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

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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 that it is 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 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
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 (Fiege and Pfaff, 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 S′ 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, 2008; 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.
Table 1
Some applications of (RT)-qPCR in food microbiology.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Target gene</th>
<th>Application</th>
<th>Test characteristics</th>
<th>Food matrix</th>
<th>Reference</th>
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<tr>
<td><strong>qPCR studies</strong></td>
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<tr>
<td>Salmonella spp.</td>
<td>invA</td>
<td>Detection</td>
<td>Enrichment + qPCR – TaqMan®, IAC®&lt;sup&gt;Dl&lt;/sup&gt;: &lt; 2.5 CFU/25 g salmon and minced meat, 5 CFU/25 g chicken meat, 5 CFU/25 ml milk</td>
<td>Artificially contaminated chicken, minced meat, salmon, raw milk</td>
<td>(Hein et al., 2006)</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>invA</td>
<td>Detection</td>
<td>Enrichment + qPCR – LightCycler® hybridization probes, IAC&lt;sup&gt;Dl&lt;/sup&gt;: &lt; 5 cells/25 g</td>
<td>Artificially contaminated fish, minced beef, raw milk</td>
<td>(Perelle et al., 2004)</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>invA</td>
<td>Detection</td>
<td>Enrichment + qPCR – TaqMan®, IAC&lt;sup&gt;Dl&lt;/sup&gt;: 0.08 or 0.2 CFU/g (24 h-enrichment or 48 h-enrichment)</td>
<td>Naturally contaminated raw milk and meat</td>
<td>(Cheng et al., 2009)</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>ssrA</td>
<td>Detection</td>
<td>Enrichment + qPCR – TaqMan®, IAC&lt;sup&gt;Dl&lt;/sup&gt;: 1–10 CFU/cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Artificially contaminated fresh meat carcases</td>
<td>(McGuinness et al., 2009)</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>iagA</td>
<td>Detection</td>
<td>Enrichment + qPCR – Molecular Beacon&lt;sup&gt;Dl&lt;/sup&gt;: 2.5 CFU/25 g</td>
<td>Artificially contaminated cantaloupe, mixed-salad, cilantro, alfalfa sprouts</td>
<td>(Liming and Bhagwat, 2004)</td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>invA</td>
<td>Detection</td>
<td>Enrichment + qPCR – TaqMan®, IAC&lt;sup&gt;Dl&lt;/sup&gt;: &lt; 3 CFU/25 g</td>
<td>Artificially contaminated chicken carcass rinses, ground beef, ground chicken</td>
<td>(Chen et al., 1997)</td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>ssrN</td>
<td>Detection</td>
<td>Enrichment + qPCR – TaqMan®, IAC&lt;sup&gt;Dl&lt;/sup&gt;: 1 CFU/10 g</td>
<td>Naturally contaminated chicken, liquid egg, peanut butter</td>
<td>(Chen et al., 2010)</td>
</tr>
<tr>
<td>Salmonella spp., S. enterica Typhimurium,</td>
<td>aceK, flhC, sefA, sdf</td>
<td>Detection</td>
<td>Enrichment + multiplex qPCR – TaqMan®, IAC&lt;sup&gt;Dl&lt;/sup&gt;: 6 × 10&lt;sup&gt;3&lt;/sup&gt; CFU/ml</td>
<td>Naturally contaminated chicken, naturally contaminated chicken</td>
<td>(O'Grady et al., 2008)</td>
</tr>
<tr>
<td>Salmonella enterica</td>
<td>oriC, STM4492, STM2745</td>
<td>Detection</td>
<td>Enrichment + multiplex qPCR – TaqMan&lt;sup&gt;Dl&lt;/sup&gt;: 5.4–16.5 CFU/ml</td>
<td>Artificially contaminated ground turkey</td>
<td>(McCarthy et al., 2009)</td>
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<tr>
<td>Salmonella spp. including S. enteritidis</td>
<td>16S rRNA, flhC, sefA</td>
<td>Detection</td>
<td>Enrichment + multiplex qPCR – NoRox&lt;sup&gt;Dl&lt;/sup&gt;: 5 × 10&lt;sup&gt;3&lt;/sup&gt; CFU/ml</td>
<td>Artificially contaminated beef, pork</td>
<td>(Lee et al., 2009)</td>
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<tr>
<td>Salmonella enterica, Listeria monocytogenes</td>
<td>invA, prfA</td>
<td>Detection, (viably only)</td>
<td>Filtration + qPCR – SYBR® Green&lt;sup&gt;Dl&lt;/sup&gt;: 10 cells/10 g</td>
<td>Artificially contaminated yogurt</td>
<td>(D'Urso et al., 2009)</td>
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<tr>
<td>Listeria monocytogenes</td>
<td>prfA</td>
<td>Detection</td>
<td>Enrichment + qPCR – TaqMan®, IAC&lt;sup&gt;Dl&lt;/sup&gt;: 7.5 CFU/25 ml milk, 9 CFU/15 g salmon, 1 CFU/15 g pâté and cheese</td>
<td>Artificially contaminated raw milk, salmon, pâté, green-veined cheese</td>
<td>(Rossmanith et al., 2006)</td>
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<tr>
<td>Listeria monocytogenes and other species</td>
<td>ssrA</td>
<td>Detection</td>
<td>Enrichment + qPCR – hybridization probes, IAC&lt;sup&gt;Dl&lt;/sup&gt;: 1–5 CFU/25 g</td>
<td>Naturally contaminated fish, meat products, and dairy products</td>
<td>(O'Grady et al., 2008)</td>
</tr>
<tr>
<td>Listeria monocytogenes and other species</td>
<td>ssrA</td>
<td>Detection</td>
<td>Enrichment + qPCR – LightCycler® hybridization probes, IAC&lt;sup&gt;Dl&lt;/sup&gt;: 1–5 CFU/25 g</td>
<td>Artificially contaminated soft cheese, meat, milk, vegetables (coleslaw), smoked salmon</td>
<td>(O'Grady et al., 2009)</td>
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<tr>
<td>Listeria monocytogenes</td>
<td>16S rRNA</td>
<td>Detection</td>
<td>Enrichment + qPCR – SYBR® Green&lt;sup&gt;Dl&lt;/sup&gt;: 1–5 CFU/50 g</td>
<td>Artificially and naturally contaminated collard green, cabbage, lettuce, mixed parsley and spring onion bunches, Chinese cabbage, arugula, chicory, watercress</td>
<td>(Aparecida de Oliveira et al., 2010)</td>
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<tr>
<td>Staphylococcus</td>
<td>16S rRNA</td>
<td>Detection, Growth dynamics</td>
<td>qPCR – SYBR® Green&lt;sup&gt;Dl&lt;/sup&gt;: Combined with DGGE</td>
<td>Milk from grazing cows</td>
<td>(Hagi et al., 2010)</td>
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<thead>
<tr>
<th>Microorganism</th>
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<th>Application</th>
<th>Test characteristics</th>
<th>Food matrix</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>nuc</td>
<td>Detection</td>
<td>qPCR – SYBR® Green</td>
<td>Naturally contaminated raw milk</td>
<td>(Hein et al., 2005)</td>
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<tr>
<td></td>
<td></td>
<td>Quantification</td>
<td>QL: 10 CFU/ml</td>
<td>Artificially contaminated cheese</td>
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<tr>
<td><em>Staphylococcus aureus</em></td>
<td>nuc</td>
<td>Detection</td>
<td>qPCR – SYBR® Green, TaqMan®</td>
<td>(depending on the type of cheese)</td>
<td>Artificially contaminated beef, naturally contaminated fresh salmon</td>
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<td></td>
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<td>Quantification</td>
<td>QL: 20–300 CFU/2 g</td>
<td>(depending on the type of cheese)</td>
<td>Artificially contaminated beef, naturally contaminated fresh salmon</td>
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<tr>
<td><em>Staphylococcus aureus</em></td>
<td>nuc</td>
<td>Detection</td>
<td>qPCR – SYBR® Green, TaqMan®</td>
<td>5 x 10² CFU/g</td>
<td>Artificially contaminated beef, naturally contaminated fresh salmon</td>
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<td></td>
<td></td>
<td>Quantification</td>
<td></td>
<td>(depending on the type of cheese)</td>
<td>Artificially contaminated beef, naturally contaminated fresh salmon</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>nuc</td>
<td>Detection</td>
<td>qPCR – TaqMan®</td>
<td>Milk from cows with intramammary infection</td>
<td>(Studer et al., 2008)</td>
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<td></td>
<td>Quantification</td>
<td></td>
<td>Artificially contaminated milk, pork</td>
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<td>Enterobacteriaceae</td>
<td>lacZ</td>
<td>Detection</td>
<td>qPCR – SYBR® Green</td>
<td>Artificially contaminated cheese</td>
<td>(Martín et al., 2010)</td>
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<td>Escherichia coli</td>
<td>uidA</td>
<td>Detection</td>
<td>qPCR – TaqMan®</td>
<td>Artificially contaminated minced beef, tuna, raw oyster</td>
<td>(Takahashi et al., 2009)</td>
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<td>Escherichia coli O157:H7</td>
<td>eae</td>
<td>Detection</td>
<td>Enrichment + qPCR – Scorpion</td>
<td>Artificially contaminated milk, naturally contaminated liquid</td>
<td>(Singh et al., 2009)</td>
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<td>D/QL: 10³ CFU/ml</td>
<td>egg and infant formula</td>
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<td></td>
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<td></td>
<td>(without enrichment)</td>
<td>Natural baby cereal, rice cereal, wheat flour samples</td>
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<td>Bacillus cereus group</td>
<td>16S rRNA</td>
<td>Detection</td>
<td>qPCR (TaqMan®)</td>
<td>Artificially contaminated gelatine, naturally contaminated gelatine</td>
<td>(Reekmans et al., 2009)</td>
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<tr>
<td>Bacillus cereus group</td>
<td>pc-plc</td>
<td>Detection</td>
<td>qPCR – SYBR® Green, TaqMan®</td>
<td>16–40 CFU/ml (depending on food matrix)</td>
<td>Artificially contaminated chicken, naturally contaminated chicken samples</td>
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<td></td>
<td></td>
<td>Quantification</td>
<td></td>
<td>(depending on the type of cheese)</td>
<td>Artificially contaminated chicken, naturally contaminated chicken samples</td>
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<td>Bacillus cereus</td>
<td>nheA, hbd, cysK1, ces</td>
<td>Detection</td>
<td>Enrichment + Multiplex qPCR – TaqMan®</td>
<td>Artificially contaminated baby food (rice pudding, carrot puree, cereal)</td>
<td>(Wehle et al., 2010)</td>
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<td>Campylobacter coli, C. jejuni</td>
<td>ceeE, hipO</td>
<td>Detection</td>
<td>Multiplex qPCR – TaqMan®</td>
<td>Artificially contaminated chicken, naturally contaminated chicken samples</td>
<td>(Hong et al., 2007)</td>
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<tr>
<td>Pectinatus, Megasphaera, Selenomonas, Zygomycota species</td>
<td>16S rRNA</td>
<td>Detection</td>
<td>qPCR – SYBR® Green, TaqMan®</td>
<td>Artificially contaminated beer, naturally contaminated beer, real brewery samples</td>
<td>(Juvonen et al., 2008)</td>
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<td></td>
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<td>Identification</td>
<td></td>
<td>Artificially contaminated chicken, naturally contaminated chicken samples</td>
<td>(Amoako et al., 2010)</td>
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<tr>
<td></td>
<td></td>
<td>Quantification</td>
<td></td>
<td>Artificially contaminated beer, naturally contaminated chicken, naturally contaminated beer, real brewery samples</td>
<td>(Amoako et al., 2010)</td>
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<td>Yersinia pestis</td>
<td></td>
<td>Detection</td>
<td>qPCR – TaqMan®</td>
<td>Artificially contaminated beer, naturally contaminated beer, real brewery samples</td>
<td>(Amoako et al., 2010)</td>
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<tr>
<td></td>
<td></td>
<td>Quantification</td>
<td></td>
<td>Artificially contaminated chicken, naturally contaminated chicken samples</td>
<td>(Amoako et al., 2010)</td>
</tr>
<tr>
<td>Genera and species of spore-forming food bacteria</td>
<td>16S rRNA, specific genes</td>
<td>Detection</td>
<td>Enrichment + multiparametric qPCR, TaqMan®</td>
<td>Artificially contaminated and naturally contaminated samples of cream cheese, curd, milk powder, fish soup, sausage, lentils, couscous, pasteurized whole liquid egg, egg white, whole egg powder</td>
<td>(Postollec et al., 2010)</td>
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<td></td>
<td></td>
<td>(commercial biochip)</td>
<td></td>
<td>Artificially contaminated and naturally contaminated samples of cream cheese, curd, milk powder, fish soup, sausage, lentils, couscous, pasteurized whole liquid egg, egg white, whole egg powder</td>
<td>(Postollec et al., 2010)</td>
</tr>
<tr>
<td>Clostridium tyrobutyricum spores</td>
<td>fla</td>
<td>Detection</td>
<td>qPCR – TaqMan®; IAC</td>
<td>Artificially contaminated raw milk, heat-treated milk</td>
<td>(López-Enriquez et al., 2007)</td>
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<td>Campylobacter, Salmonella spp.</td>
<td>16S rRNA, invA</td>
<td>Detection</td>
<td>Multiplex qPCR – hybridization probes</td>
<td>Artificially contaminated chicken skin rashes</td>
<td>(Wolffs et al., 2007)</td>
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<tr>
<td></td>
<td></td>
<td>Quantification</td>
<td>DL: 3 x 10³ CFU/ml</td>
<td>Artificially contaminated chicken skin rashes</td>
<td>(Wolffs et al., 2007)</td>
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<tr>
<td></td>
<td></td>
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<td>(commercial biochip)</td>
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</table>
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, 10^5 for S. aureus
(SYBR® Green) Artificiarily 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 Artificiarily contaminated ground beef (Suo et al., 2010)

Salmonella spp., Listeria monocytogenes, E. coli O157
Tlr, hlyA, rfbE Detection Enrichment + multiplex qPCR – DNA binding dye + melting curve analysis, hydrolysis probes, IAC
DL: 1 CFU/125 ml; 10^4 CFU/ml without enrichment

Aspergillus ochraceus, A. westerdijkiae
ITS 1 region of rRNA Detection Enrichment + qPCR – SYBR® Green
QL: 10^6 spores/ml
Penicillium camemberti, P. roqueforti
ITS 1 region of rRNA, Beta-tubulin Detection Quantification qPCR – SYBR® Green
DL: 0.25–4 μg mycelium/g

Candida albicans, C. glabrata, C. parapsilosis, C. tropicalis, Clavispora lusitaniae, Filobasidiella neoformans, Issatchenka orientalis, Trichosporon asahii, T. jiroveci
Aspergillus carbonarius pks Detection Quantification qPCR – SYBR® Green, TaqMan®
QL: 10^3 (SYBR® Green) – 10^7 (TaqMan®) conidia/ml

Brettanomyces rad4 Detection Quantification qPCR – SYBR® Green
QL: 31 CFU/ml
Saccharomyces spp., Hanseniaspora spp.
ITS 2 region, 5.8S rRNA Detection Quantification qPCR – SYBR® Green
DL: 10^2 cells/ml

Zygosaccharomyces bailii D3/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)

Enterococcus gilvus pheS Detection Quantification qPCR – TaqMan®,
DL: 10^2 CFU/g

Corynebacterium casei 16S rRNA Detection Quantification qPCR – SYBR® Green
QL: 10^3 CFU/g

Streptococcus thermophilus, Lactobacillus delbrueckii, L. casei, L. paracasei, L. rhamnosus, L. acidophilus, L. johnsonii
16S rRNA Detection Quantification qPCR – SYBR® Green
QL: 5 × 10^2–4 × 10^5 CFU/ml

Bifidobacterium 16S rRNA, recA Detection Quantification qPCR – SYBR® Green
DL: 10^2 cells/g (16SrRNA primers)–10^3 cells/g (recA)

Streptococcus thermophilus rimM Detection Quantification qPCR – TaqMan®,
DL: 10^3–10^4 CFU/ml

Lactococcus lactis subsp. cremoris 16S rRNA Detection Quantification qPCR – SYBR® Green
DL: 200 CFU/ml milk in mixed culture

Histamine-producing lactic acid bacteria hdcA Detection Quantification qPCR – SYBR® Green
DL: 2–4 × 10^4 CFU/ml

Histamine-producing Oenococcus, Lactobacillus, Pediococcus hdcA Detection Quantification qPCR – SYBR® Green
DL: 1 CFU/ml

Probiotic products

Artificiarily contaminated milk yogurt products
Artificiarily inoculated fermenting milk (Gratapanche et al., 2005)

Artificiarily inoculated milk, curd, cheese Natural cheeses
Red wine

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### Table 1 (continued)

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* IAC, an internal amplification control was included.

* 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.

* QL, lower quantification limit obtained in food matrix.
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). Intra-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 ≥ 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 ≥2 (Khot et al., 2008) or ≥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 ≥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 spooling 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 10^4 CFU/ml Saccharomyces 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 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 (McGuiness 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 10^4 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).
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 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 (Elizagüivel 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 21714:2005 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; Rossmannith 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 10^5 and 10^8 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* (pehnylalanyl-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 10^3 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 10^4 CFU/g and a linear range between 10^2 and 10^7 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 10^5 CFU/g for the monocopy recA gene (Masco et al., 2007). This is illustrating the well-known higher sensitivity of multipoly genes, which may also result in inaccurate quantification 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
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 (Beltram 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. 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These preliminary results suggested that pre-adaptation of malolactic starters to acidic conditions could enhance their resistance and viability in wine (Beltram 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 (Beltram et al., 2001).

In a risk assessment study, Lee et al. (2007) applied RT-qPCR to mate the microbiological risks and factors in food safety. 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 detect 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 pathogen. A PCR method 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 mp (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...
Table 2
Recommendations for the use of (RT-)qPCR in food microbiology analyses.

<table>
<thead>
<tr>
<th>Step</th>
<th>Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample preparation</td>
<td>- Check quantity and quality using for instance NanoDrop (Thermo Scientific) and Bioanalyzer 2100 (Agilent)</td>
</tr>
<tr>
<td>Reverse transcription</td>
<td>- Gene expression analysis: consider a preparation method that will have limited impact on de novo gene expression</td>
</tr>
<tr>
<td>qPCR</td>
<td>- 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</td>
</tr>
<tr>
<td>Detection chemistry</td>
<td>- Take into account analysis cost, PCR equipment, standard or multiplex reaction, PCR specificity to be reached</td>
</tr>
<tr>
<td>Quantification</td>
<td>- DNA binding dye (e.g. SYBR® Green): validate specificity of amplification by running a dissociation curve analysis</td>
</tr>
<tr>
<td>Amplification efficiency</td>
<td>- Calculate amplification efficiency coefficient and include it in results. Consider acceptable range between 85 and 110% (1.85–2.1)</td>
</tr>
<tr>
<td>Controls, normalization</td>
<td>- Include negative extraction control, positive PCR control, non-template control, control for environmental contamination</td>
</tr>
<tr>
<td>Experimental variation</td>
<td>- Make all measurements in triplicate. Give priority to biological replicates over technical replicates</td>
</tr>
</tbody>
</table>

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 during ripening of 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-
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.

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