., 2009)
Salmonella spp.	*ssrA*	Detection	Enrichment + qPCR – TaqMan®, IAC DL: 1–10 CFU/cm^2	Artificially contaminated fresh meat carcasses	(McGuinness et al., 2009)
Salmonella spp.	*iagA*	Detection	Enrichment + qPCR – Molecular Beacon DL: 4 CFU/25 g	Artificially contaminated cantaloupe, mixed-salad, cilantro, alfalfa sprouts	(Liming and Bhagwat, 2004)
Salmonella enterica	*invA*	Detection	Enrichment + qPCR – TaqMan® DL: <3 CFU/25 g	Artificially contaminated chicken carcass rinses, ground beef, ground pork, raw milk Naturally contaminated chicken carcass rinses, raw milk	(Chen et al., 1997)
Salmonella enterica	*ssaN*	Detection	Enrichment + qPCR – TaqMan®, IAC DL: 1 CFU/10 g	Artificially contaminated chicken, liquid egg, peanut butter	(Chen et al., 2010)
Salmonella spp., *S. enterica* Typhimurium, *S.* Kentucki, *S.* Dublin, *S. enteritidis, S. gallinarum*	*aceK, fliC, sefA, sdf*	Detection	Enrichment + multiplex qPCR – TaqMan®, IAC DL: 1–10 CFU/25 g	Artificially contaminated chicken Naturally contaminated chicken	(O'Regan et al., 2008)
Salmonella enterica Typhimurium, *S.* Heidelberg	*oriC*, STM4492, STM2745	Detection	Enrichment + multiplex qPCR – TaqMan® DL: 6×10^1 CFU/ml	Artificially contaminated ground turkey	(McCarthy et al., 2009)
Salmonella spp. including *S. enteritidis* and *S.* Typhimurium	*16S rRNA, fliC, sefA*	Detection	Enrichment + multiplex qPCR – NoRox (Qiagen) 5' nuclease assay DL: 5.4–16.5 CFU/ml	Artificially contaminated beef, pork	(Lee et al., 2009)
Salmonella enterica, Listeria monocytogenes	*inv*A, *prf*A	Detection Quantification (viable only)	Filtration + qPCR – SYBR® Green QL[c]: 10 cells/10 g	Artificially contaminated yogurt	(D'Urso et al., 2009)
Listeria monocytogenes	*prfA*	Detection	Enrichment + qPCR – TaqMan®, IAC DL: 7.5 CFU/25 ml milk, 9 CFU/15 g salmon, 1 CFU/15 g pâté and cheese	Artificially contaminated raw milk, salmon, paté, green-veined cheese Naturally contaminated fish, meat, meat products, and dairy products	(Rossmanith et al., 2006)
Listeria monocytogenes and other species	*ssrA*	Detection	Enrichment + qPCR – hybridization probes, IAC DL: 1–5 CFU/25 g	Artificially contaminated soft cheese, meat, milk, vegetables (coleslaw), smoked salmon	(O'Grady et al., 2008)
Listeria monocytogenes and other species	*ssrA*	Detection	Enrichment + qPCR – LightCycler hybridization probes, IAC DL: 1–5 CFU/25 g	Milk and milk products, meat and meat products, fish and fishery products	(O'Grady et al., 2009)
Listeria monocytogenes	*16S rRNA*	Detection Quantification	Enrichment + qPCR – SYBR® Green DL: 1–5 CFU/50 g	Artificially and naturally contaminated collard green, cabbage, lettuce, mixed parsley and spring onion bunches, Chinese cabbage, arugula, chicory, wild chicory, spinach, watercress	(Aparecida de Oliveira et al., 2010)
Staphylococcus	*16S rRNA*	Detection Quantification Growth dynamics	qPCR – SYBR® Green Combined with DGGE	Milk from grazing cows	(Hagi et al., 2010)

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Table 1 (*continued*)

Microorganism	Target gene	Application	Test characteristics	Food matrix	Reference
Staphylococcus aureus	*nuc*	Detection Quantification	qPCR – SYBR® Green QL: 10 CFU/ml	Artificially contaminated raw milk Naturally contaminated raw milk	(Hein et al., 2005)
Staphylococcus aureus	*nuc*	Detection Quantification	qPCR – SYBR® Green, TaqMan® QL: 20–300 CFU/2 g (depending on the type of cheese)	Artificially contaminated cheese	(Hein et al., 2001)
Staphylococcus aureus	*nuc*	Detection Quantification	qPCR – SYBR® Green, TaqMan® DL: 5×10^2 CFU/g	Artificially contaminated beef Natural fresh meat products, salads, cheese, smoked salmon, pâté, entrails, prepared egg, ready to serve dishes, ice cream, dry-cured meat products, fresh salmon	(Alarcon et al., 2006)
Staphylococcus aureus	*nuc*	Detection Quantification	qPCR – TaqMan®	Milk from cows with intramammary infection	(Studer et al., 2008)
Staphylococcus aureus	*htrA*	Detection	Enrichment + qPCR – SYBR® Green DL: 1 CFU/g; 10^3 CFU/g without enrichment	Artificially contaminated milk, pork	(Chiang et al., 2007)
Enterobacteriaceae	*lacZ*	Detection Quantification	Enrichment + qPCR – SYBR® Green DL: 1 cell/ml	Artificially contaminated cheese	(Martín et al., 2010)
Escherichia coli	*uidA*	Detection Quantification	Enrichment + qPCR – TaqMan® DL/QL: 1 CFU/g; 10^3 CFU/g without enrichment	Artificially contaminated minced beef, tuna, raw oyster	(Takahashi et al., 2009)
Escherichia coli O157:H7	*eae*	Detection	Enrichment + qPCR – Scorpion D/QL: 10^3 CFU/ml (without enrichment)	Artificially contaminated milk Natural samples of raw milk, pasteurized milk, ice cream, kulfi (frozen dessert), paneer (soft cheese), infant foods	(Singh et al., 2009)
Bacillus cereus group	*16S rRNA*	Detection	qPCR (TaqMan®)	Artificially contaminated gelatine Naturally contaminated gelatine	(Reekmans et al., 2009)
Bacillus cereus group	*pc-plc*	Detection Quantification	qPCR – SYBR® Green, TaqMan® QL: ~16–40 CFU/ml (depending on food matrix)	Artificially contaminated liquid egg and infant formula Natural baby cereal, rice cereal, wheat flour samples	(Martinez-Blanch et al., 2009)
Bacillus cereus	*nheA, hblD, cytK1, ces*	Detection	Enrichment + Multiplex qPCR – SYBR® Green DL: 10 CFU/g	Artificially contaminated baby food (rice pudding, carrot puree, cereal)	(Wehrle et al., 2010)
Campylobacter coli, C. jejuni	*ceuE, hipO*	Detection Identification Quantification	Multiplex qPCR – TaqMan® DL: >10 CFU/ml	Artificially contaminated chicken Natural chicken samples	(Hong et al., 2007)
Pectinatus, Megasphaera, Selenomonas, Zymophilus species	*16S rRNA*	Detection	qPCR – SYBR® Green DL: 1–10^3 CFU/25 ml	Artificially contaminated beer Real brewery samples	(Juvonen et al., 2008)
Yersinia pestis	Plasmid sequences (four sets of primers), *cnp60*	Detection Quantification	qPCR – TaqMan® DL: 10^1–10^3 CFU/ml (milk), 10^2–10^5 CFU/g (beef)	Artificially contaminated milk, ground beef	(Amoako et al., 2010)
Genera and species of spore-forming food bacteria	*16S rRNA* + specific genes (commercial biochip)	Detection	Enrichment + multiparametric qPCR, TaqMan® DL: ≥1 spore/25 g *B. cereus* (variable depending on complexity of food matrix)	Artificially contaminated and natural samples of cream cheese, curd, milk powder, fish soup, sausage-lentils, couscous, pasteurized whole liquid egg, egg white, whole egg powder	(Postollec et al., 2010)
Clostridium tyrobutyricum spores	*fla*	Detection Quantification	qPCR – TaqMan®, IAC QL: 25 spores/25 ml	Artificially contaminated raw milk, heat-treated milk	(Lopez-Enriquez et al., 2007)
Campylobacter, Salmonella spp.	*16S rRNA, invA*	Detection Quantification	Multiplex qPCR – hybridization probes DL: 3×10^3 CFU/ml	Artificially contaminated chicken skin rinses	(Wolffs et al., 2007)

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Escherichia coli O157:H7, Salmonella spp., *Staphylococcus aureus*	*uidA, nuc, oriC*	Detection	Multiplex qPCR – SYBR® Green + melting curve analysis, TaqMan® DL: 10^3 CFU/g for each pathogen (TaqMan®); 10^4 CFU/g for *E. coli* and *Salmonella*, 10^3 for *S. aureus* (SYBR® Green)	Artificially contaminated lettuce	(Elizaquivel and Aznar, 2008)
Salmonella spp., *Listeria monocytogenes, Escherichia coli* O157:H7	*invA, hlyA, rfbE*	Detection	Enrichment + multiplex qPCR – TaqMan®, IAC DL: 18 CFU/10 g	Artificially contaminated ground beef Natural beef, chicken, turkey, pork samples	(Suo et al., 2010)
Salmonella spp., *Listeria monocytogenes, E. coli* O157	*Ttr, hlyA, rfb*E	Detection	Enrichment + multiplex qPCR – DNA binding dye + melting curve analysis, hydrolysis probes, IAC DL: 1 CFU/125 ml; 10^4 CFU/ml without enrichment	Artificially contaminated milk	(Omiccioli et al., 2009)
Aspergillus ochraceus, A. westerdijkiae	ITS 1 region of *rRNA*	Detection Quantification	Enrichment + qPCR – SYBR® Green DL: 10^6 spores/ml	Artificially contaminated green coffee beans and grape	(Gil-Serna et al., 2009)
Penicillium camemberti, P. roqueforti	ITS 1 region of *rRNA*, Beta-tubulin	Detection Quantification Growth dynamics	qPCR – SYBR® Green DL: 0.25–4 μg mycelium/g	Inoculated model cheese curd, commercial camembert-type cheese	(Le Dréan et al., 2010)
Candida albicans, C. glabrata, C. parapsilosis, C. tropicalis, Clavispora lusitaniae, Filobasidiella neoformans, Issatchenkia orientalis, Trichosporon asahii, T. jirovecii	*26S rRNA*	Detection Quantification	qPCR – SYBR® Green DL/QL: ~10^2 cells/ml	Artificially contaminated fermented milk product	(Makino et al., 2010)
Aspergillus carbonarius	*pks*	Detection Quantification	qPCR – SYBR® Green, TaqMan® QL: 10^2 (SYBR® Green) – 10^3 (TaqMan®) conidia/ml	Artificially contaminated wine grapes	(Selma et al., 2008)
Brettanomyces	*rad4*	Detection Quantification	qPCR – SYBR® Green QL: 31 CFU/ml	Artificially contaminated white, rosé, red wine	(Tessonnière et al., 2009)
Saccharomyces spp., *Hanseniaspora* spp.	ITS 2 region, *5.8S rRNA*	Detection Quantification	qPCR – SYBR® Green DL: 10^2 cells/ml	Artificially contaminated white and red wine Natural wine samples	(Hierro et al., 2007)
Zygosaccharomyces bailii	D1/D2 variable domains of *26S rRNA*	Detection Quantification	qPCR – SYBR® Green DL: 2–22 cells/ml (depending on type of juice), 6 cells/ml (wine)	Artificially contaminated apple juice, grape juice, cranberry raspberry juice, wine	(Rawsthorne and Phister, 2006)
Enterococcus gilvus	*pheS*	Detection Quantification	qPCR – TaqMan® DL/QL: 10^4 CFU/g	Artificially inoculated pasteurized commercial cheese Naturally inoculated raw milk artisanal cheeses	(Zago et al., 2009)
Corynebacterium casei	*16S rRNA*	Detection Quantification	qPCR – SYBR® Green QL: 10^5 CFU/g	Cheese	(Monnet et al., 2006)
Streptococcus thermophilus, Lactobacillus delbrueckii, L. casei, L. paracasei, L. rhamnosus, L. acidophilus, L. johnsonii	*16S rRNA*	Detection Quantification	qPCR – SYBR® Green DL: 5×10^2–4×10^3 CFU/ml	Commercial fermented milk, fresh cheese, fermented soymilk	(Furet et al., 2004)
Bifidobacterium	*16S rRNA, recA*	Detection Quantification	qPCR – SYBR® Green DL: 10^2 cells/g (16SrRNA primers)–10^3 cells/g (recA)	Probiotic products	(Masco et al., 2007)
Streptococcus thermophilus	*rimM*	Detection Quantification	qPCR – TaqMan® DL: 10^2–10^3 CFU/ml	Artificially inoculated milk Yogurt products	(Ongol et al., 2009)
Lactococcus lactis subsp. cremoris	*16S rRNA*	Detection Quantification	qPCR – SYBR® Green DL: 200 CFU/ml milk in mixed culture	Artificially inoculated fermenting milk	(Grattepanche et al., 2005)
Histamine-producing lactic acid bacteria	*hdcA*	Detection Quantification	qPCR – SYBR® Green DL: 2-4 × 10^2 CFU/ml	Artificially inoculated milk, curd, cheese Natural cheeses	(Fernandez et al., 2006)
Histamine-producing *Oenococcus, Lactobacillus, Pediococcus*	*hdcA*	Detection Quantification	qPCR – SYBR® Green DL: 1 CFU/ml	Red wine	(Lucas et al., 2008)

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Table 1 (*continued*)

Microorganism	Target gene	Application	Test characteristics	Food matrix	Reference
Studies including RT-qPCR					
Total yeasts	D1/D2 variable domains of *26S rRNA*	Detection Quantification (total, viable only)	qPCR, RT-qPCR – SYBR® Green DL: 10^3 CFU/ml	Artificially contaminated white and red wine Natural wine samples	(Hierro et al., 2006)
Yeasts, molds	*act*	Detection Quantification	RT-qPCR – SYBR® Green DL: 10^2 CFU/g (fruit juices), 10^3 CFU/g (yogurt) with RT-PCR (lower limit not tested with RT-qPCR, but at least 10^3 CFU/g)	Spoiled yogurt, milk, cheese mousse, fruit juices, fruit preserves	(Bleve et al., 2003)
Total viable bacteria	*rnp*	Detection Quantification	RT-qPCR – SYBR® Green DL: 10^3 CFU/ml	Beef carcasses	(Dolan et al., 2009)
Salmonella typhimurium	*invA*	Detection (viable only)	Enrichment + RT-qPCR – SYBR® Green, IAC DL: 1–10 CFU/25 g. Without enrichment: 10^6 CFU/25 g	Artificially contaminated pork chop, sausage	(Techathuvanan et al., 2010)
Salmonella enterica	*invA*	Detection (viable only)	Enrichment + RT-qPCR, SYBR® Green, IAC DL: 10^4 CFU/25 g. Without enrichment 10^7 CFU/25 g	Artificially inoculated jalapeño, serrano peppers	(Miller et al., 2010)
Escherichia coli O157:H7	*stx1A, stx2A, eaeA, fliC, rpoS, sodB*	Virulence gene expression	RT-qPCR – SYBR® Green	Artificially contaminated Romaine lettuce	(Carey et al., 2009)
Staphylococcus aureus	*sea, sed*	Enterotoxin gene expression	RT-qPCR – SYBR® Green	Artificially inoculated cheeses	(Duquenne et al., 2010)
Listeria monocytogenes	IGS region between *16S* and *23S rRNA* genes	Detection Quantification	Enrichment + qPCR and RT-qPCR – TaqMan® QL qPCR: 10 CFU/g. Without enrichment: qPCR 10–10^3 CFU/g, RT-qPCR 10^4–10^5 CFU/g	Artificially contaminated soft cheese, fermented sausage, cured ham, ready-to-eat salad, minced meat and milk (Naturally contaminated) fresh meat, fresh sausages, fermented sausages, fresh cheeses, ripened cheeses	(Rantsiou et al., 2008)
Tyramine-producing Gram-positive bacteria	*tdc*	Detection Quantification Gene expression	qPCR, RT-qPCR – SYBR® Green DL: 3 copies target gene	Artificially inoculated pork meat Natural pork meat, fermented pork sausages	(Torriani et al., 2008)
Lactobacillus acetotolerans Microbial community	*16S rRNA*	Growth monitoring Metabolic activity	qPCR, RT-qPCR – SYBR® Green Combined with PCR-DGGE, 16S rDNA sequencing, FISH for microbial community analyses	Nukadoko (fermented rice bran)	(Nakayama et al., 2007)
Lactococcus lactis	*tuf, gapB, purM, cysK, ldh, cit, gyrA*	Growth Survival Metabolic activity	RT-qPCR – SYBR® Green	Ultrafiltration cheese, Cheddar cheese, commercial cheeses	(Ulve et al., 2008)
Propionibacterium freundenreichii *Lactobacillus paracasei*	*16S rRNA, tuf, groL*	Growth monitoring Metabolic activity	qPCR, RT-qPCR – SYBR® Green QL qPCR: 1.3–2.6 × 10^3 genome copies/g, RT-qPCR: 2.1–4.3 × 10^3 copies/g	Ripening Emmental cheese throughout making	(Falentin et al., 2010)
Oenococcus oeni	13 Genes involved in stress	Stress gene expression	RT-qPCR – SYBR® Green	Artificially inoculated synthetic wine	(Beltramo et al., 2006)
Norovirus	*orf1*	Detection	Concentration + RT-qPCR – TaqMan®	Artificially contaminated cheese, lettuce	(Fumian et al., 2009)
Hepatitis A virus	VP1–VP3 capsid regions	Detection	Concentration + RT-qPCR – TaqMan® DL: 14 PFU/g tomato sauce, 33 PFU/g blended strawberries	Artificially contaminated tomato sauce, blended strawberry	(Love et al., 2008)
Hepatitis A virus, rotavirus, feline calicivirus	P30 region (FCV), commercial kits (HAV, RV)	Survival Inactivation	RT-qPCR – TaqMan®	Artificially contaminated berries, herbs	(Butot et al., 2008)

[a] IAC, an internal amplification control was included.
[b] DL, lower detection limit obtained in food matrix. When not otherwise specified and when enrichment steps are included, the values correspond to lower DL with enrichment. Of note, in some studies DL values also correspond to quantification limits (QL), but in the absence of unequivocal description in the corresponding articles they were reported as being DL, in accordance with the authors' indications. DL/QL indicate obviously similar DL and QL values.
[c] QL, lower quantification limit obtained in food matrix.

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2.4. One- or two-step-RT-qPCR

RT-qPCR can be performed in one step within a single tube, or in two steps with reverse transcription performed independently of qPCR. Single-step protocols minimize the risk of DNA contamination in qPCR and the risk of experimental variation (Wong and Medrano, 2005), but the risk of RNA degradation is increased if analyses are performed over a long period of time. In this case, two-step protocols will be preferable. As RT efficiency is known to be highly variable, the choice of a two-step procedure is relevant when several qPCR analyses are to be carried out from the same RNA sample.

2.5. Experimental variations of (RT-)qPCR

All steps of (RT-)qPCR may introduce experimental errors. qPCR is a robust technique, but due to its high sensitivity, very small variations can induce non negligible differences in the results. The main causes of variations and some solutions to minimize their effects have been previously detailed (Tichopad et al., 2009; Wong and Medrano, 2005). To measure intra-assay variability, which follows a statistical distribution, RT-qPCR can be performed in triplicate (experimental replicates). Inter-assay variability can be estimated using a "reference" sample that will be included in each experiment. Variability due to biological factors can be important in food microbiology and we recommend performing biological triplicates with the target RNA, cDNA, or DNA over other replicates.

2.6. Controls and normalization

In addition to the above actions, a number of controls should be included to evaluate template contamination with DNA, RT efficiency and variations in master mix composition. To evaluate variability of the fluorescence signal, a passive dye can be incorporated in the PCR master mix. Some basic controls necessary for diagnostic PCR are also described in the EN ISO 16140 guidelines for the validation of alternative methods (Anonymous, 2002). They include a negative extraction control spiked with closely related, but non-target, nucleic acid to ensure that a positive PCR signal is actually due to the presence of target material; positive control (negative sample spiked with target material); non-template control (blank) containing water in place of the sample; control for environmental contamination during handling. When using $\geq$ 96-well plates, it is wise to distribute several blank controls on plates. All these controls should be processed throughout the entire protocol in parallel with samples to be analyzed.

Moreover, an internal amplification control (IAC) is highly recommended to enable identification of PCR inhibition by substances of food origin, therefore identifying potential false-negative PCR results (Hoorfar et al., 2004). A Cq shift $\geq$2 (Khot et al., 2008) or $\geq$3 (Hartman et al., 2005) between qPCR performed with the IAC alone and with the IAC and sample were proposed as cut-offs. However, as even partial inhibition can reduce the lower quantification limit, which is particularly important when qPCR is used for diagnostic purposes, we suggest considering a Cq delay $\geq$1 as cut-off inhibition value.

During gene expression analysis, normalization must be performed to correct for differences in RNA template (quality and quantity). mRNA from housekeeping genes, ribosomal RNA or total RNA can serve to normalization. However, all of these references are likely to fluctuate and it is recommended to validate stability of their expression in the specific study conditions. As none of the above methods is ideal, it is generally suggested to use several housekeeping genes and to calculate a normalization factor from the geometric mean of their expression levels (Wong and Medrano, 2005). The most appropriate genes can be selected from microarray results using computer tools such as geNorm (http://medgen.ugent.be/genorm) (Vandesompele et al., 2002; Derzelle et al., 2009).

2.7. Mode of expression of (RT)-qPCR data

Absolute quantification of bacterial populations is mostly expressed as CFU number/ml or genome equivalent (GE, or DNA copy)/ml. Expressing results in GE/ml involves knowledge about copy number of the target gene and total genome weight. Some differences between results expressed in CFU/ml and GE/ml can be observed, for instance in the presence of bacterial chains, dead cells, or when cell lysis is incomplete. In gene expression studies, relative quantification is often presented as the ratio of normalized expression level of experimental sample to control sample, or as fold-change. Depending on the study context other modes of data expression are used. For instance, in cheese samples containing genetic material from various species, using the ratio (targeted cDNA)/(DNA of targeted species) appeared more appropriate to follow specific gene expression during cheese making (Falentin et al., 2010). In most studies, the final gene expression results are log-transformed, in order to make the data distribution more symmetrical for the application of statistical parametric tests (Derveaux et al., 2009).

3. Applications of (RT-)qPCR in food microbiology

In the last years, and particularly in the last two or three years, real-time PCR applications in microbial ecology have strikingly developed. qPCR is now used to quantify microbial populations in the absence of specific culture medium while RT-qPCR is considered as the most accurate and specific technique to measure gene expression. This section presents an overview of the current range of applications in food microbiology. With respect to our practical experience in this field, some of the methodological choices are also commented. A (non-exhaustive) summary of the most recent studies is presented in Table 1.

3.1. Specific detection and quantification of pathogens in food by qPCR

qPCR has been evaluated for the detection and quantification of a wide variety of microorganisms, including bacteria, fungi and viruses, with emphasis on the main food-borne pathogens responsible for substantial medical and economic burden (*Salmonella*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Staphylococcus aureus*). The major advantage of this molecular method over standard methods is the shorter time required to obtain the results. For instance, detection of *L. monocytogenes* by qPCR methods including an enrichment step takes 2 working days, instead of 7 days with the standard method (Aparecida de Oliveira et al., 2010; O'Grady et al., 2009). Rapid *Salmonella* detection in meat carcasses was performed in 26 h versus 5 days with the standard ISO method (McGuinness et al., 2009). Beer-spoilage contaminants of the class *Clostridia* were identified with an enrichment time reduced from 2–4 to 1–3 days due to higher sensitivity of the PCR reaction over the standard method (Juvonen et al., 2008). Without enrichment, detection of *B. cereus* could be achieved within 2 h versus 2 days with the standard method and for a similar cost (Reekmans et al., 2009).

Several studies have reported qPCR detection thresholds similar to those obtained with standard plate counts (Alarcon et al., 2006; Aparecida de Oliveira et al., 2010; Chen et al., 2010; Hein et al., 2001; Hierro et al., 2006; Perelle et al., 2004; Takahashi et al., 2009). In artificially contaminated beef samples, *S. aureus* could

be detected by qPCR with a lower limit of 5×10^2 CFU/2 g (Alarcon et al., 2006). In baby food, about 60 CFU/ml of *Bacillus cereus* were detected (Martinez-Blanch et al., 2009). In wine, as low as 31 CFU/ml *Brettanomyces* could be measured (Tessonnière et al., 2009). Low levels of target pathogens can also be detected in matrices contaminated by other dominant microorganisms. For instance, the spoiling agent *Zygosaccharomyces bailii* could be detected with a threshold of 6 cells/ml in wine and 2–22 cells/ml in fruit juices even in the presence of 10^7 CFU/ml *Sacharomyces cerevisiae* (Rawsthorne and Phister, 2006).

However, in a number of cases, the detection and quantification limits obtained without enrichment of the food samples prior to performing qPCR were in the range of 10^2–10^3 CFU/g (or ml) of food matrix (Hierro et al., 2006, 2007; Takahashi et al., 2009). Therefore, in order to meet the microbiological criteria required by national and international legislations for foodstuffs, it is sometimes necessary to associate qPCR with an enrichment step of a few hours. Using this technique, a detection limit <5 CFU/25 g of food was easily reached for *Salmonella* (Chen et al., 2010; Hein et al., 2006; O'Regan et al., 2008; Perelle et al., 2004) and *L. monocytogenes* (Aparecida de Oliveira et al., 2010; O'Grady et al., 2009; O'Grady et al., 2008). For instance, Perelle et al. (2004) have used an 18-h enrichment step of meat, fish and raw milk samples in buffered peptone water (BPW) at 37 °C prior to DNA extraction and qPCR quantification. The proposed protocol showed 100% concordance with the ISO 6579 reference method for *Salmonella* detection. Similar results were described by McGuiness et al. who applied the same enrichment procedure to artificially inoculated meat carcasses (McGuinness et al., 2009). Various meat, fish and milk samples containing *L. monocytogenes* were enriched on half-Fraser broth for 24 h, followed by 4 h enrichment in Fraser broth at 30 °C. This procedure allowed qPCR detection as low as 1–5 CFU/25 g food matrix and showed 99% accuracy with the ISO 11290-1 standard method (O'Grady et al., 2009). It should be kept in mind that the drawback when using an enrichment step is the impossibility to quantify the initial contaminating amounts.

In order to lower the levels of detection of pathogens, other studies have proposed to combine qPCR to preliminary concentration by density gradient or by filtration. A separation and concentration method based on buoyant density gradient centrifugation was applied to naturally contaminated chicken and allowed detection of 10^1–10^2 CFU/g of *Salmonella* and *Campylobacter jejuni* within 3 h by qPCR (Fukushima et al., 2007). Using a flotation method in a discontinuous density gradient, similar results were reported for the separation and concentration of *Salmonella* and *Campylobacter* from chicken carcass rinses, in spite of the presence of background microbiota of 10^9 CFU/ml (Wolffs et al., 2007), and for *Salmonella* detection in pig carcass gauze swabs (Lofstrom et al., 2010). An adsorption–elution method was applied for viral concentration in cheese, lettuce (Fumian et al., 2009), tomato sauce, strawberries (Love et al., 2008) and mussels (Morales-Rayas et al., 2009) before RNA isolation and RT-qPCR.

When qPCR was used as a quantitative tool, linear quantifications were reported over a large range of at least 5 logs (D'Urso et al., 2009; Hein et al., 2005; Martinez-Blanch et al., 2009; Takahashi et al., 2009) and very good correlations with plate counts were obtained. In some cases, however, discrepancies between microbiological counts and qPCR estimates have been reported, with higher bacterial counts with the molecular method (Hein et al., 2005; Hierro et al., 2007; Makino et al., 2010; Martinez-Blanch et al., 2009). Several reasons are likely to explain the differences: (i) the presence of intact DNA from dead cells, (ii) the presence of viable but non culturable forms, which can both be quantified by qPCR but not by plate counts, (iii) the fact that one CFU on plate might be generated from more than one cell, and (iv) the use of PCR primers targeting varying numbers of multicopy genes (e.g. *16S rRNA*).

An important distinction should be made between (lower) detection limits (DL) and (lower) quantification limits (QL). This distinction is rarely clearly tackled and may lead to confusing comparisons between sensitivities of different methods. The lower DL, sometimes found abbreviated as lower LOD, is the lowest population of microorganisms that can be detected by the method. The lower quantification limit (also named lower LOQ) is the minimal population that can be accurately quantified. Most of the time, the DL is lower than QL: this is the case when qPCR gives a positive signal but the amount of template is too low and provides a Cq that falls out of the linear range of quantification curves. An illustration of differences between DL and QL can be found in (Tessonnière et al., 2009). When quantifying pathogens in food, the lower QL in the food matrix should be considered (and not the lower QL obtained from pure cultures) because it takes into account the efficiency of nucleic acid extraction and possible interactions of food components with PCR amplification.

As illustrated in Table 1, most qPCR protocols were applied using either the intercalating SYBR® Green (DNA binding dye technology) assay or the TaqMan® chemistry (5′ nuclease assay technology). Although some studies have reported up to 10-fold higher sensitivity of TaqMan® detection over SYBR® Green (Elizaquivel and Aznar, 2008; Hein et al., 2001; Nogva et al., 2000), the much less expensive DNA binding dye technology appeared to be satisfying in many cases, and several examples of its higher sensitivity over hydrolysis probe technology have been described as well. For instance, 10-fold higher sensitivity for *S. aureus* detection in beef (Alarcon et al., 2006) and for the fungus *Aspergillus carbonarius* in wine grapes (Selma et al., 2008) were reported. A higher sensitivity of SYBR® Green for the detection of members of the *B. cereus* group in egg and infant formula was also described (Martinez-Blanch et al., 2009). Other chemistries such as Scorpions or Molecular Beacons can be employed (Liming and Bhagwat, 2004; Singh et al., 2009). Although these technologies are well adapted to multiplex qPCR and specific enough to be used for allele discrimination, the PCR reaction cost remains prohibitive for routine microbiological analysis. The LNA (locked nucleic acid) probe technology was shown to provide more sensitive PCR assays and could be considered in optimization strategies aiming to lower detection levels (Josefsen et al., 2009; Reynisson et al., 2006), but owing to its high price it was not yet further developed for food-borne pathogen detection.

The bacterial ribosomal operon (16S rRNA, 23S rRNA and intergenic spacer (IGS) region) has been frequently used as target for PCR amplification (Table 1). It is ubiquitous, bears both variable and highly conserved sequences, is easily available from public databases for many species and often results in sensitive detection due to its multicopy nature. Detailed sequence analysis has demonstrated that the 16S rRNA gene is suitable for accurate PCR identification of many pathogens (Chakravorty et al., 2007). While its discriminating power might be sufficient for some genera and species, it is not always enough to distinguish closely related species (e.g. for enterococci *or B. cereus* group members). Therefore, other housekeeping genes have been studied, as well as functional genes involved in virulence or metabolism. For *Salmonella* spp. the *invA* (invasin) gene has been extensively used and provided good sensitivity and specificity when compared to standard methods (Chen et al., 2010; Cheng et al., 2009; Hein et al., 2006; Perelle et al., 2004). Similar results were achieved when using the *nuc* (nuclease) gene to detect and quantify *S. aureus* in foods (Alarcon et al., 2006; Hein et al., 2005; Hein et al., 2001; Studer et al., 2008). Greater availability of genome sequences, generalisation of high throughput molecular tools and progress in computational genomics

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are facilitating the set up of new qPCR protocols based on a large variety of genes, thus increasing test specificity. Recently, Chen et al. (2010) have employed a comparative genomic approach to identify a new target gene for improved detection of *Salmonella enterica.* By contrast with other sequences previously used in qPCR, the selected *ssaN* gene (putative type III secretion ATP synthase) was present in all *S. enterica* serovars, and thus, enabled more accurate pathogen detection. In order to increase qPCR specificity, a TaqMan®-MGB (minor groove binding) probe was used, and an internal amplification control (IAC) was included to detect false-negative results. Similarly, McCarthy et al. (2009) have performed in silico genome comparisons to identify new target sequences able to differentiate between *S. enterica* serovars Typhimurium and Heidelberg. The newly designed primers were combined to be used in a single multiplex qPCR run.

The current trend is moving towards identification of several pathogens in the same reaction tube, by applying multiplex amplification. Some qPCR technologies are particularly adapted to multiplexing. For instance, using TaqMan® chemistry several sequence-specific probes can be labelled with different fluorophores and different targets can be coamplified and quantified within a single reaction (Smith and Osborn, 2009; Wong and Medrano, 2005). By contrast, in theory the widely used SYBR® Green chemistry does not allow multiplexing reactions because binding takes place non-specifically in the presence of DNA. However, some authors have circumvented the problem and successfully proposed multiplex qPCR protocols with SYBR® Green, by performing subsequent melting curve analysis. For each target microbe, a distinct melt curve was obtained (Elizaquivel and Aznar, 2008; Wehrle et al., 2010). Multiplex qPCR is an interesting tool to quickly detect different genera or species which are potentially present in the same food matrices. This is the case for *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* in milk and meat samples (Omiccioli et al., 2009; Suo et al., 2010) and *Campylobacter* and *Salmonella* spp. in chicken (Wolffs et al., 2007). Another application of multiplex qPCR has been set up for the detection of closely related species that display genetic variations requiring the use of different sets of primers for reliable detection. A good example is the *B. cereus* group, which members exhibit a wide range of genotypic features which makes their discrimination difficult. Multiplex qPCR-based on four toxin genes responsible for diarrhoea and emesis was able to detect 337 potentially enterotoxigenic *B. cereus* strains (Wehrle et al., 2010). By using two target genes specific for either *Campylobacter coli* or *C. jejuni*, it was possible to detect, quantify and discriminate between these two species within a single PCR reaction (Hong et al., 2007). We recently proposed a multiparametric qPCR-based alternative method to rapidly identify the most prevalent spore-forming bacteria in food. After a multiple-condition enrichment step, qPCR was performed using a ready-to-use biochip that enables detection and identification of several spore-former genera and *Bacillus* species in three different samples within a single run (Postollec et al., 2010).

In agreement with EN ISO 22174:2005 standards for application of PCR for the detection of food-borne pathogens, an increasing number of studies have included an internal amplification control (IAC) to qPCR protocols (Chen et al., 2010; O'Grady et al., 2009; Omiccioli et al., 2009; Perelle et al., 2004; Reekmans et al., 2009; Rossmanith et al., 2006; Suo et al., 2010; Tessonnière et al., 2009).

3.2. qPCR for detection of non-pathogenic and beneficial microbial populations

Another interesting application of qPCR is the detection and quantification of microbial populations participating in fermentation processes, and thus in organoleptic properties of the final food product. Various lactic acid bacteria (LAB) were quantified in fermented milk, with detection limits between 10^2 and 10^3 CFU/ml, even in the presence of other bacteria and without enrichment (Furet et al., 2004; Grattepanche et al., 2005). *Enterococcus gilvus*, which presence in cheese could be beneficial, was identified in 40% of the cheeses analyzed by Zago et al. (2009) using the *pheS* (phenylalanyl-tRNA synthase) gene as target. Its level represented 0.1–10% of the total *enterococci*, indicating that the qPCR method was able to specifically detect sub-dominant populations of *E. gilvus* among other enterococci. The detection limit in cheese was 10^4 CFU/g (Zago et al., 2009). A protocol developed by Monnet et al. (2006) enabled direct and specific quantification of *Corynebacterium casei* in cheeses with a quantification limit of 10^5 CFU/g and a linear range between 10^5 and 10^{10} CFU/g. Although these thresholds are higher than those observed in broth medium or with other types of foods and are likely due to the cheese matrix itself, they remain sensitive enough to study the influence of bacterial populations on the final product. Indeed, *C. casei* is usually present at 10^9–10^{10} CFU/g at the surface of cheese after ripening, and is expected to have no organoleptic properties below 10^5 CFU/g. In six out of the nine cheeses analyzed in this study *C. casei* was present at $>10^5$ CFU/g, and for two cheeses this species represented 40% of the total microbiota.

Quantification of *Bifidobacterium* in probiotic products has been achieved using two different target genes. This interesting application of qPCR helps circumventing the limited availability of suitable culture media and methods for selective growth of bifidobacteria. A detection limit of 10^2 cells/g was obtained with the *16S rRNA* gene versus 10^3 CFU/g for the monocopy *recA* gene (Masco et al., 2007). This is illustrating the well-known higher sensitivity of multicopy genes, which may also result in inaccurate quantifications due to copy number variability among different species. Although less sensitive the *recA* gene does not require specific knowledge about the number of copies of individual species, hence does not require prior knowledge about bacterial content.

Recently, qPCR was applied to study mycelial growth dynamics of *Penicillium roqueforti* and *Penicillium camemberti* during cheese ripening. In this case DNA was used as a biomass indicator, and the results showed that it was possible to monitor changes in fungal populations. However, due to the presence of dead cells, viable biomass was probably overestimated in later stages of cheese ripening (Le Dréan et al., 2010). To overcome this limitation inherent to DNA-based qPCR methods, RT-qPCR is now being increasingly employed to study microbial growth.

3.3. RT-qPCR to study microbial responses to environmental conditions

RT-qPCR can be used to analyze the functioning of target genes in environmental samples (Table 1). Up to a few years ago, RT-qPCR has been much less employed than qPCR in food microbiology. This is primarily due to higher difficulties to extract intact RNA from complex matrices, in comparison with DNA. However, extraction protocols have been developed for a variety of different food matrices or are available in the form of commercial kits, and easy-to-use instruments are now available to quickly check for RNA integrity. Cheese is a complex matrix and efficiency of qPCR performed with genetic material isolated from this environment is sometimes inconsistent (Falentin et al., 2010). Recently two RNA extraction methods from cheese were published. One of the methods involved isolation of microbial cells prior to RNA extraction (Ulve et al., 2008) while the second proposed to directly isolate RNA from the cheese matrix (Monnet et al., 2008). Both methods provided high quality RNA and were suitable for gene expression analysis by qPCR. Some differences in gene expression were

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observed between the two methods for genes that are known to be modified by heat, acid or osmotic stress. This was probably due to activation of these genes during cell separation and is underlying the importance of choosing adapted extraction methods. Using transcriptomics (microarrays), Pieterse et al. (2006) have observed that expression of 42 genes or operons was significantly altered using standard extraction protocols for *Lactobacillus plantarum*. To minimize this effect they adapted a −45 °C methanol-based quenching method that improved reliability and reproducibility of transcript profiles.

The first studies using RT-qPCR to evaluate bacterial response to *in vitro* environmental conditions were published about ten years ago. For instance, in 2001 the response of *Staphylococcus epidermidis* to various stresses was studied (Vandecasteele et al., 2001). More recently, *Oenococcus oeni*'s response to acidic stress and its growth during malolactic fermentation in wine was evaluated on 13 target genes. The experiment was conducted in synthetic wine-like medium free of phenolic compounds known to prevent RNA extraction. These preliminary results suggested that pre-adaptation of malolactic starters to acidic conditions could enhance their resistance and viability in wine (Beltramo et al., 2006) and they were supported by similar conclusions from a recent proteomic study (Cecconi et al., 2009). The expression of seven *Lactococcus lactis* genes was investigated in ultrafiltration and Cheddar cheeses and demonstrated metabolic activity of lactococci even after several weeks of ripening (Ulve et al., 2008). Recently, we followed the growth, metabolic activity and stress response of two ripening strains throughout Emmental cheese making. We observed that *Lactobacillus paracasei* began to grow in pressed curd and its metabolic activity reached a maximum during the first part of ripening (in cold room). *Propionibacterium freudenreichii* began to grow from the beginning of ripening, its activity was maximum at the end of cold ripening and was stable during the first two weeks in warm room (Falentin et al., 2010). In various dairy and fruit-containing products, RT-qPCR was applied to detect and quantify yeasts and moulds. By targeting RNA expression from the *act* gene, the authors were able to analyze only the metabolically active cells (Bleve et al., 2003). Examples of RT-qPCR application to the detection of enteric viruses are also available. Norovirus was detected in cheese and lettuce and HAV was detected in tomato sauce and strawberry by combining RT-qPCR with a concentration step (Fumian et al., 2009; Love et al., 2008).

3.4. (RT-)qPCR and microbial risk assessment

Quantitative and cost-effective methods are essential to estimate the microbiological risks and factors influencing food safety. In a risk assessment study, Lee et al. (2007) applied RT-qPCR to determine the gene expression levels of enterotoxin genes from *S. aureus* isolated from *kimbap* samples, and thus, the potential poisoning ability of the strains. More recently, a method to accurately quantify expression of *Staphylococcus* enterotoxin genes in cheese was described. The geNorm application was used to calculate the gene expression stability measure (*M*) of candidate normalization genes, and to determine the most stable ones. Associated with an optimized RNA extraction protocol, the developed RT-qPCR procedure enabled gene expression quantification from as low as 10^3 CFU/ml *S. aureus* cells (Duquenne et al., 2010). *In vitro*, type A *Clostridium botulinum* neurotoxin gene expression was quantified in the presence of different concentrations of food additives (Shin et al., 2006). Application of this method to study the impact of food additives on *C. botulinum* is of interest to evaluate the risks linked to adventitious presence of this pathogen in food.

Determining bacterial viability is a key issue for the application to food risk management. One major issue that is often raised when using a DNA-based PCR diagnostic method is the (unwanted) detection of dead microorganisms, although in some cases the detection of dead forms, still causing toxicity, would be desirable. Several strategies have been proposed to differentiate between dead and viable forms or to detect and quantify only viable forms. Cell staining with ethidium monoazide bromide (EMA) has been applied prior to DNA extraction, but did not lead to reliable results due to viable cell inactivation and PCR inhibition (Rueckert et al., 2005). A DNAse I treatment allowed distinguishing between total, viable and spore content of *Anoxybacillus flavithermus* in milk (Rueckert et al., 2005). D'Urso et al. recently described a filtration-based method to select for viable *L. monocytogenes* and *Salmonella* in yogurt prior to qPCR detection (D'Urso et al., 2009). The enrichment step included in some procedures to lower the detection levels of pathogens is another mean to select for viable and cultivable forms only. Among all the available methods, RT-qPCR is often chosen to distinguish between viable and non-viable microorganisms (see some examples reported in Table 1).

PCR-based tools to assess the presence and/or viability of total microbial populations have also been developed. Recently, a qPCR procedure based on the lacZ gene was implemented to detect all coliforms in cheese in a single reaction within one day (Martín et al., 2010). Although not all coliforms are pathogenic, their presence is often used to assess the microbiological quality of dairy foods (and especially raw milk) and water, and can be an indicator of the presence of other pathogens from faecal origin. Their presence in milk after pasteurization may reveal inadequate practising during manufacture or packaging. EU legislation (2001/471/EC, 2004/379/EC) requires assessing total viable counts in food products, and especially in fresh meat to evaluate microbiological quality and predict shelf life. The universal bacterial *rnp* (RNA-component of ribonuclease-P) sequence was proposed as a target for RT-qPCR to determine viable bacterial load content in beef carcasses. Compared to the standard microbiological procedure, this alternative method is able to detect viable but not cultivable bacteria, and provides results within one working day instead of several days with the plate count method (Dolan et al., 2009). The authors of this work have recently deposited an international patent application describing the molecular method (Burgess et al., 2010). In order to control the risk of wine spoilage during industrial fermentation of wine, a RT-qPCR method to detect total viable yeasts by targeting variable regions of the *26S rRNA* was set up. Although a number of PCR inhibitors are present in wine, such as polyphenols, and tannins, especially in red wine, the detection limit reached 10^3 CFU/ml and common standard curves could be established for both white and red wine (Hierro et al., 2006). This threshold is sufficient for wine commercialization and consumption.

The influence of storage conditions of Romaine lettuce on expression of stress and virulence genes in *E. coli* O157:H7 was evaluated using RT-qPCR. The results indicated that *E. coli* may become more virulent when storage temperature is decreased (Carey et al., 2009). Survival of enteroviruses to sanitation and freezing in berries and herbs was evaluated by RT-qPCR. Freezing did not significantly affect viability while rinsing with chlorine decreased the viral load in parsley and raspberry samples (Butot et al., 2008).

LAB are non-pathogenic and participate in fermentation processes. However, in some environments certain strains may produce undesirable compounds such as biogenic amines, which are responsible for food poisoning. Histamine is one of these compounds, resulting from histidine decarboxylation. A qPCR method aiming to detect and quantify LAB carrying the histidine decarboxylase (*hdcA*) gene in milk and cheese was set up (Fernandez et al., 2006). A similar approach was described to quantify the presence of *hdcA*-positive LAB in wine (Lucas et al., 2008). As these assays

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Table 2
Recommendations for the use of (RT-)qPCR in food microbiology analyses.

Step	Recommendations
Sample preparation	- Check quantity and quality using for instance NanoDrop (Thermo Scientific) and Bioanalyzer 2100 (Agilent) - Gene expression analysis: consider a preparation method that will have limited impact on *de novo* gene expression
Reverse transcription	One or two-step-RT-qPCR? Consider two-step procedure if different analyses are to be done from the same sample, or if separate analyses are performed during a long period of time
qPCR	
Detection chemistry	- Take into account analysis cost, PCR equipment, standard or multiplex reaction, PCR specificity to be reached - DNA binding dye (e.g. SYBR® Green): validate specificity of amplification by running a dissociation curve analysis
Amplification efficiency	Calculate amplification efficiency coefficient and include it in results. Consider acceptable range between 85 and 110% (1.85–2.1)
Quantification	- Standard curve should be set up using the same matrix as the one containing samples to be analyzed, and using the same target sequence and the same type of nucleic acid - For robustness, establish standard curve with an average of at least three measurements/point. Linearity range should be $\geq$ 5 logs, and ideally of 6–8 logs - Repeat standard curve on each plate - When possible, prefer absolute quantification, especially when results are to be compared between different laboratories or experiments - Carefully consider the choice of mathematical model to be applied for relative quantification
Controls, normalization	- Include negative extraction control, positive PCR control, non-template control, control for environmental contamination - Include an IAC to identify false-negative amplification and PCR inhibition. Consider inhibition when Cq is delayed of $\geq$1 - When possible use several housekeeping genes for normalization. Validate stability of their expression in conditions specific for the experiment
Experimental variation	Make all measurements in triplicate. Give priority to biological replicates over technical replicates

detected both dead and viable bacteria, and because generated histamine levels are dependant on the amounts of histidine precursors in food samples, qPCR was not able to fully predict final histamine levels. Nevertheless, the molecular method can be used to predict a risk of histamine spoilage. Indeed, in wine, a risk of histamine production was observed when populations of histamine-producing bacteria were higher than 10^3 CFU/ml (Lucas et al., 2008). Tyramine is another biogenic amine produced from tyrosine decarboxylation by some LAB and staphylococci. A RT-qPCR assay targeting the *tdc* gene was set up and gene expression under different environmental conditions was analyzed. Results suggested enhanced tyrosine decarboxylase activity upon application of stressful conditions (Torriani et al., 2008).

3.5. *Combining (RT-)qPCR with other molecular approaches*

Microarrays are now being increasingly used for genome-scale analyses of microbial communities and activities. Complementary to microarray approaches, (RT-)qPCR is regarded as the method of choice to quantitatively validate the generated data. Indeed, displaying a larger dynamic range, qPCR is much more sensitive than microarrays (often considered as a semi-quantitative tool only) in detecting fold changes in gene expression. An example of application of the two technologies is given by Maligoy et al. (2008) who studied transcriptome changes of *L. lactis* in the presence of *Saccharomyces cerevisiae* during growth in fermentors. Using microarrays the expression of 158 genes was shown to be significantly modified in mixed versus pure cultures, which was confirmed by RT-qPCR. Although such type of study is increasingly popular in microbial ecology, it has not been applied yet to natural food samples.

Combined use of molecular tools is of interest in assisting to control of product fermentation. qPCR and RT-qPCR were used to study growth dynamics and metabolic activity of *Lactobacillus acetotolerans* during *nukadoko* fermentation from rice bran. qPCR revealed a doubling time of 12 h for *L. acetotolerans*, while that of the total bacteria population was 4 h. Targeting the 16S rRNA, RT-qPCR showed a low metabolic activity of *L. acetotolerans* throughout the fermentation and ripening process. These techniques were also combined with PCR-DGGE, 16S rDNA sequencing and FISH to analyse the global microbial community in the fermented product. FISH showed that *L. acetotolerans* became dominant during ripening (Nakayama et al., 2007). Combined DGGE and qPCR allowed analyzing the composition and growth dynamics of both *Staphylococcus* and total microbial populations in milk during inside and outside grazing periods, providing fast information for grazing management of cows and for the milk industry (Hagi et al., 2010).

4. Conclusions and recommendations

The numerous examples cited in this review and their recentness illustrate the current interest for (RT-)qPCR methods in food microbiology. However, not all fields of application are equally developed yet. Detection and quantification of pathogens have been largely investigated and the trend is now moving towards multiplex qPCR enabling faster multiple detection and increasing the potential of this molecular technique for routine analyses. In contrast, the use of RT-qPCR to study changes in growth and metabolic activities of microbial populations is only beginning. Further development in this field should provide useful information to control organoleptic characteristics during fermented food product making and for microbial risk assessment during industrial processes. For all these applications, (RT-)qPCR should not be dissociated from other classical and molecular techniques, but rather regarded as a complementary tool to be used in combination with the others.

In spite of the growing use of real-time PCR and of the striking rise in publications on the subject, there is an obvious lack of consensus on how best to perform experiments and interpret data (Bustin et al., 2009). Due to very high sensitivity of (RT-)qPCR small differences in sample preparation, amplification and data expression may have a major impact on the results. Therefore, in order to make this tool a reliable and accurate technique, a number of controls should be included at every step. Based on our experience we have summarized some practical recommendations that should be considered to obtain robust and reliable results (Table 2). For the development of low-cost qPCR microbial analyses, the choice of SYBR® Green chemistry generally appears fully reliable, sensitive and reproducible. When this detection chemistry is used in RT-

Please cite this article in press as: Postollec, F., et al., Recent advances in quantitative PCR (qPCR) applications in food microbiology, Food Microbiology (2011), doi:10.1016/j.fm.2011.02.008

qPCR, it is preferable to use a two-step protocol in order to eliminate primer-dimers by changing melting temperatures.

In the near future several interesting applications of RT-qPCR may be considered, such as studying the impact of different steps of industrial processes on the expression of target genes. The range of applications could take place at all stages, from starter cultures to conservation and storage of the final product. The detection and quantification of transcripts predicting for the presence of undesirable molecules and risk analysis are also interesting applications, as suggested in a few recent articles.

Acknowledgments

This work was supported by Bretagne-Pays de la Loire as well as BBA association which gathers several industrials, i.e. BEL, BONGRAIN, COOPAGRI BRETAGNE, DANONE, ENTREMONT ALLIANCE, LACTALIS, MAITRES LAITIERS DU COTENTIN, SODIAAL. Moreover, part of the research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007–2013) under the grant agreement no. FP7-222 654-DREAM as well as UMT 08.3 PHYSI'Opt national funding.

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Please cite this article in press as: Postollec, F., et al., Recent advances in quantitative PCR (qPCR) applications in food microbiology, Food Microbiology (2011), doi:10.1016/j.fm.2011.02.008

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Please cite this article in press as: Postollec, F., et al., Recent advances in quantitative PCR (qPCR) applications in food microbiology, Food Microbiology (2011), doi:10.1016/j.fm.2011.02.008