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Review

FISHing for bacteria in food – A promising tool for the reliable detection of pathogenic bacteria?

Alexander Rohde*, Jens Andre Hammerl, Bernd Appel, Ralf Dieckmann, Sascha Al Dahouk

Federal Institute for Risk Assessment, Department of Biological Safety, D-12277 Berlin, Germany

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ABSTRACT

Foodborne pathogens cause millions of infections every year and are responsible for considerable economic losses worldwide. The current gold standard for the detection of bacterial pathogens in food is still the conventional cultivation following standardized and generally accepted protocols. However, these methods are time-consuming and do not provide fast information about food contaminations and thus are limited in their ability to protect consumers in time from potential microbial hazards. Fluorescence *in situ* hybridization (FISH) represents a rapid and highly specific technique for whole-cell detection. This review aims to summarize the current data on FISH-testing for the detection of pathogenic bacteria in different food matrices and to evaluate its suitability for the implementation in routine testing. In this context, the use of FISH in different matrices and their pretreatment will be presented, the sensitivity and specificity of FISH tests will be considered and the need for automation shall be discussed as well as the use of technological improvements to overcome current hurdles for a broad application in monitoring food safety. In addition, the overall economical feasibility will be assessed in a rough calculation of costs, and strengths and weaknesses of FISH are considered in comparison with traditional and well-established detection methods.

1. Introduction

Zoonotic infections due to the consumption of contaminated food are still a global public health concern. Although significant efforts have been undertaken to limit the extent of foodborne bacterial infections by the implementation of higher hygiene standards and intensive testing, the number of infections remains high. In the European Union (EU), approximately 350,000 cases of foodborne bacterial infections were reported in the year 2011 (EFSA and ECDC, 2013). The estimated number of unreported cases per annum is considerably higher. *Campylobacter* spp. and *Salmonella* (*S.*) *enterica* as the most prominent pathogens might be responsible for more than 15 million cases in the EU (Havelaar et al., 2013). Likewise, acute and severe diarrhoea, with bacteria being major causative agents as well as viruses and parasites, is responsible for the death of about 1.2 million children under the age of 5 per year worldwide and a total of 1.6 billion cases of disease (Fischer Walker et al., 2012; Walker and Black, 2010). Therefore, food safety and the fast detection of frequent bacterial pathogens, for instance *Campylobacter*, *Salmonella*, *Listeria*, *Escherichia coli*, *Shigella*, *Vibrio* and *Yersinia*, are still important issues throughout the world and will also in the future retain its importance in the food industry and microbiological quality control.

The threat posed by foodborne pathogens can be monitored by a range of different detection methods. Some of them enable only the qualitative confirmation for the presence or absence of a pathogen, while others allow also for the quantification of the bacterial load (López-Campos et al., 2012). The latter feature might not be of significance for pathogens with a zero-tolerance-standard, but is especially important for pathogens which are acceptable in food products if the concentration is below a certain limit. Conventional plating and cultivation of pathogenic bacteria is still the method of choice and the gold standard to assess the degree and extent of contaminations in a variety of food products (Ge and Meng, 2009). In addition, reliable quantitative methods like the determination of the most probable number (MPN) are available as well as several modifications of standard protocols and growth media (Jasson et al., 2010). The high food safety requirements are met by the great sensitivity of these cultural methods. However, these techniques with their well-established, standardized and broadly accepted protocols are time-consuming, tedious, labour-intensive and often expensive (Velusamy et al., 2010). Depending on the

* Corresponding author. Federal Institute for Risk Assessment, Department of Biological Safety, Diedersdorfer Weg 1, D-12277 Berlin, Germany. Tel.: +49 30 18412 2012; fax: +49 30 18412 2000.
E-mail address: alexander.rohde@bfr.bund.de (A. Rohde).

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pathogen, it can take several days up to weeks to verify preliminary positive results. Furthermore, it may be difficult to recover all the sublethally injured microbes out of the food matrix in case of pathogens which have encountered rather unfavourable conditions outside their natural habitat and might enter a state with low metabolic activity in which cell division is stalled (Oliver, 2005, 2010). The often employed non-selective preenrichment might be helpful under these circumstances, but this procedure also prolongs the overall analysis time. The increase in global trade and the associated need for a fast transport of food products over large distances have demonstrated the drawbacks of these traditional methods with their inherent slowness. Thus, consumer safety and protection is hard to ensure since positive test results are often obtained after the product was put into circulation.

In contrast to the conventional methods, the development of rapid methods allows the fast detection of pathogens in food samples (Dwivedi and Jaykus, 2011; Jasson et al., 2010). Molecular methods, most notably PCR-based technologies or microarrays, have been demonstrated to detect pathogens in a highly specific manner (Malorny et al., 2009). Fluorescence *in situ* hybridization (FISH) represents a promising alternative method in food microbiology among other culture-independent techniques like denaturing gradient gel electrophoresis (DGGE) (Cocolin et al., 2013). Like other rapid techniques, FISH can be performed without relying on the cumbersome and lengthy conventional cultivation and has additional benefits as it visualizes whole cells and targets ribosomal RNAs (or other abundant structures like multi-copy genes), which provides FISH with the capability of distinguishing between viable organisms and dead material (Bottari et al., 2006; Brehm-Stecher, 2008; Jasson et al., 2010). Within a few hours and limited efforts results can be obtained in an enzyme-independent manner and, if desired, also independent of cultivation. Consequently, even viable but nonculturable (VBNC) or, in general, difficult to cultivate pathogens can be identified, which cannot be achieved by the established cultivation procedures. However, FISH is not yet routinely used to analyse and monitor food products. The use of FISH as a valuable and promising tool to address food safety issues depends on its ability to detect pathogens in a highly specific, sensitive and rapid manner. A major challenge for these goals is the crucial influence of the food matrix. In addition, characteristics like low costs per sample, the feasibility of high-throughput-analyses and, ideally, a high degree of simplicity concerning the performance of a test are desirable. These features should, at least, be comparable to recent advances in the use of conventional methods as well as of molecular and other novel tests. Therefore, the purpose of this review is to give an overview of the current state of the art of FISH-testing on diverse kinds of food and to assess the potential of FISH diagnostics with respect to food safety and the detection of foodborne bacteria. Especially the suitability for the implementation into routine testing is of great interest considering the obvious limitations of the currently employed methods.

2. Fluorescence *in situ* hybridization

During the early 1990s, FISH has gained increasing importance as a novel system to detect and identify microorganisms. Amann et al. (1990a) and DeLong et al. (1989) developed a convenient FISH method for the accurate identification of microorganisms in different settings by targeting the highly abundant ribosomal RNAs (rRNA) within bacterial cells (primarily 16S rRNA of the small ribosomal subunit or 23S rRNA of the large ribosomal subunit). Since then, FISH has become a standard method in different biological and medical fields and its establishment has produced significant new scientific insights owing to the substantial progress made in the following years (Amann et al., 1990b, 1995; Amann and Fuchs, 2008; Wagner and Haider, 2012). FISH is routinely used in medicine and diagnostics to rapidly and conveniently identify pathogens in the blood or the faeces and for cytogenetic examinations to detect chromosomal disorders or tumor cells, as well as in ecology and environmental biology to study the composition, growth and changes of complex microbial communities and biofilms (Bottari et al., 2006; Cocolin and Ercolini, 2008; Jehan et al., 2012). Due to the fact that rRNAs possess regions of high variability as well as regions which might be remarkably conserved throughout an entire domain, the differentiation is possible on several taxonomic levels, ranging from distinguishing between related species up to comprising whole kingdoms and domains (Amann and Kühl, 1998).

Although protocols for FISH might differ significantly, the general methodical procedure involves a fixation step of the sample, the permeabilisation to allow the entry of fluorescent probes, the hybridization of the probe to the target sequence, the removal of unbound and excess probes by washing and, finally, the observation of the cells by microscopy or via flow cytometry (Amann and Fuchs, 2008). In food microbiology, additional steps for the sample preparation and homogenization, preenrichment procedures or bacterial separation might be required. In Fig. 1 a flow-chart of FISH-testing of food products including special FISH adaptations is given. Especially the first step regarding food sample pretreatment might differ from FISH tests examining other types of sample material. All steps of a FISH test have been shown to require considerable efforts for optimization. It is, for example, necessary to determine the ideal hybridization time and temperature, to use proper permeabilization and fixation conditions as well as to design highly specific probes (Amann et al., 1995; Wagner et al., 2003). In recent years, free *in silico* modelling software tools have considerably simplified this optimization process (ARB-project: Ludwig et al., 2004; mathFISH: Yilmaz et al., 2011). In case of food and medical microbiology, commercially available FISH kits with

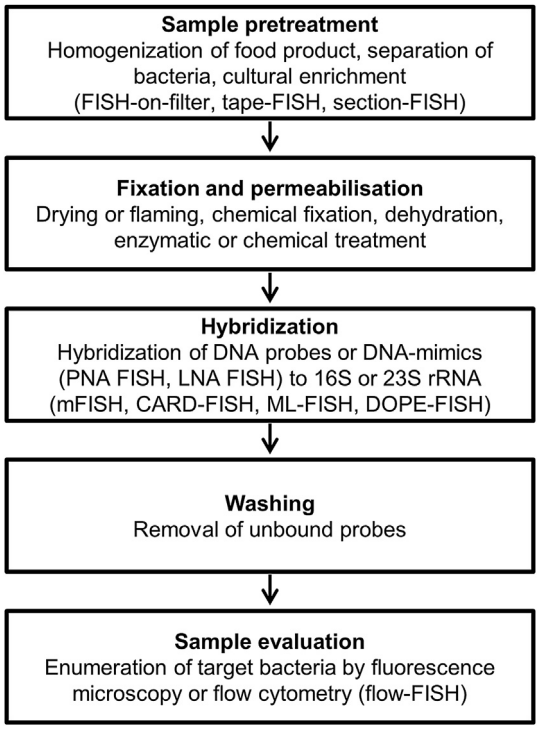

Fig. 1. Schematic representation of a FISH experiment. Overview of the five steps in a FISH experiment including special adaptations with relevance for the use in food microbiology.

simple instructions and rapid test performance have been developed (AdvanDx, BioVisible, Neogene, vermicon and others) to detect a broad range of pathogens and spoilage bacteria. Although the exact methodical steps can differ among them and principles of detection vary or are not published, most of them have a prior phase of cultural enrichment in common and do not employ a sophisticated matrix-specific pretreatment.

3. Overview of examined food matrices targeted by FISH analysis and their associated foodborne pathogens

The majority of FISH studies with the purpose to detect foodborne pathogens have focused on the most relevant pathogenic bacteria which cause foodborne infections, namely *Salmonella enterica*, *Campylobacter* spp., *Listeria* (*L.*) *monocytogenes* (Almeida et al., 2010, 2013a; Bisha and Brehm-Stecher, 2009a, 2009b, 2010; Fuchizawa et al., 2008, 2009; Oliveira et al., 2003, 2012; Schmid et al., 2003, 2005; Vieira-Pinto et al., 2005, 2007, Table 1) and a range of other pathogens found in edibles, including *Bacillus* (*B.*) *cereus*, *Cronobacter* spp. and *E. coli* (EFSA and ECDC, 2013). It could be demonstrated that the FISH method was able to reliably differentiate between the normal microbial flora, which often forms the vast majority of bacteria, and the respective target bacteria with a high specificity (Ercolini et al., 2006; Machado et al., 2013; Moreno et al., 2001; Weiler et al., 2013). Initially, these studies were mainly confined to the detection within pure or mixed culture; however, during the last ten years the fast and sensitive detection of those pathogens in complex food matrices like dairy products and meat was also demonstrated (Angelidis et al., 2011; Bojesen et al., 2003; Oliveira et al., 2012; Schmid et al., 2005; Vieira-Pinto et al., 2005).

FISH studies showing the effective application of this technique for the detection of foodborne bacterial pathogens in their respective target food matrices are summarized in Table 1. Besides, many additional studies also evaluated the presence of commensal bacteria like *Lactobacillus* spp., *Lactococcus* spp. or *Leuconostoc* spp., which are found in huge amounts in dairy products and might complicate the search for pathogenic microbes. Aside of meat and dairy products several other food matrices were examined, including fresh produce, fruit juices and alcoholic beverages like beer and wine. Furthermore, FISH in food microbiology is not restricted to the detection of pathogens, but has also been applied to identify spoilage bacteria, microbes with yet uncertain impact on human health or, in general, to monitor food production (Babot et al., 2011; Ercolini et al., 2006; Fornasari et al., 2008; Machado et al., 2013; Thelen et al., 2002, 2003). In this regard, FISH was shown to possess a similar potential for the examination of bacteria in food as PCR-DGGE (Cocolin et al., 2007). Although this review focuses on bacteria, it should be noted that FISH also enables the detection of foodborne parasites like *Giardia* and *Cryptosporidium* (Graczyk et al., 2004).

Table 1 shows that the importance of the 23S rRNA as a target for FISH analyses instead of the formerly predominant 16S rRNA increases. The 23S rRNA has roughly twice the length of 16S rRNA and has been shown to possess a similar value for species differentiation. The growing number of available 23S rRNA sequences is contributing to its increasing popularity for FISH analyses and might enable further differentiation between closely related strains which cannot be distinguished by targeting 16S rRNA sequences.

Table 1 also reveals that some pathogens causing severe foodborne outbreaks like *S. enterica* and *L. monocytogenes* were the subject of several comprehensive studies whereas for other major pathogens with a high relevance for food safety and human health like *Campylobacter* spp. or *Yersinia enterocolitica* only limited data is available.

4. Food sample pretreatment prior to FISH analysis

The pretreatment of the food matrix has been shown to be crucial for the successful detection of bacteria (Table 1). Low numbers of the target organism require the concentration of the pathogen, and huge amounts of protein and fat have to be removed since they might disturb the hybridization of the probes. Other food components with natural fluorescence activity (e.g. chlorophyll in plant material, hemoglobin in meat and other pigments) can cause strong background in the consecutive microscopic examination. The overall accessibility and the physical properties of the target pathogen have to be taken into account and adequate steps for the extraction of the microbes might be necessary, especially if culture-independent FISH analyses are performed. Moreover, dilution effects as a result of sample pretreatment have to be minimized. In conclusion, a quick and suitable sample pretreatment is as necessary as for other detection methods, for example PCR. Despite its paramount importance, there are no generally followed matrix-specific protocols for the pretreatment of food prior to FISH analysis. The most simple, but also very time-consuming, way to perform a FISH analysis is based on the plate-picking of bacterial colonies which were isolated out of food samples (Zhang et al., 2012). However, approaches which directly use the food matrix are much more preferable.

Liquid (e.g. milk) and solid (e.g. meat) samples often require different upstream sample processing. For milk and other dairy products the pretreatment may involve the use of a homogenizer, simple or multiple centrifugation steps and the addition of sodium citrate buffer to obtain a bacterial pellet with only a few interfering substances (Fornasari et al., 2008; Laflamme et al., 2009; Machado et al., 2013). Other procedures employ, additionally, a treatment with unspecific proteases (e.g. savinase or Proteinase K) for milk-clearing and to reduce the background and autofluorescence of the food matrices (Gunasekera et al., 2003a, 2003b; Yamaguchi et al., 2012). However, the reported bactericidal effects of this enzymatic treatment (Smith et al., 2003) may have an impact on the detection of pathogens by FISH. Solid food samples have to be reduced to small pieces, followed by further steps of mechanical disintegration, e.g. by stomaching, milling or sonification. A filtration of the sample through a membrane transmissible for microbes or a short centrifugation can be used to remove larger particles of the food matrix, which might disturb efficient microscopic evaluation.

Apart from the classical methodology described above with its universal aptitude, the expected location of the pathogen can call for a special sample pretreatment (Fig. 1, step 1). In this regard, FISH applications for surface contaminations, highly diluted and liquid samples or pathogens with a poor accessibility and limited extraction success have to be distinguished.

For food products with contaminated surfaces, steps to homogenize the whole sample are not necessary or even detrimental since they add to the total volume of the sample or release unwanted inhibitors. Instead, sampling by using transparent adhesive tapes and performing on tape-FISH analyses (tape-FISH) has been used for the detection of microbes on fresh produce like tomatoes with a detection limit of 10^3 colony forming units (CFU) per cm^2 (Bisha and Brehm-Stecher, 2009a, 2010). This promising method with its intriguing simplicity and fast performance has the potential to become the method of choice for any surface contamination. However, overnight washing procedures of the untreated product in adequate nutrition broth can also be employed (Ercolini et al., 2006) although this comes along with a rather long enrichment period.

In contrast to the tape-FISH use for surface contamination, section-FISH, an application which utilizes thin sections of food

Table 1
FISH studies in food microbiology to detect pathogenic bacteria.

Pathogen/reference	Target rRNA, probe type	Food matrix	Type of contamination	Enrichment period	Remarks (FISH methodology, sensitivity, specificity)
E. coli					
Regnault et al., 2000	16/23S, DNA	Ground beef	Natural	16–20 h	Co-staining of *Shigella*/*E. fergusonii*
Tortorello and Reineke, 2000	16S, DNA	Beverages (milk, juice, beer, wine, tea etc.), sprouts, vegetables	Spiked	None	FISH-on-filter, detection of 1 CFU/ml in a few hours, compared to standard total coliform membrane filter procedure, method 909A
Stender et al., 2001	16S, PNA	Milk	Natural	5 h	FISH-on-filter with formation of microcolonies, use of array scanner
***E. coli* O157**					
Almeida et al., 2013b	23S, PNA	Ground beef, milk	Spiked	18–24 h	Detection of 1 CFU/25 g in less than 24 h, 100% specificity and 97% sensitivity, compared to ISO 16654, serotype specific detection
***L. monocytogenes*/*Listeria* spp.**					
Oliveira et al., 2003	16S, DNA	Milk	Spiked	None	100-fold more sensitive than plating (not following ISO-standards)
Stephan et al., 2003	Not stated	Minced meat	Natural	48 h	Use of vermicon Listeria kit, 100% specificity and sensitivity, compared to ISO 11290/L00.00-32 (LMBG)
Schmid et al., 2003	16S, DNA	Milk	Natural	1, 2, 3 and 7 days	Positive results after 2 days of enrichment
Fuchizawa et al., 2008	23S, DNA	Smoked salmon, cheese, uncured ham	Spiked	12 h	FISH-on-filter with formation of microcolonies
Fuchizawa et al., 2009	16S, DNA	Smoked salmon, cheese, cabbage	Spiked	12 h	FISH-on-filter with formation of microcolonies
***Salmonella* spp.**					
Fang et al., 2003	23S, DNA	Various kinds of meat, fish, milk products, eggs, cheese, lettuce, butter and others	Natural and spiked	10–16 h	Detection of 2–5 CFU/25 g in 19 h, 100% specificity, compared to modified ISO 6579
Vieira-Pinto et al., 2005	23S, DNA	Pig tonsils	Natural	None	Compared to ISO 6579
Vieira-Pinto et al., 2007	23S, DNA	Mixed pig abattoir refuse	Natural	18 h	Compared to ISO 6579
Bisha and Brehm-Stecher, 2009a, 2010	23S, DNA	Tomatoes, spinach, spices	Spiked	0–5 h	Flow-FISH, tape-FISH with a detection limit of 10^3 CFU/cm^2
Bisha and Brehm-Stecher, 2009b	23S, DNA	Alfalfa sprouts	Spiked	None	Flow-FISH, detection of 10^3 CFU/ml sprout wash
Almeida et al., 2010	23S, PNA	Powdered infant formula	Spiked	8 h	Detection of 1 CFU/10 g in 12 h, 100% sensitivity and specificity
Oliveira et al., 2012	23S, DNA	Mixed pig abattoir refuse and tonsils	Natural	None	Detection in 8 h, 84% sensitivity and 69% specificity, compared to ISO 6579
Almeida et al., 2013a	23S, PNA	Eggs, milk, mayonnaise	Spiked	18–21 h	Detection of 1 CFU/25 g or ml in 24 h, 100% sensitivity and specificity, compared to ISO 6579 and real-time PCR
***Campylobacter* spp.**					
Moreno et al., 2001	16S, DNA	Various chicken products	Natural and spiked	5–22 h	
Schmid et al., 2005	16/23S, DNA	Chicken liver	Natural	None	Section-FISH
***Pseudomonas* spp.**					
Gunasekera et al., 2003a	16S, DNA and PNA	Milk	Spiked	None	Flow-FISH and conventional fluorescence microscopy
Kitaguchi et al., 2005	16S, DNA	Milk	Spiked	None	Combined with antibody and CTC staining, detection of 10^4 CFU/ml
Helicobacter pylori					
Angelidis et al., 2011	16S, DNA	Milk	Natural	None	Detection of 10^3–10^4 CFU/ml
B. cereus					
Laflamme et al., 2009	16S, DNA	Milk	Spiked	1 h	Flow-FISH and conventional microscopy, detection of 10^3 CFU/ml
***Gallibacterium* spp.**					
Bojesen et al., 2003	16S, DNA	Liver and spleen of chickens	Natural	None	Section-FISH
Cronobacter* spp./*Enterobacter sakazakii					
Almeida et al., 2009	16S, PNA	Powdered infant formula	Spiked	8 h	Detection of 1 CFU/10 g in 12 h, 100% sensitivity and specificity
Clostridium perfringens					
Shimizu et al., 2009	16S, DNA	Ground beef	Spiked	6 h	FISH-on-filter with formation of microcolonies, detection of $2*10^2$ CFU/g
Enterobacteriaceae					
Ootsubo et al., 2003	16S, DNA	Minced chicken meat and Ikura (Japanese seafood)	Natural	6 h	FISH-on-filter with formation of microcolonies, detection of 10^2 CFU/g
Multiplex FISH					
Salmonella* spp./ *L. monocytogenes (Oliveira et al., 2004)	16S/23S, DNA	Sheep milk	Spiked	None	mFISH
***Enterobacteriaceae*/ *Pseudomonas* spp.** (Yamaguchi et al., 2012)	16S, DNA	Milk	Spiked	3–5 h	mFISH, FISH-on-filter with formation of microcolonies, detection of $2*10^3$ CFU/ml

Studies stating a detection limit of 1–10 CFU in 25 g of food indicate a similar performance of FISH (in terms of sensitivity) as the current gold standard of well-established ISO-norms.

samples, similar to those used for tissue sections in immunohistochemistry, might allow for a more accurate assessment of the bacterial contamination and its spatial distribution (Bojesen et al., 2003; Khimmakthong et al., 2013; Mbuthia et al., 2001). Its potential in food microbiology has already been shown for studying microbial communities in complex food matrices like cheese (Ercolini et al., 2003). One of the rare FISH studies on *Campylobacter* has also successfully applied section-FISH (Schmid et al., 2005). Moreover, this avoids an often harsh homogenization treatment for solid products with considerable losses of bacteria. However, section-FISH might pose new technical challenges, including an impeded accessibility for the labelled probe and, therefore, the requirement of longer hybridization times.

Finally, by using the FISH-on-filter technique based on filters with a small pore size, large volumes of liquid samples with only a few particles and microbes can be analysed. The bacteria are collected and thus enriched on the filter membrane instead of passing through like the rest of the sample (including potentially interfering compounds). These filters can then be used directly for hybridization or, alternatively, may be placed on a plate with appropriate growth medium and incubated for several hours before FISH is performed (Perry-O'Keefe et al., 2001). In the latter case, microscopic evaluation of grown microcolonies can afterwards be performed much more rapidly because a lower magnification is needed. Besides, small artefacts and other single bacteria are no longer a source of uncertainty and potentially false results. However, this short cultivation step has some drawbacks because VBNC bacteria cannot be detected and the time until results can be acquired is elongated. The FISH-on-filter technique was shown to be effective and sensitive (100 CFU/g) in case of *E. coli*, *Clostridium perfringens*, *Listeria* or *Pseudomonas* spp. (Fuchizawa et al., 2008, 2009; Ootsubo et al., 2003; Shimizu et al., 2009; Stender et al., 2001; Yamaguchi et al., 2012).

5. Improvements of conventional hybridization in food microbiology and alternatives to microscopic evaluation

Not only the sample pretreatment has been optimized and further developed for the use of FISH in food microbiology, but also the hybridization procedure itself (Fig. 1, step 3) and the subsequent microscopic evaluation (Fig. 1, step 5). However, it has to be noted that these FISH adaptations are not necessarily specific for analysing food matrices: First, the switch to peptide nucleic acid (PNA) probes, the replacement of the attached fluorophore with an enzyme and further probe modifications have enabled better and faster results (Cerqueira et al., 2008; Schönhuber et al., 1999; Wagner and Haider, 2012). Secondly, the use of multiple probes allows the simultaneous detection of more than one species (Takada et al., 2004; Yamaguchi et al., 2012). Thirdly, the application of flow cytometry (flow-FISH) instead of fluorescence microscopy has been demonstrated to have several advantages such as its rapidity and high-throughput potential (Azevedo et al., 2011; Bisha and Brehm-Stecher, 2009b; Brehm-Stecher, 2008; Manti et al., 2011).

5.1. Hybridization

PNAs are one example of DNA mimics for which, in general, the same rules of base pairing apply as for DNA. However, PNA probes lack the negative charge of DNA and, therefore, less repulsive forces between two hybridizing strands occur. This enables the PNA probe to bind stronger and the length of the probe can be shortened. PNA probes have a superior performance compared to DNA probes if regions of the rRNA with complex secondary structures shall be targeted which have a poor accessibility for DNA probes (Frischer et al., 1996). PNA probes bind to their complementary strands even at low salt concentrations and high temperatures whereas secondary structures consisting out of DNA or RNA dissolve (Brehm-Stecher, 2008). Hence, PNA probes can bind to structures which are hidden from DNA probes. The accessibility of the rRNA has been shown to possess a great impact on the fluorescence intensity which might differ significantly depending on the target region (Fuchs et al., 1998), and DNA probe design thus has to take the accessibility into account. The use of PNA probes (PNA FISH) and suitable hybridization conditions can circumvent this problem enabling high fluorescence intensities. The unpolar characteristics of PNA probes also increase the general penetration properties of the probe, resulting in an enhanced permeability for the entry into the cell through cell membrane and cell walls, and, therefore, the hybridization time can be reduced while keeping high hybridization efficiency. The resistance to nucleases represents another advantage of PNA probes or other DNA mimics and RNA derivatives such as locked nucleic acids (LNA). Accordingly, the use of PNA probes for FISH analysis of food products has increased significantly in recent years (Almeida et al., 2009, 2013a, 2013b; Machado et al., 2013; Zhang et al., 2012). Lately, it was shown for *E. coli* O157 that PNA probes allow the specific detection of this particular serotype, which has not been achieved with a DNA probe (Almeida et al., 2013b). The use of other DNA-mimics (i.e. LNA-DNA hybrid structures with incorporated LNAs to enhance mismatch discrimination) may promise similar or even further benefits for FISH-testing in food microbiology (Cerqueira et al., 2008) although these mimics have only rarely been employed. Unfortunately, already published DNA probes cannot be easily converted into PNA probes or other DNA mimics since hybridization temperature, buffer stringency and the number of nucleotides have to be reevaluated and optimized in each case and self-complementarity has to be avoided (Amann and Fuchs, 2008; Cerqueira et al., 2008). The considerable higher costs for PNA probes and other DNA-mimics as well as a still inferior predictability of the results in contrast to conventional DNA probes also limit their potential for a broader diagnostic use.

To counteract weak fluorescence signals which are not caused by the bad accessibility of the target regions, but due to low ribosome contents (e.g. in very small or metabolically inactive cells), the overall brightness of the staining can be enhanced by catalyzed reporter deposition-FISH (CARD-FISH) or multi-locus-FISH (ML-FISH). CARD-FISH uses probes labelled with a peroxidase that can mediate the nearby deposition of a great amount of tyramide molecules with covalently bound fluorophores (Schönhuber et al., 1999). Thereby, an amplification of the signal is achieved, allowing the successful detection even if the food matrix causes huge background signals and autofluorescence. The rRNA might not always be sufficient to distinguish between pathogenic and apathogenic bacteria of a single species, especially if the pathogenicity depends on the recent acquisition of virulence factors or resistance genes which has not yet been reflected by a divergent rRNA evolution. Conventional FISH might fail to identify the corresponding messenger RNA (mRNA) due to a low abundance of these transcripts whereas CARD-FISH has been shown to detect mRNAs of virulence factors in *L. monocytogenes* (Wagner et al., 1998). However, the transport of enzyme-labelled probes into the cells can be more challenging since such probes require a higher permeability of the bacteria and the whole procedure takes a longer time than conventional hybridization, which is performed in only one step (Pernthaler et al., 2002; Schönhuber et al., 1999). Double-labelled probes (DOPE-FISH) or a panel of probes targeting different regions (ML-FISH) of the rRNA (Lee et al., 1993) can also help to increase the signal intensity. DOPE-FISH and ML-FISH might be simpler to perform than CARD-FISH although the

signal gain might not be equal. In contrast to PNA FISH, which has been intensively used in FISH-testing of food products, these techniques have not been broadly employed yet.

5.2. Multiplex FISH

The ability to label probes with different fluorophores enables the detection of several pathogens at the same time. Multiplex FISH (mFISH) requires the same optimization steps like multiplexing PCR. Therefore, the design of the FISH probes has to be performed in a way that the same hybridization temperatures and stringency conditions can be employed. Furthermore, the used probes should show as little mutual complementarity as possible. This sometimes tedious process is easier than the establishment of a multiplex PCR assay where for each amplification process two primers (instead of one FISH probe) have to be adapted to other primer pairs. Sequential FISH-stainings instead of simultaneous hybridization steps are also possible (Almstrand et al., 2013). mFISH has been used intensively in the field of genetics for the differential visualization of chromosomes, sometimes termed 'chromosome painting', by using combinatorial labelling of a probe with a set of different fluorophores (Liehr et al., 2004, 2013). Accordingly, mFISH should be able to simultaneously detect more than ten different pathogenic species. However, as ordinary fluorescence microscopes have only a limited set of filters (often broadband filters) and the optical equipment is not always sufficient to distinguish between closely-related fluorophores with similar emission spectra, the feasibility of the selective detection of a vast number of different species has to be estimated with caution. Therefore, many FISH approaches in microbiology target only two or three different species (Almeida et al., 2011; Almstrand et al., 2013; Yamaguchi et al., 2012). Alternatively, it is conceivable that probes targeting different pathogens are labelled with the same fluorophore and are applied as a mixture. Although this method lacks the resolution capacity to specify the kind of contamination, which is possible with mFISH using differentially labelled probes, it is sufficient to rather unspecifically detect a broad range of pathogenic bacteria. This might also be a saving of time on the long term because no channel switch is needed during microscopic evaluation. The improvement of fluorescence microscopy and the price decline of corresponding equipment will also foster the feasibility of mFISH. Up to now, only a few mFISH approaches in food microbiology have been described (Oliveira et al., 2004; Yamaguchi et al., 2012), not counting studies where the actual probe targeting a certain species was used in combination with an unspecific probe for *Eubacteria* as a control or to quantify the total amount of bacteria in the sample. Takada and colleagues showed that the simultaneous detection of seven different *Bifidobacterium* spp. in human faeces in one FISH test is possible (Takada et al., 2004), demonstrating the general feasibility of mFISH. Interestingly, Oliveira et al. have shown that mFISH in sheep milk is also possible with Gram-positive and Gram-negative bacteria like *L. monocytogenes* and *Salmonella* spp. (Oliveira et al., 2004) although Gram-positive and Gram-negative bacteria in general might require different permeabilization conditions, and for a combined detection specifically adapted protocols for the permeabilization have to be employed (Ercolini et al., 2006). An interesting extension to mFISH might be the simultaneous use of nucleotide probes together with antibody stainings and specific dyes, providing general information about the overall viability, metabolic state or the respiratory activity of the observed cells like it was shown for *Pseudomonas* spp. (Kitaguchi et al., 2005). This additional data might be extremely useful to judge the viability and thus potential perilousness of a bacterial contamination.

5.3. Evaluation of fluorescence via flow cytometry

Already in the early 1990s flow cytometry in combination with FISH (flow-FISH) was employed to count bacteria and measure their fluorescence intensity (Amann et al., 1990b). Using flow cytometry is highly convenient since it does not rely on slides and manual counting. Larger sample volumes can be analysed rapidly without human assistance (Brehm-Stecher, 2008; Gunasekera et al., 2003b). Flow cytometry in combination with FISH-staining has not yet gained huge importance in FISH-testing of food products although the composition of microbial communities can be elegantly represented (Veal et al., 2000). In addition, flow-FISH has already been applied for the quantification of different bacterial species in liquid samples like milk (Gunasekera et al., 2003a, 2003b; Laflamme et al., 2009) or in pure or mixed cultures (Connil et al., 1998). In contrast, solid food samples are more challenging for the analysis in the flow cytometer, and, depending on the food matrix, one has to cope with strong autofluorescence or interfering cell debris as a result of mechanical sample disintegration (Bisha and Brehm-Stecher, 2009b; Veal et al., 2000). As for other FISH analyses, an appropriate pretreatment of the sample is thus indispensable. Recent FISH studies to detect human pathogens like *Salmonella* in food samples were not only analysing enrichment cultures from the food matrix, but also utilized directly spiked complex food matrices for the pathogen detection via flow cytometry (Bisha and Brehm-Stecher, 2009a, 2009b, 2010). Other approaches of *Salmonella* detection in food using fluorescently labelled antibodies instead of nucleotides also demonstrate the general applicability of flow cytometry for FISH (McClelland and Pinder, 1994). The detection limit of flow-FISH is around 10^3 CFU/ml and hence similar to that obtained with conventional fluorescence microscopy (Bisha and Brehm-Stecher, 2010) although lower concentrations may be detectable as well (Gunasekera et al., 2003b). The potential of flow-FISH, especially for automated systems and high-throughput analysis, in routine testing of food products has not yet yielded many applications and should be exploited more intensively. However, this promising tool obviously requires much higher investment costs than conventional fluorescence microscopy.

6. Sensitivity and specificity of FISH and possibilities to raise the detection limit

Sensitivity, measuring the capability to detect all present target pathogens, and specificity, measuring the ability to exclusively detect the target organism, but not other microbes, are the main parameters to judge the power of a test. In order to compete with other tests for the detection of foodborne pathogens, FISH analysis has to reach similar limits of detection and should show an equal performance with respect to its specificity. In previous studies FISH was mostly compared with the gold standard of conventional cultivation and plating according to standardized procedures like norms of the International Organization for Standardization (ISO-norms); sometimes other rapid detection systems like PCR were used as reference methods (Almeida et al., 2013a, 2013b; Oliveira et al., 2012; Stephan et al., 2003; Vieira-Pinto et al., 2007). It is the aim of the conventional methods to detect as little as one CFU in a food sample of roughly 25 g; all the rapid methods have to be ultimately compared with this standard. Unfortunately, only a few studies so far have provided useful data to make a reliable comparison between FISH and other detection methods. Since in some studies enrichment periods (with differing duration) were preceding FISH and different numbers of field of views (FOV) per slide at different magnifications were analysed (Angelidis et al., 2011; Kitaguchi et al., 2005; Ootsubo et al., 2003; Regnault et al., 2000; Yamaguchi et al., 2012), a comprehensive comparison in terms of

sensitivity between these studies cannot be performed. This underlines the indispensable need for valid and extensive ring trials to obtain international standards and more reliable data.

In theory, the best possible detection limit of a FISH analysis is restricted through the amount of sample which is applied on the slide or analysed in a flow cytometer. FISH performed on glass slides usually allows for an application volume of about 10–30 μl of sample. Assuming the spotting of 10 μl of (untreated or concentrated and pretreated) sample per slide as an example, this would set the theoretical detection limit at roughly 100 cells/ml. This optimistic estimation requires the highly efficient fixation, permeabilization and hybridization as well as the complete assessment of the entire slide. Due to different kinds of losses during the preparation of the sample, statistical variations, and to enable unambiguous results for observations under the microscope, others calculate a concentration of 10^3–10^4 CFU/ml within the sample as the more appropriate detection limit because they require at least one microbe per field of view (Amann et al., 1995; López-Campos et al., 2012; Mandal et al., 2011). Since many microbial food contaminations are below this detection limit (for example *S. enterica* or *L. monocytogenes*), an enrichment of these pathogens is necessary to reach the sensitivity of the gold standard procedures. Therefore, the urgency of either a highly efficient concentration process or, alternatively or additionally, the enrichment in non-selective growth media for only weakly contaminated food products becomes evident. Of course, this consideration is valid for any rapid method, including PCR, which also has a low sample volume input.

In Table 1, studies claiming to have reached detection limits similar to those achieved by the standardized cultivation methods, thus close to 1 CFU/25 g, have been highlighted together with important specifications concerning sensitivity, specificity and the detection limit. This shows the principal suitability of FISH to replace the highly sensitive conventional methods although it has to be stressed that all of these studies relied on extended periods of cultural enrichment. It is remarkable that not only the detection limits under similar conditions vary widely, but also the total time needed for a comprehensive FISH analysis, ranging from less than 12 h to more than one day (Table 1). This indicates that in many cases much room for improvement is left although it is easily conceivable that the detection of different bacterial species by FISH might vary in time because the enrichment periods for rather slow growing bacteria like *Campylobacter* is much longer in comparison to fast dividing species like *Cronobacter* spp. (Almeida et al., 2009; Schmid et al., 2005). It is also important to note that the majority of these studies are based on artificially spiked samples. This convenient approach is probably the most feasible in practice, but it remains to be proven whether artificial spiking appropriately reflects the actual performance of FISH on naturally contaminated samples.

Whereas the sensitivity of FISH is mainly driven by the experimental set-up, specificity of FISH has been shown to be superior to other methods, at least provided that probe design was done with caution and hybridization was performed under sufficiently stringent conditions (Almeida et al., 2013a; Fang et al., 2003). Other rapid molecular methods such as PCR-derived techniques have been reported to obtain similar sensitivities, but, in direct comparison with FISH, they were more prone to errors and in general might generate more false-positive as well as false-negative results (Almeida et al., 2013a). In this regard, the growing number of published rRNA sequences in free databases will contribute to a more accurate design of FISH probes with decreasingly less cross-reactivity with other bacteria.

Interestingly, some studies emphasize also the ability of FISH analysis to detect microorganisms which cannot be recovered via conventional plating, but might still pose a threat to human health. The FISH technique is capable of efficiently detecting stressed bacteria, for example due to unfavourable conditions like a varying pH-value, an adverse salt concentration or temperature, whereas the identification and cultivation of stressed bacteria by the conventional methods have been shown to be significantly impaired (Fang et al., 2003). Therefore, cultivation and other technologies might have an intrinsic shortcoming, especially for bacteria in complex matrices like food, which can only be circumvented by cultivation-independent testing (Vieira-Pinto et al., 2007). Oliveira et al. even reported a surprising 100-fold higher count of bacteria via FISH analysis in comparison to plating (Oliveira et al., 2003). However, it has to be noted that in this study the detection by culture was performed by simple plating without prior enrichment and no ISO protocol with a presumably higher sensitivity was employed. Nevertheless, FISH analysis might in principle possess the capacity to detect food contaminations more efficiently than traditional ISO standards (Oliveira et al., 2012).

6.1. FISH after cultural enrichment

Following an extended period of non-selective enrichment, FISH was performed in some studies without any further treatment of the sample (Almeida et al., 2013a). A short cultivation period can sometimes have significant benefits because growing and dividing microbes usually have a higher number of ribosomes and, as a result, a higher amount of rRNA target structures, which increases the fluorescence signal (Amann et al., 1995; Bouvier and Del Giorgio, 2003). For larger organisms with a high ribosome content like *E. coli* this might not be of major importance whereas a cultivation period may increase the ribosome number in small pathogens such as *Campylobacter* spp., *L. monocytogenes* and *Mycobacterium* spp., which might have a positive impact on the outcome of a FISH analysis (Ehrenberg et al., 2013; Fegatella et al., 1998; Milner et al., 2001). Growing cells with many ribosomes (with a natural range between 10^3 to 10^5 ribosomes/cell) produce brighter fluorescence signals, which helps to distinguish bacteria from high background fluorescence (Amann et al., 1995). A study using the common eubacterial probe EUB338 showed that the detection efficiency can differ substantially (from 1% in rather unfavourable growth conditions to almost 100% in enrichment media), demonstrating the importance of cell size, growth stage and the number of ribosomes (Bouvier and Del Giorgio, 2003). Another advantage of enrichment steps is the dilution of interfering food matrix components, which can heavily facilitate evaluation by fluorescence microscopy as well as by flow cytometry.

Due to these reasons, most FISH studies targeting pathogens in complex food matrices were performed after enrichment periods, which ranged from a few hours up to more than a day (Table 1). Given a sufficient amount of time for the preenrichment period, FISH is able to meet the ambitious goal of the conventional methods to detect 1 CFU/25 g. After an overnight enrichment period, FISH has been shown to offer similar or even higher sensitivities compared to the conventional validated and standardized procedures, which are significantly slower (Almeida et al., 2009, 2010, 2013a, 2013b; Fang et al., 2003; Tortello and Reineke, 2000). Although these procedures are without any doubt highly effective to reach detectable concentrations and are common practice for many rapid detection methods, including PCR-based methods, they obviously prolong the overall time to obtain a result and do not improve the detection of VBNC organisms. In addition, enrichment procedures hamper the accurate determination and quantification of the initial bacterial load because the lag time before the exponential growth phase might vary (due to different sample matrices and their prior treatment) and is quite

difficult to estimate. Therefore, cultivation-independent concentration systems are more desirable.

6.2. FISH after non-cultural enrichment

A wide variety of different separation processes for bacteria in complex matrices have been developed, which are not only useful for FISH, but also for other rapid techniques or to enhance the capacity of cultivation methods (Brehm-Stecher et al., 2009; Stevens and Jaykus, 2004). For the isolation and concentration of the target organism the different physical, chemical and biological properties of a bacterial species or the adsorbance on specific materials can be employed (Brehm-Stecher et al., 2009). Approaches to concentrate the target organisms vary substantially in their specificity and costs. These methods should be chosen carefully because some rather target-unspecific techniques like filtration and centrifugation steps might also concentrate interfering substances and particles which can complicate subsequent analyses (Stevens and Jaykus, 2004). Commercially available systems which can specifically separate microbes from other parts of the sample already exist; some of them have the potential to be used for a broad range of food matrices (Pathatrix, Dynabeads and others). In contrast to DNA extraction methods, in which rather harsh conditions might be applied, enrichment procedures for FISH analyses have to be performed with much more caution to maintain the cell integrity.

Especially immunomagnetic separation (IMS) represents a powerful tool to separate bacteria from other food components. It has already been successfully implemented in PCR-derived methods as well as in conventional culturing and plating techniques. Beads coated with pathogen-specific antibodies allow for the concentration of several orders of magnitudes without the use of cultural enrichment. The specificity and rapid concentration of the target organism by IMS has been already implemented in the ISO standard 16654:2001 for the detection of *E. coli* O157:H7 (ISO, 2001). Magnetic separation (MS) using beads coated with metal hydroxides or lectins instead of antibodies is less specific and enables a broad, relatively inexpensive and fast capturing of different bacterial species (Lucore et al., 2000; Porter et al., 1998). However, IMS, MS or other promising separation techniques for FISH analyses of food products are still awaiting the broad application. Elaborated and well-thought-out separation and concentration techniques like IMS along with simple physical and biological methods like centrifugation, filtration and enzymatic treatment could easily elevate the sensitivity of FISH. However, the accomplishment of a detection limit of 1 CFU/25 g might still be quite challenging if a complete avoidance of any preenrichment step is intended.

7. Automation of FISH analysis

Since the high-throughput of samples is imperative for routine testing of food products, the use of automated or semi-automated systems is becoming more and more important for all detection methods. Automation of FISH could be achieved by establishing a modular composed system able to independently execute the whole FISH procedure. This requires first the module sample pretreatment, including the isolation of bacteria, second, the module sample processing, comprising fixation, permeabilisation and hybridization on a slide or in liquids and, third, the sample read-out by automated microscopy or in a flow cytometer, the subsequent documentation and data interpretation. Unfortunately, a system embracing all these tasks and generally adapted to various kinds of food matrices in a fully human-independent fashion has not yet been developed, but encouraging approaches for each module already exist (Daims et al., 2007; Daims, 2009; Myers, 2004, 2012; Pernthaler et al., 2003; Thiel and Blaut, 2005; Wauters et al., 2007; Zeder and Pernthaler, 2009).

Especially the process of sample pretreatment and microscopic observation of the single slides represent laborious steps of a FISH experiment. Moreover, assessment of microscopic results often demands skilled personnel with a high level of expertise. Whereas sample pretreatment might be partially performed by automated pipetting systems like those employed for automated PCR devices, and even automated slide processing devices are available, sample scanning as well as interpretation and evaluation of microscopic results require more sophisticated tools capable of distinguishing signals of bacteria from occasionally occurring strong fluorescence artefacts. In this regard, object segmentation, intensity calculation and background correction methods are necessary and the morphology of the target pathogen (together with cells sticking to each other or forming larger aggregates) has to be correctly identified. These methods are already used in molecular biology, for example for microarray data processing (Novikov and Barillot, 2007; Rueda and Rezaeian, 2011), and algorithms have been adapted to the needs of microscopic analyses like images from FISH experiments or immunohistochemistry (Theodosiou et al., 2007). Fully or at least partially automated FISH analyses from the medical field showed convincing and accurate results, similar to those obtained by manual examination and skilled personnel (Evans et al., 2006; Wauters et al., 2007). The examination and quantification of biofilms have also revealed the effectiveness of automated, motorized microscopy for FISH (Kuehn et al., 1998). Sample spiking with defined amounts of non-target bacteria prior to the analysis has been used as an internal standard and control to assess the quality of a FISH experiment and the efficiency of automated detection (Daims et al., 2001). In addition, a growing number of manufacturers like vermicon, BioVisible, miacom or AdvanDx are selling commercial kits using *in situ* hybridization approaches similar to conventional FISH settings with the potential for automation and high-throughput analyses, which underlines the principal suitability of FISH for routine diagnostics and testing.

Despite the current challenges to implement reliable and cost-effective settings, an established automated system carries enormous potential since it is fully independent of human judgement. In the field of microscopy there can be a considerable amount of uncertainty and ambiguity regarding the microscopic assessment, often resulting in a high variance, especially if rather inexperienced personnel perform such examinations (Jarvis, 1977). An estimation of the relative variance between skilled technicians in counting microorganisms under the microscope revealed a variation of more than 55% as shown for fungal contaminations (Jarvis, 2008). This illustrates a general weakness of methods based on subjective microscopic evaluation. In contrast, conventional culturing, which might imply labour-intensive steps, usually does not depend so much on personal judging. Standardized analysis software and automated systems might, therefore, uncouple FISH analyses from human interpretation, minimize staff costs and thus could largely contribute to its acceptance as a reliable alternative detection method. Fully automated slide observation with a subsequent image analysis is only one possibility; flow cytometry, which is sometimes interpreted as ‘automated fluorescence microscopy’, is equally capable of fulfilling this task. All in all, the investment costs for automated systems are usually quite high, but the general possibility for FISH automation is given, quick amortizing of costs is possible, and analytical challenges are technically feasible to solve.

8. Calculation of costs

Any emerging novel technique has to withstand a cost comparison with traditional and well-established methods. Reasonable

Table 2
Estimated costs (in Germany) for *Salmonella* FISH-testing, PCR-testing and detection via cultivation.

Cost factor	Costs per sample in €	Calculation basis	References
Staff costs (based on the salary of medical laboratory assistants)	6.22	Testing of 30 samples per day by a medical laboratory assistant, 21.94 €/h (on average 17 min of total hands-on time per sample: 5 min of sample pretreatment and enrichment culture, 4 min of sample concentration via MS or centrifugation, 2 min per sample for FISH staining assuming simultaneous sample processing, 5 min of microscopic examination, 1 min for reporting and documentation)	Salary of German public service sector; labour agreement (TvÖD) including non-wage labour costs
Technical equipment	0.40	Acquisition costs of 20,000 € for a simple fluorescence microscope and heating element, life period of 5000 working hours, total sample-throughput of at least 50,000	Nikon, Zeiss, Heratherm
Consumables			
Enrichment culture and MS	0.59		
DNA probe (use of 40 pmol)	0.60	Estimated consumption on the basis of previous FISH studies in food microbiology (Table 1)	Stellaris FISH probes, TIB Molbiol, GeneWorks, Biosearch technologies
Others	2.00		
Total costs of FISH	9.81		
Total costs of PCR	7.02–7.84	Commercially available real-time PCR kit with an internal amplification control; estimated 30% discount on catalogue price and a value added tax (VAT) of 21% (German VAT, 2014) summing up to material costs of 4.60–5.42 €/test	Applied Biosystems/Life Technologies, Qiagen
Total costs of culture	8.27		

Overhead costs, for example considering the space occupied by laboratory equipment, rent for laboratory buildings or maintenance charges have been assumed to be roughly equal and are not included in this calculation. Costs for staff training as well as the implementation and validation of a PCR test or FISH test are also not considered in this calculation. Hands-on time for PCR and traditional cultivation and bacterial enrichment were adopted from previous economic evaluations and costs were calculated according to German labour costs (Abubakar et al., 2007; Alexander et al., 2006; Lucore et al., 2000; Oliveira et al., 2003). Material costs for PCR detection depend on the price of the used real-time PCR kit; two up-to-date offers from two manufacturers of pathogen detection kits served as the calculation basis.

cost-benefit ratios are required in times of growing budget constraints and resource-poor facilities, especially in low-income countries. For high-throughput analyses a minimization of the costs per sample is desired. The actual costs per FISH sample consist of the overall materials needed, including technical equipment, as well as the labour costs, the required staff training and also the initial expenditures for the establishment of a FISH assay. Since the costs for implementation and training matters might exhibit a high degree of variation, only the expenses for established protocols shall be considered here. This is also the case for purchasing commercially available FISH kits, which are usually relatively simple to conduct, but much more expensive (Alexander et al., 2006). However, it has to be kept in mind that, due to a general lack of experience with FISH-testing in food microbiology, the implementation costs of a novel FISH test might be considerably higher than setting up a new PCR.

Table 2 summarizes the calculation of the price for a FISH system targeting *Salmonella* spp. with a sensitivity similar to cultivation methods, therefore, including a short preenrichment period, a complex separation process and without any kind of automation. Under these circumstances costs for *Salmonella* detection in a high-income country like Germany would sum up to about 9–10 € per sample. Depending on the organism (and varying preenrichment or separation conditions), it might be slightly different for other microbes. This value is above other rapid methods like ELISA-testing systems or in-house PCRs with limited validation, but similar in range compared to other established methods like commercially available and certified real-time PCRs or the conventional cultivation (Abubakar et al., 2007). The majority of the costs for this kind of FISH is to a large extent a result of the hands-on time (roughly 60% of the entire costs due to a required hands-on time of ca. 17 min per sample, Table 2), such as observation under the microscope, sample preparation or the FISH-staining procedure itself, which other publications calculate in a similar manner (Alexander et al., 2006). This is approximately twice the time required for ELISA or PCR. Therefore, any automation of this system would lead to a substantial saving of costs. Combining the detection of several pathogens into a multiplex FISH test could further decrease the costs of FISH. In contrast to that, presumptive high costs due to the microscopic equipment and usage of consumables contribute only slightly to the overall sample costs if a high sample through-put is assumed.

The conventional cultivation still has a slight cost advantage because, in contrast to FISH, training issues are rather low; on the other hand, it becomes increasingly clearer that these slow techniques are not only insufficient to evaluate potential threats of VBNC bacteria, but also are unable to detect microbial hazards before the food product is reaching the consumer. The calculations presented here consider only the current state of the art; it can be assumed that novel FISH approaches will help to reduce the costs per sample significantly.

9. Strengths and weaknesses of FISH with regard to other detection methods

For the implementation of FISH in the routine monitoring of food safety, specificity, sensitivity, test speed, the potential for high-throughput testing and economical feasibility represent major characteristics. Table 3 summarizes the features of FISH compared to PCR, presumably the most notable rapid molecular technique, and the traditional detection via cultivation. Other rapid techniques which can be also used for the detection of pathogenic bacteria in food comprise various immunological methods, biosensors or the use of microarrays (Hoorfar et al., 2011; López-Campos et al., 2012; Nugen and Baeumner, 2008). Although these techniques may be more robust and are less influenced by the food matrix and some of them have considerable potential for the simultaneous detection of multiple pathogens, they usually possess higher thresholds for a

Table 3
Summary of strengths and weaknesses of the conventional techniques, FISH and PCR-derived techniques.

Test feature	Conventional microbiological detection	FISH	PCR-derived techniques
Detection principle/test target	Pathogen multiplication, metabolic traits	Binding to bacterial rRNA (mRNA, DNA)	Amplification of bacterial DNA or RNA
Test speed	−−	+	++
Specificity	+	++	++
Sensitivity (Jasson et al., 2010)	++ (1 CFU/25 g)	+ (without enrichment, manual microscopic evaluation)	++ (real-time PCR)
Exclusion of dead material	Yes	Yes	No
Detection of VBNC bacteria	No	Yes	(Yes)
Bacterial load estimation	+	+	±
Robustness/matrix dependency	++	±	±
Multiplex feasibility	−	+	+
Costs per test	±	±	+
Test complexity	+	±	±
Potential for standardization	+	± (without automation)	+
Current state of validation and implementation	++	−	+
Potential for high-throughput analyses	−	+	++
Potential for routine testing, monitoring and risk assessment	+	+	+

++: excellent, +: good, ±: ambiguous, −: poor, −−: severe weakness.

reliable detection, might be less specific and show a higher degree of cross-reactivity compared to PCRs (Al-Khaldi, 2002; Gehring et al., 2013; Hu et al., 2012; Kupradit et al., 2013; López-Campos et al., 2012). Furthermore, comprehensive data on the evaluation of these techniques are not yet available in an equivalent extent, which is why they were not considered in our comparison.

The most prominent strengths of the conventional microbiological methods are the high sensitivity and specificity, the detection of only viable bacteria (although not VBNCs), the robustness regarding different matrices, the low test complexity and the high state of validation and standardization (Jasson et al., 2010; López-Campos et al., 2012). On the contrary, in terms of test speed, which is the main weakness of the time-consuming cultivation techniques, FISH and PCR have a superior performance with an assay duration of a few hours and an excellent specificity, although sensitivity without preenrichment does not achieve values comparable to conventional detection procedures (Cai et al., 2014; López-Campos et al., 2012). In addition, these two rapid techniques are also suitable for the simultaneous detection of more than one pathogenic bacterial species (Cai et al., 2014) and might acquire a considerable cost advantage over the cultivation techniques during the next years, especially if automation comprising sample pretreatment, test processing and the evaluation of the results is further promoted. Unlike the cultural methods and FISH, standard PCR-derived techniques usually cannot distinguish between viable and dead microbes and their genomic debris (Jasson et al., 2010). In the food industry inactivation of pathogens is the desired intention, but not necessarily the entire removal of their DNA, which can persist in detectable concentrations for at least 30 h after cell death due to heat-killing (Birch et al., 2001). Therefore, PCR techniques might be prone to detect no longer existing threats. Exact numbers of the (viable) bacterial load are thus difficult to determine, but several adaptations, e.g. sophisticated procedures like PMA-treatment (propidium monoazide) to exclude the amplification of free DNA or from dead microbes or targeting fragile RNA-structures, for example rRNAs, can be used to fulfil this task (Krüger et al., 2014). Besides, PCR-analyses have been shown to be influenced by matrix-specific inhibitors, which can cause false-negative results, and thus matrix-specific pretreatment steps for the DNA isolation might be required as well as internal amplification controls (Schrader et al., 2012). This obstacle might occur with FISH as well since potential inhibitors of hybridization in food and disturbing factors like autofluorescence are poorly characterized, but this problem can be solved by employing suitable controls like eubacterial probes and unspecific stainings of all microbes, which has the additional advantage of estimating the total bacterial count (Kitaguchi et al., 2005; Yamaguchi et al., 2012).

PCRs are suitable for rapid screening and high-throughput purposes, but every positive preliminary finding should be validated by further cultivation and confirmatory tests, which result in additional costs and considerable time losses (Ge and Meng, 2009; Havelaar et al., 2010; Hoorfar, 2011). PCR as a simple, broadly used molecular technique with the potential for high-throughput analyses of food products has been validated and implemented during the past decade (Hoorfar et al., 2011; Malorny et al., 2009), whereas comparable data for FISH is still scarce. However, one of the probably most striking strengths of FISH is its capacity to detect VBNC bacteria, which might become especially beneficial and a valuable asset in case of emerging pathogens for which effective cultivation and differentiation methods still have to be developed (Bottari et al., 2006; Brehm-Stecher, 2008; Jasson et al., 2010). Nevertheless, a lack of standardization, automation and the insufficient availability of high-throughput systems have so far limited the practicability and continue to impede the implementation in the everyday laboratory work. Summing up, none of the three approaches is without limitations and constraints; thus the method of choice depends on the particular demands.

10. Conclusions and future perspectives

During the last two decades numerous rapid methods for the identification of foodborne bacteria have been developed. Although cultivation techniques suffer from several drawbacks, their importance remains high because rapid methods continue to have limitations. Innovative techniques and methodical improvements have boosted the potential of FISH to detect foodborne pathogens although many of these beneficial advancements have not yet been adequately transferred to the routine use of FISH in food microbiology. The need for efficient pathogen concentration and separation from the food matrix to overcome or minimize cultural preenrichment, the pivotal implementation of high-performance modular systems for automated FISH analyses and the establishment of standardized protocols are future challenges, which have to be further addressed.

Like for other promising novel detection methods, the validation, harmonization of FISH and interlaboratory tests or ring trials of this method as well as the establishment and agreement on standard guidelines for the experimental procedure have to precede the implementation into routine testing of food products. In this aspect the FISH technology is still in its infancy and at the beginning of this long path whereas the PCR-techniques are years ahead; not necessarily because of their superiority, but simply due to the fact that the systematic evaluation has begun much earlier. Therefore, conducting more in-depth studies and further research efforts to establish FISH in the field of food microbiology, especially to detect pathogens at an early stage, remains highly rewarding and will help to overcome current hurdles.

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References

Abubakar, I., Irvine, L., Aldus, C.F., Wyatt, G.M., Fordham, R., Schelenz, S., Shepstone, L., Howe, A., Peck, M., Hunter, P.R., 2007. A systematic review of the clinical, public health and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in faeces and food. Health Technol. Assess. 11, 1–216.

Alexander, B.D., Ashley, E.D., Reller, L.B., Reed, S.D., 2006. Cost savings with implementation of PNA FISH testing for identification of *Candida albicans* in blood cultures. Diagn. Microbiol. Infect. Dis. 54, 277–282.

Al-Khaldi, S.F., Martin, S.A., Rasooly, A., Evans, J.D., 2002. DNA microarray technology used for studying foodborne pathogens and microbial habitats: minireview. J. AOAC Int. 85, 906–910.

Almeida, C., Azevedo, N.F., Iversen, C., Fanning, S., Keevil, C.W., Vieira, M.J., 2009. Development and application of a novel peptide nucleic acid probe for the specific detection of *Cronobacter* genomospecies (*Enterobacter sakazakii*) in powdered infant formula. Appl. Environ. Microbiol. 75, 2925–2930.

Almeida, C., Azevedo, N.F., Fernandes, R.M., Keevil, C.W., Vieira, M.J., 2010. Fluorescence *in situ* hybridization method using a peptide nucleic acid probe for identification of *Salmonella* spp. in a broad spectrum of samples. Appl. Environ. Microbiol. 76, 4476–4485.

Almeida, C., Azevedo, N.F., Santos, S., Keevil, C.W., Vieira, M.J., 2011. Discriminating multi-species populations in biofilms with peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH). PLoS One 6.

Almeida, C., Cerqueira, L., Azevedo, N.F., Vieira, M.J., 2013a. Detection of *Salmonella enterica* serovar Enteritidis using real time PCR, immunocapture assay, PNA FISH and standard culture methods in different types of food samples. Int. J. Food Microbiol. 161, 16–22.

Almeida, C., Sousa, J.M., Rocha, R., Cerqueira, L., Fanning, S., Azevedo, N.F., Vieira, M.J., 2013b. Detection of *Escherichia coli* O157 by Peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) and comparison to a standard culture method. Appl. Environ. Microbiol. 79, 6293–6300.

Almstrand, R., Daims, H., Persson, F., Sörensson, F., Hermansson, M., 2013. New methods for analysis of spatial distribution and coaggregation of microbial populations in complex biofilms. Appl. Environ. Microbiol. 79, 5978–5987.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990a. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762–770.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990b. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.

Amann, R., Kühl, M., 1998. *In situ* methods for assessment of microorganisms and their activities. Curr. Opin. Microbiol. 1, 352–358.

Amann, R., Fuchs, B.M., 2008. Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. Nat. Rev. Microbiol. 6, 339–348.

Angelidis, A.S., Tirodimos, I., Bobos, M., Kalamaki, M.S., Papageorgiou, D.K., Arvanitidou, M., 2011. Detection of *Helicobacter pylori* in raw bovine milk by fluorescence *in situ* hybridization (FISH). Int. J. Food Microbiol. 151, 252–256.

Azevedo, N.F., Jardim, T., Almeida, C., Cerqueira, L., Almeida, A.J., Rodrigues, F., Keevil, C.W., Vieira, M.J., 2011. Application of flow cytometry for the identification of *Staphylococcus epidermidis* by peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH) in blood samples. Ant. Van Leeuwenhoek 100, 463–470.

Babot, J.D., Hidalgo, M., Argañaraz-Martínez, E., Apella, M.C., Perez Chaia, A., 2011. Fluorescence *in situ* hybridization for detection of classical propionibacteria with specific 16S rRNA-targeted probes and its application to enumeration in Gruyère cheese. Int. J. Food Microbiol. 31, 221–228.

Birch, L., Dawson, C.E., Cornett, J.H., Keer, J.T., 2001. A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. Lett. Appl. Microbiol. 33, 296–301.

Bisha, B., Brehm-Stecher, B.F., 2009a. Simple adhesive-tape-based sampling of tomato surfaces combined with rapid fluorescence *in situ* hybridization for *Salmonella* detection. Appl. Environ. Microbiol. 75, 1450–1455.

Bisha, B., Brehm-Stecher, B.F., 2009b. Flow-through imaging cytometry for characterization of *Salmonella* subpopulations in alfalfa sprouts, a complex food system. Biotechnol. J. 4, 880–887.

Bisha, B., Brehm-Stecher, B.F., 2010. Combination of adhesive-tape-based sampling and fluorescence *in situ* hybridization for rapid detection of *Salmonella* on fresh produce. J. Vis. Exp. 44.

Bojesen, A.M., Christensen, H., Nielsen, O.L., Olsen, J.E., Bisgaard, M., 2003. Detection of *Gallibacterium* spp. in chickens by fluorescent 16S rRNA *in situ* hybridization. J. Clin. Microbiol. 41, 5167–5172.

Bottari, B., Ercolini, D., Gatti, M., Neviani, E., 2006. Application of FISH technology for microbiological analysis: current state and prospects. Appl. Microbiol. Biotechnol. 73, 485–494.

Bouvier, T., Del Giorgio, P.A., 2003. Factors influencing the detection of bacterial cells using fluorescence *in situ* hybridization (FISH): a quantitative review of published reports. FEMS Microbiol. Ecol. 44, 3–15.

Brehm-Stecher, B.F., 2008. Methods for Whole Cell Detection of Microorganisms. In: Microbial Surfaces, pp. 29–51. Chapter 3.

Brehm-Stecher, B.F., Young, C., Jaykus, L.A., Tortorello, M.L., 2009. Sample preparation: the forgotten beginning. J. Food Prot. 72, 1774–1789.

Cai, H.Y., Caswell, J.L., Prescott, J.F., 2014. Nonculture molecular techniques for diagnosis of bacterial disease in animals: a diagnostic laboratory perspective. Vet. Pathol. 51, 341–350.

Cerqueira, L., Azevedo, N.F., Almeida, C., Jardim, T., Keevil, C.W., Vieira, M.J., 2008. DNA mimics for the rapid identification of microorganisms by fluorescence *in situ* hybridization (FISH). Int. J. Mol. Sci. 9, 1944–1960.

Cocolin, L., Diez, A., Urso, R., Rantsiou, K., Comi, G., Bergmaier, I., Beimfohr, C., 2007. Optimization of conditions for profiling bacterial populations in food by culture-independent methods. Int. J. Food Microbiol. 120, 100–109.

Cocolin, L., Ercolini, D., 2008. Molecular techniques in the microbial ecology of fermented foods. In: Giraffa, G., Carminati, D. (Eds.), Molecular Techniques in Food Fermentation: Principles and Applications. Springer, New York, USA, pp. 1–30.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., Rantsiou, K., 2013. Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. Int. J. Food Microbiol. 167, 29–43.

Connil, N., Dousset, X., Onno, B., Pilet, M.F., Breuil, M.F., Montel, M.C., 1998. Enumeration of *Carnobacterium divergens* V41, *Carnobacterium piscicola* V1 and *Lactobacillus brevis* LB62 by *in situ* hybridization-flow cytometry. Lett. Appl. Microbiol. 27, 302–306.

Daims, H., Ramsing, N.B., Schleifer, K.H., Wagner, M., 2001. Cultivation-independent, semiautomatic determination of absolute bacterial cell numbers in environmental samples by fluorescence *in situ* hybridization. Appl. Environ. Microbiol. 67, 5810–5818.

Daims, H., Wagner, M., 2007. Quantification of uncultured microorganisms by fluorescence microscopy and digital image analysis. Appl. Microbiol. Biotechnol. 75, 237–248.

Daims, H., 2009. Use of fluorescence *in situ* hybridization and the daime image analysis program for the cultivation-independent quantification of microorganisms in environmental and medical samples. Cold Spring Harb. Protoc. 4.

DeLong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243, 1360–1363.

Dwivedi, H.P., Jaykus, L.A., 2011. Detection of pathogens in foods: the current state-of-the-art and future directions. Crit. Rev. Microbiol. 37, 40–63.

Ehrenberg, M., Bremer, H., Dennis, P.P., 2013. Medium-dependent control of the bacterial growth rate. Biochimie 95, 643–658.

European Food Safety Authority (EFSA), European Centre for Disease Prevention and Control (ECDC), 2013. The European union summary report on trends and sources of Zoonoses, Zoonotic agents and food-borne outbreaks in 2011. EFSA J. 11.

Ercolini, D., Hill, P.J., Dodd, E.R., 2003. Bacterial community structure and location in Stilton cheese. Appl. Environ. Microbiol. 69, 3540–3548.

Ercolini, D., Villani, F., Aponte, M., Mauriello, G., 2006. Fluorescence *in situ* hybridisation detection of *Lactobacillus plantarum* group on olives to be used in natural fermentations. Int. J. Food Microbiol. 112, 291–296.

Evans, M.I., Sharp, M., Tepperberg, J., Kilpatrick, M.W., Tsipouras, P., Tafas, T., 2006. Automated microscopy of amniotic fluid cells: detection of FISH signals using the FastFISH imaging system. Fetal Diagn. Ther. 21, 523–527.

Fang, Q., Brockmann, S., Botzenhart, K., Wiedenmann, A., 2003. Improved detection of *Salmonella* spp. in foods by fluorescent *in situ* hybridization with 23S rRNA probes: a comparison with conventional culture methods. J. Food Prot. 66, 723–731.

Fegatella, F., Lim, J., Kjelleberg, S., Cavicchioli, R., 1998. Implications of rRNA operon copy number and ribosome content in the marine oligotrophic ultramicrobacterium *Sphingomonas* sp. strain RB2256. Appl. Environ. Microbiol. 64, 4433–4438.

Fischer Walker, C.L., Perin, J., Aryee, M.J., Boschi-Pinto, C., Black, R.E., 2012. Diarrhea incidence in low- and middle-income countries in 1990 and 2010: a systematic review. BMC Public Health 12.

Fornasari, M.E., Rossetti, L., Remagni, C., Giraffa, G., 2008. Quantification of *Enterococcus italicus* in traditional Italian cheeses by fluorescence whole-cell hybridization. Syst. Appl. Microbiol. 31, 223–230.

Frischer, M.E., Floriani, P.J., Nierzwicki-Bauer, S.A., 1996. Differential sensitivity of 16S rRNA targeted oligonucleotide probes used for fluorescence *in situ* hybridization is a result of ribosomal higher order structure. Can. J. Microbiol. 42, 1061–1071.

Fuchizawa, I., Shimizu, S., Kawai, Y., Yamazaki, K., 2008. Specific detection and quantitative enumeration of *Listeria* spp. using fluorescent *in situ* hybridization in combination with filter cultivation (FISHFC). J. Appl. Microbiol. 105, 502–509.

Fuchizawa, I., Shimizu, S., Ootsubo, M., Kawai, Y., Yamazaki, K., 2009. Specific and rapid quantification of viable *Listeria monocytogenes* using fluorescence *in situ* hybridization in combination with filter cultivation. Microbes Environ. 24, 273–275.

Fuchs, B.M., Wallner, G., Beisker, W., Schwippl, I., Ludwig, W., Amann, R., 1998. Flow cytometric analysis of the *in situ* accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. 64, 4973–4982.

Ge, B., Meng, J., 2009. Advanced technologies for pathogen and toxin detection in foods: current applications and future directions. J. Lab. Autom. 14, 235–241.

Gehring, A., Barnett, C., Chu, T., DebRoy, C., D'Souza, D., Eaker, S., Fratamico, P., Gillespie, B., Hegde, N., Jones, K., Lin, J., Oliver, S., Paoli, G., Perera, A., Uknalis, J., 2013. A high-throughput antibody-based microarray typing platform. Sensors (Basel) 13, 5737–5748.

Graczyk, T.K., Conn, D.B., Lucy, F., Minchin, D., Tamang, L., Moura, L.N., DaSilva, A.J., 2004. Human waterborne parasites in zebra mussels (*Dreissena polymorpha*) from the Shannon River drainage area, Ireland. Parasitol. Res. 93, 385–391.

Gunasekera, T.S., Dorsch, M.R., Slade, M.B., Veal, D.A., 2003a. Specific detection of *Pseudomonas* spp. in milk by fluorescence *in situ* hybridization using ribosomal RNA directed probes. J. Appl. Microbiol. 94, 936–945.

Gunasekera, T.S., Veal, D.A., Attfield, P.V., 2003b. Potential for broad applications of flow cytometry and fluorescence techniques in microbiological and somatic cell analyses of milk. Int. J. Food Microbiol. 85, 269–279.

Havelaar, A.H., Brul, S., de Jong, A., de Jonge, R., Zwietering, M.H., Ter Kuile, B.H., 2010. Future challenges to microbial food safety. Int. J. Food Microbiol. 139, 79–94.

Havelaar, A.H., Ivarsson, S., Löfdahl, M., Nauta, M.J., 2013. Estimating the true incidence of campylobacteriosis and salmonellosis in the European Union, 2009. Epidemiol. Infect. 141, 293–302.

Hoorfar, J., 2011. Rapid detection, characterization, and enumeration of foodborne pathogens. APMIS Suppl. 119, 1–24.

Hu, Y., Liu, J., Xia, D., Chen, S., 2012. Simultaneous analysis of foodborne pathogenic bacteria by an oligonucleotide microarray assay. J. Basic Microbiol. 52, 27–34.

ISO 16654, 2001. Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Detection of *Escherichia coli* O157. International Organisation for Standardization, Geneva, Switzerland.

Jarvis, B., 1977. A chemical method for the estimation of mould in tomato products. J. Fd. Technol. 12, 581–591.

Jarvis, B., 2008. Statistical Aspects of the Microbiological Examination of Foods, second ed. Academic Press (imprint of Elsevier).

Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A., Uyttendaele, M., 2010. Alternative microbial methods: an overview and selection criteria. Food Microbiol. 27, 710–730.

Jehan, Z., Uddin, S., Al-Kuraya, K.S., 2012. *In-situ* hybridization as a molecular tool in cancer diagnosis and treatment. Curr. Med. Chem. 19, 3730–3738.

Khimmakthong, U., Deshmukh, S., Chettri, J.K., Bojesen, A.M., Kania, P.W., Dalsgaard, I., Buchmann, K., 2013. Tissue specific uptake of inactivated and live *Yersinia ruckeri* in rainbow trout (*Oncorhynchus mykiss*): visualization by immunohistochemistry and *in situ* hybridization. Microb. Pathog. 59–60, 33–41.

Kitaguchi, A., Yamaguchi, N., Nasu, M., 2005. Enumeration of respiring *Pseudomonas* spp. in milk within 6 hours by fluorescence *in situ* hybridization following formazan reduction. Appl. Environ. Microbiol. 71, 2748–2752.

Krüger, N.J., Buhler, C., Iwobi, A.N., Huber, I., Ellerbroek, L., Appel, B., Stingl, K., 2014. "Limits of control"–crucial parameters for a reliable quantification of viable *campylobacter* by real-time PCR. PLoS One 9.

Kuehn, M., Hausner, M., Bungartz, H.J., Wagner, M., Wilderer, P.A., Wuertz, S., 1998. Automated confocal laser scanning microscopy and semiautomated image processing for analysis of biofilms. Appl. Environ. Microbiol. 64, 4115–4127.

Kupradit, C., Rodtong, S., Ketudat-Cairns, M., 2013. Development of a DNA macroarray for simultaneous detection of multiple foodborne pathogenic bacteria in fresh chicken meat. World J. Microbiol. Biotechnol. 29, 2281–2291.

Laflamme, C., Gendron, L., Filion, N., Ho, J., Duchaine, C., 2009. Rapid detection of germinating *Bacillus cereus* cells using fluorescent *in situ* hybridization. J. Rapid Methods Autom. Microbiol. 17, 80–102.

Lee, S., Malone, C., Kemp, P.F., 1993. Use of multiple 16s rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. Mar. Ecol. Prog. Ser. 101, 193–201.

Liehr, T., Starke, H., Weise, A., Lehrer, H., Claussen, U., 2004. Multicolor FISH probe sets and their applications. Histol. Histopathol. 19, 229–237.

Liehr, T., Weise, A., Hamid, A.B., Fan, X., Klein, E., Aust, N., Othman, M.A., Mrasek, K., Kosyakova, N., 2013. Multicolor FISH methods in current clinical diagnostics. Expert. Rev. Mol. Diagn. 13, 251–255.

López-Campos, G., Martínez-Suárez, J.V., Aguado-Urda, M., López-Alonso, V., 2012. Microarray Detection and Characterization of Bacterial Foodborne Pathogens. In: Chapter 2: Detection, Identification, and Analysis of Foodborne Pathogens. Springer. Briefs in Food, Health, and Nutrition.

Lucore, L.A., Cullison, M.A., Jaykus, L.A., 2000. Immobilization with metal hydroxides as a means to concentrate food-borne bacteria for detection by cultural and molecular methods. Appl. Environ. Microbiol. 66, 1769–1776.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüssmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H., 2004. ARB: a software environment for sequence data. Nucleic Acids Res. 32, 1363–1371.

Machado, A., Almeida, C., Carvalho, A., Boyen, F., Haesebrouck, F., Rodrigues, L., Cerca, N., Azevedo, N.F., 2013. Fluorescence *in situ* hybridization method using a peptide nucleic acid probe for identification of *Lactobacillus* spp. in milk samples. Int. J. Food Microbiol. 162, 64–70.

Malorny, B., Huehn, S., Dieckmann, R., Krämer, N., Helmuth, R., 2009. Polymerase chain reaction for the rapid detection and serovar identification of *Salmonella* in food and feeding stuff. Food Anal. Methods 2, 81–95.

Mandal, P.K., Biswas, A.K., Choi, K., Pal, U.K., 2011. Methods for rapid detection of foodborne pathogens: an overview. Am. J. Food Technol. 6, 87–102.

Manti, A., Boi, P., Amalfitano, S., Puddu, A., Papa, S., 2011. Experimental improvements in combining CARD-FISH and flow cytometry for bacterial cell quantification. J. Microbiol. Methods 87, 309–315.

Mbuthia, P.G., Christensen, H., Boye, M., Petersen, K.M., Bisgaard, M., Nyaga, P.N., Olsen, J.E., 2001. Specific detection of *Pasteurella multocida* in chickens with fowl cholera and in pig lung tissues using fluorescent rRNA *in situ* hybridization. J. Clin. Microbiol. 39, 2627–2633.

McClelland, R.G., Pinder, A.C., 1994. Detection of *Salmonella typhimurium* in dairy products with flow cytometry and monoclonal antibodies. Appl. Environ. Microbiol. 60, 4255–4262.

Milner, M.G., Saunders, J.R., McCarthy, A.J., 2001. Relationship between nucleic acid ratios and growth in *Listeria monocytogenes*. Microbiology 147, 2689–2696.

Moreno, Y., Hernández, M., Ferrús, M.A., Alonso, J.L., Botella, S., Montes, R., Hernández, J., 2001. Direct detection of thermotolerant campylobacters in chicken products by PCR and *in situ* hybridization. Res. Microbiol. 152, 577–582.

Myers, J., 2004. Automated slide stainers for SS, IHC, and ISH: a review of current technologies and commercially available systems. MLO Med. Lab. Obs. 36, 28–30.

Myers, J., 2012. Automated IHC/ISH slide staining systems: current technologies and other considerations. MLO Med. Lab. Obs. 44, 48–50.

Novikov, E., Barillot, E., 2007. Software package for automatic microarray image analysis (MAIA). Bioinformatics 23, 639–640.

Nugen, S.R., Baeumner, A.J., 2008. Trends and opportunities in food pathogen detection. Anal. Bioanal. Chem. 391, 451–454.

Oliveira, M., Andrade, G., Guerra, M., 2003. Development of a fluorescent *in situ* hybridization protocol for the rapid detection and enumeration of *Listeria monocytogenes* in milk. Rev. Port. Ciencias Veterinarias 98, 119–124.

Oliveira, M., Blasco, L., Ferrer, S., Bernardo, F., 2004. Rapid and simultaneous detection of *Salmonella* spp. and *Listeria monocytogenes* in milk by fluorescent *in situ* hybridisation. Rev. Port. Ciencias Veterinarias 99, 215–218.

Oliveira, M., Vieira-Pinto, M., Martins da Costa, P., Vilela, C.L., Martins, C., Bernardo, F., 2012. Occurrence of *Salmonella* spp. in samples from pigs slaughtered for consumption: a comparison between ISO 6579:2002 and 23S rRNA fluorescent *in situ* hybridization method. Food Res. Int. 45, 984–988.

Oliver, J.D., 2005. The viable but nonculturable state in bacteria. J. Microbiol. 43, 93–100.

Oliver, J.D., 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol. Rev. 34, 415–425.

Ootsubo, M., Shimizu, T., Tanaka, R., Sawabe, T., Tajima, K., Ezura, Y., 2003. Seven-hour fluorescence *in situ* hybridization technique for enumeration of *Enterobacteriaceae* in food and environmental water sample. J. Appl. Microbiol. 95, 1182–1190.

Pernthaler, A., Pernthaler, J., Amann, R., 2002. Fluorescence *in situ* hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl. Environ. Microbiol. 68, 3094–3101.

Pernthaler, J., Pernthaler, A., Amann, R., 2003. Automated enumeration of groups of marine picoplankton after fluorescence *in situ* hybridization. Appl. Environ. Microbiol. 69, 2631–2637.

Perry-O'Keefe, H., Stender, H., Broomer, A., Oliveira, K., Coull, J., Hyldig-Nielsen, J.J., 2001. Filter-based PNA *in situ* hybridization for rapid detection, identification and enumeration of specific microorganisms. J. Appl. Microbiol. 90, 180–189.

Porter, J., Robinson, J., Pickup, R., Edwards, C., 1998. An evaluation of lectin-mediated magnetic bead cell sorting for the targeted separation of enteric bacteria. J. Appl. Microbiol. 84, 722–732.

Regnault, B., Martin-Delautre, S., Lejay-Collin, M., Lefèvre, M., Grimont, P.A., 2000. Oligonucleotide probe for the visualization of *Escherichia coli*/*Escherichia fergusonii* cells by *in situ* hybridization: specificity and potential applications. Res. Microbiol. 151, 521–533.

Rueda, L., Rezaeian, I., 2011. A fully automatic gridding method for cDNA microarray images. BMC Bioinforma. 12.

Schmid, M., Walcher, M., Bubert, A., Wagner, M., Wagner, M., Schleifer, K.H., 2003. Nucleic acid-based, cultivation-independent detection of *Listeria* spp. and genotypes of *L. monocytogenes*. FEMS Immunol. Med. Microbiol. 35, 215–225.
Schmid, M.W., Lehner, A., Stephan, R., Schleifer, K.H., Meier, H., 2005. Development and application of oligonucleotide probes for *in situ* detection of thermotolerant *Campylobacter* in chicken faecal and liver samples. Int. J. Food Microbiol. 105, 245–255.
Schönhuber, W., Zarda, B., Eix, S., Rippka, R., Herdman, M., Ludwig, W., Amann, R., 1999. *In situ* identification of cyanobacteria with horseradish peroxidase-labeled, rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. 65, 1259–1267.
Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors – occurrence, properties and removal. J. Appl. Microbiol. 113, 1014–1026.
Shimizu, S., Ootsubo, M., Kubosawa, Y., Fuchizawa, I., Kawai, Y., Yamazaki, K., 2009. Fluorescent *in situ* hybridization in combination with filter cultivation (FISHFC) method for specific detection and enumeration of viable *Clostridium perfringens*. Food Microbiol. 26, 425–431.
Smith, E.M., Green, L.E., Mason, D., 2003. Savinase is a bactericidal enzyme. Appl. Environ. Microbiol. 69, 719–721.
Stender, H., Oliveira, K., Rigby, S., Bargoot, F., Coull, J., 2001. Rapid detection, identification, and enumeration of *Escherichia coli* by fluorescence *in situ* hybridization using an array scanner. J. Microbiol. Methods 45, 31–39.
Stephan, R., Schumacher, S., Zychowska, M.A., 2003. The VIT technology for rapid detection of *Listeria monocytogenes* and other *Listeria* spp. Int. J. Food Microbiol. 89, 287–290.
Stevens, K.A., Jaykus, L.A., 2004. Bacterial separation and concentration from complex sample matrices: a review. Crit. Rev. Microbiol. 30, 7–24.
Takada, T., Matsumoto, K., Nomoto, K., 2004. Development of multi-color FISH method for analysis of seven *Bifidobacterium* species in human feces. J. Microbiol. Methods 58, 413–421.
Thelen, K., Beimfohr, C., Bohak, I., Back, W., 2002. Specific rapid detection test for beer-spoilage bacteria using fluorescently labelled gene probes. Brauwelt Int. 3, 155–159.
Thelen, K., Beimfohr, C., Snaidr, J., 2003. Specific rapid detection of *Alicyclobacillus* by fluorescently-labeled gene probes in fruit juices. Fruit. Process. 6, 416–418.
Theodosiou, Z., Kasampalidis, I.N., Livanos, G., Zervakis, M., Pitas, I., Lyroudia, K., 2007. Automated analysis of FISH and immunohistochemistry images: a review. Cytom. A 71, 439–450.
Thiel, R., Blaut, M., 2005. An improved method for the automated enumeration of fluorescently labelled bacteria in human faeces. J. Microbiol. Methods 61, 369–379.
Tortorello, M., Reineke, K., 2000. Direct enumeration of *Escherichia coli* and enteric bacteria in water, beverages and sprouts by 16S rRNA *in situ* hybridization. Food Microbiol. 17, 305–313.
Veal, D.A., Deere, D., Ferrari, B., Piper, J., Attfield, P.V., 2000. Fluorescence staining and flow cytometry for monitoring microbial cells. J. Immunol. Methods 243, 191–210.
Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K., Adley, C., 2010. An overview of foodborne pathogen detection: in the perspective of biosensors. Biotechnol. Adv. 28, 232–254.
Vieira-Pinto, M., Oliveira, M., Bernardo, F., Martins, C., 2005. Evaluation of Fluorescent *in situ* Hybridization (FISH) as a rapid screening method for detection of *Salmonella* in tonsils of slaughtered pigs for consumption: a comparison with conventional culture method. J. Food Saf. 25, 109–119.
Vieira-Pinto, M., Oliveira, M., Bernardo, F., Martins, C., 2007. Rapid detection of *Salmonella* sp. in pork samples using fluorescent *in situ* hybridization: a comparison with VIDAS-SLM system and ISO 6579 cultural method. Arq. Bras. Med. Veterinária Zootec. 59, 1388–1393.
Wagner, M., Schmid, M., Juretschko, S., Trebesius, K.H., Bubert, A., Goebel, W., Schleifer, K.H., 1998. *In situ* detection of a virulence factor mRNA and 16S rRNA in *Listeria monocytogenes*. FEMS Microbiol. Lett. 160, 159–168.
Wagner, M., Horn, M., Daims, H., 2003. Fluorescence *in situ* hybridisation for the identification and characterisation of prokaryotes. Curr. Opin. Microbiol. 6, 302–309.
Wagner, M., Haider, S., 2012. New trends in fluorescence *in situ* hybridization for identification and functional analyses of microbes. Curr. Opin. Biotechnol. 23, 96–102.
Walker, C.L., Black, R.E., 2010. Diarrhoea morbidity and mortality in older children, adolescents, and adults. Epidemiol. Infect. 138, 1215–1226.
Wauters, J., Assche, E.V., Antsaklis, A., Tepperberg, J., Sharp, S.M., Kilpatrick, M.W., Tafas, T., Tsipouras, P., 2007. Fully automated FISH examination of amniotic fluid cells. Prenat. Diagn. 27, 951–955.
Weiler, C., Ifland, A., Naumann, A., Kleta, S., Noll, M., 2013. Incorporation of *Listeria monocytogenes* strains in raw milk biofilms. Int. J. Food Microbiol. 161, 61–68.
Yamaguchi, N., Kitaguchi, A., Nasu, M., 2012. Selective enumeration of viable *Enterobacteriaceae* and *Pseudomonas* spp. in milk within 7 h by multicolor fluorescence *in situ* hybridization following microcolony formation. J. Biosci. Bioeng. 113, 746–750.
Yilmaz, L.S., Parnerkar, S., Noguera, D.R., 2011. mathFISH, a web tool that uses thermodynamics-based mathematical models for *in silico* evaluation of oligonucleotide probes for fluorescence *in situ* hybridization. Appl. Environ. Microbiol. 77, 1118–1122.
Zeder, M., Pernthaler, J., 2009. Multispot live-image autofocusing for high-throughput microscopy of fluorescently stained bacteria. Cytom. A 75, 781–788.
Zhang, X., Wu, S., Li, K., Shuai, J., Dong, Q., Fang, W., 2012. Peptide nucleic acid fluorescence *in situ* hybridization for identification of *Listeria* genus, *Listeria monocytogenes* and *Listeria ivanovii*. Int. J. Food Microbiol. 157, 309–313.